INHIBITION OF GENE EXPRESSION DIRECTED BY SMALL INTERFERING RNAS IN INFECTIOUS BRONCHITIS VIRUS

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Summary. – In this study, fourteen small interfering RNAs (siRNAs) were synthesized for targeting of coding gene sequences, specific 5'-leader sequence of mRNA and 3'-end sequence of the Infectious bronchitis virus (IBV) genome. The expression of viral genes in Vero-E6 cells was detected by real-time PCR. Obtained results indicated that the majority of siRNAs could effectively inhibit the expression of viral genes. The inhibition effect of siRNAs significantly differed among various genes and sites on the virus genome.

Key words: Infectious bronchitis virus; RNA interference; small interfering RNA; gene expression

Introduction

IBV is an enveloped virus belonging to the family *Coronaviridae*, genus *Coronavirus* that contains single-stranded, positive-sense RNA, which functions as mRNA (Boursnell *et al*., 1987). IBV is a highly infectious and contagious pathogen of domestic fowl that replicates primarily in the respiratory tract as well as in some epithelial cells of the gut, kidney, and oviduct (Cavanagh, 2001).

IBV produces five subgenomic mRNAs and two-thirds of the 5'-end of a coronavirus genome is encompassed in the replicase gene. The products of the replicase gene are two polyproteins, Rep1a and Rep1ab that are assembled into functional replication-transcription complexes as RdRP after the extensive posttranslational proteolytic processing (Ziebuhr *et al*., 2000). The hallmark of coronavirus replication is the production of a 3'co-terminal nested set of polycistronic subgenomic mRNAs. The subgenomic mRNAs are produced by a discontinuous transcription process, in which a 63 nt leading sequence derived from the 5'-end of the genome is fused to the 5'-end of each subgenomic mRNA (Spaan *et al*., 1983; Sawicki and Sawicki, 1998). The signals are required for the replication and packaging of the virion RNA located in the terminal regions of the genome (Parry *et al*., 2003). In general, only the 5' proximal open reading frame of each subgenomic mRNA is translated to produce one of the virus structural proteins, as spike glycoprotein (S), small membrane protein (E), integral membrane protein (M), and nucleocapsid protein (N).

RNA interference (RNAi) is a sequence-specific RNA degradation process in the cytoplasm. The process of post-translational gene silencing is initiated by siRNA, a double-stranded form of RNA that contains 21–23 bp and is specific for the sequence of its target (Elbashir *et al*., 2001). RNAi was first discovered in the nematode worm *Caenorrhabditis elegans* (Fire *et al*., 1998) and later on was detected in other organisms such as *Drosophila*, certain parasitic protozoa, plants, and vertebrates. So far, RNAi has been developed into a powerful technique for studying gene function by a generating genetic knock-downs.

Furthermore, RNAi is believed to act as a natural defense against the viruses and against the expression of transposable elements (Ketting *et al*., 1999; Li *et al*., 2002). The inhibition of virus replication by induced RNAi has now been reported for some viruses of plants, animals (Ding *et al*., 2004), and humans such as Human immunodeficiency virus 1, hepatitis

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Abbreviations: GFP = green fluorescent protein; IBV = Infectious bronchitis virus; IBV-T = IBV Australia T strain; RdRP = RNA-dependent RNA polymerase; RNAi = RNA interference; siRNAs = small interfering RNAs; RT = reverse transcription

B and C viruses, Dengue virus, poliovirus and Influenza virus A (Haasnoot *et al*., 2003).

In this study, we designed and synthesized fourteen siRNAs directed against specific 5'-leader sequence of mRNA and 3'-end sequence, and 4 IBV genes that encode RdRP, M, N and S proteins. Next, we detected the expression of three genes coding RdRP, M and N proteins after IBV infection and transfection with the synthesized siRNAs by using real-time PCR. The aim of this study was to determine the effects of RNAi on the replication of IBV with respect to different genes and sites on the viral genome.

Materials and Methods

Virus. IBV, Australia T strain (IBV-T) was provided by the Shanghai Veterinary Biotechnology Center, China. The virus was propagated in the allantoic cavity of 10-day-old embryonated (specific pathogen free) chicken eggs at 37.8°C. Allantoic fluid was harvested 36 hrs after inoculation and stored at -80°C. The virus was passed 13 times in Vero-E6 cells to adapt it to the cell culture. In each passage, the virus stock was prepared from infected cells by triple freeze-thaw cycle and used to infect the next generation of cells.

Cells. Vero-E6 (African green monkey kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were seeded on the six-well plate at the density of 5×10^5 cells per well and cultured at 37°C in 5% CO_2.

siRNAs synthesis. We focused on the 5'-leader mRNA, 3'-end genome sequences, and the genes encoding RdRP, M, N and S proteins. Thus, 14 siRNAs were designed: si5', si3', siR1-siR2, siM1-siM3, siN1-siN4, and siS1-siN3 according to a sequence of GenBank (Acc. No. NC 001451). These siRNAs took the following sites as targets: 5'-leading sequence associated with mRNA transcription, 3'-end sequence of IBV genome that represented a sequence common with other coronaviruses, and R, M, N, S genes (Brown *et al*., 1986, Jonassen *et al*., 1998). Their positions are shown in Table 1 and Fig. 1. These siRNAs target sites are required to be conservative among different strains of IBV. Specifically, the sequences could not have more than 1 mismatch in 21 nucleotides among different IBV strains and could not share the identity with any known mammalian gene. The siRNAs were synthesized by the transcription reaction (RT) according to Donzé and Picard (2002).

Table 1. Characteristics of siRNAs targeting various sites on IBV-T genome

Name	Target gene	Target site
si5'	5'-leader sequence of mRNA	46
si3'	3'-end sequences of genome virus	27489
siR1	RNA-dependent RNA Polymerase(RdRP)	14496
siR2	RNA-dependent RNA Polymerase(RdRP)	14196
siM1	membrane protein (M)	56
siM2	membrane protein (M)	478
siM3	membrane protein (M)	587
siN1	nucleocapsid protein (N)	13
siN2	nucleocapsid protein (N)	498
siN3	nucleocapsid protein (N)	1115
siN4	nucleocapsid protein (N)	769
siS1	spike glycoprotein (S)	93
siS2	spike glycoprotein (S)	760
siS3	spike glycoprotein (S)	1475

Transfection of cells. The transfection of cells was performed at 90% cell confluence with a total amount of 4 μg/well of siRNA using Lipofectamine 2000 (Invitrogen). The transfected cells were maintained in DMEM for 6 hrs. Then, the medium was removed and 0.5 ml virus suspension harvested from the viruses adapted to Vero-E6 cells that had been infected 13 times, was added to each well. After adsorption for 1.5 hr at 37°C, the virus suspension was removed and 1 ml of fresh DMEM was added to each well and the cells were maintained at 37°C with 5% CO_2 until used.

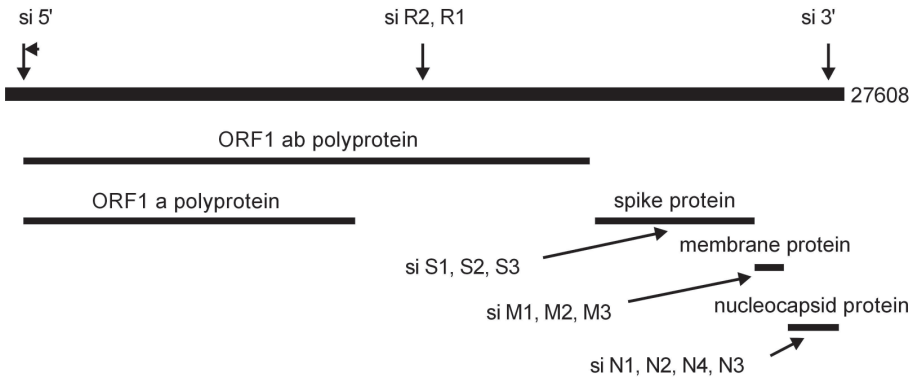

Fig. 1

IBV genome and the target regions of siRNAs

Assay of RNAi to GFP. The Vero-E6 cells were plated onto 6-well plates at a density of 5×10^5 cells/well. The cells were co-transfected with 2 μg of plasmid pEGFP-C1 (Clontech) and 3 μg siGFP or 3 μg siIRR (irrelevant siRNA) by Lipofectamine 2000. The expression of GFP was examined under the fluorescent microscope (Nikon E-600) 48 hrs after the transfection. The sequences of oligonucleotides for the siGFP (Acc. No. U55763) are: forward 5'-AAGAACGGCATCAAGGTGAACTATAGTGAGTCGTATTA-3', reverse 5'-AAGTTCACCTTGATGCCGTTCTATAGTGAGTC GTATTA-3'. The sequences of oligonucleotides for siIRR (irrelevant siRNA) are: forward 5'-AAGAACGGCATCAAGGTGAAC TATAGTGAGTCGTATTA-3', reverse 5'-AAGTTCACCTTGAT GCCGTTCTATAGTGAGTCGTATTA-3'.

RNA extraction, RT, and real-time PCR. The total RNA was isolated by TRIzol reagent (Invitrogen). RT reactions were carried out using RNA PCR Kit Ver.2.1 (TaKaRa, Japan) in a volume of 20 μl. RT primer was oligo dT-adaptor and RT reaction run for 30 mins at 48°C. The real-time PCR reaction mixture contained 1 μl product of RT reaction, 10 pmol/l of gene-specific primers, 1 μl 20x SYBR green-I dsDNA binding dye and other components. The PCR program ran as follows: one cycle at 97°C for 5 mins, 45 cycles at 95°C for 30 secs, 55°C for 30 secs and 72°C for 30 secs. The ß-actin from Vero-E6 cells was used as a control gene for the genes of IBV-T. The PCR primers were as follows: ß-actin forward 5'-CGGGAAATCGTGC GTGAC-3' and reverse 5'-TAGAAGCATTTGCGGTGG-3' (Acc. No. AB004047); IBV-M protein forward 5'-ATGATAGTGTTATGGT GCTT-3' and reverse 5'-TTCTTATTTCCGCTTTGG-3'; IBV-S protein forward 5'-CGGTCCTCTTCAAGGTG-3' and reverse 5'-CATA ACTAACATAAGGGCAA-3'; IBV-RdRP forward 5'-GTGATA GAGCAATGCCAAAT-3' and reverse 5'-ACCGTCGTCGGACA AGAT-3' (Acc. No. NC 001451).

Assay of viral genes expression. Three viral genes encoding RdRP, M and N proteins were taken as indicators (indicated genes) for the effectiveness of RNAi. The expression of these 3 indicated genes was detected by real-time PCR (Corbett Research) and quantified by Rotor-Gene software (version 6). The $2^{-\Delta\Delta CT}$ value method was used to compare the expression of the three genes from various samples treated by different siRNA (Winer *et al*., 1999) and the ANOVA procedure of SAS (SAS 6.12 version) was used for the statistical analysis.

Results

Expression of viral genes

First, the total RNA was isolated from the Vero-E6 cells infected with IBV-T after 36 hrs, then the RT-PCR reaction was performed with the specific primers of virus genes. The PCR products were cloned and sequenced. The result showed that these sequences were consistent with the published sequences of other IBV strains (data not shown) and identified the virus replicating in the Vero-E6 cells. The primers were suitable for examination of the expression of IBV genes. We have submitted and released these sequences on the web (GenBank Acc. Nos. AY820176, AY560918, AY820177, AY747109).

Expression of GFP gene with siRNAs

To evaluate the effect of the siRNA for silencing of the gene expression, we performed a preliminary experiment that examined the expression of the GFP gene in the Vero-E6 cells transfected with plasmid pEGFP-C1 and co-transfected with two siRNAs, siGFP and siIRR. The expression of GFP gene was strongly inhibited by siGFP, but the siIRR had no significant effect (Fig. 2). This finding implied that the processes such as siRNA synthesis, transfection and gene silencing were available in Vero-E6 cells.

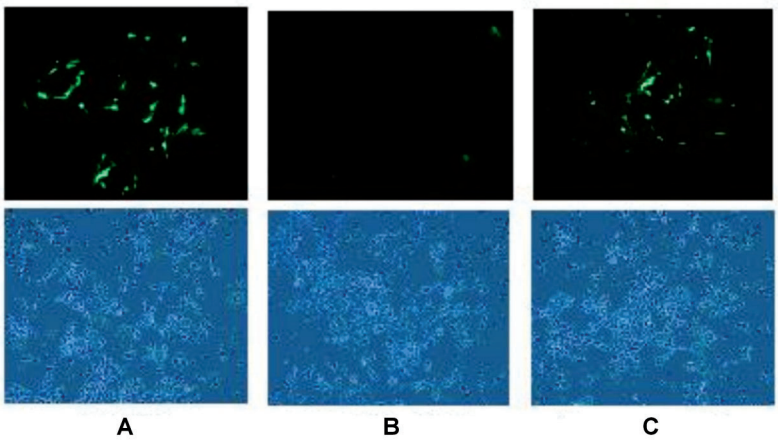

Fig. 2

Fluorescent assay of GFP gene expression in Vero-E6 cells transfected with plasmid pEGFP-C1 only (A), plasmids pEGFP-C1 and siGFP (B), and plasmids pEGFP-C1 and siIRR (C)

The lower images represented non-transfected cells.

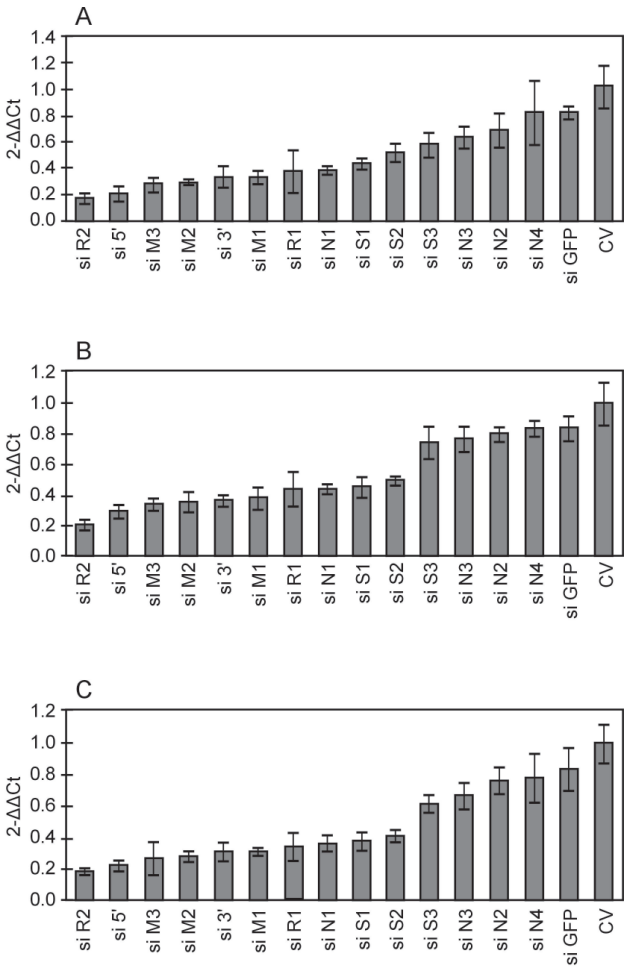

Fig. 3

Expression of M (panel A), S (panel B), and R (panel C) genes in Vero-E6 cells infected with IBV-T and transfected with a variety of siRNAs determined by real-time PCR

$y = 2^{-\Delta\Delta Ct}$ value; x = Vero-E6 cells transfected with various siRNAs; CV – (positive control) infected cells without transfection; siGFP (negative control) – irrelevant siRNA.

Expression of viral genes with siRNA

Next, we examined the expression of the 3 indicated genes coding RdRP, M and N proteins in the IBV-infected Vero-E6 cells transfected with the 14 siRNAs. In this experiment, we determined the expression of M protein, S protein and RNA-dependent RNA polymerase (RdRP) using mentioned primers. The results showed that the expression of examined genes was effectively inhibited by the majority of siRNAs (Fig. 3). The expression of the 3 indicated genes under the same siRNA was similar, but the expression of a same indicated gene under different siRNA was very different, i.e. the siR2, si5', si3', siR1, and siM3 were more effective at inhibition of expression than other siRNAs. The expression of each indicated gene was significantly different, when the siRNAs targeted different sites of the same gene (such as siM1, siM2 and siM3). In addition, the expression of the 3 indicated genes was significantly inhibited, when the siRNA targeted RdRP gene. Although the si5' and si3' did not take any coding protein as a target, they were able to inhibit the expression of the genes effectively.

Discussion

Although the RNAi is a powerful approach for studying gene function or being used in the antiviral therapy in many organisms, the design and synthesis of siRNAs is still a principal key, which has a great effect on the results of gene silencing (Holen *et al*., 2002; Miyagishi *et al*., 2003). In this study, we synthesized siRNAs based on the transcription reaction *in vitro* that was proved being effective by expression of GFP report gene.

According to the results of RNAi, the siR2 and siR1 were the most effective among the 14 siRNAs that proved again that the RdRP was the crucial element for transcription of IBV genes. In addition, some siRNAs were highly efficient in inhibiting the transcription of the IBV gene, even though they targeted only the specific 5'-leader sequences of mRNA transcription and 3'-end sequences of virus genome. This implied that the siRNA acted on the whole genome, not only on the mRNA and some flanking sequences of the virus genome (e.g. the 5'-leader sequences of mRNA).

Another key question for application of RNAi was the technique of introduction the siRNA into the targeting animal tissues. In chicken, IBV usually infects the epithelial cells of upper respiratory tract and lungs, so siRNAs could be directly and conveniently introduced via intranasal or pulmonary routes. This was a great advantage compared with other animals. In the current work, some siRNAs targeting IBV genome or mRNA genes were able to silence effectively the gene expression. It provided indirect evidence that RNAi mechanism did exist in the infection process of IBV, as well as it suggested that RNAi could be a potential tool for the prophylaxis and therapy of coronavirus diseases.

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References

Boursnell MEG, Brown, TDK, Foulds IJ, Green PF, Tomley FM, Binns MM (1987): Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J. Gen. Virol*. **68**, 57–77.

Brown TD, Boursnell ME, Binns MM, Tomley FM (1986): Cloning and sequencing of 5' terminal sequences from avian infectious bronchitis virus genomic RNA. *J. Gen. Virol.* **67**, 221–228.

Cavanagh D (2001): A nomenclature for avian coronavirus isolates and the question of species status. *Avian Pathol*. **30**, 109–115.

Ding SW, Li HW, Lua R, Li F, Li WX (2004): RNA silencing: a conserved antiviral immunity of plants and animals. *Virus Res.* **102**, 109–115.

Donzé O, Picard D (2002): RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res.* **30**, e46.

Elbashir SM, Lendeckel W, Tuschl T (2001): RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*. **15**, 188–200.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998): Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Natur*e **391**, 806–811.

Haasnoot PCJ, Cupac D, Berkhout B (2003): Inhibition of Virus Replication by RNA Interference. *J. Biomed. Sci.* **10**, 607–616.

Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H (2002): Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res*. **30**, 1757–1766.

Jonassen CM, Jonassen TO, Grinde B (1998): A common RNA motif in the 3' end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. *J. Gen. Virol*. **79**, 715–718.

Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH (1999): Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNase D. *Cell* **99**, 133–141.

Li H, Li WX, Ding SW (2002): Induction and suppression of RNA silencing by an animal virus. *Science* **96**, 1319–1321.

Miyagishi M, Hayashi M, Taira K (2003): Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. Antisense *Nucleic Acid Drug Dev*. **13**, 1–7.

Parry J (2003): SARS virus identified but the disease is still spreading. *Br. Med. J*. **326**, 897.

Sawicki SG, Sawicki DL (1998): A new model for coronavirus transcription. *Adv. Exp. Med. Biol.* **440**, 215–219.

Spaan W, Delius H, Skinner M, Armstrong J, Rottier P, Smeekens S, Van der Zeijst BA, Siddell SG (1983): Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. *EMBO J.* **2**, 1839–1844.

Winer J, Wang CK, Jung S, Shackel I, Williams PM (1999): Development and Validation of Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction for Monitoring Gene Expression in Cardiac Myocytes in Vitro. *Anal. Biochem*. **270**, 41–49.

Ziebuhr J, Snijder EJ, Gorbalenya AE (2000): Virus-encoded proteinases and proteolytic processing in the Nidovirales. *J. Gen. Virol*. **81**, 853–879.