Computer-based siRNA Design to Hepatitis B Virus and Its Expression Vector Construction

Hong Cao[1], Liang Peng[1], Zhenhua Ding[2], Zhihua Liu[3], and Xiaolu Liu[1]

[1] Department of Microbiology, [2] Department of Radiation Medicine,
School of Public Health and Tropical Medicine,
[3] Department of Infectious Diseases, Nanfang Hospital,
Southern Medical University, Guangzhou, 510515 China
{Hong Cao, Liang Peng, Zhenhua Ding, Zhihua Liu,
Xiaolu Liu} gzhcao@fimmu.com

Abstract. Scanning, comparing and designing of candidate siRNAs to HBV genes have been performed by searching the DNA databases and using network servers. The homology search among the RNAi targeted region on mRNA sequence of HBV and human genome was done with BLAST. Three candidate siRNAs to HBV were chosen to construct three shRNA expression vectors respectively. Two complementary DNA oligonucleotides linked by 9-nucleotide loop and RNA polymerase Ⅲ terminators were designed first, and the annealed hairpin siRNA was inserted into the plasmid that carries green fluorescent protein. Then these recombinant plasmids were transformed in Escherichia Coli and detected by PCR and sequencing. Finally, the plasmids were used to transfect HeGp2 cells with liposome. The results showed the molecular weight of PCR products matched predicted size, and three siRNA plasmids were sequenced as expected sequences. The transfected cells stained with green fluorescence were also observed. Thus the shRNA expression plasmid specific for interfering HBV replication was successfully constructed.

Keywords: RNAi; siRNA; HBV; bioinformatics; computer scanning

1 Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus belong to *Hepadnaviridae*. The

full-length of the viral genome is about 3.2kb, and it has four open reading frames (ORFs) including surface antigen gene S, core antigen gene C, DNA polymerase gene P and X protein gene undetermined [1]. HBV infection can cause acute and chronic type B hepatitis, and eventually leads to serious consequence, such as hepatic cirrhosis and primary hepatocellular carcinoma (PHC) etc. Although the vaccine against HBV has been widely used for several decades, the HBV prevalence rate in the population still remains high. And the commonly used medicine in clinical at present such as interferon, Lamivudine could not reach the ideal therapeutic efficacy. During a period of only seven years, RNA interference, or RNAi has grown from a biological phenomenon to one of the most widely used tools in research and medicine design in antiviral infection. A small hairpin RNA or short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. shRNA uses a vector introduced into cells and utilizes the U6 promoter to ensure that the shRNA is always expressed. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA [2,3,4]. At present the most widely used method is using plasmid or viral vector to express siRNAs. The combination of bioinformatics and RNAi approaches were applied to design siRNAs to HBV cds and construct three shRNA expression plasmids and a control plasmid containing random sequences in our study.

2. Materials and Methods

2.1 Bacterial strains, culture conditions, plasmids and cells

The expression vector pRNATU6 was a plasmid containing shRNA and used for siRNA expression. The vector containing random sequences was used as a control plasmid. *Escherichia Coli* (*E.coli*) DH5α was used as the host strain in cloning and in preparing the plasmids for shRNA vector. HepG2 cells were contributed by Dr. Zhihua Liu, from department of Infective Disease, Nanfang hospital of our university. Strains containing plasmids were grown at 37℃ in Luria broth (LB containing 10g of tryptone, 10g of NaCl, and 5g of yeast extract/L) with ampicillin (100 mg/mL) for the positive selection.

2.2 Chemicals, enzymes and primers

Taq DNA polymerase, DNA restriction enzymes (*Bam*H Ⅰ & *Hin*d Ⅲ), and dNTP were the products of TaKaRa. Plasmid isolation kit was purchased from Guangzhou

Dong-Sheng biological company, Lipofectamine^{TM}2000 used in liposome transfection was from Invitrogen. DMEM, fetal bovine serum (BSA) was purchased from Beijing Ying-Xin biological company. All other chemicals were local products of analytic grade. The primers used in PCR are the upper stream primer (5'-tacgataca aggctgttagagag-3') and the down stream primer (5'-tagaaggcacagtcgagg-3').

2.3 Bioinformatics and comparative genomic approaches

In order to obtain orf DNA sequence of HBV from GenBank, functional annotation of HBV gene has been performed by combination of bioinformatics and comparative genomic approaches. DNA databases at NCBI/NIH (http://www.ncbi.nlm.nih.gov) were used to search the genomic regions, transcripts and products in the complete cds of HBV. To define coding domain of HBV CDS start and end sites in HBV DNA sequence were determined by the Vector NTI program. The computer-based siRNA design has been performed by using siRNA Target Designer program at network servers (http://www.promega.com/siRNADesigner/default.htm). The more prevalence siRNAs have been selected to construct recombinant DNA. The homology search with BLAST was applied to compare the potential siRNA target sequences with an appropriate genome database to eliminate any sequences with significant homology to other genes. Before the introduction of U6 siRNA expression vectors, siRNA insert design was done. An unrelated random sequence was also chosen as control. Therefore, scanning, comparing and designing of candidate siRNAs specific for HBV genes from GenBank made use of bioinformatics and several conceptually different approaches to construct siRNA U6 expression system including U6 RNA-based polymerase III promoter and modified terminator for high level, precise siRNA expression inside target cells.

2.4 Design for two DNA oligonucleotides sequences

For cloning into pRNATU6 vectors in our experiment, two DNA oligonucleotides that encode the chosen siRNA sequence are designed for insertion into the vector, in which the DNA oligonucleotides consist of a 19-nucleotide sense siRNA sequence linked to its reverse complementary antisense siRNA sequence by a 9-nucleotide spacer (TTCAAGAGA). The nucleotide overhangs with *Bam*H I and *Hin*d III restriction sites are added to the 5' and 3' end of the DNA oligonucleotides, respectively.

2.5 Annealing and ligation of single-strand DNA

Annealing and ligation of single-strand DNA was carried out as follow. Twenty μl of annealing mixture containing each 1μl of two synthetic single-stranded DNA (1μg/μl), 1μl of 20 × annealing buffer and 17μl dH_2O was well mixed, heated at 95℃ for 10 min, and then diluted to 10ng/μl as final concentration after being kept at room temperature for 1h. One μl of the annealed single-strand DNA with siRNA insert was ligated into linearized vector in a ligation reaction by mixing 1μl of target DNA fragment with 1μl of linearized vector (0.1μg/μl), 2μl of 10×buffer, 0.5μl of T4 DNA ligase. The ligation reaction was incubated for 2h at 22℃.

2.6 Transformation and identification of plasmid

Each 5μl of ligated products of three pRNATU6 construct containing siRNA was transformed *E. coli* DH5α and coated on LB plate containing ampicillin, respectively at 37℃ over night. The negative control of ligation reaction was conducted with a plasmid containing random sequence.

Identification of positive clones that contain siRNA insert used PCR amplification by mixing 2.5μl of LA Buffer ($MgCl_2^+$) containing siRNA with 0.25μl of Taq (2.5U/μl), 4μl of dNTP(25mM), 0.25μl of primer F(10μM), 0.25μl of primer R(10μM) in the following condition: incubate at 94℃ for 10 min, followed by 33 cycles of 30 sec at 94℃, 30s at 55℃, 30 sec at 72℃. The PCR product was identified by 1.5% agarose gel electrophoresis. The positive clones were also confirmed by sequencing cloning site with the above primers.

2.7 Transfection and analysis of plasmid

HepG2 cells were cultured in DMEM medium containing 10% fetal bovine serum and the combination of two antibiotics, penicillin (100 IU/ml) and streptomycin (100μg/ml) with 5%CO_2 at 37℃. These cells were resuspended in LB medium without antibiotic, and plated onto 24-well plate in a density of 0.5×10^5 cell/well at 24h before transfection. The transfection was carried out by transfecting the plasmid DNA into HepG2 cells using liposome Lipofectamine^{TM}2000 at a ratio of 1 to 2 between plasmid DNA (μg) and liposome (μl). The transfection efficiency of plasmid DNA can be observed under fluorescence microscope after 24h transfection. The siRNA effect that reduces gene expression by specific siRNA construct plasmid in cells can be further assessed at both RNA and protein levels by Northern analysis, RT-PCR, Western analysis and immunofluoresence.

3. Results

3.1 Bioinformatics and computer-based design of siRNA to HBV

Our bioinformatics and comparative genomic study showed that one of the candidate siRNAs specific for HBV orf X and two of those for HBV orf P were chosen as appropriate candidate siRNAs to construct three short hairpin RNA (shRNA) expression vectors, respectively (Table 1).

Table 1. Three candidate siRNAs to HBV based on computer design

No	Designed candidate siRNAs	Targeted HBV cds
Ⅰ	ggtcttgcataagaggact	X
Ⅱ	cacttccggaaactactgt	P
Ⅲ	actcatcgggactgacaat	P

3.2 DNA oligonucleotides sequences design

Based on the chosen siRNA sequence, two DNA oligonucleotides～64 nt in size were designed for insertion into the pRNATU6 vectors in our study. In the forward oligonucleotide, the 19-nucleotide sense siRNA sequence is linked to the reverse complementary antisense siRNA sequence by a 9-nucleotide spacer (TTCAAGAGA). In the forward oligonucleotide, 5-nucleotide overhangs to the *Bam*H Ⅰ (GATCC) and *Hin*d Ⅲ (GGAAA) restriction sites were added to the 5’ and 3’ end of the 64 nt sequence complementary to the reverse oligonucleotide, respectively.

DNA oligonucleotide insert Ⅰ

Tem1:5’ GATCCcggtcttgcataagaggactTTCAAGAGAagtcctcttatgcaagacctttttGGAAA 3’,
*Bam*H Ⅰ sense siRNA spacer antisense *Hin*dⅢ

Tem2:5’agcttttggaaaaaaggtcttgcataagaggacttctcttgaaagtcctcttatgcaagaccgg 3’

DNA oligonucleotide insert Ⅱ

Tem1:5’GATCCcacttccggaaactactgtTTCAAGAGAacagtagtttccggaagtgtttttGGAAA 3’
*Bam*H Ⅰ sense siRNA spacer antisense *Hin*dⅢ

Tem2:5’agcttttggaaaaaacacttccggaaactactgttctcttgaaacagtagtttccggaagtggg 3’

DNA oligonucleotide insert Ⅲ

Tem1:5’GATCCcgactcatcgggactgacaatTTCAAGAGAattgtcagtcccgatgagttttttGGAAA 3’
*Bam*H Ⅰ sense siRNA spacer antisense *Hind*Ⅲ
Tem2:5’agcttttggaaaaaaactcatcgggactgacaattctcttgaaattgtcagtcccgatgagtcgg 3’

The resulting RNA transcript is likely fold back and form a stem-loop structure comprising 19bp stem and 9nt loop with 2-3 Us at the 3’ end.

3.3 Identification of recombinant siRNA plasmid

The single-strand DNA oligonucleotide with siRNA insert was ligated into linearized vector to construct recombinant plasmid pRNATU6. The plasmid DNA from the ligation reaction was transformed in *E.coli* DH5α. To select positive clones that contain the siRNA insert, PCR was used to detect the purified plasmid DNA from different clones, and the PCR products was identified by 1.5% agarose gel electrophoresis (Fig.1). The negative control of ligation reaction with a plasmid containing random sequence pruduced more less colonies than the ligation reaction containing the vector and the siRNA insert.

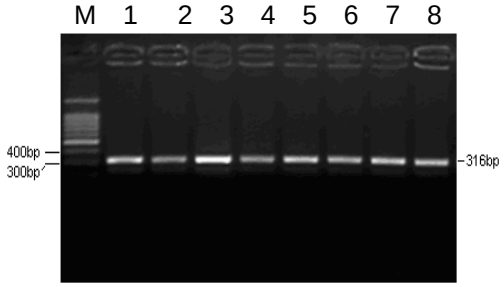
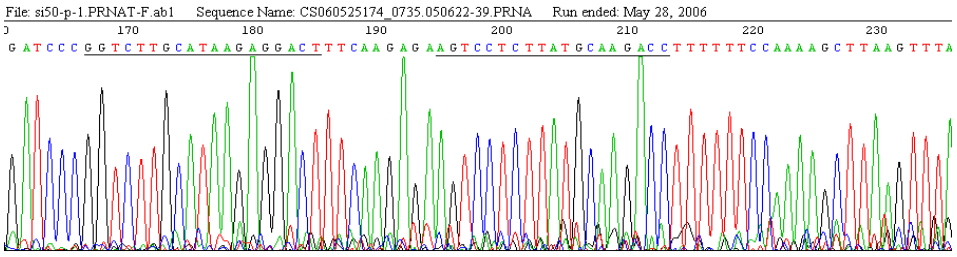
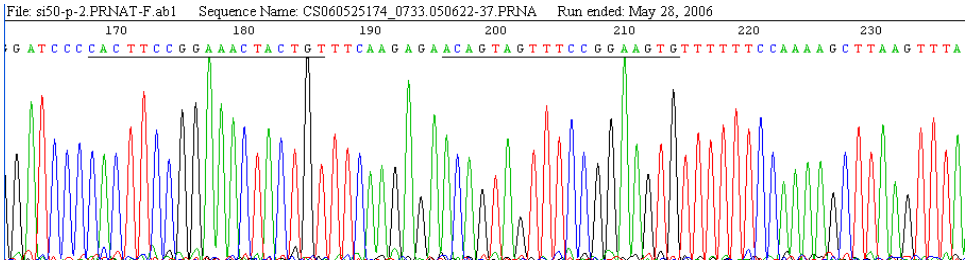


Fig. 1. The PCR amplification of the purified plasmid DNA from different clones. As expected, a 316bp PCR product was detected by 1.5% agarose gel electrophoresis. Lane M: DNA marker (100bp DNA ladder); lane 1-2: colonies of plasmid with siRNA insert Ⅰ; lane 3-4: colonies of plasmid with siRNA insert Ⅱ; lane 5-6: colonies of plasmid with siRNA insert Ⅲ; lane 7-8: colonies of plasmid with random sequence.

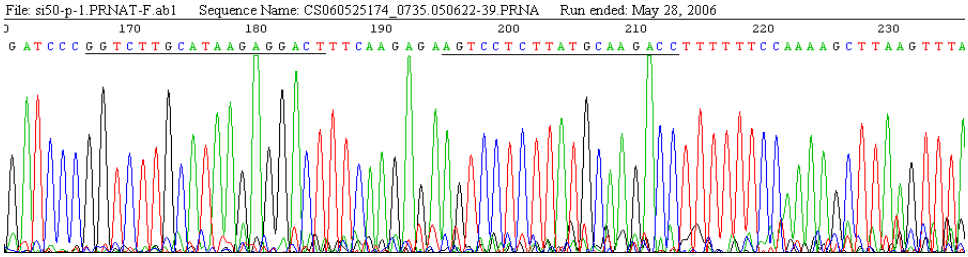
The positive clones were further confirmed by sequencing cloning site with the above primers (Fig.2).



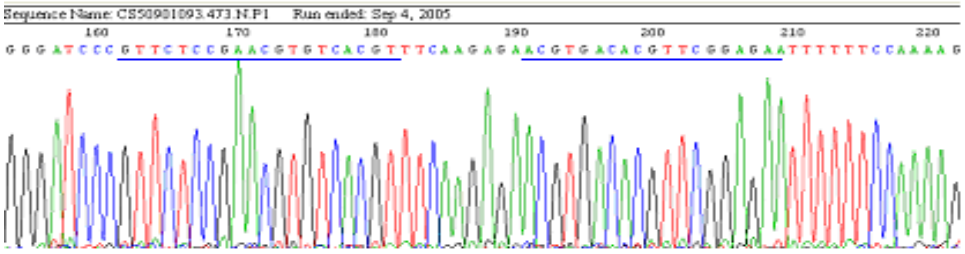
(A)



(B)



(C)



(D)

Fig. 2. The sequencing results of the purified plasmid DNA from the positive clones. (A) the plasmid with siRNA insert Ⅰ; (B) the plasmid with siRNA insert Ⅱ; (C) the plasmid with siRNA insert Ⅲ; (D) the plasmid with a random sequence.

3.4 Transfection of HepG2 cell with recombinant siRNA plasmid

Identification of the transfection efficiency of HepG2 cells was performed with fluorescence microscope. HepG2 cells were transfected by the recombinant plasmid pRNATU6 that carries green fluorescent protein (GFP) with liposome. The transfected HepG2 cells with three different recombinant siRNA plasmids were all found to be stained with green fluorescence (Fig.3).

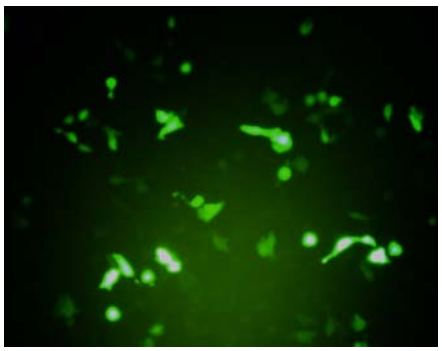


Fig. 3. HepG2 cells transfected by the recombinant siRNA plasmid with liposome. The transfected HepG2 cells by three different recombinant siRNA plasmids and a random sequence plasmid were all found to be stained with green fluorescence under fluorescence microscope. All the expression vectors used in the expriment carry green fluorescent protein (GFP). (*Magnification 200*×)

4. Discussion

RNAi via small interfering RNA (siRNA) has generated a great deal of interest in both basic and applied biology in the past several years. The hairpin siRNA sequences are the key to the RNAi process. The target design of siRNA sequences aims mainly at coding region or at non-coding region. The biologic effects of siRNA are also related to the accessibility of the siRNA targeted secondary structure on mRNA sequence or target sequence under the premise of specificity. For screening, four siRNAs per target were tested by spacing the siRNA down the length of the gene sequence to reduce the chances that we are targeting a region of the mRNA that is either highly structured or bound by regulatory proteins in our experiment, and one of

the candidate siRNAs specific for HBV orf X and two of those for HBV orf P were chosen as more prevalence candidate siRNAs to construct three shRNA expression vectors, respectively.

The current widely used methods to obtain siRNA are chemical synthesis, digestion long dsRNA with RNase Ⅲ, the construction of siRNA expression vector and PCR-based siRNA expression frame, etc [5,6,7]. Although the two formers have the merit of labelling and transfecting easilly, the scale of synthesis was limited and not suitable to long-term study, and PCR-based siRNA expression frame is not only uneasy to transfect but also not suitable for long-term study. In contrast, the siRNA expression by a plasmid or viral vector have the advantages of easy transfection and long action time, and thus they are the most common used method for siRNA expression. Due to plasmid-based siRNA expression system carrying GFP gene, it is a convenient way to observe the plasmid-based siRNA experssion in cells stained with green fluorescence.

The hairpin siRNA expression vector used in our plasmid-based siRNA experiment is a 6.5 Kb vector, containing sequence element for cloning and bacterial replication, i.e. f1 origin, ColE1 origin, ampicillin resistance gene, and multiple cloning site, and gene that encodes GFP. A U6 polymerase Ⅲ (Pol Ⅲ) promoter is cloned into the *Bam*H Ⅰ and *Hin*d Ⅲ sites to generate small RNA transcripts. The U6 Pol Ⅲ promoter was chosen because it has a well-defined transcription initiation site and Pol Ⅲ termination sequence. This hairpin siRNA expression vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved into siRNA, which is then bound to the RNA-induced silencing complex (RISC) that binds to and cleaves mRNAs which match the siRNA that is bound to it.

The HepG2 cell is the most common cell line in study of HBV infection model. Domestic and international literatures had reported that HepG2 cells were successfully transfected by adenovirus- mediated HBV DNA replicated in the cell line 293, and the viral products could be detected in the each step of replication, and it also make up for the deficiency of HepG2.2.15 that couldn't conduct point mutation *in vitro* and adjust the level of viral replication [8,9]. We introduced the hairpin siRNA recombinant plasmid into HepG2 cells by liposome following the Lipofectamine^{TM}2000 protocol, and the transfected HepG2 cells stained with green fluorescence could be seen with fluorescence microscope, which indicated that plasmid-based siRNA expressed well in host cells. It lays a foundation for the further

study on reduction of HBV genes expression by the RNAi effect that can be confirmed at both RNA and protein levels in the HBV infection model by Northern blot, RT-PCR, Western blot and immunofluorescence.

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