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Quick identification of effective small interfering RNAs that inhibit the replication of coxsackievirus A16

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ABSTRACT

Coxsackievirus A16 (CA16) is a major causative agent of hand, foot, and mouth disease (HFMD). It can cause myocarditis, pericarditis and fatal shock. There is no effective therapy against CA16. RNA interference (RNAi) is a powerful tool to silence gene expression. The small interfering RNA (siRNA) that induces RNA degradation has recently been used as an anti-virus agent to inhibit virus replication. In this study, we established the complete nucleotide sequence of CA16 strain Shzh05-1, and then compared the nucleotide sequences of Shzh05-1 with sequences of other CA16 strains in GenBank. We chose conserved regions between Shzh05-1 and the two other CA16 strains to design 30 siRNAs and construct siRNA-encoding plasmids. Thirteen siRNAs targeting conserved regions of the virus could effectively block replication of CA16 in cultured cells. Combination transfection of these 13 effective siRNAs could also produce a high inhibitory effect. These strategies and results suggest that RNAi has potential therapeutic use for suppression of CA16 infection.

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1. Introduction

Hand, foot, and mouth disease (HFMD) is a common illness in children. It can be caused by many human enteroviruses, including coxsackieviruses A16, A4, A5, A9, A10, B2, B5, and enterovirus 71 (EV71). Among these, coxsackievirus A16 (CA16) and EV71 are two major causative agents of HFMD (Li et al., 2005). Previously, CA16 was less well studied than EV71 because CA16-caused HFMD was considered to be mild and harmless due to its relatively benign symptoms. Recent studies showed that CA16 infection is not always a benign infection: it can be associated with serious myocarditis and pericarditis. The infections may cause cardiac arrhythmias, acute heart failure and fatal shock (Wang et al., 2004; Shah et al., 2003). As the most commonly detected group A coxsackievirus and the most common pathogen of HFMD, CA16 should be better studied.

CA16 is a member of the genus *Enterovirus*, which is in the family Picornaviridae. The CA16 genome is a positive single-stranded RNA molecule ~7.4 kb in length, and has a single open reading frame encoding a polyprotein, which is flanked by 5′- and 3′-untranslated regions (UTR). The polyprotein is post-translationally cleaved by CA16-encoded proteases (termed 2A and 3C) to yield individual structural proteins (termed VP1–4) and nonstructural proteins including proteases and polymerase. The RNA-dependent RNA polymerase 3D is responsible for viral RNA replication (Pöyry et al., 1994). The lifecycle of CA16 is understood, but no specific drug has been shown to provide effective protection against virus infection.

RNA interference (RNAi) is an evolutionarily conserved mechanism of sequence-specific post-transcriptional gene silence triggered by double-stranded RNA (Fire et al., 1998). This process acts via a two-step mechanism. Firstly, long dsRNAs are cleaved by a host ribonuclease-III like enzyme (named dicer) into small interfering RNA (siRNAs) of 21–23 nt. These siRNAs are associated with a multiprotein complex known as RNA-induced silencing complex (RISC) and ultimately target homologous mRNA for degradation based on complementary base pairing (Hammond et al., 2001; Martinez et al., 2002). In mammalian cells, introduction of 21–23 nt siRNAs exhibit an RNAi effect without inducing a nonspecific interferon response (Elbashir et al., 2001). Many studies have shown that RNAi could be used for suppressing gene expression when delivered into mammalian cells *in vitro* because of the specificity and efficiency of RNAi machinery (Elbashir et al., 2002; Tuschl and Borkhardt, 2002). Recently, various studies demonstrated that RNAi

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can also be used for clearing mammalian cells from viral infection, such as influenza A virus (Zhou et al., 2007), influenza B virus (Gao et al., 2006), human immunodeficiency virus type 1 (HIV-1) (Liu et al., 2007), hepatitis B virus (Wu et al., 2005), and coxsackievirus B3 (Yuan et al., 2005). There has been considerable interest in the development of siRNA as a possible treatment for CA16-induced HFMD.

This study is the first report of siRNAs interfering with CA16 reproduction in cell culture experiments. We initially established and analyzed the entire nucleotide sequence of CA16 Shzh05-1. To limit escape mutants, we designed 30 siRNAs targeting conserved regions between Shzh05-1, Tainan/5079/98, and Shzh00-1 (because of scarce genetics data, the only two Asia strains providing complete nucleotide sequences in NCBI are Tainan/5079/98, and Shzh00-1) and confirmed 13 out of 30 siRNAs could potently inhibit the replication of CA16 in cultured cells. For further preventing escape mutants, we cotransfected these 13 siRNAs targeting different regions of CA16 RNA into cultured cells and compared their inhibitory effect with single transfected siRNAs. The results show that siRNAs transfected by this method could also effectively inhibit the propagation of CA16.

2. Materials and methods

2.1. Cell cultures and Virus assays

HEK293T cells and Vero cells were propagated and maintained in DMEM (Hyclone, Thermo Fisher Scientific Inc., USA) supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific Inc., Pittsburgh, PA) at 37 °C with 5% CO_2.

The CA16 strain Shzh05-1 was obtained from the Department of Microbiology at Shenzhen Center for Disease Control and Prevention (Shenzhen, People's Republic of China) and propagated in Vero cells. The 50% cell culture infective dose ($CCID_{50}$) was calculated in Vero cells by using 96-well plates and the Reed–Muench formula (Reed and Muench, 1938). A viral suspension titrated at $10^{6.8}$ $CCID_{50}$/0.1 ml was used for the experiments.

2.2. RNA extraction and nucleotide sequences

Viral RNAs were extracted according to the Manufacturer's instructions (RNAeasy Mini kit, Qiagen, Germany). Reverse transcription (RT) and PCR amplification were performed by using standard methodologies. Briefly, viral RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) by using random primer, and the cDNA was then amplified by using segment-specific primers (Supplementary Table 1). RT-PCR products were purified use the QIAquick PCR purification kit (Qiagen, Germany). All amplicons were bidirectionally sequenced with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI3730 automated DNA sequencer (Applied Biosystems). The nucleotide sequence data were analysed by using the Bioedit sequence analysis program (version 7.0), and multiple sequence alignments were performed by DNAstar program (Lasergene Inc., Madison, WI).

2.3. Plasmid construction

To locate the target site of siRNA, we chose regions that are conserved between Shzh05-1 and two other CA16 strains and selected those sequences of 19 bp with the structure of GN18 (when choosing targeting sites with a G as the starting nt of the 19-mer region is not possible, an artificial G plus a 18-mer sequence can be used) according to empirical rules (Du et al., 2005). 22-Mer double-stranded DNA oligos (sense: AAAGN18; antisense: AAAAN18, sequences of siRNAs are shown in Supplementary Table 2) corresponding to target mRNAs were generated through annealing of complementary oligos and then subcloned into the GeneBuster vector (Genordia AB, Sweden). The GeneBuster vector with a U6 and H1 promoter and two correspondent terminator sequences arranged in convergent manner was a robust DNA based siRNA system for expression of active siRNA within cells (Chen et al., 2005). At the same time, we designed a negative control as GeneBuster-1B-SCS225, which was scrambled siRNA with same nucleotides with GeneBuster-1B-225 but a different sequence. Every clone was verified by PCR and sequence-confirmed.

The cDNAs of each gene (1B, 1C, 1D, 2A, 2C, 3A, 3C and 3D) were amplified using gene-specific primers (Supplementary Table 3) and cloned into the BglII-ApaI sites of siQuant vector (Genordia AB, Sweden) to fuse genes to *firefly* luciferase gene respectively. These fused proteins were expressed under the control of the cytomegalovirus (CMV) promoter. Using cotransfection and Dual-Luciferase reporter assay system, the inhibitory effects of the gene-specific siRNAs on corresponding gene expression could be indirectly assayed by the expression of *firefly* luciferase in the transfected HEK293T or Vero cells (Du et al., 2004; Holen et al., 2002).

2.4. siRNA efficacy validation

HEK293T cells or Vero cells were plated in 24-well plates (0.5 ml medium/well) and cultured at 37 °C with 5% CO_2 for 24 h. When the cell layer reached 60–70% confluence, the culture medium was changed into Opti-MEM I (Gibco, Carlsbad, CA), at 0.5 ml/well. siRNA-encoding GeneBuster vectors (400 ng), as well as the reporter plasmids (17 ng pRL-TK as internal control and 170 ng siQuant vectors for 1B, 1C, 1D, 2A, 2C, 3A, 3C and 3D, respectively) were cotransfected by using Lipofectamine 2000 (Invitrogen) for 4 h according to the Manufacturer's recommendations. Forty-eight hours posttransfection, cells were harvested and measured the *firefly* luciferase and *Renilla* luciferase activities on TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The inhibitory effects generated by the siRNAs were expressed in normalized ratios between the reporter and control luciferase activities.

2.5. Viral challenge assay in Vero cells

Vero cells were seeded in 24-well plates. When the cell layer reached 60–70% confluence, each siRNA-encoding GeneBuster vector (800 ng) or mixed siRNA-encoding GeneBuster vector (13 mixed-siRNA) was transiently transfected into cells. Eight hours later the cells were infected with 0.1 ml CA16 strain Shzh05-1 at 10^3 $CCID_{50}$/0.1 ml (MOI 0.02). After 1 h adsorption, the inocula were removed and cells were incubated in DMEM supplemented with 10% FBS. Sixty hours postinfection, supernatants were harvested by centrifugation at 4000 rpm for 20 min after repeated three freeze-and-thaw cycles. The viral titers ($CCID_{50}$) were determined three times on Vero cells using 96-well plates.

For therapeutic experiments, cells were infected with 0.1 ml virus (500 CCID50/0.1 ml, MOI 0.01) for 1 h and were transfected with siRNA-encoding GeneBuster vector 4 h later.

2.6. Cell viability assay

To test if 13 mixed-siRNA has cytotoxicity in the dose response experiments, Vero cells were seeded in a 24-well plate for 24 h and then transfected with different doses of 13 mixed-siRNA. Sixty hours later, cells were trypsinized and resuspended in 1 ml DMEM.

The resuspended cells (0.1 ml) were transferred into 96-well plates, and then 20 μl MTS/PMS reagent (Promega) was added into each well to measure the absorbance at 490 nm according to the manufacturer's recommendations.

2.7. Real-time reverse transcription (RT)-PCR

The following primers were used: forward, 5′-GGAAATGCGAGTTGTTTACCT-3′ and reverse, 5′-GGGGACTGACACTTGAGCTG-3′ to amplify the region of nt 2057–2274 in CA16 strain Shzh05-1 CDS, then we cloned this region into pGEM-T Easy Vector to construct the standard plasmid. Using the standard plasmid we obtained a standard curve.

RNAeasy Mini kit (Qiagen, Germany) was used for RNA extraction from every well according to the Manufacturer's instructions. A reverse transcription (RT) reaction was carried out by using Superscript III Reverse Transcriptase (Invitrogen) in a 20 μl reaction mixture by using 1.2 μg total RNA. Real-time PCR was conducted using ABI Prism 7000 Real-time PCR system (Applied Biosystems) according to the Manufacturer's instructions. Reactions were performed in a 50 μl volume that contained 2 μl cDNA, 1 μl of each primer and 25 μl Power SYBR Green PCR Master Mix (Applied Biosystems). Absolute quantitation of viral RNA was calculated using the standard curve.

3. Results and discussion

3.1. Nucleotide sequence analysis of Shzh05-1 genes

The CA16 strain used in this study was the Shzh05-1 strain. This strain was collected in Shenzhen, People's Republic of China. Shenzhen is located on the southern coast of China, has a prosperous mutual exchange with other regions of Southeast Asia, and has a relatively high incidence of HFMD (Li et al., 2005). We initially established the complete genome sequence of this strain, and submitted the nucleotide sequence to GenBank (accession number EU262658).

The complete coding sequence (CDS) of strain Shzh05-1 was compared with other CDSs of CA16 strains in GenBank. The CDS of Shzh05-1 showed 95% nucleotide identity with Tainan/5079/98 (accession number AF177911) and Shzh00-1 strains (accession number AY790926), 79% nucleotide identity with the international standard strain, termed G10 (accession number CAU05876) and 77–80% nucleotide identity with other EV71 strains. The amino acid sequence was found to be very similar; Shzh05-1 had identity scores of 99%, 98%, and 95% with Tainan/5079/98, Shzh00-1, and G10, respectively.

3.2. Design and selection of siRNAs against CA16 genes

RNAi has great potential for the treatment of viral disease by targeting either the viral genes or host genes that are essential for viral infection. Several recent studies have suggested that targeting host genes may result in cellular toxicity and affect host cell viability because these are also essential for cell growth (Saleh et al., 2004). In this study, we chose viral genes as targets of RNAi. We designed 30 siRNAs targeting conserved regions of 8 genes (1B, 1C, 1D, 2A, 2C, 3A, 3C and 3D) in Shzh05-1 using empirical rules, and constructed these siRNAs into siRNA-expressing vector, GeneBuster vector. No siRNA for 1A, 2B and 3B was designed because they contain no stretch of conserved 21 nucleotides between Shzh05-1, Tainan/5079/98 and Shzh00-1.

We cloned each target gene into BglII–ApaI sites of siQuant vector to fuse genes (1B, 1C, 1D, 2A, 2C, 3A, 3C and 3D) to *firefly* luciferase gene as report vectors; these fused proteins were expressed under the control of the cytomegalovirus (CMV) promoter. Efficacy of the siRNA was validated as previously described (Du et al., 2004; Holen et al., 2002).

The subcloned siQuant vectors were then cotransfected together with corresponding siRNA-encoding GeneBuster vectors or negative control into HEK293T or Vero cells, respectively. Using Dual-Luciferase Reporter Assay system, we selected 13 out of 30 siRNAs that could knockdown the expression of corresponding genes by greater than 80% compared with mock control (transfected subcloned siQuant vector without siRNA-encoding GeneBuster vector) and negative control (cotransfected subcloned siQuant vector with scrambled control, GeneBuster-1B-SCS225) (Fig. 1, Supplementary Table 4).

3.3. Interference of CA16 replication in cultured cells

To test if the selected 13 siRNAs could efficiently inhibit the replication of CA16 in Vero cells, the latter were challenged with CA16 after being transfected with the 13 selected siRNA-encoding GeneBuster vectors for 8 h. The supernatants were collected 60 h later and viral titers ($CCID_{50}$) calculated. As shown in Fig. 2(A), in mock control and negative control transfected cells, viral titers ($CCID_{50}$) were markedly higher than that of siRNA-encoding GeneBuster vectors-transfected cells. SiRNA-encoding GeneBuster vectors could effectively inhibit replication of CA16 and caused a 19- to 56-fold ($1.29 \log CCID_{50}$–$1.75 \log CCID_{50}$) reduction of viral titers in Vero cells compared to controls. Among those 13 siRNAs, GeneBuster-2C-550, GeneBuster-2C-165 and GeneBuster-3A-214 showed very high inhibitory activity (56.2-, 53.1- and 56.2-fold reduction).

To test if these siRNAs expressed by selected siRNA-encoding GeneBuster vectors could effectively degrade viral RNA, Vero cells were transfected with the siRNA-encoding GeneBuster vectors. Cells were infected with CA16 8 h later. Twenty hours after infection, real-time reverse transcription polymerase chain reaction (RT-PCR) analysis on RNA extracted from the cells revealed that the copy number of virus genomes significantly dropped to $11.5 \pm 1.5\%$ to $30.4 \pm 6.2\%$ (Fig. 2(B)). These real-time RT-PCR results suggested that viral RNA was degraded and the inhibitory effect was due to a post-transcriptional activity.

The results of viral titers and real-time RT-PCR confirmed that the 13 selected siRNAs obtained in the foregoing Dual-Luciferase Reporter Assay results were also effective in interfering with CA16 reproduction. It is noted that the efficiency of siRNA to repress viral infection was correlated to their efficiency for repressing corresponding reporter constructs. It shows that the strategy used to select effective siRNAs against CA16 was effective.

3.4. Inhibition effects on CA16 replication by transfection of mixed siRNA-encoding GeneBuster vectors

CA16 does not have proof-reading capabilities during replication because of the error-prone nature of virally encoded RNA-dependent RNA polymerase 3D. It can therefore escape from RNAi by introduction of nucleotide mutants. These mutants lead to nucleotide mismatch between the guide strand of siRNA duplex and target, resulting in low gene silence or even no gene silence. Two strategies have been proposed to reduce the chance of escape: targeting of conserved sequences is thought to lower the frequency of the generation of viable mutants; alternatively, the probability of generating escape mutants may be reduced by expressing two or more efficient siRNAs simultaneously (Schubert et al., 2005). In this study, we integrated these two strategies and achieved good effects.

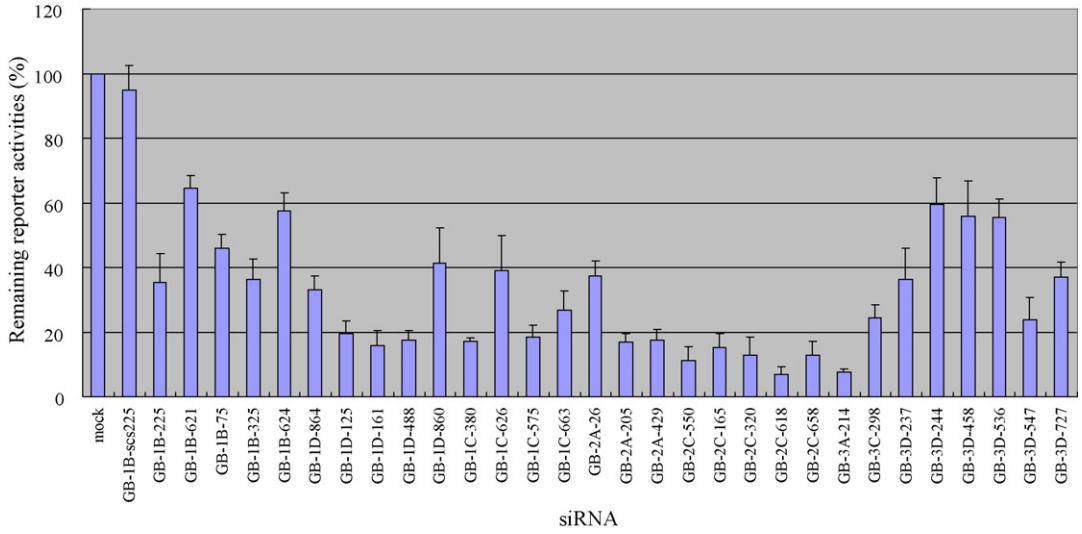

Fig. 1. siRNA efficacy validation. HEK293T cells or Vero cells were transfected with 400 ng siRNA-encoding GeneBuster vectors (30 siRNAs), 170 ng corresponding siQuant reporter vector and 17 ng internal control vector pRL-TK. Cells were harvested at 48 h posttransfection, and luciferase activities were detected. The plotted data are averages from triplicates.

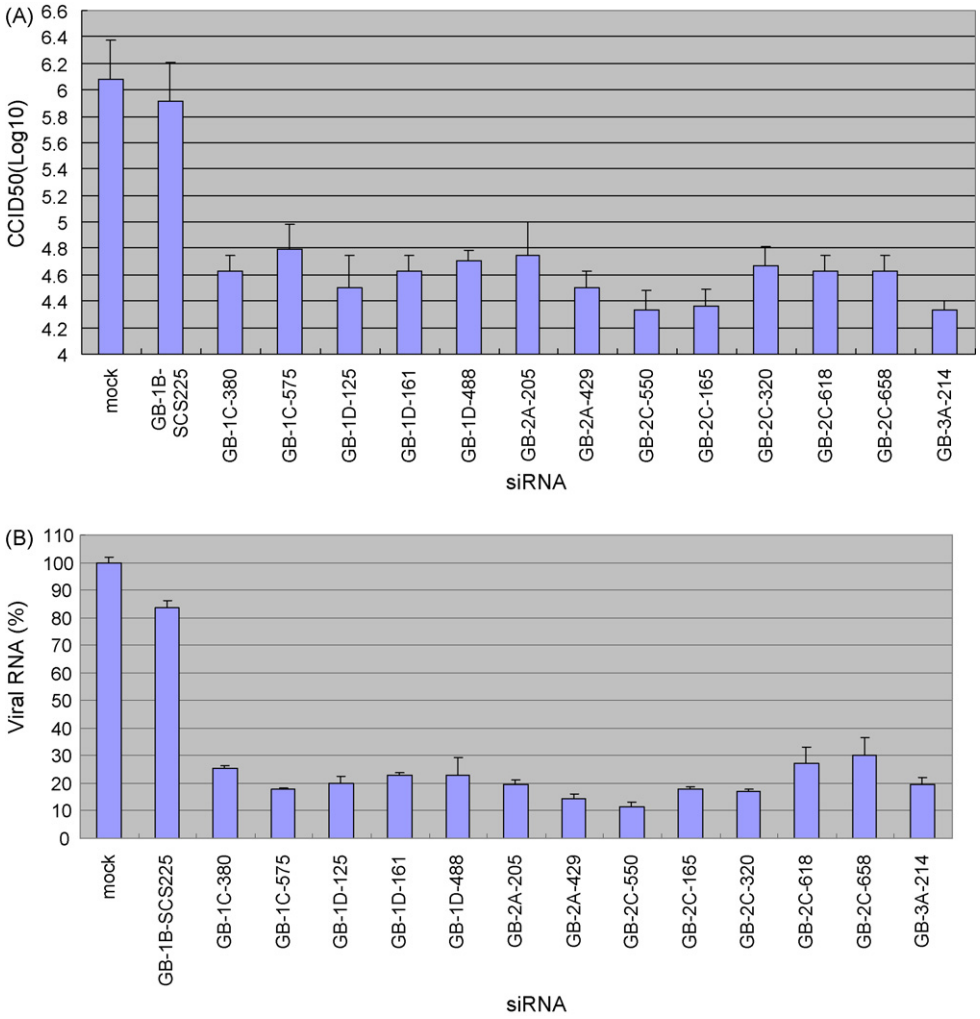

Fig. 2. The anti-CA16 effect of 13 siRNA-encoding GeneBuster vectors. Cells seeded in 24-well plate were transfected with 800 ng siRNA-encoding GeneBuster vectors for 4 h. At 8 h posttransfection, cells were infected with 0.1 ml virus at 10^3 $CCID_{50}$/0.1 ml (MOI 0.02) for 1 h. (A) Cells were infected with samples of supernant were taken and $CCID_{50}$ were determined 60 h postinfection. (B) Viral RNA was calculated using real-time RT-PCR at 20 h postinfection. The plotted data are averages from triplicates.

As described in foregoing results, the targets we chose were conserved among three CA16 strains: Shzh05-1, Shzh00-1, and Tainan/5079/98. But some of these targets may have a single-nucleotide difference when compared with other strains (e.g. G10). The optimal strategy is simultaneously targeting multiple regions using multiple highly efficient siRNAs (Yuan et al., 2005). We tested if cotransfection of cells with a combination of these 13 siRNA-encoding GeneBuster vectors targeting different regions of CA16 RNA could effectively inhibit the replication of CA16.

Cotransfection of cells with two or more siRNAs targeting different RNA sites has been reported to result in enhanced gene silencing compared to that of each single siRNA in HIV infection (Ji et al., 2003). Therefore, we also tested if mixed siRNA-encoding GeneBuster vectors would increase the antiviral effect.

As shown in Fig. 3(A) and (B), after 60 h after infection, the viral titer had a 42.2-fold (1.625 log $CCID_{50}$) reduction when cells were cotransfected with mixed siRNA-encoding GeneBuster vectors (we mixed 13 siRNA-encoding GeneBuster vectors of equal quantity to obtain an admixture, named 13 mixed-siRNA). Twenty hours after infection, viral RNA was reduced to $22.4 \pm 3.0\%$ compared with mock control and negative control. As shown in Fig. 3(C), the mock-transfected cells and negative control cells were more susceptible to CA16 infection. The results of viral titers, real-time RT-PCR and morphological changes of cells confirmed that 13 mixed-siRNA could also effectively inhibit replication of CA16 in Vero cells.

We did not find an obviously additive effect on antiviral activity between the equal-dose 13 mixed-siRNA and individual GeneBuster-2A-205, GeneBuster-3A-214 tested (Fig. 3(A)–(C)). The reason for enhanced gene silencing with multiple siRNAs has been postulated to be due to the binding of one siRNA resulting in changing the secondary structure of the target RNA so that it may offer more accessible sites for another siRNA (Ji et al., 2003). However, we did not observe enhancement effects when we used 13 mixed-siRNA. It was reported that these siRNAs may not affect the secondary structure of mRNA (Tan et al., 2007; Yuan et al., 2005), but other studies have indicated that the efficacy of siRNA is not totally dependent on secondary structure, and strongly suggest that the sequence properties of siRNA may play a major part in determining inhibitory efficacy (Hung et al., 2006; Khvorova et al., 2003; Mittal, 2004; Reynolds et al., 2004).

Although this combination transfection measure had no synergistic inhibitory effect on CA16 replication, we consider it is an optimal measure for preventing viral escape mutants from RNAi. The combination of 13 siRNAs targeting conserved regions

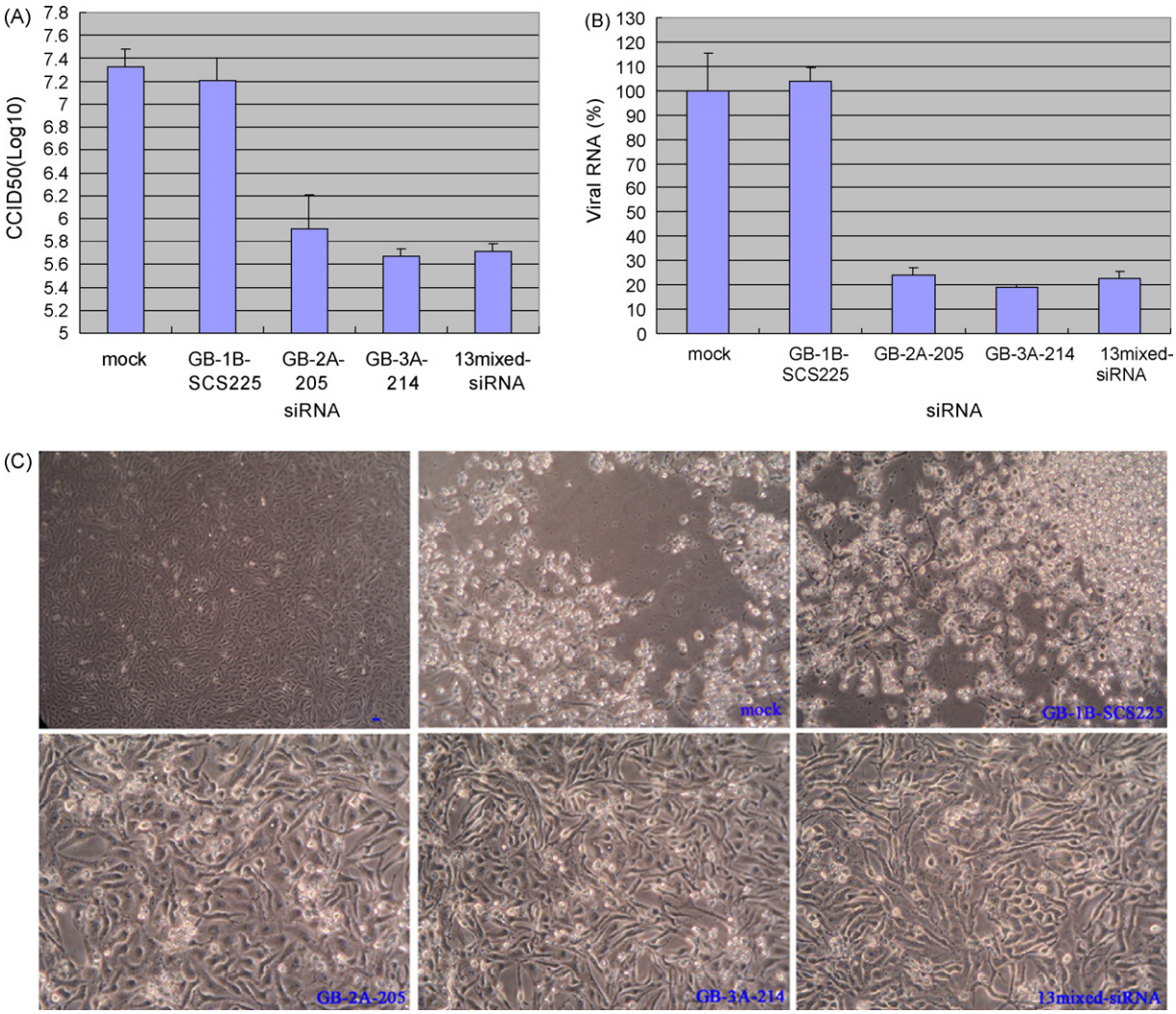

Fig. 3. Comparison of the anti-CA16 effects of 13 mixed siRNA-encoding GeneBuster vectors (13 mixed-siRNA) with GB-2A-205 and GB-3A-214. Cells seeded in 24-well plate were transfected with 800 ng plasmid for 4 h. At 8 h posttransfection, cells were infected with 0.1 ml virus at 10^3 $CCID_{50}$/0.1 ml (MOI 0.02) for 1 h. (A) Compare data of viral titers ($CCID_{50}$) at 60 h postinfection. (B) Compare data of viral RNA copies at 20 h postinfection. (C) Morphological changes of Vero cells at 60 h postinfection. (-) Uninfected Vero cells. The plotted data are averages from triplicates.

of CA16 nucleotide sequences could provide very sufficient protection against CA16. This strategy also seems feasible to conceive siRNA drugs that could cover a broader spectrum of viral strains.

The dose responses of viral suppression were studied using 13 mixed-siRNA. The virus titers and real-time RT-PCR data (Fig. 4(A) and (B)) showed that, as the amount of 13 mixed-siRNA increased (400–2400 ng), the virus titers and viral RNA also decreased. These results indicate that 13 mixed-siRNA exerts potent anti-CA16 activity in a dose-dependent manner. To confirm that there is no cytotoxicity induced by 13 mixed-siRNA, we measured the proliferation condition of 13 mixed-siRNA transfected Vero cells via a MTS assay. As shown in Fig. 4(C), we found there was no obviously increased toxicity as the amount of 13 mixed-siRNA increased from 400 to 2400 ng.

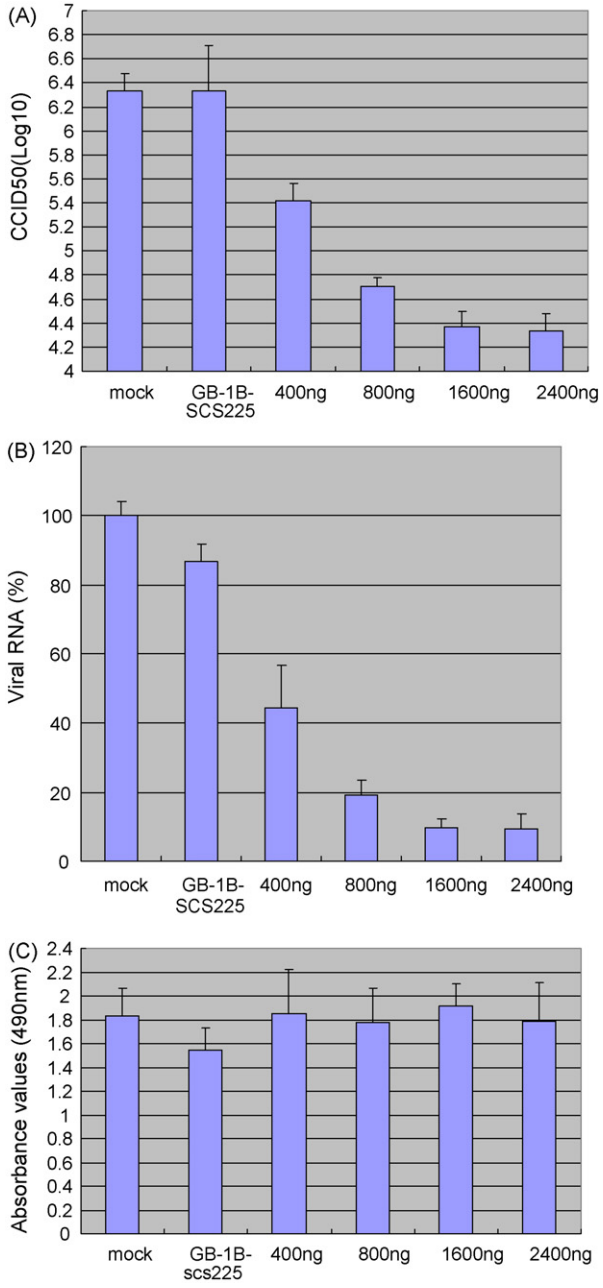

Fig. 4. Inhibition of CA16 virus production by different dosages of 13 mixed-siRNA. Cells seeded in 24-well plates were transfected with 13 mixed-siRNA (400, 800, 1600 and 2400 ng) for 4 h. (A) At 8 h posttransfection, cells were infected with 0.1 ml virus at 10^3 $CCID_{50}$/0.1 ml (MOI 0.02) for 1 h. Compare data of viral titers ($CCID_{50}$) at 60 h postinfection. (B) At 8 h posttransfection, cells were infected with 0.1 ml virus at 10^3 $CCID_{50}$/0.1 ml (MOI 0.02) for 1 h. Compare data of viral RNA copies at 20 h postinfection. (C) At 60 h posttransfection. Viability of Vero cells was determined by the MTS assay. The plotted data are averages from triplicates.

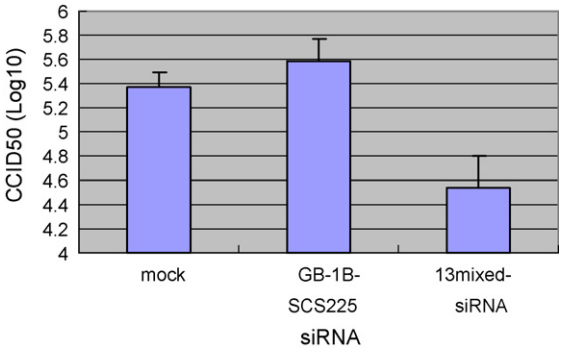

Fig. 5. The inhibitory effect of 13 mixed-siRNA on ongoing infection. Cells were infected with CA16 at 500 $CCID_{50}$/0.1 ml (MOI 0.01) for 1 h and transfected with 13 mixed-siRNA (800 ng) at 4 h postinfection. Samples of supernant were taken and $CCID_{50}$ was determined at 60 h posttransfection. The plotted data are averages from triplicates.

siRNAs must effectively inhibit an ongoing virus infection if they are to have clinical applications. To test if active siRNA could limit ongoing infection, we studied the siRNA repression of viral production in cells pre-infected by the virus. Vero cells were transfected with 13 mixed-siRNA 4 h after CA16 infection (500 $CCID_{50}$/0.1 ml was used); supernatants were collected and $CCID_{50}$ was calculated at 60 h post-transfection. The virus titers were significantly reduced by 13 mixed-siRNA treatment (Fig. 5). Thus, administration of siRNAs after viral infection can also effectively inhibit viral replication.

In conclusion, this rapid identification of active siRNAs could be very useful for confronting the sudden emergence of viral diseases. These results indicate that the 13 confirmed effective siRNA-encoding GeneBuster vectors targeting conserved regions of CA16 could be used as a potential therapy to inhibit CA16 replication in cultured cells. The strategy of simultaneously introducing multiple effective siRNAs to further prevent viral escape could be applied in the rapid development of antiviral siRNAs if the siRNA delivery issue can be resolved flawlessly. This *in vitro* study is the first step to demonstrate that siRNA technology is a very powerful approach for antiviral gene therapy. Many primary *in vivo* system delivery methods (e.g. liposome-based delivery and viral vector-based delivery) (Morris and Rossi, 2006; Zimmermann et al., 2006) have been reported recently, but further work should be conducted *in vivo* to investigate appropriate siRNA delivery strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2008.06.017.

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