Inhibition of hepatitis B virus gene expression by small interfering RNAs targeting cccDNA and X antigen

Q. XIE[1a], S. ZHANG[1a], W. WANG[1], Y. M. LI[1], T. DU[1], X. L. SU[1], Y. Q. WEI[1], H. X. DENG[1,2*]

[1]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu 610041, P.R. China; [2]Laboratory Animal Center, Sichuan University, Chengdu, Sichuan, P.R. China

Summary. – To test the possible inhibition of hepatitis B virus (HBV) replication and expression by small interfering RNAs (siRNAs) targeting simultaneously covalenthy closed circular DNA (dnacccDNA) and X antigen, corresponding recombinant plasmids were transfected into HepG2.2.15 cells and the levels of cccDNA, HBXAg, HBcAg, and HBeAg were assayed at various times post transfection. As expected, the single siRNAs showed marked inhibitory effects but their combination was even more efficient. These results provide a new insight into the development of a potential anti-HBV strategy of enhancing the efficacy of individual antivirals and overcoming the high mutation rate of HBV.

Keywords: hepatitis B virus; small interfering RNA; cccDNA; X antigen

Introduction

Hepatitis B virus (HBV) infection is a major health problem with more than 350 million chronic carriers worldwide, who are at high risk for developing liver cirrhosis and hepatocellular carcinoma (Beasley *et al.*, 1981; Lupberger and Hildt, 2007). The mechanisms of HBV pathogenicity remain unclear and molecular-biological studies are required. Although the oral cytosine nucleoside analogue lamivudine (3TC) has been clinically used for the treatment of chronic HBV infection and potently inhibited HBV replication by interfering with HBV reverse transcriptase activity (Doong *et al.*, 1991; Dienstag *et al.*, 1995), 3TC-resistant HBV strain has been reported in long-term therapy (Li *et al.*, 2007). Thus, a new potential agent for anti-HBV therapy should be evaluated with special focus on its inhibitory effect against the recurrence of HBV infection.

The HBV genome is a partially double-stranded circular DNA of 3.2 kb that is transcribed into 3.5-, 2.4-, 2.1-, and 0.7-kb viral transcripts containing four open reading frames (denoted S, C, P, and X). cccDNA serves as the template for viral transcription during HBV replication and its production is regulated and amplified by an intra-cellular pathway, in which newly synthesized genomic DNA is recycled to the nuclei (Tuttleman *et al.*, 1986; Rollier *et al.*, 1999). However, the elimination of cccDNA in infected hepatocytes remains a challenge in therapy of HBV-infected patients. The HBX gene is the smallest HBV gene, coded by 462 nt, and encodes a 17 kDa protein with 154 aa (Fujiyama *et al.*, 1983). This protein is a pleiotropic transactivator, which stimulates HBV promoters and enhancers as well as a wide range of other viral promoters through protein–protein interactions (Spandau *et al.*, 1988; Nakatake *et al.*, 1993; Fiedler *et al.*, 2006). Since the HBV X protein antigen (HBXAg) is critically implicated in HBV-mediated hepatic cell cancer (HCC) and consistently expressed in all four viral mRNAs, it seems to be an attractive target for anti-HBV siRNA development.

In this study, we constructed plasmids expressing siRNAs that targeted HBV cccDNA and X gene, respectively. We tested their possible antiviral effects in HepG2.2.15 cells individually and in combination, assuming that the latter approach would be more efficient. The obtained results on reduction of the levels

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Abbreviations: cccDNA = covalently closed circular DNA; HBeAg = hepatitis B e antigen; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HBXAg = the HBV X protein antigen; HCC = hepatic cell cancer; IFA = immunofluorescence assay; RNAi = RNA interference; siRNA = small interfering RNA; 3TC = lamivudine

of cccDNA, HBXAg, hepatitis B core antigen (HBcAg), and hepatitis B e antigen (HBeAg) confirmed this assumption.

Materials and Methods

Plasmid constructs. Briefly, 19 nt-long inverted cccDNA sequences were cloned into the plasmid pGenesil 2.4, while 19 nt-long inverted HBXAg sequences were cloned into the plasmid pGenesil 2.1. Oligonucleotides used to code the sense strand of siRNA included siRNA-cccDNA (5′-GATCTCAATCTCGGGAATC-3′), siRNA-HBX (5′-GAGGACTCTTGGACTCTCA-3′), siRNA-HBX+ siRNA-cccDNA and control siRNA-HK (5′-GACTTCATAA GGCGCATGC-3′). The control sequence HK had no homology to any mammalian sequence. All plasmid constructs were verified by restriction analysis and DNA sequencing.

Cell cultures and transfection. HepG2.2.15, the human hepatoma cell line, was maintained in DMEM with 10% fetal bovine serum in 5% CO_2 humidified air as previously described (Li *et al.*, 2007). Cells were seeded at a density of $2×10^5$ cells per well in 6-well clustered plates. 24 hrs after incubation, the cells were transfected with 2 μg siRNA-expressing plasmid using the Lipofectamine 2000 reagent according to the manufacturer′s instructions (Sells *et al.*, 1987). One group was treated with 0.5 μmol/l 3TC. Forty-eight, 72, and 96 hrs after transfection, culture media were collected and cells were harvested for HBV replication assay. All experiments were performed in triplicate.

Real-time PCR. To measure the viral DNA, DNA was extracted from the transfected cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For quantification of HBV cccDNA by real-time PCR, 250 nmol/l of the probe (5′-TTTTTCAAGCCTCCAAGC TGTGCCTTG-3′) and 900 nmol/l of each PCR primer (F-TGTC TGCGGCGTTTTATCA, R-GGTCCCGTGCTGGTAGTTG) were used. The PCR was carried out in 50 μl volume using FTC2000 (Canada). The PCR program consisted of an initial denaturing step at 94°C for 4 min, followed by 35 amplification cycles at 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec.

RT-PCR. RNA expression level was determined by semi-quantitative RT-PCR analysis. Seventy-two hrs after transfection, total RNA was extracted directly from the transfected cells using Trizol reagent. The purified total RNA was reversely transcribed with ExScript RT reagent kit (TaKaRa Biotechnology, Dalian, China) following the manufacturer′s protocol. A RT-PCR experiment targeting GAPDH gene was run as an internal control. Primer sequences specific for the HBV X gene were: 5′-T CCCCGTCTGTGCCTTCTC-3′ (forward primer), 5′-GTGG TCACCATGCGACGTG-3′ (reverse primer). GAPDH primers were: 5′-GTCGGTGTGAACGGATTT-3′ (forward primer) and 5′-ACTCCACGACGTACTCAGC-3′ (reverse primer). The PCR amplification product was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide.

Western blot analysis. Ninety-six hrs after transfection, HepG2.2.15 cells were lysed with buffer containing 1% Triton X-100, 1% deoxycholate (Jin *et al.*, 2001). Protein concentrations were determined using the BCA Kit (Thermo scientific, Fremont, CA, USA) and equalized before loading. After centrifugation, 30 μg of total proteins was separated on 15% SDS-polyacrylamide gels, followed by blotting onto the PVDF membrane. The blot was blocked with 5% non-fat dried milk, probed with specific anti-HBXAg antibodies (Abcom, Cambridge, MA, USA) (Yun *et al.*, 2000; Feng *et al.*, 2010), and blots were developed with HRP-conjugated secondary antibodies and chemiluminiscent substrate on Kodak X-Ray films.

ELISA. To assess the effects of RNAi on viral antigen expression, HBeAg and HBsAg levels were measured by ELISA following the manufacturer's instructions (Zhongshan Golden Bridge Biotech, Beijing, P.R. China).

Immunofluorescence assay (IFA). Ninety-six hrs after transfection, HepG2.2.15 cells were fixed with 4 % paraformaldehyde for 10 min and washed three times with TBS. Cells were stained for HBcAg using anti-HBcAg (Zhongshan Golden Bridge Biotech, Beijing, China) antibody overnight at 4°C and then anti-mouse Cy3-conjugated secondary antibody for 1 hr at room temperature. After counterstaining with DAPI, cells were washed with TBS three times and viewed with a fluorescence microscope (Carl Ziess Microimaging, Jena, Germany).

Results

The effects of siRNA on HBV cccDNA level

HBV cccDNA is an important parameter for the therapy of chronic HBV infection. To evaluate whether siRNAs with specific target can effectively inhibit viral cccDNA, we isolated viral cccDNA from cells at 96 hrs post-transfection. Real-time quantitative PCR and the relative standard curve method were used to analyze the results.

Quantitative assay revealed that the HBV cccDNA levels in siRNA-cccDNA, siRNA-HBX, siRNA-HBX + siRNA-cccDNA, siRNA-HK and 3TC groups were in the 30.3%, 71.3%, 21.7%, 92.1%, and 59.8%, respectively, of that in control group. The greatest inhibitory effect was exhibited in the combined therapy group (siRNA-HBX + siRNA-cccDNA) (Fig. 1).

Effects of siRNAs on HBXAg, HBsAg, HBeAg, and HBcAg expression

As the precursor of the protein, the mRNA level has been the reflector, which provides information about the protein level at a certain distance (Xin *et al.*, 2008). It has been proved that the mRNA level was markedly reduced at 96 hrs post transfection with siRNA (Dykxhoorn *et al.*, 2005). To assess whether siRNAs specifically inhibited HBX mRNA, we used semi-quantitative RT-PCR analysis to determine
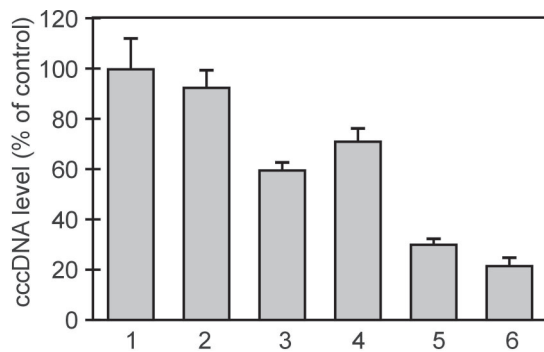
Fig. 1

Effects of siRNAs on the HBV cccDNA level

Real-time PCR. Levels of cccDNA in cells exposed to blank (1), siRNA-HK, negative control (2), 3TC (3), siRNA-HBX (4), siRNA-cccDNA (5), siRNA-HBX + siRNA-cccDNA (6).
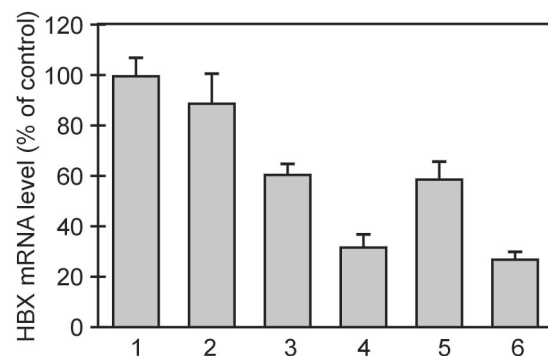

Fig. 2

Effects of siRNAs on HBXAg expression

RT-PCR. Levels of HBX mRNA in cells exposed to blank (1), siRNA-HK, negative control (2), 3TC (3), siRNA-HBX (4), siRNA-cccDNA (5), siRNA-HBX + siRNA-cccDNA (6).

the levels of HBX mRNA in HepG2.2.15 cells at 96 hrs after transfection.

The results showed that the levels of HBX mRNA were dramatically reduced in HepG2.2.15 cells after the treatment with siRNA and 3TC compared with the treatment of siRNA-HK, while the greatest reduction rate was in the combined therapy group (siRNA-HBX + siRNA-cccDNA) (Fig. 2).

To test whether specific siRNA could inhibit the expression of HBXAg, western blot was performed 96 hrs after transfection. The GAPDH was used as an internal control.

As shown in Fig. 3, the level of HBXAg was reduced to different degree in all transfected cells. The combined therapy group (Fig. 3) had the greatest decrease of HBXAg, while the protein levels in cells transfected with nonspecific siRNA showed nearly no change.

To evaluate the influence of RNAi on HBV antigen expression, HepG2.2.15 cells were transfected with HBX or cccDNA siRNA. HBsAg and HBeAg concentrations in the culture media of transfected and control cells were measured at 48, 72, and 96 hrs post transfection by ELISA. The HBeAg levels in all cells transfected with siRNA or treated with 3TC were significantly reduced compared to those transfected with an empty vector (Fig. 4b). Meanwhile, the level of HBsAg was also reduced in both the siRNA-transfected cells and the 3TC-treated cells (Fig. 4a). For both HBsAg and HBeAg, the combination therapy with siRNAs was more effective than any individual therapy.

At 96 hrs post transfection, the expression level of HBcAg in transfected cells was detected by immunofluorescent staining. The amount of HBcAg was reduced both in the siRNA-transfected cells and the 3TC-treated cells. The cells treated with combined therapy expressed approximately 14.9% HBcAg of control as measured by Image Pro-Express software (Fig. 5).
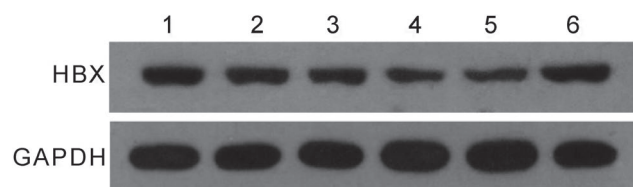

Fig. 3

Effects of siRNAs on HBXAg level

Western blot analysis, GAPDH served as an internal control. Levels of HBXAg in cells exposed to blank (1), 3TC (2), siRNA-cccDNA (3), siRNA-HBX (4), siRNA-HBX + siRNA-cccDNA (5), siRNA-HK, negative control (6).

Discussion

RNAi is an endogenous cell process that can suppress gene expression in a highly specific manner. It is believed to represent an ancestral form of nucleic acid-based elimination of intracellular pathogens. Now it is providing new approaches for the development of therapies for a variety of diseases, including tumors and viral infections (Hamasaki *et al.*, 2003; Chan *et al.*, 2006). It has been demonstrated that RNAi could attenuate the replication of HBV genome in cell culture (Shlomai *et al.*, 2003). Therapeutic interfering RNA can be directly introduced into cells as exogenously produced siRNA or can be synthesized within cells by vector-based systems. Silencing mediated by both strategies has been shown to be highly effective (Elbashir *et al.*, 2001; Morrissey *et al.*, 2005). Recent progress in chemical modification of siRNA has improved both the longevity and specificity of these agents (Seeger *et al.*, 2000; Jackson *et al.*, 2006; Zimmermann *et al.*, 2006). These reports have demonstrated that RNAi technology could be used to suppress HBV in cell cultures and animal models,
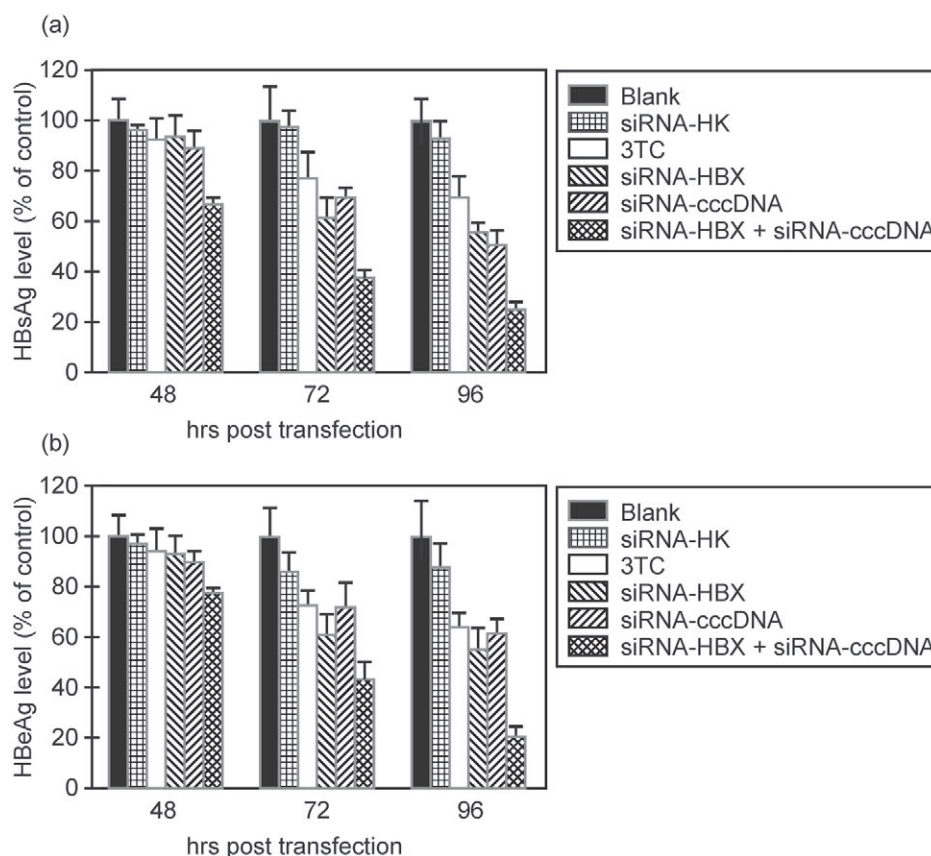
Fig. 4

Effects of siRNAs on the levels of HBsAg (a) and HBeAg (b)

ELISA. To evaluate the influence of RNAi on HBV antigen expression, HepG2.2.15 cells were transfected with siRNAs mentioned above. The concentration of HBsAg (a) and HBeAg (b) was measured in the culture media of transfected cells at 48, 72, and 96 hrs after transfection, respectively, by ELISA kit.

which provides insight into application of this technique in controlling infectious human hepatitis.

During virus replication, cccDNA is considered as the template for DNA transcription and its production is regulated and amplified by an intra-cellular pathway, in which newly synthesized genomic DNA is delivered to the nuclei. The HBXAg is a multifunctional factor related to an increasing risk of hepatocellular carcinoma in HBV patients and it is important for the transcriptional regulation of HBV genes (Feitelson *et al.*, 1993; Benn *et al.*, 1994; Wang *et al.*, 1994; Takada *et al.*, 1995). Apart from transactivation of many promoters, activities linked to HBXAg include stimulation of signal transduction (Sirma *et al.*, 1998) and binding to well-known proteins such as p53 (Takada *et al.*, 1990; Lee *et al.*, 1995), proteasome subunits (Andrisani *et al.*, 1990), and UV-damaged DNA-binding protein (Murakami, 2001). It has been reported that viral mutations could escape single synthetic siRNA treatments (Xin *et al.*, 2008). In previous

work, a therapeutic silencing vector that targeted HBV with four separate pol III-driven siRNA cassettes showed efficacy in cell culture and animal models of infection (Fu *et al.*, 1998). Recently, combination therapy has emerged as a new approach in the treatment of chronic HBV infection with the objective to decrease the viral loads. Thus, the treatment with the combination of two targets, HBXAg and cccDNA, can be prospective and effective.

To investigate the effects of selected siRNA molecules on HBV gene expression and viral replication *in vitro*, we used HepG2.2.15 (a derivative of the human HepG2 hepatoma cell line), which has been stably transformed with HBV genome and used as an *in vitro* model for HBV replication (Zhao *et al.*, 2006; Guan *et al.*, 2007). The results of our study indicated that siRNA-cccDNA, siRNA-HBX, siRNA-HBX + siRNA-cccDNA had significant effect on HBV cccDNA amplification and the expression of HBXAg in HepG2.2.15 cells, and subsequently blocked the viral protein production and
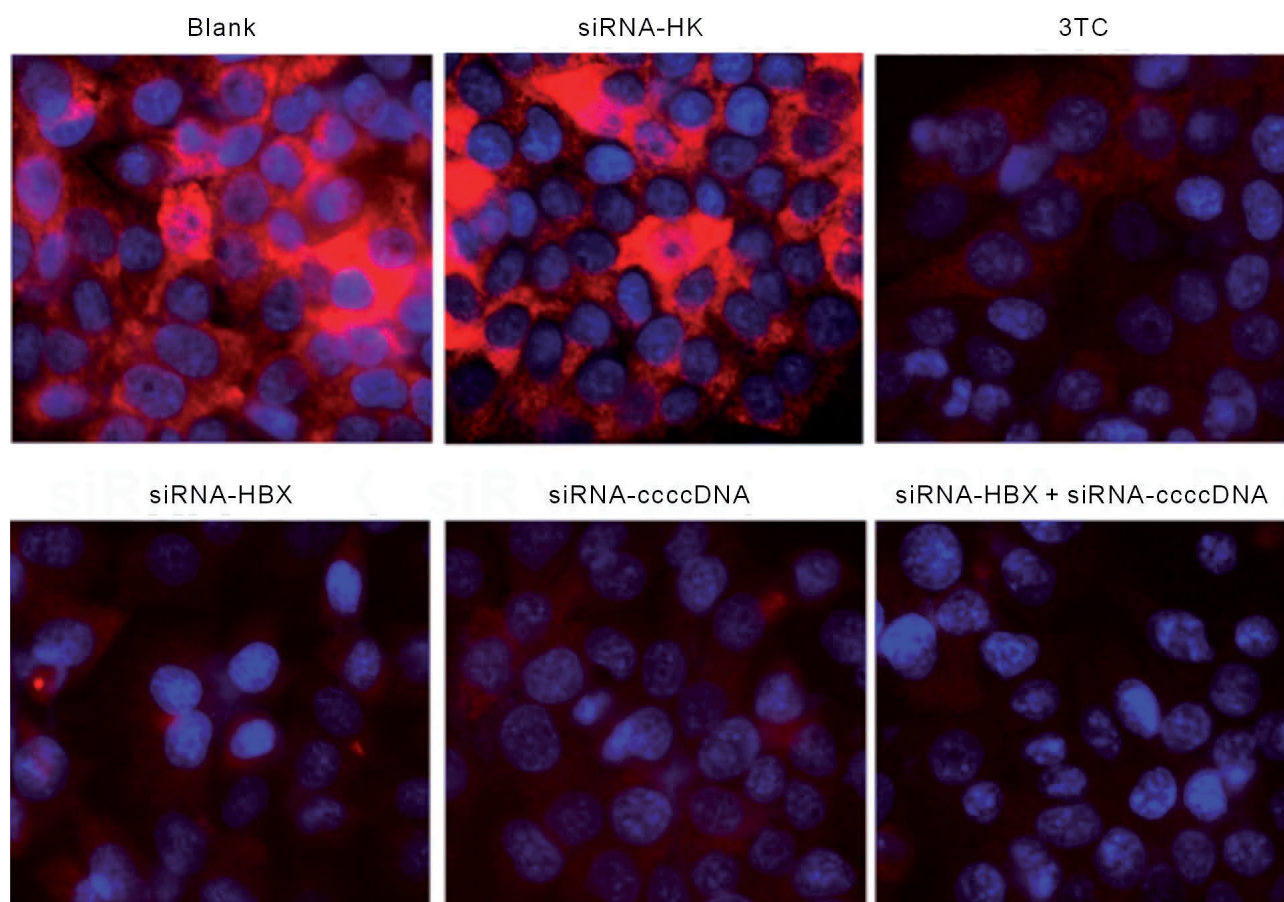
Fig. 5

Effects of siRNAs on HBcAg level

IFA. Levels of HBeAg in cells exposed to blank, siRNA-HK (negative control), 3TC, siRNA-HBX, siRNA-cccDNA, and siRNA-HBX + siRNA-cccDNA.

inhibited HBV replication. The anti-HBV effects of therapeutic siRNA were more effective than the treatment with 0.5 µmol/l 3TC in HepG2. 2. 15 cells. More importantly, dual siRNA could simultaneously inhibit the synthesis of cccDNA and HBX gene by 78.3% and 72.5%, respectively. Therefore, this dual siRNA system could provide a more powerful tool to study the gene function and could be potentially used in the treatment of viral infection.

RNAi used as an anti-HBV tool seems to have some important advantages: First, specific target of viral transcripts severely impairs virus replication and promotes its eradication, without activating nonspecific cellular responses, which can minimize undesirable side effects. Second, the numerous potential targets make it possible to target conserved regions, which can limit the ability of the virus to create escape mutants.

In the last 30 years, HBV infection affects millions of people each year worldwide. Current therapies of HBV infection include nucleoside analogs such as 3TC, which has provided some degree of cure, but the efficiency of treatment is limited due to the virus gene mutation. HBV patients currently have few therapeutic options and new approaches are needed to improve the quality of life. As a potential therapy, RNAi seems to be a promising alternative strategy. In our study, combined treatment targeting both cccDNA and HBX gene strongly inhibited not only HBV replication at the protein and mRNA level, but also the cccDNA transcription activity. Although HBV infection remains a major health problem, our experiments provide a new insight into the anti-HBV strategy, enhancing the anti-HBV efficacy and overcoming the high mutation rate of HBV.

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