Silencing of HIV-1 *gag* gene from epidemic strains among men who have sex with men (MSM) in Tianjin, China by a broad-spectrum short hairpin RNA

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Abstract RNA interference (RNAi) has been successfully used as a promising method to inhibit the replication of different viruses, including human immunodeficiency virus (HIV). Gene mutation is a hurdle for the anti-HIV by RNAi. Although prone to mutation, some genes are conserved and limited in functionally important regions. The *gag* gene is conserved in different subtypes and plays an important role in the assembly of HIV viral particle. Here, we identified subtypes and conserved sequences within forty-four *gag* genes from the epidemic strains among men who have sex with men. Three subtypes of *gag* gene, including CRF01_AE (47.7 %), CRF07_BC (40.9 %) and B (11.4 %) were analyzed by online blast. We designed five small hairpin RNAs (shRNAs) based on the conserved sequences. The gag–EGFP fusion transcript reporter system was used to select the most efficient shRNA. Among the five candidate shRNAs, gag-shRNA-3 represented a broad-spectrum inhibition against all chosen targets. This broad-spectrum shRNA diminished the titer of subtypes B and C of HIV-1 for a hundred orders of magnitude. The gag-shRNA-3 described here is a potential therapeutic agent in the HIV-1 gene therapy.

Keywords HIV-1 · MSM · *gag* · shRNA

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Introduction

Acquired immune deficiency syndrome (AIDS) which was caused by human immunodeficiency virus (HIV) [2, 8], was first found in the population of men who have sex with men (MSM) in 1981 [4, 23]. In 1990, the first HIV infection in MSM of China mainland was recorded by the Chinese Ministry of Health [34]. Nowadays, MSM has become the main vulnerable population for HIV infection in China, especially in some metropolitan areas of China such as Beijing and Tianjin [18, 32]. According to a survey by Tianjin Centers for Disease Control and Prevention (Tianjin CDC), infectious rates of MSM reach 40.2 % in the total infection events [18]. So the MSM population is a focus in the HIV prevention and therapy.

In HIV applied therapies, combination antiretroviral treatment (ART) has been accessed to more and more HIV patients especially in low-income and middle-income countries over recent years. ART works well in suppressing HIV replication and keeping the patient healthy as long as the virus is not resistant against the drugs used. Drug resistance is a serious threat to HIV patients treated by ART, so the developments of novel antiviral therapies are necessary and essential. As RNA interference (RNAi) is an evolutionary highly conserved pathway in eukaryotes whereby double-stranded RNA (dsRNA) acts as a trigger to silence gene expression, it has been popularly used as new tools for genetics research and holds much promise as novel therapeutic approach aimed at suppressing rogue cellular genes or viral genes at the post-transcriptional level [6]. Actually, RNAi has been exploited in gene therapy strategies for HIV-1 [9, 24, 33]. It has been reported that about 200 published siRNAs and shRNAs are tested against HIV-1 [17], most of HIV genes and regular sequences are successfully targeted for RNAi [1, 10, 16].

According to the rapid and non-proofreading replication of HIV, HIV is present in a patient as a virus population of variants and constantly undergoes mutation. Characterized by high genetic variability, many hundreds of genetically unique strains are classified into several major groups (M, N and O) and further into subtypes [13, 14]. It is almost impossible to find an efficient siRNA or shRNA to repress all HIV strains. However, in a particular population infected with HIV, finding an efficient broad-spectrum siRNA or shRNA is still an interesting and attractive task.

In this study, we focused on a MSM population of Tianjin which virus transmitted mainly through homosexual action. We hypothesized that HIV-1 epidemic strains among the MSM population had some common characteristics, and hoped to find some RNAi targets that could repressed all epidemic strains efficiently. The structural gene of HIV *gag* was chosen as the RNAi target. Compared with other HIV-1 structural genes, *gag* gene is more conservative than *env*, and is not the usual target in ART as *pol*. The protein Gag encoded by it plays important roles in the assembly and release of viral particle [7]. In our study, sequences of HIV-1 *gag* gene from MSM in Tianjin was amplified and analyzed. Comprehensively considered of the sequence conservation and RNAi target characteristics, broad-spectrum RNAi targets were chosen. Then the candidate shRNAs expression plasmids were constructed and verified. The effective broad-spectrum shRNA may be a potential therapeutic agent in the HIV-1 gene therapy.

Materials and methods

Sequences acquirement and phylogenetic analysis

The acquirement of *gag* genes was performed by Tianjin Centers for Disease Control and Prevention (Tianjin CDC). Nested polymerase chain reaction (PCR) was used to amplify *gag* gene from the DNA of anticoagulant peripheral blood samples derived from the HIV-infected persons. Details have been shown in the previously described methods [5]. Briefly, the *gag* genes were amplified with outer primer pairs gag-L (5'-TCGACGCAGGACTCGGCTTGC-3', 686–707 nt of HXB2) and gag-E2 (5'-TCCAACAGCCCTTTTCCTAGG-3', 2011–2032 nt of HXB2), followed by PCR with inner primer pairs GUX (5'-GGAGAGAGATGGGTGCGAGAGCGTC-3', 781–806 nt of HXB2) and GDX (5'-GGCTAGTTCCTCCTACTCCC TGACAT-3', 1836–1861 nt of HXB2). The amplification was carried out in a thermal cycler for the first-round PCR with 1 cycle (50 °C for 30 min, 94 °C for 5 min, 55 °C for 1 min, 72 °C for 2 min), followed by 35 cycles (94 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min), and a final extension at 72 °C for 10 min. From the first-round PCR

products, 5 µl was a template and was used for the second-round PCR with inner primers. Second-round PCR was carried out with 1 cycle (94 °C for 2 min, 55 °C for 1 min, 72 °C for 1.5 min), followed by 35 cycles (94 °C for 30 s, 55 °C for 45 s, 72 °C for 1.5 min), and a final extension for 10 min at 72 °C. All *gag* sequences were aligned using CLUSTALX [28]. A Neighbor-Joining (NJ) tree was constructed for all the *gag* sequences by MEGA 4.1 software [25].

Cell culture

The HEK293T cell line is derived from transformation of HEK293 cell with the SV40 large T gene [21], and is used for the expression of recombinant genes. The TZM-bl cell line (also called JC.53bl-13) is a HeLa cell derivative that is used to express CD4 and CCR5 and integrated copies of the luciferase and β -galactosidase genes under control of the HIV-1 promoter [12, 20]. These two cells lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10 % (v/v) fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin (SV30010, HyClone) at 37 °C with 5 % CO₂.

shRNA design and plasmids construction

Five shRNAs were designed based on the conservative regions of these forty-four *gag* genes. Conservative regions were got from the blast results of *gag* genes by DNAMAN V6 software (Lynnon, Canada). Sequences used in shRNA construction were synthesized as oligonucleotides by Sangon Biotech (Shanghai) as shown in Table 1. Each oligonucleotide was resuspended in Tris-EDTA (TE) buffer to a concentration of 100 µmol/l and the forward strand and the reverse strand of oligonucleotides were mixed at a 1:1 ratio up to 30 µl. The mixture was then annealed at 95 °C 30 s, 72 °C 2 min, 37 °C 2 min, 25 °C 2 min for two cycles. The annealed product was ligated into the purified RNAi-Ready pSIRENretroQ vector. Details of the protocols are available in the BDTM Knockout RNAi Systems User Manual (PT3739-1).

Generation of target reporter plasmids

The gag-EGFP fusion transcript reporter system was used to optimize shRNA. Target sequences were cloned into the pEGFP-N1 at the *Bam*HI and *Eco*RI enzyme sites. According to the phylogenetic analysis of these *gag* genes, four *gag* genes were chosen as typical representative to be used as target reporters. Five conserved sites were chosen as the targets in each *gag* gene. Target sequences were synthesized as oligonucleotides by Sangon Biotech

Table 1 Oligonucleotides used to produce shRNA expression vectors

Primer names	Primer sequences
gag-shRNA-1F	5'-GATCCG <u>GATGGCAAGCAGGGAG</u> TTTCAAGAGAACTCCCTGCTTGCCCATACCTTTTTTG-3'
gag-shRNA-1R	5'-AATTCAAAAAAGGTATGGGCAAGCAGGGAGTTCTCTTGAAACTCCCTGCTTGCCCATACCG-3'
gag-shRNA-2F	5'-GATCCG <u>CAGAAGTAATACCCATG</u> TTTCAAGAGAAACATGGGTATTACTTCTGCTTTTTTG-3'
gag-shRNA-2R	5'-AATTCAAAAAAGCAGAAGTAATACCCATGTTTCTCTTGAAAACATGGGTATTACTTCTGCG-3'
gag-shRNA-3F	5'-GATCCG <u>AGGAAGTACTAGTACCCT</u> TTTCAAGAGAAAGGGTACTAGTAGTTCCTCTTTTTTG-3'
gag-shRNA-3R	5'-AATTCAAAAAAGAGGAAGTACTAGTACCCTTTCTCTTGAAAAGGGTACTAGTAGTTCCTCG-3'
gag-shRNA-4F	5'-GATCCG <u>GAAACAAATAGCATGGATG</u> TTTCAAGAGATCATCCATGCTATTTGTTCTTTTTTG-3'
gag-shRNA-4R	5'-AATTCAAAAAAGGAACAAATAGCATGGATGATCTCTTGAATCATCCATGCTATTTGTTCCG-3'
gag-shRNA-5F	5'-GATCCG <u>AGTAAGAATGTATAGCCCT</u> TTTCAAGAGAAAGGGCTATACATTCTTACTCTTTTTTG-3'
gag-shRNA-5R	5'-AATTCAAAAAAGAGTAAGAATGTATAGCCCTTCTCTTGAAAGGGCTATACATTCTTACTCG-3'
Scramble shRNA-F	5'-GATCCGGAGGTATCGAAGTAACAAATTCAAGAGATTTGTTACTTCGATACCTCCTTTTTTG-3'
Scramble shRNA-R	5'-AATTCAAAAAAGGAGGTATCGAAGTAACAAATCTCTTGAATTTGTTACTTCGATACCTCCG-3'

Sequences marked in italic and underlined are *gag* target sequences

Table 2 Oligonucleotides used to produce target reporter plasmids

Sample no.	Target sequences	Sample no.	Target sequences
<i>gag</i> 41-1	5'-TATGCGCAAGCATGGGAGT-3'	<i>gag</i> 129-1	5'-GTATGGGCAAGCAGAGAGT-3'
<i>gag</i> 41-2	5'-CAAACTAAAAACCATGTT-3'	<i>gag</i> 129-2	5'-CAGAAGTAATACCCATGTT-3'
<i>gag</i> 41-3	5'-AGGAAGTACCACTACCTT-3'	<i>gag</i> 129-3	5'-AGGAAGTACCACTAGTACCTT-3'
<i>gag</i> 41-4	5'-GAGCAAATACGATGGATGA-3'	<i>gag</i> 129-4	5'-GAACAAATAGGATGGATGA-3'
<i>gag</i> 41-5	5'-AGTAATAGTGTAATGCCT-3'	<i>gag</i> 129-5	5'-AGTAAGAATGTATAGCCCG-3'
<i>gag</i> 91-1	5'-GTATGGGCAAGCAGGGAGT-3'	<i>gag</i> 147-1	5'-GTATGGGCAAGCAGGGAGC-3'
<i>gag</i> 91-2	5'-CAGAAGTAATACCCATGTT-3'	<i>gag</i> 147-2	5'-CAGAAGTAATACCCATGTT-3'
<i>gag</i> 91-3	5'-AGGAAGTACTAGTACCTT-3'	<i>gag</i> 147-3	5'-AGGAAGTACTAGTACCTT-3'
<i>gag</i> 91-4	5'-GAACAAATAGCATGGATGA-3'	<i>gag</i> 147-4	5'-GAACAAATAGCATGGATGA-3'
<i>gag</i> 91-5	5'-AGTAAGAATGTATAGCCCT-3'	<i>gag</i> 147-5	5'-AGTAAGAATGTATAGCCCT-3'

(Shanghai). The complementary forward and reverse oligonucleotides were used for annealing and directional cloning into pEGFP-N1. Oligonucleotides used for PCR are shown in Table 2. All plasmids were sequenced by Sangon Biotech (Shanghai).

Transfection of target reporter plasmids and shRNA plasmids into HEK293T cells

HEK293T cells were seeded at 5×10^5 cells in each well of 6-well plate. The gag-EGFP expression plasmid and corresponding gag-shRNA plasmid were co-transfected into HEK293T cells by polyethylenimine (PEI, Sigma) methods. Total plasmids (2 μ g) and PEI (1 μ g/ μ l) were mixed for 10 min in the serum-free DMEM with a mass ratio of 1:4. The mix was added to cells and incubated for 6 h. Medium was refreshed at 6 h post transfection. After 48 h, HEK293T cells were observed under fluorescence microscope and images were taken. The RNAi inhibition efficiency was evaluated by flow cytometry.

Detection of inhibition efficiency by flow cytometry

Inhibition efficiency of gag-EGFP expression was evaluated by flow cytometry. About 48 h post transfection, cells were treated in accordance with the requirements by flow cytometry. Briefly, the cells were washed twice with phosphate-buffered saline (PBS), digested and harvested. The harvested cells were centrifuged at 850g for 5 min, and resuspended with 500 μ l 1 % paraformaldehyde fix for 30 min. At last, green fluorescent intensity was checked by the flow cytometry (FACSCalibur, BD). No-transfected cells were used as a negative control.

Expression of HIV-1 molecular clone in HEK293T cells

To check the inhibition effect of efficient shRNA on the HIV-1 full-length gene expression, two subtypes of HIV-1 infectious molecular clones subtype-B pNL4-3 (GenBank: AF324493.2) and subtype-C p1084i (GenBank: AY805330.1) were used to evaluate the inhibition effect.

Fig. 1 Conservative sequences of the five shRNAs and their location in HXB2 genome. **a** Conservative sequences of each shRNA (1–5) targets in the forty-four *gag* genes. WebLogo of each shRNA target was created by the online tool (<http://weblogo.berkeley.edu/>). Genetic diversity of each site was shown in corresponding position. **b** Five

sites targeted by shRNAs were marked in the HIV-1 genome. Arrows above the enlarged *gag* gene show the sites of shRNAs (1–5), and exact coordinates to HXB2 sequence numbering are listed in parentheses

Suppression of HIV-1 gene expression was measured by determining virus titer of the supernatant. HEK293T cells (1.5×10^5) were seeded 24 h prior to transfection in each well of a 24-well culture plate. HIV-1 infectious molecular clone (pNL4-3 or p1084i) and *gag*-shRNA were co-transfected into HEK293T cells with a ratio of 1:1 (200 ng:200 ng). Approximately 50 ng of pGL3-Control (Promega) was used to control for transfection efficiency. Medium was refreshed at 8 h post transfection. Viral

supernatant was harvested at 60 h post transfection, filtered through a 0.45 μ m filter, dispensed and stored at -80°C .

The 50 % tissue culture infective dose (TCID₅₀/ml) of virus stock was determined by infecting TZM-bl cells with fourfold serial dilutions of virus [12]. Briefly, TZM-bl cells were grown in 96-well plates at 2×10^4 cells/well with 150 μ l DMEM. Firstly, dilute virus to 1:12 with 60 μ l virus and 660 μ l DMEM (40 μ g/ml DEAE-Dextran). Aspirate supernatant in 96 well plate, add 200 μ l virus (1:12) to the first

row, then 50 μ l to the following wells for serial dilution (1:4), triplicate for each dilution. Two days post infection, cultured cells were fixed and stained. Blue cells with β -galactosidase activity were counted under a light microscope.

Statistical methods

Flow cytometry assay data were analyzed by the Cell Quest software (BD Biosciences, USA). Statistical analyses and linear figures were performed using Prism 5 for Windows version 5.02 (Graph Pad Software, Inc., La Jolla, CA).

Results

Five shRNAs derived from the conservative sequences

Conservative regions of these forty-four *gag* genes were got from the blast results by DNAMAN V6 software (Lynnon, Canada). Five shRNA targets were derived from the highly conserved regions of the consensus sequences. As shown in Fig. 1a, the genetic diversity within these shRNAs was examined with the forty-four *gag* genes by the WebLogo (<http://weblogo.berkeley.edu/>). Genetic diversity of each site was shown in corresponding position. What's more, the five sites targeted by shRNA (1–5) were marked in the HIV-1 genome as shown in Fig. 1b. Arrows above the enlarged *gag* gene show the sites of shRNAs (1–5), and exact coordinates to HXB2 sequence numbering are listed in parentheses. According to the coordinates to HXB2 genome, all of these five shRNAs targets were located in the Matrix (MA) and Capsid (CA) region of Gag protein.

Phylogenetic linkage of *gag* gene in the MSM population

Subtypes of these forty-four *gag* genes were blasted from the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html) and REGA HIV Subtyping Tool Version 3.0 (<http://bioafrica.mrc.ac.za:8080/rega-genotype-3.0.2/hiv/typingtool>) [19]. A phylogenetic tree was built based on these *gag* genes, where each leaf corresponds to different *gag* genes. HIV_{NL4-3} and HIV_{1084i} were used in the inhibition on HIV replication, so the *gag* genes of HIV_{NL4-3} and HIV_{1084i} were marked in the phylogenetic tree. The results demonstrated that HIV-1 subtypes were as follows: CRF01_AE (47.7 %), CRF07_BC (40.9 %), and B (11.4 %). Details of the subtypes were shown in Fig. 2a. The target reporter plasmids were determined based on the phylogenetic analysis of these *gag* genes. Four *gag* genes (gag-41, gag-91, gag-129 and gag-147) were chosen as typical representative to be used as target reporter as marked in Fig. 2a.

Inhibition of Gag protein expression

Five gag-RNAi shRNAs including gag-shRNA-1, gag-shRNA-2, gag-shRNA-3, gag-shRNA-4 and gag-shRNA-5 were tested against the gag-EGFP fusion protein expression in HEK293T cells. As shown in Fig. 2b, the fluorescence intensity was sharply decreased in the shRNA group. On the other hand, fluorescence images showed no difference between cells transfected with gag-EGFP alone and cells co-transfected with gag-EGFP and scrambled shRNA. The inhibitory effects of shRNAs on the expression of gag-EGFP were evaluated quantitatively by flow cytometry (Fig. 2c). All shRNAs had the inhibition efficiency on the corresponding gag-EGFP protein expression. Comparing these results, gag-shRNA-3 had a broad spectrum inhibition effect. The expression of gag-91-3 was silenced up to 75 % in HEK293T cells with single gag-shRNA-3.

Silencing of HIV replication using broad-spectrum shRNA

To detect the inhibition effect of shRNA on HIV-1 replication, two different HIV-1 subtype infectious clones were used. HIV-1 infectious clone NL4-3 (subtype B) and 1084i (subtype C) which have completed matched RNAi target (5'-AGGAAGTACTAGTACCCTT-3'). Ability of the optimum gag-shRNA to inhibit cognate targets within two subtypes of HIV-1 infectious molecular clone was determined as shown in Fig. 3a. The transfection efficiency was evaluated by the luciferase assay of pGL3-Control. Virus titer was normalized to the luciferase activity of pGL3-Control. Both of these two HIV-1 infectious molecular clones were inhibited by the gag-shRNA-3. Higher virus titer was got from the scr-shRNA group of NL4-3 (2.3×10^5 TCID₅₀/ml) and 1084i (2.1×10^5 TCID₅₀/ml), and lower in the gag-shRNA group of NL4-3 (5.8×10^3 TCID₅₀/ml) and 1084i (3.6×10^3 TCID₅₀/ml). According to the changes of virus titer, the gag-shRNA-3 was effective on the virus inhibition.

Site targeted by gag-shRNA-3 was conserved in prevalent subtypes of HIV

Conservation of gene in the site targeted by gag-shRNA-3 was blasted in a total of 2,177 sequences from HIV Databases, which contains many types of HIV. Detail blast result of target gene was shown in Fig. 3b. According to the results, the target was highly conserved in HIV Database [14]. In the 2,177 *gag* sequences, 52.8 % are no mutation, 31.2 % with one mutation and 10.9 % with two mutations. In summary, the site targeted by gag-shRNA-3

Fig. 2 Phylogenetic analysis of the *gag* genes of MSM population and shRNAs silence the *gag*-EGFP fusion mRNA in HEK293T cells. **a** Phylogenetic analysis of the *gag* genes from MSM population in Tianjin. The *gag* genes of HIV_{NL4-3} and HIV_{1084i} were also marked in the phylogenetic tree. The bootstrap probability (>75 %) is indicated at the corresponding nodes of the tree. The scale bar indicates the evolutionary distance of 2 % (0.02 substitutions per site). The tree was constructed using MEGA version 4.1. **b** HEK293T cells were co-transfected with 1 µg shRNA plasmid and 1 µg corresponding target reporter plasmid for 48 h. Fluorescent images were taken by a fluorescent microscope. Experiments were done in triplicates, and one time images were shown here. Bar scale 100 µm, and applicable to other panels in (b). **c** Cells were treated following the instructions and assayed by flow cytometry. Fluorescence intensity was used to calculate inhibition efficiency of shRNA. Mean fluorescence intensity of scramble shRNA (Scr) was regarded as 100 %, and fluorescence intensity of non-transfected cells was regarded as negative control (NC). The dotted line represents the 50 % inhibition efficiency of fluorescence intensity. Error bars are standard error of triplicate experiments

Fig. 3 Inhibition of full-length HIV-1 infectious molecular clone and conservation blast result of *gag*-shRNA-3 from the HIV Databases. **a** HEK293T cells were transfected in a 1:1 ratio of HIV-1 clone (pNL4-3 or p1084i) and shRNA or scramble shRNA, and with equal amount of the pGL3-control. Supernatants were collected at 60 h post transfection. Titer assay of supernatant was performed by fourfold dilution method in TZM-bl cells. Error bars are standard error of triplicate experiments. **b** Conservation of the site targeted by *gag*-shRNA-3. Gene of the site targeted by *gag*-shRNA-3 was submitted into the online blast tools (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html). A total of 2,177 sequences which contains all subtypes of HIV were blasted in the results

was highly conserved and had a highly conservation ratio of 94.9 % within the mutation number of less than two nucleotides.

Discussion and conclusion

In recent years, HIV infection among MSM has been responsible for the tremendous increase in the outbreak of the HIV epidemic globally [30]. To prevent the epidemic of HIV infection in MSM, behavioral interventions are necessary. Meanwhile, molecular research of HIV infection in MSM is also needed. RNAi technology is a powerful tool to silence target gene expression in vitro and in vivo [27, 11], and has been successfully used as an effective approach to inhibit the HIV replication [17, 29, 35]. The use of RNAi to inhibit HIV-1 represents a novel and potentially powerful antiviral strategy.

The structural protein Gag is necessary and sufficient for the assembly and maturation of virion [7]. MA protein derived from Gag precursor plays an important role in the transportation, incorporation with envelope (Env) and early post entry events of HIV. So, gene replication and virus packaging can be suppressed by the shRNAs target the *gag* gene of HIV. In our study, expression of *gag* gene was silenced up to 75 % in HEK293T cells with single *gag*-shRNA-3. Two HIV-1 molecular clones (subtype B and C) in our lab were used to evaluate the broad inhibition effect of *gag*-shRNA-3. Viral packaging of the two subtypes of HIV-1 was sharply inhibited by the *gag*-shRNA-3. According to our results, *gag*-shRNA-3 was optimized as the broad-spectrum shRNA from the five designed shRNAs. In our study, effect of *gag*-shRNA-3 wasn't validated in the primary isolated virus because of limitation of experiments. Inhibition on the two HIV-1 molecular clones by this *gag*-shRNA-3 might not reflect the effect on the in vitro primary infection. It is better to evaluate the inhibition effect of *gag*-shRNA-3 in the primary isolates derived from the infected MSM cohort.

At present, HIV-1 standard laboratory viral isolates (mostly subtype B) were used to RNAi optimization. Our shRNA target design was derived from the *gag* sequences of HIV-1 isolated from the population of MSM of Tianjin. Fortunately, the target we found was conserved in HIV Database. So, this shRNA may be a potential candidate for gene therapy applications against the most prevalent HIV-1 subtype. Although RNAi can suppress the replication of HIV-1, a significant hurdle for the use of RNAi-based therapeutics was the mutation of target sequences [22]. A single shRNA can yield a potent antiviral effect, but the virus can escape by acquiring mutations in the RNAi-target sequence. HIV-1 can escape from RNAi by target sequences mutation or alternation of its RNA genome structure [31]. It has been suggested that combination of perhaps as few as four different siRNAs may effectively curb the emergence of viral escape mutants [26, 15, 22]. Alternatively, RNAi could be used therapeutically in combination with other antiviral approaches such as ART to successfully treat the HIV infection [3]. To get more efficient treatment effect, it is

necessary and essential to get broad-spectrum siRNA/shRNA. Our work is an experimental attempting on it. In conclusion, this study demonstrates the epidemic subtype of HIV-1 in MSM population, and provides an effective shRNA for silencing of HIV-1 *gag* gene and full-length expression. The broad-spectrum gag-shRNA-3 described here is a potential candidate for gene therapy applications against the most prevalent subtypes of HIV-1.

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