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RESEARCH

Reversing Multidrug-Resistant by RNA Interference Through Silencing MDR1 Gene in Human Hepatocellular Carcinoma Cells Subline Bel-7402/ADM

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Abstract Multidrug resistance (MDR) in hepatocellular carcinoma (HC) significantly impedes the effect of chemotherapy and is considered as a primary reason leading to its recurrences and metastasis. The aim of present study was to explore new molecular targets for the reversal of MDR in HC by screening the adriamycin (ADM)-induced, human MDR-resistant HC cell subline Bel-7402/ADM. Small interfering RNAs (siRNAs) of four (MDR1si326, MDR1si1513, MDR1si2631 and MDR1si3071) targeting MDR1 were designed and transfected into Bel-7402/ADM cell strains. The experiments involved the following: mRNA expression of MDR1 gene by RT-PCR, P-glycoprotein (P-gp) expression by Western blot, intracellular ADM accumulation flow cytometry, and IC_{50} of ADM by a cytotoxic MTT assay. Four siRNAs reversed MDR in HC mediated by MDR1 to varying degrees. The expression level of MDR1 mRNA in cells of MDR1si326 or MDR1si2631 group (0.190±0.038 or 0.171±0.011) was more decreased. The expression level of P-gp in cells of MDR1si326 group was the lowest. The accumulation of ADM in cells of MDR1si326 or MDR1si2631 group (77.0±3.5 or 75.4±2.9) was more increased. The IC_{50} of cells to ADM was lowest in MDR1si326 group (11.32±0.69 mg/L). Compared with other three siRNAs, MDR1si326 performed the optimal reversal effect of drug resistance in human HC Bel-7402/ADM.

Keywords Human hepatocellular carcinoma · siRNA · MDRl gene · Reversal · Bel-7402/ADM

Abbreviations

MDR	Multidrug resistance
siRNA	Small interfering RNA
ADM	Adriamycin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Introduction

Liver cancer is one of the major malignant tumors which pose great threats to people's health. Liver cancer in women is the seventh most frequently diagnosed cancer worldwide but the sixth most frequent cause of cancer death. In men, it is the fifth most commonly diagnosed cancer and the second leading cause of cancer death [1]. Among primary liver cancers, hepatocellular carcinoma accounts for 70 to 85 % of the total liver cancer burden worldwide, representing the major histological subtype [2]. Chemotherapy plays an important role in the treatment of hepatocellular carcinoma. However, Multidrug resistance (MDR) in hepatocellular carcinoma significantly impedes the effect of chemotherapy, and it is also considered as a key reason leading to the recurrence and metastasis of hepatocellular carcinoma [3]. Among various genesis mechanisms of MDR, the overexpression of P-glycoprotein (P-gp), the product of the MDRl gene, is generally recognized as the principal mechanism of MDR formation. The researchers

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made attempts to reserve MDR by cyclosporin A [4], verapamil [5], and COX-2 inhibitors such as celecoxib [6, 7], which generally come into effect under high concentrations. Meanwhile, the adverse reactions of drugs with high concentrations restrict their clinical practices. At present, the inhibition of the excessive expression of drug resistant gene has become a new approach in the reversal of drug resistance in chemotherapy [8].

Ribonucleic acid interference (RNAi) refers to post-transcriptional gene silencing by double-stranded RNA, one of the most effective tool in the research of gene functions [9, 10]. In the process of gene therapy, small interfering RNA (siRNA) synthesized artificially or transcribed by siRNA expressing vector was transfected into tumor cells, which could specifically inhibit gene expression [11, 12]. Compared with the gene knockdown technologies such as antisense oligonucleotide, ribozyme, or deoxyribozyme, this technology is characterized with high specificity, efficiency, stability, diffusibility, and heritability in the aspect of molecular biology; and it has been widely applied into studies of chemotherapy resistance and its reversal effects of tumors has received due attention [13, 14]. In 2001, Elbashir et al. [15] firstly reported the successful induction of the gene-specific inhibition of mammalian cells cultured in vitro by siRNAs. Since then, the treatment study of specific genetic interference in malignant tumors by siRNA such as bladder cancer, leukemia, breast cancer, and hepatocellular carcinoma has been carried out extensively [16–19]. For MDR in tumors, Neith et al. [20] applied RNAi and effectively reversed MDR in pancreatic cancer and gastric cancer mediated by MDR1 gene. In MDR research of breast cancer, Sun Y et al. [21] designed siRNA targeting MMP-2 gene and effectively inhibited the expression of this gene. In addition, progresses have been achieved in the inhibition of MDR1 of glioma, malignant tumors of the uterus, and ovarian cancer; and the drug resistance to some cells has successfully been reversed [22–24].

Studies have shown that MDR1-targeted siRNA reversed resistance to ADM, a P-gp-transportable drug, but did not affect the sensitivity to a non-P-gp substrate, such as cisplatin [25], and that the efficiency of siRNAs targeting different points in the same gene showed remarkable differences [26]. Therefore, a variety of factors affecting the effect of siRNA are taken into account when four representative siRNAs targeting different points in the MDR1 gene were designed in this research. The present study involved the screening for efficient siRNA based on four aspects such as its impact on mRNA and P-gp expressions of MDR1, changes of intracellular ADM accumulation, and reversal of the resistance to ADM; and new target points were explored for the reversal of MDR in hepatocellular carcinoma.

Materials and Methods

SiRNA Design and Synthesis

Per the design principles of siRNA, the RNA online tools from Ambion [27] (Ambion Inc., Austin, TX, USA) were applied to choose 14 potential target points for MDRl complementary deoxyribonucleic acid (cDNA) full-length sequence in GenBank [28] (NCBI, Bethesda, Maryland, USA; GenBank accession number: NM-000927.3). In addition, four targeted sequences were selected based on the integration of locations and structures of potential targets. Using basic local alignment search tool, it was identified that human genome sequence had no homology, except for MDR1. Four siRNAs were designed and the targeted sequences were: MDR1si326,5′-AAGACATG ACCAGGTATGCCT-3′(326~346), MDR1si1513,5′-AAGG AAGCCAATGCCTATGAC-3′(1513~1533), MDR1si2631,5′-AATGTTGTCTGGACAAGCACT-3′(2631~2651), and MDR1si3071,5′-AAGGCCTAATGCCGAACACAT-3′(3071~3091). The chemical synthesis was carried out by Shanghai GenePharma Company (Shanghai, China). A negative control siRNA provided by Shanghai GenePharma Company shared no homology with the genes of mammals and had the following sequences: sense strand 5′- UUCUCCGAACGUGU CACGUTT-3′, antisense strand 5′- ACGUGACACGUUCG GAGAATT -3′. This negative control was named as siRNA-nc.

Cell Culture and Transfection

Ethical Approval Animal experiments were conducted under the experimental animal ethics committee of Anhui Medical University of China. The human hepatocellular carcinoma cell line Bel-7402 was purchased from Shanghai Institute of Biological Products. The human adriamycin (ADM)-resistant hepatocellular carcinoma cell subline Bel-7402/ADM was established by nude mice liver-implanted induction [29]. Four-week-old BALB/c-nu/nu nude mice weighting 12–16 g were purchased from Shanghai Shilaike Co., Ltd., and were bred in the specific pathogen free (SPF) Animal Center, School of Life Science, University of Science and Technology of China. The nude mice aged 4 to 6 weeks were used. A 0.2-mL Bel-7402 cell suspension (density equal to 1×10^8/mL) was injected into the parenchyma of the right hepatic lobe. All animals were bred in SPF condition. From the third day, the nude mice underwent an intraperitoneal injection with ADM at a dose of 1.5 mg/kg each week for 8 weeks. When the experiment was completed, the nude mice were sacrificed. The tumor was obtained and levigated in asepsis to produce a mono-cell suspension. After multiple passages and purification, the hepatocellular implantation of drug-resistant cell

sub-lines Bel-7402/ADM were obtained. The cells were cultivated in the Rosewell Park Memorial Institute (RPMI)-1640 culture solution (10 % fetal bovine serum, 0.1 mg/mL streptomycin, and 100U/mL penicillin) and incubated in the cell incubator with 5 % carbon dioxide at 37 °C. ADM (Zhejiang Hisun Pharmaceutical Co., Ltd, Zhejiang, China) was added in the culture solutions of Bel-7402/ADM cells to form a final concentration of 0.5 μg/mL, so as to maintain the drug resistance phenotype and was removed 7 days before transfection. The transfection conditions were optimized per the instructions of Lipofectamine™ 2000 (Invitrogen Corporation, California, USA). siRNA of each group was added into Bel-7402/ADM culture solution with the final concentration of 10 nmol/L. The experiment involved the following eight groups: (1) control group: added with phosphate buffer saline (PBS) only; (2) transfection reagent group: transfection reagent + PBS; (3) MDR1si326 group: transfection reagent + MDR1si326; (4) MDR1si1513 group: transfection reagent + MDR1si1513; (5) MDR1si2631 group: transfection reagent + MDR1si2631; (6) MDR1si3071 group: transfection reagent + MDR1si3071; (7) SiRNA-nc group: transfection reagent + siRNA-nc; and (8) Bel-7402 group (parent strain group): Bel-7402 Cell + PBS.

Real-Time Polymerase Chain Reaction (PCR) Detection of mRNA Expression of MDR1

After 48 h from the cell transfection, the overall RNA was extracted with a RNA extraction kit (Invitrogen Corporation, California, USA) and verified by A260/A280. cDNA was synthesized using a reverse transcription kit (A3500, Promega, Madison, USA). The primers and probes were synthesized by Invitrogen Company with the following sequences: (A) for MDR1: the forward primer of 5′-CCCAGGAGCCCATCCT GT-3′, reverse primer of 5′-CCCGGCTGTTGTCTCCATA-3′, and probe of 5′-FAM-TGACTGCAGCATTGCTGAGAAC ATTGC-TAMRA-3′; (B) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer of 5′- GAAGGTGA AGGTCGGAGTC -3′, reverse primer of 5′-GAAGATGGTG ATGGGATTTC-3′, and probe of 5′-FAM-CAAGCTTCCC GTTCTCAGCC-TAMRA-3′. The PCR reaction was carried out on an ABI7500 fluorescent quantitative real-time PCR instrument under the following conditions: predegenerated at 95 °C for 30 s, degenerated at 95 °C for 5 s, and annealing at 60 °C for 34 s. The above process was repeated for 40 times. The circulatory threshold (Ct) of each sample was identified. The Ct value of the target gene was compared with the Ct value of the housekeeping gene GAPDH and then translated into relative expression value of MDR1gene,$2^{-\Delta\Delta Ct}$.

Western Blot Detecton of P-gp Expression

After 72 h from the transfection, the cells of each group were collected, and the total proteins of groups were extracted from protein lysis buffer (Sigma, Missouri, USA) for protein quantification using a bicinchoninic acid protein assay reagent kit (Beyotime Shanghai, China). The sample was loaded with 50 μg per pore and sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5 % separating gel and 5 % stacking gel). The proteins were transferred by water bath electrotransfer to the nitrocellulose membrane (Santa Cruz Biotechnology Inc, California, USA), blocked with 5 % nonfat milk for 1 h at room temperature, and incubated overnight at 4 °C by a monoclonal rat anti-human P-gp antibody (Santa Cruz Biotechnology Inc, California, USA with the working concentration of 1:1,000) and monoclonal rat anti-human β-actin antibody (Santa Cruz Biotechnology Inc, California, USA with the working concentration of 1:500). The nitrocellulose membrane was incubated by horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (Beyotime, shanghai, China with the working concentration of 1:3,000 and 1:4,000) at room temperature for 2 h and detected by ECL detection system (Bio-Rad) and exposed. The grayscale ratio of P-gp to β-actin was analyzed using the gel electrophoresis and image analysis system (JEDA,JD-801, Jiangsu, China), and the relative expression of P-gp was obtained.

Flow Cytometry Detection of Intracellular ADM Accumulation

After 48 h from the cell transfection of each group, RPMI-1640 culture solution (10 % fetal bovine serum and 0.5 μg/mL ADM) was used to prepare a single-cell suspension (5×10^5/L), which was cultured for 1.5 h. Then, the cells were collected and washed at 4 °C in preserved cold PBS twice and fixed on 70 % iced ethanol for 5 min. The cells were centrifuged at room temperature with the speed of 2,000 rpm for 5 min, resuspended in 1 ml cold PBS, and preserved at 4 °C to detect intracellular ADM-specific fluorescence intensity (λex= 488 nm, λem=575 nm) by flow cyotmetry (Beckman Coulter, EPICS-XL-4).

MTT Detection of Drug Susceptibility

After 48 h from the transfection, the cells of each group were collected, incubated in a 96-pore plate (5×10^5/mL, 200 μL per pore), and cultured in a culture medium without antibiotics for 24 h before adding ADM. With the reference of the maximal concentration of ADM in clinical usage, the concentration was increased and reduced by 10^2 times, respectively to form five test concentrations [30]. For each one, 5 multiple pores were built, and the cells were cultured for 48 h. Per conventional practices, MTT (3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide; 5 g/L; 20 μL; Sigma, Missouri, USA) and dimethyl sulfoxide (DMSO, Sigma,

Missouri, USA) were added in order, and the absorbance (570 nm, A value) of each pore was measured with a microplate reader (Model-450, Bio-Rad, USA). The reagent without the cells was made for blank control. The 'A' value of the control and treatment groups were detected to calculate the inhibition ratio (cell inhibition ratio=1-$A_{\text{treatment group}}/A_{\text{control group}}$). The dose survival curve was drawn to calculate the 50 % inhibitory concentration (IC_{50}).

Statistic Analysis

The measurements were performed in triplicate. All data are provided as means ± SD. The results were analyzed by one-way analysis of variance with Student-Newman–Keuls multiple comparison test. $p<0.05$ was considered significant. The statistical analysis was performed using the SPSS18.0 software (Version 18.0, SPSS Inc., Chicago, IL, USA).

Results

Efficiency Assay of Bel-7402/ADM Transfection Cell by SiRNAs

After 6 h from the transfection of the fluorescent-labeled transfection siRNAs to Bel-7402/ADM, the fluorescence microscopy was used for observation. Almost all the cells in the vision were attached with fluorescent light, and the transfection efficiency was close to 100 % (Fig. 1). After 6 h from the transfection of the fluorescent-labeled transfection siRNAs to Bel-7402/ADM, the flow cytometry detection presented the transfection efficiency of 94.0 %.

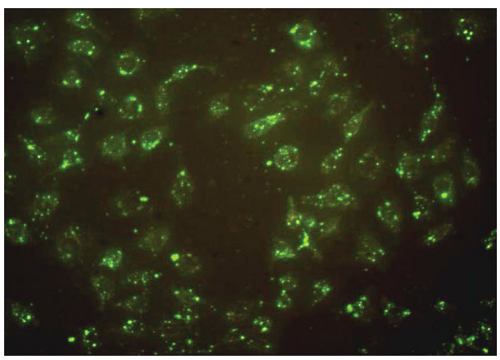

Fig. 1 After 6 h from the transfection of the fluorescent-labeled transfection siRNAs to Bel-7402/ADM, the fluorescence microscopy was used for observation. Almost all the cells in the vision were attached with fluorescent light, and the transfection efficiency was close to 100 %. The appearance of the transfected Bel-7402/ADM cells were watched using fluorescent microscope (×400)

Silence Impact of siRNAs on MDRl Gene Expression of Bel-7402/ADM

After 48 h from the siRNA transfection into Bel-7402/ADM cells, the mRNA expression of MDR1 in many groups was effectively reduced with statistically significant difference ($P<0.05$) compared with control group. The difference in statistical significance between MDR1si326 group (or MDR1si2631 group) and MDR1si1513 group (or MDR1si3071 group) was observed ($P<0.05$). No difference in the statistical significance was observed between transfection reagent group, SiRNA-nc group and control group ($P>0.05$) (Fig. 2).

P-gp Expression by Western Blot Detection

Compared with the control group, the P-gp expression of other groups was reduced 72 h after siRNA transfection into Bel-7402/ADM. Difference in statistical significance was observed between MDR1si326 group, MDR1si2631 group (or MDR1si3071 group), MDR1si1513 group and control Group ($P<0.05$) (Fig. 3). There was no difference in the statistical significance between MDR1si2631 group and MDR1si3071 group ($P>0.05$); and also between transfection reagent group, SiRNA-nc group, and control group ($P>0.05$).

FCM Detection of Intracellular ADM Accumulation

Compared with the control group, the intracellular ADM fluorescence intensity in many groups was enhanced with satistically significant difference ($P<0.05$), 48 h after transfection into Bel-7402/ADM. There was difference in statistical significance

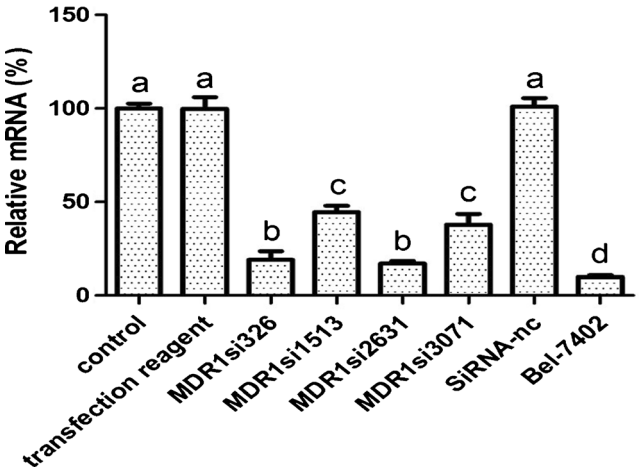

Fig. 2 Cells in duplicate wells were transfected with siRNAs or without any treatment. The cells were harvested at 48 h post-transfection and analysed by RT-PCR for MDR1 and GAPDH (normalization control). Finally, the relative expression was calculated as the ratio of MDR1 to GAPDH in each sample. Each of the eight groups shows average measurements based on three replicates of the experiment. Groups labeled with different letters (such as a, b, c, and d) are statistically different from one another at the $P<0.05$ level (SNK)

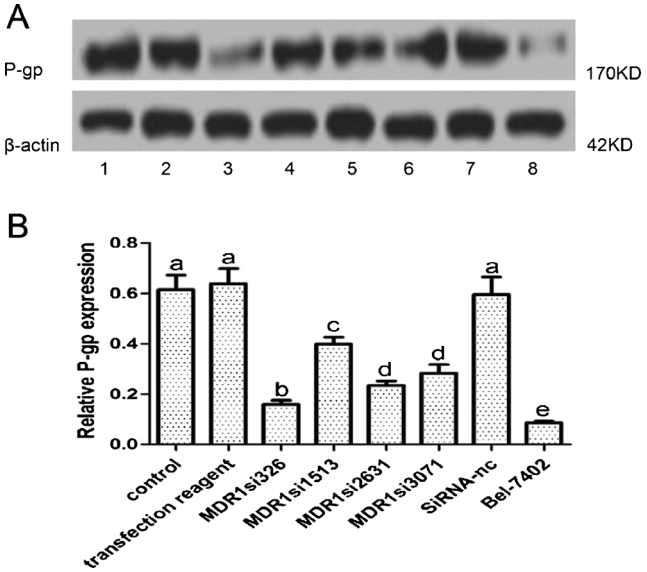

Fig. 3 Protein expression in the cells by Western blot analysis. Equal amount of protein was loaded for Western blot analysis as described in "Materials and Methods". The level of P-gp expression in Bel-7402/ADM cells and Bel-7402 cells was compared to the levels of β-actin as a house-keeping internal control protein. Independent experiments were performed at least thrice and the result from a representative experiment is shown in (A): (1) control croup; (2) transfection reagent group; (3) MDR1si326 group; (4) MDR1si1513 group; (5) MDR1si2631 group; (6) MDR1si3071 group; (7) SiRNA-nc group; and (8) Bel-7402 group (parent strain group). (B): Each of the eight bars shows average measurements based on three replicates of the experiment. The error bars represent the standard deviation of the measurements. The bars labeled with different letters (such as a, b, c, d, and e) are statistically different from one another at the $P<0.05$ level (SNK)

between MDR1si326 group (or MDR1si2631 group) and MDR1si1513 group (or MDR1si3071 group) ($P<0.05$). No difference in statistical significance was observed not only between MDR1si326 group and MDR1si2631 group, between MDR1si1513 group and MDR1si3071 group, but also between the transfection reagent group, SiRNA-nc group, and control group ($P>0.05$) (Fig. 4).

Reliability Verification of the Two Cell Models

The human ADM-resistant hepatocellular carcinoma cell subline Bel-7402/ADM and the ADM-sensitive Bel-7402 cell was veryfied by MTT assay. After the addition of ADM, they were cultured for 48 h. The difference in tatistical significance was observed between IC_{50} of the control group and that of Bel-7402 group (25.19±1.22 and 1.64±0.56, $P<0.05$). The human hepatocellular carcinoma cell subline Bel-7402/ADM was found to be resistant to a high concentration of ADM, but Bel-7402 cell was ADM-sensitive (Fig. 5).

Changes of Cell Sensitivity to ADM by SiRNAs

After 72 h from siRNA transfection into Bel-7402/ADM, the IC_{50} of each group which targeted MDR1 gene was reduced

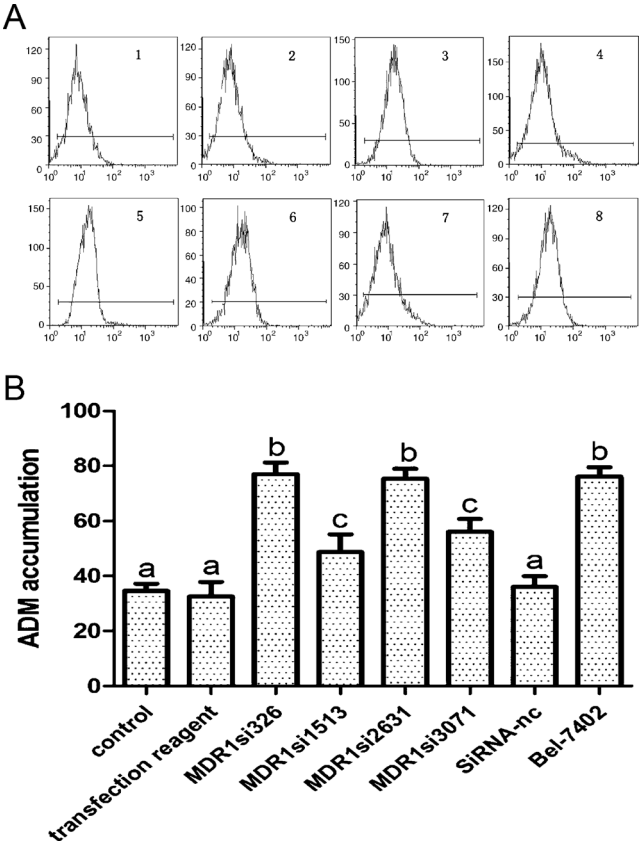

Fig. 4 Effect of siRNA on the intracellular accumulation of ADM. The accumulation of ADM was measured by flow cytometric analysis after cells were pre-incubated with or without siRNA for 48 h at 37 °C and then incubated with 0.5 μg/mL ADM for another 1.5 h at 37 °C, respectively, as described in "Materials and Methods". A: Independent experiments were performed at least thrice and the result from a representative experiment is shown. (1) control croup; (2) transfection reagent group; (3) MDR1si326 group; (4) MDR1si1513 group; (5) MDR1si2631 group; (6) MDR1si3071 group; (7) SiRNA-nc group; and (8) Bel-7402 group (parent strain group). B: Each of the eight bars shows average measurements based on three replicates of the experiment. The error bars represent the standard deviation of the measurements. The bars labeled with different letters (such as a, b, and c) are statistically different from one another at the $P<0.05$ level (SNK)

compared with the control group. There was difference in statistical significance between MDR1si326 group, MDR1si2631 group, MDR1si1513 (or MDR1si3071 group), and control group ($P<0.05$). Meanwhile, no difference in statistical significance was observed not only between MDR1si1513 group and MDR1si3071 group ($P>0.05$); but also between transfection reagent group, SiRNA-nc group, and control group ($P>0.05$) (Fig. 5).

Discussion

The inhibition of the function or expression of P-gp can enhance the sensitivty of MDR cells to chemotherapeutic drugs. SiRNA has been proved to be an effective approach

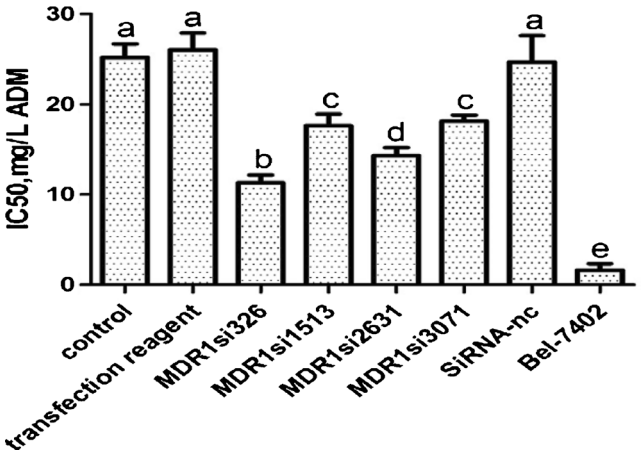

Fig. 5 Cytoroxicity of each group cells by MTT assay. Cells transfected with siRNA or without any treatment were then given different concentration of ADM as indication and incubated for an additional 48 h. The measure of the 50 % inhibitory concentration was done as described in the "Materials and Methods" Section. Each of the eight bars shows average measurements based on three replicates of the experiment. The error bars represent the standard deviation of the measurements. The bars labeled with different letters (such as a, b, c, d, and e) are statistically different from one another at the $P<0.05$ level (SNK)

for silencing gene expression that has been applied recently to inhibiting MDR1 replication in cell cultures [22–24]. Since Elbashir et al. [31] reported that the RNAi can be triggered in mammalian cells by the introduction of 21-nucleotide siRNA. The gene silencing induced by RNAi was shown to be specific and potent [13, 14], offering an alternative strategy for overcoming the drug resistances. A large number of experiments in China and other counties on the reversal of MDR mediated by MDR1 by siRNA have shown that the effects of siRNA are closely related with target sites [26].

In the present research, MDRl gene was taken as the target to synthesize four siRNAs, in order to explore the feasibility of MDR reversal and screening of efficient siRNA. It has been found in this experiment that mRNA and P-gp expressions of Bel-7402/ADM were reduced after the transinfection. Meanwhile, intracellular ADM-specific fluorescence intensity and sensibility to ADM was improved, indicating that all four siRNAs were competent to reverse MDR in Bel-7402/ADM. This study also demonstrated that the four siRNAs performed differently in the Bel-7402/ADM cells. Among them, MDR1si326 performed the optimal reversal effect while MDR1si1513 performed the most unsatisfactory effect. The four siRNAs in this study showed different RNAi efficiencies, which confirmed the "site effect" of siRNAs. This could be attributed to the secondary structure of MDR1 cDNA, stem-loop structures, and number of base pairing and hydrogen bonds outside the targeted site [32–34].

The analysis of experimental results showed that one time transfection could not reach the complete inhibition of P-gp (protein expression in MDR1si326 group vs Bel-7402 group $P<0.05$). The lack of complete inhibition is likely because of the RNAi effective time of transient transfection [35], relatively long half-life (14 to 17 h) of the protein [35–37], transfection efficiency [37], relatively high density of P-gp on surface states [36], and high expression levels [37]. The P-gp expression was detected 72 h after transfection in the present study at 4 times the time interval of the half-life of P-gp. Thus, the relatively long half-life and RNAi effective time of transient transfection are hard to be the only primary reason. The Lipofectamine™ 2000 transfection reagent was used in this study with the hige transfection efficiency of been close to 100 %, which provided the evidence that the transfection efficiency had little effect on the inhibition of P-gp. This study also suggests that high expression levels of P-gp and relatively high density of P-gp on surface states restricted the expression of P-gp. This findings is similar to the results of Ambudkar et al. [38] and Labroille et al. [39], and it explains the imbalance of the inhibition level of MDR1 mRNA and P-gp. Ambudkars' study Showed the sensitive cells could acquire the drug-resistant phenotype through the intercellular transfer of functional P-gp molecules and elevate the expression of P-gp. Labroille's research suggested that P-gp could include the cytoplasmic P-gp and surface P-gp, while the cytoplasmic P-gp pool could be a storage pool consumed for maintaining a steady-state level of surface P-gp. However, further researches are needed to explore whether continuous siRNA transfection and collaboration between multiple siRNA transfection can achieve complete inhibition of the P-gp to enhance the silencing effects of MDR1 or not.

Additional problems may arise from clinical P-gp inhibition by anti-MDR1 siRNAs. The present study has characterized the normal human tissue distribution of P-gp [40]. For example, P-gp, which is the most important and best-studied efflux transporter at the blood–brain barrier, is localized within the luminal membrane of brain capillaries [41]. They prevent the entry of xenobiotics and potentially toxic metabolites into the central nervous system and contribute to lowered drug accumulation within the brain [42]. The impact of clinical P-gp inhibition on P-gp physiological functions remains to be further explored. Therefore, more studies in animals harboring the MDR tumors will be warranted as precursors to testing this approach in humans.

Conclusions

In conclusion, the highly ADM-resistant human hepatocellular carcinoma Bel-7402/ADM cells can completely be reversed to an ADM-sensitive phenotype by siRNA. The target

sequence provides a valuable approach by gene therapy to make the MDR human hepatocellular carcinoma cells sensitive to anti-cancer drugs.

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Conflict of Interest The authors declare that they have no competing interests.

Authors' Contribution LS, CL carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. XLM, MMX conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript. MMX accepts full responsibility for the work and has accessed to the data, and overseen the decision to publish.

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