MicroRNA repertoire and comparative analysis of *Andrias davidianus* infected with *ranavirus* using deep sequencing

Yan Meng, Haifeng Tian, Qiaomu Hu, Hongwei Liang, Lingbing Zeng, Hanbing Xiao

PII: S0145-305X(18)30005-3
Reference: DCI 3138

To appear in: *Developmental and Comparative Immunology*

Received Date: 3 January 2018
Revised Date: 3 April 2018
Accepted Date: 3 April 2018


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
MicroRNA repertoire and comparative analysis of *Andrias davidianus* infected with *ranavirus* using deep sequencing

Yan Meng, Haifeng Tian, Qiaomu Hu, Hongwei Liang, Lingbing Zeng, Hanbing Xiao

Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences; Hubei 430223, China

*Corresponding author: xhb@yfi.ac.cn

Abstract

*Andrias davidianus* is a large and economically important amphibian in China. Ranavirus infection causes serious losses in *A. davidianus* farming industry. MicroRNA mediated host-pathogen interactions are important in antiviral defense. In this study, five small-RNA libraries from ranavirus infected and non-infected *A. davidianus* spleens were sequenced using high throughput sequencing. The miRNA expression pattern, potential functions, and target genes were investigated. In total, 1356 known and 431 novel miRNAs were discovered. GO and KEGG analysis revealed that certain miRNA target genes are associated with apoptotic, signal pathway, and immune response categories. Analysis identified 82 downregulated and 9 upregulated differentially expressed miRNAs, whose putative target genes are involved in pattern-recognition receptor signaling pathways and immune response. These findings suggested miRNAs play key roles in *A. davidianus*’s response to ranavirus and could provide a reference for further miRNA functional identification, leading to novel approaches to improve *A. davidianus* ranavirus resistance.

Keywords: *Andrias davidianus*; microRNA; ranavirus; deep sequencing

1. Introduction
The Chinese giant salamander, *Andrias davidianus*, belongs to order Caudata, family Cryptobranchidae, genus Andrias. It is a unique species in China, which has existed for more than 350 million years, and is termed a “living fossil” (Gao et al., 2003). In China, *A. davidianus* has been considered a good food with nutritional and medicinal value. In the evolutionary history of vertebrates, *A. davidianus* may represent animals that underwent the transition from aquatic to terrestrial life, and thus it is important in scientific research (Murphy et al., 2000). *A. davidianus* was classified as an endangered species by the International Union for Conservation of Nature (IUCN) and as class II state major protection species of China because of its diminishing population. In past thirty years, captive breeding and aquaculture of *A. davidianus* have been successful in China. Unfortunately, with the rapid development of *A. davidianus* aquaculture, the prevalence of infectious diseases has increased. As a serious emerging viral pathogen, Chinese giant salamander iridovirus (GSIV) belonged to ranavirus has spread widely in *A. davidianus* farming (Chen et al., 2013; Dong et al., 2010; Meng et al., 2014).

MicroRNAs (miRNAs) are small non-coding RNAs of about 22 nucleotides (nt) in length, which play crucial regulatory roles in gene expression by matching with and binding to a specific mRNA target site, leading to target mRNA degradation or translation inhibition, thereby affecting the level of the protein product (Wang et al., 2016). MiRNAs were firstly discovered in *Caenorhabditis elegans* (Lee et al., 1993) and then in plants, animals, and viruses. MiRNAs are involved in a range of biological processes, such as cellular development; proliferation and differentiation; metabolism; homeostasis; apoptosis; and diseases (Ameres et al., 2013; Gottwein et al., 2008). Recently, emerging evidence indicated that miRNAs play important roles in the regulation of pathological and physiological process (Omran et al., 1993), and play crucial roles during microbial infection (Wang et al., 2016). The study of miRNAs in response to pathogen infection has become hot topic in immune system research.

High-throughput sequencing has not only provides information about the expression of known miRNAs, but also has helped to identify tissue-specific and
rarely expressed miRNAs (Morin et al., 2008; Núñez-Hernández et al., 2015). To date, thousands of miRNAs have been discovered from various aquaculture species (Andreassen et al., 2017; Huang et al., 2017a; Robledo et al., 2017; Wang et al., 2017). *A. davidianus* is an important aquatic amphibian in biological evolution studies and aquaculture. Recently, sequence data of *A. davidianus*, such as transcriptome data (Fan et al., 2015), RNA-seq data (Li et al., 2015), and a small RNA library (Chen et al., 2017; Huang et al., 2017b) have been reported. These studies provided valuable genetic background knowledge for this ancient species. In the present study, to provide insights into the miRNAs expressed by *A. davidianus* in response to ranavirus infection, we constructed five small RNA libraries from the spleens of normal animals and those infected with ranavirus, and sequenced them using high-throughput sequencing technology. *A. davidianus* miRNAs, target genes, functional annotations, signal pathways, and differential expression patterns were investigated in different ranavirus infection stages. The results will help to understand the miRNA repertoire in *A. davidianus*, enrich our knowledge of regulatory mechanisms involving *A. davidianus* miRNAs under pathogen challenge, and provide a foundation resource for future functional studies of these miRNAs.

### 2. Materials and Methods

#### 2.1 Ethics statement and sample collection

Twenty-five clinically healthy *A. davidianus* (mean weight 60 g, length 20 cm) specimens were obtained from the experimental farm of the Yangtze River Fisheries Research Institute. The animals were maintained at 18–20 °C in tanks and fed daily with diced meat of bighead carp for two weeks before experimental treatment. All the animal experiment procedures were performed in accordance with the guidelines of Yangtze River Fisheries Research Institute on the use of laboratory animals. The study was approved by the state Science and Technology Commission of Hubei Province.

#### 2.2 Experimental infection

*A. davidianus* were randomly divided into two groups: five animals comprised the
control group and other 20 were the experimental group. Chinese giant salamander iridovirus (GSIV) was isolated from diseased *A. davidianus* and was preserved in our laboratory (Meng et al., 2014). The experimental groups were inoculated intraperitoneally with 0.5 ml of GSIV at a dose of $1 \times 10^6 \text{TCID}_{50} \text{ml}^{-1}$, while the control group were injected the same volume of Dulbecco’s phosphate buffered saline (DPBS, Sigma, USA). The experimental animals were maintained in tanks at 20 °C and fed normally. Then, at 0 (control), 6, 12, 24, and 36 h after inoculation, five animals were euthanized and spleen samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C.

### 2.3 RNA extraction, library construction, and sequencing

Total RNA from the spleens of the control and experimental groups at different infection stages was extracted using the Trizol reagent (Invitrogen, CA, USA) following the manufacturer’s procedure. The purity and quantity of the total RNA were checked using a NanoDrop spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and agarose gel electrophoresis. Only RNA samples with A260/A280 ratios between 1.9 and 2.1 were used for library construction. Approximately 1 µg of total RNA was used to prepare the small RNA libraries, according to protocol of the NEBNext® Multiplex Small RNA Library prep kit (NEB, USA). The RNA molecules in a size range of 16–35 nt were enriched by polyacrylamide gel electrophoresis (PAGE). Then, 3' adapters were added and the 36–44 nt RNAs were enriched. The 5' adapters were then ligated to the RNAs. The ligation products were reverse transcribed by PCR amplification, and the 140–160 bp PCR products were enriched to generate a cDNA library and sequenced using an Illumina HiSeq™ 2500 instrument at Gene Denovo Biotechnology Co. (Guangzhou, China) following the vendor’s recommended protocol.

### 2.4 Basic data processing

Sequencing reads were generated from the constructed small RNA libraries. The raw sequences were subjected to a standard analysis pipeline including several steps.
In brief, first, a filtering step was carried out to exclude reads of low quality (a tag with a quality score \( \leq 20 \) and ‘N’ nucleotides). Second, the raw sequencing data were filtered by eliminating adaptor contaminants to generate usable reads with size \( \geq 18 \) nt. The 3’ adapter null sequences, 5’ adapter contaminants sequences, polyA sequences, insert null sequences, and those with a length < 18 or length > 30 nt were removed. Third, all the clean tags were aligned with the GenBank database (Release 209.0) and Rfam database (11.0) to identify and remove rRNAs, scRNAs, snoRNAs, snRNAs, and tRNAs. Those that mapped to exons or introns might be fragments from mRNA degradation; therefore, these tags were removed. Tags that mapped to repeat sequences were also removed.

2.5 Prediction of conserved and novel miRNAs

The obtained reads were aligned to miRBase 20.0 (http://www.mirbase.org/) to identify conserved miRNAs. Currently, the reference genome of A. davidianus is unavailable; therefore, to identify novel miRNAs in A. davidianus, all the unannotated tags were aligned with the reference transcriptome data of A. davidianus (Fan et al., 2015). According to the transcriptome data and the predicted hairpin structures produced by the software Mireap_v0.2, novel miRNA candidates were identified.

2.6 Target gene prediction and pathway analysis

Based on the sequences of known miRNAs and novel miRNAs, candidate target genes were predicted using three pieces of software: RNAhybrid (v2.1.2)/svm_light (v6.01), Miranda (v3.3a), and TargetScan (Version: 7.0). The intersections of the results from this analysis were chosen as predicted miRNA target genes. Gene ontology (GO) enrichment analysis provides GO terms that are significantly enriched for target genes compared with the genome background. The related GO terms were defined using a hypergeometric test. Further analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify the biological pathways in which target genes are involved. The selected parameters for the study were the multiple test adjustment by Benjamini and Hochberg (Benjamini et al.,
1995), and the significance level was set at 0.05.

2.7 Differential expression analysis of miRNAs

To compare the miRNA expression data of the control and ranavirus infected samples, the total miRNA expression (known and novel miRNAs) was calculated and normalized to the transcripts per million (TPM) value, based on their expression in each sample. The fold change (FC) between the miRNA expression in the five libraries was determined as: FC = TPM 6 h/TPM 0 h, FC = TPM 12 h/TPM 0h, FC = TPM 24 h/TPM 0 h and FC = TPM 36 h/TPM 0 h miRNAs. Those miRNAs with a $|\log_2 FC| \geq 1$ and a p-value <0.05 (calculated from the normalized expression) were considered to be significantly differentially expressed. Thus, the upregulated and downregulated miRNAs among all the differentially expressed miRNAs were identified.

3. Results

3.1 Overview of the high-throughput sequencing data

In this study, five small RNA libraries were constructed from the spleens of A. d. treated with Chinese giant salamander iridovirus and DPBS (control) to identify and characterize miRNAs involved in the genomic level response to ranavirus infection. The five small RNA libraries were sequenced by Illumina deep sequencing technology on libraries of small RNAs from control (0 h) and infected samples at 6, 12, 24, and 36 h, and named GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h respectively. After filtering out low-quality sequences (Q-value $\leq 20$ reads), high quality reads were obtained. After removing the 3' adapter null sequences, polyA sequences, insert null sequences, 5' adapter contaminants, sequences <18 nt, the clean sequences were obtained for subsequent analysis (Table 1). A total of approximately 65,800,061 clean reads were obtained from the five small RNA libraries after high-throughput sequencing.
Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total reads</th>
<th>High quality reads</th>
<th>3' adapter null</th>
<th>polyA null</th>
<th>Insert null</th>
<th>5' adapter contaminants</th>
<th>Sequences &lt;18 nt</th>
<th>Clean reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIV-0h</td>
<td>15062524</td>
<td>13684377</td>
<td>275921</td>
<td>616</td>
<td>328106</td>
<td>22192</td>
<td>893222</td>
<td>12164320</td>
</tr>
<tr>
<td>GSIV-6h</td>
<td>13803364</td>
<td>13233203</td>
<td>24703</td>
<td>523</td>
<td>413815</td>
<td>19587</td>
<td>400204</td>
<td>12374371</td>
</tr>
<tr>
<td>GSIV-12h</td>
<td>15759116</td>
<td>15086830</td>
<td>30295</td>
<td>508</td>
<td>296596</td>
<td>20189</td>
<td>461593</td>
<td>14277649</td>
</tr>
<tr>
<td>GSIV-24h</td>
<td>15503605</td>
<td>14812238</td>
<td>28206</td>
<td>987</td>
<td>223294</td>
<td>14207</td>
<td>347053</td>
<td>14198491</td>
</tr>
<tr>
<td>GSIV-36h</td>
<td>14105214</td>
<td>13450229</td>
<td>32098</td>
<td>787</td>
<td>287102</td>
<td>14910</td>
<td>330102</td>
<td>12785230</td>
</tr>
</tbody>
</table>

Compared with the control library, the percentage of high quality reads among the total reads increased obviously in the experimental groups, representing the ratio between high quality reads and the total reads as shown in Table 1. The unique sequences <18 nt were reduced in the four infection groups compared with that in the control group. However, the number of 3' adapter null sequences increased with increasing infection time, with the the highest level in the GSIV-36h library. The size distribution of sRNAs ranged from 16–35 nt. Most of them were 19–24 nt, which was consistent with the typical sizes of dicer processed products. We determined the proportion of 16–18 nt, 19 nt, 20 nt, 21 nt, 22 nt, 23 nt, 24 nt, and 25–35 nt sequences in the small RNA libraries at GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h, respectively (Fig. 1). The sRNAs of 21 nt and 22 nt formed two major classes in the sRNA libraries. The most abundant sRNA length was 22 nt, which was present at 22.83%, 27.80%, 28.82%, 26.86%, and 24.54% in the five libraries, respectively. Analysis of the first nucleotide bias of the 19–24 nt sRNAs obtained in the five libraries indicated that (U) was preferred at the first position, especially in the 21 and 22 nt sRNAs (Supplementary File S1). After further removal of ribosomal RNA (rRNA), transfer RNAs (tRNAs), small cytoplasmic RNA (scRNA), small nuclear RNA (snRNA), small nucleolar RNAs (snoRNAs), repetitive sequence elements, the unannotated small RNA reads were retained for miRNA analysis.

3.2 Identification of known miRNAs and novel miRNA candidates

High throughput sequencing can be used to verify a large number of known miRNAs and novel specific miRNAs in organisms. The lack of a genome sequence
for *A. davidianus* meant that all of the unannotated sequencing reads were first aligned against the miRNA sequences in miRBase (http://www.mirbase.org/). The small RNA sequences were then mapped to reference transcriptome data of *A. davidianus*. Thus, the novel and known miRNAs from normal and infected *A. davidianus* were identified. In the five sRNA libraries, there were 1356, 1094, 1091, 1022, and 992 known miRNAs in GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h, respectively. In addition, compared with the reference transcriptome data of *A. davidianus*, there were 431, 435, 468, 467, and 424 novel miRNAs identified from GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h, respectively. The variability in this sRNA data is shown in Fig. 2. The results showed that the number of known miRNAs decreased with increasing infection time. The numbers of novel miRNA were increased at GSIV-6h, GSIV-12h, and GSIV-24h compared with that at 0 h. The number of novel miRNAs increased substantially after infection for 12 and 24 h. The novel miRNAs candidates also should be further validated by direct cloning using the stem-loop structure.

### 3.3 Analysis of the target genes of *A. davidianus* miRNAs

The function of a miRNA is ultimately defined by genes it targets and its effects on target mRNA translation. Based on the previous data of *A. davidianus* transcriptome sequencing, potential targets of the miRNAs were predicted using TargetScan, miRanda, and PicTar, the three most frequently used prediction algorithms. Potential targets of the 3253 *A. davidianus* miRNAs were predicted by the intersection of three prediction programs. For further functional annotation, we used GO to annotate/classify the function the predicted miRNA target genes, and KEGG to construct pathway enrichment of the predicted miRNA target genes from the five libraries. All the predicted targets were annotated in three main categories: Biological process, cellular component, and molecular function (Supplementary File S2, Fig. 3). Among the three functional categories, the GO terms with the highest number of targets were cellular process (in the biological process category), cell and cell part (cellular component), and binding (molecular function). In addition, there were some
high frequency biological processes closely related to the immune response system. These terms included response to stimulus, immune system process, biological adhesion, viral reproduction, and cell killing.

KEGG pathway annotation showed that 23712 predicted target genes were annotated into 240 biological function pathways. Among these pathways, metabolic pathways occupied the highest frequency, followed by pathways in cancer, the mitogen activated protein kinase (MAPK) signaling pathway, and endocytosis (Supplementary File S3). In addition, many immune related pathways were annotated. The immune related pathways from the KEGG functional classification are summarized in Fig. 4 and Supplementary File S4. Most of target genes in the immune related networks were found to be involved in T and B cell receptor signaling, cytokine-cytokine receptor interaction, Toll-like receptor signaling, antigen processing and presentation, NOD-like and RIG-I receptor signaling, and endocytosis. The important genes in these pathways might mediate immune response processes or activate cells with immune function, leading to control of the host defense against pathogens.

3.4 Expression analysis of A. davidianus miRNAs induced by ranavirus

High-throughput sequencing is not only a good tool to identify small RNAs, but also provides information about miRNA expression levels. The miRNAs could be expressed differentially between infected and non-infected animals in different infection phases. The expression patterns of known and novel miRNAs identified in five GSIV infection stages were profiled based on the sequencing results. The results showed that many miRNAs had a wide range of expression levels at all examined time points, and the expression quantity of most of them were more than two-fold different at 0h, 6h, 12h, 24h and 36h after infection, respectively. The detailed expression information of the known and novel miRNAs in the different stages is shown in Supplementary File S5 and S6. Based on the miRNA expression information, the differentially expressed miRNAs were identified. To analyze the differentially expressed miRNAs at each time point, we compared all the miRNAs via
pairwise comparisons between GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h. There were 835 miRNAs (including known and novel miRNAs) that were differentially expressed at all examined time points. Among them, 294 were upregulated and 541 were downregulated.

Continuous differential expression of miRNAs during the whole infection process is important and could have a role in gene expression regulation. Therefore, the upregulated and downregulated differentially expressed miRNAs at GSIV-6h, GSIV-12h, GSIV-24h and GSIV-36h were compared with their levels in GSIV-0h. The details for the differentially expressed miRNAs are listed in Supplementary File S7 and represented graphically in Fig. 5.

The Venn figure in Fig.5 shows 82 miRNAs that were downregulated in the four GSIV infection groups compared with the control group (Fig. 5A) and nine miRNAs that were upregulated after GISV infection compared with the control group (Fig. 5B). Among the 82 miRNAs showing downregulated expression, most of them showed a more than 10-fold different expression level during the infection process compared with the uninfected control, such as mir-122, mir-203, mir-206, and mir-722. For example, miR127 (upregulated) and mir-122 (downregulated) showed a 100-fold difference in their expression levels between the treatment groups and the control group. These significantly differentially expressed miRNAs targeted genes such as \textit{RIG-I}, \textit{MDA5}, \textit{TLR1}, and \textit{MHC}, are associated with the immune system.

4. Discussion

MiRNAs play a critical role in the response to biotic and abiotic stress, and have been characterized in a large number of aquatic organisms (Li et al., 2012; Wang et al., 2017). High throughput sequencing technology has been applied extensively to small RNA research, and can identify a large number of known miRNAs and novel specific miRNAs in organisms subjected biotic or abiotic stress (Ou et al., 2012; Yu et al., 2016). The study of miRNA-mediated host-pathogen interactions has emerged in the last decade because of the important role played miRNAs in antiviral defense. For example, using deep sequencing and quantitative real-time PCR, ten
polyinosinic-polycytidylic acid (pIC)-stimulated miRNAs were identified in Atlantic cod, which suggested that miRNAs are important in the antiviral immune responses of Atlantic cod macrophages (Eslamloo et al., 2018). *Marsupeneaus japonicus* miR-S5 could affect the expression of p53, tumor necrosis factor-α (TNF-α), and myosin to regulate hemocyte phagocytosis and apoptosis processes in response to white spot syndrome virus (WSSV) (Wang et al., 2018).

Ranavirus is a major pathogen in aquaculture that causes ulcerative injury in animals. Identification of miRNAs expressed by *A. davidianus* infected by ranavirus has significance in host/pathogen interaction research and in the antiviral immune response of *A. davidianus*. In this study, we investigated the miRNA expression patterns associated with ranavirus-infected *A. davidianus* using a high-throughput sequencing approach at 0 h, 6 h, 12 h, 24 h and 36 h after GSIV infection. In total, 1356 known miRNAs and 431 novel miRNAs were identified in the control group. The number of known miRNAs decreased and the number of novel miRNAs increased in infection groups compared with the control group. Using Illumina deep sequencing, Huang et al. identified 140 conserved and three novel miRNAs of *A. davidianus* when they mapped the sequence reads to the reference genome sequence of *Xenopus tropicalis* (Huang et al., 2017b). In another study, a total of 757 and 756 unique miRNAs were annotated as miRNA candidates in the ovary and testis of *A. davidianus*, respectively. Among them, 145 miRNAs in the ovary and 155 miRNAs in the testis were homologous to those in *Xenopus laevis* ovary and testis, respectively (Chen et al., 2017). In the present study we identified more miRNAs in *A. davidianus* in response to ranavirus infection compared with the studies above. This might be because these miRNAs were screened using different parameter settings or reference databases. We referenced the transcriptome data of *A. davidianus*, not the genome of *Xenopus tropicalis*. Although *Xenopus tropicalis* is often regarded as a representative animal of amphibians, in fact, *Xenopus tropicalis* belongs to the Anura and *A. davidianus* belongs to the Caudata, which have different genetic characteristics. However, our results and those of previous studies suggest that *A. davidianus* expresses abundant miRNAs.
GO is an international, standardized gene functional classification system that offers a constantly updated vocabulary and a strictly defined concept to comprehensively describe the properties of genes and their products in an organism. The KEGG pathway is a map representing knowledge of molecular interactions, reactions and relation networks, and is also a database resource to help understand the functions and utilities of biological information, especially large-scale molecular datasets generated using high-throughput technologies. The GO terms and KEGG pathways associated with the target gene of the *A. davidianus* miRNAs were analyzed. The results showed that the miRNA target genes are involved in important signaling pathways and immune response processes. For example, there were 218 target genes in T cell receptor signaling, 198 in B cell receptor signaling, 181 in natural killer cell mediated cytotoxicity, and 450 in Endocytosis. These results were consistent with the miRNA target gene research in which miRNAs were observed to play important roles in the regulation of the immune system, including the development and differentiation of B or T lymphocytes (Maryaline et al., 2017), natural killer cells (Kingsley et al., 2017), and modulation of inflammation (Neudecker et al., 2017).

In this study, we aimed to investigate the miRNA repertoire of *A. davidianus* under GSIV infection. Therefore, we compared the expression of the miRNAs between the control and four post-infection phases to identify differentially expressed miRNAs. Among the differentially expressed miRNAs, most of them showed differential expression level. Seventy-seven known miRNAs and five novel miRNAs were downregulated in all four ranavirus infection groups compared with that in the control group, while two known and seven novel miRNAs were upregulated expression in all experimental groups compared with the control. The target genes of these differentially expressed miRNAs were predicted. GO and KEGG analyses indicated that most of the target genes were related to the immune system. Among the 77 downregulated known miRNAs, some showed the significantly different expression patterns, such as mir-122, mir-203, mir-206, mir-31, mir-722, mir-72, mir-8109 and mir-8159. Taking mir-122 as a typical example, its expression level was
about 3941 in the control group, and was 758, 134, 361, and 108 at 6, 12, 24, and 36 h after infection, respectively. Analysis of mir-122 indicated that it is involved in pattern recognition receptor (PRR) signaling, including Toll-like receptor (TLRs), NOD-like receptor (NLRs), RIG-I like receptor (RLRs); complement and coagulation cascades; natural killer cell-mediated cytotoxicity; B/T cell receptor signaling pathway; chemokine signaling pathway; Fc epsilon RI signaling pathway; antigen processing and presentation; and primary immunodeficiency. The target genes of mir-122 include RIGI (retinoic acid-inducible gene I protein), Mx (myxovirus resistance), Aven (cell death regulator Aven), Apaf1 (apoptotic protease activating factor 1), IAP (inhibitor of apoptosis protein), MDA5 (melanoma differentiation-associated protein 5, TLR1, and MHCII (major histocompatibility complex II). These pathways and target genes all related to the innate and adaptive immune systems. Characterization of immune-related miRNAs is a prerequisite for a thorough understanding of the role played by miRNA-mediated post-transcriptional gene regulation in the innate immune system (Ou et al., 2012). The host limits viral infection by expressing miRNAs that target important related genes (Zhang et al., 2014). When challenged by Vibrio anguillarum, mir-122 of miiuy croaker showed dramatically reduced expression profiles. TLR4 was identified as a target of mir-122, and its expression level was increased in response to Vibrio anguillarum challenge. Therefore, mir-122 could target gene TLR14, which is involved in miiuy croaker’s inflammatory and immune response (Cui et al., 2016). Dihydroxyacetone kinase (DAK) is an inhibitor of MDA5. Mir-122 could target DAK to participate in regulating the RIG-I like receptor signaling pathway at the post-transcriptional level in miiuy croaker’s spleen and macrophages under pIC stimulation, which is a synthetic analog of double-stranded RNA (dsRNA) and has a molecular pattern associated with viral infection (Han et al., 2018). In addition, mir-203 was significantly differentially expression and it could target the Mx gene. Mx protein is an antiviral protein that has a broad antiviral function and is induced by interferon (IFN) or produced by animals infected with viruses. In our previous study, Mx gene was upregulated in gsIFN-overexpressing cells of A. davidianus after GSIV infection (Chen et al., 2015). Therefore, combining
the existing studies about mir-122 or mir-203, we could speculate that the
differentially expressed miRNAs detected in our study have important regulatory
functions in the innate and adaptive immune systems in A. davidianus in response to
ranavirus infection. These miRNAs may exert these important effects via regulating
such genes as RIGI, IFN, MDA5, TLR1, TNF, IAP, NRAMP, Mx or MHC and so on in
their different pathways.

Innate and acquired immune responses provide a critical line in defense against
pathogens. The study of miRNAs in response to pathogen infection has a hot topic in
immune system research. Identifying immune-related miRNAs will lead to a deeper
understanding of the host animal’s genetic mechanism, and will have benefits for
disease control and breeding for disease resistance. The results of the present study
provide vital insight into the immune system of A. davidianus, and will prompt further
investigations of the miRNA-mediated regulation in host-pathogen interactions.
Furthermore, the results will also aid the development of new control strategies to
prevent or treating ranavirus infections in aquatic animals.

Funding

This work was supported by the Chinese National Nonprofit Institute Research
Grant of Freshwater Fisheries Research Center [grant number 2016JBF0304].

References:


Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and

davidianus) and the response to an iridovirus infection. Mol. Immunol. 65(2), 350-359.


Zhang, B.C., et al. 2014. In-depth profiling and analysis of host and viral microRNAs in Japanese
flounder (*Paralichthys olivaceus*) infected with megalocytiviruses reveal involvement of microRNAs in host-virus interaction in teleost fish, BMC Genomics. 15, 878.
Fig.1. Analysis of sequencing reads of *A. davidianus* miRNAs infected by Chinese giant salamander iridovirus from the GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h and GSIV-36h libraries. A: The percentage of high quality reads among the total sequencing reads of *A. davidianus* at different time points of infection. B: The percentage of different read lengths in sequencing reads from 16 to 35 nt. 16–18 indicates microRNAs with a length of 16 to 18 nt, and 25–35 indicates microRNAs of 25 to 35 nt.

Fig.2. The known and novel miRNAs expressed by *A. davidianus* at five time points of Chinese giant salamander iridovirus infection. Red indicates known miRNAs and blue indicates novel miRNAs.

Fig.3. Gene Ontology (GO) Classification of target genes of miRNAs expressed in *A. davidianus*. The results are summarized in three main categories: (1) biological process, (2) cellular component, and (3) molecular function. In total, 22306 sequences with BLAST matches to known proteins could be assigned to GO terms.

Fig.4. Histogram of target gene numbers in 23 immune-related pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The letters A to W indicates the names of the 23 immune-related pathways.
A: RIG-I-like receptor signaling
B: Lysosome
C: Endocytosis
D: Natural killer cell mediated cytotoxicity
E: Complement and coagulation cascades
F: B cell receptor signaling
G: Leukocyte transendothelial migration
H: Apoptosis
I: Cytokine-cytokine receptor interaction
J: Jak-STAT signaling pathway
K: Regulation of autophagy
L: p53 signaling
M: Chemokine signaling pathway
N: Toll-like receptor signaling
O: MAPK signaling
P: mTOR signaling
Q: NOD-like receptor signaling
R: Phagosome
S: Cell adhesion molecules (CAMs)
T: Antigen processing and presentation
U: T cell receptor signaling
V: Primary immunodeficiency
W: Fc gamma R-mediated phagocytosis

Fig.5. Venn diagram of the upregulated and downregulated differentially expressed *A. davidianus* miRNAs between the control and different time post-GSIV infection. A: The number of upregulated miRNAs. B: The number of downregulated miRNAs. The numbers inside the diagram indicate the numbers of miRNAs. The colors red, purple, orange, and green represent the data for the control group compared with post-GSIV infection at 6, 12, 24, and 36 h, respectively. Nine upregulated and eighty-two downregulated miRNAs were differentially expressed at all GSIV infection stages in comparison with the control group.
Figure 1

A

Percentage of high-quality rRNA in total reads

<table>
<thead>
<tr>
<th>Stage</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIV-0h</td>
<td>96%</td>
</tr>
<tr>
<td>GSIV-6h</td>
<td>96%</td>
</tr>
<tr>
<td>GSIV-12h</td>
<td>96%</td>
</tr>
<tr>
<td>GSIV-24h</td>
<td>96%</td>
</tr>
<tr>
<td>GSIV-36h</td>
<td>96%</td>
</tr>
</tbody>
</table>

Different stage

B

Percentage (%)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSIV-0h</td>
<td></td>
</tr>
<tr>
<td>GSIV-6h</td>
<td></td>
</tr>
<tr>
<td>GSIV-12h</td>
<td></td>
</tr>
<tr>
<td>GSIV-24h</td>
<td></td>
</tr>
<tr>
<td>GSIV-36h</td>
<td></td>
</tr>
</tbody>
</table>

Different stage
Figure 2
Figure 4

A: RIG-I-like receptor signaling
B: Lysosome
C: Endocytosis
D: Natural killer cell mediated cytotoxicity
E: Complement and coagulation cascades
F: B cell receptor signaling
G: Leukocyte transendothelial migration
H: Apoptosis
I: Cytokine-cytokine receptor interaction
J: Jak-STAT signaling pathway
K: Regulation of autophagy
L: p53 signaling
M: Chemokine signaling pathway
N: Toll-like receptor signaling
O: MAPK signaling
P: mTOR signaling
Q: NOD-like receptor signaling
R: Phagosome
S: Cell adhesion molecules (CAMs)
T: Antigen processing and presentation
U: T cell receptor signaling
V: Primary immunodeficiency
W: Fc gamma R-mediated phagocytosis
Figure 5

A: Upregulated miRNAs

B: Downregulated miRNAs
Highlights:

(1) The microRNA of *Andrias davidianus* and *Andrias davidianus* responding to ranavirus were sequenced.

(2) The miRNA expression pattern, potential functions and their target genes involved in immune pathways were investigated.

(3) Differential expression miRNAs, up-regulation and down-regulation miRNAs were analyzed between ranavirus infected groups of four stages and non-infected group.