Inhibition of expression of hepatitis C virus 1b genotype core and NS4B genes in HepG2 cells using artificial microRNAs

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Abstract. The present study aimed to evaluate the silencing effect of artificial microRNAs (amiRNAs) against the hepatitis C virus (HCV) 1b (HCV1b) genotype core (C) and non-structural protein 4B (NS4B) genes. pDsRed-monomer-Core and pDsRed-monomer-NS4B plasmids, containing the target genes were constructed. A total of eight artificial micro RNA (amiRNA)-expressing plasmids, namely, pmiRE-C-mi1 to -mi4 and pmiRE-NS4B-mi1 to -mi4, were designed and constructed to interfere with various sites of the core and NS4B genes, and the amiRNA interfering plasmid and the corresponding target gene-expressing plasmid were co-transfected into HepG2 cells. At 48 h after transfection, HCV core and NS4B gene expression levels were detected using fluorescence microscopy, flow cytometry, reverse transcription quantitative polymerase chain reaction and western blot analysis. Fluorescence microscopy revealed that the target gene-transfected cells expressed red fluorescent protein, whereas the interfering plasmid-transfected cells exhibited expression of green fluorescent protein. The percentage of red fluorescent proteins and mean fluorescence intensity, as well as protein expression levels of the core and NS4B genes within the cells, which were co-transfected by the amiRNA interfering plasmid and the target gene, were significantly decreased. The results of the present study confirmed that amiRNAs may effectively and specifically inhibit the expression of HCV1b core and NS4B genes in HepG2 cells, potentially providing a novel therapeutic strategy for the treatment of HCV.

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Introduction

The hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (1,2). An estimated 185 million people around the world are infected with HCV, with three to four million new cases each year (3). However, the pathogenesis of HCV remains to be fully elucidated. The standard of care (SOC) for HCV is the combined application of pegylated interferon α and ribavirin, with a sustained virological response (SVR) rate of up to 50%; however, the various genetic subtypes of HCV exhibit differing response rates to treatment (4). In addition, the treatment cost is high and there is a significant risk of side effects (5,6). The development of small molecule inhibitors targeting HCV replication-associated enzymes has recently achieved success in HCV therapy, with a significant increase in SVR (7-10). Despite this advance, the issue of resistance remains a problem (11,12), even when used in combination therapy with the SOC. Therefore, the development of novel antiviral strategies is required in order to more effectively treat HCV infection.

RNA interference (RNAi) is a post-transcriptional cellular process mediated by short (21-25 nucleotides) double-stranded RNA molecules (13), which are capable of gene silencing (14-17). MicroRNAs (miRNAs) are members of this group of small RNAs. The primary miRNAs are processed by the Drosha ribozyme and DGCR8 into precursor miRNAs consisting of 60-70 nucleotides (18). These miRNAs are subsequently exported from the nucleus by Exportin-5 (19), and processed into mature miRNAs by the ribonuclease-III enzyme Dicer (18). The guiding strand of the mature miRNA is then loaded into the RNA-induced silencing complex (20) to form the miRNA-containing ribonucleoprotein complex (miRNP), which is able to mediate the cleavage of target mRNA or result in translational repression (16). Artificial miRNAs (amiRNAs) comprise a class of artificially synthesized RNAs (21), similar to cellular miRNAs, which may be harnessed to silence mRNAs encoding pathogenic proteins for use in therapeutic strategies and functional genomics (22).

The HCV genome is a 9.6 kb positive-stranded RNA molecule containing a long open reading frame encoding a polyprotein precursor, which is processed by cellular and viral proteases into structural (Core, E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. The

HCV core protein is a conserved protein, which comprises the viral nucleocapsid and is able to bind viral RNA. The HCV core protein is also a multifunctional, influencing gene transcription, lipid metabolism, apoptosis and a variety of signal transduction pathways in host cells (23-26). In addition, the core protein has been implicated in HCV-associated steatosis and carcinogenesis in transgenic mice (27,28). This protein is hypothesized to serve a function in the assembly of HCV (29). HCVNS4B is a hydrophobic integral membrane protein that has a central function in the formation of the membranous web; a specific membrane alteration, which serves as a scaffold for the HCV replication complex (30-32). In addition, NS4B possesses NTPase (33,34) and RNA-binding activities (35), as well as contributing to viral assembly and release (36,37). Therefore, HCV core and NS4B mRNAs may be used as targets for RNAi.

In the present study, two sets of amiRNA expression vectors were designed and constructed against the HCV1b genotype core and NS4B mRNAs, in order to evaluate the interference efficiency of amiRNAs on HCV core and NS4B gene expression in HepG2 cells. In addition, the present study aimed to screen the optimum miRNA interference vectors for use in subsequent studies on the functions and pathogenesis of HCV core and NS4B genes, as well as the examination of novel classes of inhibitors for HCV infection.

Materials and methods

Collection of serum samples. A total of 15 patients with HCV1b were enrolled in the present study from the First Affiliated Hospital of The University of South China (Hengyang, China). The patients included 7 males and 8 females, and were aged between 27 and 66 years old. The venous blood samples of the patients were collected by a trained nurse at the Research Center of Liver Diseases (First Affiliated Hospital of the University of South China). After centrifugation at 241 x gfor 10 min at room temperature, the serum was obtained and preserved at -80°C until further use. The quantification and genotyping of HCV RNA were performed by the Research Center of Liver Diseases. The present study was conducted in accordance with the Declaration of Helsinki, and with approval from the Ethics Committee of Central South University (Changsha, China). Written informed consent was obtained from all participants.

Construction of plasmids. TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract HCV RNA from the serum of HCV1b patients. The serum was then reverse transcribed into cDNA using the SuperScript III First-strand synthesis system (Invitrogen Life Technologies). Subsequently, polymerase chain reaction (PCR) amplification was performed with gene-specific primers (Shanghai Sangon Biological Technology and Services Co., Ltd., Shanghai, China). The primer sequences of the core gene amplification in the first round were as follows: HCV1b sense, 5'-GAGTAGYGT TGGGTCGCGAAAGG-3' and antisense, 5'-TTGAGTTGGAGCAGTCGTTCGTGA-3'. In the second round, the PCR primer sequences were as follows: HCV1b sense, 5'-GCCACGAAG CTTGCATGAGCACRAATCCWAAAC-3' (AAGCTT is the enzyme restriction site of *Hin*dIII) and antisense, 5'-TAGATTGGATCCAARGCGGAAGCTGGG

RTGGTC-3' (GGATCC is the enzyme restriction site of *Bam*HI). The primer sequences of NS4B gene amplification in the first round were as follows: NS4B sense, 5'-GAGTGCGCYTCRCACCTCCCT TA-3' and antisense, 5'-CGGAGCAYGGYGTGGAGCAG-3'. In the second round, the PCR sense primer sequences were as follows: NS4B sense, 5'-GTCCAGAAGCTTGCATGGCYTCRCACCTCCCTTA-3' (AAGCTT is the enzyme restriction site of *Hin*dIII) and antisense, 5'-TAGATTGGATCCAAGCAY GGYGTGGAGCAGTCCT-3' (GGATCC is the enzyme restriction site of *Bam*HI). The PCR conditions consisted of pre-denaturation, 94°C for 3 min; denaturation, 94°C for 30 sec; annealing, 61°C for 30 sec and extension, 72°C for 1 min. This cycle was repeated 35 times with a final extension at 72°C for 10 min. The amplified HCV1bCore and HCV1bNS4B genes contained the double-enzyme restriction sites of *Hin*dIII and *Bam*HI (Fermentas International Inc., Burlington, ON, Canada), which were then used to digest the two aforementioned target fragments and vector pDsRed-monomer-N1 (Clontech, Mountain View, CA, USA). Following purification, the two digested fragments were connected with the vectors using T4 DNA ligase (Fermentas International Inc.), and were then transformed into DH5α competent cells (preserved in the Laboratory of Infectious Diseases, Xiangya Hospital, Changsha, China) to construct the plasmids of pDsRed-monomer-Core and pDsRed-monomer-NS4B. The constructed plasmids were then subjected to double digestion and sequencing (Invitrogen Life Technologies, Shanghai, China).

Construction of miRNA expression vectors. The HCV core and NS4B genetic information was submitted through the BLOCK-iT™ RNAi Express database (http://rnaidesigner. invitrogen.com/rnaiexpress/rnaiExpress.jsp) to select the interference sites. The gene specificity of all interference sites was also assessed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). A total of two sets of miRNA interference sequences were designed and the single-stranded DNA oligonucleotides (Invitrogen Life Technologies) of eight pairs of miRNAs and those of a negative control miRNA, were synthesized. The sequences are shown in Table I. The eight pairs of single-stranded DNA oligonucleotides were then annealed and the double strands were formed. The vector construction kit BLOCK-iT™ Pol II miR RNAi expression vector kit with Emerald Green Fluorescent Protein (EmGFP) (Invitrogen Life Technologies, Carlsbad, CA, USA) was then used to recombine the clones. A total of eight pairs of double-stranded oligonucleotides were inserted into the miRNA expression vector pcDNA™ 6.2-GW/EmGFP-miR to construct eight miRNA expression plasmids for core silencing (pmiRE-C-mi1 to -mi4) and NS4B silencing (pmiRE-NS4B-mi1 to -mi4). The above plasmids were transformed into DH5α competent cells. Following agitation extraction using TIANprep Mini Plasmid kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) in a SHZ-88 Thermostatic Oscillator (Jintan Medical Instrument Factory, Changzhou, China), the extracted plasmids were sequenced, using the reverse sequencing primer 5'-CTCTAGATCAACCACTTTGT-3', to verify whether the fragment sequence inserted into the recombinant clones was consistent with the designed single-stranded DNA oligonucleotide sequence.

Table I. Sequences of oligonucleotides for microRNAs directed against hepatitis C virus 1b core and non-structural protein 4B genes.

Oligo name	Oligo single-stranded DNA sequence 5' to 3'
C-mi1F	TGCTGATAGGTTGTCGCCTTCCACGAGTTTTGGCCACTGACTGACTCGTGGAACGACAACCTAT
C-mi1R	CCTGATAGGTTGTCGTTCCACGAGTCAGTCAGTGGCCAAAAC<u>TCGTGGAAGGCGACAACCTAT</u>C
C-mi2F	TGCTGTATCGATGACCTTACCCAAATGTTTTGGCCACTGACTGACATTTGGGTGGTCATCGATA
C-mi2R	CCTGTATCGATGACCACCCAAATGTCAGTCAGTGGCCAAAAC<u>ATTTGGGTAAGGTCATCGATA</u>C
C-mi3F	TGCTGATTCCCTGTTGCGTAGTTCACGTTTTGGCCACTGACTGACGTGAACTACAACAGGGAAT
C-mi3R	CCTGATTCCCTGTTGTAGTTCACGTCAGTCAGTGGCCAAAAC<u>GTGAACTACGCAACAGGGAAT</u>C
C-mi4F	TGCTGCAAACAGGACAGCAGAGCCAAGTTTTGGCCACTGACTGACTTGGCTCTTGTCCTGTTTG
C-mi4R	CCTGCAAACAGGACAAGAGCCAAGTCAGTCAGTGGCCAAAAC<u>TTGGCTCTGCTGTCCTGTTTG</u>C
NS4B-mi1F	TGCTGAGTGCCTTCTGCTTGAATTGTGTTTTGGCCACTGACTGACACAATTCACAGAAGGCACT
NS4B-mi1R	CCTGAGTGCCTTCTGTGAATTGTGTCAGTCAGTGGCCAAAAC<u>ACAATTCAAGCAGAAGGCACT</u>C
NS4B-mi2F	TGCTGTAAGCCTGCTAAGTACTGTATGTTTTGGCCACTGACTGACATACAGTATAGCAGGCTTA
NS4B-mi2R	CCTGTAAGCCTGCTATACTGTATGTCAGTCAGTGGCCAAAAC<u>ATACAGTACTTAGCAGGCTTA</u>C
NS4B-mi3F	TGCTGTTAAACAGGAGGGTGTGTTGGGTTTTGGCCACTGACTGACCCAACACACTCCTGTTTAA
NS4B-mi3R	CCTGTTAAACAGGAGTGTGTTGGGTCAGTCAGTGGCCAAAAC<u>CCAACACACCCTCCTGTTTAA</u>C
NS4B-mi4F	TGCTGTTCAGTAACTGAGTGATGGTAGTTTTGGCCACTGACTGACTACCATCACAGTTACTGAA
NS4B-mi4R	CCTGTTCAGTAACTGTGATGGTAGTCAGTCAGTGGCCAAAAC<u>TACCATCACTCAGTTACTGAA</u>C
Negative-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
Negative-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAAC<u>GTCTCCACGCGCAGTACATTT</u>C

C-mi1, C-mi2, C-mi3 and C-mi4 target nt 174-194, nt 353-373, nt 484-504 and nt 535-555 of the HCV 1b core gene, respectively. NS4B-mi1, NS4B-mi2, NS4B-mi3 and NS4B-mi4 target nt 45-65, nt 181-201, nt 276-296 and nt 720-740 of the HCV 1b NS4B gene, respectively. All pre-artificial microRNAs were based on the murine miR-155 pre-microRNA and contain 2 nt central mismatches. Sequences yielding the mature antisense strands are underlined. Oligo, oligonucleotide; F, forward; R, reverse; C, core; NS4B, non-structural protein 4B; nt, nucleotides; HCV, hepatitis C virus.

Cell culture and transfection. HepG2 cells (preserved in the Laboratory of Infectious Diseases, Xiangya Hospital) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies) in a humidified 5% CO_2 atmosphere at 37˚C. HepG2 cells at the logarithmic growth phase were seeded into six-well plates at a density of ~$1.0x10^6$ cells/well. When the cell density reached ~80%, 1 μg target gene plasmid and 3 μg interference plasmid or negative control plasmid were mixed with Lipofectamine® 2000 (Invitrogen Life Technologies). The transfection was performed according to the Lipofectamine 2000 manufacturer's instructions. Following 48 h, the transfected-cellular fluorescent protein expression was observed under a fluorescence microscope (Olympus IX71; Olympus Corp., Tokyo, Japan).

Reverse transcription quantitative PCR (RT-qPCR). At 48 h post-transfection, the total RNA of each group was extracted using TRIzol reagent (Invitrogen Life Technologies). Subsequently, 5 μg of RNA was reverse transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen Life Technologies). The obtained cDNA was then used as the template for the PCR reaction. The primer sequences were as follows: HCV core sense, 5'-TACGGCAACGAGGGCTTAG-3' and antisense, 5'-ATGGTCAAACAGGACAGCAGAG-3'; NS4B sense, 5'-CTCGCCGAACAATTCAAGC-3' and anti-

sense, 5'-GTTCCCAGGCAGAGTGGATAA-3'; and GAPDH sense, 5'-GAAGGTCG GAGTCAACGGATT-3' and antisense, 5'-CGCTCCTGGAAGATGGTGAT-3' (Shanghai Sangon Biological Technology and Services Co., Ltd.). The PCR reaction mixture consisted of 1.2 μl cDNA, 2.5 μl 10X PCR buffer, 0.2 μl dNTPs (25 mM), 0.5 μl each of upstream and downstream primers (10 μM), 0.5 μl SYBR (50X), 0.3 μl of Taq enzyme (5u/ μl), 2 μl of Mg^{2+} (25 mM) and 17.3 μl of ultrapure water (Shanghai Sangon Biological Technology and Services Co., Ltd.). The total volume of the reaction mixture was 25 μl. The PCR reaction conditions were as follows: 95˚C for 2 min; 95˚C for 10 sec, 60˚C for 30 sec and 70˚C for 45 sec for 40 cycles. All RT-qPCR experiments were performed in triplicate on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Based on the RT-qPCR reaction curve, the Ct values of the target gene and reference gene of each sample were obtained using the $2^{-\Delta\Delta Ct}$ method for the relative quantification. The interference efficiency of the target gene was $1-2^{-\Delta\Delta Ct}$.

Flow cytometric analysis. The two RT-qPCR-screened miRNA vectors with the greatest interfering effects against the HCV core and NS4B genes, as well as the negative control miRNA vector, were co-transfected with the cells containing the target gene for 48 h. The cells were then digested with 0.25% trypsin (Fuzhou Maixin Biotechnology Development Co., Ltd.,
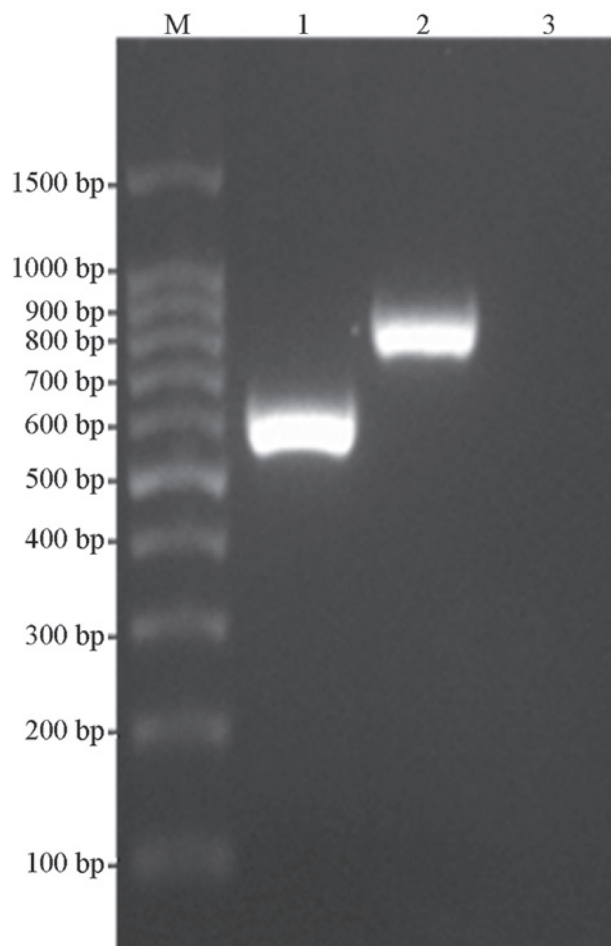
Figure 1. Gel electrophoresis of polymerase chain reaction amplification products of HCV1bCore and NS4B genes. Lanes: M, DNA relative molecular weight standards; 1, HCV1b core gene; 2, HCV1b NS4B gene and 3, empty vector control. HCV1b, hepatitis C virus 1b; NS4B, non-structural protein 4B.
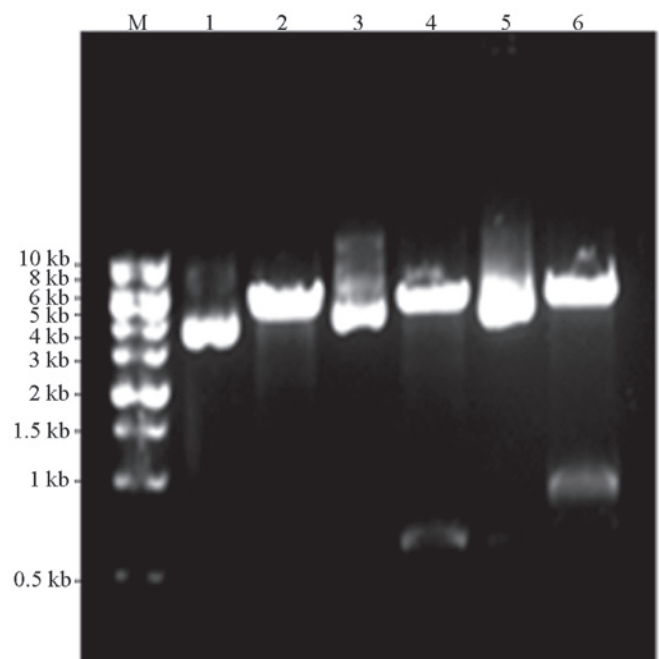

Figure 2. Enzyme digestion identification of recombinant plasmids of pDsRed-monomer-Core and pDsRed-monomer-NS4B. Lanes: M, DNA relative molecular weight standards; 1, pDsRed-monomer-N1plasmid; 2, double digestion products of pDsRed-monomer-N1 plasmid; 3, recombinant pDsRed-monomer-Core plasmid; 4, double digestion products of recombinant pDsRed-monomer-core plasmid; 5, recombinant pDsRed-monomer-NS4B plasmid and 6, double digestion products of recombinant pDsRed-monomer-NS4B plasmid. NS4B, non-structural protein 4B.

Fuzhou, China), washed twice with phosphate-buffered saline (PBS; Fuzhou Maixin Biotechnology Development Co., Ltd.) and resuspended in PBS. Samples were subsequently assessed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to calculate the percentage of fluorescent cells and the mean fluorescence intensity.

Western blot analysis. The two screened miRNA vectors with the optimum interfering effects, as well as the negative control miRNA vector, were co-transfected into the cells with the target gene for 48 h. The cells were then lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Following centrifugation at 1,048 x g for 5 min at 4°C, the supernatant was collected for protein concentration detection. Subsequently, 60 μg of protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under semi-dry conditions. Blots were blocked with 5% non-fat milk (Beyotime Institute of Biotechnology) in Tris-buffered saline with 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 2 h. The membrane was incubated overnight at 4°C with the following primary antibodies:

Mouse anti-HCV core antigen antibody (cat. no. ab2740; 1:1,000 dilution; Abcam, Cambridge, UK), mouse anti-HCV NS4B monoclonal antibody (cat. no. ab24283; 1:1,000 dilution; Abcam) and β-actin antibody (cat. no. sc-47778; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following washing the membrane, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (cat. no. A0216; 1:5,000 dilution; Beyotime Institute of Biotechnology) at room temperature for 2 h. The signals were revealed with the BeyoECL Plus enhanced chemiluminescent kit (Beyotime Institute of Biotechnology).

Statistical analysis. Statistical analyses were carried out using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Significance between groups was determined by one-way analysis of variance followed by a Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of plasmids. The target gene-expressing plasmids, pDsRed-monomer-Core and pDsRed-monomer-NS4B, were subjected to PCR amplification, double-enzyme digestion and sequencing. The results were consistent with the expected values, whereby the PCR gene products of the HCV core and NS4B proteins were ~580 and 790 bp, respectively and the digested fragment of pDsRed-monomer-N1 was ~4.7kb, as shown in Figs. 1 and 2. The DNA sequencing results
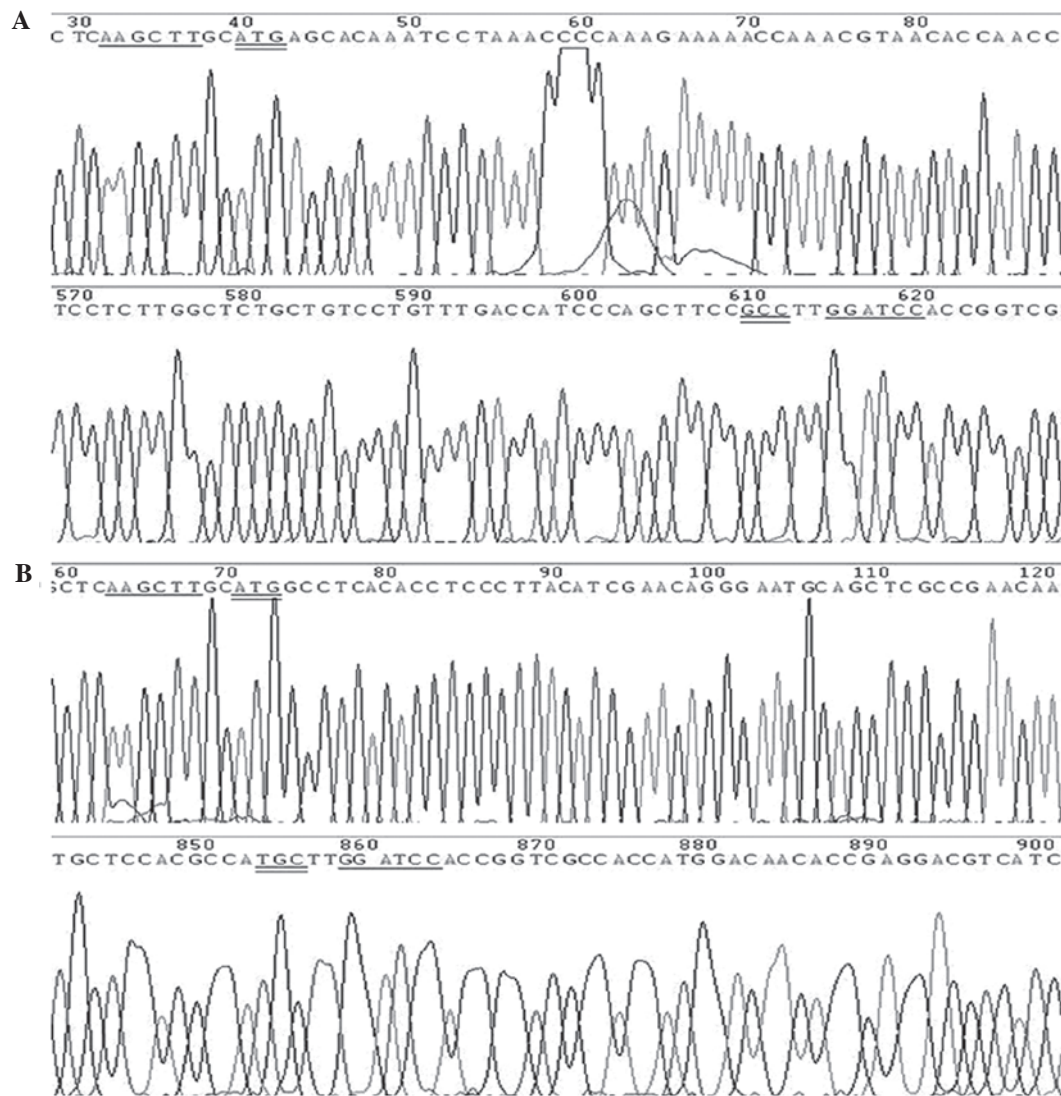
Figure 3. Sequencing peak patterns of recombinant pDsRed-monomer-Core and pDsRed-monomer-NS4B plasmids (partial). (A) Peak-figure of HCVCore gene sequencing. AAGCTT and GGATCC were the restriction sites of *Hin*dIII and *Bam*HI, respectively; ATG was the start codon of the HCVCore gene and GCC were the last three codons of HCVCore. (B) Peak-figure of HCVNS4B gene sequencing. AAGCTT and GGATCC were the restriction sites of *Hin*dIII and *Bam*HI, respectively; ATG was the start codon of HCVNS4B gene and TGC were the last three codons of HCVNS4B. HCV, hepatits C virus; NS4B, non-structural protein 4B.

confirmed that two recombinant plasmids were efficiently constructed. Partial sequencing peak patterns of the recombinant pDsRed-monomer-Core and pDsRed-monomer-NS4B plasmids are shown in Fig. 3.

Construction of miRNA expression vectors. A total of eight miRNA expression plasmids, pmiRE-C-mi1 to -mi4 and pmiRE-NS4B-mi1 to -mi4, were verified to contain identical designed single-stranded DNA oligonucleotide sequences using the sequencing test, relative to the inserted fragment sequences (data not shown).

Expression of fluorescent proteins. A fluorescence microscope was used to observe the fluorescent protein expression 48 h after the cells were transfected (Fig. 4). The fluorescence of the pDesRed-monomer-N1 (53.80±3.56%) was more evident, as compared with that of the pDsRed-monomer-Core (32.17±3.82%) and the pDsRed-monomer-NS4B (32.73±3.45%),

respectively (P<0.01). At 48 h post-transfection of the eight pairs of miRNA expression plasmids, the cells of each group exhibited green fluorescent protein expression indicating successful transfection.

Expression of HCV core and NS4B mRNAs. RT-qPCR was performed to assess the mRNA expression levels of HCV core or NS4B in the HepG2 cells. The expression levels of core or NS4B gene in HepG2 cells, which had been co-transfected by the negative control miRNA and pDsRed-monomer-Core or pDsRed-monomer-NS4B were set as 100%. pmiRE-C-mi1 to -mi4 was co-transfected into the HepG2 cells with pDsRed-monomer-Core. The relative expression levels of core mRNA were 38.0±4.0, 30.0±4.6, 48.0±7.2 and 67.0±3.0%, respectively. The pmiRE-NS4B-mi1 to -mi4 was co-transfected into the HepG2 cells with pDsRed-monomer-NS4B, and the relative expression levels of NS4B mRNA were 43.0±4.6, 58.0±4.0, 30.0±3.0 and 21.0±3.0%, respectively. The results
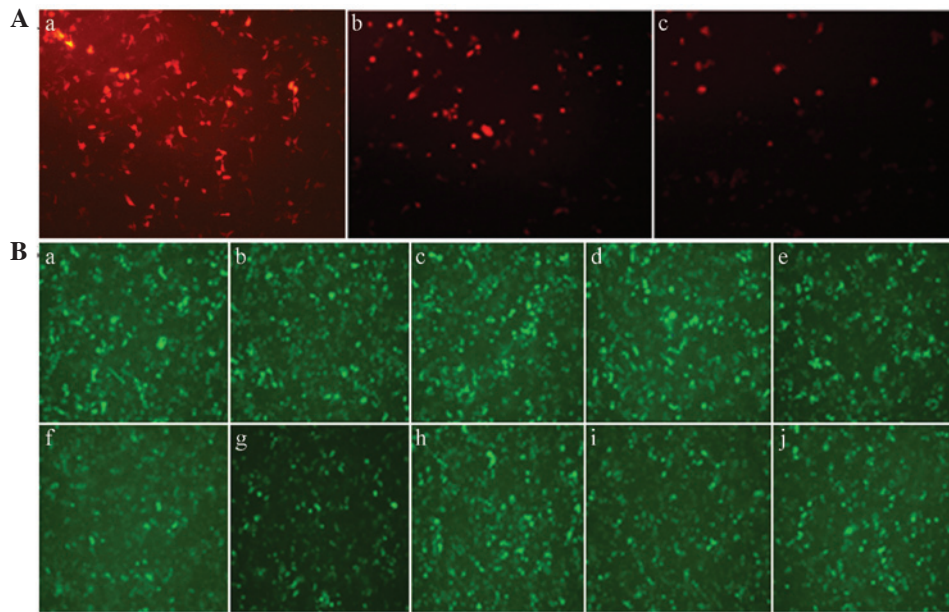
Figure 4. Expression of target proteins and their miRNAs. (A) Red fluorescent protein expression of HepG2 cells, which were transfected by target gene-expressing plasmids (magnification, x100). (a) pDesRed-monomer-N1-transfected HepG2 cells; (b) pDsRed-monomer-Core-transfected HepG2 cells and (c) pDsRed-monomer-NS4B-transfected HepG2 cells. (B) Green fluorescent protein expression by HepG2 cells, which were transfected by the miRNA interference plasmids (magnification, x100). (a-d) pmiRE-C-mi1 to -mi4-transfected HepG2 cells; (e) negative control miRNA plasmid-transfected HepG2 cells; (f-i) pmiRE-NS4B-mi1 to -mi4-transfected HepG2 cells; (j) negative control miRNA plasmid-transfected HepG2 cells. NS4B, non-structural protein 4B; miRNA, micro RNA.
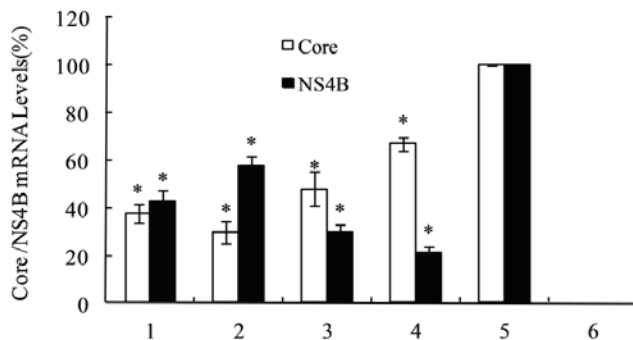

Figure 5. Inhibitory effect of miRNAs on the expression of HCV core and NS4B mRNA in HepG2 cells detected by reverse transcription quantitative polymerase chain reaction. 1: pDsRed-monomer-core/pmiRE-C-mi1 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi1 co-transfected; 2: pDsRed-monomer-core/pmiRE-C-mi2 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi2 co-transfected; 3: pDsRed-monomer-core/pmiRE-C-mi3 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi3 co-transfected; 4: pDsRed-monomer-core/pmiRE-C-mi4 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi4 co-transfected; 5: (pDsRed-monomer-core or pDsRed-monomer-NS4B)/negative control miRNA co-transfected HepG2 cells; 6: HepG2 cells without transfection. *P<0.01, compared with the cells transfected with negative control. Data are presented as the mean ± standard deviation. HCV, hepatitis C virus; NS4B, non-structural protein 4B; mRNA, messenger RNA; miRNA, microRNA.

are shown in Fig. 5. The results indicated that among the HCV core interference vectors, the optimum gene silencing effect of was a reduction of 70%, exerted by pmiRE-C-mi2; whereas, among the HCVNS4B interference vectors, the optimum gene silencing effect was a decrease of 79%, induced by pmiRE-NS4B-mi4.

Expression of HCV core and NS4B proteins. Flow cytometric analysis revealed that the single transfection of pDsRed-monomer-Core or pDsRed-monomer-NS4B into the HepG2 cells, when compared with the HepG2 cells that were co-transfected by the negative control miRNA and pDsRed-monomer-Core or pDsRed-monomer-NS4B, exhibited no significant differences in terms of red fluorescent protein percentage and average fluorescence intensity. In addition, the pDsRed-monomer-Core and pmiRE-C-mi2 co-transfected HepG2 cells exhibited a reduced percentage of red fluorescent protein expression and average fluorescence intensity by 2.5 and 2.0-fold, respectively. The red fluorescent protein percentage and average fluorescence intensity in the pDsRed-monomer-NS4B and pmiRE-NS4B-mi4 co-transfected HepG2 cells were also decreased by 3.0 and 2.6-fold, respectively, as shown in Fig. 6.

Western blot analysis revealed that, compared with the HepG2 cells that were transfected by the pDsRed-monomer-Core only, the pDsRed-monomer-Core and pmiRE-C-mi2 co-transfected HepG2 cells exhibited reduced expression of the HCV core protein; whereas expression levels in the pDsRed-monomer-Core and negative control miRNA co-transfected HepG2 cells were not decreased. Similar results were also observed in the expression of NS4B protein, as shown in Fig. 7.

Discussion

RNAi has become a powerful tool, with the potential to knock down the expression of disease-associated genes or inhibit viral gene expression (38). HCV is comprised of a positive-stranded RNA virus, containing a single-stranded RNA genome, which functions as mRNA for the translation
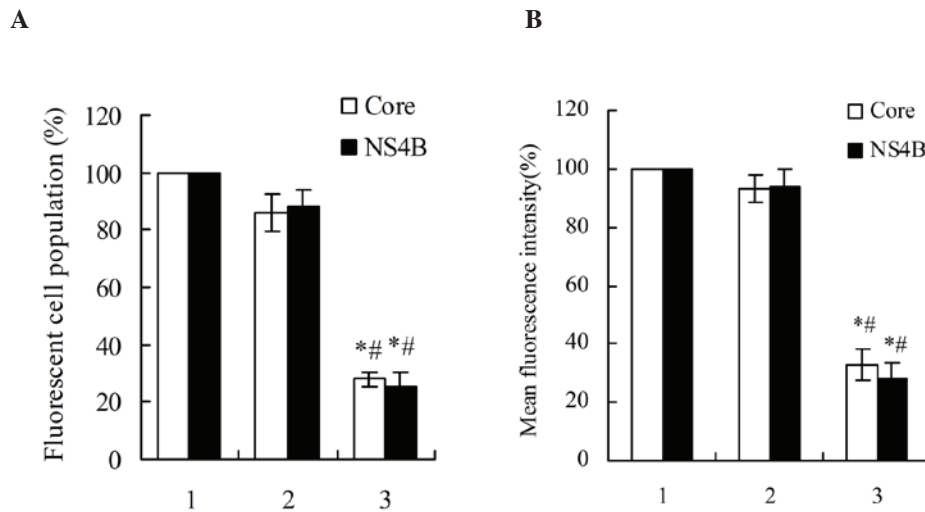
Figure 6. HCV-specific miRNA-mediated inhibition of core-DsRed-monomer or NS4B-DsRed-monomer expression in HepG2 cells, detected by flow cytometry. (A) Fluorescent cell population and (B) mean fluorescence intensity. 1: pDsRed-monomer-core or pDsRed-monomer-NS4B transfected HepG2 cells; 2: (pDsRed-monomer-core or pDsRed-monomer-NS4B)/negative control miRNA co-transfected HepG2 cells; 3: pDsRed-monomer-core/pmiRE-C-mi2 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi4 co-transfected HepG2 cells. *P<0.01, compared with pDsRed-monomer-core or pDsRed-monomer-NS4B transfected HepG2 cells; #P<0.01, compared with the (pDsRed-monomer-core or pDsRed-monomer-NS4B)/negative control miRNA co-transfected HepG2 cells. HCV, hepatitis C virus; NS4B, non-structural protein 4B; miRNA, micro RNA.
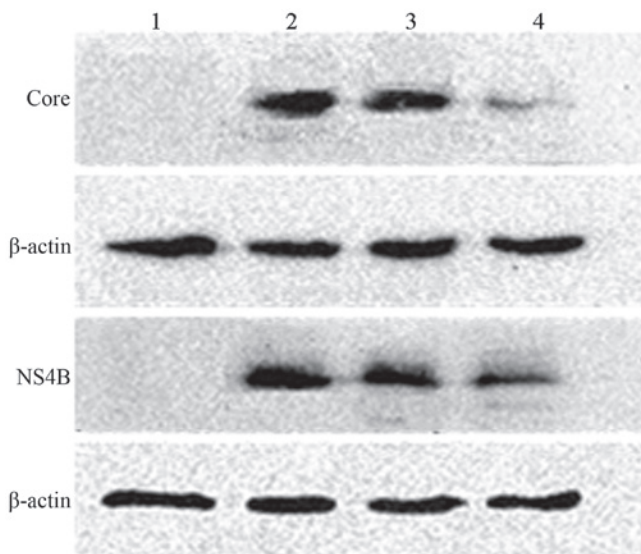

Figure 7. Inhibitory effect of miRNAs on the expression of core-DsRed-monomer and NS4B-DsRed-monomer fusion proteins. Lanes: 1, HepG2 cells without transfection; 2, pDsRed-monomer-core or pDsRed-monomer-NS4B transfected; 3, (pDsRed-monomer-core or pDsRed-monomer-NS4B)/negative control miRNA co-transfected and 4, pDsRed-monomer-core/pmiRE-C-mi2 co-transfected, or pDsRed-monomer-NS4B/pmiRE-NS4B-mi4 co-transfected. miRNA, microRNA; NS4B, non-structural protein 4B.

of viral proteins and the template for RNA replication (39). As replication occurs in the cytoplasm of liver cells without integration into the host genome DNA, HCV has emerged as a target of RNAi (40). Certain *in vivo* and *in vitro* experiments have demonstrated that exogenously administered short-interfering RNAs are able to exert replication inhibition against a variety of viruses (41-44). In the present study, two sets of amiRNA expression vectors were designed and constructed against HCV1b genotype core and NS4B mRNAs, and it was investigated whether these amiRNAs

were capable of efficiently inhibiting the expression of the HCV core and NS4B genes.

miRNAs are small non-coding RNA molecules, consisting of ~22 nucleotides, which may be incorporated into miRNP complexes and are able to induce mRNA degradation or translational repression by binding to the 3'-untranslated regions of their target mRNAs (45-48). The HCV core consists of three distinct domains: An N-terminal hydrophilic domain of ~120 amino acids, termed domain D1; a C-terminal hydrophobic domain of ~50 amino acids, termed domain D2; and the last 20 amino acids are designated as the signal peptide of the downstream protein E1 (49). The domain D1 is mainly involved in RNA binding and nucleocapsid formation. The domain D2 is responsible for core association with lipid droplets in mammalian cells and with the membrane of the endoplasmic reticulum (50). Previous studies have revealed that expression levels of the HCV core protein are correlated with the occurrence of hepatic steatosis, oxidative stress and hepatocellular carcinoma (27,28,51). The HCV core protein may also have a function in HCV assembly (29). NS4B is an integral membrane protein, which performs an essential function in HCV replication (52-54). The NS4B protein may induce the formation of the membranous web structure, which was hypothesized to represent the platform upon which HCV replication is performed (30-32). The NS4B protein also interacts with NS4A, and indirectly interacts with NS3 and NS5A (55). In addition, NS4B has been found to exhibit NTPase (33,34) and RNA binding (35) activities. Recently, NS4B has been reported to regulate HCV genome encapsidation (36). In the present study, the silencing effects of amiRNAs on the expression of HCV core and NS4B genes were investigated. The RT-qPCR method was used to detect the mRNA levels of HCV core and NS4B genes and the results demonstrated that in the miRNA-transfected HepG2 cells, the mRNA levels of HCV core and NS4B genes were effectively reduced. C-mi2 was

identified for its efficient silencing effect toward the core gene C-terminal domain (amino acids 117-124), which is required for the folding and stability of the core protein (49), reducing the expression of the core gene to 70% at the transcriptional level. NS4B-mi4 was also identified for its effective silencing effect toward the NS4B gene C-terminal domain (amino acids 240-247), which can mediate membrane association (56) and reduce the expression of the NS4B gene to 79% at the transcriptional level.

To clarify whether amiRNAs were able to effectively inhibit the expression of target gene proteins in HepG2 cells, flow cytometric analysis was applied. It was revealed that the red fluorescent protein percentage and average fluorescence intensity in the miRNA-transfected HepG2 cells were significantly reduced, compared with those of the negative control miRNA-transfected HepG2 cells. Western blot analysis revealed that the core and NS4B protein levels in the miRNA-transfected cells were also significantly reduced compared with the negative control miRNA-transfected cells, further confirming that the HCV core and NS4B-targeting miRNAs were able to effectively inhibit the expression of target genes. The results of the present study are supported by multiple studies, demonstrating that amiRNAs are able to effectively inhibit the replication of the rabies virus (57), adenoviruses (58), the human immunodeficiency virus (59) and the HCV (60).

The results of the present study also demonstrated that the amiRNAs designed against various domains of the HCV core and NS4B genes exhibited differing silencing effects against the target genes in the HepG2 cells, which may be attributed to the specific conformations between the miRNP and target mRNAs. Therefore, the biological activities varied and influenced the interference efficiency. Specific amiRNAs exhibit differing silencing effects, such that the expressed miRNAs cannot completely inhibit the expression of target genes. For this reason, targeting multiple domains of the HCV genome associated with HCV replication may be an effective method for the inhibition of viral gene expression and prevention of the generation of viral resistance.

In conclusion, the present data indicated that the expression of amiRNAs may effectively and specifically inhibit the expression of target HCV core and NS4B genes in HepG2 cells *in vitro*, providing a powerful tool for follow-up studies regarding HCV core and NS4B gene functions and pathogenesis. This approach is expected to become a novel therapeutic strategy for anti-HCV treatment.

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