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Inhibition of canine parvovirus replication in cultured cells by small interfering RNAs expressed from plasmid vectors

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ABSTRACT

Small interfering RNAs (siRNAs) target complementary mRNA for specific degradation, a mechanism many viruses are susceptible too. Thus, siRNA degradation of target RNAs can be exploited as novel therapeutics. In this report, we show that the vector-based siRNAs (psiSTRIKEs) expressed by a human U6 promoter could efficiently inhibit CPV replication in cell culture. A series of PsiSTRIKE vectors expressing siRNA were constructed that target structural protein genes or nonstructural protein genes of CPV genome. These plasmids were transfected into FK81 cells via lipofectin and the stable transfection clones were selected. The immunostaining, plaque assay, and cellz proliferation assay of the cells infected by CPV were performed. The results show that siRNAs against nonstructural protein genes effectively inhibited CPV replication. The inhibition efficiencies detected by immunostaining assay of psiSTRIKE/vp1510, psiSTRIKE/NS160, and psiSTRIKE/NS1939 were 66%, 76% and 78%, respectively at 48 h, and 69%, 46% and 67%, respectively at 96 h. Plaque assay showed that, comprising to the control, the psiSTRIKE/NS160 reduced the virion production by 100-fold, and psiSTRIKE/NS1939 or psiSTRIKE/VP1510 reduced the virion production 13-fold. When compared to control, the viability of cells transfected psiSTRIKE/NS160 increased 78% and 124%, respectively at 72 and 120 h. Our study may provide a potential therapy against CPV infection.

1. Introduction

RNA interference (RNAi), a post-transcriptional regulation mechanism, is mediated by small interfering RNAs (siRNAs) of 21–23 nucleotides that are incorporated into a multi-protein complex known as the RNA-induced silencing complex (RISC), leading to sequence-specific degradation of target mRNA that is recognized by the antisense strand of the siRNA (Fire et al., 1998; Hannon, 2002). RNAi is a valuable tool to inhibit the expression of a target gene in a sequence-specific manner, and may be used for functional genomics, target validation, and therapeutic purposes (Elbashir et al., 2001; Kamp et al., 2002). SiRNA is believed to have evolved as a host defense mechanism directed at transposable elements and infecting viruses (Waterhouse et al., 2001; Carmichael, 2002). Recently, RNAi has been applied to inhibit the replication of several pathogenic human viruses (Ge et al., 2003, 2004; Randall et al., 2003).

Canine parvovirus (CPV-2) emerged in 1978 as a pathogen causing acute hemorrhagic enteric diseases in young dogs (Parrish et al., 1985). Since then, the CPV infection with high rate of mortality in puppies has become established as an important disease of dogs. The virus has a 5 kb linear negative-sense single stranded DNA (ssDNA) genome with hairpin structures at the both ends (Reed et al., 1988). The viral genome has two major open reading frames, one encoding two nonstructural proteins (NS1 and NS2) and the other encoding the capsid proteins VP1 and VP2 (Parrish et al., 1991).

The present project was undertaken to study the effect of siRNAs on CPV replication in FK81 cells; the siRNAs were expressed from plasmid vectors directed against either the structural protein genes or the nonstructural protein genes of the CPV-2. We demonstrated that the vector-derived siRNAs targeted to the NS1 genes effectively inhibited CPV replication *in vitro*.

2. Materials and methods

2.1. Virus, plasmids, and cells

Cells of feline kidney cell line FK81 were purchased from "The Clinical Central of the Agricultural Ministry of China", and were

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Table 1
Oligonucleotides used to generate siRNA expression vectors.

oligonucleotides	Oligonucleotide sequences
NS160 A	5′-ACCGAAGAGCTAACATCTTTAACTTCCTGTCATTAAAGATGTTAGCTCTTCTTTTTC-3′
B	5′-TGCAGAAAAAGAAGAGCTAACATCTTTAATGACAGGAAGTTAAAGATGTTAGCTCTT-3′
NS1939 A	5′-ACCGACCTGAGAGCCATCTTTACTTCCTGTCATAAAGATGGCTCTCAGGTCTTTTTC-3′
B	5′-TGCAGAAAAAGACCTGAGAGCCATCTTTATGACAGGAAGTAAAGATGGCTCTCAGGT-3′
VP61 A	5′-ACCGGGAAAGATTTGATAACTTACTTCCTGTCATAAGTTATCAAATCTTTCCCTTTTTC-3′
B	5′-TGCAGAAAAAGGGAAAGATTTGATAACTTATGACAGGAAGTAAGTTATCAAATCTTTCC-3′
VP1510 A	5′-ACCGGTTATAGTGCACCATATTACTTCCTGTCATAATATGGTGCACTATAACCTTTTTC-3′
B	5′-TGCAGAAAAAGGTTATAGTGCACCATATTATGACAGGAAGTAATATGGTGCACTATAAC-3′
Random A	5′-ACCGCAGTTTCTGCTTTTCAAACTTCCTGTCATTTGAAAAGCAGAAACTGCTTTTTC-3′
Random B	5′-TGCAGAAAAAGCAGTTTCTGCTTTTCAAATGACAGGAAGTTTGAAAAGCAGAAACTG-3′

maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum in 5% CO_2 at 37 °C. The B2004 strain of canine parvovirus was from our laboratory stock, which was isolated from the stool of a sick dog in Beijing (He et al., 2009). Its genomic sequence (accession No. EF011664) indicated that B2004 is a member of the widely distributed CPV-2a subclade (He et al., 2009). The virus was propagated in feline kidney cell line (FK81) and harvested at 5 days post infection. The debris of infected cells was pelleted by centrifugation and the supernatant containing the virions was stored in a −70 °C freezer until use. The CPV suspensions were titrated using plaque assay (LaBarre and Lowy, 2001). CPV monoclonal antibody mAbc2 (against VP2 of virus capsid) was kindly provided by Prof. Sun (The Clinic Central of the Agricultural Ministry, China). Vector siSTRIKE U6 was purchased from Promega.

2.2. Selection of target sequences for hairpin siRNAs and the design of hairpin siRNAs siSTRIKEs

Small interfering RNAs against the genes of nonstructural proteins and capsid proteins of CPV were designed to target the most conserved regions of these genes using the Promega's siRNA design tool (www.promega.com/techserv/tools/siRNA). The sequences coding for the hairpin siRNAs used in these systems are under 60 bps in length (Table 1). Oligonucleotides annealed to generated double stranded DNA as the genes of designed siRNAs targeted at the indicated CPV genes in the CPV genome. A hairpin RNA with a nonspecific target sequence was used as a negative control. The plasmids psiSTRIKEs with genes of designed siRNAs were constructed using a kit from Promega.

2.3. Stable transfection of the psiSTRIKE vectors into cultured FK81 cells

DNA (2.5 μg in six-well plate) of a series of psiSTRIKEs carrying designed siRNA genes was transfected into FK81 cells using Lipofectin (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction. At 24 h after transfection, stably transfected cells were selected by adding 4 μg/ml puromycin, and transfection was confirmed by PCR with psiSTRIKE vector specific primers (U7900: 5′-GGT TTT CCC AGT CAC GAC GTT-3′, L7900: 5′-CGG CTC GTA TGT TGT GTG GAA-3′).

2.4. Infection assays

Two assays were used to detect CPV infection: immunostaining and plaque formation.

For immunostaining, cells were seeded on 24-well plate, incubated for 1 h at 37 °C, then were inoculated with CPV (1.84×10^3 plaques per well) and were incubated for 12 h after inoculation. The infected cells were continually incubated at 37 °C for 48 h in fresh growth medium. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS with 1% bovine serum albumin. Infected cells were detected with mAbc2 as primary antibody and goat anti-mice IgG-FITC as secondary antibody. The infection rate was determined by microscopy as the percentage of green-stained cells in total 200 cells (the nuclei were stained red by propidium iodide) counted in each sight field and averaged over five sight fields.

CPV infection was also analyzed by plaque assay. In the assay, FK81 cells expressing specific siRNAs or control were incubated with CPV(1.84×10^3 plaques per well) for 2 h; after replacing the medium, the CPV suspensions from both the liquid medium and cells were harvested 96 h post infection, then the plaque forming units were counted to estimate the virus titer.

2.5. MTT cell viability assay

To investigate cellular toxicity of CPV to stably transfected cells after inoculation of the virus, 2×10^4 cells were plated into each well of 96-well plates. After 12 h incubation, CPV particles(1.84×10^3 plaques per well) were added into the wells and the plate was sealed and kept at 37 °C in an atmosphere of 5% CO_2 for 72 h. About 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Inc., Japan) was added to all wells to stain the viable cells. The plate was wrapped in aluminum foil and was incubated for 1–4 h at 37 °C. The number of viable cells that stained by the dye was determined by measuring absorbance at a wavelength of 450 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader.

Cell viability was obtained using the following equation:

$$\text{Percentage of viable cells} = (\text{As} - \text{Ab}) \div (\text{Ac} - \text{Ab}) \times 100\%$$

As: absorbance of cells incubated with virus; Ab: absorbance of uninfected FK81 cells; Ac: absorbance of medium.

2.6. Statistical analysis

Data were derived from independent experiments rather than replicates within a single experiment. Data were analyzed by 1-way ANOVA using the General Linear Model procedure of SAS Statistical Software (version 9;SAS Institute). Data are presented as means ± SD. Duncan's multiple range test was performed to identify differences among groups. $P < 0.05$ was considered significant.

3. Result

3.1. Construction of psiSTRIKE/VP61, psiSTRIKE/VP1510, psiSTRIKE/NS160, psiSTRIKE/NS1939, psiSTRIKE/Random sequence, and selection of stably transfected cell lines

A series of vectors carrying genes of designed hairpin siRNAs were constructed based on pskSTRIKE from Promega according to

Table 2
CPV infection rate of stably transfected FK81 cells with different psiSTRIKEs.

Expression vector	psiSTRIKE/VP61	psiSTRIKE/NS160	psiSTRIKE/VP1510	psiSTRIKE/NS1939	psiSTRIKE/Random seq	No plasmid
48 h	19.8 ± 3.6[a,b]	7.8 ± 0.6[b]	10.9 ± 2.6[b]	6.96 ± 1.3[b]	32.0 ± 3.8	33.8 ± 2.1
96 h	60.0 ± 4.1[a,b]	45.0 ± 5.8[b]	26.3 ± 4.8[b]	27.5 ± 2.9[b]	83.8 ± 2.5	85.0 ± 5.8

[a] Data are presented as mean of 4 determinations ± standard deviation.
[b] Significantly different from control values ($P < 0.05$).

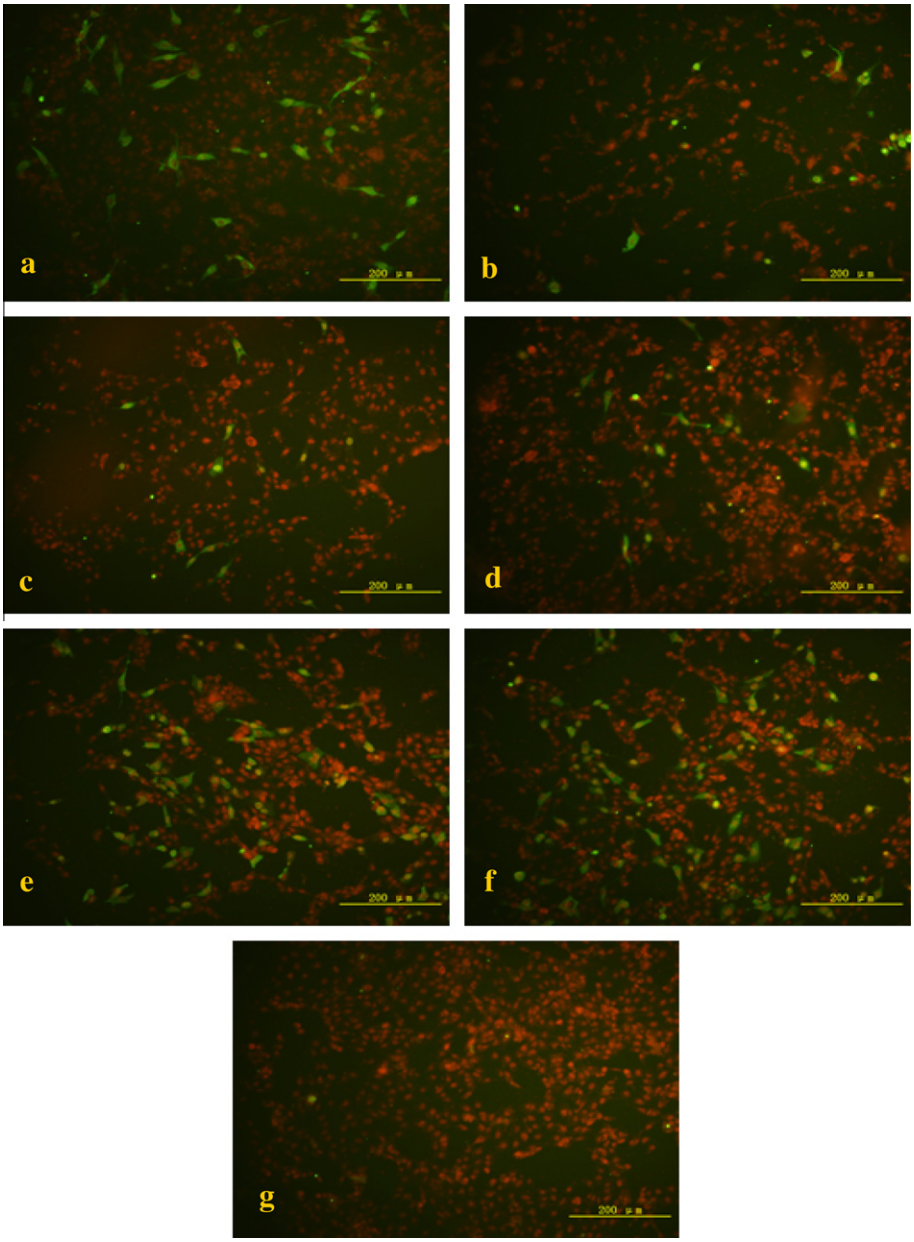

Fig. 1. Microscopic fluorescence images of stably transfected cells with different psiSTRIKEs (images a–g) incubated with CPV for 48 h; after incubation the cells were fixed, the nuclei were stained with PI (red), and infected cells were detected by incubation sequencially with CPV specific Mab and FITC conjugated anti-mouse IgG (green). The plasmids used to transfect cells are (a) psiSTRIKE/VP61, (b) psiSTRIKE/NS160, (c) psiSTRIKE/VP1510, (d) piSTRIKE/NS1939, (e) psiSTRIKE/ Random, (f) FK81 cell incubated with CPV, (g) control (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the manufacturer's instruction. The authenticity of the recombinant plasmids psiSTRIKE/vp61, psiSTRIKE/vp1510, psiSTRIKE/NS160, psiSTRIKE/NS1939, psiSTRIKE/Random sequence were confirmed by restriction enzyme analysis and sequencing. The stably transfected FK81 cell lines with psiSTRIKEs were selected by adding 4 μg/ml puromycin.

The presence of the genes for designed siRNAs in the stably transfected cell lines were confirmed by PCR resulting in 500 bp DNA fragment that is in agreement with the insertion of siRNA genes. Those results show that vectors were constructed successfully and the stably transfected cell lines were harboring psiSTRIKEs.

Table 3
CPV titers in cultured cells expressing siRNA from plasmid vectors.

Cells	psiSTRIKE/VP61	psiSTRIKE/NS160	psiSTRIKE/NS1939	psiSTRIKE/VP1510	psiSTRIKE/Random	No plasmid
Plaque number	$1.6 \times 10^3 \pm 337$[a,b]	$1.6 \times 10^2 \pm 16$[b]	$1.2 \times 10^3 \pm 365$[b]	$1.2 \times 10^3 \pm 216$[b]	$9.5 \times 10^3 \pm 1732$	$1.6 \times 10^4 \pm 4899$

[a] Data are presented as mean of 4 determinations ± standard deviation.
[b] Significantly different from control values ($P < 0.05$).

Table 4
Viability of CPV infected cells stably transfected with different psiSTRIKEs.

	psiSTRIKE/VP61	psiSTRIKE/NS160	psiSTRIKE/NS1939	psiSTRIKE/VP1510	psiSTRIKE/Random	No plasmid
72 h	70.1 ± 2.4[a,b]	92.9 ± 4.4[b]	67.7 ± 2.4[b]	69.5 ± 4.9[b]	45.5 ± 5.6	52.3 ± 4.8
96 h	65.2 ± 2.8 [a,b]	73.8 ± 3.7[b]	68.7 ± 3.3[b]	80.6 ± 4.2[b]	48.8 ± 3.7	50.1 ± 2.7
120 h	46.6 ± 3.1 [a,b]	88.6 ± 5.8[b]	57.9 ± 2.9[b]	55.0 ± 3.1[b]	39.8 ± 3.9	39.6 ± 3.0

[a] Data are presented as mean of 4 determinations ± standard deviation.
[b] Significantly different from control values ($P < 0.05$).

3.2. Target specific siRNAs expressed in stably transfected cell lines decreased CPV infection

The CPV infection rate for four cell lines expressing siRNA specifically targeted at CPV genome were dramatically lower ($P < 0.05$) than negative control or untransfected FK81 cell line 48 and 96 h after viral inoculation (Table 2). The inhibition efficiencies of psiSTRIKE/vp1510, psiSTRIKE/NS160, and psiSTRIKE/NS1939 were 65%, 75% and 78%, respectively at 48 h, and 68%, 46 % and 67%, respectively at 96 h. The microscopic images of immuno-stained cells presented a further confirmation (Fig. 1) for the inhibitory effect of siRNAs on CPV infection. Those data demonstrated that the siRNA expressed by psiSTRIKEs could inhibit the CPV infection *in vitro*.

3.3. Target specific siRNAs expressed in stably transfected cell lines suppressed replication of CPV

The CPV suspensions of the culture medium and cells expressing the target specific siRNA were harvested 96 h post infection, and then the numbers of the virions in the suspension were determined using plaque assay (Table 3). The result demonstrated that four target specific siRNAs generated with expression vectors psiSTRIKEs significantly decreased ($P < 0.05$) the number of virions in the suspension. Comprising to the control, the psiSTRIKE/NS160 reduced the virion production by 100-fold, and psiSTRIKE/NS1939 or psiSTRIKE/VP1510 reduced the virion production 13-fold. Thus, the plaque assay confirmed the suppression of viral replication by vector expressed siRNAs.

3.4. Target specific siRNAs expressed in stably transfected cell lines increased cell viability upon CPV infection

Cellular siRNAs not only depressed CPV infection and replication, but also promoted viability of the host cells when infected. After 72–120 h incubation with virus, the viabilities of transfected cells were much higher ($P < 0.05$) than control (Table 4) as assayed by staining the viable cell with a dye. When compared to control, the viability of cells transfected psiSTRIKE/NS160 increased 78% and 124%, respectively at 72 and 120 h. The results further confirmed the suppression of virus replication by siRNA expressed from plasmids.

4. Discussion

RNA interference (RNAi) of 21–23 nucleotides represents a new promising approach to develop effective antiviral strategy, which can specifically differentiate target sequence from none target up to a single nucleotide, and can induce up to 90% suppression of specific mRNAs both *in vitro* and i*n vivo* (Elbashir et al., 2001; Brummelkamp et al., 2002; McCaffrey et al., 2002). The high specificity and efficiency of RNAi encouraged the development of RNAi-based therapeutic models for possible use against infections of viruses, i.e. influenza virus (Ge et al., 2003), HCV (Randall et al., 2003), and respiratory viruses (Bitko et al., 2005). Parallel study of treating CPV infection using siRNA has not been reported both *in vitro* and *in vivo*.

In this study, we exploited siRNAs for inhibition of viral infection, which were expressed from plasmid vectors directed to the capsid proteins genes and nonstructural proteins genes of the CPV-2 in CPV infected FK81 cells. The result showed that the vector-derived siRNAs targeted to CPV genome effectively inhibited CPV replication *in vitro*, and the siRNA targeting the sequence shared by both NS1 gene and NS2 gene showed better suppression efficiency. Two important assays in virology were employed to demonstrate the efficacy of siRNA, i.e. the plaque assay measuring the propagation of infecting CPV, and immunostaining that visualized intracellular CPV proteins.

Target selection is very important for siRNA efficacy. CPV has a 5 kb linear negative-sense single stranded DNA (ssDNA) genome encoding two nonstructural proteins (NS1 and NS2) and two capsid proteins VP1 and VP2. NS1 is essential for viral replication and lytic infection, and has multifunctional properties, such as ATPase activity and DNA helicase activity (Daeffler et al., 2003; Christensen and Tattersall, 2002; Corbau et al., 2000; Christensen et al., 1997; Vanacker et al., 1996). NS2 is generated by alternative splicing and is involved in translation of viral mRNA and assembly of capsid proteins through interaction with the nuclear export factor (Naeger et al., 1990; Legrand et al., 1993). NS proteins show high sequence homology among parvoviruses and are considered to be important for cytotoxicity in permissive cells and parvoviral pathogenicity (Nuesch et al., 2003). Our result demonstrated that the siRNA (psiSTRIKE/NS160) targeting CPV RNA sequence shared by genes of nonstructural protein NS1 and NS2 had a higher efficiency; therefore, the shared region in CPV RNA by NS1 and NS2 could provide efficient therapeutic targets for siRNAs against CPV infection.

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