

## SHORT COMMUNICATION

# Inhibition of CD46 Receptor by RNAi Enhances Cell Resistance to BVDV Infection

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## Abstract

This study aimed to investigate the inhibition effect of CD46 on the replication of bovine viral diarrhoea viruses (BVDV). BVDV causes bovine viral diarrhoeal mucosal disease, and prevention of BVDV infection is very important for livestock production. Here, we have designed four pairs of effective siRNAs, and *in vitro* experiments showed that some of them can effectively inhibit the expression of BVDV receptor CD46. Further results showed that BVDV replication was efficiently inhibited and the yield decreased accordingly when expression of CD46 was decreased by siRNA. In conclusion, this study provides a new approach to defend against BVDV infection.

**Keywords:** BVDV, CD46, Replication, RNAi

## CD46 Reseptörünün RNAi İnhibisyonu BVDV Enfeksiyonuna Hücre Direncini Artırır

### Öz

Bu çalışmada, CD46'nın sığır viral diyare virüslerinin (BVDV) replikasyonu üzerindeki inhibisyon etkisinin araştırılması amaçlandı. BVDV, bovine viral diarrhoea-mukozal hastalığa neden olur ve BVDV enfeksiyonunun önlenmesi hayvan yetiştiriciliği açısından oldukça önemlidir. Bu çalışmada, dört çift etkin siRNA tasarladık ve *in vitro* denemelerde bunlardan bazılarının BVDV reseptörü olan CD46 ekspresyonunu etkili bir şekilde inhibe edebildiği saptandı. Diğer sonuçlara bakıldığında, BVDV replikasyonunun etkin bir şekilde inhibe edildiği ve bu inhibisyonun siRNA tarafından azaltılan CD46 ekspresyonuna paralel şekillendiği belirlendi. Sonuç olarak, bu çalışma, BVDV enfeksiyonuna karşı savunmada yeni bir yaklaşım sunmaktadır.

**Anahtar sözcükler:** BVDV, CD46, Replikasyon, RNAi

## INTRODUCTION

Bovine viral diarrhoea mucosal disease is a highly contagious disease caused by bovine viral diarrhoea viruses (BVDV) <sup>[1]</sup>. Animals infected with BVDV may generate a series of symptoms including fever, mucosal ulcer, diarrhoea, hematoma decreasing, immune endurance, immune inhibition and dysgenesis. Acute infection of BVDV may lead to reproductive deficiency, death of early stage embryo, abortion, and congenital malformation <sup>[2]</sup>. BVDV can not only cause disease in animals, but it is also one of the

main pollution sources of biological products (serum, vaccine, interferon). It has also caused a tremendous economic losses to the world's bovine breeding and has spread widely around the world such as America and New Zealand, especially in countries with developed bovine breeding industry <sup>[3,4]</sup>. However, the current effective methods for controlling BVDV infection are limited.

RNA interference (RNAi) is a phenomenon that double strand RNAs can specifically induce homologous gene expression silence <sup>[5]</sup>. Studies verified that RNAi can

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specifically inhibit viral gene expression to make it keep silent or dormant [6]. Moreover, to produce an accurate and effective silencing impact, a sequence identity is qualified by RNAi [7]. Research suggested that RNAi technology can inhibit the expression of viruses such as BVDV, foot-and-mouth disease virus (FMDV) and hepatitis B virus (HBV) [8-10]. BVDV can bind to the CD46 molecule, which subsequently promotes entry of the virus [11].

In order to investigate the inhibition effect of CD46 on the replication of BVDV, we screened some siRNAs which may significantly inhibit the expression of CD46 gene. BVDV can be effectively inhibited from replication when CD46-targeted siRNA reduces the expression of cell receptor CD46. In summary, our study provides a new method to prevent and treat BVDV infection.

## MATERIAL AND METHODS

### Virus and Cell Culture

BVDV (NADL [GenBank accession number M31182]) was used for viral attacks. The Madin-Darby bovine kidney (MDBK) cells were cultivated with high sugar DMEM containing 20% FBS (Fetal bovine serum) to subculture and put on a culture plate.

### SiRNA Design

According to the bovine CD46 gene sequence (GenBank accession number NM\_001242561.1), siRNA were designed using Life Technologies (<http://rnaidesigner.invitrogen.com/rnaexpress/>) and Whitehead (<http://sirna.wi.mit.edu/>) software. In order to generate small hairpin RNA (shRNA), the sequence TTCAAGAGA was added between the positive-sense strand and anti-sense strand, and the terminator sequence TTTTTT was added at the 3' end. Two restriction enzyme sites (*Bam*HI and *Hind*III) were inserted for vector construction (Table 1). Single DNA strands were synthesized by Sangon Biotech, Shanghai, China.

### Interference Vector Construction

The sense and anti-sense oligonucleotides were annealed into oligonucleotide double-stranded. The reaction contained 10  $\mu$ L of each sense and anti-sense strand (100  $\mu$ M). The reaction condition was as follows: 3 sec at 95°C, 2 min at 72°C, and 2 min at room temperature. When it was detected with 2% agarose gel electrophoresis, the

product was put on ice for 10 min and stored up at -20°C. The vector pGenesil-1 (Wuhan Genesil Biotechnology Co., Ltd, Wuhan, China) was doubly digested with *Bam*HI and *Hind*III. Small paired chains segments and the linear vector pGenesil-1 were ligated overnight at 16°C and transformed into *Escherichia coli* DH5 $\alpha$  competent cells. The transformed DH5 $\alpha$  was cultivated on LB plates containing 100  $\mu$ g/mL Kanamycin for overnight at 37°C. The obtained recombinant plasmid was confirmed by the restriction enzyme digestion with *Bam*HI and *Hind*III and DNA sequencing (Sangon Biotech).

### Interference Vector Transfection and Cell RNA Extraction

Madin-Darby bovine kidney cells were cultured in 12-well culture plate and each interference vector was transfected using the transfection kit (X-tremeGENE HP DNA Transfection Reagent, Sigma-Aldrich Trading, Shanghai, China). Then the plate was put into incubator for cultivating at 37°C with 5% CO<sub>2</sub>. A blank control was set up at the same time. Three replicates were performed for each sample. Total RNAs were extracted from MDBK cells by using Trizol (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The RT-PCR of co-expression mRNAs was performed by using a RT-PCR kit (Takara, Dalian, China) according to the manufacturer's protocol. The reverse transcription conditions were that of the reactants were at 37°C for 15 min and 85°C for 5 sec. After that, the products were stored up at -20°C for spare use.

### Interference Vector Effect Testing

Sequences of CD46 genes and  $\beta$ -actin reference genes (Accession number AY141970.1) were downloaded from Genbank and qRT-PCR primers were designed by Primer 5.0.  $\beta$ -actin was used as the reference gene. PCR products were analyzed by gel electrophoresis and qRT-PCR was carried out with following reaction system: 10  $\mu$ L SYBR, 2  $\mu$ L cDNA, 0.5  $\mu$ L upstream primer, 0.5  $\mu$ L downstream primer and 7  $\mu$ L RNase-free double distilled H<sub>2</sub>O. PCR was performed with the following thermocycling conditions: an initial 5 sec at 95°C followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec. CD46 mRNA expression amount of each group was calculated according to the method  $2^{-\Delta(\Delta Ct)}$ .

### Verification of siRNA Effect

Madin-Darby bovine kidney cells were transfected with interference vector and collected at 48 h. The expression amount of CD46 receptor was detected using real-time fluorescence PCR. Cell attack experiment was performed at the P3 laboratory belonging to TianKang Biotechnology, Shihezi, China. The virus solution was diluted to one tenth before added into the cell plate with 96 wells with MDBK cells and measured TCID<sub>50</sub>. These cells were performed by viral attack experiment with TCID<sub>50</sub> multiplying to  $2.1 \times 10^3$  per well and the virus liquid was collected when 12 h, 24 h and 48 h after cell attack experiments. The cell total RNA

Table 1. siRNA targeting target sequence

No	siRNA Target Sequence (5'-3')	Region	GC %
sh1	GATAATCCTCCAACATGTG	ORF	42
sh2	TGTGTCAACCGCTCCAGA	ORF	57
sh3	CTTGTGGAGAGAGCAAGC	ORF	52
sh4	CATGGAACGATAGTCTCAG	ORF	47

was extracted and reversely transcribed into cDNA, then they were detected on antiviral replication effect using qRT-PCR.

### Statistical Analysis

SPSS21.0 biostatistics software was used to perform single-factor t-test statistical analysis on the data,  $P < 0.05$  was considered statistically significant, and Graphpad Prism 5.0 software was used for graphing.

## RESULTS

In this study, four pairs of siRNA sequences targeting CD46 genes (Table 1) were designed. The four pairs of shRNA expression vectors which target CD46 gene and the control plasmid (shScr) were transfected into MDBK cells respectively, and the transfected cells were collected after 48 h. CD46 and reference gene primers were designed by Primer 5.0 (Table 2). We analyzed CD46 expression variation using qRT-PCR, and found that the inhibition rates of sh1, sh2, sh3 and sh4 inhibiting CD46 expression were 45%, 54%, 87% and 32%, respectively. Among them, sh3 has the most effective inhibition rate up to 87% (Fig. 1).

To investigate the inhibition effect of CD46 on virus replication, MDBK cells ( $3 \times 10^5$  cells/well) were transfected with 1.5 mg shRNA expression vectors per well. After 16 h, the transfected cells were infected with BVDV. The effect of shRNA on NADL replication was detected by RT-PCR after virus attack. The results showed that compared with wild-type control (WT), viral RNAs were significantly reduced in the transfected cells at 12 h, 24 h, and 48 h ( $P < 0.01$ ) (Fig. 2-A). Consistent with RT-PCR, virus yield of transfected cells decreased by 5 to 20-fold at 12 h, 24 h, and 48 h transfection as compared with WT (Fig. 2-B).

Gene Name	Primer Sequence (5'-3')
$\beta$ -actin F	ACCGCAACCAGTTCGCCAT
$\beta$ -actin R	CATGCCAATCTCATCTCGTTTTTC
CD46 F	GCGGAGCAGTCTTGGGTGT
CD46 R	CCAGCATCAAAAAATCGTATCTT

## DISCUSSION

As a cell surface receptor molecule, CD46 has a mRNA with 1500bp nucleotides at length, which plays a certain role in the process of BVDV invading cells, and it inhibited implication of BVDV when it was blocked by antibody [12]. The interaction between virus protein and cell receptor protein is pivotal to virus pathopoiesia that may mediate the entrance of the virus into cells. The regular biological function of the protein can be altered through their interactions which result in cell pathopoiesia and persistent infection [13]. More and more studies are related to the implication, infection and molecular biology mechanism of pathopoiesia such as HIV viruses at present while few studies are involved in BVDV yet [14]. Pre-existing studies have shown that RNAi lentiviral vector targeting FMDV receptor porcine integrin has been successfully constructed, and iav-PK-15 cell line capable of stably interfering with pig integrin  $\alpha$  subunit gene expression has been obtained, which replication has a significant inhibitory effect [15].

Therefore, in order to resist virus against infecting and replicating, it is a considerably reliable approach to screen the pivotal cell receptors of virus, and design inhibition

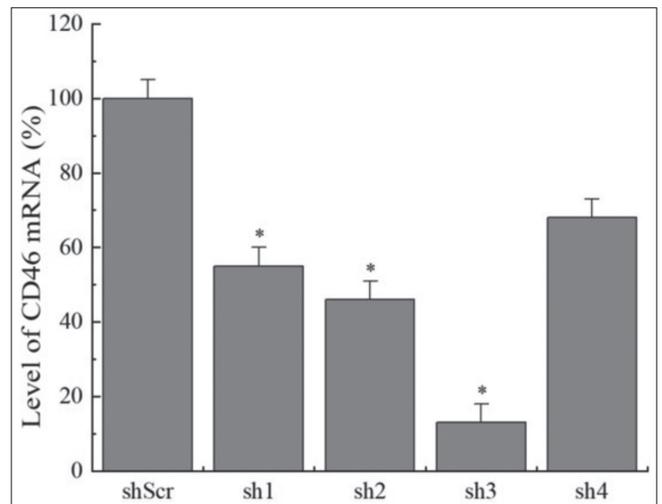
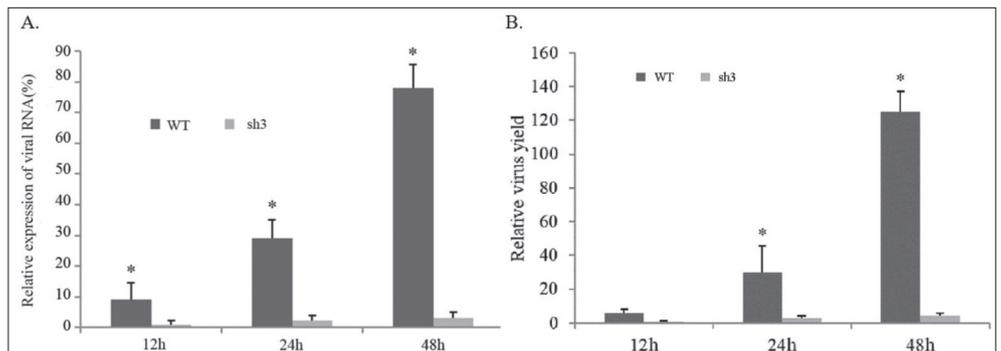


Fig 1. CD46 mRNA expression after interaction of four siRNA (sh1, sh2, sh3 and sh4) with CD46

**Fig 2.** Effect of shRNA on NADL replication. (A) Relative expression of NADL RNA in RNAi-CD46 MDBK cells after NADL infection. The relative amount of viral RNA were analyzed at different time points in cells from sh3-CD46 MDBK cells and WT. Data are presented as means  $\pm$  SD. (B) Viral titers in RNAi-CD46 MDBK cells after NADL infection. Viral titers was determined by TCID<sub>50</sub> and the data were presented as means  $\pm$  SD



siRNAs to transfect cells and establish a stable interfering cell line. In this study, we designed four pairs of siRNA sequences targeting CD46 genes and transformed the shRNA expression vectors and control plasmid into MDBK cells. The results showed that shRNA can inhibit the expression of CD46 and sh3 has a most effective inhibition rate.

However, we are not clear whether CD46 is an analogous cell receptor for BVDV, and whether the siRNA method issued above could effectively interfere with its infection. We guessed in advance that the process of its infecting cells may be interrupted by inhibiting the pivotal BVDV cell receptor, because cell receptors directly or indirectly play a vital role in the process of virus infection of cells. In order to investigate the inhibitory effect of CD46 on virus replication, we infected the transfected cells with BVDV, and detected the effect of shRNA on NADL replication. The results showed that virus RNA was significantly reduced and the virus yield also decreased in the shRNA-transfected cell.

Our results verified that siRNA designed for CD46 can significantly reduce BVDV infection. It provides a new idea for livestock antiviral breeding. Although this method does not achieve complete antiviral capacity, it may be related to the interference efficiency of RNAi. In the future, the use of genome editing techniques (such as CRISPR/Cas 9) to knock out CD46 gene expression or combine other antiviral technologies may get better results. In general, our research not only provide theoretical references for subsequent antiviral animals breeding, but also inspire more people to participate in the screening of key cell receptors of virus for siRNA-mediated antiviral.

#### CONFLICT OF INTEREST

Authors state no conflict of interest.

#### AUTHOR CONTRIBUTIONS

RH and LF participated in the design of this study, the provision of data, and the writing of this article. SH was the reviewer of this article, and other authors provided assistance in this process. The final manuscript read and approved by all authors.

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