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Gene Silence of Envelope Protein 53R and Iridovirus Resistance in Fish Cell by amicroRNA of the New Structure

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Abstract We devised artificial microRNAs (amiRNAs) that targets a viral envelope protein gene RGV 53R, by incorporating sequences encoding amiRNAs specific to 53R of RGV into pre-miRNA155 (pSM155) vectors, which could intracellularly express 53R-targeted pre-miRNAs (miR-53Rs) in fish cell line. The levels of 53R expression were analyzed through real-time PCR and RGV virions assembly were observed by electronic microscopy in fish cells transfected with or without miR-53Rs at 72h of RGV infection.

The results argue that viral envelope protein RGV 53R can be silenced and lead the virions assembly deficiency by miR-53R-1, and identify the first amiRNA of envelope protein gene from iridovirus was able to resist virus infection in fish cell. The data present evidence demonstrating that the viral infection is efficiently suppressed (58%) by miR-53R-1 targeting RGV 53R. Moreover, electron microscopic observations revealed RGV virions assembly defect or reduced virions assembly capacity closely were correlated with expression of miR-53R-1. Real-time PCR was performed, as expression of miR-53R-1 is no caught to activate IFN-related pathways correlating with Mx up-regulation.

Key words microRNA, gene silence, RGV

Introduction

Iridoviruses are one of significant viral pathogens for aquatic animals that have caused great economic losses in aquaculture industry worldwide [7]. Rana grylio virus (RGV), a member of iridoviruses, causes systemic infectious iridoviral disease in cultured pig frog (Rana grylio) [10]. To date, functional genes such as deoxyuridine triphosphatase (dUTPase), thymidine kinase (TK), 3β-hydroxysteroid dehydrogenase (3β-HSD) have been identified from RGV in our laboratory [5, 11]. Recently, an envelope protein gene, RGV 53R that has homologes in all sequenced iridoviruses was cloned and characterized from RGV. The structural conserved features shared by iridovirus envelope proteins, the intracellular distribution and dynamic changes in the transfected cells revealed that 53R involved in virus infection and assembly [12]. Therefore, RGV 53R was selected to understand gene silencing by artificial microRNAs (A miRNAs) in RGV infection.

Discovery of the RNA interference (RNAi) pathway has led to exciting new strategies for developing virus diseases treatment. To date, siRNAs and miRNAs have been used for silencing expression of the major capsid protein (MCP) encoded by red seabream iridovirus (RSIV), an iridovirus causing severe disease in fish [3, 8] but it has not been demonstrated that viral envelope protein may be used to target for RNAi. RNA interference is a process of post-transcriptional sequence specific gene silencing in many eukaryotes. The use of RNAi to inhibit virus, including siRNAs and miRNAs, offers a new approach for controlling viral infections. Artificial miRNAs expression vectors such as splicing pre-miRNA30 (pSM30) and splicing pre-miRNA155 (pSM155) vectors which

were designed to targets in ORF of endogenous and exogenous genes have been developed and used for RNA interference [2, 4, 9]. The appearance of plasmid-based expression systems that is effective and inexpensive for miRNAs generating present rational way for the design and expression of 53R targeted artificial miRNA.

In the present study, we devised artificial microRNAs (amiRNAs) with novel stem structures that targets different positions of RGV 53R to investigate whether viral envelope protein gene silencing and iridovirus resistance mediated by the amiRNAs in fish cells.

Materials and Methods

Cell lines and virus Rana grylio virus (RGV), a strain of frog iridovirus and grass carp ovaries (GCO) and epithelioma papulosum cyprinid (EPC) cells, two fish cell lines were used in this study.

AmiRNAs design and plasmids construction Three pairs of oligonucleotides encoding 53R-specific miRNAs of RGV (referred as miR-53R-1, miR-53R-2 and miR-53R-3) (Table 1), and a pairs of oligonucleotides corresponding to PB2 gene of avian influenza virus, AIV (referred as miR-PB2), were designed using the BLOCK-iTTM RNAi Designer/miR RNAi (http://rnaidesigner.invitrogen.com). Each oligonucleotide pair ("top strand" and "bottom strand" oligos) was annealed and ligated into the pre-miRNA155 (pSM155) vector[1, 4] to create plasmids (pSM155-miR-53Rs and pSM155-miR-PB2) capable of producing 53R and PB2 encoded pre-miRNAs in plasmid transfected cells. The 53R-expressing plasmid (pEGFP-N3-53R) constructed in previous studies[6, 12] was used in co-transfection experiments to express the target 53R.

Table 1. Oligonucleotides sequence encoding 53R-specific pre-miRNAs

Name	Strand	Oligo sequence	Position of target in gene
miR-53R-1	Top	TGCTGTGACAGTGTTGATAGATTCCGGTTTTGGCCACTGACTGACCGGAATCTAAACACTGTCT	36-57
	Bottom	CCTGAGACAGTGTTTAGATTCCGGTCAGTCAGTGGCCAAAAC**CGGAATCTATCAACACTGTCA**C	
miR-53R-2	Top	TGCTGTACTGAGGATGTTTGCAAACTGTTTTGGCCTCTGACTGACAGTTTCAAAATCCTCAGTT	476-498
	Bottom	CCTGAACTGAGGATTTTGAAACTGTCAGTCAGAGGCCAAAA**CAGTTTGCAAACATCCTCAGTT**C	
miR-53R-3	Top	TGCTGTTGACAGTGTTGATAGATTCCGTTTTGGCCACTGACTGACGGAATCTATACACTGTCAT	37-58
	Bottom	CCTGATGACAGTGTATAGATTCCGTCAGTCAGTGGCCAAAAC**GGAATCTATCAACACTGTCAA**C	

Bold letters represent sense sequences of engineered miRNAs derived from the target gene.

Cell culture and transfection GCO and EPC cells were seeded into 12-well or 6-well cell culture plates using 199 medium containing 5% of FBS before 24h of transfection. Cells were transfected with plasmids using Lipofectamine 2000 ("Invitrogen") following the manufacturer's protocol. The transfection mixtures were removed after 6h of transfection, and transfected cells were maintained for further processing.

amiRNAs expression and anti-RGV activity detection To select the miR-53R that has the best inhibition efficiency, the miR-53Rs were initially tested for sequence-specific silencing on the target 53R gene by employing transient transfection of a plasmid expressing 53R (pEGFP-N3-53R). GCO cells were co-transfected with pSM155-miR-53R-1/pEGFP-N3-53R, pSM155-miR-53R-2/ pEGFP-N3-53R and pSM155-miR-53R-3/pEGFP-N3-53R, respectively (Fig. 1-a). At 72h after transfection, total RNA was extracted from transfected cells and reverse transcribed to cDNA for real-time PCR analysis. Cells transfected with pSM155-miR-PB2/pEGFP-N3-53R were used as negative control.

To elucidate antiviral effects of miR-53R-1 on RGV replication, the expression of 53R gene was monitored in cells transfected with pSM155-miR-53R-1 and infected with RGV (Fig. 1-b). The pSM155-miR-PB2 was used for negative control. cDNA obtained by method as above were used for real-time PCR analysis.

To assess inhibitory effect of miR-53R-1 on RGV replication in terms of production of viral particles, monolayer of GCO cells seeded in 96-well plates was inoculated with serial 10-fold dilutions of RGV samples for TCID50 detection.(Fig. 1-c)

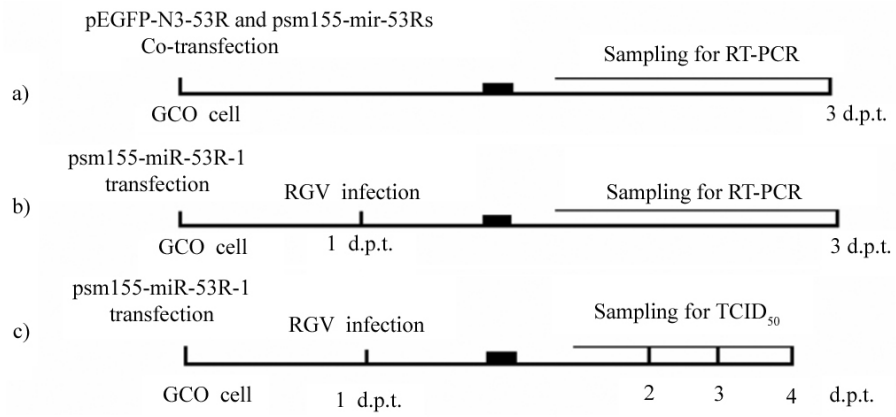

Fig.1. Experimental schemes of amicroRNA

Electron microscope observation EPC cells were transfected with pSM155-miR-53R-1 and infected with RGV. After 72h of RGV infection, cells were centrifuged at 2 000×g and the pellets were fixed in 2.5% glutaraldehyde in 0.1mol/L phosphate buffer (pH=7.4) for 1 day, rinsed in 0.1mol/L phosphate buffer containing 1% osmium tetroxide for 1h at 4℃, dehydrated in a graded ethanol series and embedded in Spurr's resin. The cell pellets were post-fixed with 2% osmium tetroxide in sodium cacodylate buffer for 1 to 2h, dehydrated in a graded acetone series, and embedded in LR white acrylic resin. Ultra-thin sections were post-stained with 2% uranyl acetate and 3% lead citrate and observed at 100kV with a "JEOL 1230" transmission electron microscope.

Mx expression detection The expression level of Mx was investigated by real time PCR in cells co-transfected with poly (I ∶ C)/pEGFP-N3-53R, pSM155-miR-PB2/pEGFP-N3-53R, pSM155-miR-53Rs/pEGFP-N3-53R, respectively. Control cells were transfected only with Lipofectamine 2000 as well as in cells transfected with pEGFP-N3-53R.

Real-time PCR RNA extracted by Trizol reagent ("Invitrogen") was treated with DNase I and added to the reverse transcription reaction solution containing 200U M-MLV reverse transcriptase ("Promega", USA) , 0.5mmol/L each of dNTP, 1×M-MLV reaction buffer, 500ng of oligo(dT)15Primer and 25U Rnasin ("TOYOBO").

Real-time PCR was performed with 2μL of cDNA in a final volume of 25μL containing 11.25μL of 2.5×RealMasterMix/20×SYBR solution("TIANGEN BIOTECH"), 300nmol/L of sense primer and antisense primer. PCR was carried out in the 7300 Fast Real-time PCR System ("Applied Biosystems"). Fragments were 244bp(53R), 120bp(β-actin), 250bp(Mx). The Fold Change(FC = $2^{-\Delta\Delta CT}$) in 53R and Mx mRNA expression levels were normalized to those of the β-actin transcripts measured with the same cDNAs.

Results

Expression of pSM155-miR-53Rs Three pairs of oligonucleotides encoding 53R-specific miRNAs of RGV (referred as miR-53R-1, miR-53R-2 and miR-53R-3), was annealed and ligated into the pre-miRNA155 (pSM155) vector to create plasmids (pSM155-miR-53Rs and pSM155-miR-PB2) capable of producing pre-miRNAs targeting 53R or PB2 gene and transfected in cells. The predicted structures of the engineered pre-miRNAs incorporated into the pSM155 backbone are shown in Fig. 2.

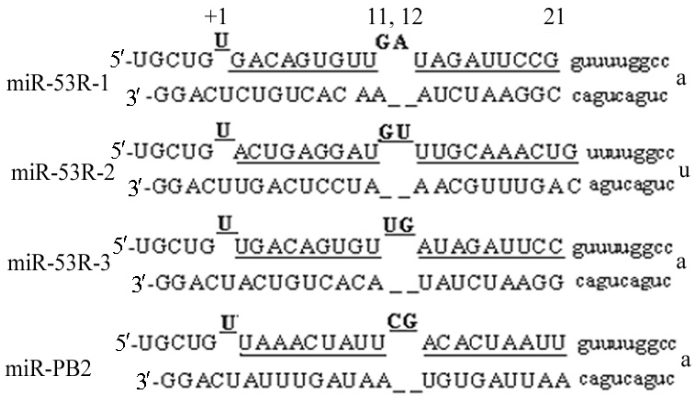

Fig. 2. Schematic presentation of predicted stem-loop sequences of pSM155-miR-53Rs

+1, 11, 12 and 21 are corresponded to positions in stem structure. The antisense of the target sequences to the ORF of 53R and the 3′ UTR of PB2 are underlined.

When pSM155-miR-53Rs were transfected into grass carp ovary (GCO) cells, they allowed co-cistronic expression of pre-miRNAs with GFP in cells under the control of the Pol II human CMV promoter. The co-cistronic expression of the pre-miRNAs was monitored microscopically under a fluorescence microscope.

Inhibitory effect of miR-53Rs on amicroRNA When GCO cells were co-transfected with pSM155-miR-53R-1/pEGFP-N3-53R, pSM155-miR-53R-2/pEGFP-N3-53R and pSM155-miR-53R-3/pEGFP-N3-53R, pSM155-miR-53Rs silenced the expression of the 53R gene in different levels with compared to the control that were co-transfected with pSM155-miR-PB2/pEGFP-N3-53R. 53R mRNA levels were evaluated in different groups using the real-time quantitative RT-PCR assay. The results indicated that expression of the 53R were reduced by 74% (FC=0.261±0.015), 56% (FC=0.443±0.043,) and 35% (FC=0.652±0.081) in cells transfected with pSM155-miR-53R-1, pSM155-miR-53R-2 and pSM155-miR-53R-3 at 72h, respectively (Fig. 3). The pSM155-miR-53R-1 was more efficiently inhibited 53R gene expression than pSM155-miR-53R-2 and pSM155-miR-53R-3. Thus, pSM155-miR-53R-1 was chose in our further studies on a miRNAs-mediated inhibition of RGV infection.

Delayed emergence of CPE of RGV in host cells by miR-53R-1 Cytopathic effect (CPE) is the most intuitionistic parameter that reflects the viral quantity of virus accumulation [19]. GCO cells were infected with RGV after transfected with pSM155-miR-53R-1 (miR-53R-1/RGV), CPE were markedly delayed than that were transfected with pSM155-miR-PB2 (miR-PB2/RGV), as well as only infected with RGV (Fig. 4).

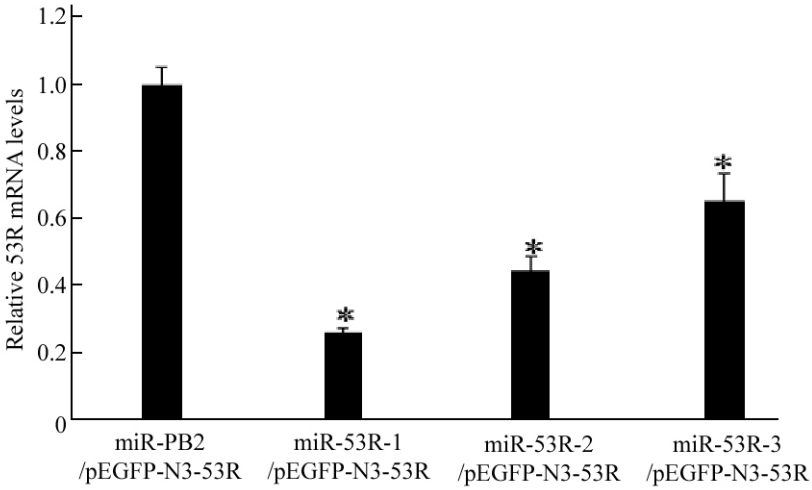

Fig. 3. Quantitative analysis of 53R mRNA levels in cells co-transfected with pSM155-miR-53R-1/pEGFP-N3-53R, pSM155-miR-53R-2/pEGFP-N3-53R and pSM155-miR-53R-3/pEGFP-N3-53R, respectively

53R mRNA from each group was measured by real-time PCR analysis 72h post transfection. Group co-transfected with pSM155-miR-PB2/pEGFP-N3-53R was used as negative controls. The value of negative control was designated as 1.0(n= 3). The values represent averages of three independent experiments, with the range indicated (±S.D).

* p<0.05 versus control

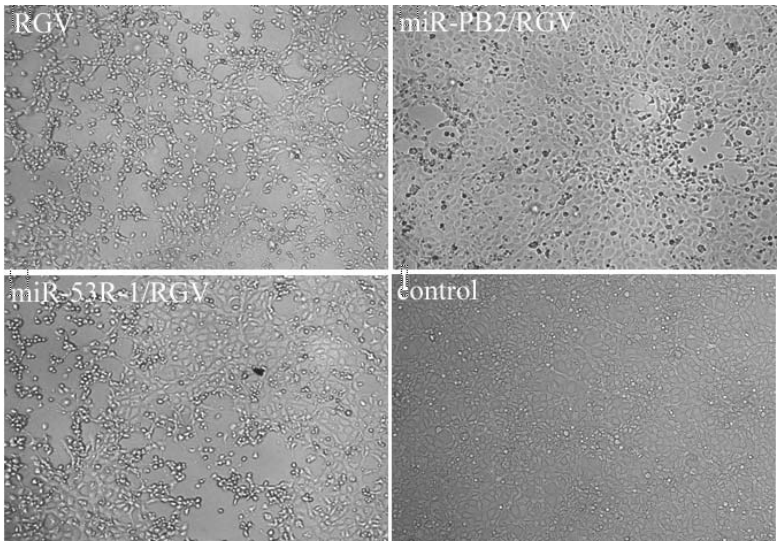

Fig. 4. Microscopical observation of RGV infected cells

Cells transfected with different pre-miRNA expression plasmids were infected with or without RGV at 24 h post transfection. Microscopy at 72h p.i. showed that few CPE existed in cells transfected with pSM155-miR-53R-1. But in cells transfected with pSM155-miR-PB2 or mock transfected, a large number of cells round up, detached from monolayer and many viral plaques appeared.

Reduction of RGV titer and virions by miR-53R-1 To test whether pSM-miR-53R-1 could impede the packaging and production of infectious RGV, pSM-miR-53R-1-transfected cells were infected with RGV at 24h of transfection. Cell supernatants were collected at 24, 48, 72 and 96 h.p.i. to determine the production of virions.

In $TCID_{50}$ assays, the average titers of RGV in pSM-miR-53R-1-transfected samples were about 21.38-fold, 12.59-fold, 46.77-fold and 4.68-fold lower than those of the transfected with pSM155-miR-PB2 at different time interval, respectively (Fig. 5).

Observation of RGV virions in ultrathin sections of epithelioma papulosum cyprini (EPC) cells by electron microscopy showed that larger numbers of RGV virions were assembled and arranged orderly in the cytoplasm of the host cells olny infected with RGV (Fig. 6-a). In pSM155-miR-53R-1-transfected EPC cells, several sporadic RGV virions were present in the cytoplasm with irregular arrangement and virions was significantly decreased (Fig. 6-b).

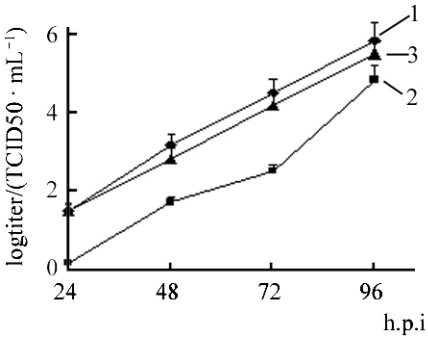

Fig. 5. Comparison of virus titers in cells with different treatment
1－RGV, 2－RGV+pSM155-miR-53R-1, 3－RGV+pSM155-miR-PB2

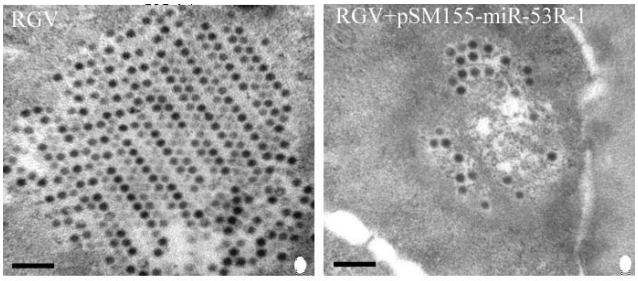

Fig. 6. Electron micrograph of RGV
In pSM155-miR-53R-1-transfected EPC cells, several sporadic RGV virions were present in the cytoplasm with irregular arrangement and RGV virions was significantly decreased (b). But in control cells only infected with RGV, crystalline aggregation contained large number of regular arranged virions (a).

RGV resistance by miR-53R-1 It has been shown that 53R silencing and RGV inhibition by pSM155-miR-53R-1, e.g. delayed emergence of CPE, or reduction of RGV titer and assembled virions in transfected fish cells, so 53R expression was assayed as the RGV inhibition efficiency in RGV infected GCO cells that transiently transfected with pSM155-miR-53R-1 or pSM155-miR-PB2 by real-time PCR. The data indicated that the 53R expression was reduced by 58% (FC=0.423±0.048) at 72h after infection of RGV in pSM-miR-53R-1 transfected cells compared with the pSM155-miR-PB2 transfected cells (Fig. 7). This showed clearly that 53R expression was suppressed by pSM-miR-53R-1.

Mx response by the expression of miR-53Rs Mx is key component of the interferon response. To determinate whether transfection of GCO cells with plasmids expressing 53R-targeted miR-53Rs and pEGFP-N3-53R resulted in induction of IFN response, mRNA levels of Mx was detected. There was no significantly change for expression of Mx gene in GCO cells that were transfected with

different pSM155-miR-53Rs (Fig. 8). These results indicated that the antiviral activity was induced by the expression of the pre-miRNAs with a novel stem structure.

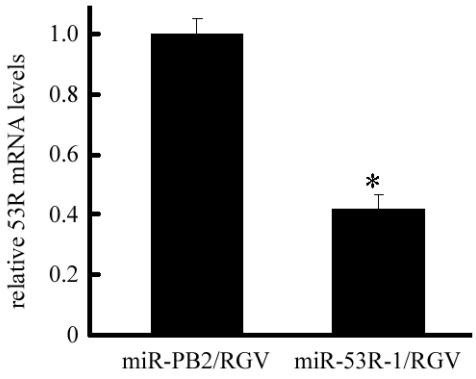

Fig. 7. Relative 53R mRNA levels in RGV infected cells

The value of pSM155-miR-53R-1 transfected cells was 0.423±0.048 when the negative control group (transfected with pSM155-miR-PB2) desigenated as 1.0. The values represent averages of three independent experiments, with the range indicated (±S.D).

* p<0.05 versus control.

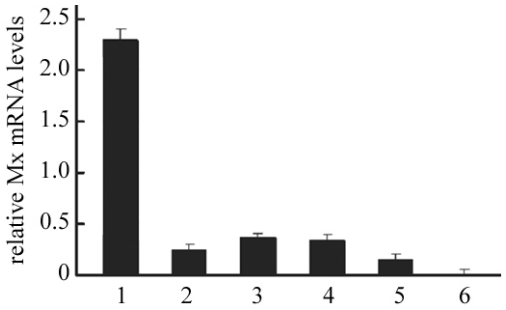

Fig. 8. Expression of the Mx in different transfected cells Quantitave real time PCR was used to measure 53R mRNA.

The value of the control (GCO cell) was designated as 0(n=3). No significant change for expression of Mx gene was observed when compared to the control. But Mx mRNA level was obviously increased in cells treated with poly I:C. The values represent averages of three independent experiments, with the range indicated (±S.D).

1－poly I:C/pEGFP-N3-53R,

2－miR-PB2/pEGFP-N3-53R,

3－miR-53R-1/pEGFP-N3-53R,

4－miR-53R-2/pEGFP-N3-53R,

5－miR-53R-3/pEGFP-N3-53R,

6－GCO cell.

Single or double stranded RNAs could active the immune response of host cells to resistant virus infection. So the AmiRNAs may active immune response, especially the interferon (IFN)-related pathways, to inhibit virus. But it was proved in this study that IFN response was not activated apparently by examining the Mx expression, which was a key component in IFN response. The pre-miRNAs used for RGV 53R inhibition had a mismatch at the +1 position and a bulge at the +11, +12 positions in its stem. It's different from the pre-miRNAs without a mismatch at the +1 position and a bulge at the +12, +13 positions which was found to activate IFN-related pathways [3, 5]. Different Mx inducing ability may be due to different structures of the pre-miRNAs.

Conclusion

AmiRNA with a novel stem structure targeting 53R gene of RGV could inhibit the expression of 53R gene by 75% and inhibit the production of virus in fish cells by 50%. There is no significant change for expression of Mx gene in fish cells that were transfected with amiRNAs(MiR-53Rs).

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