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Short communication

MiR-214 inhibits snakehead vesiculovirus (SHVV) replication by targeting host GS

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that have been reported to play important roles in virus replication. Snakehead vesiculovirus (SHVV) is a new rhabdovirus isolated from diseased hybrid snakehead and has caused heavy economical losses in cultured snakehead fish in China. Our previous study has revealed that miR-214 inhibited SHVV replication, but the underline mechanism was not completely understood. In this study, glycogen synthase (GS) gene was identified as a target gene of miR-214. Overexpression of miR-214 reduced cellular GS gene expression. Knockdown of GS by siRNA, similar to the overexpression of miR-214, inhibited SHVV replication. Moreover, we found that siGS-mediated inhibition of SHVV replication could be restored by reducing cellular miR-214 level via using miR-214 inhibitor, indicating that miR-214 inhibited SHVV replication at least partially via targeting GS. This study provided information for understanding the molecular mechanism of SHVV pathogenicity and a potential antiviral strategy against SHVV infection.

1. Introduction

MicroRNAs (miRNAs) are endogenous ~22 nt noncoding RNAs that play important role in almost all biological processes by binding to the 3′ untranslated regions (UTRs) of target mRNAs with their "seed sequences" (2–8 nt at the 5′end) [1], including cell differentiation, cell proliferation, oncogenesis, development, apoptosis, and immune defense [2–9]. Nowadays, growing evidences have suggested that miRNAs also played a pivotal role in the regulation of virus replication by targeting viral genes or host genes associated with virus replication [10–14]. Therefore, the study of miRNA-mediated virus-host interactions is important to understand the mechanism of virus pathogenesis.

Snakehead vesiculovirus (SHVV), which belongs to the genus *Perhabdovirus*, family *Rhabdoviridae* [15], has caused serious economic losses in snakehead fish culture in China [16]. The genome of SHVV consists of a ~11 Kb unsegmented negative-sense RNA molecule, which encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase protein (L) [16]. Our previous study has revealed that SHVV infection downregulated miR-214 [17], and in turn, miR-214 inhibited SHVV by targeting viral genes and host AMP-activated protein kinase (AMPK) [18,19].

In this study, glycogen synthase (GS) that mediated the synthesis of glycogen from glucose was identified as another target gene of miR-214. Knockdown of GS by siRNA (siGS) inhibited SHVV replication, which was similar to the overexpression of miR-214. Moreover, siGS-mediated inhibition of SHVV replication could be restored by reducing cellular miR-214 level via adding miR-214 inhibitor, suggesting that miR-214 inhibited SHVV replication by targeting host GS. This study will help understand the molecular mechanism of SHVV pathogenicity.

2. Materials and methods

2.1. Cells and viruses

Striped snakehead (SSN-1) cell was maintained at 25 °C in minimum essential medium (MEM) (HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, New Zealand),

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penicillin (100 μg/ml), and streptomycin (100 μg/ml). SHVV was isolated from diseased hybrid snakehead fish and stored at −80 °C.

2.2. Reagents and antibodies

The miR-214 mimic, miR-214 inhibitor, negative control (NC) mimic, and NC inhibitor were purchased from GenePharma (Shanghai, China). Their sequences were previously described [18,19]: miR-214 mimic, 5′-ACAGCAGGCACAGACAGGCAG-3' (forward) and 5′-GCCUG UCUGUGCCUGCUGUUU-3' (reverse); miR-214 inhibitor, 5′-CUGCCUG UCUGUGCCUGCUGU-3'; NC mimic, 5′-UUCUCCGAACGUGUCACG UTT-3' (forward) and 5′-ACGUGACACGUUCGGAGAATT-3' (reverse); NC inhibitor, 5′-CAGUACUUUUGUGUAGUACAA-3'. Two siRNAs for GS were synthesized from GenePharma (Shanghai, China). The sequences of the first one were: 5′-GCAUGGAGGUGGCUAAUATT-3′ (forward) and 5′-UAUUAGCCACCUCCCAUGCTT-3′ (reverse); the sequences of the second one were: 5′-CCAUCGAAGCUGAACACUUTT-3′ (forward) and 5′-AAGUGUUCAGCUUCGAUGGTT-3′ (reverse).

The antibodies against G protein of SHVV and GS were produced and stored in our laboratory. The antibody against β-actin was purchased from Bioss Biotechnology Co., LTD. (Beijing, China). The secondary antibody donkey anti-rabbit IgG antibody was purchased from Gene Co., LTD. (Shanghai, China).

2.3. Plasmids

The luciferase reporter plasmid pmirGLO-GS was constructed by amplifying the miR-214 target sequence (~200 nt) in the 3′ UTR of GS and cloning into vector pmirGLO with primers listed in Table 1. The plasmids pmirGLO-GS-MUT was generated by PCR-mediated mutation into plasmid pmirGLO-GS using primers listed in Table 1.

2.4. Transfection

The mimics, inhibitors, or plasmids were incubated with TransIntroTM EL Transfection Reagent (TransGen Biotech, China) in 500 μl Opti-MEM medium (Invitrogen, USA) for 30 min at room temperature. The incubated samples were then put onto the SSN-1 cells. At 6 h (h) post of transfection, the medium was replaced by 1 ml of MEM and continued incubation at 25 °C.

2.5. Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed as described previously [18,19]. In brief, SSN-1 cells were co-transfected with NC mimic, miR-214 mimic, NC inhibitor, or miR-214 inhibitor, together with the luciferase reporter plasmids using TransIntroTM EL Transfection Reagent (TransGen Biotech, China). At 24 h post of transfection, the *Renilla* and firefly luciferase activities were measured, and the data were expressed as relative firefly luciferase activity normalized to *Renilla* luciferase activity.

2.6. Virus infection and titration

Virus infection and titration experiments were performed as previously described [18,19]. In brief, SSN-1 cells were incubated with SHVV for 2 h, the inoculum was then removed and the cells were washed twice with PBS followed by adding MEM with 5% FBS. At 24 h post of infection (poi), the supernatants were collected for virus titration by 50% tissue culture infectious dose ($TCID_{50}$), and the cells were harvested for the detection of viral mRNA by qRT-PCR with primers listed in Table 1.

2.7. Quantitative RT-PCR

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen) according to manufacturer's instructions. The detection of viral G mRNA and host GS mRNA was performed by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method as previously described [18,19]. The data was normalized to the level of β-actin in each sample.

2.8. Western blot

Western blot was performed as previously described [18,19]. In brief, the extracted proteins were transferred onto a nitrocellulose membrane (Biosharp, China), which were blocked with 5% skim milk in tris-buffered saline with tween 20 (TBST) at 4 °C overnight, followed by incubation with the primary antibody (1:1000) for two hours at room temperature. The membranes were then washed three times with TBST and then incubated with IRDye 800CW conjugated donkey anti-rabbit antibody (1:10000) for one hour at room temperature. The signal intensity was then determined using Odyssey CLx (LI-COR, USA).

2.9. Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, CA). The statistical significance of the data was determined by Student *t*-test, and $P < 0.05$ was considered statistically significant. For data sets in which multiple comparisons were being made, the student's T-test was corrected by using false discovery rate.

3. Results and discussion

3.1. GS gene is the target gene of miR-214

Our previous study has identified host AMPK as a target gene of miR-214 [18]. In addition, the host GS gene was also predicted to be a potential target gene of miR-214 (Fig. 1A). To verify whether GS was the target gene of miR-214, we first constructed a dual-luciferase reporter plasmid pmirGLO-GS containing the wild-type sequence of the 3' UTR of GS. Based on the plasmid pmirGLO-GS, we generated a mutant plasmid pmirGLO-GS-MUT, in which the miR-214-targeted sequence was mutated (Fig. 1A). These plasmids were subsequently transfected

Table 1
Primers used in this study.

Application	Primer	Sequence (5′-3′)
qRT-PCR	SHVV-G-FW	ACACCATACATGCCAGAGGC
	SHVV-G-BW	GCCTCGCTGGGTATCCAAAT
	GS-FW	CACTCGCTCCATTCATCG
	GS-BW	AGGTAGGTCGCCTTCCAG
	β-actin-FW	CACTGTGCCCATCTACGAG
	β-actin-BW	CCATCTCCTGCTCGAAGTC
Reporter plasmids	GS-FW	CTAGCTAGCCAGAGGAACCGCACAG
	GS-BW	GCTCTAGATCACAGCAAGGCAACG
	GS-MUT-FW	CACAGAACGCCTCAGTGAGGACGACGACTGGAGATATCTGGGC
	GS-MUT-BW	GTCGTCCTCACTGAGGCCTTCTGTGCG