

Original Article

Silencing MRP1-4 genes by RNA interference enhances sensitivity of human hepatoma cells to chemotherapy

Zheng Su^{1*}, Gaojie Liu^{2*}, Tingfeng Fang³, Yang Wang⁴, Huayao Zhang⁵, Shanglin Yang⁵, Jinxing Wei⁵, Zejian Lv⁶, Langping Tan⁵, Jianping Liu³

¹Comprehensive Department, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; ²Department of Gastrointestinal Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; ³Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; ⁴Department of Gynaecology and Obstetrics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China; ⁵Shenzhen Hospital of Armed Police Frontier Corps, Shenzhen, 518029; ⁶Department of Gastrointestinal Surgery, Guangdong Provincial People's Hospital, Guangzhou 510120, China. *Equal contributors.

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Abstract: Aim: Besides surgical treatment, systematic chemotherapy plays a crucial role in HCC treatment, especially for patients with advanced HCC. However, none of the single-drug-treatment strategies have shown significant survival benefit due to a high incidence rate of chemoresistance. This study was designed to observe the effect of small interfering of RNA (siRNA) targeting multidrug resistance-related protein 1-4 (MRP1, MRP2, MRP3, and MRP4) in modulating drug resistance of HepG2/ADM and SMMC7721/ADM cells. Methods: HepG2/Adriamycin (ADM) and SMMC7721/ADM cell lines were developed by exposing parental cells to stepwise increasing concentrations of ADM. MTT assay was used to determine drug sensitivity and half inhibitory concentration (IC50) of drugs was calculated. Flow cytometry was employed to analyze cell cycle distribution. MRP1-4 mRNA expression levels were measured by quantitative real-time PCR (QRT-PCR). Expression of proteins was analyzed by Western blot. The growth curve was draw and the cell apoptosis was also observed. Animal experiment was used to compare the cell growth. Results: MTT assay showed that the values of IC50 and RI of HepG2/ADM and SMMC7721/ADM decreased after siRNA treatment in HepG2/ADM cells and SMMC7721/ADM cells. QRT-PCR analysis demonstrated the MRP1-4 mRNA expression decreased significantly in HepG2/ADM cells and SMMC7721/ADM cells after siRNA transfection. In addition, compared with parental cells, MRP1-4 protein expressions apparently decreased in SMMC7721/ADM and HepG2/ADM cells. Flow cytometry showed significantly elevated apoptosis rate following MRP1-4 siRNA transfection. Animal experiment suggested that silencing MRP1-4 gene *in vivo* inhibited tumor growth. Conclusion: Inhibition of MRP1-4 by small interfering RNA enhanced and selectively restored sensitivity of hepatoma cells to drugs. MRP1-4 siRNA might represent a new therapeutic option for HCC.

Keywords: Hepatocellular, multidrug resistance, MRP1-4, siRNA

Introduction

According to the latest statistics, there are about six hundred thousand new liver cancer cases worldwide each year, of which about 55% occur in our country. It has become a major killer that seriously threatens people's health and lives [1]. Surgery and chemotherapy are the main treatments for liver cancer currently. However, liver cancer has low liver resection rate, low sensitivity to chemotherapy, and high recurrence rate, and its five year survival rate is only 14%-30% [2, 3]. Although chemotherapy is

an important means of treatment for liver cancer and there has been new chemotherapy drugs and launch, multidrug resistant (MDR) limits the application of liver cancer chemotherapy, and it is also a major cause of liver cancer recurrence and metastasis [4-6]. Multidrug resistance refers to that tumor cells produce drug resistance to a kind of anti-cancer drug, or different cross-resistance to anti-cancer drugs with the same structure, which is a major obstacle to cancer chemotherapy. Data shows that the incidence rate of MDR in primary liver cancer is 84.6%-100%, thus solving the multidrug

MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy

Table 1. Determination of IC50 and resistance index of different anticancer drugs (mean \pm SD)

A						
	IC50		RI	IC50		RI
	SMMC7721	SMMC7721/ADM		SMMC7721	SMMC7721/ADM/MRP1 siRNA	
Adriamycin (mg/L)	0.0106 \pm 0.004	0.2657 \pm 0.003	25.43	0.0106 \pm 0.004	0.0608 \pm 0.033	5.73
Fluorouracil (mg/L)	0.1263 \pm 0.003	3.6129 \pm 0.045	28.60	0.1263 \pm 0.003	2.0031 \pm 0.004	15.86
Vincristine (mg/L)	0.0045 \pm 0.002	0.1763 \pm 0.008	39.17	0.0045 \pm 0.002	0.0112 \pm 0.002	2.49
Oxaliplatin (mg/L)	0.0063 \pm 0.004	0.1134 \pm 0.005	18.30	0.0063 \pm 0.004	0.0116 \pm 0.002	1.84
B						
	IC50		RI	IC50		RI
	SMMC7721	SMMC7721/ADM		SMMC7721	SMMC7721/ADM/MRP2 siRNA	
Adriamycin (mg/L)	0.0106 \pm 0.004	0.2657 \pm 0.003	25.43	0.0106 \pm 0.004	0.1023 \pm 0.006	9.65
Fluorouracil (mg/L)	0.1263 \pm 0.003	3.6129 \pm 0.045	28.60	0.1263 \pm 0.003	1.4417 \pm 0.023	11.41
Vincristine (mg/L)	0.0045 \pm 0.002	0.1763 \pm 0.008	39.17	0.0045 \pm 0.002	0.0452 \pm 0.010	10.04
Oxaliplatin (mg/L)	0.0063 \pm 0.004	0.1134 \pm 0.005	18.30	0.0063 \pm 0.004	0.0268 \pm 0.005	4.25
C						
	IC50		RI	IC50		RI
	SMMC7721	SMMC7721/ADM		SMMC7721	SMMC7721/ADM/MRP3 siRNA	
Adriamycin (mg/L)	0.0106 \pm 0.004	0.2657 \pm 0.003	25.43	0.0106 \pm 0.004	0.0521 \pm 0.009	4.92
Fluorouracil (mg/L)	0.1263 \pm 0.003	3.6129 \pm 0.045	28.60	0.1263 \pm 0.003	1.2740 \pm 0.018	10.09
Vincristine (mg/L)	0.0045 \pm 0.002	0.1763 \pm 0.008	39.17	0.0045 \pm 0.002	0.0146 \pm 0.002	3.24
Oxaliplatin (mg/L)	0.0063 \pm 0.004	0.1134 \pm 0.005	18.30	0.0063 \pm 0.004	0.0311 \pm 0.002	4.94
D						
	IC50		RI	IC50		RI
	SMMC7721	SMMC7721/ADM		SMMC7721	SMMC7721/ADM/MRP4 siRNA	
Adriamycin (mg/L)	0.0106 \pm 0.004	0.2657 \pm 0.003	25.43	0.0106 \pm 0.004	0.1052 \pm 0.004	9.92
Fluorouracil (mg/L)	0.1263 \pm 0.003	3.6129 \pm 0.045	28.60	0.1263 \pm 0.003	2.9576 \pm 0.006	23.41
Vincristine (mg/L)	0.0045 \pm 0.002	0.1763 \pm 0.008	39.17	0.0045 \pm 0.002	0.0532 \pm 0.001	11.82
Oxaliplatin (mg/L)	0.0063 \pm 0.004	0.1134 \pm 0.005	18.30	0.0063 \pm 0.004	0.0071 \pm 0.002	1.13

resistance during chemotherapy is of great significance to the treatment of liver cancer [7-10]. In all resistance mechanisms, ABC transporter proteins combined with the nuclear membrane are considered to be the most important factor and are most studied by researchers, such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), and breast cancer resistance protein [11-13].

RNA interference (RNAi) is a recently developed method for specific inhibition of gene expression, which refers to the degradation of double-stranded RNA (dsRNA) induced by homologous mRNA and blocking the corresponding gene expression, thus leading to specific post-transcriptional gene silencing (PTGS) [14]. Since RNAi has high sequence specificity, we can effectively and specifically block gene expression and it can be used as a simple and effective tool to replace the genetic knockout. Therefore, it was considered as one of the most

important results of Science in 2001 [15-17]. Lentiviral vectors are capable of producing high-titer lentivirus with siRNA expression. It stably expresses siRNA in periodic and aperiodic cells, stem cells, fertilized ovum and differentiated progeny cells to achieve specific and stable gene silencing, which provides better tools for the study of gene functions and gene therapy [18-21]. All of the advantages of lentiviral vectors make them one of the best tools for investigating transgenesis and RNAi.

In this study, We will first import specific expression of siRNA gene fragment into drug-resistant cell line (HepG2/ADM, SMMC7721/ADM) models by lentiviral transfection techniques to downregulate or close MRP1, MRP2, MRP3 and MRP4 genes in multidrug-resistant hepatocellular carcinoma cell strains. Then we increase the intracellular drug concentration to promote the sensitivity of liver cancer cells to chemotherapy and improve the efficacy of chemotherapy for hepatocellular carcinoma.

MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy

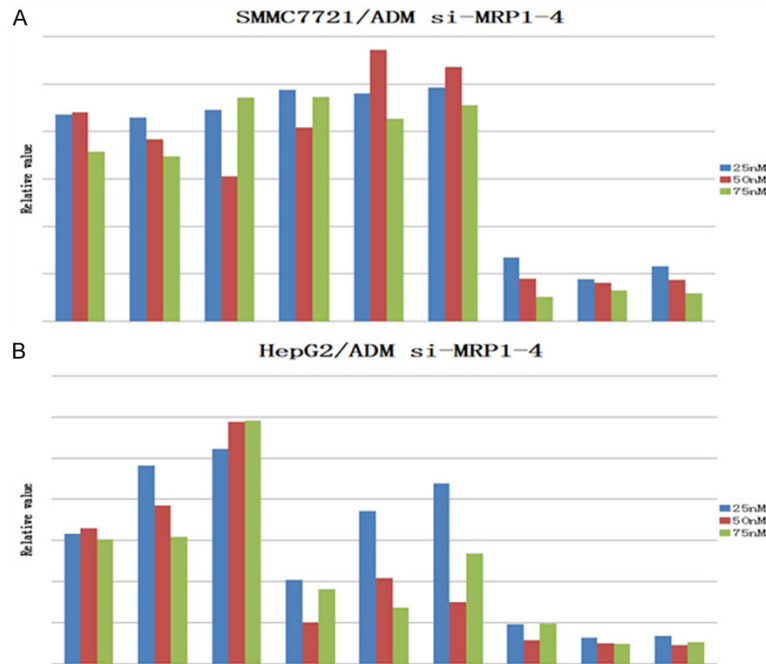


Figure 1. mRNA expression after siRNA treatment in MDR cells. mRNA levels were measured by QRT-PCR. Results were normalized by 18 sRNA mRNA expression and compared with the levels in parental cells ($n = 3$). Statistical analyses comparing MDR cells with parental cells were performed using Student's t-test. $P < 0.05$ vs parental cells (data not shown). $\Delta Ct = \text{gene Ct value} - \text{normalized gene Ct value}$; $\Delta\Delta Ct = \text{MRP1-4 genes } \Delta Ct = \text{value} - \text{normalized gene } \Delta Ct$ value; when the PCR efficacy is approaching 100%, relative value = $2^{-\Delta\Delta Ct}$.

Material and methods

Cell culture

Human HCC cell lines, HepG2 and SMMC7721, were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. HepG2 and SMMC7721 cells were cultured in DMEM medium containing 10% FBS, humidified incubator under 37°C, 5% CO₂. Cells were added with different gradients concentration of doxorubicin (ADM) (0.01~2.0 µg/ml), each successive gradient sliding 0.1 µg/ml. The cells grown in the ADM with concentration stabilized at 2.0 µg/ml were named as HepG2/ADM and SMMC7721/ADM cells.

SiRNA preparation and transfection

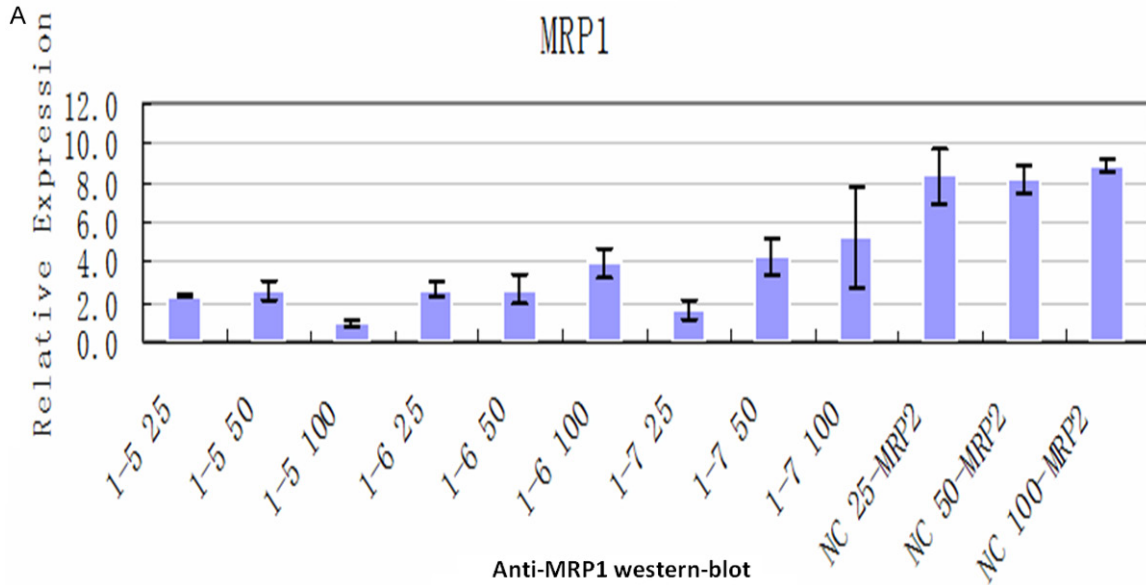
SiRNA was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Target sequences were: GCUGGUAGCCCUAGUGUGU for MRP1, CCACCCUGCUGAUACAGUA for MRP2, GGUCAAGUGUUCUACAGAU for MRP3, and UC-

UGAAAGCUCCGGUAUUA for MRP4. Approximately 5×10^4 cells per well were seeded in a six-well plate 24 h prior to transfection. Cells were transfected with 25, 50 or 75 nM siRNA using Lipofectin2000 reagent (Invitrogen Corp., Madison, Carlsbad, CA.) following the manufacturer's protocol. Cells were then incubated at 37°C in the presence of 5% CO₂ for 24 h and the culture medium was replaced 48 h after transfection before cells were ready for assay of gene knockdown.

RNA extraction and quantitative real-time polymerase chain reactions (QRT-PCR)

The cell lines were seeded in six-well plates and cultured for 24 h before the cells were collected. Total cellular RNA was extracted from the four groups by application of Trizol. 2 µg RNA was taken to synthesis reverse transcription cDNA. The 18S RNA primers were used as an internal reference. The standard curve was drawn and the relative concentrations of MRP1, MRP2, MRP3, MRP4, and 18S RNA genes were got according to the standard curve plotted. DNASIS software was used for homology analysis of the sequencing results. CLUSTALW software was used on the arrangement of sequencing. MEGA software was used for the analysis of phylogenetic tree. Each group was repeated for 3 times, and the average value was got. Primer sequences are as follows: MRP1 forward, 5'-CTTCGCTGAGTTCCTGCGTA-3', and reverse, 5'-GCTGAGCTGTCTCTGCAGTT-3'; MRP2 forward 5'-GAGCACCAGCAGCGATTCT-3', and reverse, 5'-AGCCAACAGTGTCCCACTT-3'; MRP3 forward, 5'-ATCCTGGCGATCTACTTCT-3', and reverse, 5'-TACAGCTTCAGCACCTTGAT-3'; MRP4 forward, 5'-CCTTCTCAGAGTCTTCGGTT-3', and reverse, 5'-ACCTGAGCTGCAGTGTTAGG-3'. QRT-PCR was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, at 60°C for 30 s, and at 72°C for 30 s. Data were analyzed using the Sequence detector

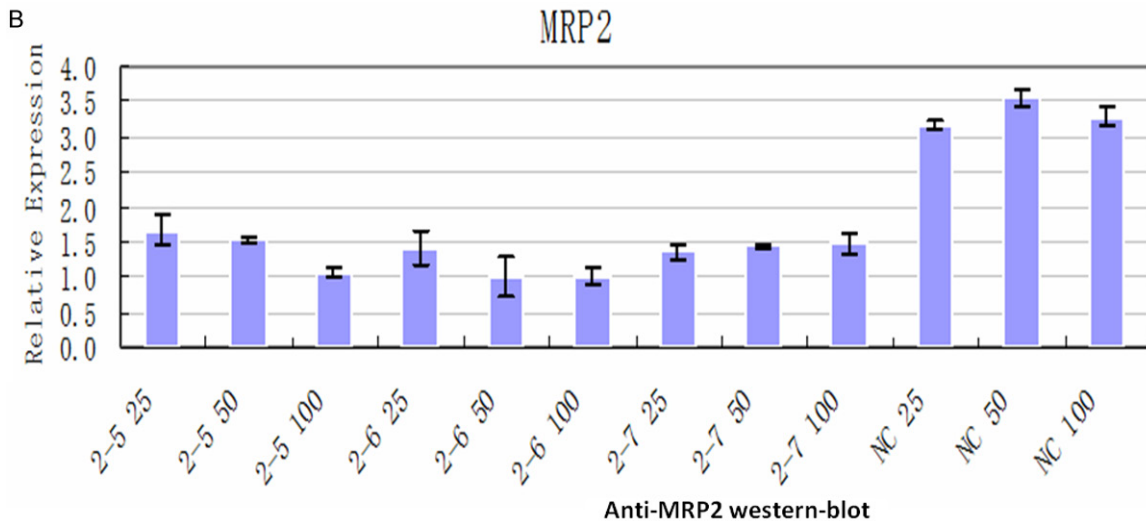
MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy



Data analysis of anti-MRP1 western blot

Sample	Ct-1	Ct-2	Ct-3	Ct-mean	Δ Ct	$\Delta\Delta$ Ct	$2^{-\Delta\Delta$ Ct}	Inhibition efficiency
1-5 25-MRP1	27.08	27.05	27.09	27.07±0.02	17.97±0.02	-1.23±0.02	2.34±0.03	72.01%
1-5 50-MRP1	26.58	26.67	27.07	26.78±0.26	17.82±0.27	-1.38±0.27	2.6±0.46	68.30%
1-5 100-MRP1	28.35	28.20	28.61	28.39±0.21	19.2±0.21	0±0.21	1±0.14	88.78%
1-6 25-MRP1	26.91	26.58	26.99	26.83±0.22	17.78±0.21	-1.42±0.21	2.67±0.41	68.09%
1-6 50-MRP1	27.15	26.73	26.46	26.78±0.35	17.81±0.38	-1.39±0.38	2.62±0.71	68.06%
1-6 100-MRP1	26.45	26.12	25.96	26.18±0.25	17.2±0.28	-2±0.28	4±0.77	54.98%
1-7 25-MRP1	28.25	27.39	27.35	27.66±0.51	18.51±0.54	-0.69±0.54	1.62±0.56	80.68%
1-7 50-MRP1	26.27	26.24	25.84	26.12±0.24	17.08±0.27	-2.12±0.27	4.34±0.87	47.13%
1-7 100-MRP1	26.66	25.79	25.50	25.98±0.6	16.79±0.7	-2.41±0.7	5.31±2.51	40.17%
NC 25-MRP1	24.95	25.25	25.39	25.2±0.23	16.14±0.22	-3.06±0.22	8.36±1.34	--
NC 50-MRP1	25.11	25.35	25.31	25.26±0.13	16.16±0.13	-3.04±0.13	8.2±0.78	--
NC 100-MRP1	25.08	25.09	25.16	25.11±0.04	16.05±0.05	-3.15±0.05	8.88±0.28	--

Δ Ct = Mean (Ct of Target Gene-Ct of Reference Gene) ±SD, $\Delta\Delta$ Ct = Mean (Δ Ct of Target Gene in Sample need to be test- Δ Ct of Target Gene in Reference Sample) ±SD (Select the biggest Δ Ct as the reference to calculate when there is no reference sample), initial template amount of relative sample = Mean of ($2^{\Delta\Delta$ Ct}) ±SD, Inhibition efficiency = (NC $2^{\Delta\Delta$ Ct}-siRNA $2^{\Delta\Delta$ Ct})/NC $2^{\Delta\Delta$ Ct *100%.



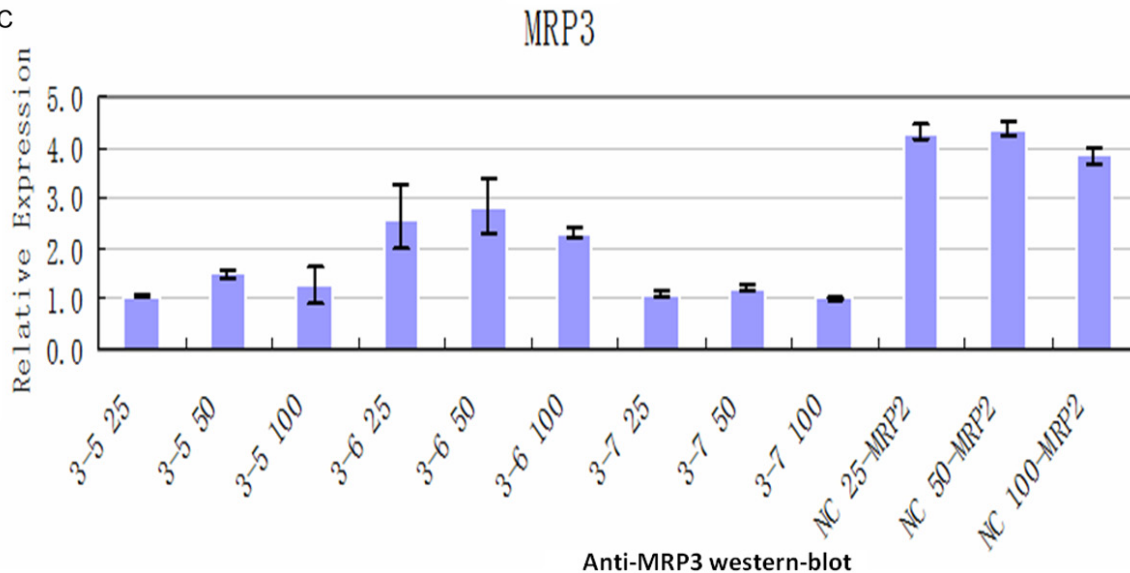
MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy

Data analysis of anti-MRP2 western blot

Sample	Ct-1	Ct-2	Ct-3	Ct-mean	Δ Ct	$\Delta\Delta$ Ct	$2^{-\Delta\Delta$ Ct}	Inhibition efficiency
2-5 25-MRP2	24.02	23.74	23.71	23.83±0.17	14.97±0.19	-0.74±0.19	1.67±0.21	47.19%
2-5 50-MRP2	24.05	23.99	24.04	24.02±0.03	15.09±0.04	-0.62±0.04	1.54±0.05	56.74%
2-5 100-MRP2	24.80	24.73	24.70	24.75±0.05	15.62±0.07	-0.09±0.07	1.07±0.05	67.52%
2-6 25-MRP2	24.12	24.58	24.29	24.33±0.23	15.21±0.25	-0.5±0.25	1.41±0.23	55.43%
2-6 50-MRP2	24.95	24.48	24.15	24.53±0.4	15.71±0.42	0±0.42	1±0.29	71.82%
2-6 100-MRP2	24.53	24.88	24.87	24.76±0.2	15.7±0.15	-0.01±0.15	1.01±0.11	69.26%
2-7 25-MRP2	24.25	24.10	24.20	24.18±0.07	15.26±0.1	-0.45±0.1	1.37±0.1	56.87%
2-7 50-MRP2	24.37	24.34	24.39	24.37±0.03	15.19±0.03	-0.52±0.03	1.44±0.03	59.57%
2-7 100-MRP2	24.31	24.23	24.35	24.3±0.06	15.14±0.13	-0.57±0.13	1.48±0.14	54.81%
NC 25-MRP2	23.20	23.20	23.22	23.21±0.01	14.05±0.02	-1.66±0.02	3.17±0.04	--
NC 50-MRP2	23.01	23.06	23.11	23.06±0.05	13.88±0.05	-1.83±0.05	3.55±0.12	--
NC 100-MRP2	23.10	23.15	23.17	23.14±0.03	13.99±0.06	-1.72±0.06	3.28±0.14	--

Δ Ct = Mean (Ct of Target Gene-Ct of Reference Gene) ±SD, $\Delta\Delta$ Ct = Mean (Δ Ct of Target Gene in Sample need to be test- Δ Ct of Target Gene in Reference Sample) ±SD (Select the biggest Δ Ct as the reference to calculate when there is no reference sample), initial templet amount of relative sample = Mean of ($2^{-\Delta\Delta$ Ct}) ±SD, Inhibition efficiency = (NC $2^{-\Delta\Delta$ Ct-siRNA $2^{-\Delta\Delta$ Ct})/NC $2^{-\Delta\Delta$ Ct} * 100%.

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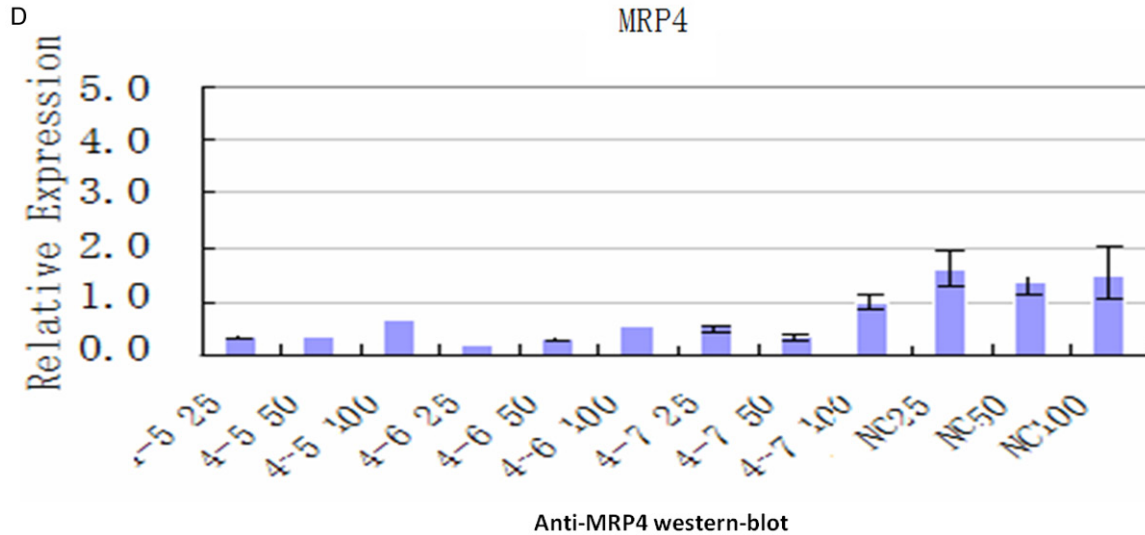


Data analysis of anti-MRP3 western blot

Sample	Ct-1	Ct-2	Ct-3	Ct-mean	Δ Ct	$\Delta\Delta$ Ct	$2^{-\Delta\Delta$ Ct}	Inhibition efficiency
3-5 25-MRP3	25.05	25.12	25.13	25.1±0.04	16.11±0.04	-0.06±0.04	1.05±0.03	75.69%
3-5 50-MRP3	24.52	24.62	24.46	24.53±0.08	15.61±0.1	-0.56±0.1	1.48±0.1	66.06%
3-5 100-MRP3	24.34	24.85	24.94	24.71±0.32	15.8±0.37	-0.37±0.37	1.29±0.36	66.47%
3-6 25-MRP3	24.30	23.65	24.10	24.02±0.33	14.78±0.32	-1.39±0.32	2.62±0.62	39.12%
3-6 50-MRP3	26.23	26.11	25.50	25.95±0.39	14.66±0.27	-1.51±0.27	2.85±0.53	34.70%
3-6 100-MRP3	24.01	24.04	24.01	24.02±0.02	14.96±0.04	-1.21±0.04	2.32±0.06	39.72%
3-7 25-MRP3	25.22	25.38	25.26	25.29±0.08	16.06±0.08	-0.11±0.08	1.08±0.06	74.98%
3-7 50-MRP3	25.06	25.04	24.97	25.02±0.05	15.89±0.05	-0.28±0.05	1.22±0.04	72.11%
3-7 100-MRP3	25.30	25.48	25.61	25.46±0.16	16.17±0.07	0±0.07	1±0.05	73.95%
NC 25-MRP3	23.23	23.31	23.22	23.25±0.05	14.06±0.06	-2.11±0.06	4.3±0.16	--
NC 50-MRP2	23.01	23.06	23.11	23.06±0.05	13.88±0.05	-1.83±0.05	3.55±0.12	--
NC 100-MRP2	23.10	23.15	23.17	23.14±0.03	13.99±0.06	-1.72±0.06	3.28±0.14	--

Δ Ct = Mean (Ct of Target Gene-Ct of Reference Gene) ±SD, $\Delta\Delta$ Ct = Mean (Δ Ct of Target Gene in Sample need to be test- Δ Ct of Target Gene in Reference Sample) ±SD (Select the biggest Δ Ct as the reference to calculate when there is no reference sample), initial templet amount of relative sample = Mean of ($2^{-\Delta\Delta$ Ct}) ±SD, Inhibition efficiency = (NC $2^{-\Delta\Delta$ Ct-siRNA $2^{-\Delta\Delta$ Ct})/NC $2^{-\Delta\Delta$ Ct} * 100%.

MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy



Data analysis of anti-MRP4 western blot

Sample	Ct-1	Ct-2	Ct-3	Ct-mean	Δ Ct	$\Delta\Delta$ Ct	$2^{-\Delta\Delta$ Ct}	Inhibition efficiency
4-5 25-MRP5	30.02	30.04	30.10	30.05±0.04	14.37±0.04	-2.04±0.04	4.12±0.13	-2.46%
4-5 50-MRP5	32.10	32.10	32.16	32.12±0.04	13.61±0.01	-2.8±0.01	6.97±0.02	-100.52%
4-5 100-MRP5	31.11	31.65	31.18	31.32±0.29	13.2±0.13	-3.21±0.13	9.27±0.89	-127.44%
4-6 25-MRP5	30.82	30.97	31.23	31.01±0.21	13.59±0.19	-2.82±0.19	7.05±0.91	-75.52%
4-6 50-MRP5	29.56	29.73	29.47	29.59±0.13	13.12±0.11	-3.29±0.11	9.76±0.76	-180.79%
4-6 100-MRP5	30.10	32.01	30.34	30.82±0.17	13.15±0.17	-3.26±0.17	9.59±1.16	-135.28%
4-7 25-MRP5	28.83	29.01	28.58	28.81±0.22	14.09±0.2	-2.32±0.2	4.98±0.7	-24.01%
4-7 50-MRP5	29.91	29.80	29.93	29.88±0.07	13.92±0.08	-2.49±0.08	5.62±0.33	-61.85%
4-7 100-MRP5	31.03	30.61	30.93	30.86±0.22	13.71±0.19	-2.7±0.19	6.51±0.88	-59.80%
NC 25-MRP5	28.30	28.03	28.35	28.23±0.17	14.4±0.19	-2.01±0.19	4.02±0.56	--
NC 50-MRP5	29.20	29.43	29.23	29.29±0.13	14.61±0.12	-1.8±0.12	3.47±0.29	--
NC 100-MRP5	29.20	29.02	29.12	29.12±0.09	14.38±0.12	-2.03±0.12	4.07±0.34	--

Δ Ct = Mean (Ct of Target Gene-Ct of Reference Gene) \pm SD, $\Delta\Delta$ Ct = Mean (Δ Ct of Target Gene in Sample need to be tes- Δ Ct of Target Gene in Reference Sample) \pm SD (Select the biggest Δ Ct as the reference to calculate when there is no reference sample), initial templet amount of relative sample = Mean of ($2^{\Delta\Delta$ Ct}) \pm SD, Inhibition efficiency = (NC $2^{-\Delta\Delta$ Ct-siRNA $2^{-\Delta\Delta$ Ct})/NC $2^{-\Delta\Delta$ Ct} *100%.

Figure 2. Expression of MRP1-4 in MDR cells.

software (v1.9, Applied Biosystems). The mean Ct value for duplicate measurements was used to detect the expression of target gene with normalization to a housekeeping gene used as an internal control (18S rRNA) according to the $2^{-\Delta$ Ct formula.

MTT assay

The cell lines were inoculated in 96-well plates. Doxorubicin, 5-FU, vincristine or oxaliplatin with different concentration gradients were added respectively after 24 hours. The gradients concentration of doxorubicin were 0.8, 1.6, 3.2, 6.4 and 12.8 mg/L, 5-FU were 0.08, 0.40, 2.00, 10.00 and 50.00 mg/L, vincristine were

0.25, 0.5, 1.0, 2.0 and 4.0 mg/L, oxaliplatin were 5, 10, 20, 40 and 80 mg/L. Each concentration of the group was repeated for three times. Meanwhile, the set of wells without drugs were used as the blank group. Each group were detected by MTT at 12, 24, 36, 48 and 60 h. Poms software was applied to calculate half inhibitory concentration of each group (IC50) and resistance index was calculated (RI) = IC50 of resistance medicines cells/IC50 of blank cells.

Western blot analysis

Protein was collected from cultured HepG2, SMMC7721, HepG2/ADM and SMMC7721/

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Table 2. Determination of IC50 and resistance index of different anticancer drugs (mean ± SD)

	IC50		RI	IC50		RI
	HepG2	HepG2/ADM		HepG2	HepG2/ADM/MRP1 siRNA	
Adriamycin (mg/L)	0.0187±0.004	0.2754±0.010	14.73	0.0187±0.004	0.0596±0.006	3.19
Fluorouracil (mg/L)	0.1319±0.002	3.5388±0.016	26.83	0.1319±0.002	2.0103±0.016	15.24
Vincristine (mg/L)	0.0057±0.007	0.1821±0.019	31.95	0.0057±0.007	0.0236±0.003	4.14
Oxaliplatin (mg/L)	0.0088±0.003	0.1956±0.008	22.23	0.0088±0.003	0.0128±0.002	1.46

	IC50		RI	IC50		RI
	HepG2	HepG2/ADM		HepG2	HepG2/ADM/MRP2 siRNA	
Adriamycin (mg/L)	0.0187±0.004	0.2754±0.010	14.73	0.0187±0.004	0.1832±0.007	9.80
Fluorouracil (mg/L)	0.1319±0.002	3.5388±0.016	26.83	0.1319±0.002	1.7210±0.019	13.04
Vincristine (mg/L)	0.0057±0.007	0.1821±0.019	31.95	0.0057±0.007	0.0307±0.005	5.39
Oxaliplatin (mg/L)	0.0088±0.003	0.1956±0.008	22.23	0.0088±0.003	0.0236±0.008	2.68

	IC50		RI	IC50		RI
	HepG2	HepG2/ADM		HepG2	HepG2/ADM/MRP3 siRNA	
Adriamycin (mg/L)	0.0187±0.004	0.2754±0.010	14.73	0.0187±0.004	0.0724±0.010	3.87
Fluorouracil (mg/L)	0.1319±0.002	3.5388±0.016	26.83	0.1319±0.002	1.2260±0.013	9.29
Vincristine (mg/L)	0.0057±0.007	0.1821±0.019	31.95	0.0057±0.007	0.0354±0.009	6.21
Oxaliplatin (mg/L)	0.0088±0.003	0.1956±0.008	22.23	0.0088±0.003	0.0291±0.006	3.31

	IC50		RI	IC50		RI
	HepG2	HepG2/ADM		HepG2	HepG2/ADM/MRP4 siRNA	
Adriamycin (mg/L)	0.0187±0.004	0.2754±0.010	14.73	0.0187±0.004	0.1106±0.013	5.91
Fluorouracil (mg/L)	0.1319±0.002	3.5388±0.016	26.83	0.1319±0.002	2.4238±0.022	18.38
Vincristine (mg/L)	0.0057±0.007	0.1821±0.019	31.95	0.0057±0.007	0.0621±0.004	10.89
Oxaliplatin (mg/L)	0.0088±0.003	0.1956±0.008	22.23	0.0088±0.003	0.0349±0.005	3.97

ADM cells and the concentration was measured (protein assay dye, Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Electrochemiluminescence (ECL) method was used to show protein bands, and gray Quantity One analysis software was applied for semi-quantitative analysis. Each group was repeated 3 times to get the mean value.

Flow cytometric analysis of cell apoptosis

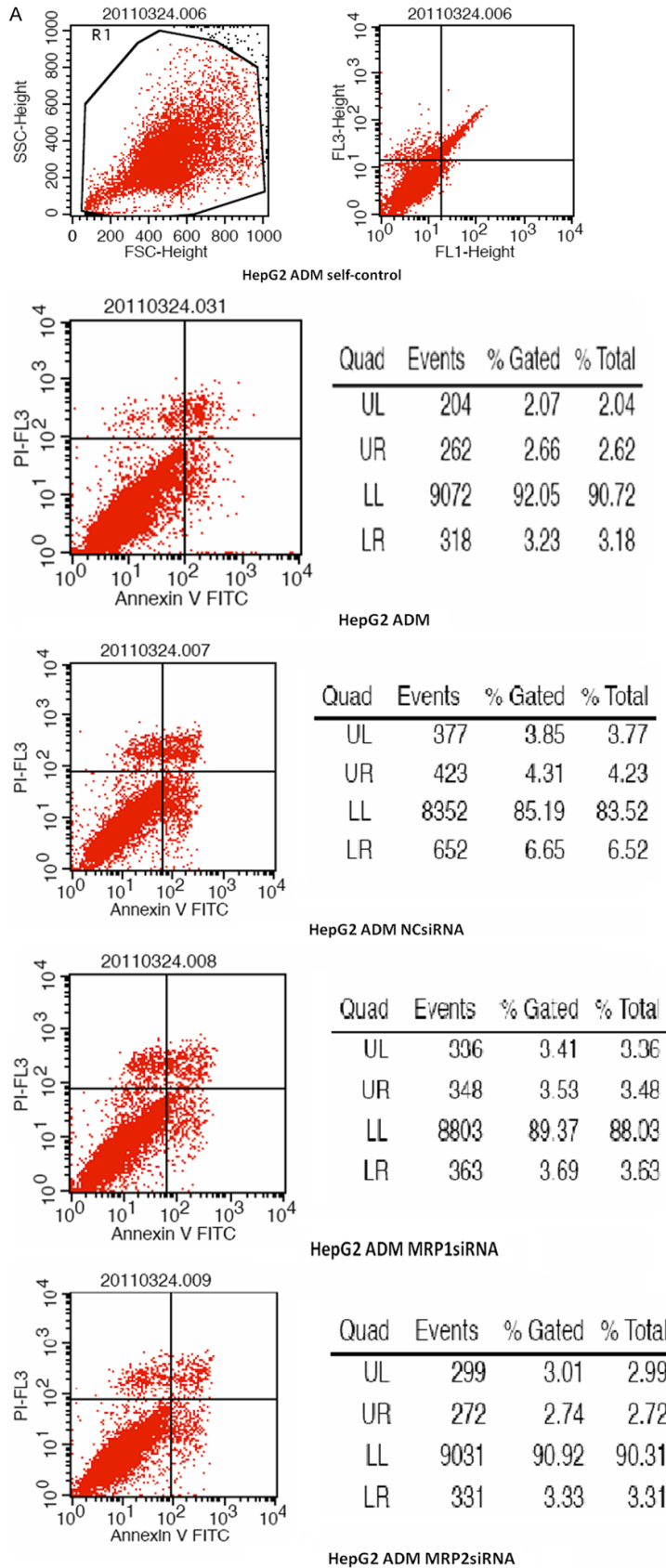
The cell lines of HepG2/ADM and SMMC7721/ADM were treated with siRNA or NS, and then were collected respectively through trypsinization, washed with ice-cold PBS, centrifuged at 2000 g for 5 min at 4°C, and washed twice with ice-cold PBS. Samples were rehydrated with PBS and then incubated with propidium iodide and Annexin V-FITC for 15 min at room temperature. More cell apoptosis showed the effect of

siRNA silencing resistant gene was remarkable, and vice versa showed resistance gene silencing was unsuccessful.

Animal experiment

Five-week-old male BALB/c mice were purchased from SHANGHAI SLAC LABORATORY ANIMAL CO. LTD. All nude mice were fed according to Chinese animal guidelines. In tumor formation experiments, siRNA was transfected with target gene fragment MRP1-4 in SMMC-7721/ADM. At 24 h after transfection, the tumor cells were resuspended in 100 µL PBS and was then injected into the right shoulder area of mice. Tumor growth was detected, and the long diameter and the wide diameter of the tumor after cell inoculation were measured. Tumor volume is calculated according to the following formula: volume (mm³) = width² (mm²) × length (mm)/2.

MRP1-4 genes enhances sensitivity of hepatoma cells to chemotherapy



Statistical analysis

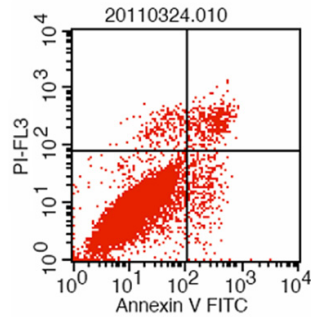
Statistical data were analyzed using the SPSS 18.0 software (SPSS, Chicago, IL, USA). The rank sum test was used to examine the differential expression of the four multidrug resistance proteins in hepatoma cells. The results were analyzed using Student's t test if two groups were compared and the Dunnett's test if multiple groups were compared. If variances were inhomogeneous in the Student's t test, the results were analyzed using the Welsh test. All data in this study were expressed as mean \pm SEM. Results were considered statistically significant when $P < 0.05$.

Results

HepG2 and SMMC7721 resistant cell lines construction

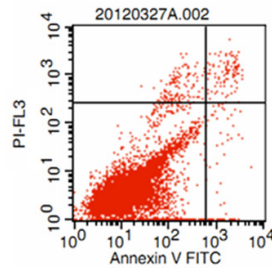
In our study, each step of developing HepG2/ADM and SMMC7721/ADM cell lines took 10 months by gradually increasing concentrations of doxorubicin (ADM) in the DM-EM medium. Each successive increasing 0.1 $\mu\text{g/ml}$ concentration gradient from 0.01 to 2.0 $\mu\text{g/ml}$, and the cells induced in the concentration 2.0 $\mu\text{g/ml}$ of ADM were called HepG2/ADM cells. When MTT assay was performed, we found that these cells were resistant not only to Adriamycin but also to multiple anticancer drugs including 5-FU, Vincristine, and Oxaliplatin. The lethal dose (IC50) and RI were significantly higher in HepG2/ADM and SMMC7721/ADM cells than in non-resistant parental cells (**Table 1**). MRP1-4 mRNA levels showed eleva-

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Quad	Events	% Gated	% Total
UL	181	1.83	1.81
UR	378	3.82	3.78
LL	9029	91.20	90.29
LR	312	3.15	3.12

HepG2 ADM MRP3siRNA



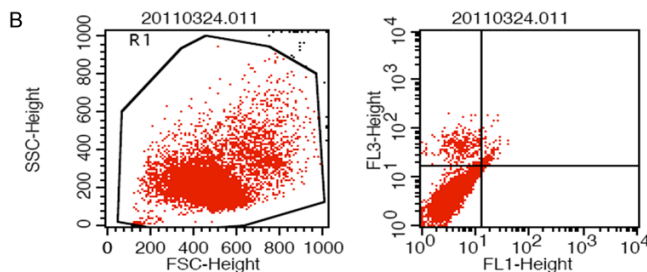
Quad	Events	% Gated	% Total
UL	60	0.62	0.60
UR	95	0.98	0.95
LL	9480	97.80	94.80
LR	58	0.60	0.58

HepG2 ADM MRP4siRNA

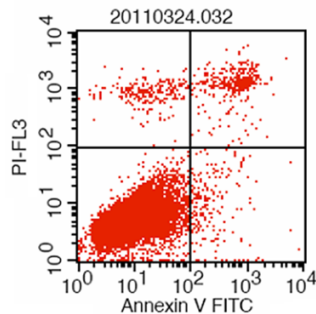
Data analysis

Cell	Treatment	UL	UR	LL	LR	UR+LR
HepG2 ADM	Normal	2.07	2.66	92.05	3.23	5.89
HepG2 ADM	NCsiRNA	3.85	4.31	85.19	6.65	10.96
HepG2 ADM	MRP1siRNA	3.41	3.53	89.37	3.69	7.22
HepG2 ADM	MRP2siRNA	3.01	2.74	90.92	3.33	6.07
HepG2 ADM	MRP3siRNA	1.83	3.82	91.2	3.15	6.97
HepG2 ADM	MRP4siRNA	1.14	0.98	97.17	1.71	1.69
		Late	Normal	Early	Apoptosis	

Compared with HepG2 ADM, After transfection of MRP1siRNA, MRP2siRNA, MRP3siRNA and MRP4siRNA, the cell apoptosis has significantly increased.



SMMC7721 ADM self-control



SMMC7721 ADM

Quad	Events	% Gated	% Total
UL	232	2.33	2.32
UR	400	4.02	4.00
LL	9105	91.46	91.05
LR	218	2.19	2.18

tion in HepG2/ADM and SMMC7721/ADM cell lines tested by QRT-PCR assay.

Silencing effects of siRNA on RNA transcription

QRT-PCR was performed to detect the alteration in mRNA levels of MRP1-4 genes after the cells were treated with siRNA. mRNA level increased significantly when multi-drug resistance was developed. However, when HepG2/ADM and SMMC7721/ADM cell lines were treated with siRNA, the levels of mRNA decreased to almost the same level as non-resistant cells. Quantities of RNA in each lane were normalized by 18S RNA expression (**Figure 1**).

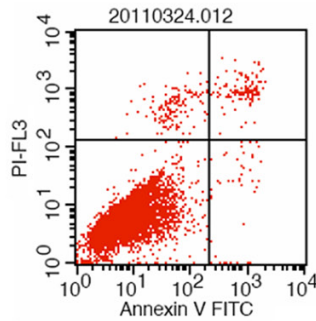
MRP1-4 protein expression after siRNA transfection

Western blot analysis was performed to assess the effects of siRNA on protein expression. Higher levels of MRP1-4 expression were detected in HepG2/ADM and SMMC7721/ADM cells. However, when these cells were treated with siRNA, the protein levels significantly decreased (**Figure 2**).

siRNA sensitizes ADM cell lines to chemotherapy drugs

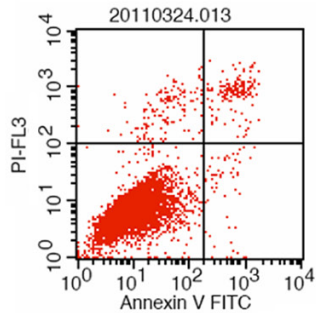
To assess whether siRNA-directed MRP1-4 suppression sensitizes MDR cancer cells to cytotoxic agents, we compared the drug sensitivity of the siRNA-treated to that of the mock-treated MDR cells using MTT assay. As shown in **Table 2**, the sensitivity of the MDR cells to doxorubicin, 5-fluorouracil, vinblastine and Oxaliplatin increased signifi-

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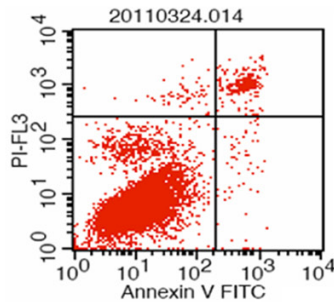
Quad	Events	% Gated	% Total
UL	138	1.39	1.38
UR	125	1.26	1.25
LL	9616	96.91	96.16
LR	44	0.44	0.44

SMMC7721 ADMNCsiRNA



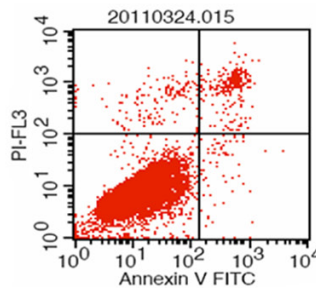
Quad	Events	% Gated	% Total
UL	139	1.40	1.39
UR	167	1.68	1.67
LL	9570	96.32	95.70
LR	60	0.60	0.60

SMMC7721 ADM-MRP1siRNA



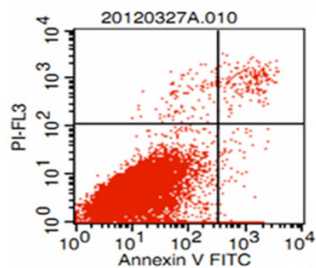
Quad	Events	% Gated	% Total
UL	60	0.61	0.60
UR	217	2.19	2.17
LL	9574	96.61	95.74
LR	59	0.60	0.59

SMMC7721 ADM-MRP2siRNA



Quad	Events	% Gated	% Total
UL	145	1.47	1.45
UR	266	2.69	2.66
LL	9421	95.19	94.21
LR	65	0.66	0.65

SMMC7721 ADM-MRP3siRNA



Quad	Events	% Gated	% Total
UL	105	1.13	1.05
UR	175	1.88	1.75
LL	8895	95.61	88.95
LR	128	1.38	1.28

SMMC7721 ADM-MRP4siRNA

cantly after the introduction of targeted siRNA ($p < 0.05$).

MRP1-4 silencing induces apoptosis

To explore whether MRP1-4 silencing could induce apoptosis, we analyzed apoptosis in ADM cells by flow cytometry. The average rates of apoptosis in SMMC7721/ADM and HepG2/ADM with siRNA were 8.50% and 8.11%, respectively (**Figure 3**). These figures were significantly higher than those with NS (0.14% and 0.11%, $p < 0.05$), indicating that MRP1-4 expression could effectively protect SMMC7721/ADM and HepG2/ADM cells from apoptosis.

MRP1-4 silencing suppresses tumor growth in vivo

After inoculated to mice for 14 days, the tumor was enucleated to calculate tumor growth. Compared with SMMC-7721/ADM, tumor growth significantly reduced after silencing MRP1-4 gene. The results suggested that silencing MRP1-4 gene in vivo could inhibit tumor growth (**Figure 4**).

Discussion

Human hepatocellular carcinoma is one of the most common cancers in males and females over the world. However, no chemotherapeutic agents have been found to provide a clinically effective treatment [1]. Studies showed that MRPs expressed in HCC may confer HCC resistance to chemotherapeutic drugs. Some reports suggested that MRPs are better candidates to mediate chemo-

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Data analysis

Cell	Treatment	UL	UR	LL	LR	UR+LR
SMMC7721 ADM	Normal	2.33	4.02	91.46	2.19	6.21
SMMC7721 ADM	NCsiRNA	1.39	1.26	96.91	0.44	1.70
SMMC7721 ADM	MRP1siRNA	1.40	1.68	96.32	0.6	2.28
SMMC7721 ADM	MRP2siRNA	0.61	2.19	96.61	0.6	2.79
SMMC7721 ADM	MRP3siRNA	1.47	2.69	95.19	0.66	3.35
SMMC7721 ADM	MRP4siRNA	1.13	1.88	95.61	1.38	3.26

Compared with SMMC7721 ADM, After transfection of MRP1siRNA, MRP2siRNA, MRP3siRNA and MRP4siRNA, the cell apoptosis has significantly increased.

Figure 3. Apoptosis of MDR cells with or without siRNA.

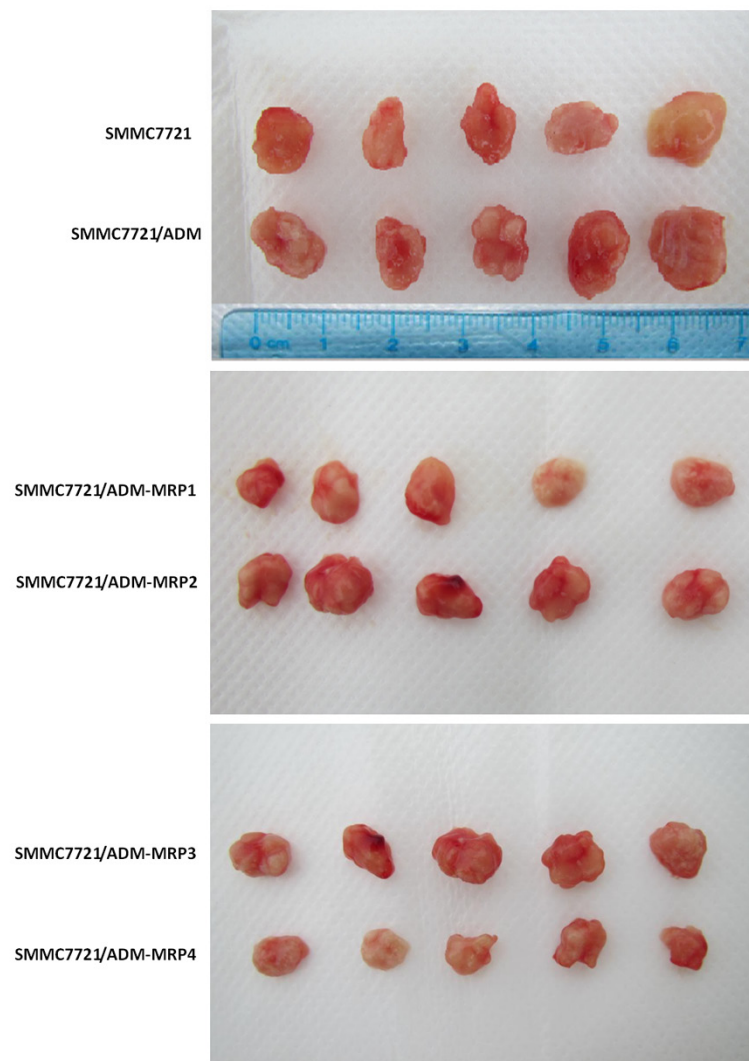


Figure 4. Animal experiment Tumor cells were injected to mice (four per group) on the right side scapular area after resuspended with 100 μ L PBS, detect the growth of the tumor cells after inoculating for 14 days and measure the major axis diameter and width. Tumor volume was calculated according to the following formula: volume (mm^3) = width² (mm^2) \times length (mm)/2.

resistance in HCC than MDR1. However, since their expression in mature hepatocytes is negligible, some researchers have ruled out the involvement of MRPs in the MDR phenotype of HCC [22-24]. However, other researchers demonstrated that MRPs expression is at significant levels in HCCs and suggested that MRPs expression is closely related to MDR in HCC [25-27]. MRPs play a major role in chemotherapy failure. In this study, we screened a variety of anticancer drugs or cytotoxic agents, including natural products to detect the impact of MRPs on the multi-drug resistance phenotype, using two human hepatocellular carcinoma-derived cell lines as an in vitro model.

As shown in **Table 1D**, the IC₅₀ of anticancer drugs to the MDR cell subclone was much higher than that to parental cells, suggesting that the acquired MDR of HepG2/ADM and SMMC7721/ADM was reliable. We also found clear differences by western blot between MDR cells and the parental cell lines. Higher levels of MRP1-4 expression were detected in MDR cell lines, indicating that MRP1-4 over-expression certainly contributed to the MDR cells [8].

Sara Vander B [28] et al declared a diffuse protein expression of MRP1-4 compared with negative hepatocytic expression observed in normal (surrounding) hepatocytes. In addition, MRP1-4 expression was high in poorly differentiated HCCs, large tumors (> 7 cm) and microvascular invasive tumors. These results corresponded to our

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findings that plenty of MRP1-4 mRNA and protein expressed in MDR cells. It may also explain the confusion that some reports deemed high P-gp expression was seen along with low MRP1-4 expression in HCC cell lines. We could detect high levels of MRP1-4 mRNA and protein expression. For the MDR cell models (as we established) tolerated the stress of anti-cancer drug for ages, it was more closed to advanced HCCs.

Since Elbashir et al. [29] have reported that RNA interference could be triggered in mammalian cells by introduction of 21-nucleotide siRNA, siRNA has been shown to be an effective approach for silencing gene expression and has been applied to inhibit oncogene in hepatoma. We now demonstrate that introduction of siRNA duplex decreased MRP1-4 expression (**Figure 1**), induced SMMC7721/ADM and HepG2/ADM cells apoptosis to drugs (**Figure 3**), and restored drug sensitivity (**Table 1D**) in human MDR cancer cells. We also found that the modulation of MDR results from the siRNA-directed degradation of MRP1-4 mRNA (**Figure 1**).

This report demonstrated the feasibility of using siRNA to specifically and effectively modulate MDR. MRP-targeted siRNA inhibits the expression of MRP1-4 RNA and MRP1-4 protein with minimum effect on 18s RNA and β -actin expression in comparison with mock treatment (**Figures 1, 2**); GAPD siRNA decreased GAPD expression but had no effect on the expression of MRP1-4 (**Figure 1**). These data suggested that silencing of MRP1-4 expression mediated by siRNA is specific.

Furthermore, SMMC7721/ADM and HepG2/ADM cell lines were selected by prolonged exposure to doxorubicin, while additional mechanisms of drug resistance are known to exist. The drug resistance of these cells was significantly restored after inhibition of MRP1-4, indicating that over-expression of MRP1-4 also contributes to MDR of HepG2/ADM and SMMC7721/ADM cells. In animal experiment, we detected tumor growth and enucleated the tumor from the mice after inoculating for 14 days. Compared with SMMC-7721/ADM, tumor growth significantly reduced after silencing MRP1-4 gene. These results suggested that silencing MRP1-4 gene in vivo experiments can inhibit tumor growth (**Figure 4**).

In summary, in this study, we successfully established SMMC-7721/ADM and HepG2/ADM multidrug-resistant HCC cell subclones. We demonstrated that the MDR cells were associated with the over-expression of MRP1-4. Our study revealed the effect of silencing MRP1-4 gene in reversing MDR. Therefore, the introduction of MRP1-4 siRNA may hold promise for the treatment of drug-resistant cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Jianping Liu, Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No. 17, Yanjiang West Road, Guangzhou 510120, China. Tel: +86-02081332020; Fax: +8602081332020; E-mail: jianpingliu@163.com

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