Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Combination of small interfering RNAs mediates greater inhibition of human hepatitis B virus replication and antigen expression*

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Received Jan. 18, 2005; revision accepted Jan. 30, 2005

Abstract: Objectives: To evaluate the inhibitory effect mediated by combination of small interfering RNAs (siRNAs) targeting different sites of hepatitis B virus (HBV) transcripts on the viral replication and antigen expression in vitro. Methods: (1) Seven siRNAs targeting surface (S), polymerase (P) or precore (PreC) region of HBV genome were designed and chemically synthesized. (2) HBV-producing HepG2.2.15 cells were treated with or without siRNAs for 72 h. (3) HBsAg and HBeAg in the cell culture medium were detected by enzyme-linked immunoadsorbent assay. (4) Intracellular viral DNA was quantified by real-time PCR (Polymerase Chain Reaction). (5) HBV viral mRNA was reverse transcribed and quantified by real-time PCR. (6) The change of cell cycle and apoptosis was determined by flow cytometry. Results: Our data demonstrated that synthetic small interfering RNAs (siRNAs) targeting S and PreC gene could efficiently and specifically inhibit HBV replication and antigen expression. The expression of HBsAg and HBeAg and the replication of HBV could be specifically inhibited in a dose-dependent manner by siRNAs. Furthermore, our results showed that the combination of siRNAs targeting various regions could inhibit HBV replication and antigen expression in a more efficient way than the use of single siRNA at the same final concentration. No apoptotic change was observed in the cell after siRNA treatment. Conclusion: Our results demonstrated that siRNAs exerted robust and specific inhibition on HBV replication and antigen expression in a cell culture system and combination of siRNAs targeting different regions exhibited more potency.

Key words: Hepatitis B virus, Combination of siRNAs, HBV replication, Antigen expression
doi:10.1631/jzus.2005.B0236 **Document code:** A **CLC number:** R730.1; R730.231. 3

INTRODUCTION

Chronic infection by hepatitis B virus (HBV) is associated with a high risk of liver cirrhosis and primary hepatocellular carcinoma (HCC). It remains an important global health problem with over 350 million chronic HBV carriers worldwide. Among these chronic carriers, about one million people die of HBV-associated liver failure or HCC annually (Kao and Chen, 2002). Although chronically infected patients have been treated with interferons and nucleoside analogs, the low efficacy and undesirable side-effects of interferons and the occurrence of nucleoside analogs resistant HBV mutations remain major obstacles in anti-HBV therapy (Lee, 1997; Lavanchy, 2004). Therefore it is important to develop new strategies to treat HBV infection.

RNA interference (RNAi) is an evolutionarily conserved process of the sequence-specific homologous (endogenous or exogenous) gene silencing, triggered by small duplexes of RNA, in a wide range of eukaryotic organisms (Plasterk, 2002; Zamore,

* Project (No. 30471943) supported partly by the National Natural Science Foundation of China

2002; Hannon, 2002). Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21- to 23-nt small interfering RNAs (siRNAs) (Elbashir *et al.*, 2001). Recently, it was reported that virus replication and gene expression of a growing number of human infectious viruses including HBV was inhibited by RNAi, which suggests that siRNA can be potentially used as a new antiviral strategy in future therapeutics (Jacque *et al.*, 2002; Kapadia *et al*., 2003; Tatsuo *et al*., 2004; Hamasaki *et al.*, 2003; McCaffrey *et al*., 2003; Ying *et al*., 2003; Hilla *et al*., 2003; Shlomai and Shaul, 2003).

Up to date, two groups have demonstrated that synthetic siRNAs targeting polyadenylation (PA), precore (PreC), and surface (S) regions of HBV genome can inhibit HBV replication and antigen expression (Hilla *et al*., 2003; Masayoshi *et al*., 2003). As HBV infection is pathological process with multiple gene involvement, it will be ideal to develop an siRNA approach that can knock down the expression of multiple pathogenic viral antigens as well as inhibit viral replication. In this study, we test the feasibility of combination treatment of siRNAs targeting various regions and the antiviral efficacy of HepG2.2.15 cell culture system (Sells *et al*., 1987).

MATERIALS AND METHOD

Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum and G418 were purchased from GIBCO BRL (USA). Oligofectamine and Trizol were from Invitrogen (USA). HBsAg and HBeAg ELISA (Enzyme Linked Immunosorbent Assay) kits were from Sino-American Biotechnology Company (China). DNase I and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase were from Promega (USA). TaqMan 2×Universal PCR Master Mix was from Applied Biosystems (USA). Propidium iodide (PI) was from Sigma (USA). Primers and Taqman probes were synthesized by Sangon (China).

siRNA design and synthesis

21-nucleotide siRNAs bearing 2-nt deoxynucleotide overhangs at the 3′-end which target S region and PreC region of the ayw HBV genome (Table 1) were designed according to the guide by Dr. Tuschl (http://www.rockefeller.edu/labheads/tuschl/sirna.html). The specificity of all siRNAs was checked by BLAST search (www.ncbi.hlm.nih.gov). All siRNAs were chemically synthesized by Dharmacon (www.dharmacon.com).

Cell culture

HepG2.2.15 cell line was kindly provided by Professor Chen (Infective Diseases Institute of Zhejiang University, China). This cell line was cultured at 37 °C in DMEM supplemented with 10% fetal bovine serum and 500 μg/ml G418 in 5% CO_2-humidified air.

Transfection

A day before transfection, cells were trypsinized and approximately 0.5×10^4 cells/well were plated in a 24-well plate. Cells were transfected with siRNA using Oligofectamine following the manufacturer's protocol. Seventy-two hours after transfection, cells were harvested and cell culture supernatant was collected for further analysis. All experiments were performed in triplicate and the results were expressed as means±*SD*.

HBsAg and HBeAg assay

HBeAg and HBsAg in the culture supernatant were measured by using ELISA kits following the

Table 1 Sequences of small interfering RNA (siRNA) and targeted sites of HBV

siRNA	Sense sequence	Antisense sequence	Targeted site of HBV genome	Targeted region of HBV genome
siRNA1	UCUUCUCGAGGACUGGGGATT	UCCCCAGUCCUCGAGAAGATT	123–143	S
siRNA2	CCUCUUGUCCUCCAAUUUGTT	CAAAUUGGAGGACAAGAGGTT	342–362	S
siRNA3	GGUAUGUUGCCCGUUUGUCTT	GACAAACGGGCAACAUACCTT	458–478	S
siRNA4	GUCUGUACAACAUCUUGAGTT	CUCAAGAUGUUGUACAGACTT	765–785	S
siRNA5	GUGUUUGCUGACGCAACCCTT	GGGUUGCGUCAGCAAACACTT	1180–1200	P
siRNA6	CUUUUUCACCUCUGCCUAATT	UUAGGCAGAGGUGAAAAAGTT	1820–1840	PreC
siRNA7	GCUGUGCCUUGGGUGGCUUTT	AAGCCACCCAAGGCACAGCTT	1876–1896	PreC

manufacturer's protocol. All experiments were performed in triplicate and the results were expressed as means±*SD*.

Intracellular HBV core-associated DNA and pre-genomic RNA quantification by real-time PCR assay

Purification of intracellular core-associated HBV DNA was performed using the method described by Turelli *et al*.(2004). Total cellular RNA was extracted with Trizol according to the manufacturer's protocol. Two μg of DNaseI-treated total RNA was reverse-transcribed by MMLV reverse transcriptase enzyme in a 20 μl reaction mix containing 100 pmol oligo (dT) for 1.0 h at 42 °C. HBV DNA and reverse transcribed cDNA were quantified by real-time PCR using HBV primers and probe specific for the C gene: sense: 5′-AGT GTG GAT TCG CAC TCC-3′; antisense: 5′-GAG TTC TTC TTC TAG GGG ACC-3′; Taqman probe: 5′-FAM-CCA AAT GCC CCT ATC CTA TCA ACA CTT C-TAMRA-3′. Amplification and detection were performed with an ABI 7700 (Applied Biosytems, USA) sequence detection system. Briefly, real-time PCR was done in 50 μl volumes containing 2 μl DNA or reverse transcribed cDNA template. Values of cDNA were normalized to that of GAPDH, which was amplified with GAPDH-specific primers and probe: sense: 5′-CTT AGC ACC CCT GGC CAA G-3′; antisense: 5′-GAT GTT CTG GAG AGC CCC G-3′; Taqman probe: 5′-FAM-CAT GCC ATC ACT GCC ACC CAG AAG A-TAMRA-3′. Plasmids containing the full-length insert of the HBV genome and the GAPDH cDNA were used to prepare the standard curve. The PCR conditions were as follows: 50 °C, 2 min; 95 °C, 10 min; 95 °C, 15 s, 60 °C, 1 min, ×40 cycles; 72 °C, 5min. Three independent experiments were performed and the amount of viral DNA or reverse transcribed cDNA in treated cultures was expressed as a percentage of the mock-treated controls. Results were analyzed with SDS 2.0 software from Applied Biosystems.

Flow cytometric analysis

Cells treated with or without 80 nmol/L siRNAs for 72 h were trypsinized, resuspended in phosphate-buffered saline and fixed with 70% alcohol. Cell cycle and apoptosis were monitored using PI staining of nuclei. The red fluorescence of DNA-bound PI in cells was measured at 488 nmol/L with FACSCalibur (Becton Dickinson, USA) and the results were analyzed using ModFit 3.0 software. Ten thousand events were analyzed for each sample.

RESULT

Effects of single treatment of siRNAs on HBsAg and HBeAg expression levels

After HepG2.2.15 cells were treated with 7 different siRNAs (Table 1) at dose of 150 nmol/L for 72 h, the expression of HBsAg and HBeAg were mainly inhibited by siRNAs targeting their own sequences (Fig.1). siRNA 4 targeting S region showed the highest efficiency for inhibiting HBsAg expression with 76.2% knock-down, and siRNA 6 and siRNA 7 targeting PreC region also displayed inhibitory effect on HBeAg expression with 73.8% and 72.8% knock-down, respectively. Other siRNAs resulted in varying degrees of inhibition for antigen expression.

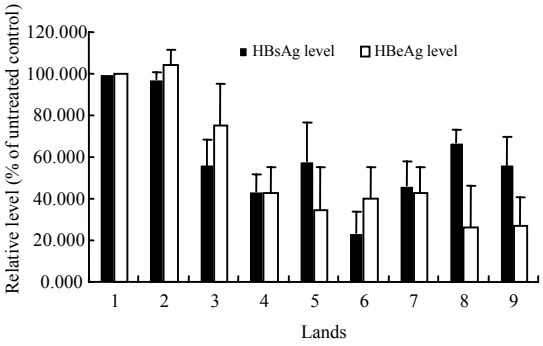

Fig.1 Effects of separate single treatment with siRNAs at the concentration of 150 nmol/L on HBV antigen expression in HepG2.2.15

HBsAg and HBeAg values are presented as the percentage of levels in untreated control cells and are expressed as means±*SD* of 3 samples. Lane 1: untreated control; Lane 2: oligofectamine alone; Lane 3–9: oligofectamine-HBV specific siRNA complex

Dose-dependent effect of single treatment with siRNAs

siRNA 4 and siRNA 6 were used to investigate dose-dependent inhibitory effect on HBsAg, HBeAg and HBV DNA level (Table 2). After cells had been treated with siRNA 4 at 10 nmol/L, 20 nmol/L, 40 nmol/L and 80 nmol/L, HBV core-associated DNA 1evels were reduced by 60.0%, 73.4%, 80.2%, and 81.9%, and HBsAg expression was reduced by 66.2%, 67.4%, 71.6%, 71.2%, respectively.

Table 2 Dose-dependent effects of siRNA targeting S and PreC regions

Dose of siRNA	HBsAg level (%)	HBeAg level (%)	HBV DNA level (%)
siRNA 4			
10 nmol/L	33.8±4.0	61.4±3.6	40±9.7
20 nmol/L	32.6±2.8	52.5±2.1	26.6±3.2
40 nmol/L	28.4±6.9	39.4±2.4	19.8±1.9
80 nmol/L	28.8±4.1	28.7±1.8	18.1±3.7
siRNA 6			
10 nmol/L	89.8±3.6	66.6±3.2	49.5±10.2
20 nmol/L	77.9±2.1	53.0±3.8	32.8±6.2
40 nmol/L	74.8±0.2	33.4±1.5	15.9±3.5
80 nmol/L	64.4±6.0	24.2±2.7	16.6±5.6

*HBsAg, HBeAg and HBV DNA values are presented as a percent of levels in untreated cells and are expressed as means±*SD* of 3 samples in each concentration

HBeAg expression, however, could not be easily knocked down by low concentrations of siRNA 4. The inhibition could only be seen by its treatment at high concentration. Treatment with siRNA 6 at 10 nmol/L, 20 nmol/L, 40 nmol/L and 80 nmol/L resulted in the reduction in the levels of the HBV DNA by 50.5%, 67.2%, 84.1% and 83.4%, and HBeAg expression was reduced by 33.4%, 47.0%, 66.6% and 75.8%, respectively. Little inhibitory effect on HBsAg expression was obtained by siRNA 6 treatment.

Inhibitory effect of combination treatment with HBV specific siRNAs on HBsAg, HBeAg and HBV DNA levels

A combined treatment of HepG2.2.15 with siRNA 4 and siRNA 6 at final concentration of 80 nmol/L (with 40 nmol/L of 4 and 6 each) not only showed more robust inhibition of HBsAg (77.5%), HBeAg (83.6%) expression, HBV DNA (91.8%), and pre-genomic RNA (89.6%) level than the single use of either siRNA 4 or 6, but exhibited the inhibitory effects at the same time (Fig.2).

Flow cytometric analysis of HepG2.2.15 cells treated with siRNAs

HepG2.2.15 cells treated with 80 nmol/L siRNA 4 and siRNA 6 or without siRNA displayed no significant changes of apoptosis (Fig.3).

DISCUSSION

HBV is a partially double-stranded DNA virus

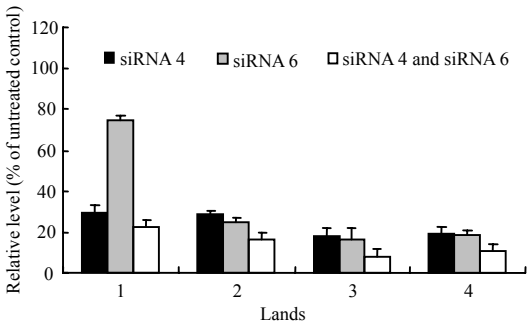

Fig.2 Inhibitory effect of combination treatment with siRNA 4 (40 nmol/L) and siRNA 6 (40 nmol/L) compared with single treatments at the concentration of 80 nmol/L
1: HBsAg level; 2: HBeAg level; 3: HBV DNA level; 4: pre-genomic RNA level. HBsAg, HBeAg, HBV DNA and pre-genomic RNA values are presented as a percent of levels in untreated cells and are expressed as means±*SD* of 3 samples in each

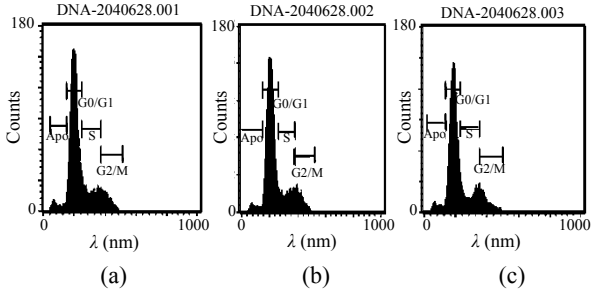

Fig.3 Flow cytometric analysis of cell cycle and apoptosis. (a) Untreated control; (b) Cell cycle and apoptosis was determined 72 h after treatment with siRNA 4 (80 nmol/L); (c) Cell cycle and apoptosis was determined 72 h after treatment with siRNA 6 (80 nmol/L). All the data were shown in Table 3

Table 3 Effect of siRNAs on cell cycle distribution and apoptosis of HepG2.2.15 cells

Groups	Apoptosis	G0/G1	S	G2/M
Untreated control	3.91	72.55	15.07	8.88
siRNA 4	2.54	74.15	14.86	8.58
siRNA 6	3.10	71.82	16.98	8.99

which replicates by reverse transcription of a pre-genomic RNA intermediate in a manner similar to that of the retroviruses. The 3.5-, 2.4-/2.1-kb and 0.7 kb transcripts generated from the HBV genome encode the core protein/HBeAg, polymerase-reverse transcriptase, HBsAg, and X protein respectively. Introduction of siRNAs into HBV-infected hepatocytes can form cytoplasmic RNA-induced silencing complex (RISC) and RISC will bind the targeted HBV mRNAs (Beverly and Davidson, 2003). The consequent cleavage of pre-genomic RNA and viral mRNAs by the RISC is attributable to the inhibition

of HBV antigen expression and HBV replication.

HepG2.2.15 cell line, a derivative of the human HepG2 hepatoma cell line was used as an in vitro model. The cell line which has been transformed with several copies of the HBV genome can stably produce HBV mRNAs, antigen and viral particles (Sells *et al*., 1987). Chemically synthetic siRNAs that target highly conserved regions of 3.5-, 2.4- and 2.1-kb HBV transcripts were screened in HepG2.2.15 cell culture. These HBV-specific siRNAs are capable of reducing HBV antigen, viral mRNA and DNA level in HepG2.2.15 cells. The siRNA 4 and siRNA 6, which target the S and PreC region respectively, exhibited the highest inhibition rate of HBsAg and HBeAg expression. In addition to inhibiting HBsAg and HBeAg expression, siRNA 4 and siRNA 6 can also dramatically reduced HBV DNA level. Our results showed that siRNAs targeting different HBV genome sites resulted in varying degrees of inhibition of HBV replication and antigen expression. The difference in inhibition among various siRNA may be attributed to the change of affinity between cellular siRNA binding protein(s) and the secondary and/or tertiary structures of target mRNA participating in the induction of RNAi activities (Hammond *et al*., 2001).

Though both siRNA 4 and 6 showed inhibitory effect on viral DNA, they were only efficient in the inhibition in its own targeted antigen expression. It should be very important if we could find the cocktail treatment with the capability of inhibiting multiple viral antigen expression and DNA replication. Our results clearly showed that combination of siRNA 4 and siRNA 6 is effective in the inhibition of HBsAg, HBeAg, HBV DNA, and HBV pre-genomic mRNA level and at the same time, is more robust than that achieved by individual use of each siRNA in the combination. This result indicates the feasibility of developing siRNA combination into an effective therapeutic treatment in the future.

Combination of siRNA 4 with siRNA 6 was more effective in the inhibition of HBV replication and antigen expression compared with the use of any one though the final concentration of siRNAs in those treatments were the same. This phenomenon was also observed in other RNAi experiment targeting endogenous gene in our laboratory. Current knowledge for the explaining this phenomenon is not available. We propose that mRNA molecules in the cell may also have a repairing mechanism like DNA when it is cleaved by biological, chemical, or physical forces. Combination of siRNAs, will cleave multiple sites in the mRNA target and make it difficult for the target to be repaired. So it will achieve greater efficiency than the individual use of siRNA.

During viral infection, HBV shows a large number of mutations in the HBV genome due to the high rate of replication and the lack of proofreading by reverse transcriptase (Fung and Lok, 2004). However, the absolute conservation of base pairing between the siRNA and its target is required for efficient RNAi, and even a single base mismatch in the targeting region can nullify its RNAi effect (Hammond *et al*., 2001). Therefore, combinations of siRNAs targeting various conserved regions in the HBV genome may delay or prevent the emergence of resistant viral mutants.

Though vector-based siRNA approach showed the same efficacy as synthetic siRNA with longer duration (Brummelkamp *et al*., 2002; Zhao *et al*., 2003; Liu *et al*., 2004), the uncertainty of its long-term toxicity will prevent them from clinical use. The combination of synthetic siRNA open a new window for developing the great technology into an effective therapy in future.

CONCLUSION

HBV infection is a serious disease with multiple viral genes involved. The ideal therapy for the disease is to identify an antiviral approach with greater efficiency and less toxicity. In this study, we found that the cocktail of siRNAs targeting various regions of viral mRNAs not only shows greater efficacy in the inhibition of viral replication, but also mediates greater suppression of multiple viral genes involved in the pathogenesis. Our findings strongly suggest the feasibility of developing combined siRNAs technology as an efficient anti-HBV approach in the future. By the way, as chemically synthetic siRNAs were cocktailed in our combination, it should exclude the potential long-term and uncertain toxicity induced by vector-based siRNA approach in vivo. It has been reported that transgene silencing can be induced by the introduction of siRNAs into adult mouse liver (McCaffrey *et al*., 2003; Hilla *et al*., 2003). Future

studies are needed to explore the specific delivery of siRNA to liver cells in vivo and assess the applicability of the therapeutic potential of siRNAs for treatment of HBV infections and liver cancer by knocking down oncogenes in hepatocytes.

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