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Antiviral Activity of Sirna UL42 against Herpes Simplex Virus Type 1 in HeLa Cell Culture

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Abstract

RNA interference (RNAi) is a process by which introduced small interfering RNA (siRNA) can cause the specific degradation of mRNA with identical sequences. In this study, we applied siRNAs targeting the UL42 gene of human herpes simplex virus type 1 (HSV-1), which encodes a multifunctional polypeptide that is vital for virus DNA replication, binding to DNA, stably associating with the virus DNA polymerase (Pol), and acting to increase the length of DNA chains synthesized by Pol. HeLa cell line was used for HSV 1 infection and SiRNA transfection was done to suppress UL-42 gene in cell culture. The decrease in titer of HSV 1 was observed by rReal Time PCR to detect the drop in HSV 1 DNA synthesis and translation. The inhibition rates of siRNA1 and siRNA2 on HSV-1 plaque formation were reported and Comparing with virus control, siRNA1 and siRNA2 reduced DNA replication HSV-1. The decision whether the decrease in the number of HSV-1 plaques was due to siRNA silencing expression of the UL42 gene, a real-time PCR indicating that UL42-specific siRNAs blocked the expression of the UL42 gene significantly. Comparing with virus control, siRNA1 and siRNA2 reduced the expression of UL42 gene. In this study the synthetic siRNA silenced UL42 mRNA expression effectively and specifically and inhibited HSV-1 replication and also our data offer new possibilities for RNAi as a genetic tool for inhibition of HSV-1 replication.

Keywords: Herpes simplex virus type1, siRNA, UL42, Transfection

Introduction

Herpes simplex virus type 1 (HSV-1) is a large, nuclear-replicating, dsDNA virus which is the member of the alpha herpes virus subfamily. The viral genome has 152 kilobase (kb) packaged into an icosahedral capsid surrounded by a tegument layer that is limited by a membranous envelope constructed with viral glycoproteins [1,2]. Herpes virus type 1 is the most common cause of cold sores around the mouth and lips (fever blisters). This virus is transmitted through oral secretions or wounds on the skin can be spread through kissing or sharing objects such as toothbrushes or eating utensils. HSV-1 is a neurotropic agent that establishes latency causing a lifelong infection in sensory neuronal ganglia which supply the primary site of infection [3]. Prophylactic oral antiviral therapy reduces recurrences of herpes simplex keratitis (HSK) by only 40% and has no sustained benefit following its discontinuation [4,5]. There are three kinetic groups of proteins playing a role in herpes simplex virus replication: immediate early, early, and late. Immediate early genes activate the host cell transcriptional machinery [6]. The seven of early (β) genes, DNA polymerase (U_L30), DNA binding protein (U_L42 and U_L29/ ICP8), ORI binding protein (U_L9), and the helicase/primase complex (U_L5, U_L8 and U_L52) have been shown to be essential in virus replication, while the rest of the (β) gene products play only a partial role . In addition to DNA polymerase activity, the U_L30 acts as a DNA-dependent polynucleotide synthesis, 3'-5' exonuclease and *RNase H* [7,8].

Different treatments against Herpes simplex virus imply the important role to develop more effective methods for HSV-1 infections. Because the unavailability of a cure for herpes infections, the expensive treatments, and the emergence of resistance are problems that the entire world is still facing off [9]. One of antiviral defense in vertebrates which includes variety of mechanisms to inhibit virus replication is RNA silencing pathways. Gene silencing, induced by RNAi, is mediated by microRNAs (mirRNA) and small interfering RNA (siRNA) forming Watson–Crick base pairing to a target mRNA, subsequently leading to its sequence-specific cleavage [10,11]. The first term of RNAi was used in the worm Caenorhabditiselegans to explain that dsRNA complementary to a particular gene was more effective in silencing than either strand individually [12]. Subsequent studies showed that silencing related with the processing of dsRNA to small 21–23 nucleotide-duplex short-interfering RNAs (siRNAs) and that a dsRNA-specific endoribonuclease III, known as Dicer, was responsible for cleavage of dsRNA triggering the silencing machinery that produces siRNA fragments (19-23 bpduplexes). And also, siRNA binds to the RNA-induced silencing complex (RISC) leading siRNA to the mRNA [13]. Then, the siRNA is unwound in an ATP-dependent manner, and the RISC complex becomes activated. So the target mRNA is cleaved to 12 nucleotides from the 30-terminus of the siRNA strand. Small interference RNAs (siRNAs), 21-23 nucleotide long double-stranded RNA molecules could specifically cleave targets mRNAs [14]. The siRNA cleavage action is not affected by the secondary structure of mRNA and the efficiency of siRNA is determined by the siRNA specific properties [15]. This feature is in contrast with the antisense, where silencing is dependent on mRNA properties such as target site accessibility, which is determined by local mRNA confirmation [16]. The siRNA

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sequence should be 21 nt long, and the sequence should preferably be 50 to 100 nt downstream of the start codon and the selected sequence site should not be in untranslated regions [17]. The selected sequence should carry 5'-AA (N19) UU, where N is any nucleotide in the mRNA sequence. So the blast searching of a respective organism ensuring the specificity of selected siRNA sequences on the cleavage of the target sequence [18]. The HSV DNA polymerase holoenzyme is a complex of the UL30 protein with the UL42 protein. The UL30 protein contains the polymerase and exonuclease activity. The polymerase accessory protein of the HSV-1 DNA polymerase UL42 plays an important role in viral replication [19]. The UL42 gene encodes a multifunctional polypeptide that is vital for virus DNA replication, binding to DNA, stably associating with the virus DNA polymerase (Pol), and acting to increase the length of DNA chains synthesized by Pol [20].

UL42 (65 kd) increases the processivity of the UL30 DNA polymerase activity. Functionally, the structure of UL42 protein linked to the C-terminal fragment of UL30 has been determined. Unusual direct binding of UL42 to DNA is monomer rather than toroid wrapping around the DNA. UL42 is capable to diffuse linear along DNA despite its high affinity binding to DNA [21]. The interaction between UL30 and UL42 is vital for viral DNA replication *in vivo*, and these communication sites have been investigated as a good target for antiviral elements. UL42 also interacts with cdc2 and topoisomerase III [22]. In this study, we mainly focused on UL42 gene of HSV-1 and designed only one small interfering RNA (siRNA) molecule that targets the mRNA of the early gene UL42 as a possible therapeutic strategy in herpes simplex virus (HSV) infections and the activity of HSV-1 *in vitro* evaluated after challenging with siRNA therapy.

Material and Methods

Cell culture

The effect of siRNA on the replication of HSV 1 was measured in cell culture. The HeLa cells were grown in the minimum essential medium supplemented with 5% calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were in the same type of medium without antibiotics and incubated in a 5% CO_2 at 37°C during transfection. Estimation of transfection efficiency to determine the optimal siRNA concentration was done in 6 well micro plate cell cultures. Confluent 3×250 ml (75 cm^2) flasks were trypsinized and cell suspensions were diluted 1:10, and then 400 µl of aliquots were transferred into each well. Before siRNA transfection, the confluent HeLa cells in 250 ml (75 cm^2) cell culture flasks were trypsinized with 5 ml of trypsin. After adding 10 ml of antibiotics, the cells were centrifuged at 2000×g for 5 minutes. The cell pellet was resuspended in 16 ml of antibiotics free medium. 1×10^5 of cell suspensions were transferred into the dishes that contained 2.5 ml of antibiotic free medium. The experiments carried out to assay the plaque forming units in the same dishes were seeded with the cells in 1:14 dilution to achieve 100 % of confluency at the time of infection and the plaque assays were done in 24 well plates. Before the infection, 2 ml of medium were poured to the dishes. Confluent cells in 250 ml (75 cm^2) cell culture flasks were trypsinized with 5 ml of trypsin. Cell proliferation assays were carried out in 96 well plates and HeLa cells grown in 250 ml (75 cm^2) cell culture flasks and trypsinized with 5 ml of trypsin.

HSV-1 virus stock

Initial virus stock was derived with direct swab from a woman with cold sore and after DNA extraction, DNA was used for Real time PCR for detection HSV-1 based on instruction kit (AmpliSense, InterLabService, Russia). After approval of positive HSV-1, the swab inoculated in 75 cm^2 sterile flask HeLa cell line for preparation Virus stock. Cells were incubated in a 5% CO_2 at 37°C for virus attachment and 25 ml minimum essential medium supplemented with 5% calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin added and cells incubated for 48-72 hours. Following days, the harvested infected cells were transferred to 250 ml bottles. Virus pellets were obtained by spinning the bottles at 10,000 rpm for 40 minutes at 4°C. The pellet was resuspended in 2 ml of medium. The stock was frozen and thawed once and was vortexed vigorously. The stocks were stored at -70°C in 200 µl aliquots. One aliquot was titrated to determine the virus titer of the stock.

Determination of virus titer

Using the Reed and Manch method, the highest dilution of virus (TCID50) that causes 50% of cells to show the cytopathogenic effect is the virus titer. The activity of the cells was measured with the MTT method. Viral titer determined on HeLa cells was 5.5 TCID50 which is calculated by Reed and Munch is equal to $10^{-5.5}$ virus/ml.

Design and labeling of siRNA

Gene sequence of UL42 of HSV 1 was achieved from NCBI web site with accession UL42_0819011, UL42_08190212, UL42_08161181, UL42_0814709, UL42_0814709, UL42_08031195, UL42_0801852, UL42_07520316, UL42_07510439, UL42_0750824, UL42_07491379, UL42_0901420, UL42_0852551, UL42_0848290, UL42_08451381, UL42_0842087, UL42_08391215, UL42_0835078, UL42_0831064, UL42_0830614, UL42_08281267, UL42_0827493, UL42_0827350, UL42_0825677, UL42_0824910, UL42_08211181. Ul-42 of the HSV 1 sequences were aligned using the EMBL-EBI web tool to identify the homologues gene sequences (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Several homologous target sites for siRNA were identified for UL42 in HSV 1. Highly effective siRNA sequence was selected and the possible target sites and siRNA sense and antisense obtained by using the Integrated DNA Technologies web tool (http://eu.idtdna.com/Scitools/Applications/RNAi). Target sites for sense and antisense oligonucleotides are shown in Figure 1. The siRNA sense and antisense oligonucletides were purchased from Vbc Biotech (VBC-Biotech Service GmbH) and Cy5 labeled siRNA was used to detect the optimal condition for the siRNA transfection. The SiRNA was labeled by Cy5 to ensure of SiRNA entry into the cells, and it will be detectable after 4 hours of transfection, by using a fluorescent microscope. Sequence of SiRNAs was shown in Table 1.

Transfection of siRNA

Cells plated 18 to 24 hours prior to transfection and the monolayer cell density reaches to the optimal 50% confluency at the time of transfection. The culture medium with serum and antibiotics is freshly added to each well 30-60 minutes before transfection. PepMute™ siRNA Transfection Reagent (Signagene Laboratories) was used as a

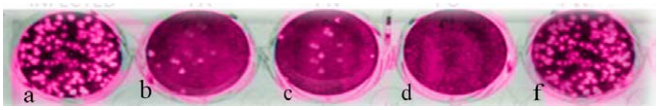

Figure 1: Effect of siRNA transfection on HSV-1 Plaque formation in HeLa cells. (a) HeLa cells were infected with HSV-1, (b) HeLa cells were treated with siRNA1 in 2.5 nM , (c) HeLa cells were treated with SiRNA2 in 2.5 nM (d)HeLa cells were treated with SiRNA1 in 3.5 nM or complete treated, (f) HeLa cells were treated with siN.C(Negative control for siRNA) or untreated. Cultures were infected with HSV-1 and plaques were counted 48 h later.

Name of SiRNA	Sequence	Position
Si N.C	Sense: UUCUCCGAACGUGUCACGUdTdT Anti-sense: ACGUGACACGUUCGGAGAAdTdT	---- -----
SiRNA1	Sense: AAUCUUCUGGGGUUUCAGGAG Anti-sense: CUCCUGAAACCCCAGAAGAUU	940 962
SiRNA2	Sense: GAGCGAGCCAACAGACAGCCAGGAC Antisense: GUCCUGGCUGUCUGUUGGCUCGCUC	1029 1053

Table 1: Design of siRNA targeting the UL42 gene.

novel peptide based siRNA delivery tool which provides more than 95% silencing efficiency at 1 nM siRNA in variety of mammalian cells. To optimize the best concentration of siRNA silencing, different concentration 0.5-50 nM siRNA were examined and the final siRNA concentration was set more than 30 nM (Figure 7). For SiRNA transfection, 6 well microplate with more than 50% confluency HeLa cells were used. SiRNA transfection was performed in two ways, before and after infecting cells with herpes virus.

The media removed before cells infection, and the cells washed two times with PBS buffer (pH=7.4), one milliliter media, 100 µl transfection buffer, 3.6 µl PepMute™ reagent and 0.5-50 nM siRNA (final concentration) were poured. To discover the maximum gene silencing, dilute siRNA and PepMute™ reagent with PepMute™ Transfection Buffer (1x) were tested.

Re-constituting siRNA stock solution had to be at 10 µM, so 0.5 or 5.0 µl siRNA stock solution added per well of 6-well plate to make final 5.0 and 50 nM siRNA respectively and mixed 3.6 µl PepMute™ reagent by pipetting up and down. Incubation for 15 minutes at RT was done to let transfection complex form and after that transfection mixture added to the cells drop wise. Gently shacked the plate back and forth and returned the plate to CO_2 incubator for 24 hours. For evaluation of silencing after infection, infected HeLa cells were inoculated with herpes viruses (MOI=3) and after 48 hours supernatant media removed and cells washed two times with PBS (pH=7.4) and the above steps are repeated. Gene silencing is usually measured 24-72 hours post transfection.

Plaque assays

To evaluate SiRNA gene silencing efficacy, HeLa cells were grown in 24-well plates to 80–90% confluence and then transfected with specific or control siRNA using PepMute™ siRNA Transfection Reagent (Signagene Laboratories). After 1 h, the cells were infected with 25 PFU of HSV-1. Cells were overlaid with 1 ml of a 1:1 mixture of NaCMC (50%) and DMEM (50%) without serum to allow only cell-to-cell spread of virus. At 48-72 h post-infection, plates were fixed with 10% paraformaldehyde for 2 min and then stained with 1% crystal violet for 20 min to count the number of plaques per well and to photograph the monolayer. Overlapping plaques and plaques at the edge of the well were counted as a single plaque.

mRNA extraction

To evaluate SiRNA gene silencing efficacy, mRNA was extracted from cells with or without herpes infection and SiRNA treatment. Total RNA was extracted by RNA easy extraction kit (Qiagen, Germany) and used to study the mRNA UL42 gene expression with or without gene silencing in different concentrations of transfected SiRNA.

DNA extraction

To evaluate replication of HSV-1 after silencing of UL-42 gene, remove of supernatant and DNA was extracted from infected cells before and after silencing UL-42 gene. DNA was extracted with DNA genomic extraction kit with following instruction manual (Bioneer, Korea).

rReal time PCR

Reverse transcription real time PCR (rReal time PCR) was carried out by using the first strand cDNA synthesis kit by Revert Aid cDNA synthesis kit (Thermo scientific, USA). Briefly, RNA samples were heated to 65°C for 10 minutes and then chilled on ice. The uniform suspension of bulk first-strand cDNA reaction mix was added according to the manufacturer's protocol. One µl of DTT solution, and 1 µl of pd $(N)_6$ primer (0.2 µg) were then added to the heat-denatured RNA. Samples were mixed properly by pipetting up and down several times and then incubated for 1 hour at 42°C. For Real time PCR, the QuantiTect Probe PCR Kit (Qiagen, Germany) used base on instruction kit. Real time PCR primers and probes were design for UL42 herpes virus type 1 after alignment of this region between all of them in EBML-EBI and as an internal control, eukaryotic 18s rRNA were purchased from Metabion company (metabion international AG ,Germany) (Table 2).

Results

Effect of siRNA transfection on HSV-1 infection

HSV-1 produces noticeable plaques on HeLa cells without serum at 48 h after infection which make central clearing as the virus spreads outward. HeLa cells were transfected with UL42-specific siRNAs (siRNA1, siRNA2) or negative control siN.C and then infected with 25 PFU of HSV-1. Comparing with virus control, transfection with siN.C had no effect upon viral plaque formation following infection ($P>0.05$) (Figure 1). On the other hand, HSV-1 infection of HeLa cells transfecting with UL42-specific siRNAs produced fewer plaques ($P<0.01$). The inhibition rates of siRNA1 and siRNA2 on HSV-1 plaque formation were 99.5% and 89.3%, respectively (Figure 2). Also the CPE

Name	Sequence
Ul42 -Forward	CAGCTCACCAAGGTCCTTAACG
UL42-Reverse	AGCAAATGTGACGCAGGTACTC
UL42-Probe	FAM-CCGATAGTGCCACGCCCACCACG-TAMARA
18sRNA -Forward	GGGAGGTAGTGACGAAAAATAA
18sRNA-Reverse	TTGCCCTCCAATGGATCCT
18sRNA-Probe	JOE- CGAGGCCCTGTAATTGGAATGAGTCCACT-TAMARA

Table 2: UL42 sequence-specific primers and probe.

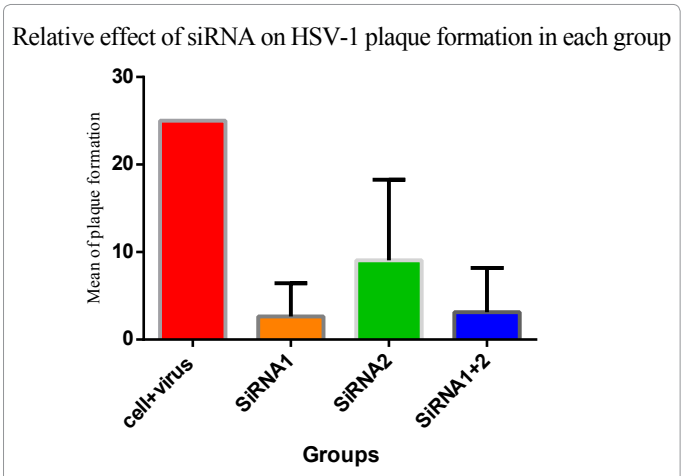

Figure 2: Effect of siRNA from 0 .5 to 30 nM on plaque formation of HSV-1 in HeLa cell line. Data represent the mean ± SD of three plates.

(Cytopathtic effect) results showed that no morphological changes in the cells were detected in titer above 3 nmol and after 12 days with one cell's passage, the cells showed no CPE (Figure 3).

Effect of siRNA on the DNA replication of HSV-1

To study of the decrease in the number of HSV-1 was due to siRNA silencing expression of the UL42 gene, a real-time PCR analysis was used. The results indicated that UL42-specific siRNAs inhibited the DNA replication of HSV-1 considerably. Compared with virus control, siRNA1 and siRNA2 reduced DNA replication HSV-1 to levels that were 98.5% ($P<0.01$) and 93.2% ($P<0.01$) lower, respectively, than those illustrated in cells treated with negative control siN.C (Figure 4) and the activity of the cells was measured with the MTT method (Figure 5).

Effect of siRNA on the expression of the UL42 gene

To detect mRNA UL42, the Real Time PCR was done on extracted RNA samples and determined that from 3 nmol/ul SiRNA or higher titers, the UL42 gene blocked with sharp reduction in the titer. These experiments have the same results after six repetitions and apparently became clear that the target gene UL42 decomposed from 3 nmol or higher. Also the examination based on CPE (Cytopathtic effect) showed that no morphological changes in the cells were detected in titer above 3 nmol and after 12 days with one cell's passage, the cells showed no CPE. To decide whether the decrease in the number of HSV-1 plaques was due to siRNA silencing expression of the UL42 gene, a real-time PCR analysis was performed. The results indicated that UL42-specific siRNAs blocked the expression of the UL42 gene significantly. Compared with virus control, siRNA1 and siRNA2 reduced the expression of UL42 gene to levels which were 98.3% ($P<0.01$) and 96.5% ($P<0.01$) lower, respectively, than those seen in cells treated with negative control siN.C (Figure 6).

Figure 3: Effect of siRNA UL42 transfection in HeLa cell culture. **A**: Normal HeLa cell line(uninfected or transfected with siN.C), **B**: Treated HeLa cell line with siRNA1 and no effect was seen after 48 hour , **C**: Flourescent microscopic illustration for siRNA UL42 transfected HeLa cell line, **D**:infected HeLa cell line with HSV-1 after 24 hours, **E**: infected HeLa cell line with HSV-1 after 48 hours, **F**: infected HeLa cell line with HSV-1 after 72 hours.

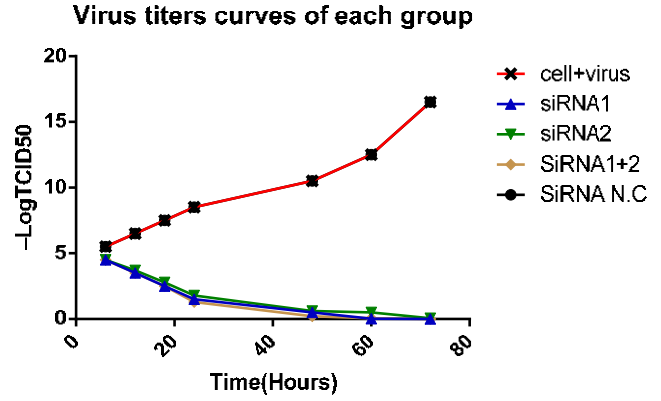

Figure 4: Effect of siRNA UL42 transfection on HSV-1 titer in each group in 30 nM concentration of SiRNAs, during 72 hours.

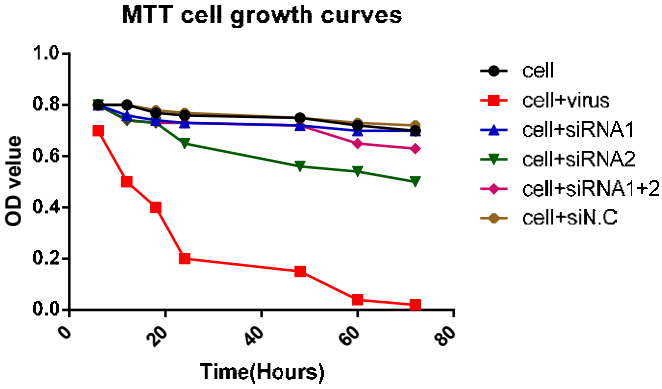

Figure 5: Effect of siRNA transfection UL42 on HeLa cell growth in each group in 30 nM concentration of SiRNAs, during 72 hours(MTT assay).

Discussion

Despite the effect of RNAi in probing gene function and in the development of novel therapeutics and antivirals, there are different studies exploring the potential for RNAi approaches to HSV-1 but variable finding have been published [23]. These studies mostly have focused on the α and γ genes of HSV-1 [24]. UL42 as a β gene, is a well-recognized object for antiviral agents has a role in controlling DNA replication and regulating other enzymes in the DNA synthesis pathway by means of control of the nucleotide pool [21]. In our study, we showed that HSV-1 was liable to the RNAi pathway. The siRNA-1 and siRNA-2 showed in HSV-1 UL42 gene silencing and a reduction in viral plaque formation. Since assays with infectious viruses are time-consuming and labor-intensive and have to be executed under elevated safety conditions, a simple method for the pre selection of active siRNA species is desirable. In this study, we designed two 21 and

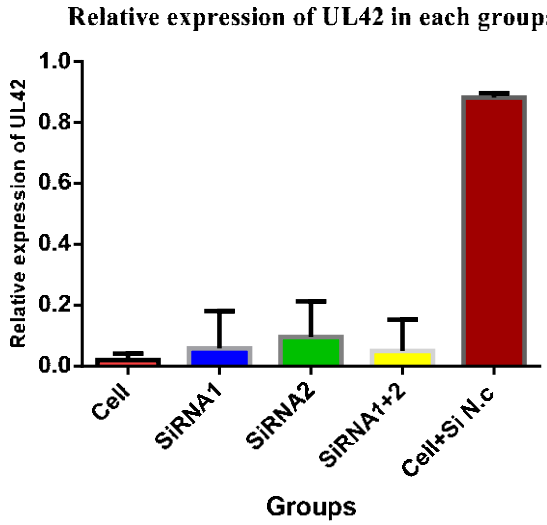

Figure 6: Effect of siRNA UL42 transfection on expression UL42 gene in HeLa cell line in 30 nM concentration of SiRNAs after 72 hours.

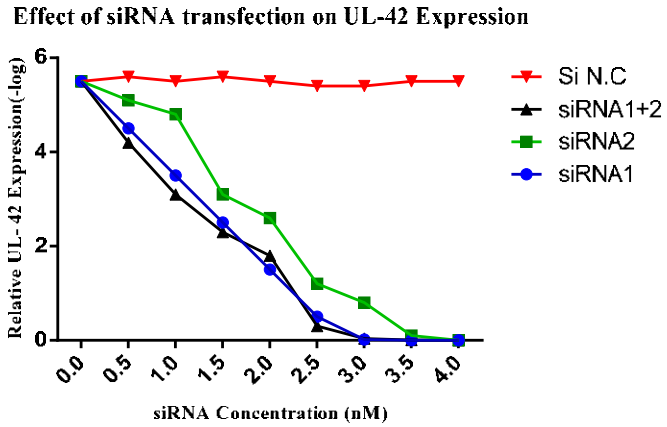

Figure 7: Effect of different concentration of siRNAs on expression UL42 gene in HeLa cell line after 72 hours.

27nt siRNAs to different regions on the HSV-1 UL42 mRNA, termed siRNA1, siRNA2, which were synthesized chemically. To facilitate screening of active siRNA, we constructed the reporter dye (Cy5) for SiRNA2 , therefore provides a simple and fair model for estimation the efficacy of gene silencing by analysis at the protein level by fluorescent microscopy. Although these two siRNAs had sequences homology to the HSV-1 UL42 mRNA, they differed in their efficacy for founding DNA catalytic Polymerase protein silencing. In several experiments, siRNA1 consistently restrainedUL-42 expression more efficiently than the others have done, showing that the difference in efficacy cannot be attributed solely to differences in transfection efficiency [22,25]. Diversity in the silencing activity of several siRNAs targeting the same mRNA has also been reported previously [18,19]. The reason for the differences in RNAi efficacy are not well understood, but has been supposed to be due to differences in the thermodynamic properties of the siRNAs as well as the secondary and tertiary RNA structure of the target site, which disables recognition by the siRNA and we think materials for transfection are important for transfection and siRNA entry to the cells. There was significant difference in HeLa cell line infected with HSV-1 when treated and untreated with siRNA, by main changes in the relative amounts of HSV-1 DNA synthesis, expression of UL42 gene and plaque formation. During the experiment, we designed two pairs of UL42-specific siRNA according to the same principles show different efficiency. The siRNA-1 is presenting better results than the siRNA-2. The possible reasons were: there are more complex mechanisms of RNAi; some protein-binding sites exist in target sequences, or some siRNA sequences form a stem-loop structure easily and prevent the combination of siRNA and mRNA; some specific sequences of siRNA are more unstable and vulnerable to degradation of RNA enzymes, which needs further research. The siRNA designed in accordance with our principles can achieve good transfection efficiency, using suitable transfection reagents. Our experiment introduced an effective siRNA to inhibit UL42 gene expression specifically, which confirmed that UL42 specific siRNA can inhibit viral replication, while is not harmful for host cells. This result provided a preliminary theory for treating HSV-1 by RNAi. Our experiment also verified that UL42 plays an important role in viral replication and lays the foundation for UL42 to be a new target. Our results showed that siRNA1 could efficiently inhibit HSV-1 replication in serum-starved HeLa. This is harmonious with their finding. The evolution of siRNA-mediated antiviral therapy has the greatest benefit among populations infected with virus strains resistant to routine antivirals and in cases of severe or recrudescent disease. The *in vitro* system depicted will be enable us to more easily follow up further studies of SiRNA transfection and provides a foundation for the development of *in vivo* applications of siRNA. *In vivo* activity of RNAi has been demonstrated for both viral and host genes of mice. These data suggest that siRNA can be developed as a drug.

Conclusion

In our study by designing new siRNA1 and siRNA2 against UL42 of HSV-1, the viral infection in HeLa cells stopped and the inhibition rates of on HSV-1 plaque formation were 99.5% and 89.3%, shown respectively. The sharp inhibition for DNA replication of HSV-1 considerably reported and expression of the UL42 gene significantly blocked by UL42-specific siRNAs.

Acknowledgments

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