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Identification and characterization of hemocyte microRNAs in mud crab *Scylla paramamosain* in response to *Vibrio parahemolyticus* infection

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ABSTRACT

The microRNAs (miRNAs) are known to regulate immune functions in crustaceans, but little is known about the role of miRNAs against bacterial infection. We performed small RNA sequencing to characterize the differentially expressed microRNAs in *V. parahemolyticus* infected crab, in comparison to that in control uninfected crab, at 12 h and 24 h post infection. In total, 23 host miRNAs were up-regulated in response to the infection and 23 host miRNAs were down-regulated at both the time-points. Among these miRNAs, miR-125, PC-3p-11,483, miR-281-5p, miR-10-3p and PC-5p-50,686 were significantly up-regulated by above 10 folds, and stem-loop RT-qPCR confirmed this result. Further, gene ontology analysis revealed that many signaling pathways, especially phagocytosis, were mediated by these miRNAs. Golgi apparatus and Magnesium ion binding are important biological processes, endocytosis and phagosome are important pathways in the immune response to *V. parahemolyticus*. This study is one important attempt at characterizing crab miRNAs that response to *V. parahemolyticus* infection, and will help unravel the miRNA pathways involved in antibacterial immunity of crab.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level and play an important role in development, homeostasis, and immunological defense function (Bartel, 2004; Filipowicz et al., 2008; Ryan et al., 2010; Wienholds and Plasterk, 2005). Mature miRNAs, which are between 18 and 25 bp in length, are transcribed as primary-miRNA molecules which contain a characteristic stem loop structure (Bartel, 2004). In animals, miRNAs regulate gene expression through imperfect sequence-specific binding to the 3′-untranslated regions (3'UTR) of target mRNAs and usually causing translational repression (He and Hannon, 2004). Till date numerous miRNAs have been discovered in variety of organisms including crustaceans. The crustaceans such as shrimps are cultured and are economically valuable; hence they need to be protected from infection caused by pathogens such as bacteria and viruses. Therefore, efforts are targeted towards understanding the role of shrimp miRNAs which regulate target genes during host pathogen interactions. Firstly, 35 miRNAs were identified from *Marsupenaeus japonicus* and fifteen miRNAs exhibited high homology to the known miRNAs present in the arthropods (Ruan et al., 2011). Further, 24 signature miRNAs were confirmed to take great effects on the innate immunity like phagocytosis, apoptosis and phenoloxidase in *M. japonicus*, and 21 of these miRNAs are known to be conserved in animals too (Yang et al., 2012). Then 55 miRNAs were identified from *M. japonicus* that were either up-regulated or down-regulated following *Vibrio alginolyticus* infection (Zhu et al., 2015). In crabs, the microRNA transcriptome response to *Spiroplasma eriocheiris* infection firstly was identified from *Eriocheir sinensis* (Ou et al., 2012). Then salinity-related microRNAs and growth-related microRNAs were identified from *Portunus trituberculatus* (Lv et al., 2016; Ren et al., 2016), and differential expressed microRNAs were identified in *P. trituberculatus* in response to *Hematodinium* parasites (Li et al., 2018). Further, the effects of *E. sinensis* microRNA-217 and microRNA-7 on the replication of white spot syndrome virus were deeply studied (Huang et al., 2017; Huang et al., 2018). Gonadal microRNA expression profiles and their roles in sex differentiation and gonadal maturation of *S. paramamosain* were discoveried (Waiho et al., 2019). The bacterium *Vibrio parahemolyticus*, which usually causes infection, occurs when host immunity is compromised. It is considered a secondary and opportunistic pathogen which causes high mortality of

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marine crustacean in stressful environments (Yingkajorn et al., 2014; Kewcharoen and Srisapoome, 2019; Ananda Raja et al., 2017). Hence, the objective of this study was to identify and characterize the differentially expressed miRNAs in mud crab *S. paramamosain* in response to *V. parahemolyticus* infection.

Our study revealed that 47 and 49 host miRNAs were differentially expressed in response to *V. parahemolyticus* infection at 12 and 24 h post infection. Our results extend the knowledge of crustacean miRNA regulation, providing clues for further research on crab immunity against *Vibrio* infection.

2. Materials and methods

2.1. Crabs and tissue preparation

The normal adult *S. paramamosain* (approximately 100 g) were obtained from a aquatic product market of Hangzhou. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). The hemolymph was collected from healthy or challenged crabs according to the previous report (Sun et al., 2017). The samples were used immediately for RNA extraction, aiming to prevent total RNA degradation. *Vibrio parahemolyticus* (GenBank accession no. AF 332093.3) was purified and used in challenge experiments, as described previously (Zhang et al., 2019).

2.2. Sequencing of small RNAs

Total RNA were isolated from the hemolymph of the *Vibrio*-free and *Vibrio* -infected crabs at different time points (12 h and 24 h) post infection by using a mirVana miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's instructions. The quantity and purity of total RNAs were monitored using a NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington, USA) at a 260/280 ratio $>$ 2.0. The integrity of total RNAs was analyzed using an Agilent 2100 bioanalyzer system and an RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with an RNA integrity number (RIN) $>$ 8.0. The extracted RNA samples were immediately stored at -80 C. Subsequently 200 μg of total RNAs was separated onto a denaturing 15% polyacrylamide gel. The small RNAs (16–30 nt) were excised and dephosphorylated by alkaline phosphatase. After recovery by ethanol precipitation, the purified small RNAs were ligated sequentially to RNA adapters (5′- ACAGGUUCAGA GUUCUACAGUCCGACGAUC-3′ and 5′-UCGUAUGCCGUCUUCUGC UUG-3′). Reverse transcription and polymerase chain reaction (PCR) amplification were preformed after ligation. The resulting products were sequenced on the Genome Analyzer GA-II (Illumina, San Diego, USA) in accordance with the manufacturer's instructions.

2.3. Small RNA sequence analysis

Illumina's Genome Analyzer Pipeline software and the ACGT V3.1 program developed by LC Sciences (Houston, USA) were used for small RNA sequence analysis, as described in an earlier report (Huang et al., 2012). The following sequences were removed: (1) sequences of the vector and adaptor, (2) low-quality sequences, (3) low-copy sequences (counts $<$ 3), (4) sequences containing more than 80% A, C, G, or T, (5) sequences containing only A and C or only G and T, (6) sequences shorter than 16 nt and longer than 26 nt, (7) sequences containing 10 repeats of any dimer, 6 repeats of any trimer, or 5 repeats of any tetramer, (8) sequences matching mRNAs, rRNA, tRNA, snRNA, snoRNA. After these sequences were removed, all the remaining high-quality sequences were used for miRNA identification. To identify conserved miRNAs that were homologous with those of other species, all high-quality sequences were mapped to known mature and precursor arthropod miRNAs in miRBase 15.0 with an Evalue similarity cutoff of 1e-10. To characterize novel miRNA candidates in crab, the remaining high-quality sequences with no homologs in miRBase 15.0 were analyzed by a BLASTN search against the crab EST database in the National Center for Biotechnology Information. To reveal the differentially expressed miRNAs, the hybridization signals with the microRNA microarray were analyzed using the statistical calculation of the relevant *P*-values. The fold-change and P -value were calculated and used as the threshold to determine significant differences between the differentially expressed miRNAs. Statistical significance was analyzed by multiple *t*-test method. The significantly differential miRNAs ($P < .05$) were identified the differentially expressed miRNAs.

2.4. Quantitative real-time PCR of miRNA

Total RNA was extracted from the hemolymph of the *Vibrio*-free and *Vibrio* -infected crabs at 12 and 24 h post-infection and quantified using a spectrophotometer (NanoDrop, Wilmington, USA). The qPCR of miRNAs was based on stem-loop real-time PCR technology and was performed as described before (Wang and Zhu, 2017). Less than 200 μg total RNA was used for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover Code: FSQ-301 (Toyobo, Japan), The cDNA was kept at -20 ˚C. The SYBR Green RT-qPCR assay was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data was calculated according to the $2 - \Delta\Delta CT$ comparative CT method by office EXCEL, with the amplification of GAPDH as internal control. Designing and synthesizing of the RT q-PCR primers were entrusted to Generay Shanghai Company, based on the open read frame (ORF). The primer sequences for SYBR Green RT-PCR were shown in Table 1.

2.5. Gene ontology (GO) analysis

The coding sequences of the crab ESTs were extracted and used as queries to search the protein sequences collected by the GO database with the blast E value $<$ 1e-5 (http://www.geneontology.org). The best hit GO IDs were assigned to the crab EST sequences. The *P*-values were corrected by false discovery rate (FDR).

3. Results

3.1. Sequence analysis of miRNAs

Based on the small RNA sequencing, the small RNA sequences of crab infected with *V. parahemolyticus* were analyzed. The Vibrio-free crabs were considered as controls for the analysis. The small RNA sequencing generated a total of 10–16 million raw reads. Above 80% raw reads were present at least twice and their lengths were ranged from 18 to 26 nucleotides. After removal of mRNA, rRNA, tRNA, snRNA and snoRNA, the high throughput sequencing generated a total of 2 million sequences. The data analysis showed a low proportion of long RNAs (over 2% by count), indicating that the sequencing samples were not contaminated by degraded RNA and were of high integrity. Size distribution of small RNAs of the indicated length (%) showed that 22.63% of miRNAs were of length 22 bp (Fig. 1).

3.2. Host miRNAs involved in V. parahemolyticus infection

The expression profiles of miRNAs *V. parahemolyticus*-infected crab at various times post infection were compared with that of control, and the miRNAs involved in the infection were identified and characterized. A *P*-value $<$ .05 indicated that differences expression profiles in the miRNA counts were statistically significant. The results showed that the expression patterns of many miRNAs did not significantly change in response to *V. parahemolyticus* infection. However, 47 and 49 host miRNAs were differentially expressed in response to *V. parahemolyticus* infection at 12 and 24 h post infection (Fig. 2). Among these, 23 miRNAs were up-regulated and 24 miRNAs were down-regulated at 12 h post infection (Fig. 2). While, 23 miRNAs were up-regulated and

Table 1
Expression patterns of up-regulated or down-regulated ($P < .05$) crab miRNAs after *Vibrio parahemolyticus* infection at 12 or 24 h post infection. The expression levels were compared with that of control, then get the fold change. The expression level of control was designated as 1.

miRNA name	miRNA sequence	Fold change	
		12 hpi	24 hpi
tcf-miR-125	TCCCTGAGACCCTAACTTGTGA	214.28	172.32
PC-3p-11,483	GGCCTCGCGCCGCGCTCCC	202.1	10.98
bmo-miR-281-5p	AAGAGAGCTATCCGTCGACAGT	243.66	267.62
pte-miR-10-3p	CAAATTCGGTTCTAGAGAGGTT	39.61	35.10
PC-5p-50,686	TGAAAAGGTTTATTGTGC	10.64	40.50
dpu-miR-133	TTGGTCCCCTTCAACCAGCTGT	28.89	6.11
pte-miR-10-5p	TACCCTGTAGATCCGAATTTGT	10.48	8.81
tur-miR-92-3p_1ss10CT	TATTGCACTTGTCCCGGCCTGT	11.63	8.24
dqu-mir-3759-p3_1ss5CG	TTGAGTGGGCGTCCGCACTAA	13.33	6.23
mja-mir-6494-p3_1ss12AC	CTGGAGCCGCCCCTGGTGC	4.7	2.4
bmo-mir-6497-p5	AAGGATTGGCTCTGAGGA	6.33	3.48
bmo-miR-305-5p_1ss22TG	ATTGTACTTCATCAGGTGCTCGG	2.66	2.73
bmo-mir-6497-p5_1ss9AG	AACTTCGGGATAAGGATTGGCTCT	4.96	3.77
PC-3p-94603_18	TGGGACTGGCATTTCAGTGGGC	2.43	2.00
PC-5p-59798_39	TTGTCTGGAAGGTTGTGTCGAG	3.70	3.91
mja-mir-6489-p5_1ss12CT	CGGACTGGCGCTCTTGGA	3.48	3.22
isc-miR-278_1ss12TA	TCGGTGGGATTATCGTCCGTTT	2.61	2.90
PC-3p-224738_4	TGTATTGGGCGTGTGTTGGCCA	7.74	7.88
bmo-miR-2779_L-2_1ss9CT	ATCCGGTTCGAAGGACCA	5.60	3.76
pca-miR-316-5p_1ss11CT	TGTCTTTTTCTGCTTTGCTGCCG	7.03	5.93
bmo-mir-6497-p3_1ss12AG	CGTAACTTCGGGATAAGG	7.76	3.99
mja-mir-6491-p3_1ss12CT	CCGGGGAAGAGTTTTCTT	3.09	2.06
isc-miR-2b_L-3R-2	CACAGCCACCTTTGATGA	7.36	4.59
tcf-miR-2944a_R-1_1ss11GC	TATCACAGCCCTAGTTACCTAG	0.12	0.40
PC-5p-28739_114	GGCGGCGCTCTCCGGGGCC	0.22	0.21
PC-5p-132743_10	TTCAGGAGCCCAGAAGTCAGGT	0.15	0.32
PC-5p-315387_3	AAAGGCCGCGGAATGTCA	0.18	0.49
PC-5p-147453_8	TTTGGACACGGGAACGATTTGG	0.35	0.28
PC-3p-249211_4	GGAGAAACAGACTTGAGAAC	0.30	0.11
PC-5p-79645_24	TTTAACAAGATTTCAAGTTGCAT	0.08	0.20
dpu-miR-153	TTGCATAGTCACAAAAGTGATG	0.14	0.30
bdo-miR-283_R-4_1ss17TA	AAATATCAGCTGGTAAAT	0.06	0.16
tur-miR-745-3p_R-3_2ss1CG11GA	GAGCTGCCCAATGAAGGG	0.32	0.41
dpu-miR-12_R-1	TGAGTATTACATCAGGTACTGG	0.27	0.28
PC-3p-12157_315	TATCACTGGGAGAAAACACTCA	0.06	0.09
tcf-miR-995_L-1R-1_1ss20TA	AGCACCACAGGATTCAGCA	0.15	0.14
dpu-miR-9-3p_R-2_1ss11GA	ATAAAGCTAGATTACCAAAGT	0.04	0.04
isc-miR-279	TGACTAGATCCACACTCATCCA	0.23	0.29
isc-miR-263a_R + 2	AATGGCACTGGAAGAATTCACGG	0.18	0.16
dan-miR-79_L + 1R-3	ATAAAGCTAGATTACCAAAG	0.03	0.03
dqu-miR-2788-3p	CAATGCCCTTGGAAATCCCAAA	0.14	0.14
tcf-miR-995_1ss20TA	TAGCACCACAGGATTCAGCAT	0.18	0.20
ame-miR-29b-3p_R + 1	TAGCACCATTTGAAATCAGTG	0.26	0.36
dpu-miR-100_R-2	AACCCGTAGATCCGAACTTGT	0.31	0.43
pte-miR-2d-3p_L-2R-2	TCACAGCCAGCTTTGATGA	0.21	0.25
pte-miR-2d-3p_R-5	TATCACAGCCAGCTTTGA	0.31	0.44
dpu-miR-137_L + 1R-2	TTATTGCTTGAGAATACACGT	0.23	0.52

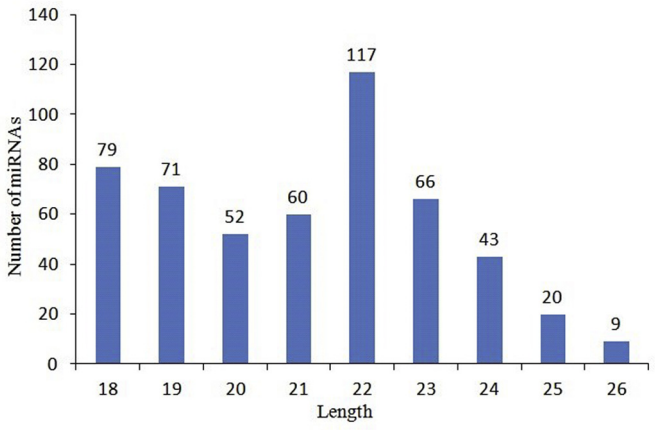

Fig. 1. Size distribution of small RNAs found by RNA sequencing. The number of miRNAs in a certain length (18–26 nucleotides).

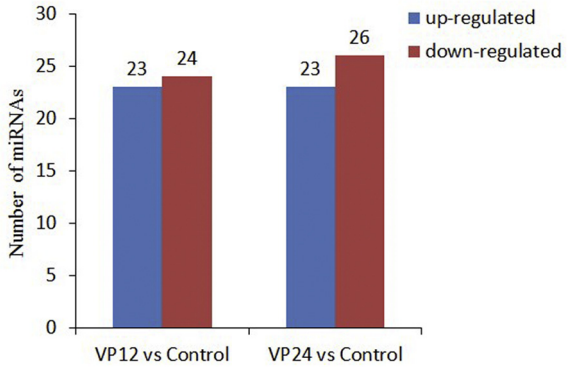

Fig. 2. The numbers represented the miRNAs up-regulated or down-regulated ($P < .05$) compared with the control in the *V. parahemolyticus*-infected *S. paramamosain*.

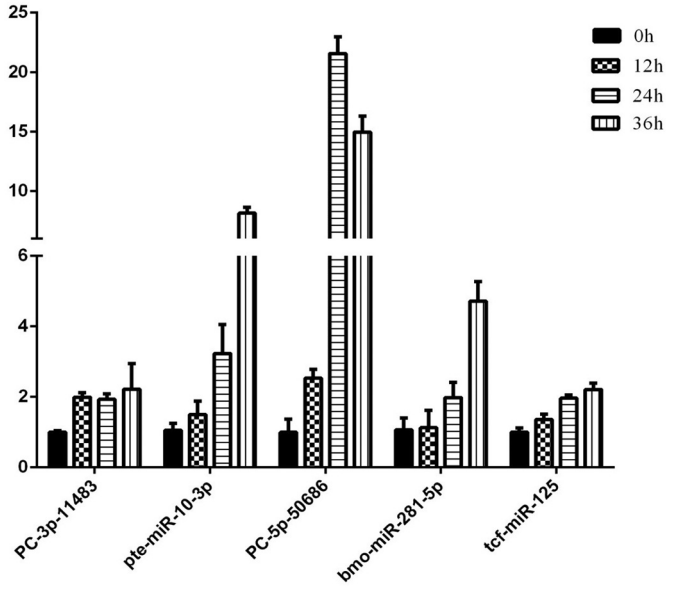

Fig. 3. The qRT-PCR results of five differentially expressed miRNAs in the immune response to *V. parahemolyticus* in *S. paramamosain*. The miRNA expression was normalized to U6 expression level, miRNA expression of 0 h was normalized as one.

26 miRNAs were down-regulated at 24 h post infection (Fig. 2). 23 miRNAs were significantly up-regulated by > 2 folds: like miR-125, PC-3p-11,483, miR-281-5p, miR-10-3p and PC-5p-50,686 with a statistical significance of $P < .05$ at both time intervals post infection (Table 1). Other 23 miRNAs (like miR-153, miR-279, miR-12_R-1, miR-100_R-2 and PC-3p-12157_315) were significantly down-regulated by > 2 folds ($P < .05$) at both time intervals post infection (Table 1). To confirm the involvement of these miRNAs in *V. parahemolyticus* infection, five significantly up-regulated (over 10 fold) miRNAs were selected for stem-loop RT-qPCR. These miRNAs showed similar expression patterns (significantly up-regulated or down-regulated) to the sequencing results (Fig. 3).

3.3. Gene ontology analysis and KEGG pathway analysis

The target genes of the 46 differentially expressed miRNAs were predicted by using TargetScan and miRanda, and many miRNAs have more than three predicted target gene and pathways (Table S1). Gene ontology classification of target genes of miRNAs showed that transcription is the most represented biological process, nucleus and integral component to membrane are significant in cellular component, and ATP binding is over represented in molecular function (Fig. 4). To explore the potential pathways regulated by these miRNAs, we performed Gene Ontology (GO) analysis by using "GoStat" analysis tool (http://gostat.wehi.edu.au/). We took the total list of predicted and validated targets of the differentially expressed miRNAs and determined if any GO categories were over-represented within our list. Magnesium ion binding and golgi apparatus are important biological processes in the immune response to *V. parahemolyticus* by GO enrichment scatter plot analysis (Fig. 5). In addition, KEGG enrichment scatter plot analysis showed that endocytosis is the most important pathway in the immune response to *V. parahemolyticus* in *S. paramamosain*. And the following pathways are phagosome, Wnt signaling pathway and tight junction (Fig. 6). Among the 46 differentially expressed miRNAs, 25 miRNAs target the pathway of endocytosis, 14 miRNAs target the pathway of lysosome, 9 miRNAs target the pathway of phagosome, and 5 miRNAs target Wnt signaling pathway (Table S1).

4. Discussion

In the present investigation, we infected mud crab *S. paramamosain* with *V. parahemolyticus* and compared the expression profiles of miRNAs in *Vibrio* free and *Vibrio* infected crabs, and characterized the differentially expressed miRNAs. Our study provides the large-scale characterization of *S. paramamosain* miRNAs in response to *V. parahemolyticus* infection. We identified 47 and 49 host miRNAs were differentially expressed in response to *V. parahemolyticus* infection at 12 and 24 h post infection. It was revealed that there was only a small difference in miRNA expression at two points of time. Among these miRNAs, miR-125, PC-3p-11,483, miR-281-5p, miR-10-3p and PC-5p-50,686 were significantly up-regulated by above 10 folds. In mammals, a lot of studies confirmed that miR-125 showed its important role in

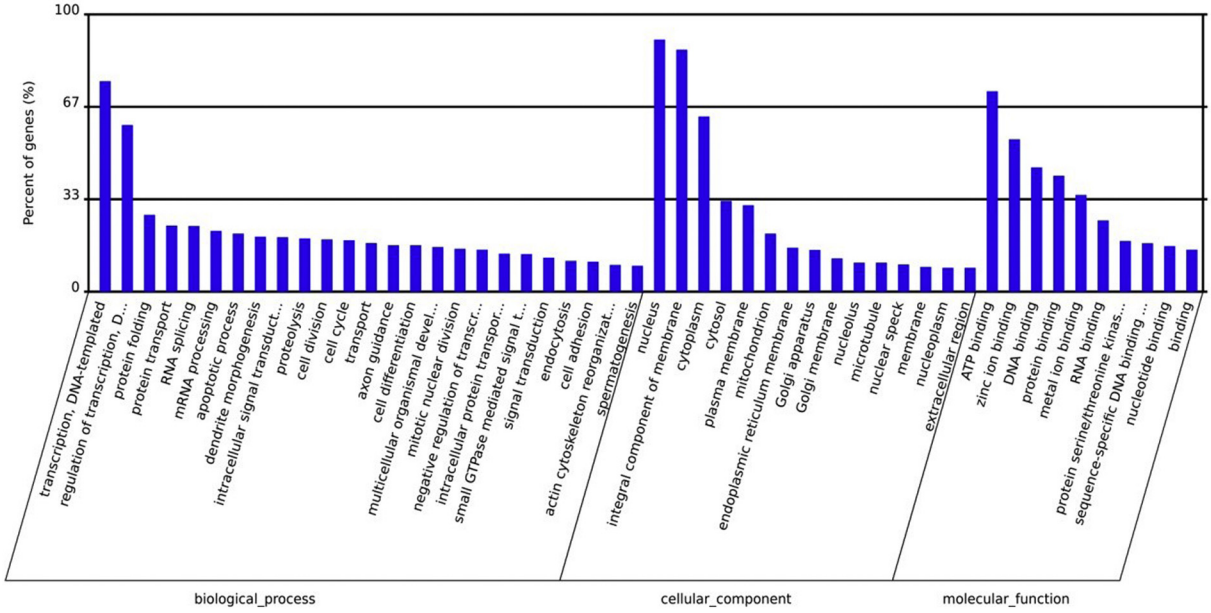

Fig. 4. Gene ontology classification of target genes of miRNAs in *S. paramamosain*. By alignment to GO terms, the target genes were mainly divided into three categories with 50 functional groups: biological process (25 functional groups), cellular component (15 functional groups), and molecular function (10 functional groups). The left y-axis indicates the percentage of a specific category of genes existed in the main category.

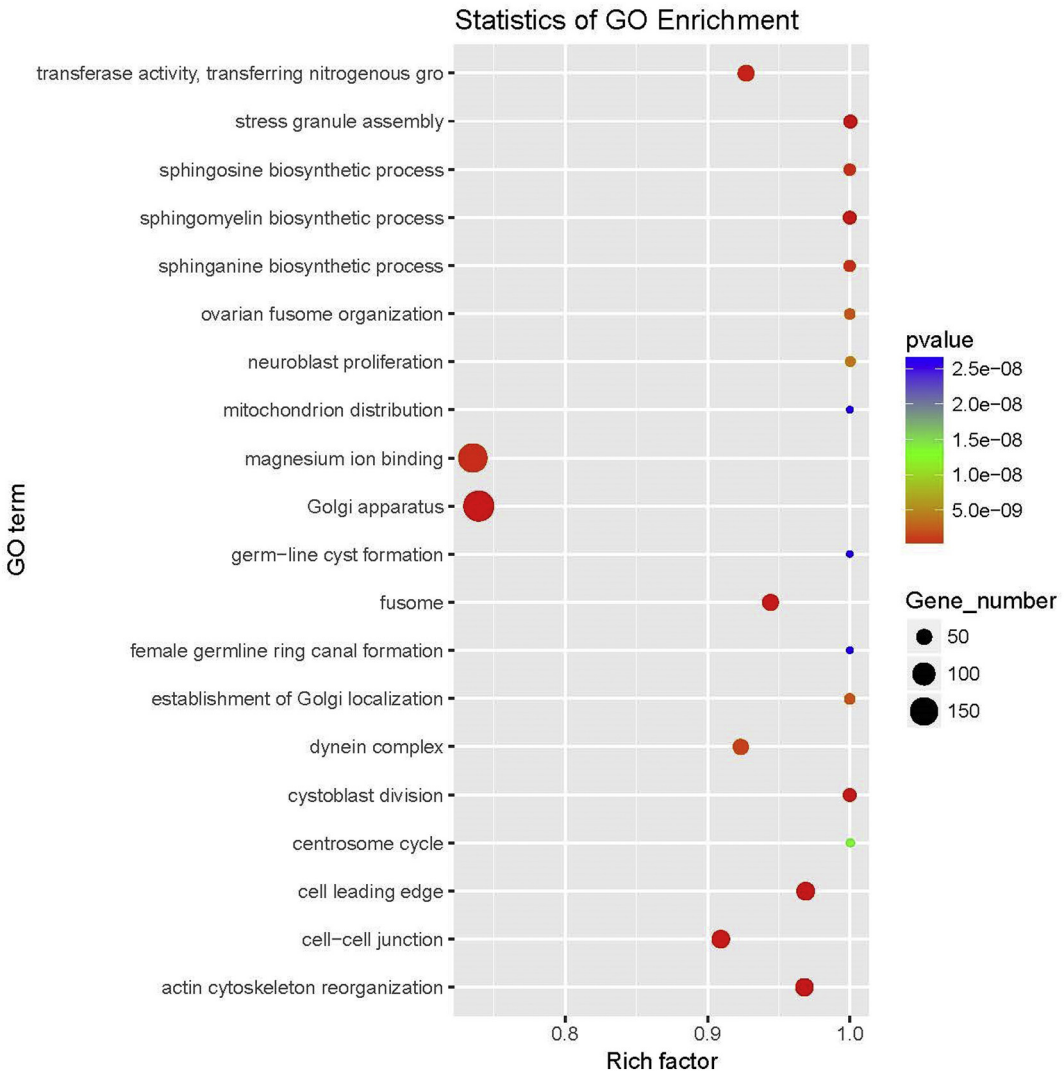

Fig. 5. GO enrichment scatter plot analysis of target genes of differentially expressed miRNAs output for GO biological process (GOBP) terms.

development (Boissart et al., 2012), hematopoiesis (Shaham et al., 2012), cancer and immunity (Wang et al., 2019), and directly participate in the immune response to both bacterial (Staedel and Darfeuille, 2013) and viral pathogens (Ren et al., 2019; Ruiz et al., 2016). In invertebrate, limited researches also showed that miR-125 regulate development and innate immunity in *Drosophila* (Yatsenko and Shcherbata, 2018; Chawla et al., 2016; Caygill and Johnston, 2008; Garbuzov and Tatar, 2010), and it was related to the skin ulceration syndrome of sea cucumber (Sun et al., 2016). The miR-153 was significantly down-regulated in this study. And down-regulation of miR-153 is also related to cancer (Xu et al., 2013), Alzheimer's disease (Long et al., 2012) and Parkinson's disease in mammals (Mouradian, 2012). The miR-153 exerted anti-tumor activity in many kinds of cancer through inhibiting migration and invasion of cancer (Yuan et al., 2015; Shan et al., 2015). So down-regulation of miR-153 in *V. parahemolyticus* infection is a manifestation of disease development. Other significantly differentially expressed microRNAs should also play important roles in immune regulation, and they deserves for further study.

This gene ontology classification of target genes is closed to the transcriptomic analysis of crab hemocytes in response to *V. alginolyticus* infection (Zhu et al., 2018). In this study, endocytosis and phagosome were found to be the important pathway in the immune response to *V. parahemolyticus*. These pathways were all the components of phagocytosis. And phagocytosis is very important for antibacterial immunity of invertebrate (Rämet et al., 2002; Kong et al., 2018; Yang et al., 2017). Interesting, the Wnt signaling pathway also were found to be an important pathway in the immune response to *V. parahemolyticus* (Zhang et al., 2016). In our previous study, the Wnt signaling pathway is involved in the regulation of phagocytosis of virus in *Drosophila* (Zhu and Zhang, 2013). So we can deduce a conclusion that miRNAs would regulate the phagocytosis process to fight *V. parahemolyticus* infection. Golgi apparatus and Magnesium ion binding are important biological process. Golgi apparatus was found to be very important in disease and immunity (Park et al., 2015; Pourcelot et al., 2016). Magnesium ion channels and transporters are very important in lymphocyte function and immunity in mammals (Feske et al., 2015; Feske et al., 2012). The target genes of miRNAs showed that transcription is the most represented biological process, nucleus and integral component to membrane are significant in cellular component, and ATP binding is over represented in molecular function. Although *S. paramamosain* miRNAs after *V. parahemolyticus* infection has been reported (Li et al., 2013), this study reveals more information about target genes and pathways of miRNAs and provides the expression profiles of miRNAs at 12 h post infection.

Our study shows that most of the miRNAs targeted the genes involved in immune response to *V. parahemolyticus* infection. Further studies should investigate the molecular events in bacteria-host interactions mediated by miRNAs, which will help to control the bacterial

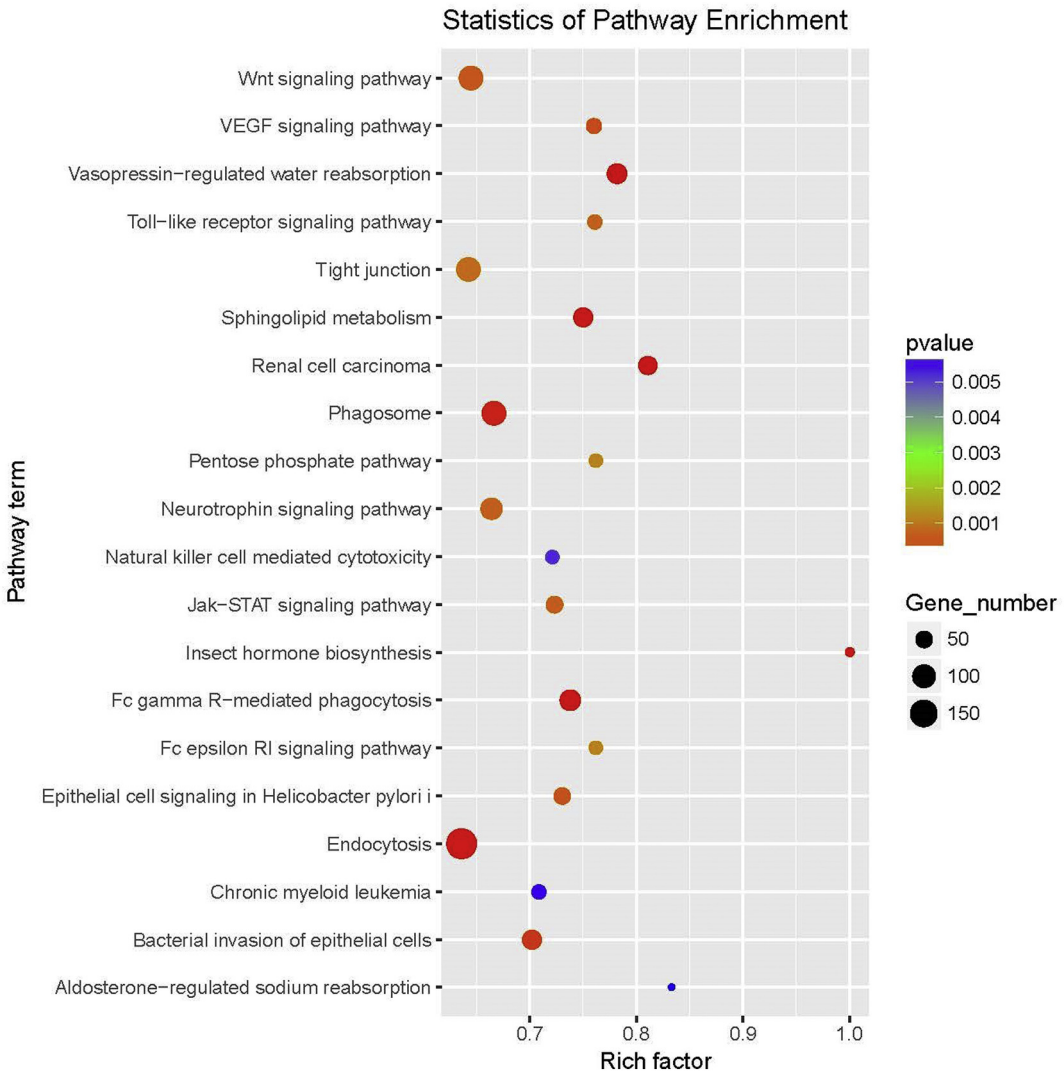

Fig. 6. KEGG enrichment scatter plot analysis for pathway enrichment of target genes of differentially expressed miRNAs.

infections.

Declaration of Competing Interest

There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the gene policies on sharing data and materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735288.

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