

RAPID COMMUNICATION

Combination of small interfering RNA and lamivudine on inhibition of human B virus replication in HepG2.2.15 cells

Gui-Qiu Li, Wei-Zhen Xu, Jing-Xia Wang, Wen-Wei Deng, Di Li, Hong-Xi Gu

Gui-Qiu Li, Wei-Zhen Xu, Di Li, Hong-Xi Gu, Department of Microbiology, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

Jing-Xia Wang, Department of Histology and Embryology, Jiamusi Medical College, Jiamusi University, Jiamusi 154002, Heilongjiang Province, China

Wen-Wei Deng, Department of Anesthesiology, Second Affiliated Hospital, Jiamusi University, Jiamusi 154002, Heilongjiang Province, China

Supported by PhD Foundation of Education Ministry, China, No. 2005006; Youth Foundation of Heilongjiang Province, No. QC060061; and Foundation of Health Hall, Heilongjiang Province, No. 2005-009

Correspondence to: Hong-Xi Gu, Department of Microbiology, Harbin Medical University, Harbin 150081, Heilongjiang Province, China. hxgu2432@163.com

Telephone: +86-451-86685122

Received: 2007-01-18

Accepted: 2007-03-28

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Key words: Hepatitis B virus; RNA interference; siRNA with lamivudine; HepG2.2.15 cell

Li GQ, Xu WZ, Wang JX, Deng WW, Li D, Gu HX. Combination of small interfering RNA and lamivudine on inhibition of human B virus replication in HepG2.2.15 cells. *World J Gastroenterol* 2007; 13(16): 2324-2327

<http://www.wjgnet.com/1007-9327/13/2324.asp>

Abstract

AIM: To observe the inhibition of hepatitis B virus (HBV) replication and expression by combination of siRNA and lamivudine in HepG2.2.15 cells.

METHODS: Recombinant plasmid psil-HBV was constructed and transfected into HepG2.2.15 cells. The transfected cells were cultured in lamivudine-containing medium (0.05 μ mol/L) and harvested at 48, 72 and 96 h. The concentration of HBeAg and HBsAg was determined using ELISA. HBV DNA replication was examined by real-time PCR and the level of HBV mRNA was measured by RT-PCR.

RESULTS: In HepG2.2.15 cells treated with combination of siRNA and lamivudine, the secretion of HBeAg and HBsAg into the supernatant was found to be inhibited by 91.80% and 82.40% (2.89 ± 0.48 vs 11.73 ± 0.38 , $P < 0.05$; 4.59 ± 0.57 vs 16.25 ± 0.48 , $P < 0.05$) at 96 h, respectively; the number of HBV DNA copies within culture medium was also significantly decreased at 96 h (1.04 ± 0.26 vs 8.35 ± 0.33 , $P < 0.05$). Moreover, mRNA concentration in HepG2.2.15 cells treated with combination of siRNA and lamivudine was obviously lower compared to those treated either with siRNA or lamivudine (19.44 ± 0.17 vs 33.27 ± 0.21 or 79.9 ± 0.13 , $P < 0.05$).

CONCLUSION: Combination of siRNA and lamivudine is more effective in inhibiting HBV replication as compared to the single use of siRNA or lamivudine in HepG2.2.15 cells.

INTRODUCTION

Chronic HBV infection is one of the most severe infectious diseases in humans. More than 400 million people worldwide suffer from chronic HBV infection and the infected individuals are at high risk of developing liver cirrhosis and hepatocellular carcinoma^[1]. Current efforts to treat HBV infection have only limited success. Only 20% of the patients benefit from anti-HBV therapy with recombinant interferon and nucleotide analogues such as lamivudine^[2-5]. Thus, there is an urgent need to develop more effective strategies to combat HBV infection.

RNA interference (RNAi) is a cellular process in which double-stranded RNA of 21-23 nt induces the post-transcriptional gene silencing of homologous mRNA^[6-10]. Based on the important role on the reverse transcription step in HBV replication^[11-15], several researches have demonstrated that RNAi could suppress the replication of HBV through silencing the expression of the viral genes and provide a new method against chronic HBV infection^[16-19]. Considering that lamivudine has been a leading drug which is highly effective in inhibiting HBV DNA synthesis^[20] and RNAi is a newly discovered antiviral mechanism^[6,7], we hypothesize that combination of siRNA and lamivudine may exhibit greater inhibitory effect on HBV replication. To address this question, we systemically measured the anti-HBV effect of siRNA combined with lamivudine on HepG2.2.15 cells at HBeAg, HBsAg, DNA and mRNA levels. Our findings suggested that combination of siRNA and lamivudine exerted a pronounced inhibition of HBV replication.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and G418

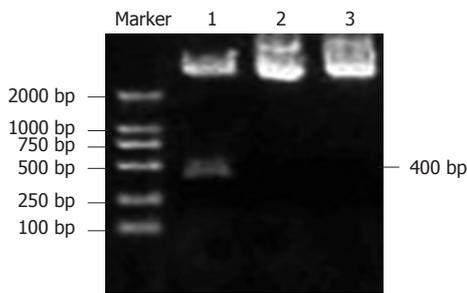


Figure 1 Electrophoresis analysis of digestion using *Sal*I/*Pst*I. Lane 1: psi-HBV/*Sal*I; lane 2: psi-HBV/*Pst*I; lane 3: psi-HBV.

were purchased from GIBCO BRL (USA). HepG2.2.15 cells were maintained in our laboratory. Plasmid psi/U6 was supplied by Wuhan JS Biotech (China). All PCR primers were synthesized by Shanghai Boya Biological Company (China). Trizol, M-MLV reverse transcriptase, Lipofection 2000 reagents were purchased from Invitrogen Company (USA). Restriction enzymes were purchased from Beijing New England Biolab (China).

Construction of plasmid psi-HBV

HBV siRNA expression plasmid was generated by inserting a 21-nt specific sequence encoding corresponding siRNA into *Bam*H I-*Hind*III sites of plasmid psi/U6 as previously described^[21] and further analyzed by BLAST to ensure that it does not have sequence homology with human genome sequence. The oligonucleotide pairs used to coding for the same strand of siRNA were as follows: 5'-GATCCGCACTTCCGGAAACTGTTTCAAGACGACAGTAGTTTCCGGAAAGTGTTTTTTGTGCGACA-3' (sense) and 3'-GCGTGAAGGCCTTTGATGACAAAGTTCTGCTGTCATCAAAGGCCTTCACAAAAACAGCTGTTTCGA-5' (antisense). The recombinant plasmid, named as psi-HBV, was transformed into *E. coli* strain DH5 α and identified by restricted endonuclease digestion and DNA sequence analysis. Construction and identification of an irrelevant control plasmid HK were essentially the same as described above.

Cell culture and transfection

HepG2.2.15 cells were cultured in DMEM supplemented with 100 mL/L fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL streptomycin and 200 μ g/mL G418 and incubated at 37°C in a humidified atmosphere containing 50 mL/L CO₂ in air. Cells were cultured in 6-well plates until they were 60%-70% confluence 24 h prior to transfection. Transfection was performed using Lipofection 2000 reagent and psi/HSV complex at 2.5:1 ratio following the manufacturer's instructions. The transfected cells were cultured in lamivudine-containing medium (0.5 μ mol/L) and harvested at 48 h, 72 h and 96 h for further analysis. All experiments were performed in triplicate and divided into five groups.

Quantitative assay of HBeAg and HBsAg

The levels of HBeAg and HBsAg in culture supernatants were measured using ELISA kits (SA Biotech, Shanghai,

China) at 48, 72 and 96 h according to the manufacturer's instructions.

Quantitative assay of HBV DNA

HBV DNA was extracted from the culture medium and examined by real-time PCR using HBV diagnostic kit (PG Biotech, Shenzhen, China) at 48, 72 and 96 h according to the manufacturer's protocol.

Assay of HBV mRNA

Total RNA of the transfected cells was isolated with Trizol reagent and reverse transcribed into cDNA with 200 U of M-MLV reverse transcriptase at 48, 72 and 96 h.

Samples were amplified through 30 cycles, each amplification cycle consisting of denaturation at 94°C for 15 s, primer annealing at 52°C for 30 s, and extension at 72°C for 30 s. Cycles were preceded by incubation at 94°C for 3 min to ensure full denaturation of the target gene and were followed by an extra incubation at 72°C for 10 min to ensure full extension of the products. Primers for PCR were as follows: HBV, 5'-ACCTCTGCCTAATCATCTC-3' (forward) and 5'-GTAAGACAGGAAATGT GAAAC-3' (reverse); and GAPDH 5'-GTCGGTGTGAACGGATTT-3' (forward) and 5'-ACTCCACGACGTACTCAGC-3' (reverse). The PCR amplification product was analyzed by 1.2% agarose gel electrophoresis.

Statistical analysis

Data were expressed as mean \pm SD. Data were compared using *F*-test and one-way Anova. All statistical analyses were performed using the SPSS 12.0 software. *P* < 0.05 was considered statistically significant.

RESULTS

Identification of recombinant plasmid psi-HBV

The *Pst*I site in the plasmid psi-HBV was replaced by *Sal*I site that we designed in the inserted DNA sequence. The fragment digested by *Sal*I was about 400 bp in size (Figure 1). The DNA sequence analysis of positive clones confirmed the results as expected.

Inhibition of HBeAg and HBsAg in cultured HepG2.2.15 cells

The concentrations of HBeAg and HBsAg in the culture supernatant were measured at 48, 72 and 96 h by ELISA. As is shown in Figure 2 and Table 1, there was no significant inhibition on the expression of HBeAg and HBsAg at 48 and 72 h; an obvious inhibition (*P* < 0.05) was, however, observed at 96 h, with the reduction in levels of HBsAg and HBeAg by 91.8% and 82.4%, respectively, in the supernatant of cells treated with combined siRNA and lamivudine as compared to the single use of siRNA or lamivudine.

Inhibition of HBV DNA replication in cultured HepG2.2.15 cells

The level of HBV DNA was examined by real-time PCR. This approach can detect HBV DNA in the range of

Table 1 Concentration of HBeAg and HBsAg in HepG2.2.15 cells detected by ELISA

Group	HBeAg			HBsAg		
	48 h	72 h	96 h	48 h	72 h	96 h
siRNA	6.54 ± 0.30 (15.43)	6.23 ± 0.27 (55.78)	4.90 ± 0.29 (70.92) ^a	6.94 ± 0.14 (13.88)	14.00 ± 0.64 (15.06)	7.68 ± 0.70 (60.57) ^a
LAM	6.80 ± 0.22 (10.48)	10.02 ± 0.45 (15.20)	9.53 ± 0.44 (22.85) ^a	7.10 ± 0.19 (11.03)	14.39 ± 0.45 (12.38)	11.93 ± 0.36(30.53) ^a
siRNA+LAM	6.01 ± 0.42 (25.52)	8.40 ± 0.20(32.55)	2.89 ± 0.48 (91.8) ^{a,c}	6.47 ± 0.27 (22.24)	11.26 ± 0.77 (34.62)	4.59 ± 0.57(82.40) ^{a,c}
HK	7.25 ± 0.27 (0)	11.06 ± 0.21 (0)	10.92 ± 0.33 (0)	7.52 ± 0.18 (0)	15.93 ± 0.44(0)	15.43 ± 0.47 (0)
psi/U6	7.35 ± 0.34	11.44 ± 0.17	11.73 ± 0.38	7.72 ± 0.22	16.11 ± 0.41	16.25 ± 0.48

The inhibitory ratio is shown in bracket. ^a*P* < 0.05 vs HK or psi/U6 group; ^c*P* < 0.05 vs siRNA or LAM group.

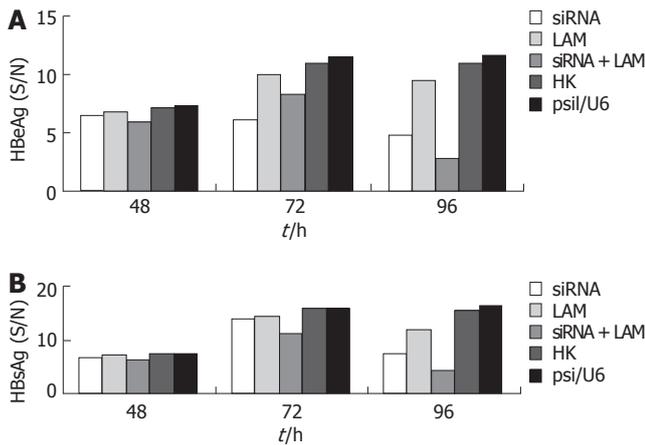


Figure 2 Decrease in the concentration of HBeAg and HBsAg in HepG2.2.15 cells. **A:** Reduction of HBeAg level; **B:** Reduction of HBsAg level (S/N: sample to negative ratio).

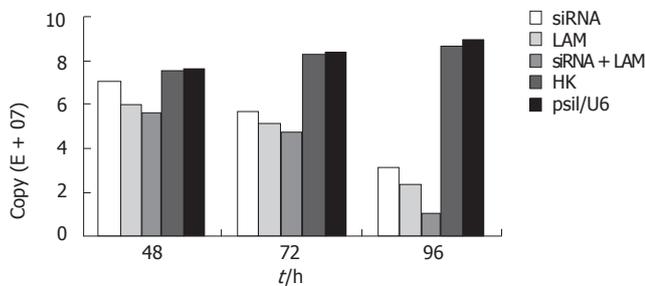


Figure 3 Inhibition of HBV DNA replication in HepG2.2.15 cells.

10⁴ to 10⁸ copies. As shown in Figure 3 and Table 2, the copies of viral DNA in cells treated with siRNA combined with lamivudine reduced by 88.42% at 96 h (*P* < 0.05) compared to controls. The inhibitory effect was much higher than that obtained with single use of siRNA or lamivudine.

Inhibition of HBV mRNA expression in cultured HepG2.2.15 cells

The level of HBV mRNA was examined by RT-PCR. The results revealed that the use of siRNA combined with lamivudine yielded the greatest reduction by 80.56% at 96 h compared to either siRNA or lamivudine alone (Figure 4).

DISCUSSION

RNAi regulates gene expression *via* a unique mechanism

Table 2 Real-time PCR detection of the level of HBV DNA in HepG2.2.15 cells

Group	48 h	72 h	96 h
siRNA	7.02 ± 0.33 (7.63)	5.69 ± 0.18 (31.86)	3.10 ± 0.27 (65.48) ^a
LAM	6.03 ± 0.41 (20.66)	5.19 ± 0.18 (37.84)	2.40 ± 0.33 (73.27) ^a
siRNA + LAM	5.60 ± 0.37 (26.32)	4.72 ± 0.21 (43.47)	1.04 ± 0.26 (88.42) ^{a,c}
HK	7.50 ± 0.33 (0)	8.26 ± 0.20 (0)	8.65 ± 0.21 (0)
psi/U6	7.60 ± 0.31	8.35 ± 0.33	8.98 ± 0.33

The inhibitory ratio is shown in bracket. ^a*P* < 0.05 vs HK or psi/U6 group; ^c*P* < 0.05 vs siRNA or LAM group.

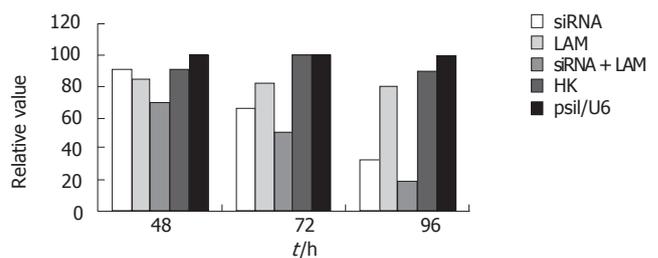


Figure 4 Reduction of mRNA level in HepG2.2.15 cells.

by degradation of target mRNA in a sequence-specific manner^[6,9]. There has been considerable reports on the use of RANi to inhibit viral replication, such as HCV, HIV and poliovirus, *etc*^[22-24]. Although synthetic or vector-based siRNA has been shown to efficiently suppress HBV replication^[11-19], the possibility of the combination of siRNA and lamivudine to inhibit HBV replication has not been systemically studied. In this paper, we showed that the use of siRNA combined with lamivudine markedly reduced RNA level in HepG2.2.15 cells. Furthermore, the results indicated that combination of siRNA and lamivudine exhibited a stronger anti-HBV effect than the single use siRNA or Lamivudine.

During HBV replication, HBV genome shows a rapid mutation due to the high rate of replication and the lack of proofreading by reverse transcription^[5]. Thus, drug resistant mutations appeared upon completing of antiviral therapy with lamivudine, which concentrated on the inhibition of reverse transcriptase activity^[4]. During RNAi process, siRNA first recognizes and degrades the corresponding sequence of viral RNA, consequently, depleting template for reverse transcription. Decreased synthesis of HBV DNA then further influences the life-cycle of viral genome, especially in transcription and

translation, thereby resulting in the overall inhibition of viral replication. In addition, the numerous potential targets for RNAi along HBV genome make it possible to target conserved regions, limiting the viral ability to create escape mutations. The ability of siRNAs to reduce the level of viral transcripts and proteins even in the absence of active viral replication makes it a good candidate as an assistant therapy to lamivudine, which acts only on the replication-competent HBV.

Although the development of a more effective anti-HBV therapy is still a challenge, our findings provide solid evidence for the feasibility of a cocktail treatment, which may enhance anti-HBV efficacy and overcome the shortcomings of current therapy. Therefore, this new method which combined gene therapy and chemotherapy opens a new avenue for treating chronic HBV infection. The precise mechanism by which combination of siRNA and lamivudine exerts more inhibitory efficacy on HBV replication needs to be clarified in further studies.

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S- Editor Liu Y L- Editor Kumar M E- Editor Lu W