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A transcriptome analysis focusing on splenic immune-related mciroRNAs of rainbow trout upon *Aeromonas salmonicida* subsp. *salmonicida* infection

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ABSTRACT

MicroRNAs (miRNAs) are a class of small non-coding RNAs that can regulate the immune responses during pathogen infection. *Aeromonas salmonicida* (*A. salmonicida*) *subsp. salmonicida* is the causative agent of furunculosis in salmon and trout. To identify the miRNAs and investigate the specific miRNAs in rainbow trout upon *A. salmonicida* subsp. *salmonicida* infection, we performed high throughput sequencing using the spleens of rainbow trout infected with and without an *A. salmonicida* subsp. *salmonicida* clinical isolate. A total of 381 known miRNAs and 926 novel miRNAs were identified. Eleven known and 16 novel miRNAs were found to be differentially expressed upon infection. The results of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses indicated that the target genes of the differentially expressed miRNAs were closely associated with immune responses and biological regulations. Additionally, over- and suppressed expression of miR-155–5p significantly enhanced and reduced the IL-2 and IL-1β expressions in RTG-2 cells induced by *A. salmonicida*, respectively. To our knowledge, this is the first experimental study on the miRNAs of rainbow trout upon *A. salmonicida* infection. The results here might lay a foundation for the further understanding of the roles of miRNAs in the immune responses during *A. salmonicida* infection in rainbow trout.

1. Introduction

Aeromonas salmonicida (*A. salmonicida*) *subsp. salmonicida* can cause a classical furunculosis, which is a global infectious disease of marine and freshwater fish that especially impacts the salmonid species [1]. When the fish were acutely infected by *A. salmonicida* subsp. *salmonicida*, the necrotic lesions in the skin and haemorrhages in the internal organs could be observed. These infections are often fatal within as little as two or three days, and usually bring major economic losses [2,3].

Due to furunculosis caused by *A. salmonicida* is a threat to aquaculture, extensive work have been done to understand the bacterial virulence factors and the host defense responses. For example, expression of A-layer protein coding gene of *A. salmonicida* was reported to be regulated by the AI-2 quorum sensing, which could influence the bacterial survival ability when suffered from attack of the host immune system [4]. *A. salmonicida* has been proved to suppress the immune response of rainbow trout (*Oncorhynchus mykiss*) by its type III secretion system [5]. With the development of high throughput sequencing technologies, the overall immune responses to *A. salmonicida* infection were also characterized by transcriptome or proteomic analysis [6]. Although the current knowledge of the transcriptome can benefit the fish diseases [7,8], the lack of the information on the regulation of the transcriptome will still limit the better understanding the immune mechanism during *A. salmonicida* infection.

MicroRNAs (miRNAs) are non-coding small RNAs of approximately 22 nucleotides in length, defined as the important regulators in numerous biological progresses of animals. MiRNA genes are transcribed as large primary transcripts (pri-miRNA) that will be cleaved by RNase III Drosha to form a precursor miRNA (pre-miRNA). Subsequently, this pre-miRNA is processed by RanGTP/Exportin-5 and cleaved by RNase III Dicer to generate miRNA duplexes. Then, the miRNAs are loaded onto an argonaute family protein (AGO) to form the miRNA-induced silencing complex (miRISC). Finally, the miRNA will guide miRISC to

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the target mRNA containing a complementary sequences, thus inhibiting gene translation [9].

The miRNAs profiles during bacterial infections were recently identified in kinds of fish, such as Half-smooth tongue sole (*Cynoglossus semilaevis*) upon *Vibrio anguillarum* infection [10], grass carp (*Ctenopharyngodon idella*) upon *Aeromonas hydrophila* infection [11], miiuy croaker (*Miichthys miiuy*) upon *Vibrio anguillarum* infection [12], Nile tilapia (*Oreochromis niloticus*) upon *Streptococcus agalactiae* infection [13], and Common Carp (*Cyprinus carpio*) upon *Flavobacterium columnare* infection [14]. More important, fish miRNAs were also proved to be functional in the regulation of signaling induced by bacteria. For example, cid-miRn-115 or miR-142a-3p resulted in a visible change in *Ctenopharyngodon idella* kidney (CIK) cells immune effector activity by directly regulating the *tlr5* expression [15]. Miiuy croaker miR-192 was involved in the immune and inflammatory responses by modulating IL-1RI expression [16]. MiR-200a-3p regulated TLR signaling pathways by targeting TLR1 in bacterial challenged miiuy croaker [17]. Therefore, the above evidences have revealed that miRNAs responding to bacterial infection play important roles in the fish innate immune responses. At present, only three potent miRNAs were predicted by computational analysis of 1119 ESTs from macrophages of Atlantic salmon (*Salmo salar*) infected with *A. salmonicida* [18]. However, the actual miRNAs responses of rainbow trout upon *A. salmonicida* infection were still unknown until now.

In this study, we use rainbow trout (*Oncorhynchus mykiss*) as a model to evaluate the host miRNAs responses to *A. salmonicida* subsp. *salmonicida* infection by the Solexa deep sequencing. In total, 11 known and 16 novel miRNAs were identified to be differentially expressed. Additionally, the validity of four known miRNAs and six novel miRNAs were confirmed by RT-PCR. In order to further understand the potential functions of these miRNAs, target prediction, GO enrichment, and KEGG pathway were analyzed. Finally, the effects of four selected miRNAs on the inflammatory response induced by *A. salmonicida* subsp. *salmonicida* were investigated on RTG-2 cells. This study will contribute to the better understanding of miRNA mediated regulation during the *A. salmonicida* infection.

2. Materials and methods

2.1. Fish

Rainbow trout weighing approximately 10 g were cultivated at 12.0–12.5 °C in aerated fiberglass tanks under a 12 h:12 h light:dark photoperiod with circulating water. Investigations were conducted according to Animal Care and Experiments Ethics Committee of Heilongjiang River Fisheries Research Institute.

2.2. Experiment design

Aeromonas salmonicida subsp. *salmonicida* 2014–182 was isolated from a rainbow trout farm in China, causing > 70% mortality during 3–7 days post infection (dpi) in the challenge experiment. The fish in experimental group were intraperitoneally injected with *A. salmonicida* subsp. *salmonicida* at a dose of 2×10^7 cells/ml. The control group was intraperitoneally injected with 100 μl of sterilized phosphate buffered saline (PBS). At 2, 4 and 6 day post infection (dpi), the spleens from three individuals of each group were collected, pooled separately. All the samples were stored at −80 °C until to use.

2.3. Construction of small RNA libraries and sequencing

The small RNA libraries construction and sequencing were carried out according to the typical procedures of Illumina sequencing. Briefly, total RNAs from the infected or uninfected group at 2 dpi were extracted using TRIzol reagent (Life Technologies, USA) and digested with DNase I. The quantities and purities of RNAs were monitored by A260/A280. Then low molecular weight RNAs were exacted by denaturing PAGE (7 M urea/15% PAGE). A pair of Solexa proprietary adaptors as PCR primer was ligated to both 5′ and 3′ ends of the RNAs. After amplification cDNA for the production of sequencing libraries by reverse PCR, the quality and output of the sequencing libraries were evaluated by Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System. The cDNA library was sequenced using the Hiseq 2500 platform.

2.4. Sequencing reads analysis

The initial reads were processed by eliminating the low quality reads, adaptor sequences, and redundancy contaminants from raw reads. The clean reads were aligned with Rfam and Repbase database to discard the degenerated fragments of mRNA or other non-coding RNAs, such as rRNA, tRNA, and snoRNA using Bowtie tools software. The clean short reads were mapped to the Atlantic salmon (*Salmo salar*) genome (NCBI: GCA_000233375.4) using SOAP [19]. Then, the unique small RNA sequences were BLASTN searched against the mature miRNA sequences of *Salmo salar* in miRBase (http://www.mirbase.org/), and sequences with perfect match were identified as known miRNAs. Novel miRNAs were predicted to find step-loops or hairpin structures using the miRDeep2 software.

2.5. Differential miRNA expression analysis

Numbers of reads from each miRNA were normalized to the total number of reads in each library, respectively. The DE miRNAs were identified using the MA-plot-based method (DEGseq) [20]. Poisson Distribution formula were selected to calculated the random error of sequencing, while the comparison between groups was done based on the read counts. False discovery rate (FDR) was used to adjust the p-value. The miRNAs with reads number > 1000, FDR < 0.01, and $|\log_2(\text{fold change})| \geq 1$ were defined to be significantly differentially expressed (DE).

2.6. Target gene prediction and functional annotation

In order to analyze the potential functions of DE miRNAs during *A. salmonicida* infection, the target genes were predicted using TargetScan (default parameters) and MiRanda (free energy < −20 kcal/mol) based on the 3′ UTRs of *Salmo salar* that were prepared in our previous work [21]. Finally, the predicted miRNA target genes were conducted with KEGG (http://www.genome.jp/kegg/) and Gene ontology (GO) analyses (http://david.abcc.ncifcrf.gov/).

2.7. Validation and measurement of miRNAs

In order to confirm the deep sequencing results, the expression levels of four DE known miRNAs were detected by qRT-PCR using the same RNA samples used for the construction of the miRNA library. RT-PCR was performed to confirm the existence of 6 selected novel miRNAs in rainbow trout. Additionally, we also used qRT-PCR to profile the DE miRNAs based on the RNA samples from fish at 2, 4, 6 dpi. qRT-PCR was performed according to the All-in-One™ miRNA qRT-PCR Detection System User Manual (GeneCopoeia). The primers used in the PCR were shown in Table 1. Briefly, RNAs were reverse-transcribed using a mixture containing 8 μl extracted total RNAs, 1 μl Poly A Polymerase, 1 μl RTase Mix, 5 μl 5 × PAP/RT Buffer and 10 μl dd H_2O (RNase/DNase free). The condition of reverse transcription was 37 °C for 60 min, followed by 85 °C for 5 min. Then, the relative quantification real-time PCR was performed using an Applied Biosystems 7500. The real-time PCR is consisted of 10 μl 2 × All-in-One qPCR Mix, 2 μl Universal Adaptor PCR Primer, 2 μl First-strand cDNA, 0.1 μl ROX Reference Dye, 3.9 μl dd H_2O. The condition was 95 °C for 10 min, 40 cycles of two steps (95 °C for 10 s, 60 °C for 30 s). The analysis of

Table 1
Primers for the analysis of miRNA expression by qRT-PCR or RT-PCR.

Primer name	Primer sequences (5′ to 3′)
miR-146a-3p sense	TCGGCAGGATCTATGGGCTCAGT
miR-155–5p sense	GCCGAGTTAATGCTAATCGTG
miR-499b-5p sense	GCCGAGTTAAGACTTGCAGT
miR-125a-2-3p sense	TACACAGAGACGGGTTAGGCTCT
novel-1	TACACATAGGTGCGAAGCGGGGCT
novel-51	TCGGCAGGTGTAAACATCCCCGA
novel-154	GCCGAGTGAGGTAGTTGTTTG
novel-420	TATATATACGAGCCGCGGCTGGG
novel-564	ATATATAGCGAAGCGGGGCTG
novel-721	TACACATAGTGCGAAGCGGGGCT
18S rRNA sense	CGGAGGTTCGAAGACGATCA
18S rRNA antisense	TCGCTAGTTGGCATCGTTTAT

relative expression was done using $2_{T}^{-\Delta\Delta C}$ method. The expression of 18s rRNA were used to normalize the miRNAs expression. Comparisons between two groups were made by one-way ANOVA. Results were considered significant when $P < 0.05$.

2.8. Effects of four DE miRNAs on the inflammation induced by A. salmonicida subsp. salmonicida

RTG-2 cells were cultured in six-well plates until cell density was 60%–80% confluent. Four pairs of mimics and inhibitors for miR-125a-2-3p, miR-146a-3p, miR-155–5p and miR-499b-5p were synthesized by GenePharma (Shanghai, China). Then these molecular were transfected into RTG-2 cells at 50 nM for mimics or 100 nM for inhibitors using Lipofectamine 3000, respectively (Invitrogen, USA). The control cells were transfected with control mimic or inhibitor. At 24 h post transfection, the cells were collected for either determination of miRNA expression or infected with *A. salmonicida* subsp. *salmonicida* at 2×10^7 cells/ml for 4 h at 18 °C. Then, total RNAs were extracted from cells to evaluate the expression levels of IL-2 and IL-1β by relative quantification real-time PCR, normalized by the expression of β actin.

3. Results

3.1. Overview of the high throughput sequencing

In order to identify the miRNAs responses to *A. salmonicida* subsp. *salmonicida* infection in rainbow trout, small RNA libraries derived infected samples and mock samples were constructed and sequenced using Illumina/Solexa deep-sequencing technology. More than 11 million raw reads, 100% high-quality reads were identified from the infected and mock groups. A total of 10,736,342 (95.61%) and 11,720,956 (96.05%) clean reads of *A. salmonicida* group and mock group were obtained by elimination of the 5′ adapter, insert, 3′ adapter, below 18 nt, and polyA segments, respectively (Table 2). The length distribution and frequency percentage of the majority of the clean reads from the two groups both followed the typical length range of miRNAs (Fig. 1A). After BLASTN search against the Rfam, 37.79% and 66.94% miRNAs for infected and uninfected groups were mapped to the genome of *Salmo salar*, respectively (Fig. 1B).

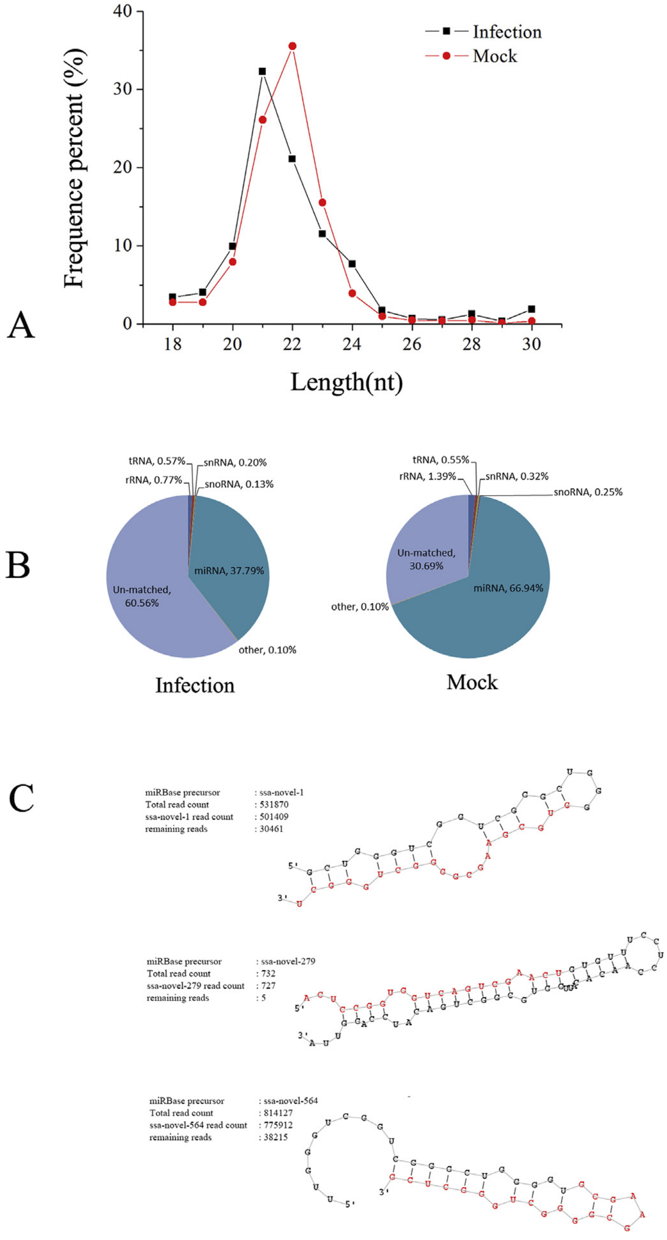

Fig. 1. Characterization of the small RNAs of rainbow trout and the structures of the novel miRNAs. (A) Size distribution of clean reads derived from sequencing results based on rainbow trout libraries. (B) Annotation of small RNAs. (C) The secondary structures of novel miRNAs predicated using the miRDeep2 software.

3.2. Identification of known miRNAs and novel miRNA candidates

A total of 381 known miRNAs were identified from the experimental and control groups after alignment with the *Salmo salar* miRNAs in miRBase 21.0 (Excel S1). Among these, the read numbers of 102 miRNAs were above 1000 (e.g., miR-22a-3p, miR-100a-5p, miR-146a-5p and miR-26a-5p), while some other miRNAs were expressed at quite

Table 2
Preliminary analysis of the high throughput sequencing results.

Group	Total reads	High quality	clean reads	3′adapter	insert	5′adapter	< 18 nt	polyA
Infection	11358362	11229835 (100%)	10736342 (95.61%)	78809 (0.70%)	13294 (0.12%)	3983 (0.04%)	397394 (3.54%)	13 (0.00%)
Mock	12306193	12203024 (100%)	11720956 (96.05%)	131374 (1.08%)	30765 (0.25%)	8497 (0.07%)	311415 (2.55%)	17 (0.00%)

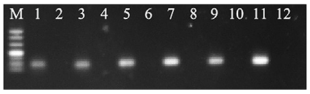

Fig. 2. RT-PCR analysis of the selected six novel miRNAs. M. DL 500 DNA marker; Lane 1, 3, 5, 7, 9, 11: PCR products of novel-1, novel-51, novel-154, novel-420, novel-564 and novel-721; Lane 2, 4, 6, 8, 10, 12: PCR products of the negative control for the corresponding miRNAs, respectively.

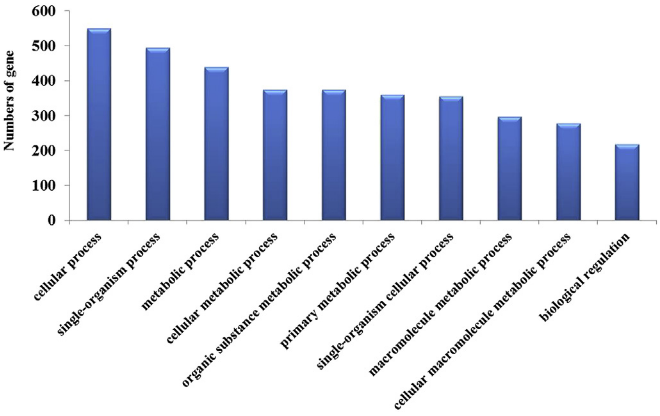

Fig. 4. Analysis of the top 10 most-enriched GO terms.

low level (e.g., miR-9b-5p, miR-202–5p, miR-101b-5p and miR-455–3p). Sixteen miRNAs (e.g., miR-10b-2-3p, miR-132-3-5p and miR-725–5p) were only found in infected group, while 31 miRNAs (e.g., miR-8162–5p, miR-456–5p and miR-137–3p) only existed in uninfected group. The remaining 926 putative candidate miRNAs were predicted as novel miRNAs (Excel S2), which could form the hairpin structure as shown in Fig. 1C. Additionally, RT-PCR was performed to validate six novel miRNAs (novel-1, novel-51, novel-154, novel-420, novel-564 and novel-721). The results shown in Fig. 2 confirmed the existence of these novel miRNAs in rainbow trout.

3.3. Analysis of the differentially expressed (DE) miRNAs

In order to investigate the DE miRNAs upon *A. salmonicida* infection, the expression of eleven known miRNAs (10 upregulated and 1 downregulated miRNAs) and sixteen novel miRNAs (11 upregulated and 5 downregulated miRNAs) were found to be different in response to *A. salmonicida* infection (Excel S3). Then, qRT-PCR were performed to profile four DE miRNAs (miR-125a-2-3p, miR-146a-3p, miR-155–5p, miR-499b-5p) during *A. salmonicida* infection based on the RNA samples from the spleen samples collected at 2, 4, and 6 dpi. The qRT-PCR results were consistent with those obtained by sequencing. Additionally, the most significant expression of miR-125a-2-3p was observed at 4 dpi and decreased to the similar level of that at 2 dpi. MiR-146a-3p displayed acute induction at 2 dpi and returned to the normal level at 6 dpi. The expression of miR-155–5p increased consecutively during the course of infection, while that of miR-499b-5p decreased (Fig. 3). These results indicated *A. salmonicida* infection could indeed change the expression patterns of trout miRNAs.

3.4. Target prediction and gene ontology of DE miRNAs

The 3′ UTRs of rainbow trout were obtained in our previous work [21]. Based on these 3′ UTRs, the target genes of DE miRNAs were predicted using TargetScan and miRanda. As a result, a total of 8133 and 9993 targets were predicted for the known and novel miRNAs, respectively (Excel S4). The 8133 targets of known miRNAs were clustered into 1222 terms by Gene ontology (GO) analyses (Excel S5). The top ten most-enriched Go terms including biological regulation, cellular process and regulation of biological process were shown in Fig. 4. Additionally, KEGG pathway analysis grouped the targets into 288 pathways (Excel S6). The top three were Wnt signaling pathway, mTOR signaling pathway and TGF-beta signaling pathway for signal transduction, while Fc gamma R-mediated phagocytosis, Fc epsilon RI signaling pathway and RIG-I-like receptor signaling pathway were found to be significantly enriched for immune system (Fig. 5).

In order to evaluate the potential functions of DE miRNAs in the regulation of immune response, we focused on the target genes of DE known miRNAs that were closely associated with Immune system, Signaling molecules and interaction and Signaling molecules and interaction. Finally, 63 target genes were found to cover the immune system (Excel S7). The numbers of immune related genes targeted by miR-146a-3p were the most, followed by that of miR-200b-3p. The same immune pathway or even the same gene might be targeted by multiple miRNAs. For example, RAC serine/threonine-protein kinase gene was predicted to be the target gene of miR-125a-2-3p, miR-146a-3p, miR-181a-4-3p, miR-200a-3p, and miR-200b-3p. Of interest, MAP kinase kinase 7 which is involved in the immune response to bacterial challenge is both targeted by miR-146a-3p (an up-regulated miRNA) and miR-499b-5p (a down-regulated miRNA) [22]. Additionally, 204 and 57 target genes of DE miRNAs were predicted to be local in the Signaling molecules and interaction pathway and Signaling molecules and interaction pathway, respectively. Therefore, the immune

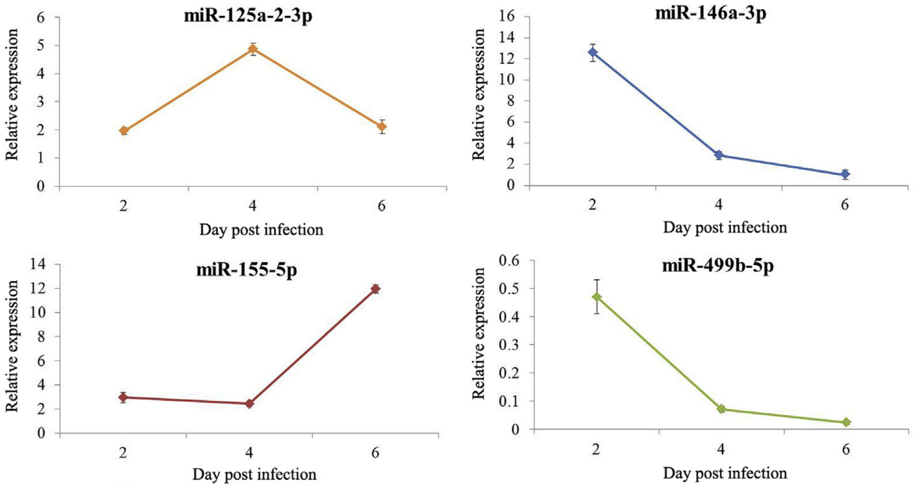

Fig. 3. Time-course expression patterns of four DE miRNAs at 2, 4, 6 days post the *A. salmonicida* infection. The expression levels of miR-146a-3p, miR-125a-2-3p, miR-155–5p and miR-499b-5p were normalized by that of 18S rRNA. Mock-infected fish were used as controls. *, $P < 0.05$.

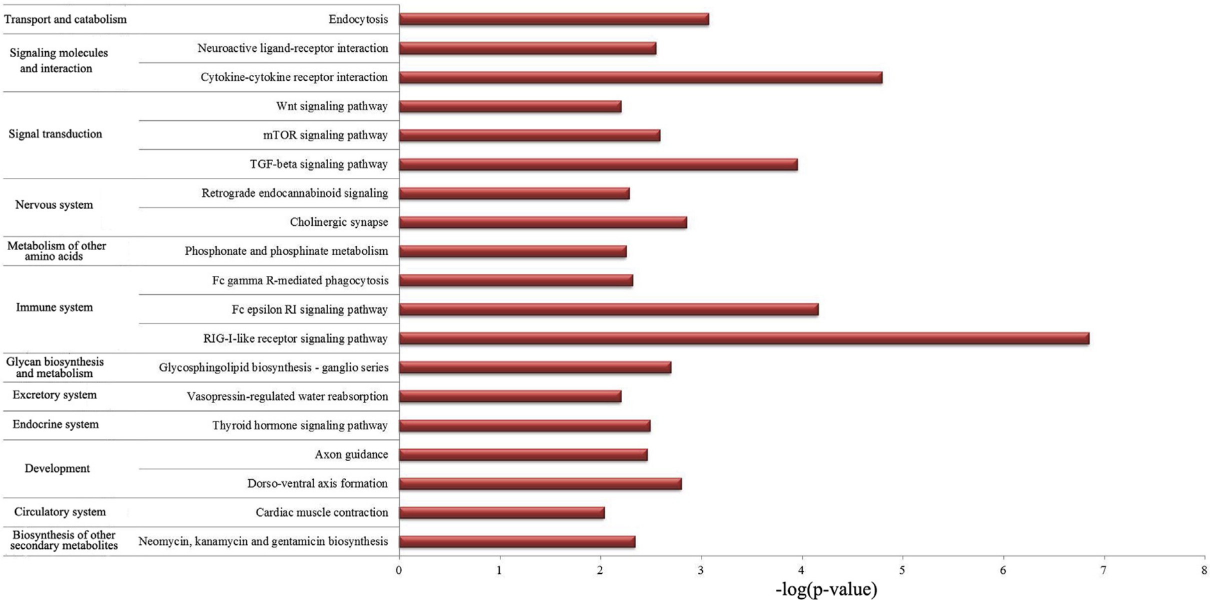

Fig. 5. Analysis of the top 10 most-enriched KEGG pathways.

responses of rainbow trout upon *A. salmonicida* infection seemed to be complexly regulated by miRNAs.

3.5. Effects of four DE miRNAs on the inflammation induced by *A. salmonicida*

Four DE miRNAs (miR-146a-3p, miR-125a-2-3p, miR-155–5p and miR-499b-5p) were selected to evaluate their potential functions in the inflammation during the *A. salmonicida* infection. First of all, miRNA mimics, inhibitors and control miRNAs were transfected into RTG-2 cells according to our previous method. As a result, all the four miRNAs were significantly over-expressed or suppressed successfully in RTG-2 cells ($P < 0.05$) (Fig. 6). Based on this, the cells transfected with miRNA mimics or inhibitors were infected by *A. salmonicida*. The effects of four DE miRNAs on expressions of two pro-inflammatory genes (IL-2 and IL-1β) were assessed by qRT-PCR. The results showed that the over-expression of miR-146a-3p could significantly reduce the expression of IL-2 ($P < 0.05$). The over-expressed miR-155–5p would both significantly enhance the expressions of IL-2 and IL-1β ($P < 0.05$). However, either miR-125a-2-3p or miR-499b-5p mimic transfection seemed to do not affect the expressions of the two pro-inflammatory genes (Fig. 7A). Of interest, suppressed expressions of the above four miRNAs all resulted in the down-regulated expressions of IL-2 and IL-1β ($P < 0.05$) (Fig. 7B).

4. Discussion

Numerous reports have made it clear that the immune responses play critical roles during the bacterial infections in fish. The investigations into the immune actions and identification of immunity-related genes during the pathogenic bacterial infection in fish might not only provide basis for development of effective control methods (e.g., improved vaccines, adjuvants and immune stimulants), but also might be applied in the selective breeding for disease-resistant strains [23,24]. Fish possess the similar immune system as vertebrates. At the early stages of embryogenesis, fish mainly rely on the innate immune responses to fight against pathogen. For adult fish, the innate immune responses serve as the first line of defense against infection, then will active the adaptive immune system that is essential for the protection against recurrent infections [25]. When Atlantic salmon (*Salmo salar*) were infected with pathogenic *A. salmonicida*, the innate immune responses including lysozyme and alkaline phosphatase activities were enhanced in serum, mucus and skin, while the decreased antioxidant capacities of the challenged fish were also observed [26]. Additionally,

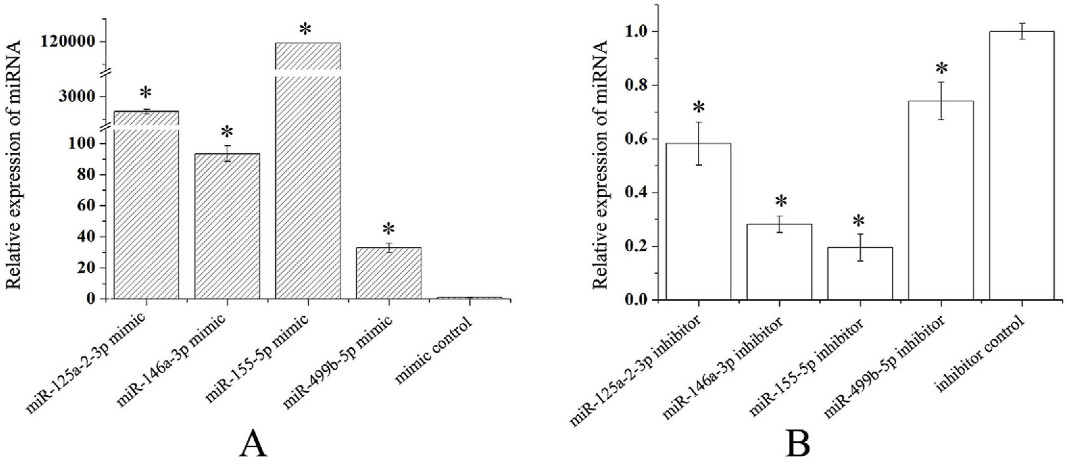

Fig. 6. The expression levels of four selected miRNAs in RTG-2 cells after mimic or inhibitor transfection at 24 h.

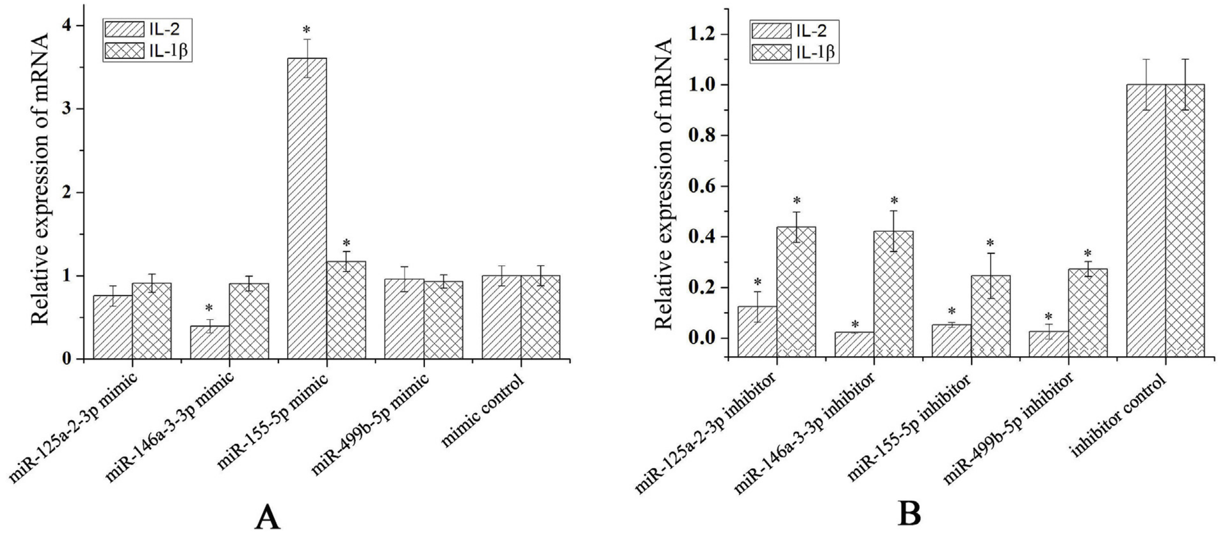

Fig. 7. Effects of four DE miRNAs on the inflammatory response induced by *A. salmonicida* subsp. *salmonicida* infection. Four pairs of mimics and inhibitors for miR-125a-2-3p, miR-146a-3p, miR-155–5p and miR-499b-5p were transfected into RTG-2 cells at 50 nM for mimics or 100 nM for inhibitors, respectively. The cells transfected with the control mimic or inhibitor serves as controls. Twenty four hours post-transfection, cells were infected with *A. salmonicida* subsp. *salmonicida* at 2×10^7 cells/ml for 4 h at 18 °C. Then, total RNAs were extracted from cells to evaluate the expression levels of IL-2 and IL-1β by relative quantification real-time PCR, normalized by the expression of β actin. *, $P < 0.05$.

A. salmonicida infection also elicited the adaptive immune responses of Arctic charr (*Salvelinus alpinus, L.*) including the accumulation of B cells and the existence of IgM-positive cells around ellipsoid arterioles in spleen [27]. However, *A. salmonicida* could suppress the immune responses by type III secretion system-effectors [5]. Hence, there are complex interactions between *A. salmonicida* and host.

MiRNAs act as important regulators in animals by cleaving or repressing the target mRNAs. Host might regulate its own miRNA expressions to augment antibacterial defense by regulating inflammatory responses [28], while bacteria could also utilize host miRNA to inhibit the immune responses in turn [29]. Although several miRNAs that are likely to have evolutionary known immune regulatory functions in teleost fish, miRNA responses to bacterial infection between different fish specifies seemed to be quite different, even miRNA responses of the same fish species responding to different bacterial pathogen might be also different. Three *A. salmonicida* infection-related miRNAs have been predicted previously by computational approach based on the ESTs from macrophages of Atlantic salmon (*Salmo salar*) [18]. However, the overall *A. salmonicida* infection-related miRNAs responses and their functions are still lack of experimental supports. In order to better understand the potential roles of miRNAs in the interaction of *A. salmonicida* subsp. *salmonicida* and host, the expression patterns of trout miRNAs responding to *A. salmonicida* subsp. *salmonicida* infection were characterized for the first time.

Bacterial infections were widely believed to change the expressions of host miRNA. These differentially expressed (DE) miRNAs might be functional in the regulation of immune responses during the course of infection. The identification of DE miRNAs in pathogen challenged animal is critical for the further functional study. Pathogenic *A. salmonicida* subsp. *salmonicida* 2014–182 used in this work might cause > 70% mortality during 3–7 dpi (unpublished). Therefore, the sampling time for sequencing was chose at 2 dpi to preliminarily identify the DE miRNAs in rainbow trout upon *A. salmonicida* infection. In the present study, eleven known miRNAs and 16 novel miRNAs were found to be differentially expressed in response to *A. salmonicida* infection. Two evolutionary conserved miRNA genes (miR-146 and miR-181) that associated with immune response during bacterial infection were also found. This supports the supposition that several miRNAs that probably have evolutionary conserved immune regulatory functions in teleost fish [30]. MiRNA-155 serves as an important regulator in immune cells responding to bacterial pathogens. Up-regulations of miRNA-155 were both found in our work and in Nile tilapia (*Oreochromis niloticus*) challenged with *Streptococcus agalactiae* [31]. Teleost-specific miRNA-7132 was also identified here as the DE miRNAs in response to *A. salmonicida*. Additionally, miR-200 that has been proved to regulate the TLR1 expression in miiuy croaker challenged by bacteria was also found to be upregulated [17]. Of interest, an upregulated miR-125 in this study was found to reduce significantly in the anterior kidneys of Nile tilapia at 3 days post *Streptococcus* infection [32]. Therefore, the DE miRNAs identified here and the previous work on other fish miRNAs associated with bacterial infection might facilitate the following study on the roles of miRNAs during *A. salmonicida* infection.

MiRNAs regulate the immune responses via multiple target genes. The results of GO analysis and enriching KEGG pathway indicated that the targets of the eleven DE miRNAs here were all involved in the immunity pathway, the signaling molecules and interaction pathway and signaling molecules and interaction pathway. Human miR-200a-3p has been reported to inhibit tumor proliferation and promote apoptosis by regulating the SPAG9 expression in renal cell carcinoma [33]. Whereas miR-200a-3p in fish was proved to participate in the expression modulation of TLR1 in miiuy croaker [17]. However, neither SPAG9 nor TLR1 is predicted to be the target gene of miR-200a-3p in rainbow trout according to our results. On the other hand, although the gene sequences between human and fish actually existed, the same target genes of known miRNAs could be predicted. For example, Ras-related protein Rab-8A (Rab8a) was predicted to be the target of miR-125a from human and rainbow trout. According to a previous study, the enriched Rab8a would recruit phosphatidylinositol 3-kinase (PI3Kγ) to regulate Akt signaling generated by surface TLR4, thus constrain the inflammation to avoid the robust responses [34]. Therefore, more studies should be performed to confirm the target genes of the *A. salmonicida* infection-related DE miRNAs for better understanding their functions. This would provide important information for the comparative immunity study. Additionally, the confirmed target genes might serve as potential biomarkers and therapeutic targets for fish diseases.

Inflammation is one of the most important reactions induced by *A. salmonicida* infection. In order to preliminarily analyze the roles of DE miRNAs in the inflammatory response, the expressions of two proinflammatory cytokines (IL-2 and IL-1β) were evaluated and compared between DE miRNAs enabling miRNA overexpression and suppression in RTG cells. It is reported that miR-155 have the inverse functions. For example, miR-155 promoted the inflammation by driving IL-8 expression via reducing the expression of SHIP1 to activate the PI3K/Akt signaling pathway [35]. MiR-155 also enhanced the productions of proinflammatory cytokines IL-6 and TNF-α by inhibiting the SOCS1 expression [36]. In contrast, miR-155 could repress calcium-regulated heat stable protein 1 and thereby relieving the chronic inflammation responding to lipid accumulation [37]. Among the four selected DE miRNAs, miR-155–5p was proved to promote the inflammation induced by *A. salmonicida* by our results. When miR-155–5p was overexpressed, the enhanced productions of IL-2 and IL-1β induced by *A. salmonicida* were observed. However, the suppressed expression of miR-155–5p inhibited the two proinflammatory cytokines expressions. Additionally, up-regulations of miR-155–5p were 2–3 fold-changes compared to control at 2 and 4 dpi, while the 11.9 fold-change of miR-155–5p was observed at 6 dpi. The kinetic profile of miR-155–5p correlated well with the course of *A. salmonicida* infection. Therefore, we speculated that *A. salmonicida* might promote the inflammatory response via miR-155–5p. Of interest, a fish DNA vaccine with miR-155–5p as adjuvant increased the specific antibodies and classical complement pathway compared to that only containing the antigen [38]. Based on our work, miR-155–5p might also be used as a potential molecular adjuvant for bacterial vaccine in rainbow trout. MiR-146a-3p has been reported to inhibit the inflammation induced by mycobacteria and promote bacterial replication [39]. However, the effect of miR-146a-3p was not discernible in this study because either overexpressed or suppressed this miRNAs inhibited the expression of IL-2. Unexpected, suppressed expressions of miR-125a-2-3p or miR-499b-5p resulted in the down-regulated expression of IL-2 and IL-1β, while over-expressed these two miRNAs could not significantly enhance the expressions of proinflammatory cytokines. Therefore, the regulation of inflammation by miR-146a-3p, miR-125a-2-3p or miR-499b-5p during *A. salmonicida* infection should be confirmed in the following work.

In this study, the miRNA profiles responding to *A. salmonicida* subsp. *salmonicida* infection were characterized in rainbow trout for the first time. Eleven known miRNAs were found to be differentially expressed and their target genes were closely associated with immune response. Among four selected DE miRNAs, only miR-155–5p was proved to promote the inflammation induced by *A. salmonicida*. The current study preliminarily elucidated the importance of the miRNAs in the immune regulation during *A. salmonicida* infection and might contribute to the development of possible therapeutic methods.

Conflicts of interest

There is no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.048.

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