

Optimized Tandem amiRNA Mediates Stronger Inhibitory Effects on Hepatitis B Virus Infection

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Abstract

Background & Aims. RNA interference (RNAi) has emerged as a potential new approach against hepatitis B virus (HBV) infection but unfortunately it also selects resistant virus mutants. In this study we combined the advantages of artificial micro RNAs (amiRNAs) reported previously with the purpose of constructing a more practical amiRNA with high inhibition effects against HBV. **Method.** Aiming at conserved sites, we constructed singular-sequence vectors amiRNA-HBV1, amiRNA-HBV2, amiRNA-HBV3 and amiRNA-HBV4. We chose the two sequences of high efficiency, then built the tandem-sequence vector amiRNA-HBV3-HBV4. These vectors were transfected into HepG2.2.15 transiently. The secreted HBV surface antigen (HBsAg) and HBV 'e' antigen (HBeAg) were measured with a chemiluminescent microparticle immunoassay, and intracellular and extracellular HBV DNA was quantified by real-time PCR. **Results.** Our results demonstrated that amiRNA-HBV1, amiRNA-HBV2, amiRNA-HBV3, and amiRNA-HBV4 achieved a maximum inhibition of HBV mRNA expression of 29.3%, 14.9%, 61.2%, and 75.6%, respectively, while the tandem amiRNA-HBV3-HBV4 vector led to an inhibition of 87.2%. **Conclusion.** Taken together, our data suggest that vector-based multiple artificial microRNAs are a promising therapeutic approach for chronic HBV infection.

Key words

HBV – DNA – therapy – RNAi – amiRNA – multi-amiRNA.

Introduction

Chronic hepatitis B virus (HBV) infection is associated with a high risk of liver cirrhosis and primary hepatocellular carcinoma. It is known that three quarters of all liver cancers are caused by HBV infection [1]. According to World Health Organization reports, two billion people have been exposed to HBV and more than 350 million are chronically infected worldwide, accounting for over one million deaths each year [2].

Currently, interferons and nucleoside analogs, which act as immunomodulators and viral polymerase inhibitors respectively, are the principal treatment options for chronic HBV infection. However, these therapies have many limitations, including low efficacy and harmful side-effects, emergence of nucleoside analog-resistant HBV mutants, poor patient tolerance, and induction of complicating disorders, high expense, and lengthy treatment regimens [3]. Therefore, novel and improved strategies are in urgent demand for the treatment of HBV infection.

RNA interference (RNAi) has emerged as a potential new approach against HBV infection, in cell culture and animal models [4]. The RNAi method has proven effective against HBV infection in various transient assays, as long lasting suppression must be achieved in order to be clinically relevant. However, many RNA and DNA viruses escape RNAi suppression through mutation of the targeted region. For example, small interfering RNAs (siRNAs) tend to exert selection pressure on pre-existing resistant mutants, as illustrated in a recent report on HBV [5]. Even a single point mutation (mismatch) in the virus is sufficient to confer escape from siRNA inhibition. In contrast, microRNAs (miRNAs) do not require exact sequence complementation, and thus miRNA-based therapeutic gene silencing is more desirable for combating highly mutable viruses.

For example, Chen et al [6] tested both the feasibility of a combination treatment of chemical synthetic siRNAs targeting various regions, and the antiviral efficacy of the HepG2.2.15 cell culture system. Xia et al [7] reported that multiple short hairpin RNAs (shRNAs) expressed by an inducible pol II promoter could knockdown the expression of

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multiple target genes. Liu et al [8] demonstrated the efficient inhibition of HIV-1 replication by inserting multiple effective anti-HIV siRNA sequences using the miRNA polycistron strategy. Ely et al [9] inserted three anti-HBV miRNA sequences into the miRNA polycistron, but they did not test the interference effect at HBV DNA and protein levels.

HBV is a DNA virus which replicates by reverse transcription of a pre-genomic RNA intermediate. Therefore, using a pool of miRNAs to simultaneously target multiple sites in the viral genome could achieve better gene silencing to inhibit HBV DNA. We believe that this strategy may prevent the emergence of resistant virus mutants. To this end, we generated plasmid vectors to target transcripts from conserved regions of the HBV genome. We constructed expression plasmid vectors of four distinct monomeric miRNAs and one heterodimeric tandem miRNA. The miRNA method caused no detectable toxicity, resulting from an interferon (IFN) response induction, or disruption of the endogenous miR pathway [9]. In transient transfection assays, the inhibition at the RNA level was 29.3%, 14.9%, 61.2%, 75.6% for the four monomers, and 87.2% for the tandem dimer. We also analyzed miRNA-mediated HBV suppression at the DNA and protein (viral antigen) levels in order to find whether multiple miRNAs in tandem aimed at multiple targeting sites are more effective than single

ones. This may be a better way to prevent the occurrence of resistant viruses.

Materials and Methods

Design and construction of miRNA expression vectors

For the design of artificial microRNA (amiRNA) sequences, 15 HBV genomes were aligned with the online basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the conserved region, which was then selected as the target sequence for the miRNAs. These 15 HBV genomes included: U95551, AJ131956, AJ344117, DQ315776, AB210819, EU594436, Z35716, EU594409, AB109477, AB222713, X02496, AY161157, AY945307, AY721605, and AB188243. Four miRNAs were designed (pcDNA6.2-GW/EmGFP-ami-HBV1, 2, 3, and 4, abbreviated ami-HBV-1, ami-HBV-2, ami-HBV-3, and amiHBV-4) using Invitrogen miRNA design online software and checked by BLAST to prevent non-specific off-target inhibition (Block-IT™ RNAi Designer --<http://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=mirna&pid=-8543560688424874295>). The synthesized oligonucleotides (oligos) for sense and anti-sense strands of the four ami-HBV miRNAs are listed in Table I. The

Table I. The designed and synthesized miRNA oligos

No.	Oligo Type	Oligo Sequence
ami-H1	Top Strand	5'- TGCTGTTTGAAGTATGCCTCAAGGTCGTTTTGGCCACTGACTGACGACCTTGACATACT TCAAA -3'
	Bottom Strand	5'- CCTGTTTGAAGTATGTCAAGGTCGTCAGTCAGTGGCCAAAACGACCTTGAGGCATACT TCAAAC -3'
	ds Oligo	5'- TGCTGTTTGAAGTATGCCTCAAGGTCGTTTTGGCCACTGACTGACGACCTTGACATACT TCAAA -3'
		3'- CAAAC TTCATACGAGTTCAGCAAACCGGTGACTGACTGCTGGAAGTGTATGAAGT TTGTCC -5'
ami-H2	Top Strand	5'- TGCTGAGGTGAAGCGAAGTGCACACGGTTTTGGCCACTGACTGACCGTGTGCATCGC TTCACCT -3'
	Bottom Strand	5'- CCTGAGGTGAAGCGATGCACACGGTTCAGTCAGTGGCCAAAACCGTGTGCACCTTCGCT TCACCTC -3'
	ds Oligo	5'- TGCTGAGGTGAAGCGAAGTGCACACGGTTTTGGCCACTGACTGACCGTGTGCATCGC TTCACCT -3'
		3'- CTCCACTTCGCTTACGCTGTGCCAAAACCGGTGACTGACTGGCACACGTAGCGAAGTG GAGTCC -5'
ami-H3	Top Strand	5'- TGCTGATTGAGAGAAGTCCACCACGAGTTTTGGCCACTGACTGACTCGTGGTGCTTCT CTCAAT -3'
	Bottom Strand	5'- CCTGATTGAGAGAAGCACCACGAGTTCAGTCAGTGGCCAAAACCGTGGTGACTTCTC TCAATC -3'
	ds Oligo	5'- TGCTGATTGAGAGAAGTCCACCACGAGTTTTGGCCACTGACTGACTCGTGGTGCTTCT CTCAAT -3'
		3'- CTAACTCTTTCAGGTGGTGTCAAACCGGTGACTGACTGAGCACCACGAAGAGAGT TAGTCC -5'
ami-H4	Top Strand	5'- TGCTGAATGGCACTAGTAACTGAGCGTTTTGGCCACTGACTGACGCTCAGTTCTAGT GCCATT -3'
	Bottom Strand	5'- CCTGAATGGCACTAGAACTGAGCGTTCAGTCAGTGGCCAAAACCGCTCAGTTTACTAGTG CCATTC -3'
	ds Oligo	5'- TGCTGAATGGCACTAGTAACTGAGCGTTTTGGCCACTGACTGACGCTCAGTTCTAGT GCCATT -3'
		3'- CTTACCGTGATCATTTGACTCGCAAACCGGTGACTGACTGCGAGTCAAGATCACGGT AAGTCC -5'

oligos were cloned into pcDNATM6.2-GW/ \pm EmGFP-miR (Invitrogen) to construct four miRNA expression vectors (ami-HBV-1, ami-HBV-2, ami-HBV-3, and ami-HBV-4), which were verified by DNA sequencing.

Construction of tandem heterodimer miRNA expression vector

To construct the tandem heterodimeric miRNA expression vector that bears the sequences of both ami-HBV-3 and ami-HBV-4, the ami-HBV-3 miRNA insert was excised with BamH I/Xho I, and the ami-HBV-4 miRNA plasmid (vector+insert) was linearized with Bgl II/Xho I. The double-digested fragments were then ligated together directionally at the BamH I/Bgl II and Xho I sites. The potential positive clones were selected by polymerase chain reaction (PCR) and verified by DNA sequencing as tandem heterodimeric miRNA vector pcDNA6.2-GW/EmGFP-ami-HBV3-HBV4. The sequences of ami-HBV-3 and ami-HBV-4 were validated as efficient in the inhibition of HBV mRNA.

The miR-neg control vector is not an empty vector. It allows formation of a pre-miRNA hairpin sequence predicted not to target any known vertebrate gene (only for the pcDNATM6.2-GW/ \pm EmGFP miR-neg control). The sequence without 5' overhangs is: 5'-GAAATGTAAGTGC GCGTGGAGACGTTTTGGCCACTGACTGACGT CTCCACGCAGTACATTT-3'.

Cell culture and transfection

HepG2.2.15 cells (a gift from Prof. A. Miao, Affiliated Changzheng Hospital of the Second Military Medical University of China) were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone), 400 μ g/mL G418 sulfate (Invitrogen), and incubated at 37°C in 5% CO₂-humidified air. For transient transfection, cells were cultured in 24-well plates until 60-70% confluent, 24 h prior to transfection. Plasmid DNA was prepared using the PureLink Ultra-pure plasmid DNA purification kit (Invitrogen). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

All experiments were performed in triplicate and divided into several groups. The mock group was treated only with Lipofectamine 2000 reagent. The negative control group was transfected with negative control plasmid, which has no known RNAi effect on normal human genes. After transfection, the medium was partly removed for analysis every 24 h and the cells were replenished with fresh medium. The cell culture supernatant was collected for detection of hepatitis B surface antigen (HBsAg), hepatitis B 'e' antigen (HBeAg), and HBV-DNA. Transfection efficiency was monitored by green fluorescent protein (GFP) expression.

Quantitative real time PCR (Q-PCR) analysis of HBV mRNA

HepG2.2.15 cells were collected 48 h post-transfection. Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Complimentary DNA (cDNA) was synthesized from total RNA using the

Reverse Transcription System (Promega) according to the manufacturer's protocol. The 1st-strand cDNA product was used as a template for Q-PCR, performed in a single reaction of 20 μ L volume.

HBsAg and HBeAg assay

The culture supernatant from transfected cells at different time points was collected and HBsAg and HBeAg were measured by the Abbott Architect HBsAg and HBeAg assay kit using the automated immunoassay instrument Architect i2000 SR. Assays were performed in triplicate, and the results were expressed as mean \pm standard deviation (SD).

Detection of HBV DNA by Q-PCR

Genomic DNA was isolated from transfected HepG2.2.15 cells using a genomic DNA miniprep kit (AxyPrep). HBV DNA in genomic DNA or the supernatant of transfected cells was detected using an HBV DNA Q-PCR kit (Shanghai CloneTech). Amplification and detection were automated on an ABI7500 Q-PCR detector.

Statistical analyses

Data were expressed as mean \pm SD and analyzed by a univariate test. A probability (P)-value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 13.0 software.

The inhibitory rate of HBV was calculated according to the following formula:

$$\text{inhibitory rate (\%)} = [(C_{\text{control}} - C_{\text{tester}}) / C_{\text{control}}] \times 100\%$$

where C is the quantity of HBV DNA, HBsAg, or HBeAg. C_{control} is the quantity detected from the cell that was transfected by the neg-control plasmid.

Results

Transfection efficiency of miRNA vectors

The pcDNATM6.2-GW/EmGFP-miR vector was designed to accept engineered pre-miRNA sequences targeting the gene of interest. The engineered pre-miRNA sequence structure was based on the murine miR-155 sequence and the stem-loop structure was optimized to obtain a high knockdown rate. The addition of emerald green fluorescent protein (EmGFP) allowed monitoring the miRNA expression due to a strong correlation between EmGFP expression and the miRNA expression of the target gene. As shown in Fig. 1, the post-transfection expression of EmGFP which peaked at 48 h declined afterwards, but was still detectable at 96 h.

The tandem miRNA vector is more efficient at inhibiting HBV infection than the single miRNA vector

Expression of HBV mRNA was assessed using Q-PCR analysis. RNA was prepared 72h post-transfection and subjected to reverse transcription and quantitative PCR analysis. When compared to negative controls, percent inhibition of HBV mRNA expression in the monomeric ami-HBV1, ami-HBV2, ami-HBV3, and ami-HBV4 vector transfected-HepG2.2.15 cells was 29.3%, 14.9%, 61.2%*, and 75.6%*, respectively (*P < 0.01; Fig. 2). The inhibition

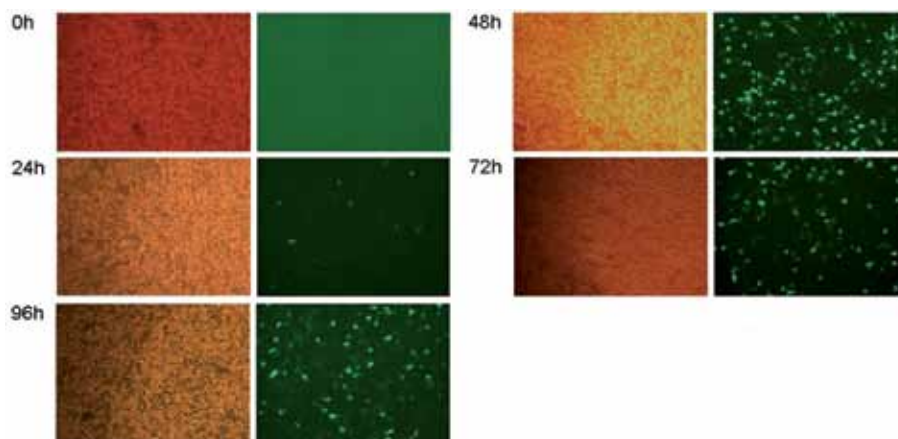


Fig 1. HEPG2.2.15 cells at 60-70% confluency were transfected with pcDNA6.2-GW/EmGFP-ami-HBV4-HBV3 co-cistronic miRNA expression plasmid using Lipofectamine 2000 reagent. To monitor transfection efficiency, light phase and green fluorescence pictures of the same view field were taken before transfection (0 h) and 24 h, 48 h, 72 h and 96 h after transfection.

rate of sequences (HBV3 and HBV4) at the RNA level was more effective. HBV mRNA inhibition in the ami-HBV4-HBV3 tandem vector-transfected HepG2.2.15 cells reached as high as 87.2%.

Next we evaluated the inhibitory effect of miRNA vectors on HBV protein expression. Since ami-HBV1 and ami-HBV2 demonstrated only moderate effect on HBV mRNA expression (29.3% or 14.9% inhibition, respectively), they were dropped from further analysis. As shown in Figs. 3A and 3B, the tandem ami-HBV3-HBV4 amiRNA demonstrated stronger inhibitory effects on HBsAg and HBeAg expression than either the single ami-HBV3 or ami-HBV4 amiRNA.

Lastly, we examined the inhibitory effect of miRNA vectors on HBV DNA levels as a representation of HBV replication. When we measured the HBV DNA level in the supernatants of the HepG2.2.15 culture, there was as much as 94.3% reduction

in HBV DNA level detected in tandem amiRNA-HBV3-HBV4 transfected cells, but only 60.5% and 68.0% reduction was detected in amiRNA-HBV3 and amiRNA-HBV4 transfected cells, respectively (Fig. 4A). Furthermore, we measured intracellular HBV DNA levels in amiRNA transfected cells, and observed 71.6%, 80.2%, and 79.7% reduction of HBV DNA levels in amiRNA-HBV3, amiRNA-HBV4, and amiRNA-HBV3-HBV4 transfected cells at 96 h post-transfection, respectively. Overall, at 24 to 72 h post-transfection, tandem amiRNA-HBV3-HBV4 transfected cells demonstrated the strongest inhibition of DNA replication (Fig. 4B).

Discussion

In clinical practice, the evaluation of antiviral effects is largely based on HBV DNA testing. If a low viral HBV DNA load could be maintained by prolonging treatment, the patients would have the best outcome. RNAi is a promising therapeutic approach to human diseases, acting through the degradation of mRNA. HBV is a double-stranded DNA virus which replicates by reverse transcription of a pre-genomic RNA intermediate and thus is perfect for RNAi-mediated inhibition. RNAi has been demonstrated to be a powerful tool for suppressing viral infection in various transient, short-term assays. However, few studies have applied RNAi with the goal of developing a therapy for HBV infection and which observed the HBV DNA. RNA interference results in the degradation of RNA, therefore we used the measure of mRNA levels by real-time PCR in selecting candidates for concatenating constructs. We next verified that inhibition is effective at both the protein and DNA levels, consistent with the unique DNA replication characteristics of HBV.

Recent studies have shown that some viruses (including HBV) escape RNAi-mediated inhibition through mechanisms such as mutations in the targeted region, under siRNA selection pressure [5]. Viral resistance due to mutation still presents a difficult problem in medical treatment. Viral RNA replication in infected cells is mediated by a viral

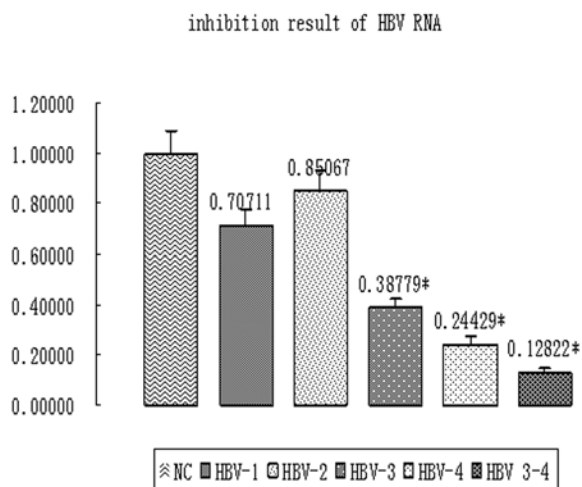


Fig 2. Effect of amiRNA-HBV on HBV RNA levels. HBV RNA levels were expressed as mean \pm SD. NC group was transfected by negative control plasmid. Other groups were compared to NC. Inhibition rate of tandem amiRNA was 87.2%, which was the best.

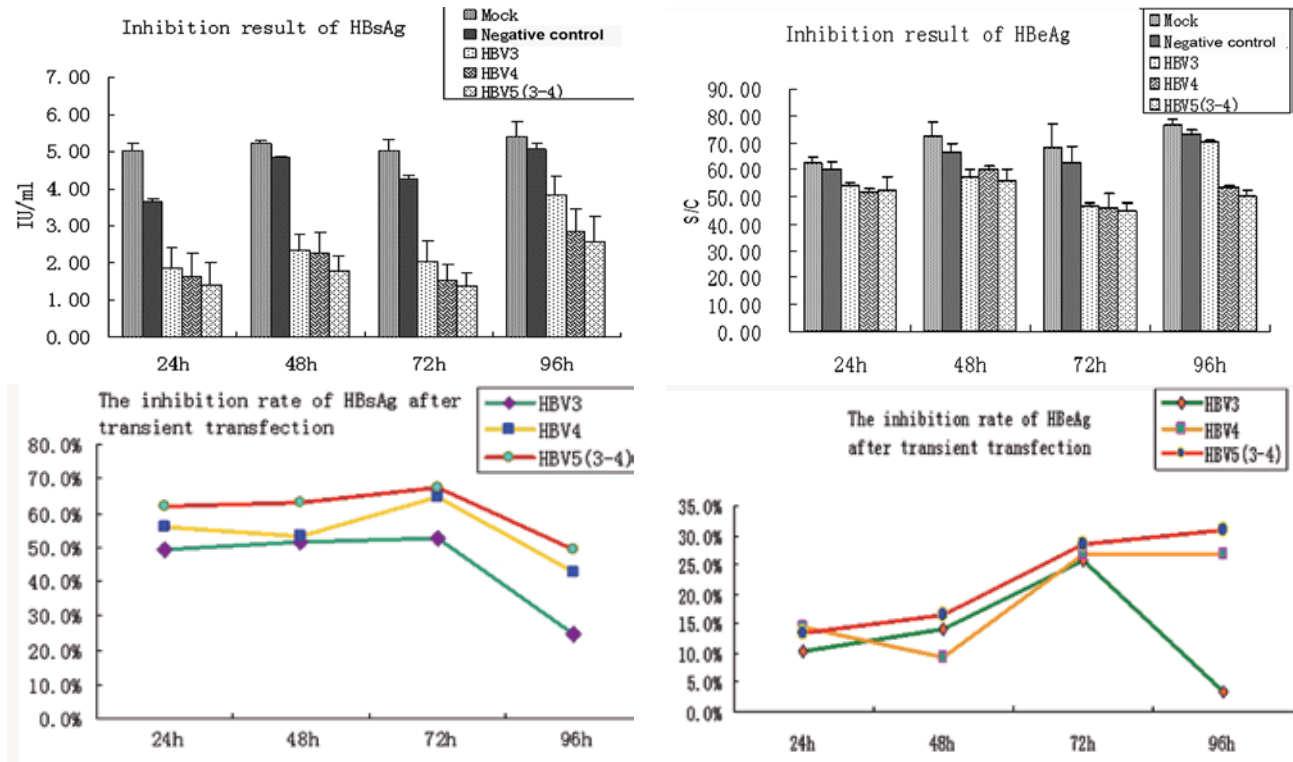


Fig 3A. Effect of amiRNA-HBV on HBsAg level. HBsAg level was expressed as mean ± SD; **3B.** Effect of amiRNA-HBV on HBeAg level. HBeAg level was expressed as mean ± SD.

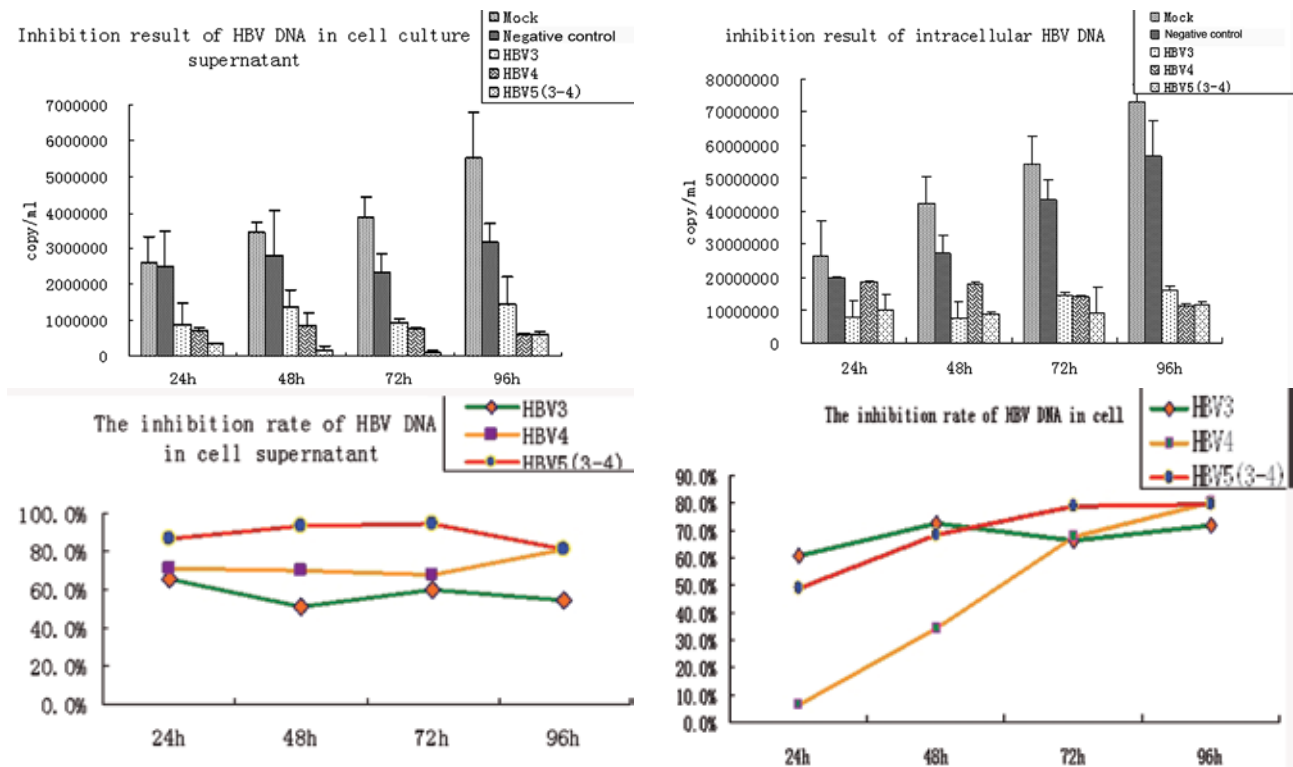


Fig 4 A. Effect of amiRNA-HBV with transient transfection on HBV DNA level in cell culture supernatant. The amount of HBV DNA was expressed as mean ± SD. Compared with the negative control, all three plasmids had a significant inhibitory effect on HBV DNA levels at 24h, 48h, 72h and 96h after transfection ($P < 0.05$); **4B.** Effect of amiRNA-HBV with transient transfection on intracellular HBV DNA level. The amount of HBV DNA was expressed as mean ± SD. Compared with the negative control, all three plasmids had a significant inhibitory effect on HBV DNA levels at 24 h, 48 h, 72 h and 96 h after transfection ($P < 0.05$).

RNA-dependent RNA polymerase. Due to the lack of a proofreading mechanism, this polymerase replicates the RNA genome with low fidelity, resulting in a mutation rate on the order of 10^{-4} per incorporated nucleotide [10, 11]. HBV is a DNA virus, making its genome relatively stable in comparison, with a low mutation rate of 10^{-8} to 10^{-11} per incorporated nucleotide. However, HBV replication utilizes a virally-encoded reverse transcriptase, and siRNA may exert selection pressure on pre-existing resistant mutants, as shown recently [5]. Even a single point mutation resulting in a G:U mismatch is sufficient for the virus to escape siRNA inhibition [12]; otherwise one site mismatch does not escape miRNA. It is supposed that amiRNA can also play a role when the target sequences of miRNA match the mRNA backbone. The design of amiRNAs is a technique used to produce microRNA spontaneously.

Viral resistance has brought difficulties to medical treatment. We know that the anti-HBV drug entecavir has one more resistance site compared to lamivudine (used to treat HIV infections), and the incidence of HBV virus resistance to entecavir has been significantly reduced. Now entecavir is widely used. To overcome this problem, multiple synthetic siRNAs targeting different regions were used to exert robust and specific inhibition of HBV replication and antigen expression in a cell culture system [13]. It has been proposed that using a pool of siRNAs to simultaneously target multiple sites in the virus genome might be a good choice to prevent the emergence of resistant viruses. In this respect, our design of multi-amiRNAs targeting different regions of the virus genome simultaneously will be a new approach that avoids the escape of HBV mutants.

Wilson et al [14] found that the inhibition efficiency of siRNA 24 h and 72 h after transfection is the highest, but after 96 h weakened in efficiency, and at 120 h almost disappeared. Therefore, using a carrier to transfer siRNA into cells to ensure its continuous expression will become an inevitable choice. Plasmid- or viral vector-based delivery of siRNA has been shown to be effective in the inhibition of HBV [15]. In addition, plasmid vector-mediated miRNA expression is superior to synthetic RNAi, in that it is propagatable *in vivo* and longer lasting, and thus ideal for the treatment of chronic diseases such as HBV infection.

Although efficient HBV gene silencing by shRNA expressed from RNA pol III promoters has been reported, constitutive high-level transcription may cause harmful side effects [16]. The knockdown efficiency of the modified miR-framework of natural sequences was generally lower when expressed from pol III promoters as compared with pol II promoters [17, 18]. These disadvantages of expressing miRNA from pol III promoters may reflect the fact that natural miRNAs are expressed from pol II promoters [19, 20]. In this study, we applied the pol II (CMV) promoter, which is more effective in driving miRNA synthesis and hence producing better interference [21]. Overall, we designed a plasmid vector with an RNA pol II-driven gene cassette that leads to efficient expression and processing of multiple, user-designed miRNAs from a single transcript.

The expression of GFP under a bicistronic promoter facilitates real-time monitoring of transfection efficiency.

To prevent viral escape through mutation, our study incorporated anti-escape mechanisms when designing RNAi. We aligned all 15 kinds of HBV genomes and selected only conservative regions for miRNA targeting. In clinic, lamivudine is commonly used. The drug resistant loci of the resistant hepatitis B virus (YMDD) are about 540-542 site (aa180), locus 612-614 site (aa204). In our study, we designed the target sequence to avoid these sites. Tandem miRNA simultaneously targets separate positions in the viral genome and thus has a much better chance of defeating the viral mutation-avoidance scheme. We proved that the tandem miRNAs targeting two sequences were more effective in inhibiting HBV replication and RNA and protein expression, in comparison with their corresponding singular amiRNAs. In addition, the expression of tandem miRNAs was high under one cistronic promoter, and so the effort to construct and transfect multiple plasmids was unnecessary.

HepG2.2.15 is a good cell model to study HBV infection *in vitro*. The entire HBV-DNA genome was transfected into HepG2 (human hepatoblastoma) cells. HBV DNA is released into the cell culture medium after replication and the secreted proteins (including HBsAg and HBeAg), can also be detected in cell supernatants. Therefore we can evaluate the inhibitory effects of miRNAs on HBV without destroying cells.

HBV has four mRNA transcripts, 3.5 Kb, 2.4 Kb, 2.1 Kb and 0.7 Kb in length. Among these, the 3.5 Kb mRNA is pre-genome RNA, containing all the genetic information and is the genome reverse transcription template of HBV DNA. The S region encodes S protein, which is a membrane protein mediating HBV infection of cells. The C region encodes HBcAg and HBeAg proteins. The P region encodes DNA polymerases, and the X region encodes proteins called HBxAg. Cheng et al [13] proposed that HBsAg is an important risk factor for HBV infection of the liver, and its mutations contribute to the difficulty in HBV treatment. Therefore, effectively silencing the specific gene, is the key to inhibiting HBV. In our study, the two sequences with better interference effect were used to target HBsAg.

The primer for RT-PCR was at nucleotide position 1383. The reason we picked this site was because all 4 of our amiRNA targets share this common sequence. We used RT-PCR instead of Northern-blotting to detect the mRNA degradation. Efficiency of interference was actually lower with amiHBV1 and amiHBV2 which target the x-protein, compared to amiHBV3 and amiHBV4, which target the S zone. We postulated that due to the critical role of HBV surface antigen in infecting host cells, the rate of mutation must be suppressed. We designed the amiRNA sequence according to the U95551 gene; however, during long-term culture, mutations in high-occurrence areas such as the X zone can accumulate. The S zone where amiHBV3 and amiHBV4 targeted is relatively stable in terms of mutation.

There are four overlapping open reading frames in the HBV genome, coding for four known proteins, i.e.,

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1376..1840 /codon_start=1/product="x-protein",          atggc
1381 tgcctaggctg tgcctgccaac tggatcctgc gcggagcgc ctttgtttac gtccctcgg
1441 cgcctgaatcc tgcggagcgc ccttctcggg gtgccttggg actctctcgt ccccttctcc
1501 gtctgcccgtt ccgacccgacc acggggcgca cctctcttta cggagcctcc ccgtctgtgc
1561 cttctcatctt gccggaccg atgcaattca ctgcacgtgca tggagaccac
1621 cgtgaaccgc caocgaatgt tgcacaagt ctacataag aggactcttg gactctctgc
1681 aatgtcaacg accatgcaatg cctcctcctc gactctt tgttttaag actgggagga
1741 gttggggag gagattagat taanagtctt tctactagga ggcctgtagc ataantgtt
1801 ctgcgcaca gcaccatgca actttttcac ctctgcctaa

157..837/notes="HBsAg" /codon_start=1/product="surface antigen",
                                atgg agaacatcac atcaggattc
181 ctaggaccoc tctctgttt acagccgggg tttttctgt tgacaagaat cctcacata
241 ccgcagagtc tagaccatg atgctctctc tttctttc tagggggaac taocgtgtgt
301 cttggccaaa attgcagtc cccaacctcc aatcactcac caacctctgt tctccaaact
361 tctctcgttt atcgtctgat gtgtctcggg cgtttttaca tcttctctt catctctgt
421 ctatgctcca tctctctgtt ggttctctcg gactatcaag gtatgttgc cgtttctct
481 ctaattccag gatcctcaac caaccagcag ggaccatgoc gaacctgcat gactactgt
541 caaggaaact ctatgtatcc ctctctgttc tgaaccaac cttcggagc aaattgcacc
601 tgtattccca tcccatcacc ctggccttcc gaaaattcc tatggagtg ggcctcagcc
661 cgtttctctc gctctctctc atgctctcct ttttctctc gtttctctc gctttctctc
721 actgtttggc tttcagttat atggatgat tggattggg ggcacaact gtcacagatc
781 ttgagtcctt ttttaccgct gttaccaatt tctttttgct tttgggata catttaa

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Fig 5. HBV genes targeted by each miRNA. Conserved regions were confirmed with BLAST. The product of region 1376-1840nt is HBxAg, and the product of region 157-837nt is HBsAg. The design of interference target regions are amiHBV-1 (1694-1714bp), amiHBV-2 (1578-1898 bp), amiHBV-3 (256-276 bp), amiHBV-4 (672-692 bp).

DNA polymerase, HBsAg, X-protein, and the core antigen precursor HBeAg. The four miRNAs designed in this study were specifically aimed at the following targets: HBV-1(1694)-X protein, HBV-2(1578)-X protein and polymerase, HBV-3(256)-polymerase and HBsAg, and HBV-4(672)-polymerase and HBsAg.

Detection of HBsAg and HBeAg is already widely used in clinics to measure HBV infection and replication. The Architect HBsAg assay uses chemiluminescent microparticle immunoassay technology for the quantitative determination of HBsAg. It is superior in sensitivity and precision to the conventional ELISA method. Although amiHBV-3 and amiHBV-4 target the HBsAg coding area, our results showed that they also produced a moderate inhibitory effect on HBeAg expression. Our hypothesis is that HBeAg expression was inhibited through secondary or indirect degradation of the 3.5 kb RNA. This explains why the inhibition of HBsAg was as high as 67.4%, but that of HBeAg was only 31%. Highest inhibition was detected in cells at 72 h post-transfection, with tandem amiRNA. As we know, the inhibitory effect of exogenously introduced siRNA usually declines beyond 72h. Therefore, our next objective is to select stable cell lines and incorporate an amiRNA expressing cassette to investigate the long-term suppression of HBsAg and HBeAg.

The secreted HBV DNA level in culture supernatants could indicate the inhibition of HBV replication by amiRNA. Tandem amiRNA also showed better suppression over its singular component amiRNAs. The inhibition of cellular DNA synthesis was not as great as the inhibition of supernatant DNA. Our explanation is that taking the measure of cellular viral DNA does not exclude nuclear (non-viral) DNA content and was less indicative of viral replication

inhibition. Therefore measuring supernatant HBV DNA content is a better and more effective monitoring method.

Previous studies have assessed the activation of the IFN response upon transfection of miRNA expression plasmids [22]. Measurement of IFN-mRNA concentration in cells transfected with these expression cassettes showed no elevation [9]. This indicates that little or no immunostimulation is caused by IFN pathway induction. We used miR-155 shuttles. It has also been proved that by utilizing miR-155 shuttles, the processing of endogenous miRNA (miR-181 and miR-16) and the Stat1 mRNA levels associated with the IFN response were not affected [23]. Therefore, the miRNA pathway is safer than the siRNA pathway.

Tandem amiRNAs also showed stronger inhibitory effect than their single component amiRNAs. Overall, from 24h to 96h post-transfection, tandem amiRNA-HBV3 - HBV4 showed the strongest inhibition of DNA replication. We postulate that the high inhibition efficiency of tandem amiRNAs may be because: 1) two separate sequences aimed at the same target have a much better chance in preventing resistance, and 2) two cistronically expressed and processed amiRNAs can work synergistically in destroying the target mRNA.

Ely et al [9] transfected pri-miR-31/9-8-5 and primiR-31/5-9-8 into cells. But interestingly, no mature primiR-31/8 was detected in RNA extracted from the cells. Unfortunately, they did not examine HBV DNA and proteins after transfection. In this study we combined the advantages of designing amiRNAs found from other studies and clinical experiences with the aim of constructing a more practical amiRNA with a high inhibition effect against HBV.

Conclusion

Our results demonstrate the superior efficiency of tandem artificial miRNA in inhibiting HBV mRNA and protein expression as well as viral DNA replication, in comparison with singular-sequence target miRNAs. The methodology developed in this study will help pave the way for the future application of miRNAs in clinical antiviral therapy.

Conflicts of interest

None to declare.

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