

Inhibition of genes expression of SARS coronavirus by synthetic small interfering RNAs

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ABSTRACT

RNA interference (RNAi) is triggered by the presence of a double-stranded RNA (dsRNA), and results in the silencing of homologous gene expression through the specific degradation of an mRNA containing the same sequence. dsRNA-mediated RNAi can be used in a wide variety of eucaryotes to induce the sequence-specific inhibition of gene expression. Synthetic 21-23 nucleotide (nt) small interfering RNA (siRNA) with 2 nt 3' overhangs was recently found to mediate efficient sequence-specific mRNA degradation in mammalian cells. Here, we studied the effects of synthetic siRNA duplexes targeted to SARS coronavirus structural proteins E, M, and N in a cell culture system. Among total 26 siRNA duplexes, we obtained 3 siRNA duplexes which could sequence-specifically reduce target genes expression over 80% at the concentration of 60 nM in Vero E6 cells. The downregulation effect was in correlation with the concentrations of the siRNA duplexes in a range of 0~60 nM. Our results also showed that many inactive siRNA duplexes may be brought to life simply by unpairing the 5' end of the antisense strands. Results suggest that siRNA is capable of inhibiting SARS coronavirus genes expression and thus may be a new therapeutic strategy for treatment of SARS.

Keywords: SARS, small interfering RNA, Vero E6 cells, EGFP fusion protein, antiviral therapy.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a newly emerging infectious disease. It is usually characterized by fever, dry cough, myalgia, and mild sore throat, which progresses to a typical pneumonia. Over 8000 SARS cases and 770 SARS-related deaths were reported to WHO from over 26 countries around the world (<http://www.who.int/csr/sars/country/en>). SARS is caused by a newly identified virus within the family *Coronaviridae* [1-3]. This virus has been designated as the SARS coronavirus (SARS-CoV) [4].

RNAi is a natural process by which double-stranded RNA directs sequence-specific silencing of homologous genes. This process is evolutionarily conserved and is found in a wide range of eukaryotic organisms (see for reviews [5, 6]). Specific inhibition of cellular mRNA by RNAi can be triggered in mammalian cells by the introduction of synthetic 21- to 23-nucleotide duplexes of RNA

(siRNA) [7, 8]. This discovery prompted the use of RNAi for specifically inhibiting gene expression and replication of infectious viruses. The replication of a growing number of human pathogenic viruses in cell culture was shown to be inhibited by RNAi, including poliovirus, HIV-1, HCV replicons, hepatitis B virus and influenza virus [9-18]. The successful use of siRNA in mammalian cells encouraged us to apply RNAi to the exploration of anti-SARS strategies.

SARS-Cov is an enveloped, single-stranded, plus-sense RNA virus with a genome of about 30 kb [19, 20]. The SARS genome encodes 23 putative proteins which are typical of coronavirus such as 5'-replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3'. The structural proteins S, E, M and N are common to all known coronavirus, Playing a role in host cell entry and viron morphogenesis and release [21, 22]. During viron assembly, N binds to a defined packaging signal on viral RNA to form the helical nucleocapsid. M is localized at intracellular membrane structures. The interaction between the M and E proteins and nucleocapsids results in budding through the membrane. These structural proteins were attractive targets for anti-SARS agent development.

Here, we synthesized 26 siRNAs to target the E, M, and

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N genes of SARS-CoV and evaluated their effects on viral genes expression in Vero E6 cells. We conclude that the synthetic siRNAs could effectively and sequence-specifically downregulate SARS-CoV genes expression in a dose-dependent manner. We obtained 3 siRNAs which could sequence-specifically reduce target genes expression over 80% at the concentration of 60 nM.

MATERIALS AND METHODS

Preparation of siRNAs

Sense and antisense strands of siRNA oligonucleotides were synthesized and annealed at 95 °C for 1 min, followed by incubating at 37°C for 1 h in annealing buffer, pH 7.4, containing 2 mM Mg(AC)₂ [23]. To confirm the specificity of the inhibition, the experiments included negative control siRNA duplexes directed against other genes of SARS-CoV. Such as siRNA targeting *M* gene used as negative controls for siRNA targeting *E* gene (Tab. 1).

Vectors expressing SARS-CoV genes

The *E*, *M* and *N* genes expression vectors (pCDNA3.1/*E*, pCDNA3.1/*M*, and pCDNA3.1/*N*) were constructed and donated by Dr. Youhua XIE (Institute of Biochemistry and Cell Biology, SIBS, CAS). The *E*, *M* and *N* segments were released and inserted into the pEGFP-N3 N-terminal 'protein fusion mammalian expression vector' (Clontech) to generate 3 new constructs: pEGFP/*E*, pEGFP/*M*, pEGFP/*N*. All inserts were sequenced to ensure the orientation and correct reading frame.

Cell culture and transfection

The Vero E6 cells (from the cell bank of the Institute of Biochemistry and Cell Biology, SIBS, CAS) were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose (Gibco), supplemented with 10% heat inactivated fetal calf serum (Hyclone). Transfections of the siRNA duplexes and the plasmids were carried out in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. 100 nM of the construct and 30-240 nM of siRNA were transiently transfected into Vero E6 cells. The viability of the cells was tested by removing the cells from the culture plate and counting with a haemocytometer after trypan blue staining. The total number of living and dead cells was then calculated.

RT-PCR analysis

The total RNA of the cells were extracted using TRIZOL reagent (Invitrogen) and digested by RQ1 DNase (RNase free) (Promega) at 37°C for 30 min. The DNase was then heat-inactivated at 65°C for 10 min. The sequences of the primers for the detection of *E* gene expression are: 5'ATGTAATCATTCGTTTCGGAA3' (forward) and 5'TTAGACCAGAAGATCAGGAAC3' (reverse). The primers for *M* gene are: 5'ATGTTACTACAATTTGCCTATTC3' and 5'ACGCTCCTAATTTGTAATAAGA3'. The primers for *N* gene are: 5'ATGCTGATAATGGACCCAA3' and 5'GCCAGGAGTGAATTTCTTGA3'. The primers for β-actin are: 5'GTGCCACAGACAGCACTGTGTTG3' and 5'TGGAGAAGAGCTATGAGCTGCCTG3'. Single-tube and one-step RT-PCR was performed with the One-step RNA PCR Kit (TaKaRa). Each 50 μl reaction mixture contained 1 μg extracted RNA, 1×buffer, 1 mM each deoxynucleotide triphosphate, 0.4 μM primer, 5 mM MgCl₂, 40 U

RNase inhibitor, 5 U AMV reverse transcriptase, and 5 U AMV-optimized Taq. After an initial incubation for 30 min at 50°C followed by denaturation at 94°C for 4 min, 28 cycles of amplification were performed by using a thermo profile of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec. The final cycle included an extension at 72°C for 10 min. The amplification products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Fluorescence microscopy

The Vero E6 cells were seeded on coverslips-mounted of 6-well plates (5 × 10⁵ cells/well) and transfected as described above. After 48 h, cells were fixed with freshly made 4% paraformaldehyde in PBS for 30 min at room temperature and imaged using an Olympus BX-50 fluorescence microscope with 10 × objective. The fields were randomly chosen by a sweep across the coverslips under phase contrast illumination. Randomly chosen fields from differently treated cultures at the same experiment were photographed using the same time of exposure. The proportion of Vero E6 cells expressing GFP fusion proteins was calculated by counting the number of green fluorescent cells against the total number of cells.

Statistical analyses

Statistical analyses were performed using the student's *t*-test; *P* values of less than 0.05 were considered as statistically significant.

RESULTS

siRNAs targeting the E, M, and N genes of SARS-CoV

siRNAs were designed to target the open reading frames of the *E*, *M*, and *N* genes of SARS-CoV (Tab. 1). The target sequences were directed to a single-stranded region (Fig. 1A), according to the secondary structure of the SARS-CoV RNA predicted by using RNA structure 3.71. Sequences of the form N21 (where N indicates any nucleotide) and with a GC content of less than 70% were selected from this region [24]. The selected 21-nucleotide RNAs, followed by TT, were synthesized chemically by Institute of Biochemistry and Cell Biology, SIBS, CAS.

Downregulation effect of 26 siRNA oligonucleotides

First, we evaluated the 26 SARS-directed siRNAs for their abilities to suppress SARS-CoV genes expression. The vectors, pCDNA3.1/*E*, pCDNA3.1/*M*, and pCDNA3.1/*N*, expressing the structural proteins *E*, *M*, and *N*, were cotransfected with each of the siRNA duplexes into Vero E6 cells. The expression levels of SARS-CoV genes were determined 48 h later by RT-PCR. The results show that all siRNA duplexes specifically reduced SARS-CoV genes expression to different extents compared with the control (Fig. 1). No. 5, No. 6, and No. 16 siRNA duplexes could suppress SARS-CoV genes expression over 70% when used at 30 nM (Fig. 1).

Dose-dependent inhibition of SARS-CoV genes expression in cell culture by siRNAs

We conducted a dose response analysis by co-transfected

Tab. 1 Sequences of siRNA duplexes targeting the exons of E, M, and N proteins of SARS-CoV

Name	Sequence*	Target gene
No. 1	5'-uuuauaguuaauagcguacuTT-3' 3'-TTaauuaucauuuauagcgaugaa-5'	Small envelope protein (E)
No. 2	5'-uuucgugguauuucugcuaguTT-3' 3'-TTaaagcaccuauaagaacgauca-5'	
No. 3	5'-uucgauugugcgucacugcuTT-3' 3'-TTaagcuaacacacgcaugacga-5'	
No. 4	5'-aacgguuacgucucacugcgTT-3' 3'-TTuugcacaauagcagaugagcgc-5'	
No. 5	5'-ugaaggaguuccgacuucuTT-3' 3'-TTacuuccuaggaagacuagaaga-5'	
No. 6	5'-agcuuaaacaacuccuggaacTT-3' 3'-TTucgaauuugugaggaccuug-5'	
No. 7	5'-ugcugcugucacagaauuaaTT-3' 3'-TTacgacacagaugucuuaauu-5'	
No. 8	5'-uauuguaggcuugaugugcuTT-3' 3'-TTauaacaucgaaucacaccga-5'	
No. 9	5'-uagcuacuucguucuccuTT-3' 3'-TTaucgaugaagcaacgaaggaa-5'	
No. 10	5'-ccagaccgcucauggaagugTT-3' 3'-TTggucggcgaguuuuuac-5'	
No. 11	5'-uugcgauggccggacacuccTT-3' 3'-TTaacgcuuaccggccugagg-5'	
No. 12	5'-agagauacuguggcuaucTT-3' 3'-TTucucuagugacaccgaugaag-5'	
No. 13	5'-cgucgcagcugugaaggcagTT-3' 3'-TTgcagcugcgcacauccgugac-5'	
No. 14	5'-uugcugcauacaaccgcuaccTT-3' 3'-TTaacgagcuauuugcggaugg-5'	
No. 15	5'-aaacuuaaaauaaauacagaTT-3' 3'-TTuugauuuuuuuuuugucu-5'	
No. 16	5'-gauauggaccccaucaaacTT-3' 3'-TTcuuuaccuggguuaguuuug-5'	Nucleocapsid protein (N)
No. 17	5'-aaggccaaaacagcgcgaccTT-3' 3'-TTuuccgguuuugucgcgug-5'	
No. 18	5'-aauaauacugcguuugguucTT-3' 3'-TTuuuuuagcgcgagaaccaag-5'	
No. 19	5'-aaggaggaacuagauccuTT-3' 3'-TTuucccuugaaucaaggga-5'	
No. 20	5'-aagagcuaccgacgaguucgTT-3' 3'-TTuucugaugggcugcuacc-5'	
No. 21	5'-aacaagaaggcaucguaugTT-3' 3'-TTuuguuucuccguagcauacc-5'	
No. 22	5'-aaucacccaaagaccacauTT-3' 3'-TTuuuuggguuucugguuaa-5'	
No. 23	5'-aaucuaaauaauugcugccTT-3' 3'-TTuuaggauuuuuuacgacgg-5'	
No. 24	5'-ucaaggaacaauugccaaaTT-3' 3'-TTaguuccuuguuagcgguuu-5'	
No. 25	5'-ugaggcaucuaaaagccucgTT-3' 3'-TTacuccguaguuuuucggagc-5'	
No. 26	5'-aacguacugccacaaacaguTT-3' 3'-TTuugcaugacgguuuuuca-5'	

*Uppercase letters indicate deoxyribonucleotides.

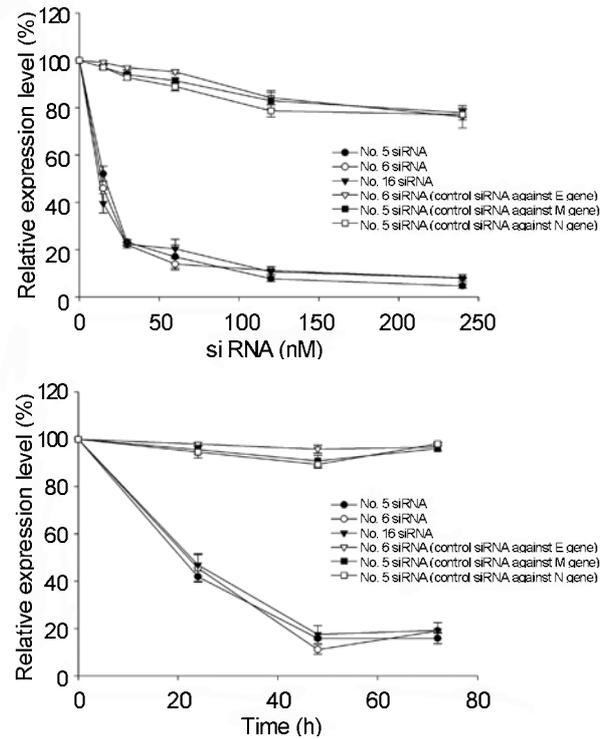
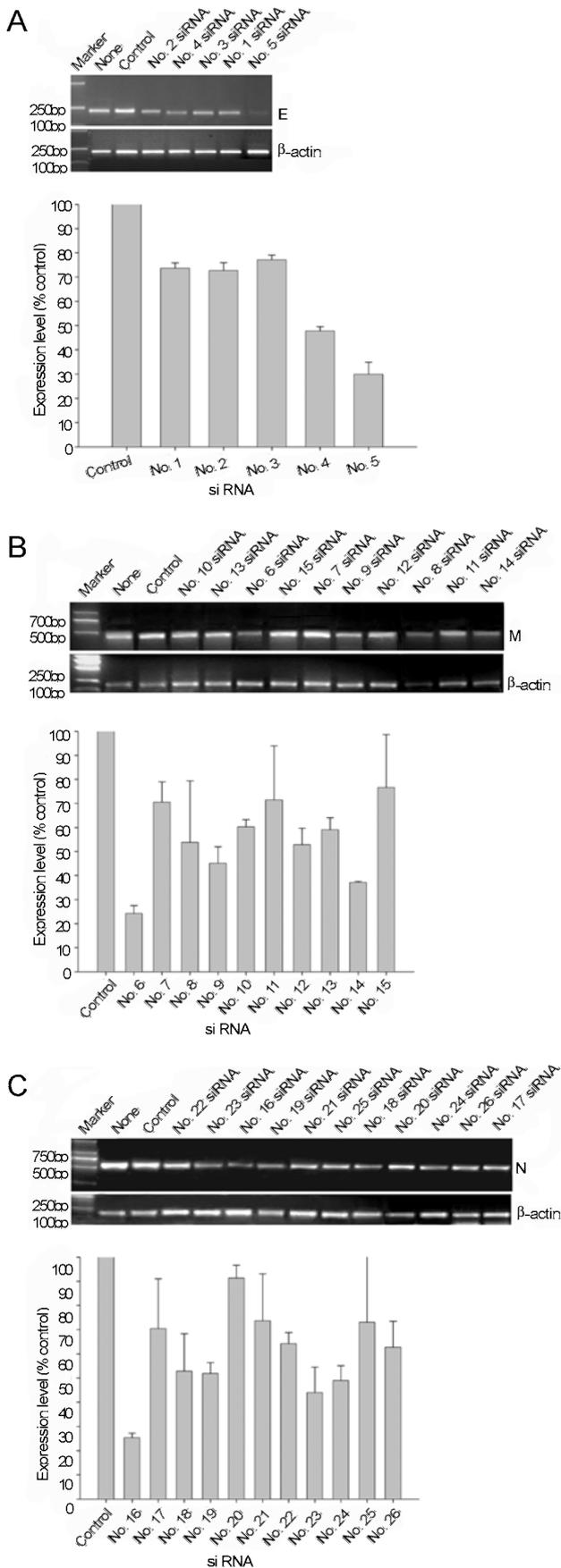


Fig. 2 No. 5, No. 6, and No. 16 siRNA duplexes interfere with expression of viral *E*, *M*, and *N* genes. **(A)** Dose-dependent inhibition of *E*, *M*, and *N* genes expression. Vero E6 cells (5×10^4) were co-transfected by 50 ng pCDNA3/*E*, pCDNA3/*M*, or pCDNA3/*N* together with the indicated amounts of the specific siRNA or the irrelevant siRNA as a control and assayed for the expression level of viral *E*, *M*, and *N* gene after 48 h by RT-PCR. **(B)** Kinetics of inhibition effects of No. 5, No. 6, and No. 16 siRNA duplexes. The expression levels of SARS-CoV *E*, *M*, and *N* genes in Vero E6 cells co-transfected with 50 ng pCDNA3/*E*, pCDNA3/*M*, or pCDNA3/*N* together with 60 nM specific siRNA or the irrelevant control siRNA were measured 24, 48, and 72 h posttransfection. The expression levels of SARS-CoV genes are shown as percentages of the levels secreted by mock-transfected cells (control). The data represent the mean \pm SD of three independent experiments.

Fig. 1 Inhibition of SARS-CoV genes expression by different siRNA duplexes. Vero E6 cells (5×10^4) were transfected with 50 ng plasmid pCDNA3/*E*, pCDNA3/*M*, or pCDNA3/*N* alone (none) or together with 30 nM of each siRNA or with a negative control siRNA (NC), and the expression levels of SARS-CoV genes was measured 48 h later by RT-PCR. β -actin served as an internal control. The bar charts below show the results of densitometric analysis shown as percentages of the siRNA negative control. The data represent the mean \pm SD of two independent experiments performed in duplicate. **(A)** The effects of No. 1 ~ No. 5 siRNA duplexes on expression of SARS-CoV *E* gene. **(B)** The effects of No. 6 ~ No. 15 siRNA duplexes on expression of SARS-CoV *M* gene. **(C)** The effects of No. 16 ~ No. 26 siRNA duplexes on expression of SARS-CoV *N* gene.

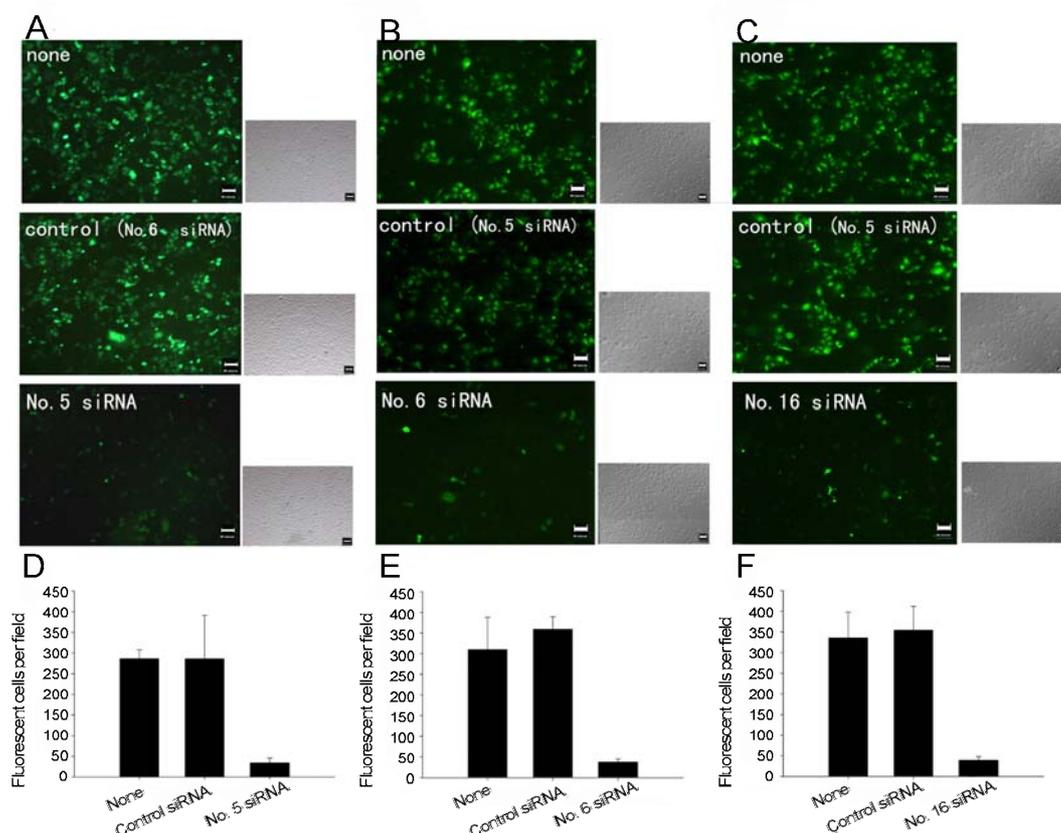


Fig. 3 Effects of No. 5, No. 6, and No. 16 siRNA duplexes on expression of E-EGFP, M-EGFP, and N-EGFP fusion proteins in Vero E6 cells. Fluorescence micrographs of cells (about 1×10^5) co-transfected with GFP-fusion protein expression constructs (100 ng) together with irrelevant control siRNA (control), specific siRNA, or without siRNA (none) at the final concentration of 60 nM were taken 48 h posttransfection. Phase contrast micrographs of the same fields are shown in the right-hand row. (A) Downregulation of expression of E-EGFP fusion protein by No. 5 siRNA. (B) Downregulation of expression of M-EGFP fusion protein by No. 6 siRNA. (C) Downregulation of expression of N-EGFP fusion protein by No. 16 siRNA. (D, E, F) Fluorescent cells per field of view expressing detectable EGFP fusion protein. An average from four fields is shown for each transfection. Data are from a representative experiment. Scale bar: 100 μ m

Tab. 2 Cellular toxicity of the siRNA duplexes

Type and concentration of siRNA	cells/culture**
None*	3.68×10^5
15 nM No.5 siRNA	3.40×10^5
30 nM No.5 siRNA	3.53×10^5
60 nM No.5 siRNA	3.31×10^5
120 nM No.5 siRNA	3.86×10^5
240 nM No.5 siRNA	3.15×10^5
15 nM No.6 siRNA***	3.38×10^5
30 nM No.6 siRNA	3.14×10^5
60 nM No.6 siRNA	3.55×10^5
120 nM No.6 siRNA	3.52×10^5
240 nM No.6 siRNA	3.32×10^5

*Transfection was carried out with pCDNA3.1/E alone.

**Number of cells seeded in each well of a 6-well cell culture plate was 2.0×10^5 . Viability staining showed not more than 10% dead cells in all cultures at the end of the experiment (48 h).

***Here No. 6 siRNA served as a negative control for E gene.

fection Vero E6 cells with increasing amounts of No. 5, No. 6, No. 16 siRNA or negative control siRNA together with pCDNA3.1/E, pCDNA3.1/M, or pCDNA3.1/N. SARS-CoV genes expression levels 48 h post-transfection were measured. Here the No. 6 siRNA targeting *M* gene was used as a negative control for detection of *E* gene expression, whereas the No. 5 siRNA targeting *E* gene was used as a negative control for that of both *M* and *N* genes. The No. 5, No. 6 and No. 16 siRNA duplexes dose-dependently inhibited target genes expression in the range 0~60 nM (Fig. 2A). At higher doses of siRNA, we observed a slight nonspecific inhibitory effect of negative control siRNA duplexes. We also tested for different toxicities of the siRNA duplex in a set up using 15 nM, 30 nM, 60 nM, 120 nM, and 240 nM No. 5 siRNA. The total number of living and dead cells revealed no significant

differences between the No. 5 and the negative control siRNA (No. 6 siRNA) treated cultures (Tab. 2), indicating that the observed downregulation of SARS-CoV genes expression is not due to toxic effects of these duplexes.

Kinetic study results (Fig. 2B) revealed a continuous increase in the specific inhibition of SARS-CoV genes expression by No. 5, No. 6, and No. 16 siRNA from 24 to

72 h after transfection. Inhibition of SARS-CoV genes expression reached maximal at 48 h and started to decline at 72 h. However the effect of siRNA is known to be transient and could last 3-4 days [5].

Effect of siRNA duplexes on the expression of EGFP fused SARS-CoV proteins

To confirm the effect of No. 5, No. 6, and No. 16 siRNA duplexes on SARS-CoV genes expression, we constructed vectors containing the exon of *E*, *M*, or *N* cloned in frame with the EGFP reporter gene and cotransfected Vero E6 cells with these vectors together with the siRNA duplexes. The cells were examined 48 h post-transfection with fluorescence microscopy. Microscopic examination of the green fluorescence revealed a distinctly reduced expression of the fusion protein in the cells treated with the No. 5, No. 6, and No. 16 siRNA duplexes, compared with cells treated with the negative control siRNA duplexes (Fig. 3).

Effect of siRNA duplexes designed to place the 5' end of the antisense siRNA strand in a mismatch

Recent studies suggested that the thermodynamic properties of siRNA play a critical role in determining the molecule's function [25, 26]. Functional siRNA duplexes display a lower internal stability at the 5'-antisense terminal base pair end than nonfunctional duplexes. According to this rule, the nucleotides at position 21 of the sense strands of No. 4, No. 13, and No. 26 siRNA duplexes, which exhibited low inhibition activity, were changed to unpair the 5' end of the antisense strands (Fig. 4A). These mutated siRNA duplexes were transfected into Vero E6 cells together with the expression vectors of the target genes and the expression levels were measured 48 h post-transfection by RT-PCR. The results showed that the mutated siRNA could reduce target genes expression more effectively (Fig. 4B, 4C). Many inactive siRNA duplexes may be brought to life simply by modifying the sense strands of the siRNA duplexes.

DISCUSSION

Gene silencing mediated by double-stranded RNA (dsRNA) is a sequence-specific, highly conserved mechanism in eukaryotes. In plants, it serves as an antiviral defence mechanism [27-29]. Recently, the mammalian RNAi machinery has been successfully programmed with siRNAs targeting viral sequences to induce an effective antiviral response. This prompted us to evaluate the effect of siRNA on SARS-CoV. Viruses, especially RNA viruses, are variable due to high mutation rates during replication. The mutated viral proteins can help the viruses to escape immune-system defence mechanisms [30]. These mutations

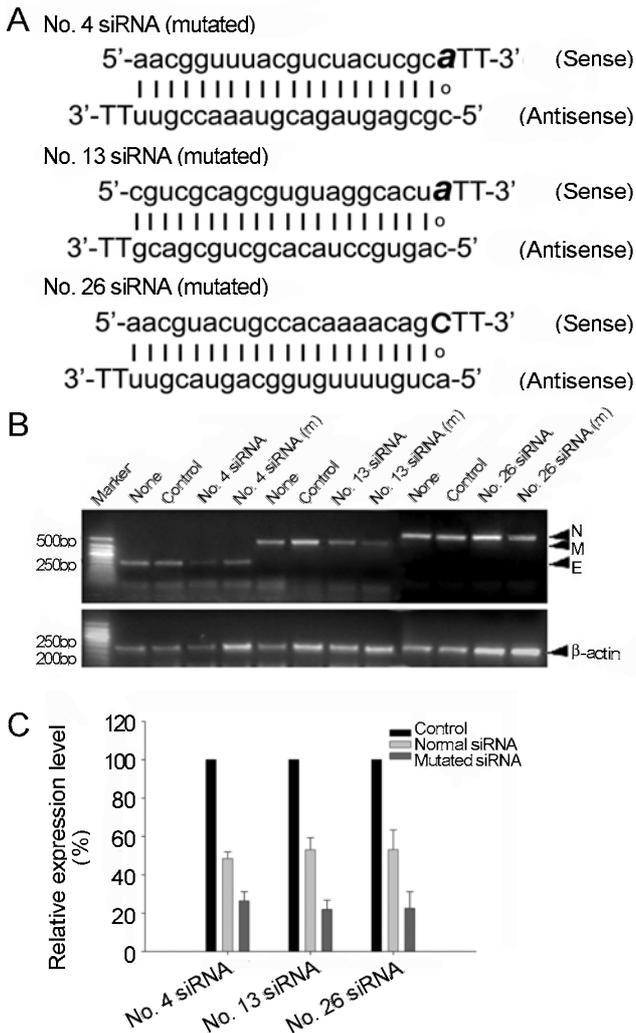


Fig. 4 Effects of mutated siRNA duplexes with a mismatch at the 5' end of antisense strands. (A) The sequences of mutated No. 4, No. 13, and No. 26 siRNA duplexes. The bold italic letters indicate the mutated nucleotides. Uppercase letters indicate deoxyribonucleotides. (B) Vero E6 cells (5×10^4) were cotransfected by 50 ng pCDNA3/E, pCDNA3/M, or pCDNA3/N together with 60 nM of the mutated siRNA [siRNA (m)] or the irrelevant siRNA as a control and assayed for the expression level of viral E, M, and N gene after 48 h by RT-PCR. (C) The bar chart shows the results of densitometric analysis shown as percentages of the siRNA negative control. The data represent the mean \pm SD of three independent experiments.

may also escape attack by siRNAs. However, the emergence of escape variants resistant to siRNA can be minimized by using siRNA directed against multiple conserved RNA target sequences. The genome of SARS-CoV encoded 23 putative proteins, including four major structural proteins; nucleocapsid (N), spike (S), membrane (M), and small envelope (E) which played essential roles in host cell entry, viral morphogenesis and release [21, 22]. These structural proteins were attractive targets for anti-SARS agent development. Among these proteins, we selected E, M, and N as the targets of RNAi. The spike protein, a glycoprotein projection on the viral surface, was crucial for viral attachment and entry into the host cell. In addition, variations of S protein among strains of coronavirus are responsible for host range and tissue tropism [31]. The results of genetic sequencing of samples from the latest patient with SARS show variation of S gene in this SARS-CoV strain [32]. Since the S1 subunit of the spike protein is the major antigenic moiety for coronaviruses and is not an essential structural protein, it is prone to have high mutation rates as the virus evolves in host populations [33]. Therefore we don't chose S protein as a target. In this work, we have evaluated the down-regulation effects of 26 siRNA duplexes targeting different sites along the open reading frames of E, M, and N proteins. As shown in Fig. 1, three siRNA duplexes exhibit over 70% inhibition efficiency, 11 siRNA duplexes showed 40~70% inhibition efficiency, and 12 siRNA duplexes reduced target genes less than 40% at the concentration of 30 nM. By modifying the sense strand of siRNA duplex as shown in Fig. 4, the inhibition efficiency of many siRNA duplexes may be improved. The combined use of siRNA duplexes targeting different regions of the virus may increase the efficiency of the treatment and, in turn, will prevent the appearance of viral revertants resistant to the treatment.

Human coronaviruses are usually difficult to culture *in vitro*, whereas most animal coronaviruses and SARS-CoV can be easily cultured in African Green Monkey Kidney (Vero E6) cells [1]. Direct cytopathic effects of SARS-CoV could be demonstrated on inoculating the viral isolates into Vero E6 cells [3, 19, 34], which make Vero E6 cells a suitable model for the study of the effect of siRNA duplex. Because SARS is dangerous for its high morbidity and mortality rates, it is more safe and convenient to study anti-SARS agent by using Vero E6 cells transfected with SARS-CoV genes expression vectors. In this cell model, we obtained sequence-specific downregulation of SARS-CoV genes expression by synthetic siRNA as seen from the results of RT-PCR (Fig. 1) and from a reduced fluorescence intensity of GFP to which the SARS-CoV protein E, M, or N was fused (Fig. 3). Inhibition was dose dependent in the range of 0~60 nM and was sustained for

3 d after administration of the siRNA (Fig. 2). In addition, this model system can be exploited to further study the pathological functions of SARS-CoV genes.

At present, several potential SARS therapies are under development, such as vaccines [35, 36] and interferons [37, 38]. The use of RNAi in the therapy of SARS would be advantageous in its specificity for the target gene with minimized side effects. However, the interference of SARS-CoV genes expression induced by the use of siRNA duplexes is transient. It will be essential to prolong the expression of siRNA and to ensure its delivery. Current methods for prolonging siRNA expression include the use of plasmids that express endogenously the siRNAs. An alternative approach is to express the siRNA from an integrating viral vector to achieve continuous and prolonged expression. A recent article [39] published during the preparation of our work for report on the anti-SARS activity of a short hairpin RNA (shRNA) targeting the S gene expressed from a U6 promoter suggest that RNAi has potential to inhibit the replication of SARS-CoV from the infected Vero E6 cells. In some cases there may be an advantage to using siRNA because of safety issues regarding the administration of foreign DNA into a patient.

In conclusion, we show that siRNA could be designed to induce an antiviral effect on SARS-CoV genes expression in cells. This approach might provide an effective therapeutic strategy for SARS-CoV infection.

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