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Short communication

Effective siRNAs inhibit the replication of novel influenza A (H1N1) virus

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ABSTRACT

In March and April 2009, an entirely novel influenza A (H1N1) virus (NIAV) emerged in Mexico and the USA. During the subsequent months, the virus rapidly spread all over the world by person-to-person transmission. In this report, RNA interference (RNAi) was used as an antiviral agent to inhibit NIAV replication in A549 cells. Ten small interfering RNAs (siRNAs) targeting extremely conserved regions among multiple NIAV genomes could effectively block the replication of NIAV strain A/Beijing/01/2009 (H1N1) in A549 cells. This study may be useful to confront the sudden emergence of NIAV infection.

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The novel influenza A (H1N1) virus (NIAV) is a new pandemic flu virus that was first detected in Mexico and the USA in March and April 2009 (Smith et al., 2009). As of 6 September 2009, over 277,607 cases of NIAV infection, including at least 3205 deaths, were officially reported to the World Health Organization (WHO). WHO has raised its pandemic alert to the highest phase, phase 6, to announce that the world is facing the challenge of a 2009 influenza pandemic (http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html). Four antiviral drugs used against influenza (oseltamivir, zanamivir, amantadine and rimantadine) could provide only limited protection, and may lead to resistance and/or side effects. It has been reported that adding exogenous, synthetic small interfering RNAs (siRNAs) to mammalian cells could induce RNA interference (RNAi) and clear mammalian cells from influenza A virus infection by degrading viral mRNAs (Ge et al., 2003; Hui et al., 2004; Sui et al., 2009). This study shed light on the development of anti-NIAV siRNA drugs by reporting that effective siRNAs (Table 1) targeting conserved regions of mRNA sequences could potently inhibit the replication of NIAV *in vitro*.

Eight RNA segments (PB1, PB2, PA, HA, NP, NA, MP and NS) of the NIAV genome were aligned respectively based on the pandemic (H1N1) 2009 virus sequences available in GenBank. Extremely conserved regions among NIAV genomes were chosen to design siRNAs. Thirty-five siRNAs were designed and synthesized according to empirical rules. For enhancing target specificity, maximum care was taken to verify that these 35 siRNAs had as little homology as possible to other mRNAs in the expressed sequence tag (EST) database of the human genome. We simultaneously designed a negative control (NC), which was a scrambled siRNA with the same nucleotides component as PB2-912. Efficacy of these siRNAs was validated using siRNA-to-target reporter assay as described previously (Du et al., 2004; Wu et al., 2008). Ten out of 35 siRNAs that could knock down the expression of corresponding targets by greater than 90% were chosen for the test of inhibitory effects on NIAV replication in cultured cells (data not shown).

Using NIAV strain A/Beijing/01/2009 (H1N1) (a China strain isolated from a hospitalized patient in Beijing with a laboratory confirmation of NIAV infection; the complete genome sequence of strain A/Beijing/01/2009 (H1N1) was established by overlap sequencing and submitted to GenBank, accession number GQ183617–GQ183624), the inhibitory efficacy of the 10 siRNAs were tested. A549 cells pre-transfected with 40 nM siRNAs were infected with NIAV at a multiplicity of infection (MOI) of 0.001. Twenty-four hours post-infection, viral RNA and viral protein were detected, respectively, using real-time Reverse Transcription (RT)-PCR and In Cell Western assay.

For detecting viral RNA, total RNA was extracted from cultured cells at 24 h post-infection. The mRNA level of the target gene and NP gene (a single-stranded RNA-binding protein that encapsidates the virus genome and has essential functions in viral-RNA synthesis) was simultaneously determined using SYBR Green real-time RT-PCR (relative quantification). All 10 siRNAs inhibited the mRNA transcription of target genes by greater than 70% compared with NC, and simultaneously inhibited the accumulation of NP mRNA

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Table 1
Sequence of synthesized siRNAs and their genomic positions in NIAV strain A/Beijing/01/2009 (H1N1).

siRNA	Sequence (all sequence from 5′ to 3′)	ORF position	Target gene
PB2-912	GGAACAAGCCGUAGACAUAtt UAUGUCUACGGCUUGUUCCtt	912–930	PB2
PB1-851	UGGCAAAUGUUGUGAGAAAtt UUUCUCACAACAUUUGCCAtt	851–869	PB1
PB1-1067	GGUACAUGUUCGAGAGUAAtt UUACUCUCGAACAUGUACCtt	1067–1085	PB1
PB1-1789	GGACCAAACUUAUACAAUAtt UAUUGUAUAAGUUUGGUCCtt	1789–1807	PB1
PA-1274	GGAUAGAACUUGAUGAAAUtt AUUUCAUCAAGUUCUAUCCtt	1274–1292	PA
NP-574	GAAAGGAGUUGGAACAAUAtt UAUUGUUCCAACUCCUUUCtt	574–592	NP
NP-571	GGUGAAAGGAGUUGGAACAtt UGUUCCAACUCCUUUCACCtt	571–589	NP
NP-1494	GAGACAAUGCAGAGGAGUAtt UACUCCUCUGCAUUGUCUCtt	1494–1512	NP
MP-969	CAACAUAGAGCUAGAGUAAtt UUACUCUAGCUCUAUGUUGtt	969–987	M2
MP-559	CGGCAAAGGCUAUGGAACAtt UGUUCCAUAGCCUUUGCCGtt	559–577	M1

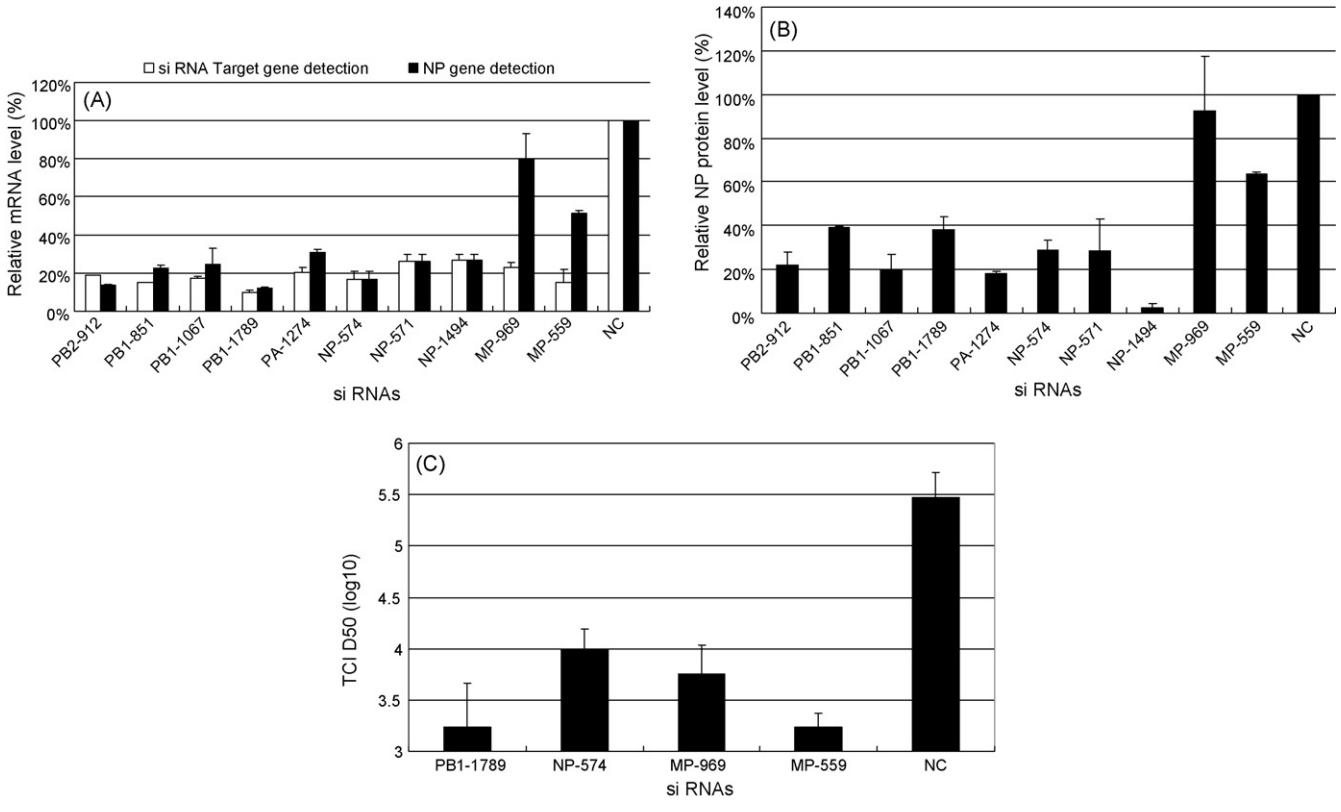

Fig. 1. Comparison of the inhibitory effects of 10 siRNAs and a negative control (NC). A549 cells were transfected with 40 nM siRNAs or NC, and 6 h post-transfection, cells were infected with the NIAV strain A/Beijing/01/2009 (H1N1) at a MOI of 0.001. (A) Viral RNAs were isolated from cultured cells at 24 h post-infection, the target genes and NP gene were detected simultaneously by SYBR Green real-time RT-PCR. The mRNA expression level of NC-transfected cells was defined as 100% (white bar represents the siRNA target gene detection and black bar represents the NP gene detection). The amount of viral RNA was normalized by the amount of cellular GAPDH transcripts. (B) Cells were fixed by paraformaldehyde 24 h post-infection and subjected to an In Cell Western assay using anti-NP, anti-β-tubulin antibody and fluorescent secondary antibodies. The NP protein level of NC-transfected cells was defined as 100%. The amount of NP protein was normalized by the amount of cellular β-tubulin expression. Data shown were from three independent experiments with the mean and standard errors shown. (C) Culture supernatants of siRNAs (PB1-1789, NP-574, MP-969, MP-559 and NC) transfected cells were taken and $TCID_{50}$ were determined at 48 h post-infection.

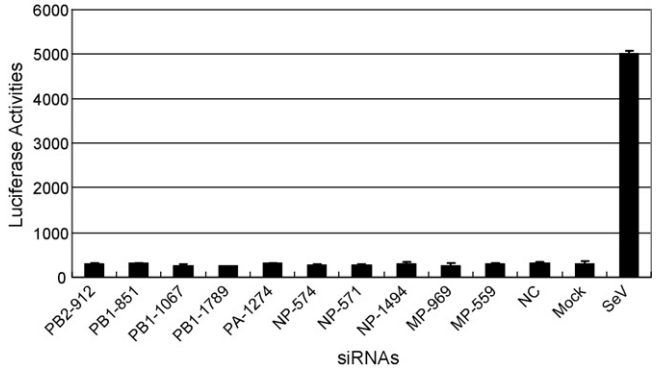

Fig. 2. Interferon (IFN) response detection. The IFN-β promoter luciferase reporter expressing stable A549 cells were transfected with siRNAs or infected with Sendai virus (SeV). Cells were harvested 24 h later, and luciferase activities were detected. Mock is mock-transfected cells.

(Fig. 1(A)). siRNAs targeting PB2, PB1 and PA could inhibit the transcription of NP by greater than 70%. Two siRNAs, MP-969 and MP-559, had a relatively slight inhibitory effect on NP. This finding suggested that, as RNA-dependent RNA polymerase, newly synthesized PB2, PB1 and PA proteins play critical roles in viral-RNA transcription.

For detecting viral protein by In Cell Western assay at 24 h post-infection, NP protein was detected by an immunoassay with the Li-COR Odyssey system and quantified using Odyssey infrared imaging software. The selected siRNAs inhibited the expression of NP protein by greater than 60% compared with NC except for MP-969 and MP-559 (Fig. 1(B)). It seemed that MP-969 targeting M2 failed to effectively inhibit NP expression. This finding and foregoing real-time RT-PCR results were different from a previous report (Sui et al., 2009), which showed that siRNAs targeting M2 not only decreased the level of M2 mRNA, but also the level of NP mRNA. The reason why our finding contradicts the report by Sui et al. needs further study. Although siRNAs targeting M1 and M2 had a relatively slight inhibitory effect on NP expression, we found that they are also effective for the inhibition of NIAV propagation from the results of viral titers (Fig. 1(C)). This proves that M1 and M2 are also important for viral propagation (Betakova, 2007; Itoh and Hotta, 1997; Takeda et al., 2002).

There is a possibility that the inhibitory efficacy of the 10 siRNAs is due to the triggered interferon (IFN) response. To exclude this possibility, A549 cells stably expressing IFN-β promoter luciferase reporter were used to detect IFN response after being transfected with siRNAs. As a positive control for the induction of IFN response, cells were infected with Sendai virus (SeV) (Strähle et al., 2007). The results showed that no IFN was produced in response to our siRNAs and NC when compared with the positive control (Fig. 2). This finding supported the conclusion that viral inhibition was mediated through RNAi.

In conclusion, 10 siRNAs obtained in the present study could effectively inhibit the propagation of NIAV in cultured cells. This report may be useful to confront the sudden emergence of NIAV infection.

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