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Transcriptomic analysis of microRNAs–mRNAs regulating innate immune response of zebrafish larvae against *Vibrio parahaemolyticus* infection

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ABSTRACT

In recent years, microRNAs (miRNAs) have been shown to play important roles in immunity. Analyses of the functions of miRNAs and their targets are useful in understanding the regulation of the immune response. To understand the relationships between miRNAs and their targets during infection, we used zebrafish as an infection model in which to characterize the miRNA and mRNA transcriptomes of zebrafish larvae infected with *Vibrio parahaemolyticus*. We identified the differentially expressed miRNAs and mRNAs. Overall, 37 known zebrafish miRNAs were differentially expressed in the infection group and 107 predicted target genes of 26 miRNAs were differentially expressed in the mRNA transcriptome. These targets with specific Gene Ontology (GO) terms, such as peripheral nervous system neuron axonogenesis, organophosphate metabolic process, heme binding, protein binding, tetrapyrrole binding, protein dimerization activity, and aromatase activity, which regulate nerve conduction, energy metabolism, hematopoiesis, and protein synthesis. They were also associated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways such as phototransduction, tryptophan metabolism, notch signaling, and purine metabolism. Our findings indicate that miRNAs regulate the innate immune response via complex networks, and zebrafish (*Danio rerio*, dre)-miR-205-3p, dre-miR-141-5p, dre-miR-200a-5p, dre-miR-92a-2-5p, dre-miR-192, and dre-miR-1788 may play important roles in the innate immune response by regulating target genes.

1. Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate mRNA expression to affect biological processes [1], molecular functions, and cell development [2,3]. Recent studies have shown that miRNAs play important roles in the regulation of cellular proliferation, differentiation and maturation, metabolism, apoptosis, and immunity [1,4]. Teleost miRNAs were first reported in zebrafish and since then, a number of miRNAs have been identified in other teleosts. Information on the functions of miRNAs has largely been obtained from studies performed in zebrafish [5]. At present, 38,589 miRNAs have been annotated, among which 2,654 mature miRNAs have been identified in *Homo sapiens* and 373 mature miRNAs in *D. rerio* (http://www.mirbase.org/, Release 22.1).

In the past 20 years, zebrafish larvae have emerged as a new model to study bacterial infection, clarify host–pathogen interactions and inflammatory processes, and study the innate immunity of vertebrates, because it has the advantage that the larvae have only an innate immune system in the first 3 weeks after fertilization [6]. *Vibrio parahaemolyticus*, a Gram-negative marine bacterium, is one of the most commonly reported species causing serious foodborne diseases, leading to hospitalization and death in China [7]. It has adapted to various lifestyles.

In recent years, analyses of the functions of miRNAs and their targets have extended our understanding of the regulation of developmental and other biological processes. There are two ways in which miRNA–mRNA interactions regulate these processes. First, the usual mechanism involves the downregulation of protein expression by translational repression, mRNA cleavage, or the promotion of mRNA decay [8]. Second, under certain circumstances, miRNAs can also upregulate the translation of target mRNAs or even directly interfere with gene transcription [9]. It has been shown that miRNAs function in

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various biological and pathological processes by regulating more than 30% of protein-coding genes, but the physiological impact of this regulation remains unclear.

Some miRNAs have been identified as regulatory elements in the immune system, and are involved in the control of cellular development, homeostasis, and highly specific responses [10]. In recent years, the miRNAs of some aquatic animals and cell lines have been challenged with viruses or bacteria and analyzed, including epithelioma papulosum cyprini cells infected with spring viraemia of carp virus [11], Pacific white shrimp (*Litopenaeus vannamei*) infected with white spot syndrome virus [12], grass carp (*Ctenopharyngodon idella*) infected with *Aeromonas hydrophila* [13], and half-smooth tongue sole (*Cynoglossus semilaevis*) infected with *Vibrio anguillarum* [14]. These studies investigated the functions of miRNAs in the infection process and many miRNAs have been shown to be closely associated with the innate or adaptive immunity. However, because there is a lack of whole-genome information for many fish and shrimp species, it remains difficult to determine the specific roles and underlying functional downstream target genes of miRNAs differentially expressed during the immune response. The aim of this study is to clarify the functions of miRNAs that are associated with the innate immune response and to gain insight into the regulatory network of miRNAs and mRNAs in the zebrafish. miRNA and mRNA transcriptome data were analyzed simultaneously. Differentially expressed miRNAs and their target genes, which were also differentially expressed in the mRNA transcriptome and correlated negatively with the differentially expressed miRNAs, were both detected. The data from this show that miRNAs play important roles in defending zebrafish against *V. parahaemolyticus* infection, and that miRNA–mRNA networks participate in the innate immune response by regulating energy metabolism, cell communication, signal transduction, nerve conduction, hematopoiesis, and protein synthesis.

2. Materials and methods

2.1. Sample collection and challenge

The wild-type adult zebrafish AB line was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Zebrafish were handled according to the procedures of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (Shanghai, China) and maintained according to standard protocols (http://zfin.org). The procedures and research methodology were approved by the Shanghai Ocean University Experimentation Ethics Review Committee (SHOU-DW-2016-002). Zebrafish larvae were infected with Vp13 using the immersion method, as previously described [15]. In brief, the bacteria were incubated to mid-logarithmic stage at 28 °C in trypticase soy broth (TSB) containing 3% NaCl, collected by centrifugation, and resuspended to a final concentration of 3.63×10^7 colony forming units (CFU)/mL in egg water. The 3 dpf larvae were then immersed in this suspension for 2 h, which has been shown to activate the innate immune response [15], whereas the control larvae were immersed in egg water only (each group contained three independent replicates, designated I1, I2, and I3, and C1, C2, and C3, respectively, with each replicate containing ten larvae). All samples of whole larvae were snap-frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction.

2.2. miRNA library construction and deep sequencing

Six miRNA libraries were constructed from the control group (C1, C2, and C3) and the infection group (I1, I2, and I3). Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the isolated RNA was checked by gel electrophoresis on 2% agarose gel and by absorption spectroscopy. The RNA concentration of each sample was measured with a NanoDrop 2000c and its quality was measured with an Agilent 2100 Bioanalyzer (Agilent, San Diego, CA, USA). The miRNAs (18–32 nt) were gel-purified with 6% polyacrylamide gel electrophoresis on Novex TBE gels (Invitrogen, Carlsbad, CA, USA), and then eluted from the gel according to the supplier's protocol. The libraries were then deep sequenced with the Illumina HiSeq 4000 System (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. The mRNA library construction and deep sequencing were as previously described [15].

2.3. Sequence cleaning, filtering small RNA (sRNA) reads, and miRNA identification

The initial raw reads were filtered with the SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle software (https://github.com/najoshi/sickle) [16]. The clean reads were then analyzed statistically using the Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/ version 0.0.14). The unique reads were then screened against the Rfam database (http://rfam.janelia.org/, Version v12.3) [17] with BLAST (http://blast.ncbi.nlm.nih.gov/, Version 2.9.0) to annotate the sRNAs and remove non-miRNAs (rRNA, scRNA, snoRNA, snRNA, and tRNA), and then with the program Bowtie (http://bowtie-bio.sourceforge.net/index.shtml, Version 1.1.2) [18] to map the annotated miRNAs to the zebrafish genome (http://www.ensembl.org/Danio_rerio/Info/Index, GRCz10). We subjected the sRNAs (without rRNA, scRNA, snoRNA, snRNA, and tRNA) to a BLASTx search of the zebrafish miRNAs in the miRBase database (http://www.mirbase.org/, Release 22.1) to identify the sequences that could be mapped to the pre-miRNAs and mature miRNAs.

To predict novel miRNAs, the unmatched datasets were aligned with the zebrafish genome. To determine whether a sequence could form a suitable hairpin (the secondary structure of the miRNA precursor), the sequences surrounding the matched sequence were extracted, and the secondary structure was predicted with miRDeep2 (https://www.mdc-berlin.de/content/mirdeep2-documentation, version 2.0.0.5). These miRNAs were considered to be novel miRNAs based on the prediction results of the dicer digestion sites, energy values, and other characteristics using the Bowtie and RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) software [19–21].

2.4. Analysis of differentially expressed miRNAs

The differentially expression of known and novel miRNAs were analyzed with the DESeq2 package (http://bioconductor.org/packages/stats/bioc/DESeq2/, version 1.10.1) [22,23]. The criteria we used to screen for up- and downregulated miRNAs were a 1.5-fold change between the infected and control groups and a corrected P value (p-adjust) < 0.05.

2.5. Prediction of the target genes of differentially expression miRNAs, GO, and KEGG enrichment analyses

The miRanda (http://www.miRNA.org/miRNA/home.do, version v3.3a), TargetScan (http://www.targetscan.org, version 7.0), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/, Version 2.1.2) software were used to predict the target genes, with the criterion that at least two software packages supported the results. The functional categories of the target sequences were annotated in the GO database (http://www.geneontology.org/) using the BLAST program.

To investigate the pathway perturbations in zebrafish larvae infected with Vp13, we performed GO and KEGG pathway analyses of the target genes of differentially expressed miRNAs, which were also differentially expressed in mRNA transcriptome. The GO and KEGG pathway enrichment analyses were conducted with hypergeometric distribution testing using the software BLAST2go (https://www.blast2go.com/, version 2.5) and KOBAS (http://kobas.cbi.pku.edu.cn/download.php, version 2.1.1), respectively. Bonferroni, Holm, Sidak,

and false discovery rate (FDR) corrections were used to adjust the P values. A functional cluster was deemed significantly enriched when the corrected P value (q value) < 0.05 [24–27].

2.6. Construction of the miRNA–mRNA network

A general miRNA–mRNA regulatory network was constructed to represent the putative functional regulatory effects of the miRNAs on their targets, based on the negative correlations between miRNAs and mRNAs expression. Specifically, Pearson's correlation coefficients of the differentially expressed miRNAs and their target genes that were also differentially expressed were calculated. An index of corrected correlation < −0.5 was used to evaluate the negative correlations. The Cytoscape software (https://cytoscape.org, Version 3.7.1) was used to visualize the differentially expressed miRNAs and their differentially expressed target genes [28].

2.7. Reverse transcription–quantitative real-time PCR (RT–qPCR) of differentially expressed miRNAs

The miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, Nanjing, China) was used to reverse transcribe the total RNA, according to the manufacturer's instructions. In brief, 100 ng of RNA was added to 2 μl of 5 × gDNA Wiper Mix, and RNase-free water was added to a total volume of 10 μl. The mixture was then incubated at 42 °C for 2 min. Stem-loop primer (1 μl), 2 μl of 10 × RT Mix, 2 μl of HiScript II Enzyme Mix, and 5 μl of RNase-free water were added to the reaction mix. The total reaction mixture was incubated at 25 °C for 5 min, then at 50 °C for 15 min, and at 85 °C for 5 min. The stem-loop primer and RT–qPCR primers were designed with the Vazyme miRNA design software (http://www.vazyme.com/download_detail/downloadsId=386.html). RT–qPCR was performed in the Roche LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland), according to the manufacturer's instructions. Zebrafish U6 snRNA was the reference RNA used for normalization [29]. The results were analyzed with the $2^{-\Delta\Delta Ct}$ method [30]. The sequences of the primers are listed in Table 1.

3. Results

3.1. Overview of transcriptome sequencing results and sRNA mapping to genome

We sequenced the mRNAs and miRNAs from the same total RNA samples. The control group (C1, C2, and C3) and infection group (I1, I2, and I3) generated 11,438,951, 9,525,642, 15,121,749, 11,407,446, 11,532,549, and 14,860,607 raw reads, respectively (Table 2). We then analyzed the repeated sequences and the sequences common to the different samples, generating 10,662,180, 8,850,166, 13,988,256, 10,636,765, 10,774,874, and 13,684,069 clean reads in C1, C2, C3, I1, I2, and I3, respectively. There were 8,105,054, 7,200,267, 11,328,814, 9,072,469, 7,558,680 and 10,801,339 useful reads (18–32 nt) in C1, C2, C3, I1, I2, and I3, respectively.

Table 2
Summary of small RNA next-generation sequencing data processing.

Sample	Total reads	Error Rate (%)	Q20 (%)	Q30 (%)	Clean reads	Useful reads 18-32 bp
C1	11,438,951	0.01	99.63	98.18	10,662,180	8,105,054
C2	9,525,642	0.01	99.65	98.22	8,850,166	7,200,267
C3	15,121,749	0.01	99.66	98.26	13,988,256	11,328,814
I1	11,407,446	0.01	99.60	98.03	10,636,765	9,072,469
I2	11,532,549	0.01	99.63	98.13	10,774,874	7,558,680
I3	14,860,607	0.01	99.63	98.16	13,684,069	10,801,339

The distribution of useful reads was analyzed and showed that reads of 22 or 23 nt predominated (Fig. 1a). We then mapped the useful reads to the zebrafish genome to determine the mapping rate (Table 3) and chromosomes 9, 23, 6, 3, 2, 22, 8, 21, 5, and 12 contained the most reads (Fig. 1b). In addition, 61% (5,519,223) of the useful reads were matched to the genome including known miRNAs (47.37%), novel miRNAs (13.25%), tRNAs (1.83%), rRNAs (8.96%), snRNAs (0.27%), repbase (3.66%), exon (3.26%), intron (6.79%), and unknown (14.62%) (Fig. 1c).

3.2. miRNAs identified in the two groups

The main purpose of this study was to identify known miRNAs and novel miRNAs that were differentially expressed in zebrafish during infection with Vp13. Firstly, we performed a BLASTx search of the zebrafish miRNAs in the miRBase database and identified 643 known miRNAs in the C (C1, C2, and C3) and I (I1, I2, and I3) groups (Supplementary Table 1). Based on the secondary structures, dicer digestion sites, energy values, and scores, 590 novel miRNAs were predicted (Supplementary Table 2). A correlation analysis, principal components analysis (PCA), and miRNA (known and novel) distribution analysis were performed on the different samples (Fig. 1d–f) and the repeatability of samples in control group and infection group were both acceptable. According to the changes in the relative miRNA abundances between the two groups, 37 known miRNAs (Supplementary Table 3) and 255 novel miRNAs (Supplementary Table 4) were significant differentially expressed in the infection groups. The top 10 differentially expressed known and novel miRNAs were listed (Table 4). Compared with the control group, nine known miRNAs and 127 novel miRNAs

Table 1
Primers used for miRNA 1st strand cDNA synthesis of stem-loop method and RT-qPCR.

miRNA	stem-loop primer	miRNA sequence ID
dre-miR-205-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACACT	LM383315.1
dre-miR-141-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAAGC	LM383363.1
dre-miR-200a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCAGC	LM383373.1
dre-miR-92a-2-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCATT	LM383343.1
dre-miR-192	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAGG	NR_029930.2
dre-miR-1788	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTCGT	NR_030651.1

	Forward primer	Universal reverse primer
dre-miR-205-3p	CGCGGATTTCAGTGGTGTGA	AGTGCAGGGTCCGAGGTATT
dre-miR-141-5p	CGCGCATCTTACCTGACAGT	
dre-miR-200a-5p	GCGCATCTTACCGGACAGT	
dre-miR-92a-2-5p	GGTTGGGATCGGCCGC	
dre-miR-192	TGTCAGTTCTGTAGGCCACTGC	
dre-miR-1788	TCTGGGAGGCCAGAGACAAC	

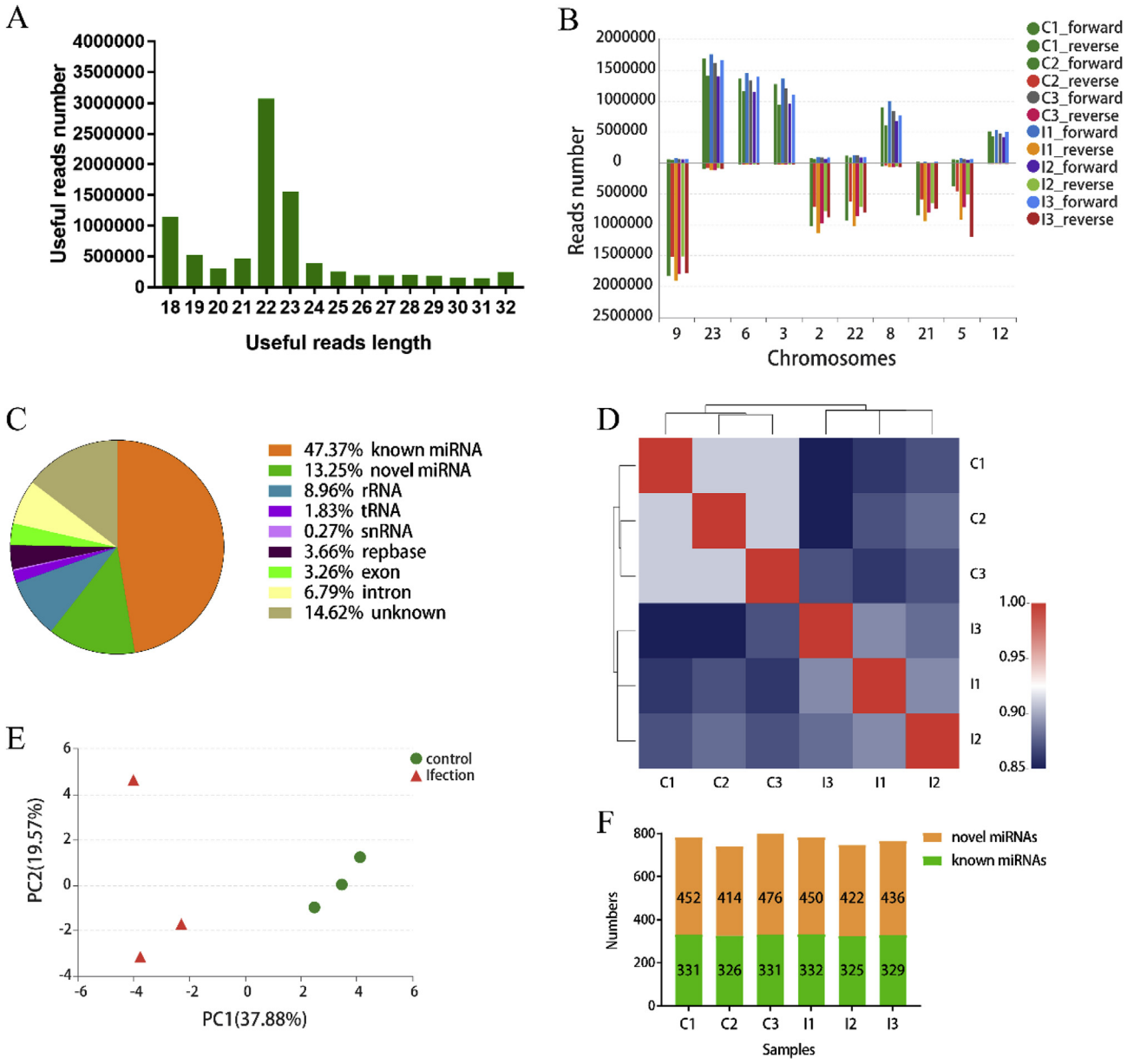

Fig. 1. Summary of small RNA next-generation sequencing data processing. (A) Distribution of different length useful reads. (B) Result of useful reads mapping to genome. forward represents mapped to sense strand and reverse represents mapped to antisense strand. (C) Distribution ratio of miRNA, rRNA, tRNA, snRNA, repbase, exon, intron and unknown useful reads. (D) Correlation analysis of samples. The color of cross square represents the correlation degree between samples. (E) PCA analysis of transcriptome differences between infection and control groups. (F) The distribution of known and novel miRNAs in different samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Summary of useful reads mapping to genome.

Sample	Total reads	Mapped reads	Mapped reads Forward	Mapped reads Reverse	Mapped Rate (%)
C1	8,105,054	5,415,681	4,744,553	3,837,752	0.67
C2	7,200,267	4,525,681	3,847,060	3,181,406	0.63
C3	11,328,814	6,541,672	5,280,541	4,744,583	0.58
I1	9,072,469	5,816,349	4,817,290	4,174,202	0.64
I2	7,558,680	4,605,817	3,838,639	3,325,637	0.61
I3	10,801,339	6,210,140	4,743,144	4,567,072	0.57
average	9,011,104	5,519,223	4,545,205	3,971,775	0.61

were significantly upregulated in the infection group, and 28 known miRNAs and 128 novel miRNAs were significantly downregulated. Among the up-regulated known miRNAs, zebrafish (dre) miR-735-5p had the highest change (log (I/C) = 5.04), followed by dre-miR-23a-5p (4.50), dre-miR-30a-5p (3.43), dre-miR-22b-5p (1.55), dre-miR-7145 (1.00), dre-miR-738 (0.79), dre-miR-2187-5p (0.76), dre-miR-144-3p (0.70), and dre-miR-7a (0.65). Among the down-regulated known miRNAs, dre-miR-7149-5p had the highest fold change (−5.17), followed by dre-miR-2185-3p (−4.64), dre-miR-733 (−4.46), dre-miR-202-5p (−2.13), dre-miR-1788-5p (−2.05), dre-miR-7147 (−1.33), dre-miR-459-3p (−1.06), dre-miR-725-5p (−1.05), dre-miR-15a-3p (−1.03), dre-miR-194b (−1.03), dre-miR-2189 (−0.95), dre-miR-459-5p (−0.94), dre-miR-34c-3p (−0.86), dre-miR-31 (−0.84), dre-miR-142b-5p (−0.80), dre-miR-19c-5p (−0.69), dre-miR-122 (−0.68), dre-miR-193a-5p (−0.66), dre-miR-219-5p (−0.66), dre-miR-192

Table 4
Top ten differentially expressed known miRNAs and novel miRNA in control (C) and infection (I) groups.

MiR-name (known miRNA)	C (FPKM)	I (FPKM)	Log2 (Fold-change)	P-value	Regulated	Significance
dre-miR-7149-5p	18	0	−5.17	1.14E-05	down	**
dre-miR-2185-3p	12.5	0	−4.64	3.20E-04	down	**
dre-miR-733	10.99	0	−4.46	8.21E-04	down	**
dre-miR-202-5p	102.97	23.52	−2.13	2.39E-13	down	**
dre-miR-1788-5p	54.47	13.18	−2.05	2.06E-07	down	**
dre-miR-7145	1.56	6.16	1.01	6.45E-04	up	**
dre-miR-22b-5p	44.43	129.85	1.55	3.88E-11	up	**
dre-miR-30a-5p	5.83	99.34	3.43	9.59E-56	up	**
dre-miR-23a-5p	0	11.29	4.50	6.81E-04	up	**
dre-miR-735-5p	0	16.46	5.04	2.86E-05	up	**
MiR-name (novel miRNA Top ten)	C (FPKM)	I (FPKM)	Log2 (Fold-change)	P-value	Regulated	Significance
7_34222	40.34	0	−6.35	4.08E-11	down	**
19_13885	33.71	0	−6.10	1.41E-09	down	**
15_8378	33.14	0	−6.07	1.93E-09	down	**
24_22262	28.99	0	−5.88	1.89E-08	down	**
13_5765	26.7	0	−5.76	6.81E-08	down	**
16_9488	0	28.23	5.80	3.62E-08	up	**
15_8570	0	36.62	6.17	3.86E-10	up	**
9_38207	0	38.98	6.26	1.11E-10	up	**
12_4495	0	43.71	6.43	9.55E-12	up	**
4_28888	0	52.17	6.68	1.32E-13	up	**

* represents fold change (log2) > 0.5 or fold change(log2) < −0.5, and P-value < 0.01.
** represents fold change (log2) > 1 or fold change(log2) < −1, and P-value < 0.01.
FPKM: Fragments per kilobase of exon per million reads mapped.

(−0.65), dre-miR-194a (−0.63), dre-miR-125b-3-3p (−0.62), dre-miR-141-5p (−0.62), dre-miR-200a-5p (−0.61), dre-miR-92a-2-5p (−0.61), dre-miR-451 (−0.61),dre-miR-205-3p (−0.61), and dre-miR-25-5p (−0.59), for all of which the change was greater than 1.5-fold.

3.3. miRNA target prediction

We used the genomic sequence of zebrafish as a reference to map the target genes of the zebrafish miRNAs. A total of 38,362 (Supplementary Table 5) target genes were identified for the known miRNAs and 332,518 (Supplementary Table 6) target genes were predicted for the novel miRNAs. Repeating our predictions with Miranda (score > 140) did not affect the general results of this study. Two hundred sixty-seven genes were significantly differentially expressed in mRNA transcriptome data, in which 78 were up-regulated and 189 were down-regulated (Supplementary Table 7). A GO enrichment analysis of these differentially expressed genes showed that nine GO terms were significantly enriched (after Bonferroni correction, P < 0.05) (Table 5): single-organism process, biological process, peripheral nervous system neuron axonogenesis, organophosphate metabolic process, heme binding, protein binding, tetrapyrrole binding, protein dimerization activity, and aromatase activity. A KEGG pathway annotation analysis was used to better understand the potential functions of the miRNAs by determining the functions of their target mRNAs. Four pathways were significantly enriched: phototransduction, tryptophan metabolism, notch signaling, and purine metabolism (Table 6). The GO network (Fig. 2) showed the differentially expressed GO terms and the relationships between the different GO terms. The main GO terms that were altered by infection were peripheral nervous system neuron axonogenesis, protein binding, heme binding, and organophosphate metabolic process.

3.4. miRNA–mRNA network

The entire analysis led to the identification 26 miRNAs and 107 target genes in the miRNA–mRNA regulatory network based on the negative correlations between the miRNAs and mRNAs (Supplementary Table 8). The number of target genes per miRNA ranged from one to 17 (Fig. 3): dre-miR-205 regulated 17 genes, dre-miR-144 regulated 14 genes, dre-miR-22b regulated ten genes, dre-miR-2187 regulated eight genes, dre-miR-1788 regulated seven genes, dre-miR-459 and dre-miR-34c each regulated six genes, dre-miR-7a regulated five genes, dre-miR-34c, dre-miR-19c, dre-miR-7149, and dre-miR-23a each regulated four genes, dre-miR-200a and dre-miR-735 regulated three genes, dre-miR-141, dre-miR-31, dre-miR-15a, and dre-miR-142b regulated two genes, and dre-miR-92a, dre-miR-725, dre-miR-25, dre-miR-2189, dre-miR-202, dre-miR-192, dre-miR-125b, and dre-miR-122 each regulated one gene. Two genes (*cyp1b1* and *fgf7*) were regulated by five miRNAs; 15 genes (*cyp1c1*, *cyp1a*, *cyb5a*, *syt8*, *nmrk2*, *pfkfb3*, *chme*, *plek*, *kynu*, *lpin1*, *wnt5a*, *tspeare*, *prph2b*, *si:dkey-94e7.2*, and *her4.2*) were regulated by

Table 5
GO enrichment of differentially expressed miRNAs' targets which were also differentially expressed.

ID	Description	Sample number	Background number	P-Value	Corrected P-Value
GO:0044699	single-organism process	41	8213	3.61E-06	2.50E-03
GO:0008150	biological_process	56	13685	6.55E-06	4.60E-03
GO:0048936	peripheral nervous system neuron axonogenesis	3	15	7.52E-06	5.30E-03
GO:0019637	organophosphate metabolic process	10	750	2.49E-05	1.75E-02
GO:0020037	heme binding	5	147	4.19E-05	2.95E-02
GO:0005515	protein binding	17	2199	4.25E-05	2.98E-02
GO:0046906	tetrapyrrole binding	5	152	4.91E-05	3.45E-02
GO:0046983	protein dimerization activity	7	378	5.89E-05	4.14E-02
GO:0070330	aromatase activity	2	5	6.63E-05	4.66E-02

Table 6
KEGG enrichment of differentially expressed miRNAs' targets which also differentially expressed.

KEGG Term	ID	Sample number	Background number	P-value	Corrected P-value
Phototransduction	ko04744	3	49	3.96E-04	1.75E-02
Tryptophan metabolism	ko00380	3	56	5.74E-04	1.75E-02
Notch signaling pathway	ko04330	3	72	1.16E-03	2.35E-02
Purine metabolism	ko00230	4	215	3.11E-03	4.74E-02

two miRNAs; and other genes were regulated by one miRNA. Various subnetworks can be derived from the global network identified here, such as those accounting for the targeting relationships of specific miRNA signatures associated with distinct infection groups. The most positive network consisted of 11 miRNAs and 36 anticorrelated targets, and approximately 28% of the genes (ten genes) were targeted by at least two miRNAs.

3.5. Analysis of differentially expressed miRNAs with RT–qPCR

To validate the differentially expressed miRNAs identified from the transcriptome data, six miRNAs (dre-miR-205-3p, dre-miR-141-5p, dre-miR-200a-5p, dre-miR-92a-2-5p, dre-miR-192, and dre-miR-1788) were selected for RT–qPCR analysis. The RT–qPCR results for the miRNAs examined matched the expression patterns observed in the transcriptome data. We wondered whether the differentially expressed miRNAs were transiently or sustainably induced by infection, we then tested these miRNAs at 4 h post infection (hpi). The expression of dre-miR-205-3p, dre-miR-141-5p, dre-miR-200a-5p, dre-miR-92a-2-5p, and dre-miR-192 was consistent with the expression patterns at 2 hpi, but dre-miR-205-3p, dre-miR-141-5p, dre-miR-200a-5p, and dre-miR-92a-2-5p were more significantly downregulated. However, dre-miR-1788 had the opposite expression pattern at 4 hpi than that detected with RT–qPCR and the transcriptome analysis at 2 hpi (Fig. 4).

4. Discussion

In this study, we used next-generation sequencing to survey the miRNA and miRNA expression profiles in a control group and an infection group of zebrafish larvae. The innate immune response can be activated in zebrafish larvae infected with the Vp13 strain of *V. parahaemolyticus* for 2 h [15]. This allowed us to determine the degree to which changes in gene expression could be attributed to changes in miRNA expression. These results also allowed us to analyze the distinct

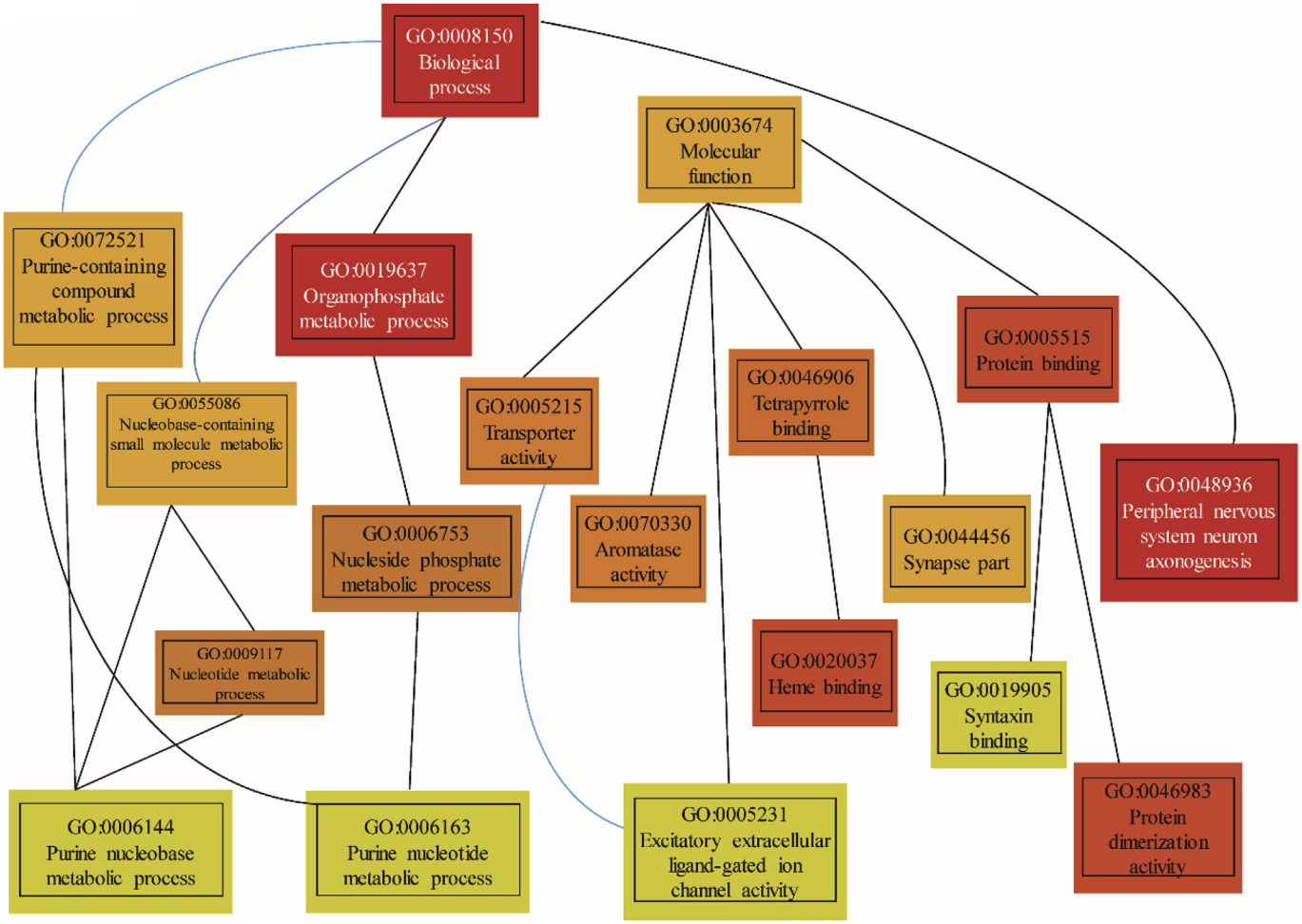

Fig. 2. GO enrichment of the biological process, cellular component and molecular function. The rectangle represents GO terms, the color of rectangle represents the P value of GO term enrichment degree. Yellow represents $P < 0.05$ and red represents $P < 0.001$. The line represents relationship of different GO terms. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

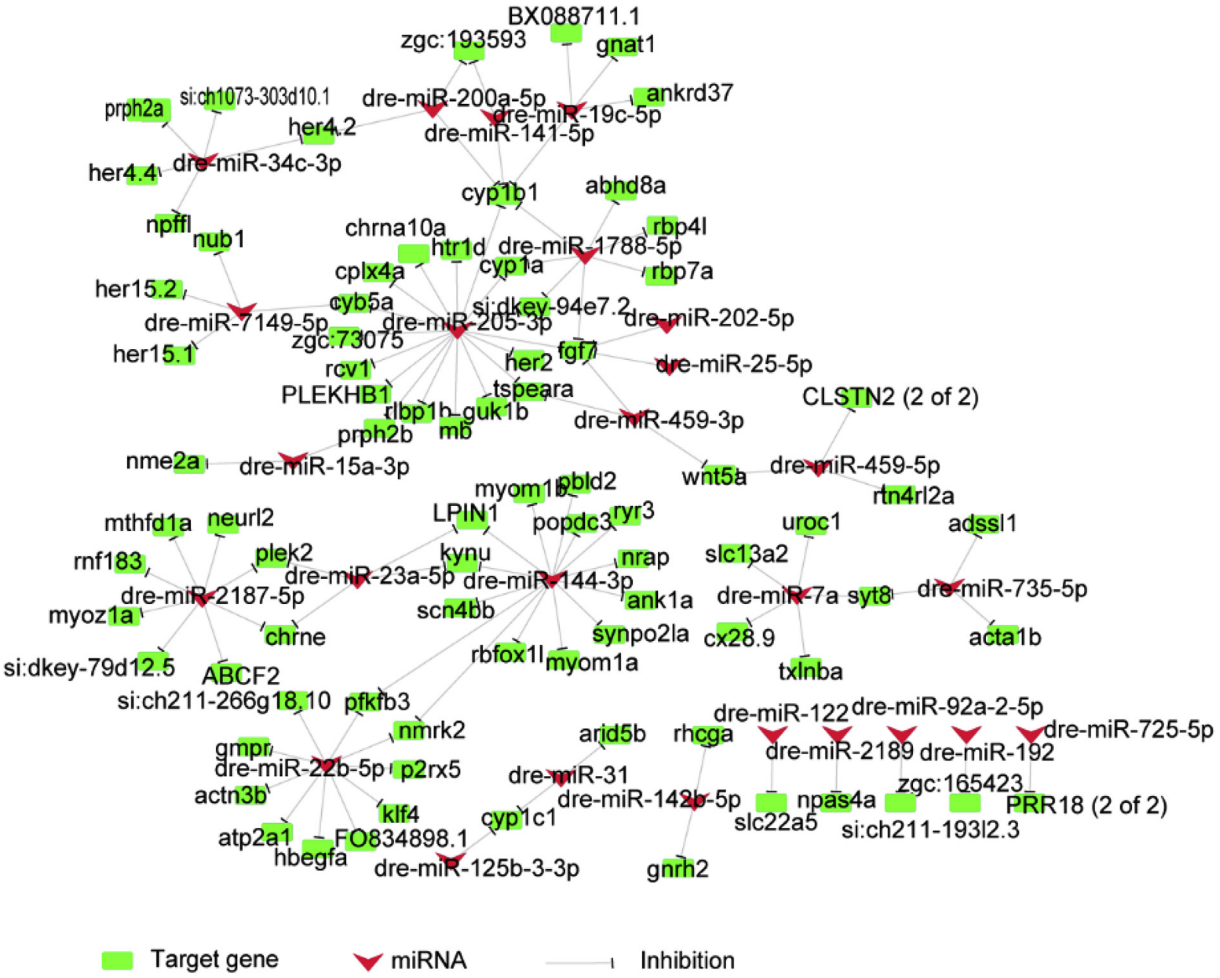

Fig. 3. Regulation of miRNA-mRNA network. The red triangle represents differentially expressed miRNAs, green rectangle represents differentially expressed mRNAs and the line between triangle and rectangle represents the regulation relationship of miRNA and mRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

patterns of miRNA deregulation and predict the miRNA–mRNA regulatory network of zebrafish larvae.

Most of the 26 miRNAs that were differentially expressed in the infected larvae have been previously shown to be present in the zebrafish genome. The most extensively investigated are miR-200a, miR-205, and miR-141. The miR-200 family (including miR-200a and miR-141) and miR-205 regulate the epithelial to mesenchymal transition (EMT) [31] and are highly expressed in the epidermis during skin morphogenesis [32]. The knockdown of these miRNAs reduced E-cadherin expression and increased cell motility, indicating that these miRNAs play important roles in EMT-dependent processes, such as mesoderm development and tumor metastasis [33]. The miR-205 has also been shown to regulate *il24* and *il32* expression to suppress tumorigenesis [34]. Collectively, these observations suggest that the miR-200 family and miR-205 are involved in establishing an epithelial cell barrier and defending the organism against bacterial invasion during Vp13 infection.

Some miRNAs also directly regulate the processes and genes of the innate immune response. It has been shown that miR-17–92 regulates the immune system [10]. Tili *et al.* used lipopolysaccharide to stimulate Raw 264.7 mouse macrophages, which then showed downregulated miR-125b levels. The miR-125b levels in Raw 264.7 cells were altered in response to TNF-α [35]. Wu *et al.* screened the zebrafish miRNA responses to bacterial infection, and showed that miR-122, miR-192, and miR-194a play important roles in zebrafish immunology [36]. It also has been demonstrated that miR-2187 is expressed only in the spleen, an immune organ, in the common carp [37]. However, the functions of miR-1788, a fish-specific miRNA, and miR-19c are unclear. When the olive flounder was infected with viral hemorrhagic septicemia virus, the genes *cxcr3*, *il11b*, *il1r*, *il10*, *il8*, *tlr14*, *tnfr1*, and *mst1* were the targets of miR-1788 whereas genes *c9*, *il10*, *tlr14*, and *tnfr1* were the targets of miR-19c [38]. However, when zebrafish larvae were infected for 2 h, the targets of miR-1788 were *cyp1b1*, *abhd8a*, *rbp4l*, *rbp7*, *fgf7*, and *si:dkey-g4e7.2*, which are important in cell morphogenesis, adhesion, and migration. These data suggest that miR-92a, miR-125b, miR-122, miR-192, miR-2187, miR-1788, and miR-19c are essential to the innate immune system when it is induced by Vp13 and regulate cell morphogenesis and migration in the initial stage of infection. Interestingly, miR-1788 had opposite expression patterns at 2 hpi and 4 hpi. Further research is required to determine the function of miR-1788 in the innate immune response.

Furthermore, miRNAs influence the blood system response to Vp13 infection. It has been demonstrated that miR-31 can regulate the effector function of CD8 T cells [39], promote mammary stem cell expansion and breast tumorigenesis [40], and negatively regulate lymphatic development in vertebrates [41]. In zebrafish, miR-23a regulates angiogenesis [42]; miR-144 mainly induces hematopoiesis and vascular development [43] to produce erythrocytes and macrophages; and miR-142 regulates the formation and differentiation of hematopoietic stem cells to influence hematopoiesis [44]. The miR-142 also acts as an essential modulator of neutrophil development [45]. The lymphatic vascular system maintains the homeostasis of tissue fluids, helps to

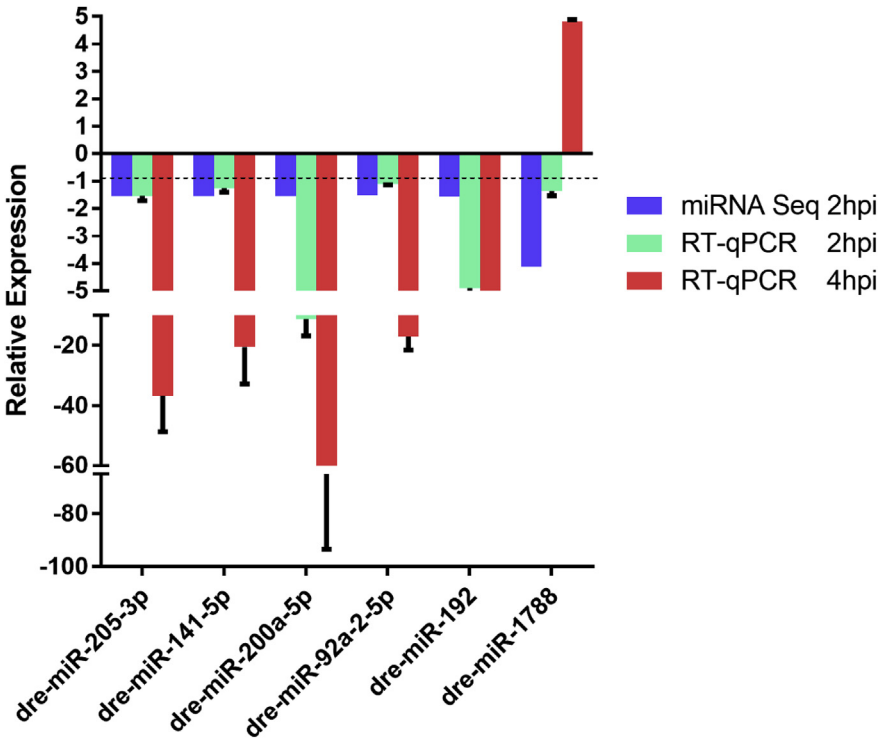

Fig. 4. Validation of transcriptome data by RT-qPCR. The 2 hpi samples used in RT-qPCR experiment were previously used for the transcriptome experiment. The results at 2 hpi and 4 hpi were used $2^{-\triangle\triangle Ct}$ method to get the relative expression between the control and infection group, respectively. The RT-qPCR results of all genes were normalized to the zebrafish U6 snRNA.

mediate the afferent immune responses, and promotes cancer metastasis [41]. Our results suggest that in zebrafish larvae infected by Vp13, the potential functions of dre-miR-31 and dre-miR-142b-5p, which are down-regulated during infection, are to promote the function of lymphatic vascular and neutrophils, to defend the organism against infection. The dre-miR-23a-5p and dre-miR-144-3p, which are up-regulated during infection, may limit vascular development.

In addition, miRNAs may mediate the activities of the hypothalamus and neurons to regulate signal transduction when zebrafish larvae were infected with Vp13. Several miRNAs, such as dre-miR-7a, which was up-regulated, dre-miR-34c-3p, dre-miR-2189 and dre-miR-192, which were downregulated were differentially expressed in infection group. It has been demonstrated that miR-7a is enriched in the hypothalamus and is involved in the specification and maintenance of oligodendrocyte precursor cells from neural progenitor cells [46]. miR-34c is essential for normal brain development and motile ciliogenesis [47], and the predicted target gene of miR-2189, *npas4a*, is restricted to the brain, where it is up-regulated in response to neuronal activity [48]. Our results suggest that these miRNAs regulate signal transduction in the hypothalamus and neuronal activity to activate the innate immune response to Vp13 infection.

The functions of several miRNAs in the zebrafish are unclear, including dre-miR-15a, dre-miR-202, dre-miR-22b, dre-miR-25, dre-miR-459, dre-miR-7149, and dre-miR-735, whose target genes were differentially expressed in the transcriptomic analysis. However, these miRNAs have demonstrated functions in lung cancer [49], hepatocellular carcinoma [50], the response to endocrine-disrupting chemicals [51], cardiac contractility [52], carcinogenesis [53], and cell communication [54]. Whether they play roles in the innate immune response is still unclear.

It is thought provoking that, when we analyzed the targets of these differentially expressed miRNAs, which were also differentially expressed in the transcriptome analysis, few of the genes were involved in the category immunity-related biological process, molecular function, or cellular component. The most significant GO terms were peripheral nervous system neuron axonogenesis, organophosphate metabolic process, heme binding, protein binding, tetrapyrrole binding, protein dimerization activity, and aromatase activity, which regulate nerve conduction, energy metabolism, hematopoiesis, and protein synthesis. The most significant KEGG pathways were phototransduction, tryptophan metabolism, notch signaling pathway, and purine metabolism. And phototransduction is known as an important G protein signaling pathway transducin, and activates cGMP phosphodiesterase, which is necessary for signal transfer [55]. Recent research has shown that tryptophan metabolism is an inflammatory marker in patients with chronic kidney disease [56], and also has immunosuppressive effects on dendritic cells [57]. The notch signaling pathway is important in the development of vertebrates, and this pathway can influence diverse developmental processes, is implicated in many cancers [58], and regulates the innate and adaptive immune responses [59]. Our results indicate that when zebrafish larvae were infected for 2 h, miRNAs played a complex role in regulating the induction of the innate immune response.

We selected 3 dpf zebrafish larvae as the developmental stage for study, because this is when the mouth open and the gut has begun to be colonized by environmental bacteria. Our results suggest that infection for 2 h activated the immune response of the zebrafish larvae, which was regulated by miRNAs, influencing energy metabolism, cell communication, signal transduction, and so on.

5. Conclusion

In summary, infection by immersion with *V. parahaemolyticus* Vp13 for 2 h was the initial stage of the infection of zebrafish larvae. Larvae first defend themselves against infection with their skin and intestinal mucosa, and miRNAs could participate in this process. In particular, miRNAs regulate the expression of their target genes to modify the blood system, lymphatic system, energy metabolism, cell migration and communication, protein synthesis, the transmission of neural signals, and the preparation of the innate immune response. Our results provide evidence that the expression of a proportion of mRNAs is altered during infection in response to regulation by miRNAs. The miRNA–mRNA expression network can help us understand the interactions between miRNAs and their target genes. Some miRNAs are specific to fish, such

as miR-1788, which was significantly differentially expressed during infection, although there has been no clear identification of its role in the innate immune response. A detailed investigation of its function in the future is warranted. Our findings provide valuable information about the composition, expression, and functions of miRNAs in zebrafish larvae, and will extend our understanding of the functional roles of miRNAs in the innate immune system.

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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.2019.05.050.

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