Fish and Shellfish Immunology 84 (2019) 299–303

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

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

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ARTICLE INFO

Keywords:
Nakehead vesiculovirus
microRNA
miR-214
Replication
Glycogen synthase

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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https://doi.org/10.1016/j.fsi.2018.10.028
Received 25 May 2018; Received in revised form 19 August 2018; Accepted 8 October 2018
Available online 09 October 2018
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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

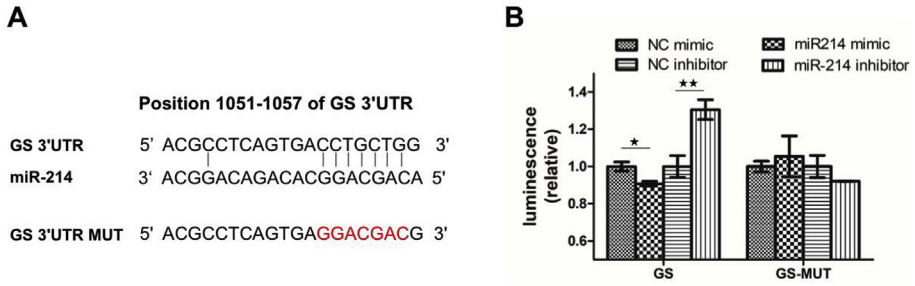

Fig. 1. MiR-214 targets the 3′ UTR of GS mRNA. (A) Alignment of miR-214 with the predicted target sequences in the 3′ UTR of GS mRNA. (B) SSN-1 cells were transfected with pmirGLO-GS or pmirGLO-GS-MUT, together with NC mimic, miR-214 mimic, NC inhibitor, or miR-214 inhibitor. Luciferase activity was measured at 24 h post of transfection, and the data was expressed as relative firefly luciferase activity normalized to Renilla luciferase activity. All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

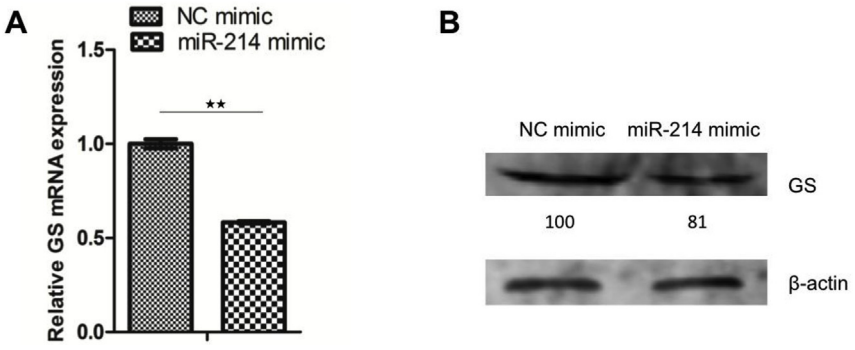

Fig. 2. The effect of miR-214 on the expression of cellular GS. SSN-1 cells were transfected with NC mimic or miR-214 mimic for 24 h (A) GS mRNA in SSN-1 cells was measured using qRT-PCR. β-actin was used as the internal control. All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$). (B) GS Protein was determined by western blot. β-actin was used as the internal control. The integrated optical densities of the protein bands were measured using Image-Pro Plus 6.0. The value of GS protein band was normalized to that of β-actin. The value of the GS protein band in cells transfected with NC mimic was set as 100.

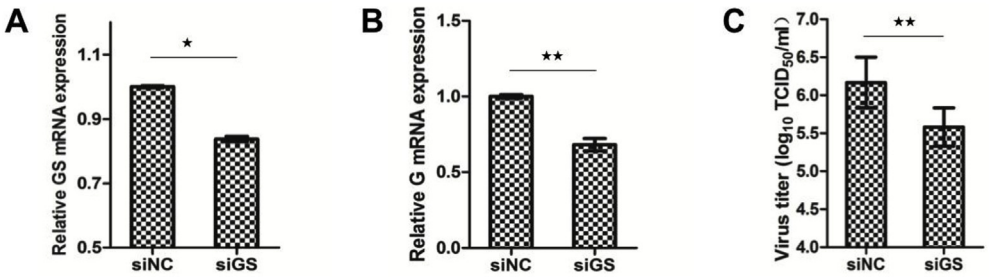

Fig. 3. Knockdown of GS inhibits SHVV replication. (A) SSN-1 cells were transfected with siNC or siGS, the GS mRNA in SSN-1 cells was measured at 24 h post of transfection using qRT-PCR. β-actin was used as the internal control. (B, C) SSN-1 cells were transfected with siNC or siGS, followed by SHVV infection. G mRNA (B) in SSN-1 cells was measured using qRT-PCR at 24 h poi. β-actin was used as the internal control. The SHVV titers (C) in the supernatants were measured using $TCID_{50}$ at 24 h poi. All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

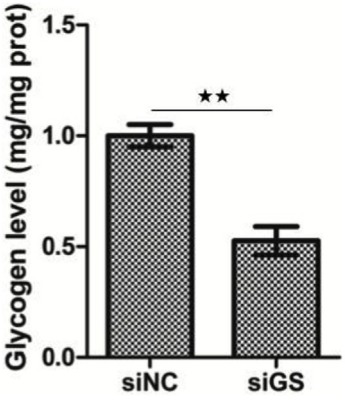

Fig. 4. Knockdown of GS inhibits glycogen production. SSN-1 were transfected with siNC or siGS, the glycogen production in SSN-1 cells was measured at 24 h post of transfection using Glycogen Assay Kit (Solarbio No: BC0340). All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

into SSN-1 cells with miR-214 mimic, NC mimic, miR-214 inhibitor, or NC inhibitor, respectively. Significant reduction in luciferase activity was observed in cells co-transfected with miR-214 mimic and the plasmid pmirGLO-GS, whereas significantly increased luciferase activity was detected when transfected with miR-214 inhibitor (Fig. 1B). However, the luciferase activity was not significantly altered when miR-214 mimic or inhibitor was co-transfected with the mutant plasmid pmirGLO-GS-MUT (Fig. 1B). These results indicated that GS was a target gene of miR-214. To further confirm that GS was the target gene of miR-214, we determined the effect of miR-214 on cellular GS expression (Fig. 2). SSN-1 cells were transfected with miR-214 mimic or NC mimic, followed by the detection of cellular GS mRNA or protein (Fig. 2). We found that transfection with miR-214 mimic significantly reduced cellular GS mRNA and protein levels (Fig. 2), suggesting that GS was indeed a target gene of miR-214.

3.2. Knockdown of GS inhibits SHVV replication

Our previous studies have revealed that overexpression of miR-214 inhibited SHVV replication [17–19]. In this study, we use siRNA to knockdown GS expression and then evaluated the effect on SHVV replication. SSN-1 cells were transfected with siGS or siNC, followed by the detection of GS mRNA at 24 h post of transfection. The results showed that transfection of siGS significantly reduced the expression of GS compared to that transfected with siNC (Fig. 3A). The effect of siGS on SHVV replication was further determined. SSN-1 cells were transfected with siGS or siNC, followed by SHVV infection. The viral G

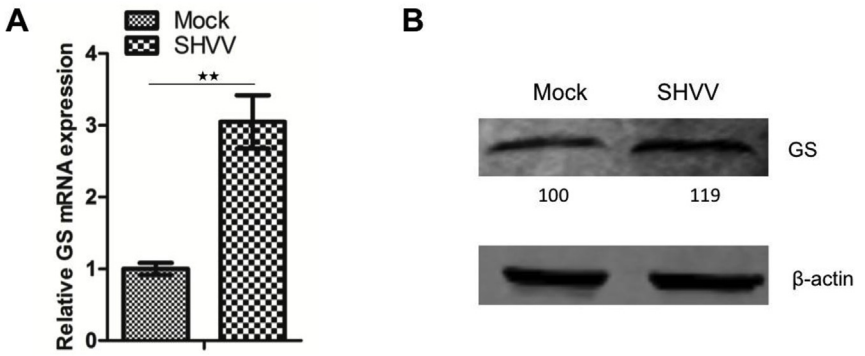

Fig. 5. SHVV infection upregulates GS. SSN-1 cells were infected with SHVV and the cells were harvested at 24 h poi. (A) The GS mRNA was determined by qRT-PCR, β-actin was used as the internal control. All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$). (B) The GS protein was determined by western blot, The integrated optical densities of the protein bands were measured using Image-Pro Plus 6.0. The value of GS protein band was normalized to that of β-actin. The value of the GS protein band in mock-infected cells was set as 100.

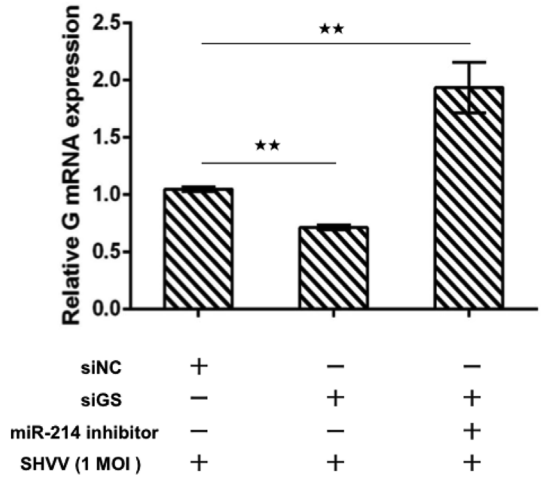

Fig. 6. Reducing cellular miR-214 can restore siGS-mediated inhibition of SHVV replication. SSN-1 cells were transfected with siNC, siGS, or siGS with miR-214 inhibitor, followed by SHVV infection. The G mRNA was determined by qRT-PCR at 24 h poi. β-actin was used as the internal control. All the data are representative of at least two independent experiments, with each determination performed in triplicate (mean ± SD). The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

mRNA and viral titer were detected at 24 h poi. As shown in Fig. 3B and C, the viral G mRNA was significantly reduced in siGS transfected cells than in siNC transfected cells. Similarly, the viral titer was also significantly decreased in siGS group than in siNC group (Fig. 3C). It can thus be suggested that knockdown of GS, similar to the overexpression of miR-214, inhibited SHVV replication.

GS mediates the metabolism of glucose that has been reported to be involved in virus replication [20]. In this study, the effect of siGS on glycogen production was evaluated. The results showed that the glycogen production was significantly reduced in siGS group than in siNC group (Fig. 4). However, whether the siGS-mediated inhibition of SHVV replication was due to the reduction of glycogen production was unclear and the related mechanism needed further investigation.

3.3. SHVV infection upregulates GS

To study the effect of SHVV infection on GS expression, SSN-1 cells were infected with SHVV and the cells were harvested at 24 h poi. The GS mRNA and protein were determined by qRT-PCR and western blot. We found that cellular GS mRNA and protein were both significantly increased upon SHVV infection (Fig. 5). As the knockdown of cellular GS inhibited SHVV replication (Fig. 3B and C), it suggested that lower level of cellular GS was disadvantageous for SHVV replication. Thereby, the increased expression of GS upon SHVV infection was beneficial for SHVV replication. Taken into consideration of our previous study, in which SHVV infection downregulated miR-214 [17], it was hypothesized that SHVV infection downregulated miR-214, and then the downregulated miR-214 led to the upregulation of GS expression.

3.4. Reducing cellular miR-214 can restore siGS-mediated inhibition of SHVV replication

In order to figure out whether miR-214-mediated inhibition of SHVV replication was associated with targeting GS, SSN-1 cells were transfected with siNC, siGS, or siGS with miR-214 inhibitor, followed by SHVV infection. The viral G mRNA was determined at 24 h poi. As shown in Fig. 6, siGS reduced G mRNA level to about 60% compared to that in siNC group. However, addition of miR-214 inhibitor restored the G mRNA level to 180%. Overall, these findings indicate that miR-214 inhibits SHVV replication at least partially due to its targeting host GS.

Our previous study has revealed that miR-214 could inhibit SHVV replication by targeting host AMPK [18]. Similar with GS, AMPK was also a regulator of energy metabolism. In addition, AMPK was a regulator of IFN-α expression even through the underline mechanism was unclear. Here, we also evaluated the effect of GS on the expression of IFN-α during SHVV infection. The results showed that knockdown of GS did not significantly alter IFN-α expression (data not shown). Overall, although the underline mechanism on how GS regulated SHVV replication was unclear, it was solid that miR-214 could inhibit SHVV replication by targeting host GS.

4. Conclusion

In this study, we identified host GS as another target gene of miR-214 and the positive regulator of SHVV replication. Moreover, our data suggested that the targeting host GS gene by miR-214 could be the cause of miR-214-mediated inhibition of SHVV. However, further *in vivo* studies are needed to understand the relationship between miR-214 and GS during SHVV infection in snakehead fish.

Acknowledgments

This work was jointly supported by National Natural Science Foundation of China (31602195, 31572657), the Fundamental Research Funds for the Central Universities (2662018QD017), the Open Project Program of the Key Laboratory of Fishery Drug Development, Ministry of Agriculture (201501), and Natural Science Foundation of Hubei Province (2016CFB233).

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