Developmental and Comparative Immunology 82 (2018) 1–6

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Differential expression of miRNA in *Carassius auratus gibelio* in response to cyprinid herpesvirus 2 infection

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ARTICLE INFO

Article history:
Received 7 November 2017
Received in revised form
28 December 2017
Accepted 29 December 2017
Available online 2 January 2018

Keywords:
Carassius auratus gibelio
Cyprinid Herpesvirus-2
miRNA
Immune regulation

ABSTRACT

miRNAs (microRNAs), a small endogenous non-coding RNAs, play crucial roles in post-transcriptional regulator of genes expression in various biological processes. Cyprinid Herpesvirus-2 (CyHV-2) is a highly pathogenic member of the alloherpesviridae that causes acute mass mortalities in populations of *Carassius auratus gibelio* and *Carassius auratus auratus*. However, the molecular mechanisms underlying the pathogenicity of CyHV-2 have not been fully determined. Here, miRNA expression profiles were identified via high-throughput sequencing in the kidney of *Carassius auratus gibelio* infected or uninfected with CyHV-2. The results showed that a total number of 840 known miRNAs and 48 putative novel miRNAs were identified. Then we compared the expression patterns of miRNAs in the two groups, 23 miRNAs were significantly differentially expressed between the uninfected and infected groups. Further, the expressions of 23 miRNAs were validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR), the results showed that the expression patterns were basically the same with the sequencing. Prediction of targets of differentially expressed miRNAs revealed that the miRNAs participated in the regulation of multiple immune-related signaling pathways, including Chemokine signaling pathway, Apoptosis, Jak-STAT signaling pathway and MAPK signaling pathway. Taken together, these data provide insight into the regulatory mechanisms of miRNA and highlight the function of miRNA in the regulation of the immune response during the interaction between host and virus pathogens.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs involved in post-transcriptional regulation of target genes in eukaryotic organisms (Ambros, 2004; Wizman et al., 2016; Yates et al., 2013). In the canonical miRNA biogenesis pathway, miRNAs are transcribed by RNA polymerase II in nucleus as long primary miRNA precursors (pri-miRNA), then the nuclear RNase III enzyme Drosha combines with these stem-loops, termed a pre-miRNA intermediate (Han et al., 2006; Kim et al., 2009). After export to the cytoplasm, the pre-miRNA is recognized by Dicer, a second RNase III enzyme, which cleaves ~22 bp from the base of the pre-miRNA, to release the ~22 bp mature double-stranded miRNA duplex (Lee et al., 2003). A single strand of the mature miRNA is incorporated subsequently into a cellular argonaute (ago) protein to form the RNA-induced silencing complex, RISC (Huntzinger and Izaurralde, 2011). Finally, the mature miRNA strand of this duplex associates with argonaute family proteins and is loaded into miRNA-induced silencing complex (miRISC) (Bartel, 2004). The mature miRNA guides miRISC to bind with partially complementary sequences which are mostly localized within the 3' untranslated region (UTR) of mRNAs, and results in translation blockage and/or mRNA decay (Bartel, 2009; Huntzinger and Izaurralde, 2011). It has been well known that miRNAs play essential roles in the regulation of pathogen infection, and an increasing number of miRNAs associated with host-pathogen interactions have been identified in recent years (W. Chen et al., 2017; Li et al., 2017; Qiao et al., 2016; Wang et al., 2016). However, few researches have been done on the miRNAs functions in the host-virus interactions in teleost.

Silver crucian carp (*Carassius auratus gibelio*), a major freshwater aquaculture species in China (Kong et al., 2017; Wang et al, 2016), also a widely distributed cyprinid throughout Europe (Yang et al.,

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https://doi.org/10.1016/j.dci.2017.12.027
0145-305X/

2001). However, in 2011, a novel communicable viral disease caused by CyHV-2 has resulted in high mortality of silver crucian carp in China (Podok et al., 2014; Xu et al., 2014). CyHV-2 is a member of the alloherpesviridae, epizootics caused by CyHV-2 infection have been reported in juvenile goldfish in many countries, including Asia (Sahoo et al., 2016; Wang et al., 2012), Europe (Boitard et al., 2016), North America (Goodwin et al., 2006), and Oceania (Becker et al., 2014). Therefore, strategies to control CyHV-2 infections are required urgently to reduce the serious losses to aquaculture caused by this viral disease. Currently, many immune-related genes have been reported can respond to CyHV-2 infection in silver crucian carp (Podok et al., 2014; Podok et al, 2016). Meanwhile, the transcriptome of the silver crucian carp has been completed, which was facilitated to demonstrate the various roles of viral miRNAs in the immune defense against pathogens (Lu et al., 2017). The identification and comparative analyses of immune-related miRNAs are indispensable to further investigate their physiological or pathological functions and regulation of innate and adaptive immunity response to diverse pathogens infection.

In this study, we constructed two groups of small RNA (sRNA) libraries from the kidney of silver crucian carp under uninfected and CyHV-2 infected conditions using the high-throughput sequencing technology. Totally 888 miRNAs were identified, including 840 known miRNAs and 48 novel miRNAs. Meanwhile, the expression profiles of 23 differentially expressed miRNAs were further investigated. Target gene and function prediction of 23 differentially expressed miRNAs were illuminated by bioinformatics. Collectively, the integrated analysis of miRNAs from this research could provide more insight into the mechanism of miRNA-mediated regulation of the immune response.

2. Materials and methods

2.1. Fish and virus challenge

Healthy silver crucian carp (approximately 10 cm in body length) were obtained from the Wujiang National Farm of Chinese Four Family Carps, Jiangsu Province, China. Initially, fish were temporarily reared in aquarium at 23 °C for adaptation. After seven days of acclimation, fish were divided into two groups (30 fish per group/aquarium) for the intraperitoneal injection. One group was intraperitoneally injected with CyHV-2 suspended in PBS at a dose of 1×10^6 TCID50/g, the control group was injected with PBS at the same dosage (Lu et al., 2017). After injection, all the fish were reared under the same conditions, fed with a diet according to standard feeding scheme. Samplings kidney of control fish (T1K, T2K, and T3K) and moribund fish (T4K, T5K, and T6K, 72 h post-challenge), each made up of three biological replicates, and three different individual kidney tissues, and immediately frozen in liquid nitrogen.

2.2. Small RNAs library construction and deep sequencing

For six small RNA library constructions, the RNA preparation, library construction and high-throughput sequencing were performed by LC-BIO (Hangzhou, China). Total RNAs were extracted using the Trizol reagent (Invitrogen, CA, USA), following the manufacturer's instructions. The sRNA data was sequenced by Illumina Hiseq2500 50SE at the LC-BIO. The raw reads were subjected to the Illumina pipeline filter (Solexa 0.3), and then the dataset was further processed with an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18–26 nucleotide were mapped to specific species precursors in miRBase 20.0 by BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. The unmapped sequences were blasted against the specific genomes, and the hairpin RNA structures containing sequences were predicated from the flank 80 nt sequences using RNAfold software (http://rna.tbi.univie.ac. at/cgi-bin/RNAfold.cgi).

2.3. Analysis of differential expressed miRNAs

miRNA differential expression based on normalized deep-sequencing counts was analyzed by selectively using Fisher exact test, Chi-squared 2X2 test, Chi-squared nXn test, Student *t*-test, or ANOVA based on the experiments design. The significance threshold was set to be 0.01 in each test.

2.4. Validation of miRNA expression by qRT-PCR

The infection methods were same as section 2.1, and samples were collected at 0, 12, 24, 48 and 72 h post infection. The Mir-X™ miRNA First-Strand Synthesis and SYBR® qRT-PCR Kit (Clontech, USA) was employed to quantify mature miRNAs according to the manufacturer's instructions. Total RNA was isolated from kidney organs of CyHV-2 infected or uninfected fish, 5 μg of total RNAs were used for cDNA synthesis. U6 RNA was used as an internal standard. The relative expression levels of miRNAs were calculated by the comparative Ct method. At least three independent biological replicates were used for each miRNA. All reactions were performed in triplicate on the CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). $2^{-\Delta\Delta CT}$ method was adopted to analyze the expression of the different miRNAs. All the expression data were subjected to a one-way ANOVA, and statistical significance was assumed at $P < .05$ and $P < .01$.

2.5. The prediction of miRNA target genes

miRNA target prediction algorithms TargetScan 50 (http://www.targetscan.org/) and miRanda3.3a (http://www.microrna.org) were employed to identify miRNA binding sites. Finally, the data predicted by both algorithms were combined and the overlaps were calculated. KEGG pathway (http://www.genome.jp/kegg/) of these miRNAs and miRNA targets were also annotated.

3. Results

3.1. Overview of the high-throughput sequencing data

To identify the miRNAs associated with CyHV-2 infection in kidney of silver crucian carp, the small RNA libraries from moribund and healthy kidney of silver crucian carp were constructed and sequenced by Illumine Hiseq2500 platform. A total of 10,714,657 reads were generated in the uninfected and infected groups. After reads processing and filtering, over 90% of the sequences were obtained (Table S1). Among them, about 4.02% of the sequences mapping to Rfam. After searching against Rfam and GenBank databases, other non-coding RNAs (rRNA, tRNA scRNA, snRNA and snoRNA) and repeat sequences were annotated (Table S1).

3.2. Identification of conserved miRNAs and novel miRNA candidates

Taken together, a total of 888 silver crucian carp miRNAs were identified. Among the 888 miRNAs, the expression level of 84 miRNAs were in a relatively high level. However, the expression levels of 497 miRNAs were rather low (Table S2). These results