Fish miR-146a promotes Singapore grouper iridovirus infection by regulating cell apoptosis and NF-κB activation

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Abstract

miR-146a was reported to participate in various pathophysiological conditions in mammals, such as inflammation and immune responses, oncogenesis and cell damage. However, its function in low vertebrates has not been well elucidated. In this study, we characterized the expression profiles and functions of miR-146a in fish cells during iridovirus infection. We found that the reported fish miR-146a genes encoded an identical mature sequence, which shared high similarity with its mammalian orthologues, suggesting a putative functional conservation of miR-146a between fish and other vertebrates. Using a well-established infection model of Singapore grouper iridovirus (SGIV) in fathead minnow cells, we found that SGIV infection induced the expression of miR-146a to a dramatic extent. More importantly, we found that miR-146a promoted SGIV propagation, as demonstrated by higher expression of viral genes and increased virus titres in miR-146a-overexpressing cells. Mechanistically, we found that miR-146a overexpression suppressed, while miR-146a knockdown promoted, NF-κB activation and SGIV-induced cell apoptosis, two major cellular events involved in SGIV infection. Our study suggested that the induction of miR-146a by SGIV infection may function through a feed-forward mechanism to promote viral infection by restraining anti-viral cellular responses.

INTRODUCTION

MicroRNA (miRNA) is a class of small non-coding RNAs (ncRNAs), 18–25 nucleotides in length, which post-transcriptionally regulates gene expression through binding to the 3’UTRs (untranslated regions) of target mRNAs [1, 2]. Since its first discovery in the late 1990s, there has been increasing evidence demonstrating that this class of ncRNAs participates in various biological processes, including cell proliferation, differentiation, oncogenesis, immuno-inflammatory responses and cell apoptosis [3–7]. miR-146a is one of the first reported miRNAs to negatively regulate the immune signalling pathways [8–10]. For example, in chronic hepatitis B miR-146 is induced significantly, which causes hypo-responsiveness in T-cells by targeting STAT1, a key mediator of IFN-α signalling activation, and thus results in immune defects and immunopathogenesis during chronic viral infection [11]. Park et al. found that miR-146a actively participated in the determination of the fate of dendritic cells (DCs), the key cell type responsible for the activation of lymphocytes to fight against exogenous infection as well as subsequent restriction of excessive autoimmunity. They found that during the differentiation of human monocytes from DCs, the upregulation of miR-146a can repress the expression of Bcl-2, which in turn promotes DC apoptosis [12]. While most previous studies concerning miR-146a have focused on its regulation in immuno-inflammatory responses and cell fate determination during tumour development, its roles in viral infection and virus–cell interactions remain largely unclear.

Singapore grouper iridovirus (SGIV) is a high pathogenic double-stranded DNA virus isolated from cultural groupers [13]. It belongs to genus Ranavirus, family Iridoviridae, and has a broad infectivity in various cell types of different grouper tissues. After infection, SGIV can cause high mortality and it has caused huge economic losses to the aquaculture industry in China and Southeast Asia [14]. In the last decade, extensive studies have been performed to characterize the pathogenic viral genes related to SGIV infection, and these have greatly improved our understanding of the molecular characteristics of this viral pathogen [15–17]. However, the molecular mechanisms underlying this viral pathogen and the host/cell interactions still require extensive investigation.
Recently, by employing Solexa deep-sequencing technologies and miRNA microarray strategies, we found that a number of grouper miRNAs were differentially regulated after SGIV infection, which represented another layer of complexity of host and virus interactions [18, 19]. However, how these small RNA molecules participate in the regulation of the SGIV infection and the pathogenesis of this viral disease is still unknown.

In this study, we focused on miR-146a, given the high importance of its orthologues in mammals’ immune systems. We found that the grouper mature miR-146a sequence was identical to other reported fish miR-146a, and shared high homology with its mammalian orthologues. We confirmed that miR-146a was significantly upregulated in fish cells after SGIV infection. More importantly, overexpression of miR-146a promoted SGIV infection in fish cells dramatically, while miR-146a knockdown presented an anti-viral effect. Mechanistically, we found that miR-146a could inhibit cell apoptosis and repress the activation of NF-κB after SGIV infection. Our study provided new insights into the cellular response to SGIV infection, and suggested that manipulating cellular miRNA levels may represent a novel strategy to control SGIV infection.

RESULTS

miR-146a is upregulated by SGIV infection in fish cells

Our previous study found that miR-146a stood out as one of the most upregulated miRNAs after SGIV infection by performing a customized miRNA microarray on SGIV-infected grouper cells; however, its role in SGIV infection has not been studied [18]. In this study, we initially compared the grouper miR-146a sequence with other reported fish miR-146a, and found that fish miR-146a genes encoded an identical mature sequence which shared high similarity with its mammalian orthologues (Table 1). These findings suggested that fish miR-146a may possess conserved functions, as observed in other vertebrates. Meanwhile, it also offered a reason to study grouper miR-146a and SGIV interactions in non-host fish cell systems.

To further characterize the expression profile of miR-146a after SGIV infection, we infected fathead minnow (FHM) cells with SGIV, a well-established infection model in our laboratory, and examined the miR-146a expression by bulge–loop qRT-PCR assay. We found that miR-146a was induced up to 7-fold at 12 h post-SGIV infection, and up to >50-fold after 48 h of viral infection in FHM cells (Fig. 1). These data, together with our previous findings, demonstrated that miR-146a was a robustly upregulated miRNA during SGIV infection in fish cells.

**miR-146a facilitates SGIV replication in fish cells**

To examine whether miR-146a regulates SGIV infection in fish cells, we manipulated the miR-146a level in FHM cells by transfection of synthesized fish miR-146a mimics or miR-146a inhibitors. As shown in Fig. 2(a, b), the expression level of miR-146a increased or decreased significantly after transfection with fish miR-146a mimics (100 nM) or inhibitors (100 nM), respectively. After incubation with SGIV for 36 h, we found that transfection with miR-146a mimics at both 50 and 100 nM significantly increased the severity of the cytopathic effect (CPE) induced by SGIV, while transfection with miR-146a inhibitors at both 50 and 100 nM weakened the severity of the CPE.

Next, we quantitatively examined the expression levels of several SGIV functional genes after transfection with miR-146a mimics or inhibitors. The results showed that the expression levels of all detected viral genes, including SGIV ORF072 (MCP), ORF019R (dUTPase), ORF086R (ICP18) and ORF136 (LITAF), were significantly increased in 50 or 100 nM miR-146a mimic-transfected cells at 24 and 48 h after infection (Fig. 3a). Moreover, the expression levels of the viral genes between the 50 and 100 nM miR-146a mimic-transfected groups were similar at the same time points. In contrast, transfection of miR-146a inhibitors significantly inhibited the transcription of these viral genes in a concentration-dependent manner (Fig. 3b). Given that 100 nM of miR-146a mimics and inhibitors both showed no toxicity in FHM cells, we chose 100 nM in this study. As shown in Fig. 3(c, d), Western blotting assays showed that the protein synthesis of SGIV MCP was significantly enhanced in 100 nM miR-146a mimic-transfected cells, but significantly reduced in 100 nM miR-146a inhibitor-transfected cells.

Finally, we evaluated the effect of miR-146a on virus production. After transfection with control mimics, miR-146a mimics (100 nM), control inhibitors or miR-146a-specific inhibitors (100 nM), cells were incubated with SGIV for further virus titre assay. As shown in Fig. 3(e), the virus production of SGIV in miR-146a mimic-transfected cells

<table>
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miR-146a regulates SGIV-induced cell apoptosis through caspase-9- and caspase-3-dependent mechanisms

It was reported that cell apoptosis could be triggered by caspase-dependent and caspase-independent pathways, while in most cases caspases played vital roles in the initiation and progression of cell apoptosis [26]. Our previous studies found that SGIV infection induced intracellular caspase activity [23]. We next sought to determine whether miR-146a-regulated SGIV-induced apoptosis through affecting caspase activation. As shown in Fig. 6, SGIV infection markedly activated caspase-9 activity by 13–26 % (Fig. 6b), and caspase-3 activity by 9–26 % (Fig. 6c) 12 h to 48 h p.i. In contrast, miR-146a inhibitor transfection enhanced SGIV-activated caspase-9 activity by 13–38 % (Fig. 6b), and caspase-3 activity by 10–16 % (Fig. 6c). These data suggest that miR-146a regulated SGIV-induced cell apoptosis through caspase-9- and caspase-3-dependent mechanisms.

**DISCUSSION**

SGIV infection can activate multiple cellular signalling pathways, such as NF-κB, type I interferons (IFNs) and mitogen-activated protein kinases (MAPKs), in fish cells [27, 28]. By employing high-throughput sequencing technologies and other cellular and molecular strategies, we recently identified a number of host genes involved in SGIV infection [29–31]. These greatly improved our knowledge of apoptosis and thus regulates SGIV infection in fish cells, we transfected fish cells with miR-146a mimics or inhibitors (100 nM), infected them with SGIV and evaluated their apoptotic status. As shown in Fig. 6(a), SGIV infection induced the formation of apoptotic bodies (positive Hoechst 33342 staining spots) in FHM cells. In the miR-146a mimic-transfected group, apoptotic bodies decreased visibly compared to the control, but when miR-146a was mostly eliminated by specific inhibitors, an obvious aggregation of apoptotic bodies could be observed.

Next, we evaluated the effect of miR-146a on SGIV-induced cell apoptosis quantitatively by performing flow cytometric assays. In detail, we transfected FHM cells with control mimics and miR-146a mimics, and control inhibitors and miR-146a inhibitors at a concentration of 100 nM, and then infected cells with SGIV. At 24 and 48 h p.i., cells were stained with propidium iodide (PI) and flow cytometry analysis was applied. As shown in Fig. 5, in contrast to the control mimic group, there was a smaller population of apoptotic cells (M4 region) in the miR-146a mimic-transfected group, while more apoptotic cells were seen in the miR-146a inhibitor-transfected FHM cells, as compared to its control group after SGIV infection. These results quantitatively indicated that the induction of miR-146a by SGIV infection may negatively regulate apoptosis to achieve a beneficial cellular condition for efficient SGIV replication.
the cellular responses to SGIV infection. However, the underlying mechanisms by which this signalling is regulated, and what outcomes will arise from manipulating these pathways to control SGIV infection, remain far from clear.

In the present study, we found miR-146a was upregulated by SGIV infection in fish cells, and provided experimental evidence that upregulation of miR-146a may aggravate SGIV infection, mechanistically by affecting cell apoptosis and NF-κB activation.

Virus-induced cell apoptosis represents an important type of interaction between a virus and its host [32]. Our recent studies found that SGIV infection can induce typical apoptosis in FHM cells through diverse mechanisms. First, SGIV infection
can activate multiple MAPK pathways, such as extracellular-signaling-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 protein kinase, all of which were experimentally verified to contribute to SGIV-induced cell apoptosis [23, 27, 30]. Second, SGIV-encoded functional genes, exemplified by the lipopolysaccharide-induced TNF-α (LITAF)-like factor, can induce cell apoptosis through a death-receptor-mediated (or extrinsic) pathway [17]. More recently, we identified an SGIV-encoded miRNA, SGIV-miR-homoHSV, acting as a fine-tuning regulator of SGIV-induced cell apoptosis by repressing SGIV-LITAF expression [33]. All these findings showed cell apoptosis to be a stringently regulated cellular response after SGIV infection.

In this study, we found that miR-146a functions as an additional layer to regulate SGIV-induced cell apoptosis. This finding contributed to our knowledge of the mechanisms underlying cell apoptosis and SGIV infection. As is known, cell apoptosis plays complicated roles in the modulation of viral infection, which is largely attributable to its double-edged sword effects, as afforded by the mechanisms...
whereby apoptosis will not only weaken the holistic immunocompetence of the host cells, but also shrink the virophere of the virus [34–36]. Thus, delineation of the consequences of virus-induced apoptosis during viral infection is crucial for the development of apoptotic interventions based anti-viral approaches. Here we found that miR-146a inhibited cell apoptosis and demonstrated a beneficial effect on SGIV infection, as shown by higher viral replication and the production of infectious progeny viruses in miR-146a-introduced cells. These findings suggested that SGIV-induced expression of miR-146a functioned through a negative feedback mechanism to tone down the apoptosis, and thus provided a facilitating environment for continuous viral replication and production of progeny virions.

Unlike protein-coding genes that primarily function in one or several ways in a certain cellular event, miRNA can serve as a master regulator of multiple signalling pathways by targeting various target genes in the cell [37, 38]. In the present study, in addition to its role in regulating cell apoptosis, miR-146a possessed an anti-inflammatory capacity, as demonstrated by its inhibitory effects on NF-κB activation, the key mediator of pro-inflammatory and immune responses under various stress conditions [39]. Several previous studies have demonstrated that upregulated miR-146a caused by viral infection generally results in host immunosuppression, which in turn aggravates the development of viral lesions [40–42]. Our data were in accord with some previous reports, such as studies in chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection of hepatocytes [43, 44], and Japanese encephalitis virus (JEV) infection of human microglial cells [41], where the virus-induced miR-146a repressed the expression of genes involved in NF-κB activation, thus forming a negative feedback loop to dampen the anti-viral immune response, and ultimately facilitating immune evasion of the virus.

Meanwhile, fish miR-146a regulation of cell apoptosis and NF-κB activation may not be two parallel events after SGIV infection, as there is mounting evidence demonstrating the mutual interaction between NF-κB activation and apoptosis initiation [45, 46]. Whether fish miR-146a regulates these two processes independently or synergistically, and whether these two cellular events have a mutual influence on each other in the context of SGIV infection, requires further study. Further, given that NF-κB signalling is broadly involved in diverse pathophysiology processes, such as cell proliferation and differentiation, and given the complexity of effector cells during SGIV infection in vivo, there is also a possibility that other cellular events mediated by the NF-κB signalling pathway may also be regulated by miR-146a in fish cells during SGIV infection.

Our finding that fish miR-146a possesses a similar capacity to regulate cell apoptosis and NF-κB signalling compared to its homologues in other species, suggests an evolutionarily functional conservation of miR-146a in virus-induced cellular responses. However, we have to acknowledge that we have not identified the direct target(s) of miR-146a accounting for its regulation of the above cellular events in fish cells. With increasing knowledge of fish biology genomics, further exploration of these targets will help us to better understand the regulatory mechanism of miR-146a in respect of SGIV infection. On an additional note, other cell signalling pathways, such as cell proliferation and differentiation, type I IFNs responses, autophagy, etc., which have been reported to be regulated by miR-146a in mammalian cells, may also be regulated by fish miR-146a during SGIV infection. Therefore, the integrative role of miR-146a in SGIV infection is still an open question that is worth further investigation.

In conclusion, we identified miR-146a as a regulatory molecule in SGIV infection. We found that miR-146a is upregulated by SGIV infection in fish cells. miR-146a overexpression promotes SGIV replication and propagation, while miR-146a knockdown suppresses SGIV infection. Mechanistically, we found that induction of miR-146a promoted SGIV infection in a feed-forward manner by restraining anti-viral cellular responses, such as cell apoptosis and NF-κB activation. Our data provide experimental evidence that cellular miRNAs are involved in the cellular responses to SGIV infection, and moreover, our study suggests that the manipulation of cellular miRNA levels may represent a novel remedy to control SGIV infection.
METHODS

Cell and virus

Fathead minnow (FHM) cells were maintained in our laboratory and grown in Leibovitz’s L-15 medium containing 10% fetal bovine serum (Gibco, USA) at 25°C. The SGIV used in this study was originally separated from diseased Epinephelus tauvina and was propagated as described previously [19, 47]. Virus-infected cells were collected and stored at −80°C until use. Virus was titrated by the 50% tissue culture infectious dose (TCID50) assay [48], and an m.o.i. of 0.1 was used for viral-infection experiments.

miRNA and plasmid transfection

Fish miR-146a mimics and inhibitors were purchased from Ambion (catalogue nos 4464066 and 4464084, Applied

Fig. 5. miR-146a inhibits SGIV-induced cell apoptosis in FHM cells. FHM cells in 6-well plates were transfected with control mimics or miR-146a mimics, and control inhibitors or miR-146a inhibitors, and infected with SGIV or left uninfected (mock). (a) Infected cells were collected and assessed by cytometric analysis, and the primary data were processed by FlowJo software, with the M4 region representing the apoptotic peak. (b) The percentage of apoptotic cells was calculated from three independent experiments and is shown as mean±SD. *P<0.05 compared to the respective control group.
Fig. 6. miR-146a inhibits the formation of apoptotic bodies and caspase activation after SGIV infection. (a) FHM cells in 24-well plates were transfected with control mimics or miR-146a mimics, and control inhibitors or miR-146a inhibitors, and infected with SGIV at an m.o.i. of 0.1 or left uninfected (mock). At 36 h after infection, cells were incubated with DNA fluorescent stain Hoechst 33342 and apoptotic bodies containing nuclear chromatin fragments (arrows) were visualized using a fluorescence microscope.
Hoechst 33342 (Sigma-Aldrich, USA) (1 µg ml⁻¹) FHM cell DNA was stained with fluorescent DNA stain, Hoechst 33342 DNA staining (Promega, USA). were collected and intracellular luciferase activities were followed by SGIV infection for another 12 and 24 h. Cells transfection, cells were retransfected with miRNA mimics as previously described [49].

**RNA extraction and cDNA synthesis**

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. Regular cDNAs were prepared using the ReverTra Ace kit (TOYOBO, Japan) according to the manufacturer’s instructions. miR-146a-specific reverse transcription was performed by a customized miRNA RT kit (RiboBio, China). Briefly, 1 µg RNA was incubated at 70°C for 10 min to be denatured and then cooled on ice. A 20 µl reaction containing 4 µl 5× RT buffer, 5 pmol miR-146a-specific RT primer, 1 µl RT polymerase and the denatured RNA was carried out by incubation at 42°C for 60 min, followed by 70°C for 10 min and 4°C cooling down.

**Bulge–loop miRNA qRT-PCR**

miR-146a levels were determined by real-time quantitative PCR using a bulge–loop qRT-PCR starter kit (RiboBio, China) and performed on the LightCycler 480 detection system (Roche, Switzerland). A 10 µl reaction contained 5 µl SYBR qPCR mix, 0.4 µl forward and reverse bulge–loop miRNA primer, respectively, 3.2 µl PCR-grade water and 1 µl diluted miR-146a-specific cDNA. PCR amplification was performed in quadruplicate wells using cycling parameter: 95°C for 5 min, followed by 40 cycles of 5 s at 94°C, 10 s at 56°C and 15 s at 72°C. 18S rRNA was used as the reference.

**NF-κB activation analysis**

FHM cells seeded in 24-well plates were firstly transfected with 100 ng pGL3-luc-NF-KB reporter plasmid (Promega, USA) and 50 ng pRL-TK normalizing plasmid. 24 h after transfection, cells were retransfected with miRNA mimics followed by SGIV infection for another 12 and 24 h. Cells were collected and intracellular luciferase activities were measured by the Dual-Luciferase Reporter assay system (Promega, USA).

**Hoechst 33342 DNA staining**

FHM cell DNA was stained with fluorescent DNA stain, Hoechst 33342 (Sigma-Aldrich, USA) (1 µg ml⁻¹ in L-15 medium) for about 2 min. Cells were then washed with L-15 three times and observed under a fluorescence microscope (Leica, Germany) to visualize nuclear morphology changes.

**Flow cytometric analysis of cell apoptosis**

Flow cytometry was used to analyse the cell apoptosis by determining the population in the sub-G0/G1 phase. In brief, cells in six-well plates were transfected with miRNAs and then infected with SGIV. At 24 and 48 h post-infection, cells were collected and washed with PBS. Cells were then fixed and permeabilized in 70% ethanol overnight at −20°C. Before detection, cells were washed twice with PBS to remove ethanol, and then resuspended in a PBS solution containing 50 µg ml⁻¹ PI (Sigma-Aldrich, USA) and 100 µg ml⁻¹ RNase in the dark for 30 min. Apoptotic cells were analysed using a FACSScan flow cytometer (Becton-Dickinson, USA) and FlowJo software.

**Caspase-9/3 activity assay**

Intracellular caspase-9 and caspase-3 activity was determined by the Caspase Fluorometric assay kit (BioVision, USA) according to the manufacturer’s instructions. Briefly, cells were collected and trypsinized and centrifugation, and lysed in 25 µl cold lysis buffer for 10 min on ice. Before measurement, the lysate was centrifuged at 1000 g for 1 min to remove sediment. For a 40 µl reaction, 20 µl cell lysate was mixed with 20 µl 2× reaction buffer, 0.2 µl fresh DTT and 2 µl caspase-9/3 fluorogenic substrate (LEHD-AFC). After incubation at 37°C for 1 h, fluorescence was measured (excitation 400 nm, excitation 505 nm) by a fluorescence microplate reader (PerkinElmer, USA).

**Western blotting**

To detected the protein synthesis of SGIV MCP, Western blotting was performed as described previously [50].

**Virus titre assay**

To detect the effect of miR-146a on SGIV production, we assessed the virus titres using the TCID50 assay. Briefly, cells were transfected with miRNA mimics and 24 h later infected with SGIV at an m.o.i. of 0.1. Cell lysates were collected at 12, 24, 36 and 48 h post-infection, and freeze–thawed three times at −80°C. Cell lysates were then serially diluted and applied for the infection of FHM cells in 96-well plates. After 5–7 days of infection, viral titres were calculated using the TCID50 assay.

**Statistical analysis**

Data were expressed as mean±SD, and statistical differences between groups were analysed using the Student’s t-test. P<0.05 was considered statistically significant.

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