Original Article

RNA interference targeting glycoprotein D inhibits infectious bovine rhinotracheitis virus replication in MDBK cells

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Abstract

Infectious bovine rhinotracheitis (IBR) is an acute, febrile, contagious disease caused by infectious bovine rhinotracheitis virus (IBRV). IBRV vaccine is considered to be partially effective but not yet completely successful. Therefore, establishing a new antiviral approach is critical. RNA interference (RNAi) has been rapidly developed in recent years as an antiviral therapy for several viruses. This study showed that RNAi could suppress IBRV replication via knockdown of a virion glycoprotein. Two recombinant lentiviral vectors containing short hairpin RNAs (shRNAs) (H1-RNA-121, H1-RNA-304) against the glycoprotein gD of IBRV and a pcDNA3-gD vector containing a FLAG tag were constructed. pcDNA3-gD and the individual shRNA recombinant lentiviral vectors were co-transfected into 293T cells, and the efficiency of RNAi was verified using Western blotting. H1-RNA-304 strongly suppressed the transient expression of the FLAG-tagged gD fusion protein. The recombinant H1-RNA lentivirus was packaged by transfecting the 293T cells with the recombinant H1-RNA vector and two helper plasmids using Lipofectamine, and the lentivirus was then used to infect MDBK cells. When the MDBK cells were infected with IBRV after infection with the H1-shRNAs, H1-shRNA-304 was more effective at markedly silencing viral gD gene expression and it inhibited IBRV replication. These results indicate that shRNAs targeting the gD gene have substantial antiviral properties and inhibit IBRV replication in a sequence-specific manner, demonstrating their potential for clinical application.

Keywords: RNAi, infectious bovine rhinotracheitis virus, shRNA, Gd

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Introduction

Infectious bovine rhinotracheitis virus (IBRV) is a critical pathogen causing bovine respiratory disease (also known as shipping fever). In addition to direct pathogenicity, IBRV induces immune suppression resulting in secondary bacterial infection, which subsequently weakens cattle reproduction and causes mortality in severe cases (Romera et al., 2014). Some vaccines are now available; however, there are no specific pharmacological interventions for infectious bovine rhinotracheitis. Thus, there is an urgent need for effective prevention and therapeutic strategies.

RNA interference (RNAi) using short hairpin RNA (shRNA) can block target gene expression by inducing homologous mRNA degradation without affecting the expression of other genes. Therefore, RNAi has become a powerful and widely used tool to study any specific gene's function (Jaber et al., 2010; Narute et al., 2009; Workman and Jones, 2010). Moreover, RNAi can be utilized as an antiviral therapeutic approach to protect both plant and animal species from different viruses (Benitez et al., 2015; Kumar and Arankalle, 2010; Russo et al., 2015; Tan et al., 2012; Uddin et al., 2014; Yang et al., 2015).

The IBRV genome is an approximately 138-kb linear double-stranded DNA (Zhen and Jinghua, 1997). It encodes approximately 30 to 40 structural proteins, including 11 glycoproteins, among which gD, a major protein located at the virus particle surface, is associated with virus infection. It is mainly involved in viral entry and can induce humoral and cellular immunity in host cells (van Drunen Littel-van den et al., 1998). Compared with other glycoproteins such as gB and gC, gD provokes stronger and long-lasting cellular immunity, with both a higher neutralization antibody titer and better antiviral effects (AbdelMagid et al., 1992). Hence, gD was chosen as the target protein in IBRV antiviral research. This study demonstrated that shRNA targeting gD could inhibit IBRV replication in a sequence-specific manner. Here, an approach to studying the role of gD in IBRV replication as well as a new anti-IBRV strategy is provided.

Materials and Methods

Cell lines and viruses: MDBK cells and 293T cells were purchased from the China center for type culture collection and were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Utah, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Utah, USA). The culture medium was replaced every two days. IBRV strain SD was isolated and identified in our laboratory and was propagated in the MDBK cells.

Titration of virus: The MDBK cells in 96-well plates were infected with 10-fold dilutions of the IBRV strain SD in four replicates per dilution. After 48 hr, viral cytopathic effect was assessed, and the 50% tissue culture infective dose (TCID50) of the virus was calculated using the Reed-Muench method (Poch et al., 1990).

Construction of gD shRNA recombinant lentiviral vectors and gD recombinant pcDNA3: Lentivirus-based shRNA vectors were constructed as previously described (He and Sun, 2007). The lentiviral vector, which contains the U6 promoter, was used for the expression of single short hairpin molecules. The sequences of these gD shRNA oligonucleotides were as follows: H1-shRNA-121-P1: 5′- CTA CAC TGA ACG CTG GCA CTT CAA GAG AGT GCC AGC GTT CAG TGT AGT TTT TTG T -3′ and H1-shRNA-121-P2: 5′- CTA GAC AAA AAA CTA CAC TGA ACG CTG GCA CTC TCT TGA AGT GCC AGC GTT CAG TGT AG -3′; H1-shRNA-304-P1: 5′- CGC CAC GGT CAT ATG GTA CTT CAA GAG AGT ACC ATA TGA CCG TGG CGT TTT TTG T -3′ and H1-shRNA-304-P2: 5′- CTA GAC AAA AAA CGC CAC GGT CAT ATG GTA CTC TCT TGA AGT ACC ATA TGA CCG TGG CG -3′. The control shRNA sequences were as follows: LacZ-P1: 5′- CAG TTG CGC AGC CTG AAT GTT CAA GAG ACA TTC AGG CTG CGC AAC TGT TTT TTG T-3′ and LacZ-P2: 5′-CTA GAC AAA AAA CAG TTG CGC AGC CTG AAT GTC TCT TGA ACA TTC AGG CTG CGC AAC TG-3′. These oligonucleotides were annealed to each other to form the first fragment with the XbaI cleavage site. After the vector was cut by XbaI and AgeI (Promega, Wisconsin, USA), the second fragment (12.5 bp) was obtained. After the vector was cut by SmaI and AgeI, the third fragment (1.5 bp) was obtained. The three fragments were ligated with T4 DNA ligase (Promega, Wisconsin, USA), then used to transform the E.coli DH5α strain (Transgene Biotechnology Co., Ltd, Beijing, China), followed by DNA sequence confirmation. The sequence of LacZ shRNA is not homologous to any viral or cellular genes. Therefore, LacZ shRNA recombinant lentivirus served as the negative control. The PCR primer pair used was LT1 and LT2 (Table 1). The positions in the gD gene (NC 001847) sequence that the gD shRNA oligonucleotides (H1-shRNA-121, H1-shRNA-304) targeted were 121 and 304.

The PCR primer pair used for pcDNA3-FLAG-gD (pcDNA3 vector, Invitrogen, V790-20) construction was gD-P1 and gD-P2 (Table 1). The PCR fragments were digested with HindIII and EcoRI and subcloned into previously digested pcDNA3, followed by DNA sequence confirmation.

Co-transduction of lentiviral shRNAs and FLAG-tagged gD gene in 293T cells: pcDNA3-FLAG-gD (4 μg) was co-transfected along with 4 μg of individual recombinant shRNA lentiviral vector into the 293T cells using Lipofectamine 2000 (Invitrogen, California, USA) according to the manufacturer's instructions. After 48 hr, transient expression of the FLAG-gD fusion protein was verified by Western blotting, as previously described (He and Sun, 2007). Briefly, the 293T cells were lysed with lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA, 5 mM EDTA, 1 mM NaF, 1 mM Na3VO4 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) with a freshly added proteinase inhibitor tablet), and supernatant was collected by centrifugation. Proteins were separated on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and were electrophoretically transferred onto nitrocellulose

membranes. The membranes were blocked with 5% Blotto in TBS-T (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20; Sinopharm Chemical Reagent Co., Ltd) for 1 hr at room temperature and were probed with antibodies against FLAG and β-actin (Sigma-Aldrich (Shanghai) Trading Co, Ltd, Shanghai, China). Specific proteins were visualized using ECL (Amersham Biosciences, Connecticut, USA). gD gene expression was quantified relative to the LacZ shRNA control.

Table 1 Sequences of primers used in this study

Primer	Primer sequence (5'-3')
LT-1	TGT CGC TAT GTG TTC TGG GA
LT-2	GGT ACA GTG GAG GGG AAA GA
gD-P1	CTC AAG CTT GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG ATG CAA GGG CCG ACA TT
gD-P2	CGC GAA TTC GAG GAG GGC CTA GAC CGC
β-actin-P1	GAT ATG GAG AAG ATC TGG CA
β-actin-P2	GTT GAA TGT AGT TTC GTG GA
gB-P1	GAC TAC AAG GAC GAT GAC AAG
gB-P2	CTT CYY CCA CGA TGC AGC TTG TCA T

Packaging of recombinant shRNA lentivirus: The recombinant shRNA lentiviral vectors were co-transfected along with gag- and env-expressing plasmids into the 293T cells as previously described (He and Sun, 2007). Supernatant was collected 48 hr post-transfection. Transduction of the MDBK cells was performed in 12-well plates with 10-fold serial dilutions of the supernatant from one of the recombinant H1 lentiviruses. After 4 hr of incubation, the medium was refreshed. Two days later, the cells were removed and fixed in 2% paraformaldehyde. Transduction efficiency of the shRNA recombinant H1 lentivirus encoding enhanced green fluorescent protein (eGFP) was determined using the fluorescence-activated cell sorting (FACS) analysis, and transduction titers (TU/ml) were calculated using the Reed-Muench method.

IBRV infection after transduction of MDBK cells with recombinant shRNA lentivirus: The MDBK cells in 12-well plates were transduced with 1×10^6 TU of the gD shRNA recombinant lentivirus (H1-shRNA-121 and H1-shRNA-304) or LacZ shRNA in eight replicates for each shRNA. After 24 hr, the cells from four replicate wells were removed for FACS-based assessment of shRNA transduction. The medium in the remaining four wells was changed, and each well was inoculated with 100 $TCID_{50}$ of the IBRV SD strain. After 48 hr, the cytopathic effect (CPE) was measured in each well. The cells and supernatants were collected for assessment of the shRNA gene silencing efficiencies and IBRV titers, respectively.

Total RNA extraction, real-time quantitative RT-PCR and Western blotting: mRNA expression levels of the viral genes in the cells infected with recombinant shRNAs were detected by RT-PCR. Total RNA was isolated from the cells with TRIzol reagent (Promega, Wisconsin, USA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was conducted using the QuantiTect SYBR green RT-PCR kit (Qiagen, Dusseldorf, Germany). Amplification was performed using the following primer pairs: gD-P1 and gD-P2 for gD, and β-actin-P1 and β-actin-P2 for β-actin (Table 1). The cycling program was set as follows: initial activation at 95℃ for 10 min, 40 cycles of denaturation at 95℃ for 15 sec, and annealing and extension at 60℃ for 1 min. Data were analyzed according to the Ct method, and the amount of gD RNA in the samples was normalized to β-actin. The samples were run in triplicate.

To verify the sequence specificity of the shRNA, relative quantification of the gB gene was performed by RT-PCR as mentioned above. The primers used were gB-P1 and gB-P2 (Table 1).

Expression of the gD protein was evaluated by Western blotting, as previously described (He and Sun, 2007). The cells were lysed with lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA, 5 mM EDTA, 1 mM NaF, 1 mM Na3VO4 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) with a freshly added proteinase inhibitor tablet), and supernatant was collected by centrifugation. Proteins were separated on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% Blotto in TBS-T (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20; Sinopharm Chemical Reagent Co., Ltd) for 1 hr at room temperature and were probed with antibodies against gD and β-actin (Sigma-Aldrich (Shanghai) Trading Co, Ltd, Shanghai, China). Specific proteins were visualized by ECL (Amersham Biosciences, Connecticut, USA).

Titration of virus: The cells and supernatants were collected 48 hr after infection with shRNA recombinant lentiviruses. After three freeze-thaw cycles, the cultures were diluted 10-fold from 10-1 to 10-10 and were used to infect a confluent monolayer of MDBK cells in 96-well plates. Each dilution was added to four wells. After 48 hr, CPE was assessed, and TCID50 of the virus was calculated using the Reed-Muench method.

Results

gD shRNA recombinant lentiviral vectors inhibit transient gD expression in 293T cells: Two shRNA recombinant lentiviral vectors targeting gD (H1-shRNA-121 and H1-shRNA-304) were individually co-transfected with pcDNA3-gD into the 293T cells. Expression of the FLAG-gD fusion protein was evaluated by Western blotting. As shown in Figure 1, two of the shRNAs significantly inhibited the transient expression of FLAG-gD compared with the LacZ-control shRNA. H1-shRNA-304 had the stronger effect, almost completely eliminating the gD expression. These results strongly suggest a direct role of shRNAs in specifically blocking the transient expression of the gD protein in 293T cells.

gD shRNAs silence viral gD during IBRV replication in MDBK cells: It was determined whether these shRNAs could block the natural expression of the viral gD gene during IBRV replication. The shRNA recombinant lentivirus was packaged and titered (TU/ml) using the FACS analysis based on eGFP expression. The transduction efficiency of the shRNA recombinant lentiviral vectors was determined by the FACS analysis based on eGFP co-expression from the viral vector. As shown in Figure 2A, over 96% of the IBRV-susceptible MDBK cells transduced with 1×10^6 TU H1-shRNA vector expressed eGFP after 24 hr. The MDBK cells were inoculated with 100 $TCID_{50}$ of the IBRV SD strain after gD shRNA recombinant lentiviral infection using eight wells for each shRNA. The IBRV infection of the MDBK cells served as the control. Relative quantification of the targeted gene, gD, and an untargeted gene, gB, was performed using real-time quantitative RT-PCR. The relative amount of the gD gene in control cells infected with IBRV alone was regarded as 1.000. As shown in Figure 2B, the relative amounts of the gD gene in cells infected with IBRV after inoculation with H1-shRNA-121, H1-shRNA-304 or H1-shRNA-LacZ were 0.371, 0.072 and 0.941, respectively. H1-shRNA-304 had the stronger effect, reducing the amount of the gD gene by approximately 96%.

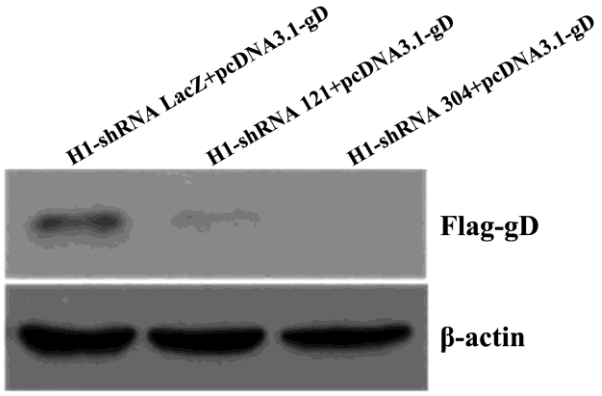

Figure 1 Analysis of shRNA-mediated silencing of transiently expressed gD gene by Western blotting
Lysates of 293T cells co-transfected with pcDNA3-FLAG-gD and individual shRNA recombinant lentiviral vectors (H1-shRNA-121 and H1-shRNA-304) were separated by SDS-PAGE and then analyzed by Western blotting using antibodies against FLAG and β-actin. The experiment was independently repeated three times.

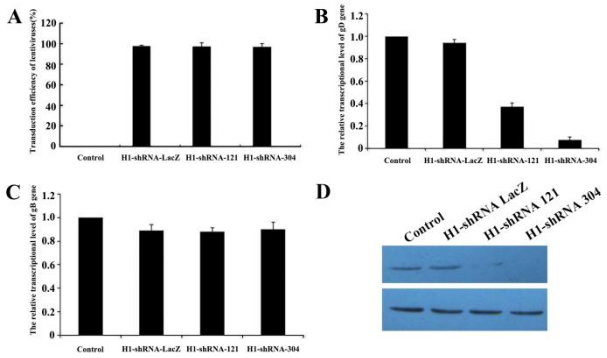

Figure 2 gD shRNA recombinant lentiviruses inhibiting viral gD gene expression in MDBK cells
To directly demonstrate viral RNA degradation by shRNAs, MDBK cells were inoculated with 100 TCID50 of the IBRV SD strain after gD shRNA recombinant lentiviral infection, using ten wells for each shRNA. The MDBK cells were inoculated with shRNA recombinant lentivirus. After 24 hr, the cells were removed from five wells. Viral samples after IBRV infection of the MDBK cells were used as controls. Transduction efficiency of the shRNA recombinant lentiviruses was evaluated by FACS, and over 96% of the cells transduced with H1-shRNA expressed GFP (a). The results from three independent experiments in quintuplicate are presented as percentages of the average transduction efficiencies in treated cells ± one standard deviation. At 48 hr after IBRV infection, the viral samples were collected. The viral samples after IBRV infection of the MDBK cells served as the control. Relative quantification of both the targeted gD gene (b) and the untargeted gB gene (c) were performed by real-time quantitative RT-PCR. The expression levels of gD and β-actin were determined by Western blot. β-actin was used as the loading control to ensure equal protein loading in all wells.

To verify the sequence specificity of H1-shRNA, relative quantification of the gB gene was performed using real-time quantitative RT-PCR. The relative amount of the gB gene in control cells infected with IBRV alone was regarded as 1.000. As shown in Figure 2C, there was no obvious change in the relative amount of the gB gene. Thus, shRNAs against the gD gene significantly degraded the viral RNA.

As shown in Figure 2D, two of the shRNAs significantly inhibited the expression of gD compared with the LacZ-control shRNA. H1-shRNA-304 had the stronger effect.

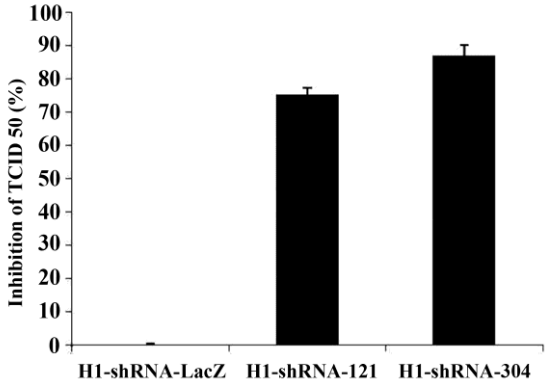

Figure 3 gD shRNA recombinant lentiviruses inhibiting IBRV replication in MDBK cells
To directly demonstrate viral RNA degradation by shRNAs, MDBK cells were infected with IBRV after gD shRNA recombinant lentiviral infection. After 48 hr, the viral samples were collected. The viral samples after IBRV infection of the MDBK cells served as the control. IBRV viral replication is presented by TCID50. The results from three independent experiments in quintuplicate are presented as percentages of the average TCID50 in treated cells compared to that in IBRV-infected control cells ± one standard deviation.

gD shRNAs inhibit IBRV replication in MDBK cells: The $TCID_{50}$ assay was used to examine the capacity of shRNAs to inhibit IBRV replication. As shown in Figure 3, regardless of H1-shRNAs being inoculated before IBRV infection, H1-shRNA-304 achieved maximal inhibition, and H1-shRNA-121 achieved partial suppression of viral replication.

Discussion

For many years, there has been an ongoing search for a veterinary vaccine or antiviral agent for the treatment of IBRV infection. Although several vaccines are currently available, their efficacies are variable. Moreover, there is no fully effective antiviral treatment against IBRV infection. Therefore, there is an urgent need for rapid and potent preventive and therapeutic strategies against this infection. RNAi has become a powerful tool for the development of gene-based therapies for many viral infections (Perrimon et al., 2010). RNAi-mediated targeting of viral genes is known to silence viral RNA, inhibiting virus replication. For example, two siRNAs were found to inhibit BHV-1 multiplication in MDBK cells to different extents, namely by 11% and 40%, respectively, as demonstrated by virus titers measured in cell culture (Narute, Raut, Saini, Rai and Gupta, 2009). Although RNAi has been used as an effective antiviral strategy, presently, there is no clinical application of RNAi targeting approaches on farms. Moreover, no further studies of IBRV RNAi in experimental animals have been published.

IBRV is a DNA virus that encodes approximately 11 glycoproteins. Among these glycoproteins, the gD glycoprotein is essential for viral replication. This protein is highly expressed on the surface of the virus capsule and in infected cells and is mainly involved in the transmission of the virus through the cell membrane and into the cell (Chowdhury and Sharma, 2012). Therefore, gD was chosen as the target gene in this study.

Pivotal issues for RNAi therapeutics include reagent delivery, specificity and stability. Of these, delivery is currently considered the biggest hurdle (Perrimon, Ni and Perkins, 2010). RNAi can be introduced into host cells by various methods. The first method is chemically synthesized siRNA, which can effectively silence viral RNA. Because siRNAs are easily degraded, the effects are provisional. The second method is shRNA carried by plasmids or viral vectors, which can produce siRNAs in host cells. Lentiviral vectors are one of most useful tools currently available for delivering and stably expressing shRNAs in target cells (Carbonaro Sarracino et al., 2014). Conditionally replicating lentiviral vectors with limited tissue tropism can be specifically pseudotyped (Delzor et al., 2013; Sandrin et al., 2003) to effectively deliver shRNA into experimental animals. The present study demonstrated the use of RNAi against IBRV via shRNA delivered by a pseudotyped lentivirus under the U6 promoter to silence the gD gene, distinct from the siRNAs used in previous studies (Cuadras et al., 2006; Diaz-Salinas et al., 2013; Gutierrez et al., 2010; Silva-Ayala et al., 2013). Unlike the siRNAs targeting the UL25 gene of BHV-1 to inhibit viral multiplication, shRNA can be loaded with the GFP gene to identify positive cells. gD shRNA recombinant lentiviral vectors are constructed and packaged into viruses. The viruses infect cells to resolve the defect of the low efficiency of plasmid transfection. In our study, shRNAs were better at inhibiting virus multiplication *in vitro*, as demonstrated by the virus titers ($TCID_{50}$) determined in cell culture. In this experiment, these shRNAs were screened for the inhibition of transient expression of a gD-FLAG fusion protein in the 293T

cells. It was observed that H1-shRNA-121 partially blocked the expression of a gD fusion protein, while H1-shRNA-304 completely silenced gD in the 293T cells.

Biotechnology and pharmaceutical companies have focused on the development of RNAi therapeutics for viral infections, cancer, hypercholesterolemia, cardiovascular disease, macular degeneration, and neurodegenerative diseases (Perrimon, Ni and Perkins, 2010). Based on our results, lentivirus-encoded shRNA (H1-shRNA-304) could be further evaluated for the prevention of IBRV in IBRV-sensitive calves. Safety, duration of the silencing effect, and potency should be evaluated in larger animal models.

Consequently, this study demonstrated that shRNAs targeting the gD gene degraded viral RNA and inhibited viral replication. This approach merits further investigation as a potential therapeutic option to prevent the morbidity and mortality associated with rotavirus infections in livestock and humans.

Acknowledgements

This study was partially supported by grants from Taishan Scholar and Distinguished Experts from Overseas (H.H.), an earmarked fund for the China Agriculture Research System (CARS-37, H.H.), the National Natural Science Fund of China (31272586 to H.H., 31302129 to S.L., 31302095 to W.J., 31502064 to H.P.), the Shandong province Key R&D program Fund (2015GNC113006), the Open Subjects for the Major Basic Research Program of Science and Technology Department of Inner Mongolia Autonomous Region (H.H.), the Cultivation of Major Scientific and Technological Achievements Foundation of Shandong Academy of Agricultural Sciences (2015CGPY02), the technological innovation key projects of Shandong Academy of Agricultural Sciences (2014CXZ08-3), the Shandong Agricultural Significant Application and Technological Innovation Fund (H.H., W.H., Z.J.), the State Key Laboratory of Veterinary Biotechnology (SKLVBF201510), the National Major Breeding Program of Genetically Modified Organisms (2014ZX08008-004), the Promotive Research Found for Excellent Young and Middle-aged Scientists of Shandong Province (BS2014SW008), the Youth Scientific Research Found of Shandong Academy of Agricultural Sciences (2014QNM17), the Shandong Provincial Natural Science Foundation of China (BS2015SW014) and the Natural Science Fund of Shandong Province (ZR2010CM012, ZR2010ZR029, ZR2014CP029, ZR2015PC007).

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บทคัดย่อ

RNA interference สำหรับ glycoprotein D ยับยั้งการเพิ่มจำนวนของเชื้อไวรัส infectious bovine rhinotracheitis virus ในเซลล์เพาะเลี้ยงเชื้อชนิด MDBK

หลิง หลิง เซิน[1] หุย จาง[2] ฮอง เหมย วาง[2] เปย หลี ฮัว[2] ม่าน สือ หยา[3*] ฮอง บิน ฮี[3*]

โรค Infectious bovine rhinotracheitis (IBR) เป็นโรคติดเชื้อไวรัสเฉียบพลัน เกิดจาก เชื้อไวรัส infectious bovine rhinotracheitis virus (IBRV) เนื่องจากการใช้วัคซีนป้องกันโรค IBRV ให้ผลในการป้องกันโรคไม่เต็มประสิทธิภาพ ดังนั้นการรักษาโดยใช้การต้านไวรัส เช่น RNA interference (RNAi) จึงมีการพัฒนาและนำไปใช้ในเชื้อไวรัสหลายชนิด การศึกษาครั้งนี้พบว่า RNAi สามารถยับยั้งการเพิ่มจำนวนของเชื้อ IBRV โดยหยุดการสร้าง glycoprotein ของเชื้อไวรัส การศึกษาครั้งนี้ได้สร้าง recombinant lentiviral vector จำนวน 2 ชนิด ได้แก่ vector ที่ประกอบด้วย short hairpin RNAs (shRNAs) (H1-RNA-121, H1-RNA-304) สำหรับยับยั้ง glycoprotein gD ของเชื้อไวรัส IBRV และ pcDNA3-gD ที่ประกอบด้วย FLAG tag จากนั้นนำ vector ทั้ง 2 ชนิดมานำเข้าในเซลล์ชนิด 293T และได้ตรวจสอบประสิทธิภาพของ RNAi ด้วยวิธี Western blotting ผลการศึกษาพบว่า H1-RNA-304 สามารถยับยั้งการสร้างโปรตีน FLAG-tagged gD และเมื่อนำ recombinant lentiviral vector มาเข้าในเซลล์ชนิด MDBK และทดสอบกับเชื้อไวรัส IBRV พบว่า H1-shRNA-304 สามารถยับยั้งการแสดงออกของยีน gD และยับยั้งการเพิ่มจำนวนของเชื้อไวรัส IBRV ผลการศึกาาครั้งนี้แสดงให้เห็นว่า shRNAs สำหรับยีน gD มีคุณสมบัติในการต้านไวรัส โดยยับยั้งการเพิ่มจำนวนของเชื้อไวรัส IBRV ดังนั้นจึงมีความเป็นไปได้ในการนำ RNAi มาใช้ทางคลินิกในอนาคต

คำสำคัญ: RNAi เชื้อไวรัส infectious bovine rhinotracheitis virus shRNA Gd

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