

株化細胞におけるSiniperca chuatsi rhabdovirusのRNA干渉法による複製阻害

誌名	魚病研究
ISSN	0388788X
著者名	Zhou,G.Z. Zhu,R. Gui,J.F. Zhang,Q.Y.
発行元	日本魚病学会
巻/号	47巻1号
掲載ページ	p. 30-32
発行年月	2012年3月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Inhibition of *Siniperca chuatsi* Rhabdovirus by RNA Interference in a Fish Cell Line

Guang-Zhou Zhou^{1,2,*}, Rong Zhu¹,
Jian-Fang Gui¹ and Qi-Ya Zhang^{1,*}

¹State Key Laboratory of Freshwater Ecology and
Biotechnology, Institute of Hydrobiology,
Chinese Academy of Sciences,
Wuhan 430072, China

²College of Bioengineering, Henan University of
Technology, Zhengzhou 450001, China

(Received September 17, 2011)

ABSTRACT—We evaluated the antiviral activity of several small interfering RNAs (siRNAs) corresponding to the *Siniperca chuatsi* rhabdovirus (SCRV) nucleoprotein (N) gene against the SCR in a fish cell line, EPC. Among the siRNAs we examined, si983 was most effective at reducing virus titer compared to the mock group. Western blot analysis suggested that si983 inhibited N gene expression and virus replication in a dose-dependent manner. These results indicate that N gene 983 sites are potential targets for antiviral therapy. Furthermore, RNAi targeting of the N gene may represent a viable method of treating against SCR infection.

Key words: Mandarin fish, rhabdovirus, nucleoprotein, RNA interference, SCR

Fish rhabdoviruses are some of the most important virus pathogens in aquaculture industry. *Siniperca chuatsi* rhabdovirus (SCRV) was isolated from diseased mandarin fish which is an economically important fish widely cultured in China¹. Genomic sequencing analysis suggested that it belongs to one strain of rhabdoviruses which encoded the five structural proteins: the nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, and RNA-dependent RNA polymerase protein L².

Currently, the effective prevention of rhabdovirus infection in aquaculture remains to be developed. It has been suggested that the preferred method for controlling the virus is to interfere with replication of the viral genome and the expression of viral genes. RNAi is a phenomenon induced by double-stranded RNA (dsRNA) in which gene expression is inhibited through

specific degradation of mRNA^{3,4}. For instance, inhibition of virus replication and gene expression by directly introducing small interfering RNAs (siRNAs) into the cells has been reported for a number of negative-strand RNA viruses, including respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), human parainfluenza virus (HPIV) and so on⁵. Recently, much research has further confirmed that engineered virus-encoded pre-microRNA could also induce antiviral response and lead to virus resistance in cell cultures^{6,7}. The results of these studies suggested the possibility of using RNAi as an antiviral tool. This study aims to determine the efficacy of the siRNAs targeting the SCR N gene against the virus infection.

Materials and Methods

The SCR was propagated in EPC cells which were grown at 25°C in TC199 medium supplemented with 10% fetal bovine serum (FBS). The virus was purified directly from the supernatant fluid as described previously⁸. Four siRNAs targeting the regions starting at positions 447, 533, 707 and 983 of SCR N gene from the start codon AUG were designed for transfection, respectively. One mismatched siRNA molecule served as negative control for nonspecific inhibition (shown in Table 1). siRNAs were chemically synthesized by Shanghai Gene Pharma Co., Ltd, China. N-GFP fusion plasmids was generated by fusing PCR fragment of SCR N gene with in-frame restriction sites into pEGFP-N3 vector (Clontech) using standard cloning procedures with XhoI and KpnI. The relative sense and anti-sense primers were shown in the Table 1.

The day before transfection, EPC cells were trypsinized, diluted with the fresh medium and seeded into 24-well plates at 4×10^5 cells per well. Co-transfection with the N-GFP plasmids or siRNAs was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 1 µg DNA were complexed with 2 µL Lipofectamine™ 2000 per well in 100 µL TC199 serum-free medium. After 20 min incubation at room temperature, the complexes were added to cell-culture wells containing 500 µL

Table 1. Primers used for all the experiments and sequences of N template cDNA for siRNA. Con-siRNA denotes the mismatched siRNA molecule served as negative control for nonspecific inhibition.

Primer name	Sequence (5'–3')
SCRV N F	F primer: CTCGAGATGGAACACCAAATCATC
(aa 1–429)	R primer: GGTACCTCTCAAAGCTTGGTGCTT
si447 F	TATCACAACGATGCGTATAA
si533 F	TCAGAGTACTAGCTGGCAATA
si707 F	TCGCATCATCTACTGGATTAA
si983 F	ACCTCAAGCTCAACGCTGAAA
Con-siRNA F	TCTCCGAACGTGTACCGT

* Corresponding author

E-mail: zhouguangzhou@hotmail.com

serum-free medium. Plates were incubated for 5 h at 25°C and then the well supernatant was replaced with 1 mL fresh medium with 5% FBS. Transfected cells were examined under a bright-field illumination and fluorescence microscope (Leica) after 24 h. Infection of SCR V (300 TCID₅₀/well) was performed 6 h after siRNA's transfection at a final concentration of 100 nM. Controls were done by using scrambled siRNA and non-transfected cells. SCR V titers were determined using the above cell culture supernatants with standard procedure¹⁾.

And then, the most effective siRNA was further used to investigate the effect of inhibiting virus production. Indirect immunofluorescence assays (IFA) were performed with infected cells in six-well chamber slides at 48 h post-infection (h.p.i) with previous procedures⁹⁾. Moreover, Western blotting analysis was also carried out to test whether RNAi could impede the packaging and production of infectious viruses on the protein suppression effect level accord to previous method²⁾.

Results and Discussion

Analysis of CPE in EPC cells were carried out at four time intervals after siRNAs transfection and SCR V infection from 24 h.p.i to 72 h.p.i. The results showed that si983-transfected cells didn't show apparent CPE until 72 h.p.i, which was similar to the control cells at 24 h.p.i, while other three siRNAs almost had no (si707 and si533) or weak (si447) inhibition effects of CPE. It suggested that si983 could apparently down-modulate the propagation of SCR V and postpone the emergence of CPE. Fig. 1 showed that overtly less N protein-positive cells were seen in the monolayers pretreated with si983 than in the control positive monolayers under the identical capturing image conditions (exposure time and aperture). In addition, no N protein-positive cells were seen in the blank EPC monolayer with the same anti-N protein antiserum (Fig. 1).

Western blotting showed that purified virus, mismatched siRNA control and infected cells lysates were all shown to express the expected 47 kDa N protein band. Lysates from cells transfected with si447 showed a similar pattern of reactivity. In contrast, in cells that were previously transfected with si983, the specific band could not be seen. No N protein band was detected from uninfected cell lysates (Fig. 2a). It showed that the average titer of si983-treated cells (10^{3.7} TCID₅₀/mL, the mean of three experiments') was 10^{5.6}-fold, 10^{5.1}-fold lower than those of the mock-transfected (10^{9.3} TCID₅₀/mL) and mismatched siRNA controls (10^{8.7} TCID₅₀/mL), respectively (Fig. 2b).

To investigate whether the inhibitory effect of si983 was dose-dependent, EPC cells were transfected with 0.4, 0.8, 1.2 and 1.6 µg, respectively. Subsequently, the cells were infected with SCR V and the whole cell lysates were examined by Western blotting. It presented that the N protein bands became fainter when the amount of si983 increased (Fig. 2c), which indicated the obvious dose-dependent silencing effect in cell cultures.

Finally, the N-GFP plasmid vector and si983 were co-transfected into EPC cells. The fluorescence intensity of EPC cells co-transfected with N-GFP and scrambled siRNA showed no significant difference in comparison with that of those transfected with N-GFP alone, but was much stronger than that of co-transfected with N-GFP and si983. Subsequent Western blotting analysis also suggested that N protein expression was reduced in cells co-transfected with si983, but not in cells co-transfected with scrambled siRNA, as compared with cells transfected with N-GFP plasmid alone (Fig. 2d).

The rhabdovirus N protein forms a viral nucleocapsid by interacting with its viral RNA. The resultant nucleoprotein complex associates with the RNA-dependent RNA polymerase subunits to initiate intracellular virus replication. So the N protein of rhabdoviruses would play essential roles in viral RNA protection and

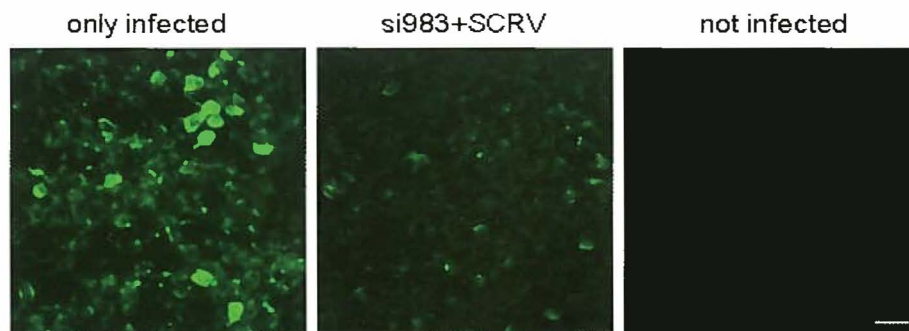


Fig. 1. Inhibition of virus replication in EPC cells by si983. The transfected cells were infected with SCR V (200 TCID₅₀/well) and immunostained by IFA with anti-N sera after 48 h (Scale bar = 50 µm). SCR V-infected EPC without si983 and blank cells were as positive and negative controls. The photographs were obtained at the unified exposure time (1.2 s) and aperture (1.6).

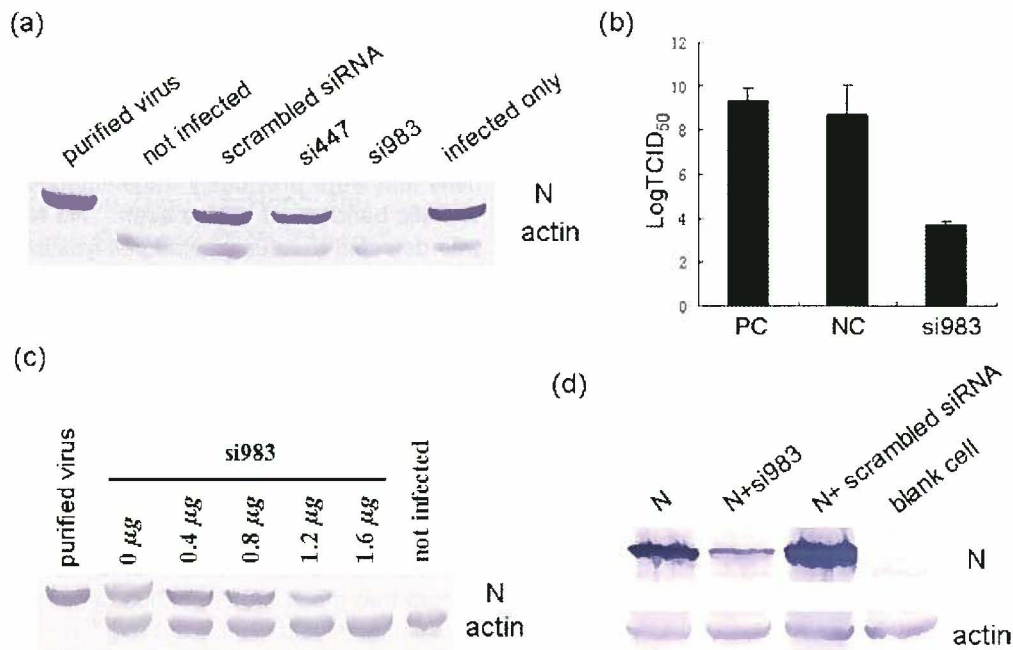


Fig. 2. Inhibitory effect of si983 on expression of N protein and virus production. (a) Western blotting was performed on equal amounts of proteins harvested from mock or siRNA-transfected EPC cells at 48 h post infection with SCR. β -actin was used as a loading control; (b) Viral titers of siRNA-transfected cells after SCR infection. The scrambled siRNA (NC) and si983 were transfected and thereafter, the cells were infected with SCR (300 TCID₅₀/well) and incubated for 72 h, viral titers in these wells were measured and shown by log TCID₅₀/0.1 mL. The infected cell supernatants without transfection served as positive control (PC). Error bars represent the standard deviation; (c) Dose-dependent inhibition in reduction of N protein levels by si983. Cells were transfected with the si983 at increasing amount and then infected with SCR for 72 h. The cell lysates were collected for immunoblotting; (d) Downregulation of viral N protein expression via co-transfection with si983 and N-GFP vector. β -actin served as internal control.

virus replication. In this study, SCR N gene expression was inhibited by RNAi and the inhibitory effect on virus production was determined. Among the four target sequences for RNAi, si983 was found to be the most effective, which could effectively block expression of the viral target gene and inhibit viral replication. It also suggested a potential candidate to study further SCR gene functions. Future studies are necessary to determine the antiviral mechanism of RNAi on SCR replication.

Acknowledgements

This work was supported by grants from the National Major Basic Research Program (2009CB118704), the National 863 High Technology Research Foundation of China (2007AA09Z429, 2006AA09Z445 and 20060110A4013), the National Natural Science Founda-

tion of China (30871938, 30800854 and 31101931) and High-level talents Fund from Henan University of Technology (2010BS016).

References

- 1) Tao, J. J., J. F. Gui and Q. Y. Zhang (2007): *Aquaculture*, **262**, 1–9.
- 2) Tao, J. J., G. Z. Zhou, J. F. Gui and Q. Y. Zhang (2008): *Virus Res.*, **132**, 86–96.
- 3) Hannon, G. J. (2002): *Nature.*, **418**, 244–251.
- 4) Denli, A. M. and G. J. Hannon (2003): *Trends Biochem. Sci.*, **28**, 196–201.
- 5) Barik, S. (2004): *Virus Res.*, **102**, 27–35.
- 6) Dang, L. T., H. Kondo, T. Aoki and I. Hirono (2008): *Antiviral Res.*, **80**, 316–323.
- 7) Kim, Y. S., F. Ke, X. Y. Lei, R. Zhu and Q. Y. Zhang (2010): *PLoS One.*, **5**, e10308.
- 8) Zhang, Q. Y., J. J. Tao, L. Gui, G. Z. Zhou, H. M. Ruan, Z. Q. Li and J. F. Gui (2007): *Dis. Aquat. Organ.*, **74**, 95–105.
- 9) Zhou, G. Z., L. Gui, Z. Q. Li, X. P. Yuan and Q. Y. Zhang (2008): *J. Virol. Methods.*, **148**, 205–210.

株化細胞における *Siniperca chuatsi* rhabdovirus の
RNA 干渉法による複製阻害

G.-Z. Zhou · R. Zhu · J.-F. Gui · Q.-Y. Zhang

ケツギョ *Siniperca chuatsi* ラブドウイルス (SCRV) N 遺伝子に対応する siRNAs を合成し、その抗 SCR V 活性について EPC 細胞を用い検討した。供試 siRNAs のうち、si983 が最も高い抗 SCR V 活性を示し、またウエスタンプロット解析で si983 により SCR V N 遺伝子の発現ならびに SCR V 複製の阻害が確認された。従って、N 遺伝子の983領域が RNA 干渉法の重要な標的であり、本遺伝子を標的とした RNA 干渉法が SCR V 感染に対する一つの有効な治療法になり得ることが示唆された。

魚病研究, 47 (1), 30–32 (2012)