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Inhibition of Hepatitis B virus gene expression by single and dual small interfering RNA treatment

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Abstract

RNA interference (RNAi) has been successfully applied in suppression of Hepatitis B virus (HBV) replication. To circumvent the problem that mutation in HBV genome may result in resistance when siRNA is further developed as an anti-viral drug, in this study, we established a dual small interfering RNA (siRNA) expression system, which could simultaneously express two different siRNA molecules that can specifically target two genes. To test the effectiveness of this system, we applied this new approach to express simultaneously two different 21-bp hairpin siRNA duplexes that specifically attack the HBs and HBx genes of HBV, respectively, in Bel-7402 and HepG2.2.15 cells. Results indicated that dual siRNA could simultaneously inhibit the expression of HBs and HBx gene by 83.7% and 87.5%, respectively, based on luciferase assays. In addition, dual siRNA molecules were able to significantly reduce the amount of HBV core associated DNA, which is considered as an intracellular replicative intermediate, and the viral DNA in culture supernatant. Therefore, this dual siRNA system provides a more powerful tool for the study of gene function and implicates a potential application in the treatment of viral infection.
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Keywords: Dual siRNA; HBV; Gene expression

1. Introduction

RNA interference (RNAi) is a natural process of eukaryotic cells by which double-stranded RNA initiates and directs the degradation of homologous mRNA (Hannon, 2002). This RNA silencing mechanism was first described in *Caenorhabditis elegans* and *Drosophila melanogaster* (Fire et al., 1988). It has many similarities to the post-transcriptional gene silencing in plants. Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21- to 23-nucleotide double-stranded small interfering RNA (siRNA) (Elbashir et al., 2001; Paul et al., 2002) or, alternatively, by the transcription of siRNA from a DNA construct driven by the RNA polymerase cassette (Brummelkamp et al., 2002). These findings open up a new field for the analysis and control of the processes of gene expression, and perhaps pathogen infection.

The replication of a growing number of human pathogenic viruses in cell culture was shown to be inhibited by RNAi, including poliovirus (Coburn and Cullen, 2002), HIV-1 (Jacque et al., 2002; Lee et al., 2002), flock house virus (FHV) (Dector et al., 2002), Rous sarcoma virus (Hu et al., 2002) dengue virus (Adelman et al., 2002), hepatitis C virus (HCV) (Kapadia et al., 2003) replicons, influenza virus (Ge et al., 2003), hepatitis B virus (HBV) (Hamasaki et al., 2003; McCaffrey et al., 2003), HPV (Jiang and Milner, 2002). Recently, it was reported that RNAi could also induce transcriptional silencing of SARS coronavirus (He et al., 2003). In most above studies, synthetic 21-nucleotide double-stranded siRNAs were applied. However, vector based RNAi

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techniques were used more frequently in recent studies. Each vector expresses unique siRNA that can degrade a specific target.

Eight genotypes (A–H) of HBV have been described. The number of HBV carriers worldwide has been estimated to be more than 400 million. These individuals have a 15–25% risk of developing liver diseases such as liver cirrhosis and hepatocellular carcinoma (Kao and Chen, 2002). Although a few drugs were developed against HBV infection, the success rate of these treatments, however, is low and frequently infections reoccur (Carreno et al., 1992; Lai et al., 1997). The fact that RNAi can be applied for blocking the replication of HBV in several reports provided insights into the field of controlling infectious human hepatitis. Nevertheless, mutations in HBV genome may result in viral resistance to siRNA. It has been reported that HIV-1 can escape from RNAi-mediated inhibition due to nucleotide change in the genome (Das et al., 2004). One strategy to circumvent the problem is to choose target in the relatively conserved DNA sequence. The other approach is to produce multiple siRNAs that target different sites or genes on the viral genome. We here established a system that can express two siRNA duplexes simultaneously and target the S and X genes of HBV, respectively. To study the effects of dual RNAi on HBV gene expression in a cell culture model, we used a derivative of the human HepG2 hepatoma cell line, HepG2.2.15, which has been stably transformed with several copies of the HBV genome and used as an in vitro model for HBV replication. The effects of dual siRNA system on HBV gene expression were investigated in this study.

2. Methods

2.1. Cell lines and condition of transfections

Two human hepatoma cell lines, Bel-7402 and HepG2.2.15 were maintained in Dulbecco's modified Eagle medium (GIBCO/BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37 °C under 5% CO_2. Cells were seeded onto 24-well plates at a density of 1.0×10^5 or 4.0×10^5 cells per 24-well plate or 6-well plate and grown to the confluence reaching approximately 60% at the time of transfection. Cells were transfected with 0.1 or 0.4 μg of plasmid pCMV-HBS together with 0.45 or 1.2 μg pSliencer-2.1-U_6-siRNA, using Sofast^{TM} transfection reagent (Xiamen Sunma Biotechnology Co. Ltd., China) according to the protocol provided by the manufacturer. The cells were harvested 48 h after transfection.

2.2. Plasmid construction

Full-length HBV genomic DNA (subtype ayw) was cloned into the *Hin*dIII and *Sac*I sites of pBluescript (Stratagene) to generate the plasmid pBlue-HBV. HBs gene was cloned into the *Hin*dIII and *Sac*I sites of vector pCMV-tag2A (Stratagene) to yield plasmid pCMV-HBs. HBx gene was cloned into pCMV-Tag2A at *Eco*RI and *Xho*I sites to generate pCMV-HBx.

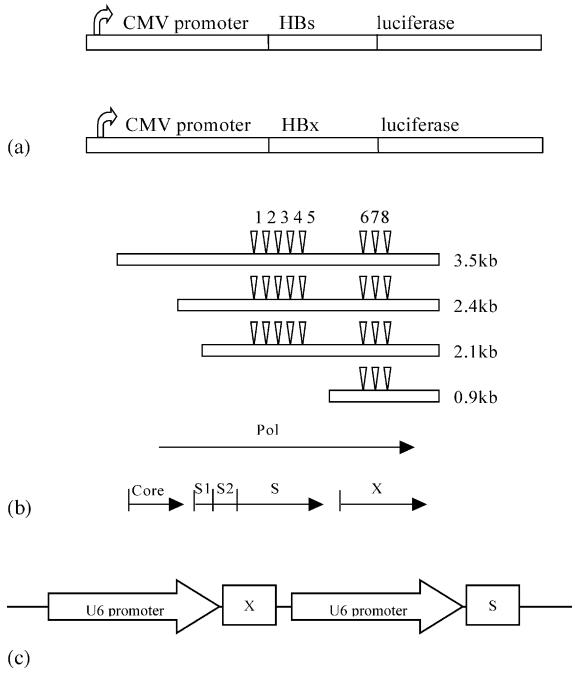

Fig. 1. Schematic diagrams of luciferase fusion genes, siRNA Targeting sites and dual siRNA expression cassettes. (a) Diagram of the two reporter fusion vectors, which contain targeted sequences of HBs or HBx gene and the luciferase report gene driving by the CMV promoter. (b) Locations of RNAi targeted sites and structure of the HBV genome. Downward arrows indicate the locations of RNAi target sites within the four HBV transcripts. The 3.5-kb transcript is the pregenomic RNA that serves as the template for HBV viral DNA replication. The HBV open reading frames are shown below aligned with the HBV mRNAs. Pol, polymerase; core, HBcAg; S1, large presurface antigen; S2, mid pre-surface antigen; S, HBsAg; X, X gene. The numbers above the arrows indicate the siRNA target sites. 1 = HBS_1siRNA, 2 = HBS_2siRNA, 3 = HBS_3siRNA, 4 = HBS_4siRNA, 5 = HBS_5siRNA, 6 = HBX_1siRNA, 7 = HBX_2siRNA, 8 = HBX_3siRNA. (c) Diagram of dual siRNA expression cassettes.

Two-pair of primers 5′-CTGCGAGATCTATGGAGAGC-TCACATCAGGATTC-3′ (sense), 5′-GTTAGGTCGACAA-TGTATACCCAAAGACAAAAAGAA-3′ (antisense) or 5′-GATCATACGCGTAAGCTTTTCATTTATTGATCAT-3′ (sense), 5′-GTCGGGGCTTCATTCACTCGTCTAGAAC-TGAT-3′ (antisense) were used to amplify the HBs and HBx gene, respectively. The PCR products were then cloned into *Sal*I and *Bgl*II sites of plucF to generate plasmid plucF-HBs and plucF-HBx (Fig. 1a), in which the HBs or HBx were fused in frame with the luciferase gene and the expression of the fusion gene was drove by the CMV promoter (Fig. 1a).

2.3. Generation of siRNA expression vectors

Five regions of the HBs gene and three regions of the HBx gene were selected as the targeted sequences of siRNA in this study (Fig. 1b).

To construct single siRNA expression vector, two 64nt primers, each containing a 19nt target sequence in the sense and antisense forms from different regions of the HBs gene or HBx gene as indicated below, were systhesized (Invitrogen):

5′-GCTCCCGCGTGTCTTGGCC-3′ (HBS_1siRNA);
5′-GGTGGACTTCTCTCAATTT-3′ (HBS_2siRNA);
5′-GCCAAAATTCGCAGTCCC-3′ (HBS_3siRNA);
5′-GTTGCTGTACCAAACCTT-3′ (HBS_4siRNA);
5′-GCTCAGTTTACTAGTGCCA-3′ (HBS_5siRNA);
5′-GCACTTCGCTTCACCTCTG-3′ (HBX_1siRNA);
5′-GCAATGTCAACGACCGACC-3′ (HBX_2siRNA);
5′-GTTTAAAGACTGGGAGGAG-3′ (HBX_3 siRNA).

Sense and antisense primers were then cloned into pSilence-2.1-U_6 plasmid (Amibion) at *Bam*HI and *Hin*dIII sites after annealing according to the manufacturer's instructions.

To generate the dual siRNA expression plasmid, two primers 5′-GCTGATGACGTCAGTGGAAAGACGCG-3′-(sense) and 5′-TCAGCGAATTCACGCCAAGCTTTTCC-3′ (antisense) were designed to amplify a DNA fragment containing U6 promoter and HBX_2 siRNA expression cassette from recombinant plasmid pSilencer-2.1-U6-HBX_2. The PCR product was then cloned into *Aat*II and *Eco*RI sites of plasmid pSilencer-2.1-U6-HBS_2 to generate recombinant plasmid pSilencer-2.1-U6-HBSX, which carries two independent siRNA expression cassettes (Fig. 1c).

2.4. Luciferase assay

Bel-7402 cells were co-transfected with reporter plasmids and siRNA expression plasmids. Cells were washed with PBS and lysed with luciferase cell culture lysis reagent (Promega). Ten microliters of the cell lysates and 100 μl of luciferase assay substrate (Promega) were mixed and fluorescence intensity was detected by the luminometer (Turner T20/20). Assays were performed in triplicate, and expressed as means ± S.D. relative to vector control as 100%.

2.5. Hepatitis B surface antigen (HBsAg) assay

Bel-7402 cells and HepG2.2.15 cells were transfected with siRNA expression plasmids, the level of HBsAg protein in culture media from transfected cells were then determined by enzyme-linked immunosorbent assay using a HBV diagnostic kit (Shanghai Kehua Biotech Co. Ltd.). Assays were performed in triplicate independent experiments.

2.6. RNA isolation and RT-PCR assay

Bel-7402 cells and HepG2.2.15 cells were transfected with siRNA expression plasmids, total RNA were then extracted from transfected cells by Trizol Reagent (Invitrogen) according to the method described in the manufacturer's manual. Reverse transcription were performed with total RNA as the template. The cDNAs were synthesized with HBs or HBx gene specific primers, 5′-GCGGGGTTTTTCTTGTTGAC-3′ (sense), 5′-CTACGAACCACTGAACAAAT-3′ (antisense) or 5′-CCTGCGCGGGACGTCCTTTG-3′ (sense), and 5′-CAGTCTTTGAAGTATGCCTC-3′ (antisense).

2.7. Assays for HBV core associated DNA and HBV DNA by real time-PCR

To assay the effect of siRNAs on HBV replication, intracellular core-associated HBV DNA was extracted by the method described previously (Pugh et al., 1988). Briefly, 1×10^5 transfected HepG2.2.15 cells were lysed and centrifuged at 25 °C. Magnesium chloride was added to the supernatant. DNA not protected by HBV core was treated digested with deoxyribonuclease (DNase I). Then the lysates were treated with proteinase-K and, after phenol/chloroform extraction; core-associated HBV DNA was recovered by ethanol precipitation, and quantified by real time-PCR (RT-PCR) as described by the manufacturer (PG BIOTECH, Shenzhen, China). The HBV DNA in the supernatants was also quantified following the procedure provided by the manufacturer (PG BIOTECH, Shenzhen, China). Primers used in RT-PCR were: P1, 5′-ATCCTGCTGCTATGCC-TCATCTT-3′ and P2, 5′-ACAGTGGGGAAAGCCCTA-CGAA-3′. The probe was 5′-TGGCTAGTTTACTAGTGC-CATTTTG-3′. PCR reaction was carried out and analyzed by a PE Gene Amp 7700 (Perkin-Elmer, USA).

3. Results

3.1. Effects of HBV-specific siRNA treatment on the expression of HBx-luciferase and HBS-luciferase fusion genes

To efficiently screen siRNA molecules, selected targeting DNA sequences were fused in frame with that of luciferase gene, in which luciferase activity was supposed to represent the level of HBs or HBx mRNA expression. Cells were co-transfected with pLucF-HBs or pLucF-HBx and eight single siRNA expression vectors, respectively. Luciferase activities were then determined from those transfected cells. Result showed that HBS_1siRNA, HBS_2siRNA and HBX_2siRNA strongly inhibited luciferase activities by 81.5%, 80.5%, and 76.5%, respectively, comparing to that of vector control (Fig. 2a and b). These results indicated that the three siRNAs could efficiently degrade the mRNA of HBs-luciferase or HBx-luciferase fusion gene.

To evaluate the effects of dual siRNA expression plasmid on the inhibition of HBs-luciferase or HBx-luciferase fusion gene expression, cells were co-transfected with pLucF-HBs or pLucF-HBx and the dual siRNA expression plasmid pHBSXsiRNA. Result from luciferase activity assays indicated that there was a further reduction in luciferase activity by dual

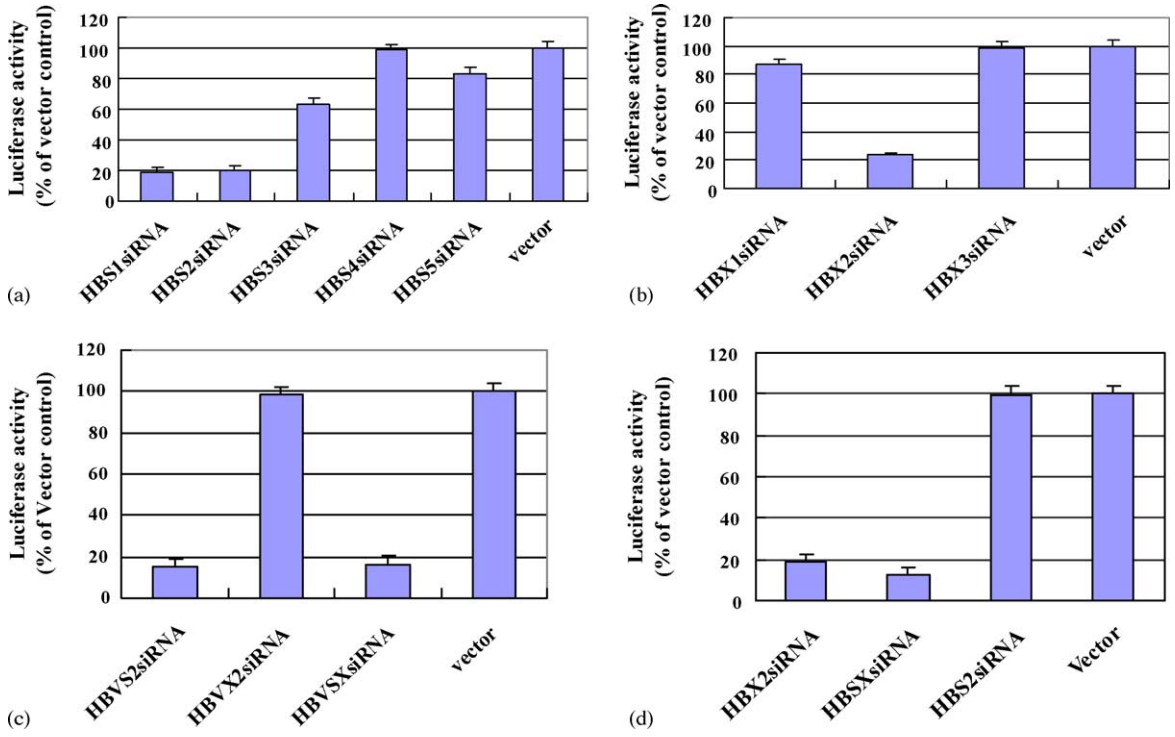

Fig. 2. Quantitative analysis of luciferase activity in cells after transfected with siRNA expression plasmids. (a) Bel-7402 cells were co-transfected with pLucF-HBs plasmid and pSliencer-2.1-U_6-siRNA (HBS_1siRNA, HBS_2siRNA, HBS_3siRNA, HBS_4siRNA, HBS_5siRNA) plasmids; pSliencer-2.1-U_6 plasmid was used as a control. (b) Bel-7402 cells were co-transfected with pLucF-HBx plasmid and pSliencer-2.1-U_6-siRNA (HBX_1siRNA, HBX_2siRNA, HBX_3siRNA) plasmids; pSliencer-2.1-U_6 vector was used as a control. (c) Bel-7402 cells were co-transfected with pLucF-HBs and pSliencer-2.1-U_6-siRNA (HBS_2siRNA, HBX_2siRNA, HBSXSiRNA) plasmids; pSliencer-2.1-U_6 was used as vector control. (d) Bel-7402 cells were co-transfected with pLucF-HBx and pSliencer-2.1-U_6-siRNA (HBX_2siRNA, HBSXSiRNA, HBS_2siRNA); pSliencer-2.1-U_6 was used as control. Forty-eight hrs after transfection, cells were lysed and luciferase activities were determined by luminometer.

siRNA duplexes (HBSXsiRNA) comparing to that of single siRNA expression vectors (HBS_2siRNA or HBX_2siRNA). The reduction rate of luciferase activity caused by HBSXsiRNA was 83.7% to HBs and 87.5% to HBx, respectively (Fig. 2c and d).

3.2. Effects of HBs specific siRNA treatment on the levels of HBsAg production

To evaluate the influence of RNAi on HBS gene expression, Bel-7402 cells were transfected with pSilence2.1-U6-siRNA, pCMV-HBs or HBSXsiRNA and HepG2.2.15 cells were transfected with pSilence2.1-U6-siRNA or HBSX siRNA. HBsAg concentrations in the culture media of transfected and control cells were measured 2 days after transfection by ELISA using HBV diagnostic kit. Results showed that HBsAg level was decreased in the Bel-7402 cells after transfection with HBS_1siRNA, HBS_2siRNA or HBSXsiRNA with reduction rate of 91.5%, 88.5% ,and 83.7%, respectively (Fig. 3a and d). In HepG2.2.15 cells, transfection with HBS_1siRNA, HBS_2siRNA, or HBSXsiRNA reduced HBsAg level by 75.4%, 85.7%, and 87.6%, respectively (Fig. 3b and c). In addition, transfection with HBX_2siRNA reduced the level of HBsAg production by 65.3% in HepG2.2.15 cells (Fig. 3e).

3.3. Inhibition of HBs and HBx gene expression by siRNA treatment

To determine whether siRNAs specificly degrade HBs or HBx mRNA, we used semi-quentitation RT-PCR analyses to determine the levels of HBs or HBx mRNA in two different cell lines, Bel-7402 (Fig. 4a and b) and HepG2.2.15 (Fig. 4c and d), 2 days after transfection. Results indicate that the levels of HBs mRNA were significantly decreased by the treatment of HBSXsiRNA (Fig. 4a, lane 1), HBS_1siRNA (Fig. 4a, lane 2), HBS_2siRNA (Fig. 4a, lane 3) in Bel-7402. The levels of HBx mRNA were also decreased by the treatment of HBSXsiRNA (Fig. 4b, lane 1), HBX_2siRNA (Fig. 4b, lane 2), but not by that of pSliencer-2.1-U_6 or untreated cells (Fig. 4b, lanes 3 and 4). Similar results were also obtained in HepG2.2.15 cell lines under the same conditions of siRNA treatments (Fig. 4c and d). Results showed that the levels of HBs mRNA were dramatically reduced in HepG2.2.15 cells after the treatment of HBSXsiRNA (Fig. 4c, lane 6), HBS_1siRNA (Fig. 4c, lane 7) and HBS_2siRNA (Fig. 4c, lane 8), respectively. The levels of HBx mRNA in HepG2.2.15 cells were also reduced by the treatment of HBSXsiRNA (Fig. 4d, lane 2), HBX_2siRNA (Fig. 4d, lane 3), but not by that of pSliencer-2.1-U_6 or untreated cells (Fig. 4d, lanes 1 and 4). In addition, our results showed that the inhibition

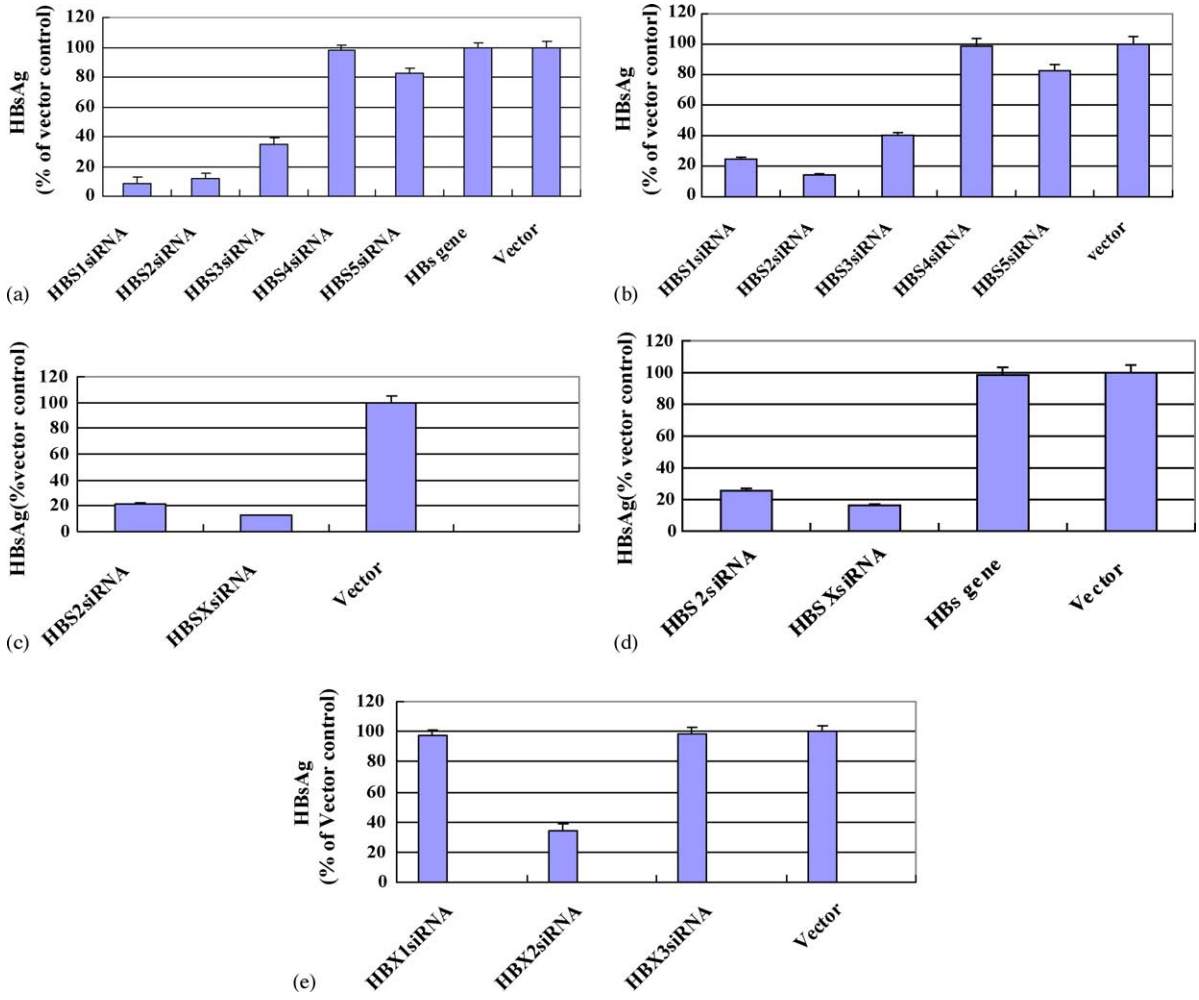

Fig. 3. Determination of the effectiveness of siRNAs on levels of HBsAg expression. pSliencer-2.1-U_6 plasmid was used as vector control in all transfection experiments. HBsAg levels were determined by ELISA using a HBV diagnostic kit. (a) Bel-7402 cells were co-transfected with pCMV-HBs and pSliencer-2.1-U_6-siRNA plasmids expressing HBS_1siRNA, HBS_2siRNA, HBS_3siRNA, HBS_4siRNA and HBS_5siRNA. (b) HepG2.2.15 cells were transfected with pSliencer 2.1-U_6-siRNA plasmids expressing HBS_1siRNA, HBS_2siRNA, HBS_3siRNA, HBS_4siRNA and HBS_5siRNA. (c) HepG2.2.15 cells were transfected with pSliencer-2.1-U_6-siRNA plasmids expressing HBS_2siRNA and HBSXsiRNA. (d) Bel-7402 cells were co-transfected with pCMV-HBs and pSliencer-2.1-U_6-siRNA plasmids expressing HBS_2siRNA and HBSXsiRNA. (e) HepG2.2.15 cells were transfected with pSliencer2.1-U_6-siRNAs plasmids expressing HBX_1siRNA, HBX_2siRNA, HBX_3siRNA.

effects of dual siRNA, HBSXsiRNA on the levels of HBs and HBx mRNA (Fig. 4a (lane 1), b (lane 1), c (lane 6), and d (lane 2)) were more sever than that of single siRNA (Fig. 4a, (lanes 2 and 3), b (lane 2), c (lane 7 and 8), and d (lane 3)).

3.4. Effects of siRNA on the viral DNA replication

3.4.1. Analysis HBV core associated DNA and HBV DNA in the supernatants

To determine the effectiveness of siRNAs on viral DNA replication, HBV core associated DNA (as an intracellular replicative intermediate) and HBV DNA were extracted from HepG2.2.15 cells transfected with HBSXsiRNA, HBS1siRNA, HBS2siRNA, HBX2siRNA, and vector, respectively. The levels of HBV core associated DNA and HBV DNA were determined by real time PCR. Results indicated that the levels of HBV core associated DNA were significantly decreased in the cells transfected by HBSXsiRNA, HBS_1siRNA, HBS_2siRNA, and HBX_2siRNA with reduction rate of 90.2%, 85.7 %, 81.3%, and 60.4%, respectively, compared with that of vector control (Fig. 5a). In HepG2.2.15 cells, transfection with HBSXsiRNA, HBS_1siRNA, HBS_2siRNA and HBX_2siRNA reduced the level of viral DNA in supernatants media by 88.7%, 82.6%, 78.4%, and 58.3%, respectively (Fig. 5b).

4. Discussion

It has been attracted considerable attentions in the use of RNAi as therapeutics to treat a variety of diseases, including tumors and viral infections. Hamasaki et al. (2003) demonstrated that RNAi could attenuate the replication of HBV genome in cell culture. Shlomai and Shaul (2003) used

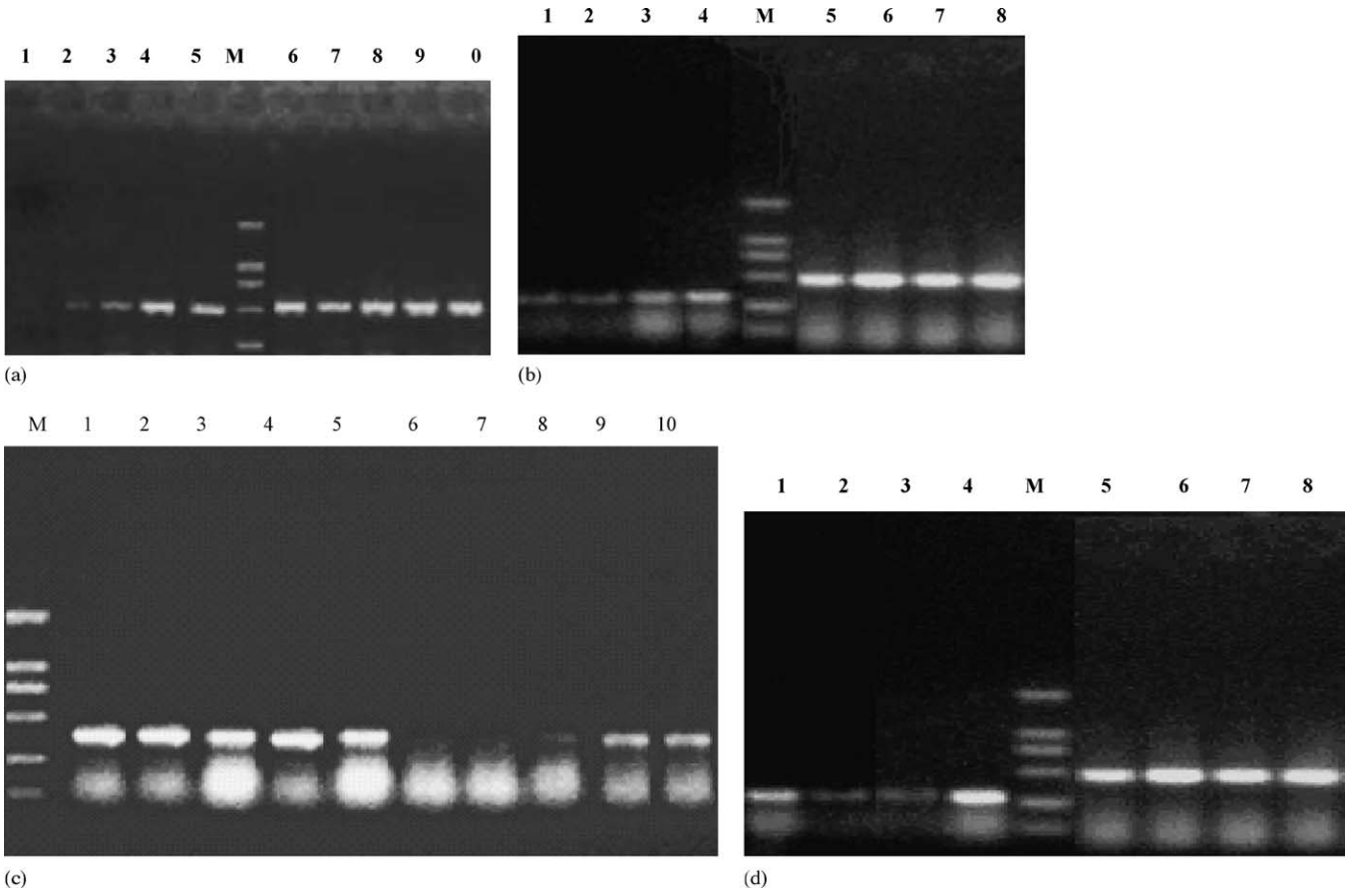

Fig. 4. RNA expression level determined by semi-quentitated RT-PCR analysis. Total RNA was used as template to synthesize cDNA. HBs specific primers were then applied for detection in (a and c), and HBx specific primers in (b and d). (a) Bel-7402 cells were co-transfected with pCMV-HBs and HBSXsiRNA (lane 1), HBS_1siRNA (lane 2), HBS_2siRNA (lane 3), and pSliencer-2.1-U_6 (lane 5, as a vector control). As control, Bel-7402 cells were also transfected with pCMV-HBs only (lane 4). (b) Bel-7402 cells were co-transfected with pCMV-HBx and HBSXsiRNA (lane 1), HBX_2siRNA (lane 2) or pSliencer-2.1-U_6 (lane3, as a vector control). As control, Bel-7402 cells were also transfected with pCMV-HBx only (lane 4). Lanes 5–8 were β-actin controls. Lane M indicates DNA markers. (c) HepG2.2.15 cells were transfected with HBSXsiRNA (lane 6), HBSsiRNA (lane 7), HBS_1siRNA (lane 8) or pSliencer-2.1-U_6 (lane 9, as a vector control). Cells without transfection were used as controls (lane 10). Lanes 1–5 were β-actin controls. (d) HepG2.2.15 cells were transfected with HBSXsiRNA (lane 2), HBX_2siRNA (lane 3), or pSliencer-2.1-U_6 (lane 1, as a vector control). Cells without transfection were used as controls (lane 4). Lanes 5–8 were β-actin controls.

a similar approach to inhibit the replication and expression of HBV in HepG2.2.15 cell line, in which all HBV proteins could be expressed. Recently, McCaffrey et al. (2003) went further in this field by showing that RNAi were function well in transgenic mice. These reports demonstrate that siRNA treatments can be used to suppress HBV in cell cultures and animal models as well as provided insights into the application of controlling infectious human hepatitis.

In this study, we applied a different approach by designing a pair of 64nt primers that contain a specific 19nt target sequence from HBV genome to create recombinant pSilencer-U6 plasmid. Primers were annealed and cloned into *Bam*HI–*Hin*dIII sites of the pSilencer2.1-U6 vector. In order to construct a useful tool to choose the most effective siRNA molecules, we created a quick screening vector plucF by fusing the targeted sequence and the reporter luciferase gene together to produce recombinant plucF plasmid, which could express HBs-luciferase or HBx-luciferase fusion mRNAs. Therefore, we can initially select the suitable siRNA duplexes rapidly by simply analyzing the activities of lucifearse. By using this approach, we have identified two siRNA molecules (HBS_1siRNA and HBS_2siRNA) having significant impact on the HBs-luciferase fusion gene expression and one siRNA duplex (HBX_2siRNA) having effects on the expression of HBx-luciferase fusion gene. This provides a quick approach to select effective siRNA in the study of gene expression and function analysis.

To further study the effects of selected RNAi molecules on HBV gene expression and viral replication in a cell culture models, we used a derivative of the human HepG2 hepatoma cell line, HepG2.2.15, which has been stably transformed with several copies of the HBV genome and used as an in vitro model for HBV replication. The effects of dual siRNA system on HBV gene expression and viral replication were studied thoroughly by the analyzing the levels of viral protein production through enzyme-linked immunosorbent assay and

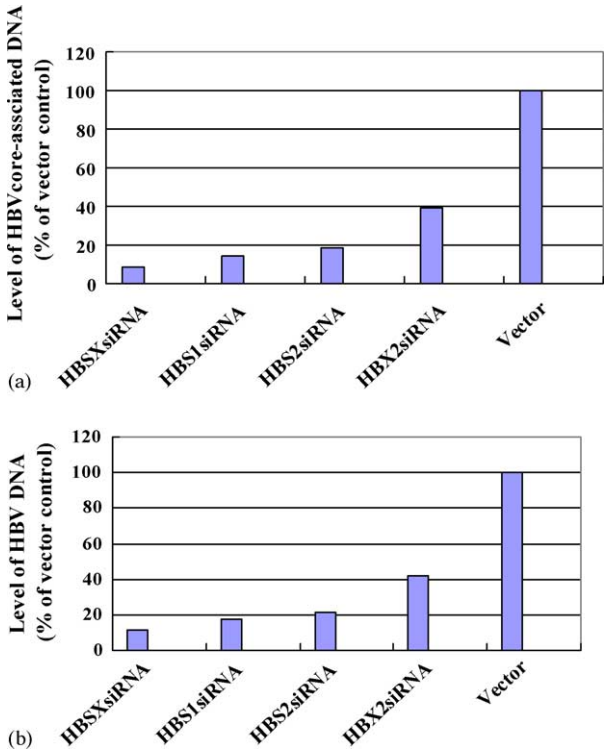

Fig. 5. Determination of the effectiveness of siRNAs on HBV DNA replication by RT-PCR. HepG2.2.15 cells were transfected with pSliencer 2.1-U_6-siRNAs (HBSXsiRNA, HBS_1siRNA, HBS_2siRNA and HBX_2siRNA). pSliencer-2.1-U_6 was used as vector control. (a) HBV core associated DNA in HepG2.2.15 cells was detected by RT-PCR. (b) The viral DNA in the transfected culture media was measured by RT-PCR.

the levels of viral RNA expression by semi-quantitated RT-PCR analysis. All results indicated that HBS_1siRNA, HBS_2siRNA, and HBX_2siRNA had significant reduction effects on viral mRNA expression, and viral protein production.

The fact that mutation in HBV genome may result in resistance if siRNA molecules were further developed as antiviral drugs raised our concerns. Our strategies to address such potential problems are to choose targets in the relatively conserved DNA sequences and to generate multiple siRNA molecules that can target different sites or genes on the viral genome. To test our approach, in this study we established a system that can simultaneously express two siRNA duplexes from a single vector that can attack the S and X genes of HBV, respectively. Results from luciferase activity assay, enzyme-linked immunosorbent assay and semi-quantitated RT-PCR analysis were consistently showed that the dual siRNA molecules had synergetic effects or more efficient on the targeted viral protein production and HBs and HBx gene expression comparing to that of the single siRNA molecules. More importantly, dual siRNA could simultaneously inhibit the expression of HBs and HBx gene by 83.7% and 87.5%, respectively. Therefore, this dual siRNA system could provide a more powerful tool for the study of gene function and could be used as a potential application in the treatment of viral infection.

In the last 20 years, HBV infection affects millions of people each year worldwide. Current therapies of HBV infection including immune modulators such as interferon Alfa, or nucleoside analogs such as lamivudine have provided some degree of cures, but the efficiency of treatment was limited. As a potential therapy, siRNA seems to be a hopeful alternative strategy. We believe that our approach presented in this study could be broadly used. For example, it could be used to generate more than two siRNAs duplexes that could silent more genes in order to study the interactions of genes and their functions. Such strategies of constructing multiple-siRNA vectors can confront the evading mechanism of virus infections. Obviously, this cocktail approach would be benefit to application of siRNA therapy in viral infections, especially to those viruses with high mutation rate. In addition, this approach could also used to deal with two or more viruses, which are especially useful in the treatment of co-infections by two or more pathogens, such as HBV–HIV and HCV–HIV. We are in the process of testing these approaches.

Acknowledgments

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References

Adelman, Z.N., Sanchez-Vargas, I., Travanty, E.A., Carlson, J.O., Beaty, B.J., Blair, C.D., Olson, K.E., 2002. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J. Virol. 76, 12925–12933.

Brummelkamp, T.R., Bernards, R., Agami, R., 2002. Asystem for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.

Carreno, V., Castillo, I., Molina, J., Porres, J.C., Bartolome, J., 1992. Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. J. Hepatol. 21, 6041–6048.

Coburn, G.A., Cullen, B.R., 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. 76, 9225–9231.

Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R., Berkhout, B., 2004. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol. 78 (5), 2601–2605.

Dector, M.A., Romero, P., Lopez, S., Arias, C.F., 2002. Rotavirus gene silencing by small interfering RNAs. EMBO Rep. 3, 1175–1180.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1988. Potent and specific genetic interference by double-stranded RNA in *Caenorbabditis elegans*. Nature 391, 806–811.

Ge, Q., McManus, M.T., Nguyen, T., Shen, C.H., Sharp, P.A., Eisen, H.N., Chen, J., 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 100, 2718–2723.

Hamasaki, K., Nakao, K., Matsumoto, K., Ichikawa, T., Ishikawa, H., Eguchi, K., 2003. Short interfering RNA-directed inhibi-

tion of hepatitis B virus replication. FEBS Lett. 543, 51–54.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

He, M.L., Zheng, B., Peng, Y., Peiris, J.S., Poon, L.L., Yuen, K.Y., Lin, M.C., Kung, H.F., Guan, Y., 2003. Inhibition of SARS-associated coronavirus infection and replication by RNA interference. JAMA 290, 2665–2666.

Hu, W.Y., Myers, C.P., Kilzer, J.M., Pfaff, S.L., Bushman, F.D., 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr. Biol. 12, 1301–1311.

Jacque, J.M., Triques, K., Stevensom, M., 2002. Modulation of HIV-1 replication by RNA interference. Nature 418, 435–538.

Jiang, M., Milner, J., 2002. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 21, 6041–6048.

Kao, J.H., Chen, D.S., 2002. Global control of hepatitis B virus infection. Lancer Infedt Dis. 2, 395–403.

Kapadia, S.B., Brideau-Andersen, A., Chisari, F.V., 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc. Natl. Acad. Sci. U.S.A. 100, 2014–2018.

Lai, C.L., Ching, C.K., Tung, A.K., Li, E., Young, J., Hill, A., Wong, B.C., et al., 1997. Lamivudine is effective in suppressing hepatitis B virus DNA in Chinese hepatitis B surface antigen carriers: a placebo-controlled trial. Hepatology 25, 241–244.

Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsami, A., Salvaterra, P., et al., 2002. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat. Biotechnol. 20, 500–505.

McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L., Kay, M.A., 2003. Inhibition of hepatitis B virus in mice by RNA interference. Nat. Biotechnol. 21, 639–644.

Paul, C.P., Good, P.D., Winer, I., Engelke, D.R., 2002. Effective expression of small interfering RNA in human cells. Nat. Biotechnol. 20, 505–508.

Pugh, J.C., Yaginuma, K., Koike, K., Summers, J., 1988. Duck hepatitis B virus(DHBV) particles produced by transient expreesion of DHBV DNA in a human hepatoma cell line are infectious in vitro. J. Virol. 62, 3516–3531.

Shlomai, A., Shaul, Y., 2003. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 37, 764–770.