Reversal of Adriamycin Resistance of Hepatocellular Carcinoma by Targeting with Recombined Adenovirus Carring Antisense mdr1 RNA

Xiong Ding

Ying Mei

Yujun Shi

Jianping Gong

Xuhong Li

Yong Peng

Yong Liu

Chang'an Liu

Department of Hepatobiliary Surgery, the Second Affiliated Hospital, Chongqing Medical University. Chongqing 400010, China.

Correspondence to: Chang'an Liu
Tel: 86-23-6378-4175
Fax: 86- 23-6371-1527
E-mail: dxiong@ yeah.net.

OBJECTIVE Chemotherapy is an important therapy for hepatocellular carcinoma (HCC). However, it is not effective in many cases due to recurrence and metastasis even if the initial treatment produces a response. Multidrug resistance (MDR) is considered to be one of the considerable causes. The aim of this study was to reverse MDR of HepG2/ADM cells by blocking mdr1 with an adenovirus vector carrying antisense mdr1 in a tumor transplantated in athymic mice.

METHODS $p_{CMV\ IE}$ was removed from the pshuttle vector. A 0.3 kb AFP promoter was inserted into the pshuttle vector and pCMV changed into pAFP. The pAFP and asmdr1 PCR products were doubly digested with Kpnl and Apal, the digested products were ligated by T4 ligase, the as-mdr1 gene was inserted into pAFP and a newly plasmid pAFP-asmdr1 was constructed. Following digestion with PI-SceI/I-Ceu I, pAFP-asmdr1 was ligated with Adeno–X genome DNA and amplified in E.coli XL1–Blue. The HEK293 cells were transfected and virus collected. The HepG$_2$ MDR cells (HepG$_2$/ADM) were induced by graded resistance to ADM and were inoculated into athymic mice. After adeno–asmdr1 was injected, the expression of mdr1–mRNA and the volume of the transplantated tumor and its cells were observed.

RESULTS Following injection with Adeno–asmdr1, the tumor volume in the ADM+Adeno–asmdr1 group did not increase. However the tumor volume in the PBS plus ADM group did significantly increase (P<0.05). In the tumor xenograft cells, mdr1 mRNA in the xenografts was assessed by RT–PCR and was found to be reduced at 1 week and 4 weeks in the ADM+asmdr1 group, but it was stable in the ADM group. It was only 20% in the ADM+asmdr1 group compared to the ADM group at the 4th week (P<0.05). Evidence of apoptosis was observed in the tumor xenograft cells treated with Adeno–asmdr1, but there was rare or no apoptosis in the group treated with ADM and PBS.

CONCLUSION Adenovirus carrying antisense mdr1 RNA can partially reverse the MDR of HepG$_2$/ADM cells and inhibit tumor growth by down-regulating mdr1 mRNA resulting in tumor cell apoptosis.

KEYWORDS: hepatocellular carcinoma, multidrug resistance, adenovirus, antisense technology, mdr1.

P rimary hepatic carcinoma (PHC) is one of the most common, highly malignant tumors throughout the entire world. Of these tumors 90% are hepatocellular carcinoma (HCC). Many methods of treatment such as surgery, chemotherapy, radiotherapy and interventional therapy have been employed, but often with poor success. [1,2] Besides surgery, although chemotherapy is an important therapy ,the results are not ideal as many patients have a recurrence and metastasis even though the initial chemotherapy appeared successful. One of the

—

main causes of poor results is that the tumors develop multidrug resistance (MDR).[3] In recent years, it has been shown that the expression product of the mdr1 gene, P-gp170, is related to MDR. So, it may be possible to prevent MDR if the tumor phenotype can be changed and thus increase sensitivity to chemotherapeutics. The aim of this study was to attempt to alter the MDR of HepG$_2$/ADM cells by blocking mdr1 with adenovirus vector carrying antisense mdr1 in a tumor transplantated in athymic mice.

MATERIALS AND METHODS

Animals and reagents

Eighteen male Balb/c athymic mice 4 weeks of age and weighting 20±22 g were purchased from the Chinese Academy of Science, Shanghai Center of Experimental Animals. EX *Taq* DNA polymerase, T$_4$ ligase, and a DNA isolation and purification kit were purchased from Promega (USA). Restriction endonucleases and the DNA marker were from Takara Biotechnology, Dalian (China). Lipofectamin TM 2000 was obtained from Invitrogen (USA). RPMI-1640 medium and fetal calf serum were purchased from Hyclone (USA). MTT and PBS were procured from Sigma (USA). DMEM was purchased from GIBCO (USA) and P-gp monoclonal antibody was obtained from Chemicon (USA).

Cell lines and vectors

The hepatocellular carcinoma cell line, HepG$_2$, was kindly denoted by Prof Weixue Tang (Department of Pathophysiology, Chongqing Medical University). Low-passage HEK 293 cells were obtained from the Institute for Cytobiology, Chinese Academy of Sciences, Shanghai. All cells were cultured in RPMI-1640 medium containing 10% fetal calf serum at 37°C in saturated humidified air with 5% CO$_2$. The cells were subcultured once every 3 days. An adenovirus vector Adeno-XTM expression system was purchased from Clontech Corp (USA).

Establishment of HepG$_2$/ADM cells with MDR

The HepG$_2$ MDR cell line (HepG$_2$/ADM) was induced by graded resistance to ADM. Concentrations of 0.01~2.0 μg/ml ADM were added to the DMEM medium containing HepG$_2$ cells until the HepG$_2$ cells could tolerate 2 μg/ml of ADM with 90% survival. Induction of drug resistance was stopped and the cells preserved in DMEM medium containing 0.01 μg/ml ADM. These cells were preserved for future use by freezing. They

maintained MDR and were named HepG$_2$/ADM cells. In order to observe drug tolerance of the HepG$_2$/ADM cells, ADM, 5-FU, MMC and MTX were employed. The MTT assay was used to measure cellular proliferative activity. Development of color (OD) was evaluated at 490 nm 72 h later. The inhibitory rate of tumor cell growth (%) = (OD in control-OD in study)/OD in control×100%.

Construction of the adenoviral vector using AFP and asmdr1

To remove the p$_{CMV\ II}$ from the *pshuttle* vector, PCR was used to amplify the 99-744 region of the *pshuttle* vector. Specific primers were as follows: 5'-AGC CAG TAT CTG CTC CCT GCT TGT G-3' (sense), 5'-ATG CTA GCG GTG CCA AAA CAA ACT CCC A-3' (antisense). A NheI site was introduced into the antisense primer. The pshuttle vector and the PCR products of the 99-744 region were both doubly digested with MluI and NheI, and the digested products were ligated with T$_4$ ligase. The MluI site was 256, and the NheI was 921 in the pshuttle vector. The 256-921 region of the pshuttle vector was removed from the plasmid, and replaced by the region of 256-744. Then the 744-921 region containing the pCMV IE was removed from the pshuttle vector. Thus, a new plasmid *pCMV* (-) was constructed.

Subsequently, the *pCMV*(-) and AFP PCR products were doubly digested with NheI and ApaI. The 0.3 kb AFP promoter was inserted into *pCMV* (-), which was called *pAFP*. Then the p$_{CMV\ II}$ was replaced by the AFP promoter. Both *pAFP* and asmdr1 PCR products were doubly digested with *KpnI* and *ApaI*, and the digested products were ligated by T$_4$ ligase. Then the asmdr1 gene was inserted into the *pAFP* to construct the *pAFP*-asmdr1.

The newly constructed plasmid *pAFP*-asmdr1 was then doubly digested with *PI-SceI/I-Ceu I* (New England Biolabs, UK), and the purified product ligated with Adeno-X genome DNA. It was then amplified in *E.coli XL1-Blue*. The HEK293 cells were transfected with recombinant adenovirus which was linearized with *PacI*, as described in the manual. In brief, the HEK293 cells were cultured in a 60 mm plate, and 10 μl *PacI* digested Adeno-X DNA was added to the culture medium when the cells were 50~70% confluent. The cells then were transfected with Lipofectamin and incubated. A week later, the cells were lysed with three consecutive freeze-thaw cycles, and the virus was collected from the supernatant. The titer of the virus was about 1×10^7 pfu/ml, which was determined by an

end-point dilution assay.

Reverse transcriptase polymerase chain reaction (RT— PCR)

RT-PCR was employed to amplify the mdr1-mRNA from mouse transplantated tumor cells. A specific mdr1 gene primer was obtained from the Shanghai Boya Biologic Corporation: 5'-ATG CTC GTG TTT GGA GA-3' (sense), 5'-AGC CAG AAC ATT CTT CC-3' (antisense). RT-PCR was performed in a 50 μl volume consisting of 1 μM of each primer, 0.2 mM of each dNTP, 1 μl 5×AMV/Tfl buffer, 2 μl 25 mM Mg-SO₄, 1 μl Tfl DNA polymerase, 1 μl AMV reverse transcriptase and 2 μl DNA template. The sample was heated to 48 °C for 45 min, 94 °C for 2 min for the first chain of cDNA, followed by amplification for 40 cycles at 94 °C for 45 s, 60 °C for 30 s, 68 °C for 90 s and 1 cycle at 68 °C for 7 min and 4 °C for 8 min for the second chain. Then 5 μl of each product was analyzed by 1% agarose gel (containing 0.5 μg/ml EB) electrophoresis.

Transplantated tumor model in athymic mice

Satisfactorily growing HepG₂/ADM cells were digested with 0.25% trypase and washed twice with PBS, and then inoculated subcutaneously in the left armpit of the mice using 1×10⁷/0.1ml. After 1 week, the transplantated tumor was examined and 2 weeks later the diameter reached 5~6 mm.

Observation of reversed MDR activity by recombined adenovirus in HepG₂/ADM cells

Eighteen athymic mice bearing tumors for 2 weeks were randomly used for the following 3 groups: PBS, ADM (2.0 μg/ml) and ADM+Adeno-asmdr1 (ADM was 2 μg/ml, Adeno-asmdr1 was 1×10⁹ pfu/ml). The

mice were injected with 0.1 ml PBS, ADM and ADM+Adeno-asmdr1 and the long and short diameters (A and B) of the tumors were measured each week. Four weeks later, the athymic mice were sacrificed and tumor tissues were collected. The tumor volume was calculated ($A \times B^2/2$) mm³,[4] the tissue examined under transmission electron microscopy and RT-PCR conducted on the tissue samples.

Statistical analysis

Data were treated with SPSS 11.0 software and expressed as the mean±SD. Differences between groups were analyzed using the t-test with $P<0.05$ considered as statistically significant.

RESULTS

Inhibitory effects of ADM, 5–FU, MMC and MTX on HepG₂/ADM cells and HepG₂ cells

The concentrations of the HepG₂/ADM cells or HepG₂ cells were 5 ×10⁴/ml. ADM, 5-FU, MMC and MTX were added at the dosage of HepG₂ cells: IC₅₀×1, IC₅₀× 10, IC₅₀×20 and IC₅₀×30, respectively. With the increase in drug concentrations, the inhibitory rate in the 2 groups also increased (P<0.01). In the HepG₂/ADM cells, only the high concentration of ADM had an obvious inhibitory effect, but in the HepG₂ cells, the inhibition was obvious at a low concentration (Table 1). Therefore the HepG₂/ADM cells had features of MDR and tolerance to ADM.

Reversal of MDR by recombined adenovirus in HepG₂/ADM cells

Tumor tissues were obtained from athymic mice bearing tumors for 7 days and then examined by electron microscopy. Apoptotic bodies were observed in the

Table 1. The inhibitory rate of ADM, 5–FU, MMC and MTX on HepG₂/ADM and HepG₂ cells (%)

Drug	Concentration of drug							
	IC₅₀×1		IC₅₀×10		IC₅₀×20		IC₅₀×30	
	HepG₂/ADM	HepG₂	HepG₂/ADM	HepG₂	HepG₂/ADM	HepG₂	HepG₂/ADM	HepG₂
ADM	1.04 ± 0.01*	48.3 ± 0.02	12.4 ± 0.01*	96.6 ± 0.02	14.8 ± 0.01*	98.1 ± 0.03	66.7 ± 0.02*	99.2 ± 0.02
5-FU	6.01 ± 0.01*	53.9 ± 0.02	36.7 ± 0.02*	97.4 ± 0.01	90.3 ± 0.01*	98.4 ± 0.02	97.6 ± 0.01	99.8 ± 0.02
MMC	2.13 ± 0.01*	50.1 ± 0.01	37.5 ± 0.01*	99.2 ± 0.02	88.5 ± 0.03*	99.5 ± 0.02	98.3 ± 0.22	99.9 ± 0.03
MTX	3.15 ± 0.02*	52.4 ± 0.02	59.4 ± 0.01*	95.8 ± 0.02	89.6 ± 0.01*	99.2 ± 0.01	95.2 ± 0.02	99.5 ± 0.01

*: Compared with HepG₂ cells treated with the same drug and dosage, P<0.01. With an increase in the drug concentration, 5-FU, MMC and MTX displayed a gradual inhibitory effect, but ADM only had obvious inhibitory effect on the HepG₂/ADM cells at IC₅₀× 30 (P<0.01). ADM, 5-FU, MMC and MTX had an obvious inhibitory effect on the HepG₂ cells from IC₅₀× 1 to IC₅₀× 30 (P<0.01).

xenograft tumor cells treated with Adeno-asmdr1, but there was little or no apparent apoptosis in the groups treated with ADM or PBS (Fig.1).

Fig.1. Many apoptotic bodies were detected by transmission electron micrograph analysis in the Adeno-asmdr1 group (× 8000).

The tumor volume in the ADM+Adeno-asmdr1 treated group did not increase, but did obviously increase in the PBS and ADM groups ($P<0.05$), especially in the PBS group (Fig.2).
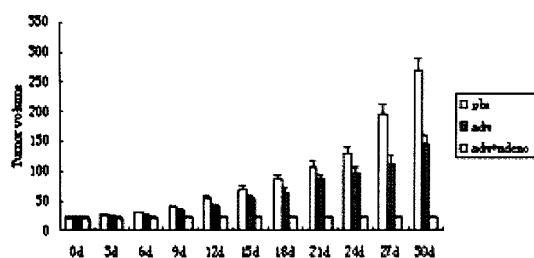

Fig.2. The tumor volume in the groups treated with PBS, ADM and ADM + adeno-asmdr1 ($P<0.05$).

mdr1 mRNA in xenografts examined by RT–PCR

From the electrophoresis and semi-quantitative mRNA analysis of the tumor xenografts, strips of 5 s, 18 s and 28 s were observed and the total RNA was complete. The mdr1 RNA was reduced at 1 week and 4 weeks in

the ADM+asmdr1 group and found to be nearly unchanged in the ADM group. At 4 weeks, the mdr1 RNA in the ADM+asmdr1 group was only 20% compared to the ADM group (Fig.3).

DISCUSSION

Chemotherapy is an important mode of therapy for HCC, however, many patients do not fully respond. MDR is considered to be one of the main causes. It is known that the MDR gene family includes mdr1 and mdr2, of which mdr1 is the functional gene but mdr2 is non-functional. [5] Activation of the mdr1 gene and overexpression of its product, P-glucoprotein (P-gp 170), play an important role in the mechanism of MDR. [6,7] Now, many drugs are used to reverse MDR, such as the calcium channel blocker verapamil, [8] quinine, [9] naphthyridine [10] and pyronaridine, [11] but these drugs are not totally effective and have obvious side effects themselves, so their clinical use is limited.

With the development of gene transfer techniques, gene therapy is considered to be of considerable interest for HCC therapy. [12] The distinctive expression of a unique gene is one of the important goals in tumor gene therapy. AFP is a widely accepted serologic marker for HCC. In theory, the vector carrying the AFP promoter will induce a high expression of the target gene in cells that express AFP but cause little or no expression in other cells. [13] In our study, antisense technology was employed by inserting an inverted structural gene of 200 bp from the initiation site of the coding region of mdr1. Using adeno virus as a carrier, antisense mdr1 RNA was transcribed into HepG$_2$/ADM cells, which displayed the MDR phenotype in athymic mice. This gene transfer blocked the transcription and expression of the mdr1 gene resulting in
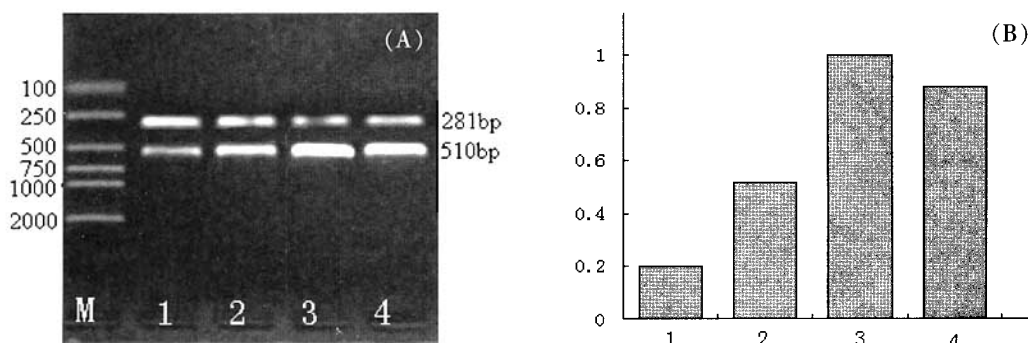

Fig.3. The mdr1 mRNA of xenografts detected by RT-PCR (A) and by semi-quantitative mdr1 mRNA analysis (B). M: marker; lane 1, ADM+asmdr1 at 4 weeks; lane 2, ADM+asmdr1 at 1 week; lane 3, ADM at 4 weeks; lane 4, ADM at 1 week.

recovery of the sensitivity of the transplantated tumor to chemotherapic agents.

Our studies showed that the volume of the transplantated tumors failed to increase when injected with a high titer of restructured adenovirus and ADM. Down-regulation of the mdr1 gene transcription assessed by RT-PCR was significant compared to administration of only PBS or ADM. In the group receiving the restructured adenovirus plus ADM, apoptosis occurred early in the transplantated tumor. Compared with the group receiving only ADM or PBS, one can conclude that the sensitivity of the transplantated tumor to ADM had recovered as the tumor celluar MDR was reversed by adeno-asmdr1. But this reconstructed adenovirus did not completely reverse MDR. Possible reasons for this partial reversal are: 1) The mdr1 gene is not the only gene participating in MDR, since it has been shown that a multidrug resistance-associated protein (MRP) gene [14,15] and an apoptotic regulator gene Bcl-2 family [16] have an important role in MDR. Thus blocking only the mdr1 gene can not totally reverse MDR. 2) There are many concerns with regard to gene blocking technology including antisense technology, as any single therapy can not block the target gene completely.[17,18] 3) It is possible that the antisense titer was too low to block the transcription of the mdr1 gene. 4) The priming efficacy of the AFP promoter was limited and could not promote down-stream transcription. 5) In this study, the treatment period was short. So, the total effect of adeno-asmdr1 on MDR in the transplantated tumor and the post-effect of ADM were not observed.

REFERENCES

1 Yujun S, Jianping G, Changan L, et al. Construction of a targeting adenoviral vector carrying AFP promoter for expressing EGFP gene in AFP-producing hepatocarcinoma cell. World Journal of Gastroenterology. 2004;10:186 – 189.

2 Di MM, De ME, Perrone F, et al. Hepatocellular carcinoma:systemic treatments. J Clin Gastroenterol. 2002;35: S109–114.

3 Liang G, Zhang S, Huang ZM, et al. MDR-reversing effect of two components of catechin on human hepatocellular carcinoma BEL-7404/Adr in vitro. Ai Zheng. 2004; 23:401–405.

4 Tang ZY. Hepatocellular carcinoma –cause, treatment and metastasis. World J Gastroenterol. 2001;7:445–454.

5 Licht T, Pastan I, Gottesman MM, et al. The multidrug-resistance gene in gene therapy of cancer and hematopoietic disorders. Ann Hematol. 1996; 72:184–193.

6 Kang Y, Perry RR. Effect of alpha interferon on p-gp expression and function and on verapmil modulation of doxorubicin resistance. Cancer Res.1994;54:2952–2958.

7 Brenner SS, Klotz U. P-glycoprotein function in the elderly. Eur J Clin Pharmacol. 2004;60:97–102.

8 Wang J, Goh B, Lu W, et al. In vitro cytotoxicity of stealth liposomes co-encapsulating doxorubicin and verapamil on doxorubicin –resistant tumor cells. Biol Pharm Bull. 2005;28:822–828.

9 Solary E, Drenou B, Campos L, et al. Quinine as a multidrug resistance inhibitor: a phase 3 multicentric randomized study in adult de novo acute myelogenous leukemia. Blood. 2003;102:1202–1210.

10 Misbahi H, Brouant P, Hever A, et al. Benzo [b]–1,8– naphthyridine derivatives: synthesis and reversal activity on multidrug resistance. Anticancer Res. 2002; 22:2097– 2101.

11 Qi J, Yang CZ, Wang CY, et al. Function and mechanism of pyronaridine: a new inhibitor of P-glycoprotein-mediated multidrug resistance. Acta Pharmacol Sin. 2002;23: 544–550.

12 Huang TG, Savontaus MJ, Shinozaki K, et al. Telomerase-dependent oncolytic adenovirus for cancer treatment. Gene Ther. 2003;10:1241–1247.

13 Ohashi M, Kanai F, Tateishi K, et al. Target gene therapy for alpha –fetoprotein producing hepatocellular carcinoma by E1B55k–attenuated adenovirus. Biochem Biophys Res Commun. 2001;282:529–535.

14 Bonin S, Pascolo L, Croce LS, et al. Gene expression of ABC proteins in hepatocellular carcinoma, perineoplastic tissue and liver diseases. Mol Med. 2002;8:318–325.

15 Atkinson DE, Greenwood SL, Sibley CP, et al. Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer. Am J Physiol Cell Physiol. 2003; 285: C584–591.

16 Schaich M, Illmer T, Seitz G, et al. The prognostic value of Bcl–x L gene expression for remission induction is influenced by cytogenetics in adult acute myeloid leukemia. Haematologica. 2001;86:470–477.

17 Lebedeva I, Stein CA. Antisense oligonucleotides:promise and reality. Annu Rev Pharmacol Toxicol. 2001;41:403– 419.

18 Okino T, Onda M, Matsukura N, et al. Sequential histopathological changes in vivo after suicide gene therapy of gastric cancer induced by N–methyl– N´–nitro–N–nitrosoguanidine in rats. Jpn J Cancer Res. 2001;92:673– 679.