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1 # MicroRNA repertoire and comparative analysis of *Andrias* 2 *davidianus* infected with *ranavirus* using deep sequencing

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11 ## Abstract

12 *Andrias davidianus* is a large and economically important amphibian in China. 13 Ranavirus infection causes serious losses in *A. davidianus* farming industry. 14 MicroRNA mediated host-pathogen interactions are important in antiviral defense. In 15 this study, five small-RNA libraries from ranavirus infected and non-infected *A.* 16 *davidianus* spleens were sequenced using high throughput sequencing. The miRNA 17 expression pattern, potential functions, and target genes were investigated. In total, 18 1356 known and 431 novel miRNAs were discovered. GO and KEGG analysis 19 revealed that certain miRNA target genes are associated with apoptotic, signal 20 pathway, and immune response categories. Analysis identified 82 downregulated and 21 9 upregulated differentially expressed miRNAs, whose putative target genes are 22 involved in pattern-recognition receptor signaling pathways and immune response. 23 These findings suggested miRNAs play key roles in *A. davidianus*'s response to 24 ranavirus and could provide a reference for further miRNA functional identification, 25 leading to novel approaches to improve *A. davidianus* ranavirus resistance.

26

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

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29 ## 1. Introduction

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30 The Chinese giant salamander, *Andrias davidianus*, belongs to order Caudata, 31 family Cryptobranchidae, genus Andrias. It is a unique species in China, which has 32 existed for more than 350 million years, and is termed a "living fossil'' (Gao et al., 33 2003). In China, *A. davidianus* has been considered a good food with nutritional and 34 medicinal value. In the evolutionary history of vertebrates, *A. davidianus* may 35 represent animals that underwent the transition from aquatic to terrestrial life, and 36 thus it is important in scientific research (Murphy et al., 2000). *A. davidianus* was 37 classified as an endangered species by the International Union for Conservation of 38 Nature (IUCN) and as class II state major protection species of China because of its 39 diminishing population. In past thirty years, captive breeding and aquaculture of *A.* 40 *davidianus* has been successful in China. Unfortunately, with the rapid development 41 of *A. davidianus* aquaculture, the prevalence of infectious diseases has increased. As a 42 serious emerging viral pathogen, Chinese giant salamander iridovirus (GSIV) 43 belonged to ranavirus has spread widely in *A. davidianus* farming (Chen et al., 2013; 44 Dong et al., 2010; Meng et al., 2014).

45 MicroRNAs (miRNAs) are small non-coding RNAs of about 22 nucleotides (nt) 46 in length, which play crucial regulatory roles in gene expression by matching with and 47 binding to a specific mRNA target site, leading to target mRNA degradation or 48 translation inhibition, thereby affecting the level of the protein product (Wang et al., 49 2016). MiRNAs were firstly discovered in *Caenorhabditis elegans* (Lee et al., 1993) 50 and then in plants, animals, and viruses. MiRNAs are involved in a range of 51 biological processes, such as cellular development; proliferation and differentiation; 52 metabolism; homeostasis; apoptosis; and diseases (Ameres et al., 2013; Gottwein et 53 al., 2008). Recently, emerging evidence indicated that miRNAs play important roles 54 in the regulation of pathological and physiological process (Omran et al., 1993), and 55 play crucial roles during microbial infection (Wang et al., 2016). The study of 56 miRNAs in response to pathogen infection has become hot topic in immune system 57 research.

58 High-throughput sequencing has not only provides information about the 59 expression of known miRNAs, but also has helped to identify tissue-specific and

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60 rarely expressed miRNAs (Morin et al., 2008; Núñez-Hernández et al., 2015). To
61 date, thousands of miRNAs have been discovered from various aquaculture species
62 (Andreassen et al., 2017; Huang et al., 2017a; Robledo et al., 2017; Wang et al.,
63 2017). *A. davidianus* is an important aquatic amphibian in biological evolution studies
64 and aquaculture. Recently, sequence data of *A. davidianus*, such as transcriptome data
65 (Fan et al., 2015), RNA-seq data (Li et al., 2015), and a small RNA library (Chen et
66 al., 2017; Huang et al., 2017b) have been reported. These studies provided valuable
67 genetic background knowledge for this ancient species. In the present study, to
68 provide insights into the miRNAs expressed by *A. davidianus* in response to ranavirus
69 infection, we constructed five small RNA libraries from the spleens of normal animals
70 and those infected with ranavirus, and sequenced them using high-throughput
71 sequencing technology. *A. davidianus* miRNAs, target genes, functional annotations,
72 signal pathways, and differential expression patterns were investigated in different
73 ranavirus infection stages. The results will help to understand the miRNA repertoire in
74 *A. davidianus*, enrich our knowledge of regulatory mechanisms involving *A.*
75 *davidianus* miRNAs under pathogen challenge, and provide a foundation resource for
76 future functional studies of these miRNAs.

77 2. Materials and Methods

78 *2.1 Ethics statement and sample collection*

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

86 *2.2 Experimental infection*

87 *A. davidianus* were randomly divided into two groups: five animals comprised the

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88 control group and other 20 were the experimental group. Chinese giant salamander
89 iridovirus (GSIV) was isolated from diseased *A. davidianus* and was preserved in our
90 laboratory (Meng et al., 2014). The experimental groups were inoculated
91 intraperitoneally with 0.5 ml of GSIV at a dose of 1×10^6 $TCID_{50}$ ml^{-1}, while the
92 control group were injected the same volume of Dulbecco's phosphate buffered saline
93 (DPBS, Sigma, USA). The experimental animals were maintained in tanks at 20 °C
94 and fed normally. Then, at 0 (control), 6, 12, 24, and 36 h after inoculation, five
95 animals were euthanized and spleen samples were collected, immediately frozen in
96 liquid nitrogen, and stored at −80 °C.

97 *2.3 RNA extraction, library construction, and sequencing*

98 Total RNA from the spleens of the control and experimental groups at different
99 infection stages was extracted using the Trizol reagent (Invitrogen, CA, USA)
100 following the manufacturer's procedure. The purity and quantity of the total RNA
101 were checked using a NanoDrop spectrophotometer (Thermo Fischer Scientific,
102 Wilmington, DE, USA) and agarose gel electrophoresis. Only RNA samples with
103 A260/A280 ratios between 1.9 and 2.1 were used for library construction.
104 Approximately 1 μg of total RNA was used to prepare the small RNA libraries,
105 according to protocol of the NEBNext® Multiplex Small RNA Library prep kit
106 (NEB, USA). The RNA molecules in a size range of 16–35 nt were enriched by
107 polyacrylamide gel electrophoresis (PAGE). Then, 3' adapters were added and the
108 36–44 nt RNAs were enriched. The 5' adapters were then ligated to the RNAs. The
109 ligation products were reverse transcribed by PCR amplification, and the 140–160 bp
110 PCR products were enriched to generate a cDNA library and sequenced using an
111 Illumina HiSeq™ 2500 instrument at Gene Denovo Biotechnology Co. (Guangzhou,
112 China) following the vendor's recommended protocol.

113 *2.4 Basic data processing*

114 Sequencing reads were generated from the constructed small RNA libraries. The
115 raw sequences were subjected to a standard analysis pipeline including several steps.

116 In brief, first, a filtering step was carried out to exclude reads of low quality (a tag
117 with a quality score ≤ 20 and 'N' nucleotides). Second, the raw sequencing data were
118 filtered by eliminating adaptor contaminants to generate usable reads with size ≥ 18
119 nt. The 3' adapter null sequences, 5' adapter contaminants sequences, polyA
120 sequences, insert null sequences, and those with a length < 18 or length > 30 nt were
121 removed. Third, all the clean tags were aligned with the GenBank database (Release
122 209.0) and Rfam database (11.0) to identify and remove rRNAs, scRNAs, snoRNAs,
123 snRNAs, and tRNAs. Those that mapped to exons or introns might be fragments from
124 mRNA degradation; therefore, these tags were removed. Tags that mapped to repeat
125 sequences were also removed.

126 *2.5 Prediction of conserved and novel miRNAs*

127 The obtained reads were aligned to miRBase 20.0 (http://www.mirbase.org/) to
128 identify conserved miRNAs. Currently, the reference genome of *A. davidianus* is
129 unavailable; therefore, to identify novel miRNAs in *A. davidianus*, all the unannotated
130 tags were aligned with the reference transcriptome data of *A. davidianus* (Fan et al.,
131 2015). According to the transcriptome data and the predicted hairpin structures
132 produced by the software Mireap_v0.2, novel miRNA candidates were identified.

133 *2.6 Target gene predication and pathway analysis*

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

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144 1995), and the significance level was set at 0.05.

145 *2.7 Differential expression analysis of miRNAs*

146 To compare the miRNA expression data of the control and ranavirus infected
147 samples, the total miRNA expression (known and novel miRNAs) was calculated and
148 normalized to the transcripts per million (TPM) value, based on their expression in
149 each sample. The fold change (FC) between the miRNA expression in the five
150 libraries was determined as: FC = TPM 6 h/TPM 0 h, FC = TPM 12 h/TPM 0h, FC =
151 TPM 24 h/TPM 0 h and FC = TPM 36 h/TPM 0 h miRNAs. Those miRNAs with a
152 $|\log2 \text{FC}| \geq 1$ and a p-value <0.05 (calculated from the normalized expression) were
153 considered to be significantly differentially expressed. Thus, the upregulated and
154 downregulated miRNAs among all the differentially expressed miRNAs were
155 identified.

156 **3. Results**

157 *3.1 Overview of the high-throughput sequencing data*

158 In this study, five small RNA libraries were constructed from the spleens of *A.*
159 *davidianus* treated with Chinese giant salamander iridovirus and DPBS (control) to
160 identify and characterize miRNAs involved in the genomic level response to ranavirus
161 infection. The five small RNA libraries were sequenced by Illumina deep sequencing
162 technology on libraries of small RNAs from control (0 h) and infected samples at 6,
163 12, 24, and 36 h, and named GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and
164 GSIV-36h respectively. After filtering out low-quality sequences (Q-value ≤ 20 reads),
165 high quality reads were obtained. After removing the 3' adapter null sequences, polyA
166 sequences, insert null sequences, 5' adapter contaminants, sequences <18 nt, the clean
167 sequences were obtained for subsequent analysis (Table 1). A total of approximately
168 65,800,061 clean reads were obtained from the five small RNA libraries after
169 high-throughput sequencing.

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172 **Table 1**

173 Summary of high throughput sequencing data

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

174 Compared with the control library, the percentage of high quality reads among the
175 total reads increased obviously in the experimental groups, representing the ratio
176 between high quality reads and the total reads as shown in Table 1. The unique
177 sequences <18 nt were reduced in the four infection groups compared with that in the
178 control group. However, the number of 3' adapter null sequences increased with
179 increasing infection time, with the the highest level in the GSIV-36h library. The size
180 distribution of sRNAs ranged from 16–35 nt. Most of them were 19–24 nt, which was
181 consistent with the typical sizes of dicer processed products. We determined the
182 proportion of 16–18 nt, 19 nt, 20 nt, 21 nt, 22 nt, 23 nt, 24 nt, and 25–35 nt sequences
183 in the small RNA libraries at GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and
184 GSIV-36h, respectively (Fig. 1). The sRNAs of 21 nt and 22 nt formed two major
185 classes in the sRNA libraries. The most abundant sRNA length was 22 nt, which was
186 present at 22.83%, 27.80%, 28.82%, 26.86%, and 24.54% in the five libraries,
187 respectively. Analysis of the first nucleotide bias of the 19–24 nt sRNAs obtained in
188 the five libraries indicated that (U) was preferred at the first position, especially in the
189 21 and 22 nt sRNAs (Supplementary File S1). After further removal of ribosomal
190 RNA (rRNA), transfer RNAs (tRNAs), small cytoplasmic RNA (scRNA), small
191 nuclear RNA (snRNA), small nucleolar RNAs (snoRNAs), repetitive sequence
192 elements, the unannotated small RNA reads were retained for miRNA analysis.

193 *3.2 Identification of known miRNAs and novel miRNA candidates*

194 High throughput sequencing can be used to verify a large number of known
195 miRNAs and novel specific miRNAs in organisms. The lack of a genome sequence

196 for *A. davidianus* meant that all of the unannotated sequencing reads were first
197 aligned against the miRNA sequences in miRBase (http://www.mirbase.org/). The
198 small RNA sequences were then mapped to reference transcriptome data of *A.*
199 *davidianus*. Thus, the novel and known miRNAs from normal and infected *A.*
200 *davidianus* were identified. In the five sRNA libraries, there were 1356, 1094, 1091,
201 1022, and 992 known miRNAs in GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and
202 GSIV-36h, respectively. In addition, compared with the reference transcriptome data
203 of *A. davidianus*, there were 431, 435, 468, 467, and 424 novel miRNAs identified
204 from GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and GSIV-36h, respectively. The
205 variability in this sRNA data is shown in Fig. 2. The results showed that the number
206 of known miRNAs decreased with increasing infection time. The numbers of novel
207 miRNA were increased at GSIV-6h, GSIV-12h, and GSIV-24h compared with that at
208 0 h. The number of novel miRNAs increased substantially after infection for 12 and
209 24 h. The novel miRNAs candidates also should be further validated by direct cloning
210 using the stem-loop structure.

211 *3.3 Analysis of the target genes of A. davidianus miRNAs*

212 The function of a miRNA is ultimately defined by genes it targets and its effects
213 on target mRNA translation. Based on the previous data of *A. davidianus*
214 transcriptome sequencing, potential targets of the miRNAs were predicted using
215 TargetScan, miRanda, and PicTar, the three most frequently used prediction
216 algorithms. Potential targets of the 3253 *A. davidianus* miRNAs were predicted by the
217 intersection of three prediction programs. For further functional annotation, we used
218 GO to annotate/classify the function the predicted miRNA target genes, and KEGG to
219 construct pathway enrichment of the predicted miRNA target genes from the five
220 libraries. All the predicted targets were annotated in three main categories: Biological
221 process, cellular component, and molecular function (Supplementary File S2, Fig. 3).
222 Among the three functional categories, the GO terms with the highest number of
223 targets were cellular process (in the biological process category), cell and cell part
224 (cellular component), and binding (molecular function). In addition, there were some

225 high frequency biological processes closely related to the immune response system.
226 These terms included response to stimulus, immune system process, biological
227 adhesion, viral reproduction, and cell killing.

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

241 *3.4 Expression analysis of A. davidianus miRNAs induced by ranavirus*

242 High-throughput sequencing is not only a good tool to identify small RNAs, but
243 also provides information about miRNA expression levels. The miRNAs could be
244 expressed differentially between infected and non-infected animals in different
245 infection phases. The expression patterns of known and novel miRNAs identified in
246 five GSIV infection stages were profiled based on the sequencing results. The results
247 showed that many miRNAs had a wide range of expression levels at all examined
248 time points, and the expression quantity of most of them were more than two-fold
249 different at 0h, 6h, 12h, 24h and 36h after infection, respectively. The detailed
250 expression information of the known and novel miRNAs in the different stages is
251 shown in Supplementary File S5 and S6. Based on the miRNA expression
252 information, the differentially expressed miRNAs were identified. To analyze the
253 differentially expressed miRNAs at each time point, we compared all the miRNAs via

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254 pairwise comparisons between GSIV-0h, GSIV-6h, GSIV-12h, GSIV-24h, and
255 GSIV-36h. There were 835 miRNAs (including known and novel miRNAs) that were
256 differentially expressed at all examined time points. Among them, 294 were
257 upregulated and 541 were downregulated.

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

264 The Venn figure in Fig.5 shows 82 miRNAs that were downregulated in the four
265 GSIV infection groups compared with the control group (Fig. 5A) and nine miRNAs
266 that were upregulated after GISV infection compared with the control group (Fig.
267 5B). Among the 82 miRNAs showing downregulated expression, most of them
268 showed a more than 10-fold different expression level during the infection process
269 compared with the uninfected control, such as mir-122, mir-203, mir-206, and
270 mir-722. For example, miR127 (upregulated) and mir-122 (downregulated) showed a
271 100-fold difference in their expression levels between the treatment groups and the
272 control group. These significantly differentially expressed miRNAs targeted genes
273 such as *RIG-I*, *MDA5*, *TLR1*, and *MHC*, are associated with the immune system.

274 4. Discussion

275 MiRNAs play a critical role in the response to biotic and abiotic stress, and have
276 been characterized in a large number of aquatic organisms (Li et al., 2012; Wang et
277 al., 2017). High throughput sequencing technology has been applied extensively to
278 small RNA research, and can identify a large number of known miRNAs and novel
279 specific miRNAs in organisms subjected biotic or abiotic stress (Ou et al., 2012; Yu et
280 al., 2016). The study of miRNA-mediated host-pathogen interactions has emerged in
281 the last decade because of the important role played miRNAs in antiviral defense. For
282 example, using deep sequencing and quantitative real-time PCR, ten

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283 polyinosinic-polycytidylic acid (pIC)-stimulated miRNAs were identified in Atlantic
284 cod, which suggested that miRNAs are important in the antiviral immune responses of
285 Atlantic cod macrophages (Eslamloo et al., 2018). *Marsupeneaus japonicus* miR-S5
286 could affect the expression of p53, tumor necrosis factor-α (TNF-α), and myosin to
287 regulate hemocyte phagocytosis and apoptosis processes in response to white spot
288 syndrome virus (WSSV) (Wang et al., 2018).

289 Ranavirus is a major pathogen in aquaculture that causes ulcerative injury in
290 animals. Identification of miRNAs expressed by *A. davidianus* infected by ranavirus
291 has significance in host/pathogen interaction research and in the antiviral immune
292 response of *A. davidianus*. In this study, we investigated the miRNA expression
293 patterns associated with ranavirus-infected *A. davidianus* using a high-throughput
294 sequencing approach at 0 h, 6 h, 12 h, 24 h and 36 h after GSIV infection. In total,
295 1356 known miRNAs and 431 novel miRNAs were identified in the control group.
296 The number of known miRNAs decreased and the number of novel miRNAs
297 increased in infection groups compared with the control group. Using Illumina deep
298 sequencing, Huang et al. identified 140 conserved and three novel miRNAs of *A.*
299 *davidianus* when they mapped the sequence reads to the reference genome sequence
300 of *Xenopus tropicalis* (Huang et al., 2017b). In another study, a total of 757 and 756
301 unique miRNAs were annotated as miRNA candidates in the ovary and testis of *A.*
302 *davidianus*, respectively. Among them, 145 miRNAs in the ovary and 155 miRNAs in
303 the testis were homologous to those in *Xenopus laevis* ovary and testis, respectively
304 (Chen et al., 2017). In the present study we identified more miRNAs in *A. davidianus*
305 in response to ranavirus infection compared with the studies above. This might be
306 because these miRNAs were screened using different parameter settings or reference
307 databases. We referenced the transcriptome data of *A. davidianus*, not the genome of
308 *Xenopus tropicalis*. Although *Xenopus tropicalis* is often regarded as a representative
309 animal of amphibians, in fact, *Xenopus tropicalis* belongs to the Anura and *A.*
310 *davidianus* belongs to the Caudata, which have different genetic characteristics.
311 However, our results and those of previous studies suggest that *A. davidianus*
312 expresses abundant miRNAs.

313 GO is an international, standardized gene functional classification system that
314 offers a constantly updated vocabulary and a strictly defined concept to
315 comprehensively describe the properties of genes and their products in an organism.
316 The KEGG pathway is a map representing knowledge of molecular interactions,
317 reactions and relation networks, and is also a database resource to help understand the
318 functions and utilities of biological information, especially large-scale molecular
319 datasets generated using high-throughput technologies. The GO terms and KEGG
320 pathways associated with the target gene of the *A. davidianus* miRNAs were
321 analyzed. The results showed that the miRNA target genes are involved in important
322 signaling pathways and immune response processes. For example, there were 218
323 target genes in T cell receptor signaling, 198 in B cell receptor signaling, 181 in
324 natural killer cell mediated cytotoxicity, and 450 in Endocytosis. These results were
325 consistent with the miRNA target gene research in which miRNAs were observed to
326 play important roles in the regulation of the immune system, including the
327 development and differentiation of B or T lymphocytes (Maryaline et al., 2017),
328 natural killer cells (Kingsley et al., 2017), and modulation of inflammation
329 (Neudecker et al., 2017).

330 In this study, we aimed to investigate the miRNA repertoire of *A. davidianus*
331 under GSIV infection. Therefore, we compared the expression of the miRNAs
332 between the control and four post-infection phases to identify differentially expressed
333 miRNAs. Among the differentially expressed miRNAs, most of them showed
334 differential expression level. Seventy-seven known miRNAs and five novel miRNAs
335 were downregulated in all four ranavirus infection groups compared with that in the
336 control group, while two known and seven novel miRNAs were upregulated
337 expression in all experimental groups compared with the control. The target genes of
338 these differentially expressed miRNAs were predicted. GO and KEGG analyses
339 indicated that most of the target genes were related to the immune system. Among the
340 77 downregulated known miRNAs, some showed the significantly different
341 expression patterns, such as mir-122, mir-203, mir-206, mir-31, mir-722, mir-72,
342 mir-8109 and mir-8159. Taking mir-122 as a typical example, its expression level was

343 about 3941 in the control group, and was 758, 134, 361, and 108 at 6, 12, 24, and 36 h
344 after infection, respectively. Analysis of mir-122 indicated that it is involved in pattern
345 recognition receptor (PRR) signaling, including Toll-like receptor (TLRs), NOD-like
346 receptor (NLRs), RIG-I like receptor (RLRs); complement and coagulation cascades;
347 natural killer cell-mediated cytotoxicity; B/T cell receptor signaling pathway;
348 chemokine signaling pathway; Fc epsilon RI signaling pathway; antigen processing
349 and presentation; and primary immunodeficiency. The target genes of mir-122 include
350 *RIGI* (retinoic acid-inducible gene I protein), *Mx* (myxovirus resistance), *Aven* (cell
351 death regulator Aven), *Apaf1* (apoptotic protease activating factor 1), *IAP* (inhibitor of
352 apoptosis protein), *MDA5* (melanoma differentiation-associated protein 5, *TLR1*, and
353 *MHCII* (major histocompatibility complex II). These pathways and target genes all
354 related to the innate and adaptive immune systems. Characterization of
355 immune-related miRNAs is a prerequisite for a thorough understanding of the role
356 played by miRNA-mediated post-transcriptional gene regulation in the innate immune
357 system (Ou et al., 2012). The host limits viral infection by expressing miRNAs that
358 target important related genes (Zhang et al., 2014). When challenged by *Vibrio*
359 *anguillarum*, mir-122 of miiuy croaker showed dramatically reduced expression
360 profiles. *TLR4* was identified as a target of mir-122, and its expression level was
361 increased in response to *Vibrio anguillarum* challenge. Therefore, mir-122 could
362 target gene *TLR14*, which is involved in miiuy croaker's inflammatory and immune
363 response (Cui et al., 2016). Dihydroxyacetone kinase (DAK) is an inhibitor of MDA5.
364 Mir-122 could target *DAK* to participate in regulating the RIG-I like receptor
365 signaling pathway at the post-transcriptional level in miiuy croaker's spleen and
366 macrophages under pIC stimulation, which is a synthetic analog of double-stranded
367 RNA (dsRNA) and has a molecular pattern associated with viral infection (Han et al.,
368 2018). In addition, mir-203 was significantly differentially expression and it could
369 target the *Mx* gene. Mx protein is an antiviral protein that has a broad antiviral
370 function and is induced by interferon (IFN) or produced by animals infected with
371 viruses. In our previous study, *Mx* gene was upregulated in gsIFN-overexpressing
372 cells of *A. davidianus* after GSIV infection (Chen et al., 2015). Therefore, combining

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373 the existing studies about mir-122 or mir-203, we could speculate that the
374 differentially expressed miRNAs detected in our study have important regulatory
375 functions in the innate and adaptive immune systems in *A. davidianus* in response to
376 ranavirus infection. These miRNAs may exert these important effects via regulating
377 such genes as *RIGI*, *IFN*, *MDA5*, *TLR1*, *TNF*, *IAP*, *NRAMP*, *Mx*or *MHC* and so on in
378 their different pathways.

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

388

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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.

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Figure 1

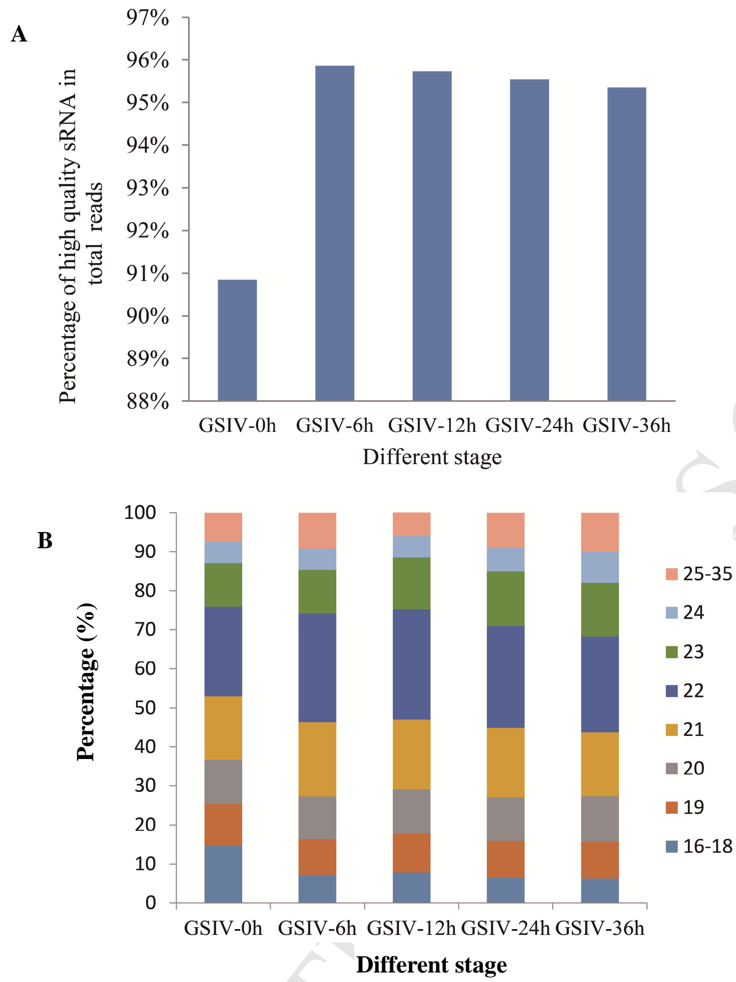

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Figure 2

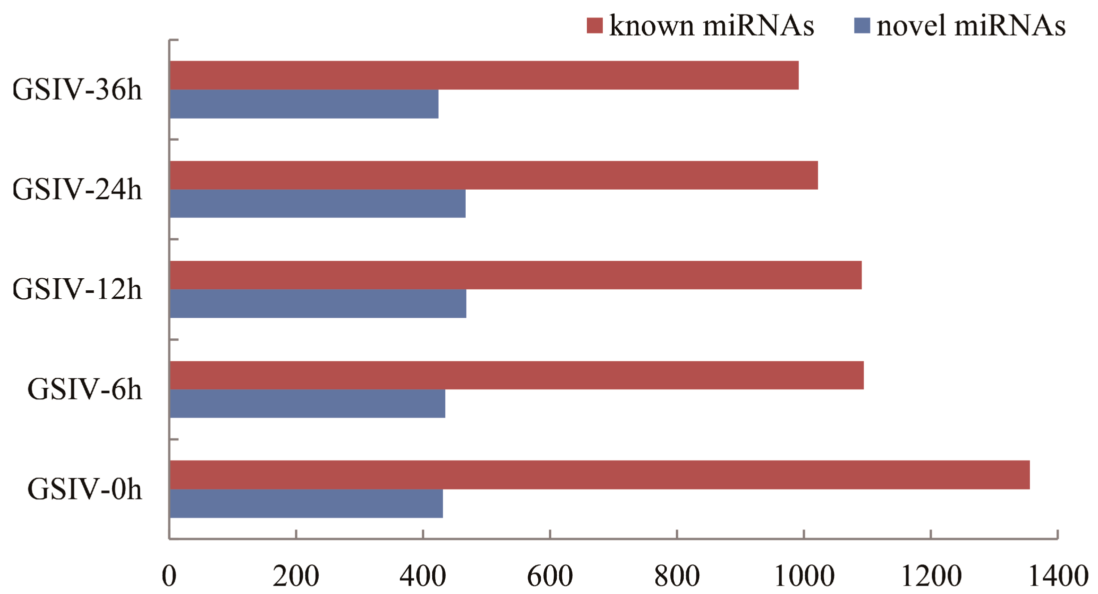

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Figure 4

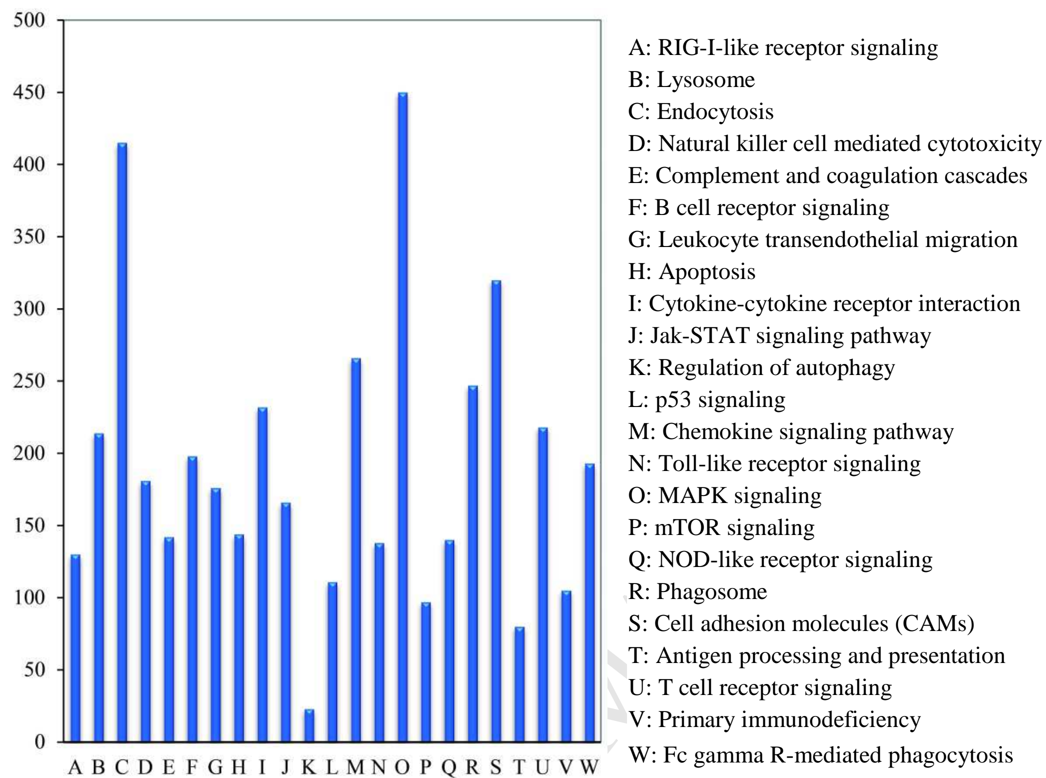

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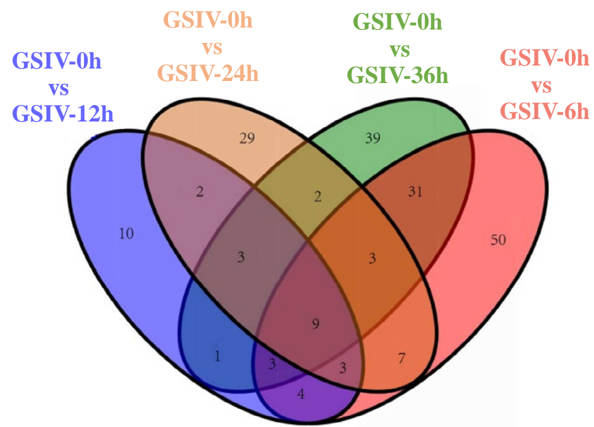
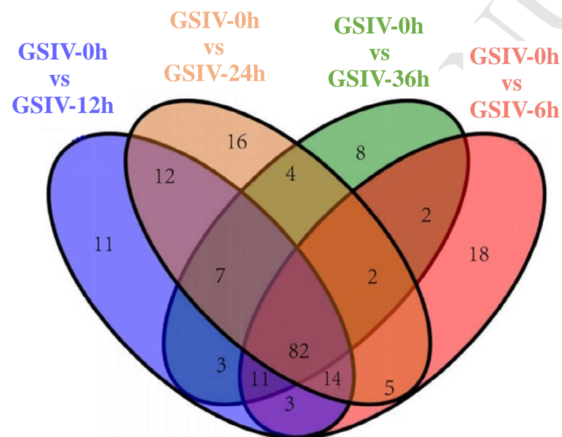
Figure 5



A: Upregulated miRNAs



B: Downregulated miRNAs

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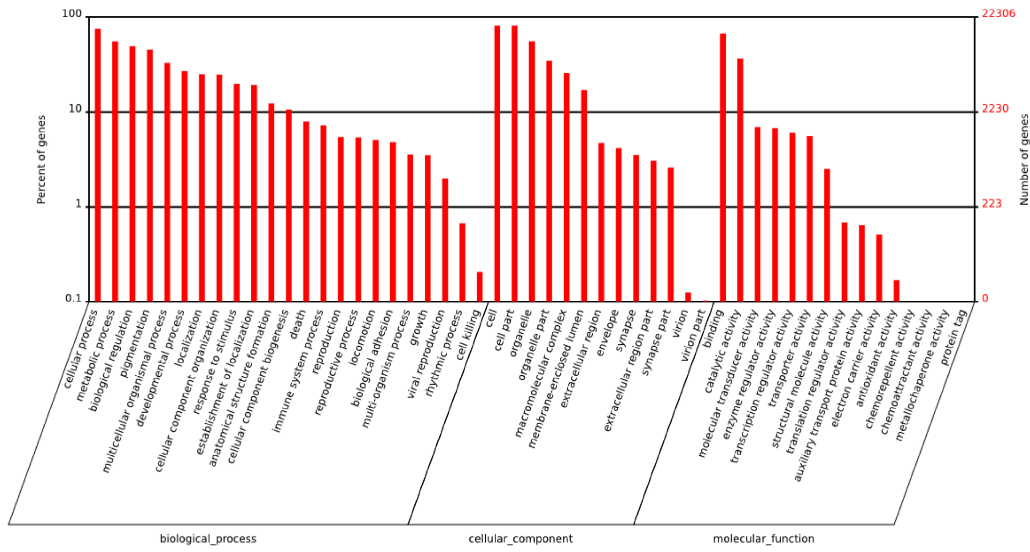

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Highlights:

(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.

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