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Changes of microRNAs expression profiles from red swamp crayfish (*Procambarus clarkia*) hemolymph exosomes in response to WSSV infection

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ABSTRACT

MicroRNAs (miRNAs) as short noncoding RNAs play important regulatory roles in diverse biological processes by degrading the target mRNAs, and could be delivered by exosomes. WSSV is a highly pathogenic and prevalent virus, and has brought high mortality of *P. clarkia.* Till present, no studies focus on the miRNAs changes in exosomes during WSSV infection. To understand the different virulence of WSSV on miRNAs expression in *P. clarkia* hemolymph exosome, the deep sequencing was performed to compare the small RNA libraries from the hemolymph exosome of *P. clarkia* individuals with or without WSSV infections. From the TEM observations, NTA and Western Blot analysis, the extracted exosomes were well identified with classic characteristics. The 209 conserved miRNAs and 250 novel miRNAs were identified from the small RNA libraries. In response to WSSV infection, there were about 98 miRNAs significantly up-regulated and 59 miRNAs significantly down-regulated. The target genes prediction, GO and KEGG enrichment analysis revealed that some target genes of *P. clarkia* miRNAs were grouped mainly into the categories of biological regulation, immune system process, signal pathway and other more functions. This is the first report of comprehensive identification of *P. clarkia* hemolymph exosome miRNAs being differentially regulated in response to WSSV infection. These results will help to understand the hemolymph exosome miRNAs response to different virulence WSSV infection.

1. Introduction

Exosomes are endosome-derived vesicles (size about 50–150 nm) releasing from multivesicular bodies (MVBs) within many cell types [1]. They contain many small molecular substances such as proteins, lipids, mRNAs and microRNA (miRNAs), and could deliver these from one cell to other cells [2]. In many researches, exosomes are reported to play important roles in mediating the intercellular communication. These communications among immune cells could regulate the host immune response. Some studies have demonstrated exosomes participate in the immune-stimulation and immune effector molecule secretion events [3,4]. The miRNAs delivered by host exosomes are considered as novel regulators of cellular function [5]. It has been proved that the transferred miRNAs could repress target mRNAs and cause physiological changes in recipient cells [6–8]. During the immune response to lipopolysaccharide, the host miRNAs delivered by exosomes could regulate the immune gene expression [9]. The exosomes also play a crucial role for antiviral innate immune response for generating immunological memory [10]. So exosomes act as molecule carriers and play multi-functions in the host innate immune response by delivering the small molecular substances.

miRNAs are found in eukaryotes, which are a class of endogenous non-coding small RNA molecules (19–25 nucleotides) [11]. Through binding to 3′ UTR of target mRNA, miRNA could repress gene expression at the post-transcriptional level [12,13]. miRNAs participate in lots of biological process like homeostasis, apoptosis, tissue development, cellular proliferation and differentiation, and immunological defense function [14–16]. During the bacterial and viral infections to hosts, miRNAs also play a complex role in pathological process [17,18]. miRNAs can help regulate the development of immune cells and modulate the innate and adaptive immune responses [19]. Till present, many studies have demonstrated viral infections can alter the cellular miRNA expression profile. miRNAs are generally thought to function within the cells in which they are made, however, recently, miRNAs have been observed in secreted exosomes [6–8]. Immune cells can both secrete and take up exosomal miRNAs to regulate some important gene

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expressions by intercellular communication ways [20]. In parent cell, the exosomal miRNA signatures do not simply reflect the miRNA composition but are composed of a distinct set of miRNAs [21,22]. Some certain miRNAs have finally evolved to be packaged into exosomes to carry out their biological functions. Despite the recognized importance of miRNAs in host immune response, the specific exosomal miRNAs in regulating the immune response to the pathogens infections are still in preliminary stages of study.

The red swamp crayfish *Procambarus clarkia* is a kind of freshwater crayfish with a highly adaptation and reproductive ability [23,24]. In recent years, because of these characteristics, *P. clarkia* is becoming an important economic aquaculture shrimp species in China with the intensive artificial culture. White spot syndrome virus (WSSV) is a highly pathogenic and prevalent virus, and has brought high mortality of farmed shrimp [25]. It has a broad host range within different kinds of shrimp, and could infect most organs in shrimp. *P. clarkia* could be infected by WSSV and used as a laboratory model [26]. Till present, with the great development of artificial culture and environmental deterioration, WSSV has brought great economic lose in *P. clarkia*. It is necessary to investigate the immune defense mechanisms of *P. clarkia* for control of WSSV disease. Great researches have focused on the host immune response to the WSSV infection, including transcriptome and small RNAs analysis in hepatopancreas and hemolymph [27–29], but no studies focus on the miRNAs changes in exosomes during WSSV infection. Exosomes can deliver miRNAs from cell to cell and play important roles in the intercellular communication mediating [2]. To have a better understanding of the function of miRNAs changes in exosomes during WSSV infection, we have collected and identified the exosomes in hemolymph, and tested the different miRNAs expression in exosomes during host response to WSSV. The present data is helpful to explore the immune response mediated by miRNA to WSSV infection.

2. Materials and methods

2.1. Shrimp, WSSV challenge and detection

Red swamp crayfish (about 10–15 g each) were collected from a crayfish farm in Yangling, Shaanxi Province, China. They were cultured in water tanks in fresh water at room temperature (20 °C) in the laboratory with adequate aeration and a natural photoperiod. The shrimp were fed with a commercial crayfish diet once per day. Shrimps were randomly selected for routine WSSV screening. DNA extracted from gills was used as the template for WSSV PCR detection, which was performed with the primer pair VP28F/VP28R (forward: AAACCTCC GCATTCCTGTGA; reverse: TCCGCATCTTCTTCCTTCAT) and 40 amplification cycles. Using a 100 μl syringe, 5×10^5 copies virus particles were injected into the abdominal segment of each crayfish. The preparation and quantification of viral inocula followed a previously described method [30]. 60 h after injection, the hemolymph was collected from the ventral sinus of twenty crayfish and mixed with 1 mL anticoagulant buffer (10% sodium citrate, pH 7). Then the hemolymph was centrifuged at 1000 × *g* for 5 min at 4 °C to abandon the hemocytes. The supernatant of hemolymph was used to extract the exosome. The control samples (uninfected shrimp) of hemolymph were also collected as above descriptions. Three repeats for experimental and control hemolymph samples were collected for deep sequencing. The gills of crayfish were also collected for DNA extraction to test the WSSV particles.

2.2. Hemolymph exosomes collection and identification

Exosomes were isolated according to published reports [31,32], with minor modifications. Briefly, hemolymph supernatants were collected. Then the hemolymph samples were subjected to sequential centrifugation steps at 1000 g for 10 min, 2400 g for 30 min, and 12,000 g for 30 min, with each step at 4 °C, which removed hemocytes and debris fraction. The supernatant containing exosomes was diluted in PBS, followed by filtration through 0.45-μm and 0.22-μm filters. Then it was ultra-centrifuged at 100,000 g for 90 min. The pellets containing exosomes and contaminating proteins were re-suspended in PBS buffer and underlayered with a 30% sucrose cushion, followed by centrifugation at 100,000 g for 90 min. The sucrose cushion was washed by re-suspension in PBS, followed by ultra-centrifugation at 100,000 g for 90 min. The pellets containing exosomes were re-suspended in PBS and frozen at −80 °C until use.

2.3. Identification of the exosomes

To visually confirm the presence of exosomes, the exosomes derived from hemolymph samples were observed under transmission electron microscope (TEM). Briefly, the exosome pellet was fixed in 2.5% glutaraldehyde in PBS buffer overnight at 4 °C. The samples were rinsed 3 times in PBS and fixed in 1% osmium tetroxide for 60 min at room temperature. Then they were embedded in 10% gelatin, fixed in glutaraldehyde at 4 °C and observed on a Hitachi H-600 transmission electron microscope at 70,000 magnification.

The size distribution of exosomes was measured using Zetaview (Particle Metrix) nanoparticle tracking analyzer. NTA analytical software version 2.3 was used for capturing and analyzing the data.

The western blot analysis was also used to test the protein markers of exosomes. Briefly, the protein lysates of exosomes (3 μg) were run on 15% SDS-PAGE electrophoresis and transferred to PVDF membrane (Millipore, USA). The membranes were blocked using 5% skim milk for 1 h and washed three times in TBST. Then the membranes were incubated separately with mouse monoclonal anti-rabbit CD63 antibody at a dilution of 1:2000, mouse monoclonal anti-rabbit Calnexin antibody at a dilution of 1:1500, mouse monoclonal anti-rabbit Hsp70 antibody at a dilution of 1:1000, mouse monoclonal anti-rabbit Hsp90 antibody at a dilution of 1:1500 (Wanleibio, China) at room temperature for 4 h followed by washing with TBST. Then the membranes were incubated with HRP conjugated goat anti-rabbit IgG antibody for 2 h at room temperature. The immunoreactive bands were visualized using the enhanced chemiluminescence reagents (Advansta, California, USA) according to manufacturer protocol, and quantified by chemiluminescence imaging system (Bio-Rad, California, USA).

2.4. Small RNA library construction and sequence analysis

The different RNA samples from hemolymph exosomes were extracted using Trizol according to the manufacture's instructions. Nanodrop, Qubit 2.0, the Agilent 2100 bioanalyzer were used respectively to detect the purity of RNA samples, concentration and integrity to ensure that the use of qualified samples for sequencing. Small RNA libraries were constructed using a TruSeq Small RNA Library Prep Kit (Illumina) according to the manufacturer's protocol. The generated libraries were sequenced on an Illumina HiSeq X Ten platform, and single-end reads were generated.

The miRNAs high-throughput dates were aligned with miRBase21.0 to search for the miRNA and examine the conservative situation. Randfold tools soft was used for novel miRNA secondary structure prediction. The matched sequences that could form stable secondary structures predicted by miRDeep2.0 were identified as novel miRNAs [33].

2.5. Analysis of different miRNA expressions

To compare differentially expressed miRNAs between multiple samples, reads per million reads (RPM, miRNA counts/total counts of each sample × 1 million) were used as the value of normalized miRNA expression levels. The microRNAs with the RPM value less than 100 were excluded for comparison due to low expression levels. The log transformed data were normalized using Quantile normalization method [34]. The fold change between miRNA expression in two

Table 1
Details of the primer sequence used for qPCR.

Primers	Sequences (5′–3′)
VP28-F	AAACCTCCGCATTCCTGTGA
VP28-R	TCCGCATCTTCTTCCTTCAT
novel_miR_151-F	GCAGTAAGGCTTTTTAAACTACTC
aga-miR-7-F	CGCAGTGGAAGACTAGTGA
dme-miR-7-5p-F	CGCAGTGGAAGACTAGTGA
tca-miR-7-5p-F	GTGGAAGACTAGTGATTTTGTTG
novel_miR_242-F	AGGGGGGTGAGTGTG
mmu-miR-1943-5p-F	CGCAGAAGGGAGGATCT
isc-miR-750-F	GCCAGATCTAACTCTTCCAG
novel_miR_92-F	GCAGTGGGGATGTGCT
U6-F	CGCAAGGATGACACGCAAATT
Reverse Primer	Qiagen universal primer

groups was determined as follows: fold changes = $\log_2$(WSSV group/control group). With a Bonferroni correction for multiple comparisons and a p-value < 0.05, results of the Chi-squared test and |Log2Fold Change| ≥ 1 indicated that differences in the miRNA counts were statistically significant.

2.6. qPCR of differentially expressed microRNAs

The expression profiles of different microRNAs were detected by qPCR method according to previous study [35]. The RNA was reverse-transcribed using miScript II RT Kit (Qiagen). The detections of mature miRNAs were done using miScript SYBR Green PCR Kit (Qiagen). The reaction was carried out in Bio-Rad CFX96 in a 20 μl reaction volume. The program for qPCR was 95 °C for 15 min, followed by 45 cycles of 95 °C for 15s, 55 °C for 30s, and 70 °C for 30s. The U6 small nuclear RNA (snRNA) was used as internal reference gene. The different sequences of primers were shown in Table 1. The relative expression level of target gene was calculated using the comparative Ct method with the formula $2^{-\triangle\triangle Ct}$. The results were expressed as mean ± S.D.

2.7. Target gene prediction of differentially expressed miRNAs and gene ontology (GO) analysis

The mRNA sequences of *P. clarkia* from the GenBank database and our previous transcriptome (unpublished) were used to predict miRNA target genes. The corresponding 3′UTR sequences were determined using the UTRScan program (http://itbtools.ba.itb.cnr.it/utrscan) and incomplete 3′UTRs were discarded. The target prediction algorithms was done using miRanda (https://www.microrna.org/) and RNA22 (https://cm.jefferson.edu/rna22/). TargetScan was used to search for miRNA seed matches (nucleotides 2–8 from the 5′ end of miRNA) in 3′UTR sequences. miRanda was used to match the entire miRNA sequences. The parameter for miRanda were a score > 120 and a free energy < −20 kcal/mol, and for RNA22 the free energy was < −20 kcal/mol. The predicted results by the two tools were combined and the overlaps were calculated. Gene Ontology (GO) enrichment analysis was carried out by using Fisher's exact test with a *p*-value below 0.05. The GO enrichment results were exhibited using Web Gene Ontology Annotation Plot (WEGO, http://wego.genomics.org.cn/cgi-bin/wego/index.pl) [36]. The best hit GO IDs were assigned to the shrimp EST sequences.

3. Results

3.1. WSSV detection of experimental shrimp by one-step PCR

The experimental crayfish were cultured in the lab normally with no clinical signs. Different DNA templates of gills were extracted to test the WSSV infection from random selected shrimp. From the result (shown in Fig. 1A), no obvious band was detected verifying the healthy state of crayfish. Then manual infection was performed by muscular injection of WSSV inocula. At about 36 h and 60 h after WSSV infection, the crayfish were random selected to test the virus. From the results (shown in Fig. 1B), the viral load in gills assumed a rising trend from 36 hpi to 60 hpi, indicating that it was the moderate infection stage corresponding to the logarithmic phase of WSSV replication [30].

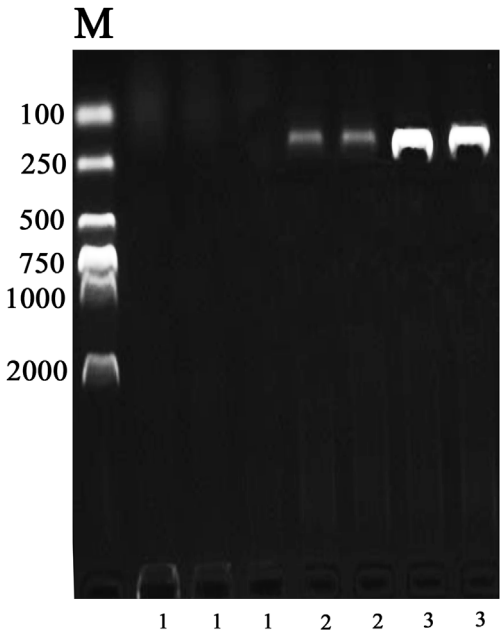

Fig. 1. WSSV detections from different *P. clarkia*. Lane M represents the D2000 marker. Lane 1 represents different samples from *P. clarkia* before WSSV injection. Lane 2 represents the WSSV detections after 36 h post injection. Lane 3 represents the WSSV detections after 60 h post injection. Gills from three *P. clarkia* were used as one sample. The VP28 primer pairs were used in PCR amplification.

3.2. Detection analysis of hemolymph exosome

To confirm the presence of hemolymph exosomes, western blot and TEM were used to detect the exosomes. From the results of TEM (shown in Fig. 2A), it showed that hemolymph exosomes samples were membranous vesicles containing lipid bilayer-bound membranes, with size distributions peaking at 100 nm diameter according to NTA (Fig. 2B). The western blot was also conducted to detect the exosome-marker proteins, including Hsp70, Hsp90, CD63 and Calnexin. The results showed (Fig. 2C) that these exosome-marker proteins were validated by western blot. All these data indicated that the successful isolation and purification of exosomes from hemolymph of *P. clarkia*.

3.3. Identification and annotation analysis of microRNAs in P. clarkia hemolymph exosome

From the results of high-throughtput sequencing, approximately 25,000,000–35,000,000 clean reads from each small RNA library were obtained. The sequence length distribution among different libraries had a broad scope ranging from 15 nts to 30 nts, while the major parts of reads were distributed between 20 and 25 nt (shown in Fig. 3). Removed the same sequences, the Unique Sequences were left, which the small RNAs of 22 nts were the most, followed by 21 nts and 23 nts. The trimmed reads were blasted to the Rfam 12.0 database, GtRNAdb database, Silva database and Repbase database to filter the ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (snRNA), nucleocapsid (snoRNA), and repeat sequences, then miRNA unannotated reads were harvested. To identify conserved microRNAs, the small RNA reads were blasted against the microRNAs of shrimp retrieved from miRbase

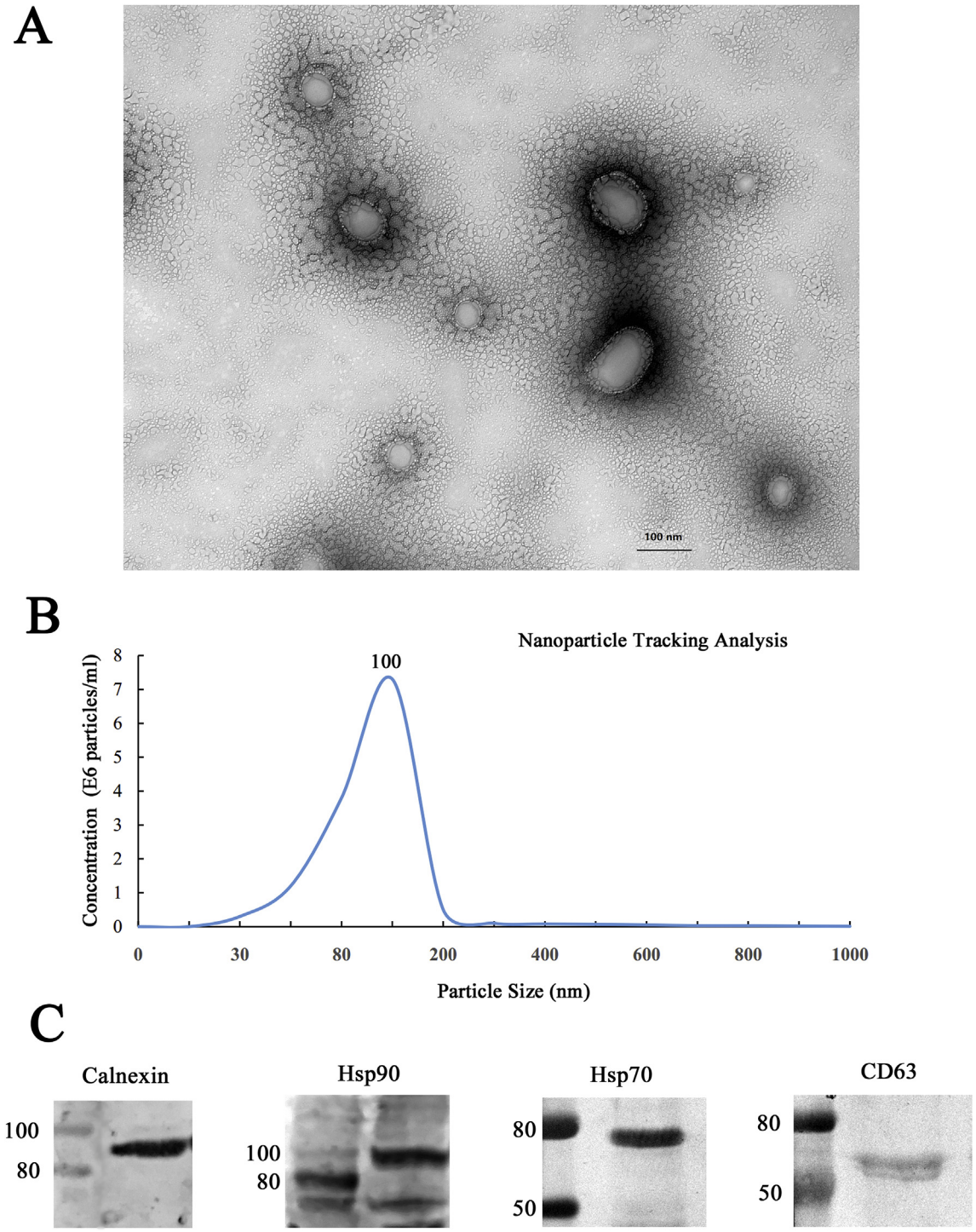

Fig. 2. Detections of hemolymph exosomes from *P. clarkia*. (A) TEM observation of the extracted exosomes. Bar is 100 nm. (B) Nanoparticle tracking analysis (NTA) of the exosome particle size. E6 particles/ml indicated the amount of exosomes per ml solutions. (C) Western blot analysis of the protein markers from exosomes.

database. A total of 459 mature miRNAs were harvested including 209 conserved miRNAs and 250 novel predicted miRNAs.

3.4. Differential expression of microRNAs in response to WSSV infection

To investigate the differentially expressed microRNAs in response to WSSV infection in hemolymph exosome of crayfish, the fold changes (FC) of microRNAs between different samples (control groups and WSSV infected groups) were calculated. During the analysis, $|\log2(FC)| \geq 1$, $FDR \leq 0.01$ and P-value < 0.01 indicated that differences expression profiles in the miRNA counts were statistically significant. From the results, the expression of 157 microRNAs was altered by WSSV infection, with 98 microRNAs up-regulated and 59 microRNAs down-regulated. According to the expression quantity, the most significantly expressed miRNAs were shown in Table 2 and Table 3. Of these, 4 miRNAs (novel_miR_151, novel_miR_145, novel_miR_123, novel_miR_26) were highest up-regulated, while 13 miRNAs (novel_miR_242, novel_miR_182, novel_miR_180, novel_miR_13, novel_miR_16, novel_miR_119, novel_miR_154, novel_miR_228, novel_miR_29, novel_miR_95, tur-miR-745-3p, mmu-miR-

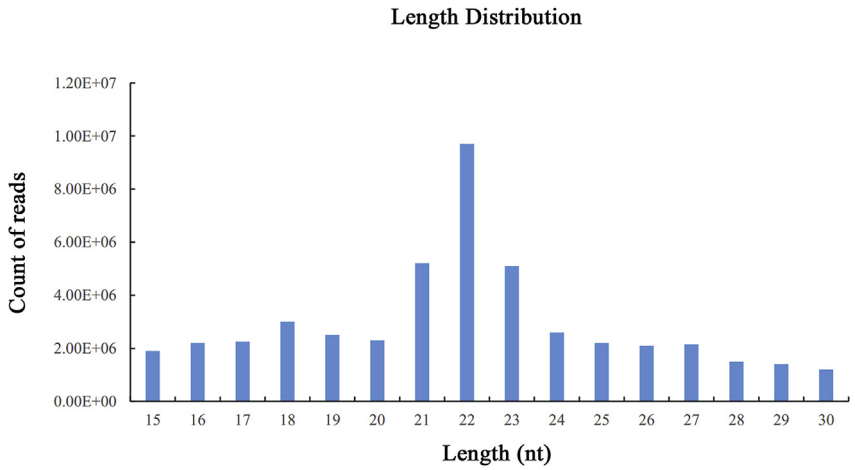

Fig. 3. Length distribution of small RNAs after discarding junk reads, trimming the adapter sequence, removing reads < 17 or > 30 nt, and mapping to Rfam database.

1943-5p, novel_miR_66) were down-regulated mostly. The other different miRNAs of hemolymph exosome have a certain changes after WSSV infection (shown in Supplemental Table 1 DEG miRNAs sheet). These results revealed the differential miRNAs expression changes in response to WSSV infection.

The target genes of the differentially expressed miRNAs were predicted by using TargetScan and miRanda. There are more than one predicted target genes for some miRNAs. The sequence and target gene of higher differentially expressed miRNAs are shown in Table 2. All the predicted target genes were shown in Supplemental Table 1 miRNA target genes sheet. The sequence of the top 20 DEGs target genes were shown in Supplemental Table 2. From the results, the target genes of microRNAs have different functions, while no target genes were found to some microRNAs.

The gene ontology analysis based on the biological processes showed the predicted target genes could be clustered into different GO terms. The top 10 GO terms were shown in Fig. 4. The mostly target genes of these DEG miRNAs were related to biological regulation, immune system process, response to stimulus, biological adhesion, immune response, signaling, multicellular organismal process, inflammatory response, developmental process and cellular component organization or biogenesis. The top 10 KEGG pathways were shown in Fig. 5. The predicted target genes are mostly involved in Hippo signaling pathway, Jak-STAT signaling pathway, Wnt signaling pathway,

Table 2
Putative target genes of top up-regulated expressed miRNAs after WSSV infection.

Number	miRNA_name	miRNA_Sequence	Length	Target_gene	Fold Change (log2 FC)
1	novel_miR_151	UAAGGCUUUUUAAACUACUCCAUC	24	–	+31.44
2	novel_miR_145	CCAUACACAGUUUCAAGUGU	20	Exocyst complex component 1 Y + L amino acid transporter 2	+29.56
3	novel_miR_123	AGCAGUCUGUUAUUUGAGAACC	22	–	+28.88
4	novel_miR_26	AAAUAUCAGCUGGUAAAUUUGG	22	calcium/calmodulin-dependent protein kinase I	+7.28
5	aae-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	–	+3.62
6	aga-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	FERM and PDZ domain-containing protein 4	+3.62
7	api-miR-7	UGGAAGACUAGUGAUUUUGUUGUU	24	–	+3.62
8	bmo-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGU	23	RNA-binding protein fusilli	+3.62
9	chi-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	24	Down syndrome cell adhesion molecule-like protein Dscam	+3.62
10	cqu-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	–	+3.62
11	csa-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	FoxO protein	+3.62
12	dan-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	Transmembrane 9 superfamily member 4	+3.62
13	der-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	Molybdenum cofactor sulfurase	+3.62
14	dme-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGU	23	heparan sulfate 2-*O*-sulfotransferase pipe	+3.62
15	dmo-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	–	+3.62
16	dpe-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	legumain-like Ufm1-conjugating enzyme	+3.62
17	dse-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	–	+3.62
18	dsi-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	ferrochelatase, mitochondrial RNA-binding protein fusilli	+3.62
19	dvi-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUU	24	–	+3.62
20	dwi-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	–	+3.62
21	lgi-miR-7	UGGAAGACUAGUGAUUUUGUUGUU	24	Fem-1-like protein B	+3.62
22	dpu-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	cathepsin L serine/threonine-protein kinase PAK 2-like	+3.59
23	efu-miR-7a	CUGGAAGACUAGUGAUUUUGUUGUU	25	TAR RNA-binding protein isoform 1	+3.59
24	isc-miR-7	UGGAAGACUAGUGAUUUUGUUGU	23	Apoptosis regulator BAX	+3.59
25	tca-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGUUC	25	Guanine nucleotide-binding protein	+3.59
26	novel_miR_136	GACGACAAUGUUCAAUCCCUGAAC	24	Na(+)/H(+) exchange regulatory cofactor NHE-RF2	+3.19
27	novel_miR_210	GGUGGACGUGUAGUUUGCCUAG	22	reverse transcriptase transcription factor AP-2 HSP70	+2.75
28	novel_miR_28	CAUCACAGUGAUAGUACCUUACU	23	Protein cappuccino	+2.25

Table 3
Putative target genes of top down-regulated expressed miRNAs after WSSV infection.

Number	miRNA_name	miRNA_Sequence	Length	Target_gene	Fold Change (log2 FC)
1	novel_miR_242	GGGGGUGAGUGUGGGCCUG	19	zinc metalloproteinase endonuclease/reverse transcriptase NK2-3/5 transcription factor Rab effector Noc2, partial hypoxia-inducible factor alpha Pro-resilin Hippo BDNF/NT-3 growth factors receptor peritrophin-44-like protein nuclear export mediator factor NEMF homolog c-jun N-terminal kinase thioredoxin reductase 2 Heparan sulfate 2-*O*-sulfotransferase	−30.67
2	novel_miR_182	CUGACGUGUGGUUUGGUCAU	20	–	−30.38
3	novel_miR_180	UCAGGAACCACGGAGGCU	18	nucleosome-remodeling factor	−30.21
4	novel_miR_13	GUGUGCUACUGUACCUUC	18	–	−29.79
5	novel_miR_16	AGGUCUUUGAGUAUGUGGU	19	–	−29.79
6	novel_miR_119	CAGUGGGAGCUGUUGGAUGAAAGG	24	Hsp90 co-chaperone Cdc37 C-jun-amino-terminal kinase-interacting protein 3 Supporter of activation of yellow protein	−29.53
7	novel_miR_154	AUCUGCACUCUCCAUGAAGG	20	–	−29.53
8	novel_miR_228	GCGGAGAGAUGUGCAAGAA	19	–	−29.53
9	novel_miR_29	GACGACGUUUCGGCUGUG	18	–	−28.79
10	novel_miR_95	CUCGUGCAUCUGUGGGCC	18	ras association domain-containing protein	−28.79
11	tur-miR-745-3p	CAGCUGCCCAGUGAAGGGCUG	21	Transient receptor potential cation channel subfamily A member 1-like protein Cytoplasmic FMR1-interacting protein plexin domain-containing protein 2	−28.21
12	mmu-miR-1943-5p	AAGGGAGGAUCUGGGCACCUGGA	23	rap1 GTPase-activating protein 1 MOG interacting and ectopic P-granules protein 1 homeodomain transcription factor isoform ankyrin-2 NO-sensitive soluble guanylyl cyclase beta 1 short isoform	−27.21
13	novel_miR_66	GGGAGUCUAGGAGACGAGGG	20	Transforming growth factor-beta-induced protein ig-h3 transcription initiation factor TFIID subunit 6-like serine protease chitinase 2	−27.21
14	novel_miR_10	GAAGUAUGGGAUCAUAGGGCC	21	signal sequence receptor beta-like protein Rab GDP dissociation inhibitor alpha autophagy-related protein 2 homolog A peritrophin-44-like protein Rab GTPase RAS-like protein, partial	−6.44
15	novel_miR_19	UUGUUCUGAUGAAGGUGAAUU	21	C-type lectin	−5.96
16	ame-miR-750	CCAGAUCUAACUCUUCCAGCUC	22	–	−5.86
17	cte-miR-750	CCAGAUCUAACUCUUCCAGCUCA	23	–	−5.86
18	isc-miR-750	CCAGAUCUAACUCUUCCAGCUCA	23	Apoptosis regulator BAX E3 ubiquitin-protein ligase Insulin-like peptide receptor erythroid differentiation-related factor 1 ADP-ribosylation factor-like protein 6-interacting protein 1	−5.86
19	novel_miR_93	GUGGGGGGGCGGCGACGU	18	octopamine receptor-like GATA transcription factor Regulator of G-protein signaling 3	−5.44
20	novel_miR_92	UGGGGAUGUGCUGGAGUU	18	leucine-rich repeat-containing protein 4	−5.30

mTOR signaling pathway, ECM-receptor interaction, Ubiquitin mediated proteolysis, Endocytosis, Regulation of autophagy, FoxO signaling pathway, Notch signaling pathway.

3.5. qPCR of differentially expressed microRNAs

To confirm the involvement of differential expression of miRNAs, eight different miRNAs including up- and down-regulated miRNAs were selected and a poly(T) RT-PCR was conducted. From the results, these miRNAs showed a similar fold changes to the high throughput sequencing results (shown in Fig. 6), indicating the right sequence analysis.

4. Discussion

WSSV is a disease with extremely high mortality and brings huge economic losses to the shrimp farming industry [25]. It is of great significance to study the immune mechanism of host response to WSSV invasion for the prevention of WSSV. Till present, a large number of literature have studied the transcriptome and miRNAs expression profiles of *Macrobrachium rosenbergii* [28,29,37], *Fenneropenaeus chinensis* [38], *Litopenaeus vannamei* [39], and *P. clarkia* [40] after WSSV infection, but no studies have reported the type and role of miRNAs in shrimp hemolymph exosome. Studies have shown that exosomes play an important role in cellular communication regulation [5]. The inclusions of exosomes by immune cells, including proteins and miRNAs, can regulate other cells to produce appropriate immune response strategies [3,4]. In *Drosophila*, this exosome-mediated cellular

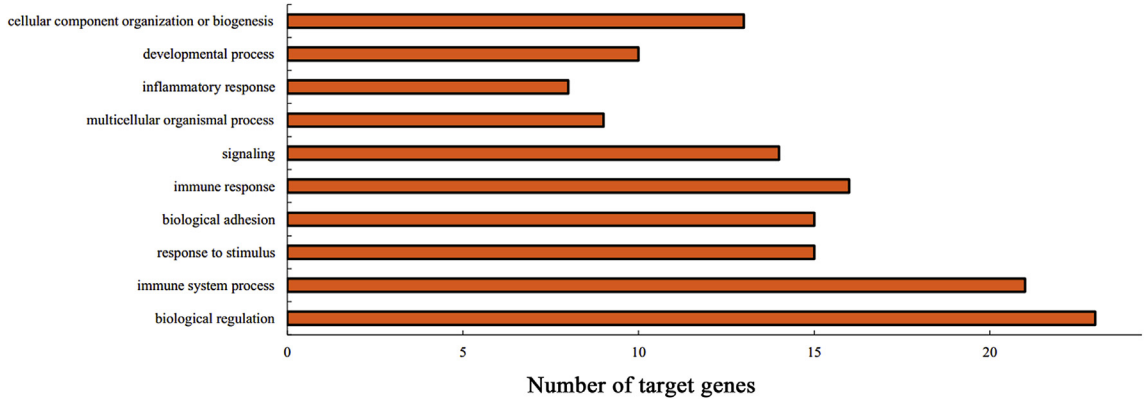

Fig. 4. Top ten GO terms of biological process of target genes. The predicted target genes of differentially expressed miRNAs in *P. clarkia* were blasted, annotated and the GO analysis was done using Blast2Go software.

communication can produce certain adaptive RNAi-mediated antiviral immune signatures for viral invasion [10]. Exosomes contain some specific miRNAs, which play an important role in regulating gene expression. Hemolymph is an important immune defense organization for shrimps and plays a key role in innate immune defense [41]. Therefore, it is important to study the main immune cells of shrimp in the process of coping with WSSV infection, and the expression of miRNAs in secreted exosomes is important and helpful to understand the signal transmission of shrimp immune cells in antiviral process.

In this study, we extracted exosomes from the hemolymph of *P. clarkia*. The extracted exosomes were detected by TEM, NTA and Western blot. Similar to other previous studies, the exosomes we extracted have typical vesicular structural features under TEM analysis [42,43]. CD63, HSP70, HSP90 and calnexin are protein markers of exosomes that can be used to detect and identify exosomes [42,44]. In accordance with other previous studies, the results of WB detection confirmed the good quality of exosome extraction. Exosomes could be secreted within many cell types [1]. Compared with the other exosome results secreted by different cells, it can be seen that the particle size is similar among different species [43,45]. These detections indicate that exosomes also exist in the hemolymph of *P. clarkia*, and the extracted exosomes could be used in the following experiment.

Many studies have confirmed that miRNAs could play a significant role in the specific biochemical reactions, such as regulation of the immune system [19]. From the results of high-throughtput sequencing in hemolymph exosomes, 209 conserved miRNAs and 250 novel predicted miRNAs were identified. The amount of miRNAs is almost consistent with the microRNAs of *L. vannamei* in response to WSSV infection [29]. The 22 nts in length small RNA were enriched for the whole small RNAs, this is similar with other miRNAs library of *Blattella germanica* [46]. But in some other studies, the 21 nts small RNAs were the most abundant [47]. This is due to the lack of genomic data from some species and the alignment method of miRBase 21.0. 170 different-expression miRNAs were altered by WSSV infection, with 98 microRNAs up-regulated and 72 microRNAs down-regulated, suggesting they are involved in the different virulence viral replication. Among these, novel_miR_151 was the most significant up-regulated miRNA, but its target gene was not found. This miRNA may be a biomarker for the WSSV infection. The expression fold changes of chi-miR-7-5p was up-regulated to +3.62, and its target gene is Down syndrome cell adhesion molecule-like protein (Dscam). Dscam is highly expressed in crayfish hemocytes and located in these immune-related cells membrane [48]. Dscam may act as a phagocytic receptor and play important role in the phagocytosis [49]. The up-regulation of chi-miR-7-5p indicated that phagocytosis was involved in the immune response to WSSV infection. dan-miR-7 and dme-miR-7-5p that their target genes were Transmembrane 4 and heparan sulfate 2-*O*-sulfotransferase, were up-regulated respectively. Transmembrane 4 and heparan sulfate 2-*O*-sulfotransferase (HS chain) were located on the cell membrane and related to the pathogens infections. Previous studies have demonstrated that dsRNA was used to interfere the gene expression of transmembrane and lead to a decrease of the WSSV copy number [50]. The up-regulated

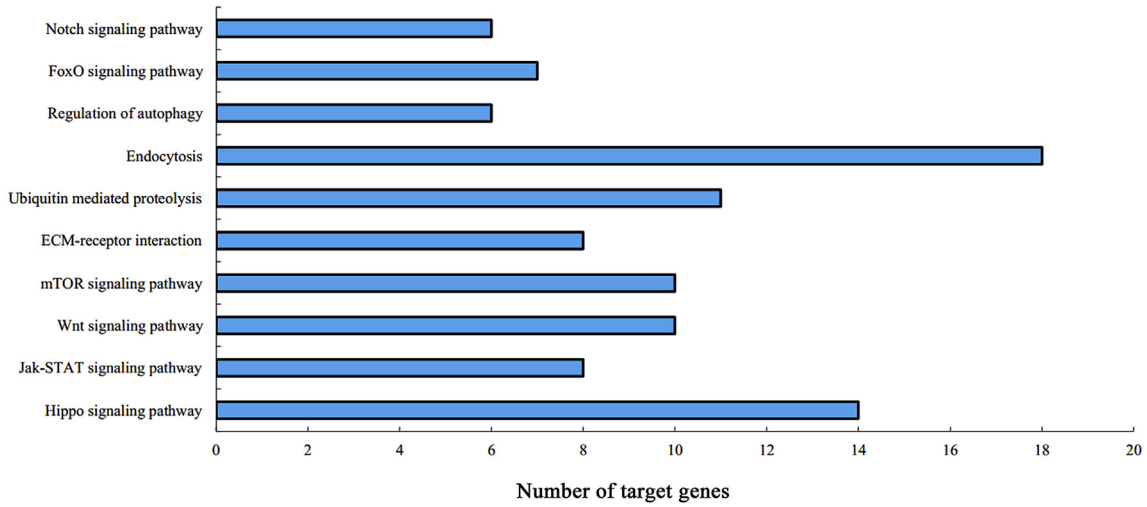

Fig. 5. Top ten abundant KEGG pathways of the target genes of differentially expressed miRNAs in *P. clarkia.* The KEGG pathways analysis was done using the KEGG Automatic Annotation Server and their abundance was analyzed.

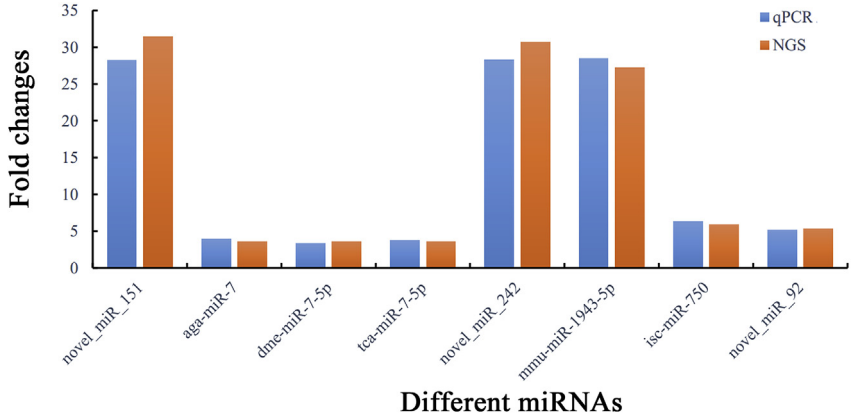

Fig. 6. Expression of miRNAs during WSSV infection. miRNAs expression level was measured using qPCR after synthesis of cDNA. The relative expression of miRNA was calculated using the $2^{-\triangle\triangle Ct}$ method and normalized against small nuclear RNA U6.

dan-miR-7 may be a strategy of host to defense WSSV infection. The heparin sulfate proteoglycan is an important attachment factor for pathogeny entry into cells [51]. Though the direct interaction between HS chain and WSSV has not been detected, the block of syndecan could depress WSSV replications in *L. vannamei* [52]. Some other target genes, like hsp70, cathepsin, and Apoptosis regulator BAX, which act as the effector molecules, could directly participate in the immune defence or regulate the immune response [53–55]. The up-regulated miRNAs indicated the target genes may paly vital role during WSSV infection.

Despite the up-regulated miRNAs, the significant down-regulated miRNAs may also participate in regulation of immune response. In this study, some miRNAs were down-regulated of the fold changes about 30, such as novel_miR_242, novel_miR_180, tur-miR-745-3p, mmu-miR-1943-5p and novel_miR_66. These miRNAs could be indicative of WSSV infection, as the plasma exosome miRNAs of breast cancer [43]. The novel_miR_19 that its target gene was C-type lectin, was significantly down-regulated. C-type lectin was reported to possess anti-WSSV activity [56]. Down-regulated novel_miR_19 could increase the expression of C-type lectin. This could be the host immune response to WSSV infection, indicating the important of C-type lectin during defence of WSSV. In *Marsupenaeus japonicas*, the ADP ribosylation factors4 (Arfs4) might be involved in WSSV infection for its mRNA level of MjArf4 was up-regulated significantly as WSSV propagated [57]. isc-miR-750 predicted to target ADP-ribosylation factor-like protein was down-regulated in response to WSSV, indicating its regulation of the immune response gene. In accordance with previous study in *L. vannamei* [29], one miRNA named cte-miR-750 was down-regulated in hepatopancreas to WSSV infection, that was also found the similar expression tendency in the present study. Though its target gene Eka-PIP kinase protein was not studied during WSSV infection, it could have some certain important functions. Some regulated target genes such as Rab GTPase, E3 ubiquitin-protein, peritrophin, chitinase [58], were also reported to play multifunction during the immune response to viral infections. In *Drosophila*, Jak-STAT signaling pathway was required but not sufficient for the antiviral response [59]. In hemolymph exosomes miRNAs, the predicted target genes are mostly involved in Jak-STAT signaling pathway, indicating its critical functions in defensing WSSV.

In conclusion, the present research is the first study to focus on the crustacean hemolymph exosomes miRNA expressions changes in response to WSSV infections. These data suggested the hemolymph exosomes in crustacean also shared the similar characteristic with other species exosomes from plasma, urine, milk and so on. The significantly changed miRNAs could also be an indicative of WSSV proliferation in host. The changed miRNAs indicated the importance of their target genes are involved in host immune response or signaling pathways in response to WSSV infection. And the specific functions of these target genes are needed for further research.

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Appendix A. Supplementary data

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