Letter to the Editor

Gene Knockdown in Human Rhinovirus 1B Using 2′-OMe-modified siRNAs Results in the Reactivation of the Interferon Response

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The aim of this study was to investigate the knockdown efficiency of 2′-O-methylated (2′-OMe)-modified small interfering RNAs (siRNAs) on human rhinovirus 1B (HRV1B) replication and the interferon response. Thus, 24 2′-OMe-modified siRNAs were designed to target HRV1B. The RNA levels of HRV1B, Toll-like receptor 3, melanoma differentiation-associated gene 5, retinoic acid inducible gene-I, and interferons were determined in HRV1B-infected HeLa and BEAS-2B epithelial cells transfected with 2′-OMe-modified siRNAs. The results revealed that all 2′-OMe-modified siRNAs interfered with the replication of HRV1B in a cell-specific and transfection efficiency-dependent manner. Viral activation of Toll-like receptor 3, melanoma differentiation-associated gene 5, retinoic acid inducible gene-I, and the interferon response was detected. In conclusion, the 2′-OMe-modified siRNAs used in this study could interfere with HRV1B replication, possibly leading to the reactivation of the interferon response.

Human rhinovirus (HRV) is a single-stranded positive-sense RNA virus belonging to the genus *Enterovirus* of the *Picornaviridae* family. It was first discovered in the 1950s, while investigating the etiology of the common cold. Almost 60 years later, more than 100 serotypes/genotypes of the virus have been identified (*www.picornaviridae.com*). HRVs (HRV-A, -B, and -C) are the most common cause of human respiratory infections in all age groups, especially in children[1], however effective therapeutic agents against HRVs have not been developed yet. HRVs are small non-enveloped viruses with genomes of approximately 7.2 kb. The single open reading frame (ORF) encodes a large polyprotein containing capsid proteins (VP1-VP4) and non-structural proteins (2A-2C, 3A-3D)[2].

RNA-mediated interference (RNAi) is a biologically conserved mechanism of post-transcriptional gene knockdown that triggers degradation of specific RNAs by double-stranded RNAs (dsRNAs) or microRNAs (miRNAs). dsRNAs are initially processed by Dicer (a ribonuclease-III-like enzyme) into 21-25 nucleotides long RNAs known as small interfering RNAs (siRNAs)[3]. RNAi has been successfully used to inhibit the replication of many viral pathogens, although the resistance of siRNAs to nucleases remains a problem. Chemical modifications, such as 2′-O-methylation (2′-OMe) and 2′-fluorine (2′-F), add desirable properties to siRNAs. Whether these chemically modified siRNAs can effectively interfere with the replication of HRVs is unknown. The innate immune response is the first line of defense against virus infection. Specific pattern-recognition receptors, such as Toll-like receptor 3 (TLR3), retinoic acid inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5), recognize pathogen-associated molecular patterns. These include dsRNAs produced during viral replication, whose spreading the organism tries to restrict by inducing interferon and cytokines[4]. In this study, we evaluated gene knockdown efficiency of 24 2′-OMe-modified siRNAs targeting HRV1B replication.

The HRV1B virus (VR-1645) was purchased from the American Type Culture Collection (ATCC). HRV1B was propagated in HeLa and BEAS-2B epithelial cells; the supernatant was obtained by centrifugation at 5000 ×g. for 15 min, filter sterilized, and stored at -80 °C. The 50% tissue culture infective dose (TCID$_{50}$) of HRV1B was determined using the Reed-Muench formula.

Twenty-four 21-nt double-stranded siRNAs containing 19-mer core sequences and two dT units

at the 3'-terminus were designed to target HRV1B (GenBank accession no., D00239). Briefly, the 1B, 1C, 1D, 2A, 2B, 2C, 3C, and 3D regions of the HRV1B genome were selected, and three siRNAs were designed for each region. All siRNAs were chemically modified by the addition of 2'-OMe moieties to the uracils and cytosines on the complementary strands. siRNA containing an unmatched sequence was used as a negative control (siRNA-NC). A 5'-carboxyfluorescein (FAM)-labeled siRNA (FAM-siRNA) was used as a positive control to determine the transfection rate.

Various amounts of FAM-siRNA and 1 μL of the transfection reagent Entranster-R™ (Engreen, Beijing, China) were added to 100 μL aliquots of serum-reduced Opti-MEM (Gibco, Grand Island, USA). After 5 min of incubation at room temperature, the FAM-siRNA and Entranster-R™ solutions were gently mixed and incubated for 30 min at room temperature to form transfection complexes. Next, 200 μL of transfection complexes were added to each well and the plate was rocked gently. The final concentration of FAM-siRNA was 100, 150, or 200 nmol/L. The transfection rate of FAM-siRNA was determined after 6 h using a NucleoCounter NC-3000™ (Chemometec, Allerod, Denmark).

Twenty-four 2'-OMe-modified siRNAs were transfected into HeLa and BEAS-2B cells for 6 h, the transfected cells were then washed three times with PBS, and infected with HRV1B at a multiplicity of infection (MOI) of 1. Cell viability was assessed using a NucleoCounter NC-200™ (Chemometec, Allerod, Denmark). In addition, HeLa cells were infected with HRV1B for 3 h followed by transfection with the 24 2'-OMe-modified siRNAs. After 36 h, total RNA was extracted from HeLa and BEAS-2B cells using RNAiso-Plus (TaKaRa, Dalian, China), and 2 μg was used for cDNA synthesis. Gene knockdown efficiencies of the 24 2'-OMe-modified siRNAs against HRV1B were analyzed using real-time TaqMan PCR (RT-PCR). SYBR green-based RT-PCR using SYBR Premix DimerEraser mix (TaKaRa, Dalian, China) was performed to evaluate the mRNA transcriptional levels of TLR3, MDA5, RIG-I, IFN-α1, IFN-α2, IFN-β, IFN-λ1, and IFN-λ2/3. All RT-PCRs were performed using the ABI Prism 7500 platform (Applied Biosystems).

All presented data were based on the results of at least three independent experiments and were analyzed using SPSS 13.0 statistical software (IBM). All values are expressed as mean±standard deviation (SD). One-way ANOVA was used for statistical analysis.

Results revealed that FAM-siRNA was transfected into HeLa cells at a 150 nmol/L concentration, and the transfection rate was 79% (Figure 1A). Thus, 150 nmol/L was selected as the transfection concentration for 2'-OMe-modified siRNAs in subsequent experiments. Cell viability was determined to further explore whether 2'-OMe-modified siRNAs could protect HeLa cells from HRV1B-induced cytopathogenicity. Average cell viability was over 60%. The highest (88%) was found in cells transfected with 2'-OMe-modified siRNAs and then infected with HRV1B for 36 h (Figure 1B). These findings indicate that the 24 2'-OMe-modified siRNAs could interfere with the replication of HRV1B. We then determined the $TCID_{50}$ of HRV1B in HeLa and BEAS-2B cells. Results showed that the titers of HRV1B in both cell lines were more than 6 Log10 $TCID_{50}$/mL (Figure 1C). Viral gene copies indicated that HeLa and BEAS-2B cells supported HRV1B replication. Transfection of negative siRNA-NC did not affect the replication of HRV1B (Figure 1D).

To further determine the gene knockdown efficiency of the 24 2'-OMe-modified siRNAs, the levels of HRV1B RNA transcripts were analyzed using RT-PCR. The results showed that HRV1B replication ability was down-regulated in infected HeLa cells transfected with 2'-OMe-modified siRNAs compared with the siRNA-NC control. 2'-OMe-modified siRNAs such as 1C-1, 1C-3, 1D-1, 1D-2, 2C-3, and 3C-1, were most effective against HRV1B replication (Figure 2A). The same 2'-OMe-modified siRNAs interfered with HRV1B replication also in BEAS-2B cells. In the latter, 1B-3, 1C-3, 2A-1, 2B-1, 2C-1, and 3D-3 were particularly effective (Figure 2B).

We also determined the knockdown efficiency of the 24 2'-OMe-modified siRNAs in HeLa cells that were first infected with HRV1B for 3 h. The 24 2'-OMe-modified siRNAs could interfere with HRV1B replication, particularly when targeting regions 1B-3, 2A-3, 3C-3, 3D-1, and 3D-3 (Figure 2C).

Next, we examined the involvement of MDA5, RIG-I, and TLR3 in the recognition of dsRNA by selecting five 2'-OMe-modified siRNAs: 1C-3, 2A-3, 2B-2, 3C-3, and 3D-1. TLR3, MDA5, and RIG-I were significantly up-regulated in HeLa and BEAS-2B cells transfected with the five 2'-OMe-modified siRNAs, especially 2B-2 (Figure 3A). Similarly, reactivation of the IFN response, measured as fold change in IFN-α1, IFN-α2, and IFN-β, was highest in HeLa and BEAS-2B cells transfected with 2B-2 2'-OMe-modified siRNA (Figure 3B). Reactivation of type III IFNs, IFN-λ1,

and IFN-λ2/3, was again higher in BEAS-2B cells transfected with 2B-2 than in those transfected with 1C-3, 2A-3, 3C-3, and 3D-1 (Figure 3C).

Development of prophylactic vaccines against

HRVs is difficult, due to the existence of several serotypes. Thus, considerable effort has been devoted to the design of efficient treatments for HRV infection. Initial attempts to use IFN to control
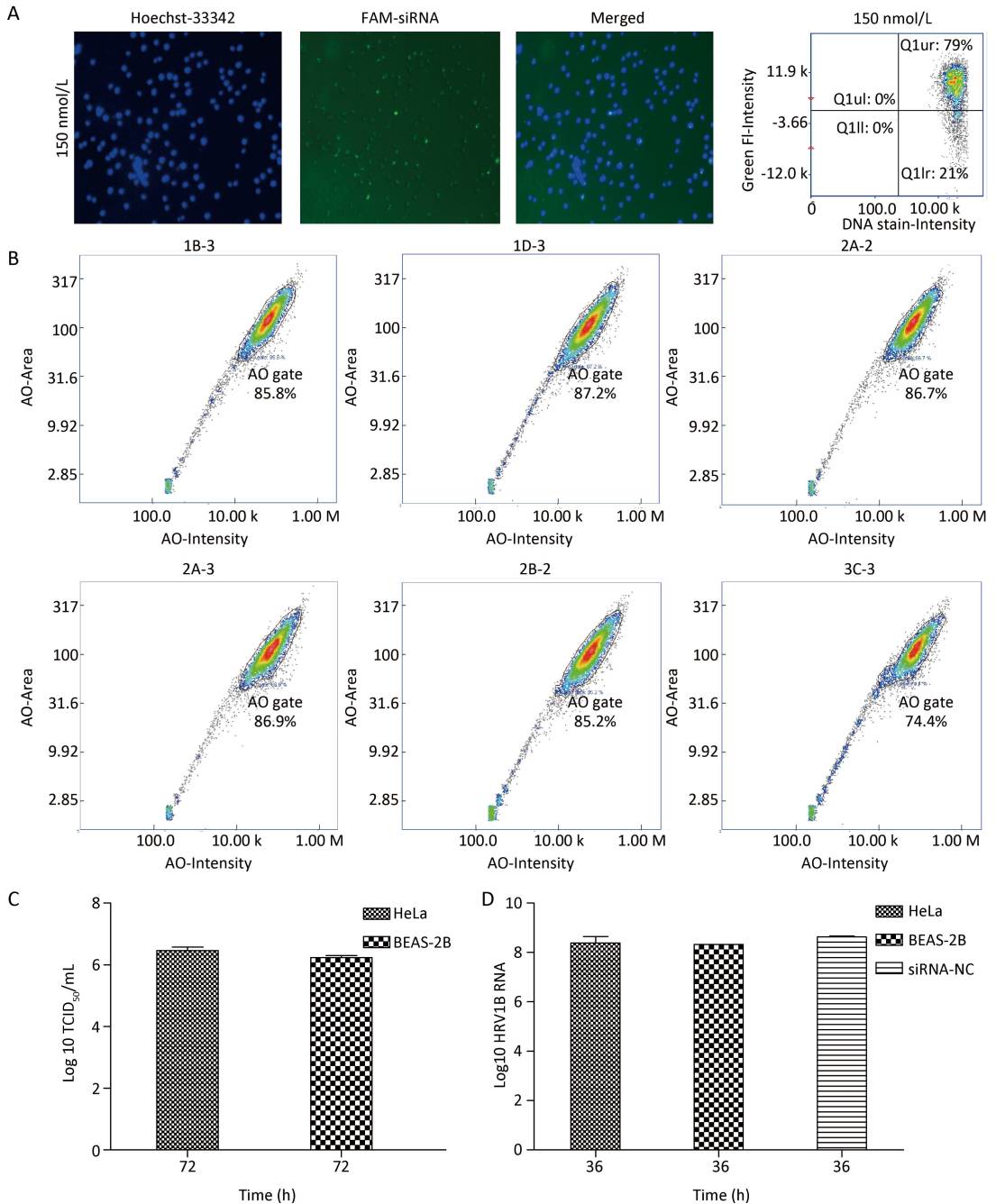

Figure 1. Effect of 2'-OMe-modified siRNAs against HRV1B infection, and HRV1B viral load in HeLa and BEAS-2B cells. (A) Representative fluorescent images and transfection rate using 150 nmol/L FAM-siRNA in HeLa cells. (B) Viability of HeLa cells transfected with 150 nmol/L siRNAs following HRV1B infection for 36 h. (C) Supernatants from infected HeLa and BEAS-2B cells were collected at 72 h post infection with HRV1B at a MOI of 1, and the viral titer was measured by $TCID_{50}$ analysis. (D) Viral gene copies were quantified by RT-PCR. HRV1B RNA was extracted at 36 h post infection with HRV1B at a MOI of 1. Data shown are representative of at least three independent experiments.

HRV infections have been ineffective. The use of antibodies or a soluble form of the intracellular adhesion molecule ICAM-1 to disrupt viral attachment to the host cell surface has been also disappointing. Antiviral molecules that interact with capsid proteins have not yielded good clinical results. Specific molecules targeting the viral 3C protease

have been developed in recent years[5]. Previous studies revealed that siRNAs targeting different regions of the coxsackievirus genome affected viral replication to varying extents[6-8]. We demonstrated that 24 different 2'-OMe-modified siRNAs interfere with the replication of HRV1B to varying degrees. Therefore, siRNA could be a potential therapeutic
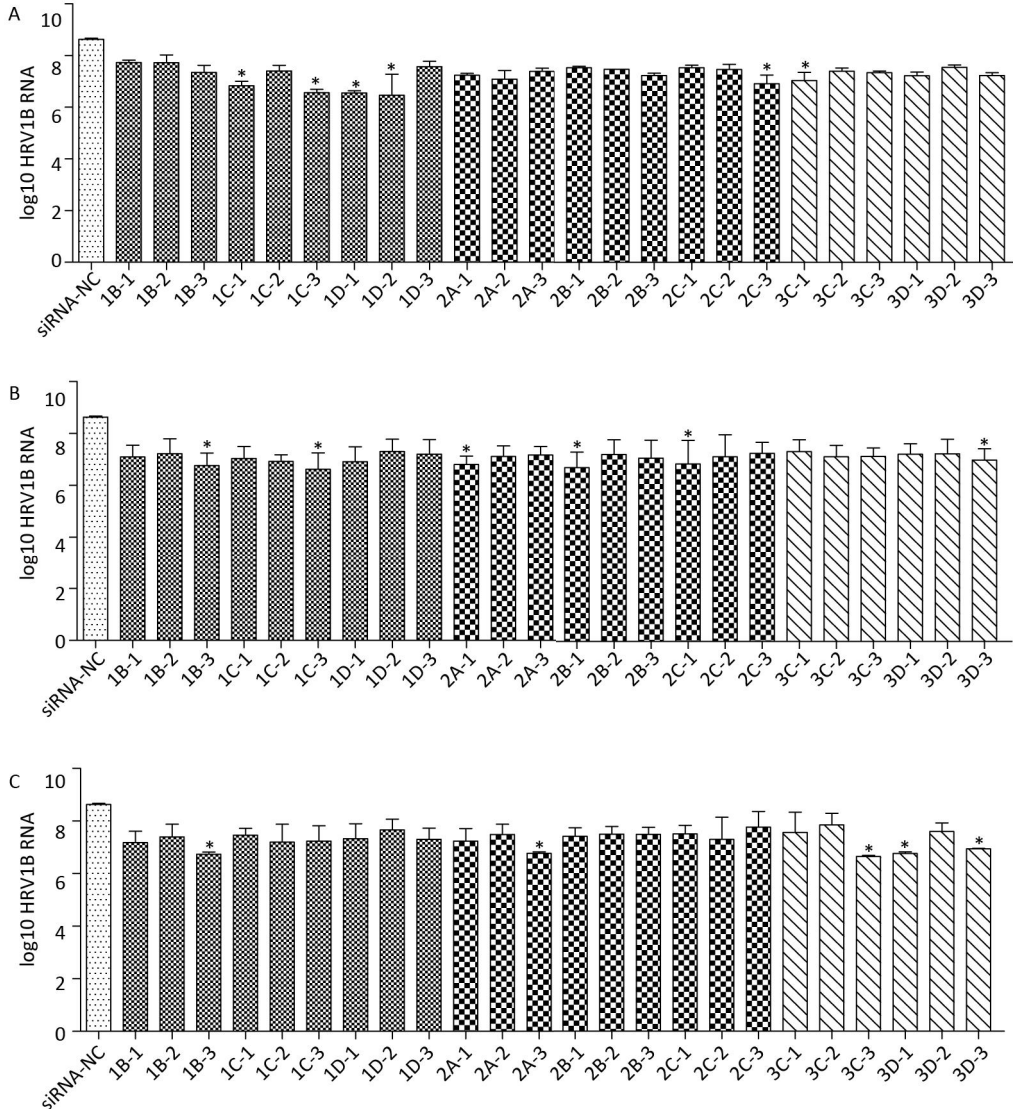

Figure 2. Gene knockdown efficiency of 24 2'-OMe-modified siRNAs against HRV1B in HeLa and BEAS-2B cells. (A) HeLa and (B) BEAS-2B cells were first transfected with 24 2'-OMe-modified siRNAs and then infected with HRV1B. Viral RNA was extracted at 36 h post-infection, and viral gene copies were quantified by RT-PCR. (C) HeLa cells were infected with HRV1B for 3 h and were then transfected with the 24 2'-OMe-modified siRNAs. Viral RNA was extracted at 36 h post-infection, and viral gene copies were quantified by RT-PCR. Data shown are representative of at least three independent experiments. *A P value of <0.05 was considered to be statistically significant.

measure to prevent HRV infection. Indeed, a long-lasting HRV viral gene knockdown effect is unnecessary because HRV infections are self-limiting in nature. Any long-lasting effect might trigger non-specific adverse complications. The use of synthetic siRNAs to inhibit HRV replication may be an optimal antiviral therapy because the process is straightforward and the siRNAs are amenable to chemical modification. Available modifications, such as 2'-OMe and 2'-F, increase the binding affinity and nuclease resistance, and are well tolerated[9]. A recent study found that 2'-OMe-modified siRNAs exhibited better RNAi activity against enterovirus 71 than their unmodified counterparts.
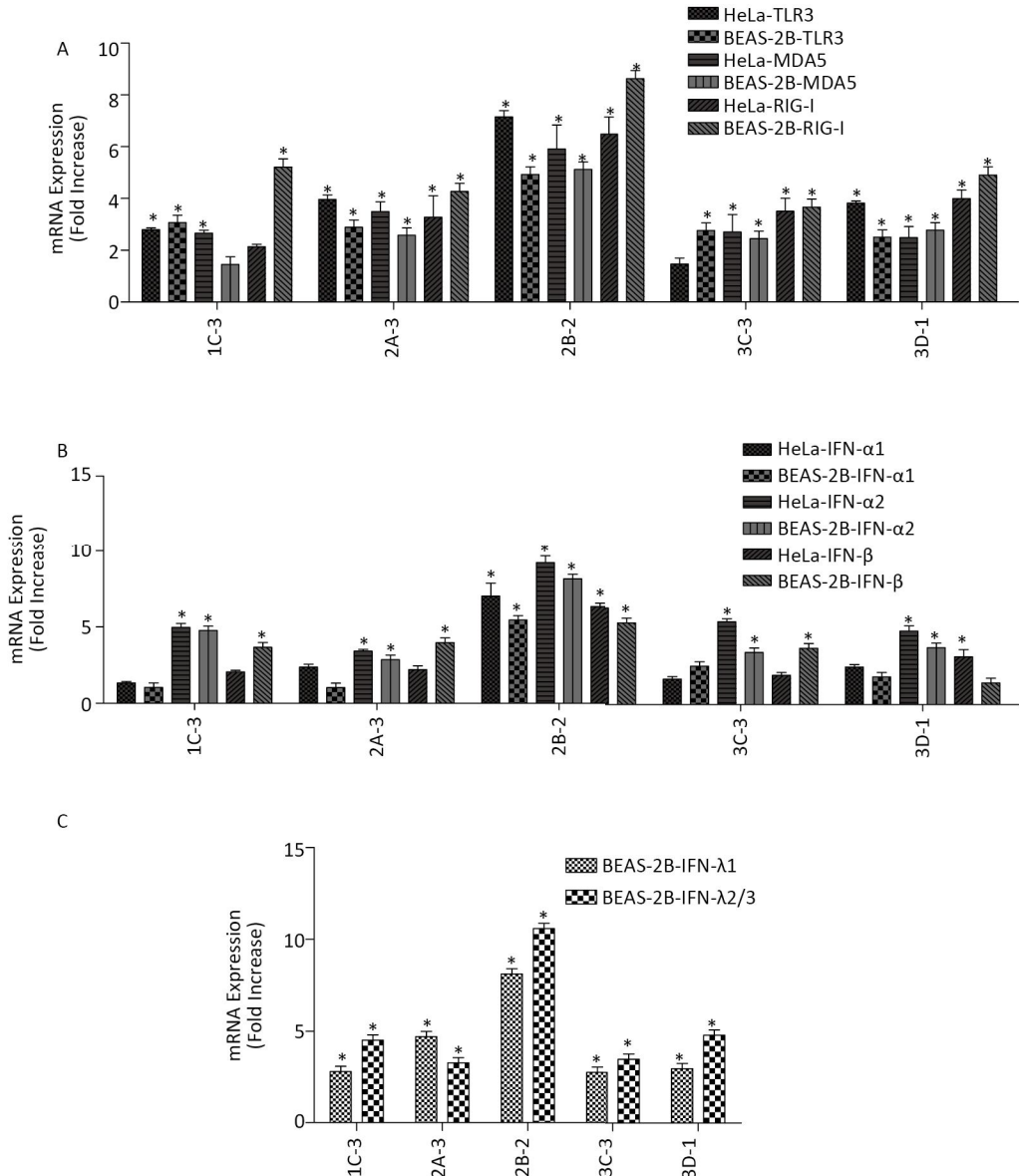

Figure 3. Fold changes in pattern-recognition receptors and IFNs in HeLa and BEAS-2B cells. (A) Fold changes in TLR3, MDA5, and RIG-I in HeLa and BEAS-2B cells. (B) Fold changes in IFN-α1, IFN-α2, and IFN-β in HeLa and BEAS-2B cells. (C) Fold changes in IFN-λ1 and IFN-λ2/3 in BEAS-2B cells. HeLa and BEAS-2B cells were transfected with 2'-OMe-modified siRNA, followed by infection with HRV1B for 36 h. Total RNA was extracted and the fold changes of the respective genes were determined by RT-PCR. Data shown are representative of at least three independent experiments. *A P value of <0.05 was considered statistically significant.

Our study shows that 2'-OMe-modified siRNAs could knockdown the replication of HRV1B and reactivate the IFN response. Further research on the effect of these 2'-OMe-modified siRNAs will be required to explore the potential of 2'-OMe-modified siRNAs as a prophylactic agent.

Competing Interests   The authors declare that they have no competing interests.

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