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Short communication

Differential expression of microRNAs in shrimp *Marsupenaeus japonicus* in response to *Vibrio alginolyticus* infection

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ABSTRACT

Till date numerous microRNAs (miRNAs) have been discovered from various organisms, including mammals, plants, insects, nematodes and viruses. They are known to have antiviral functions in crustaceans such as shrimp *Marsupenaeus japonicas.* However, little is known about the role of miRNAs against bacterial infection in this shrimp caused by *Vibrio alginolyticus*. We performed small RNA sequencing to characterize the differentially expressed microRNAs in *V. alginolyticus* challenged shrimp, in comparison to that in control uninfected shrimp, at 24 h and 48 h. In total, 55 host miRNAs were differentially expressed in response to the infection and most of these were downregulated at both the time-points. TargetScan and miRanda algorithms showed that the target genes of these down-regulated miRNAs were related to innate immune functions such as production of phenoloxidase enzyme, apoptosis and phagocytosis. Further, gene ontology analysis revealed that many immune signaling pathways were mediated by these miRNAs. This study is one of the earliest attempts at characterizing shrimp miRNAs that respond to *V. alginolyticus* infection, and will help unravel the miRNA pathways involved in antibacterial action in shrimp.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level (Bartel, 2004) and play an important role in development, homeostasis and immunological defense function (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 (pri-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 virus. Therefore, efforts are targeted towards understanding the role of shrimp miRNAs which regulate target genes during host pathogen interactions. 35 miRNAs were firstly 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). There are reports of virus infection altering the expression profile of shrimp miRNAs. In *M. japonicas,* 63 shrimp miRNAs were identified in response to white spot syndrome virus (WSSV) infection, 48 of which were conserved in other animals, representing 43 distinct families (Huang et al., 2012). Among these miRNAs, 31 were differentially expressed in response to viral infection and the analysis showed that most target genes of these miRNAs were related to immune responses. Shrimp miR-7 could target the 3'-untranslated region (3′UTR) of the WSSV early gene wsv477, and cause a large effect on virus infection (Huang and Zhang, 2012).

Although shrimp miRNAs were discovered and studied recently, but few studies were performed in shrimp that response to

Abbreviations: miRNA, microRNAs; pri-miRNA, primary-miRNA; 3′-UTR, 3′-untranslated regions; WSSV, white spot syndrome virus; GO, Gene Ontology; RIN, RNA integrity number; DIG, digoxigenin; PCR, polymerase chain reaction.

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bacterial infection. The bacterium *Vibrio alginolyticus*, which usually causes infection, occurs when host immunity is compromised. It is considered a secondary and opportunistic pathogen which causes high mortality of shrimps in stressful environments, when grown in the aquacultures (Liu et al., 2004; Wang and Chen, 2005). The investigation of miRNAs that responds to *Vibrio* infection has not yet been performed. Hence, the objective of this study was to identify and characterize the differentially expressed miRNAs in shrimp *M. japonicas* in response to *V. alginolyticus* infection.

Our study revealed that 55 miRNAs which were differentially expressed lead to various innate immune responses. Our results extend the knowledge of crustacean miRNA regulation, providing clues for further research on shrimp immunity against *Vibrio* infection.

2. Materials and methods

2.1. Shrimps and tissue preparation

Total of healthy adult *M. japonicus* were obtained from Jinjiang seafood market of Hangzhou. They were divided into two groups viz *Vibrio* infected and *Vibrio* uninfected (free) shrimps. The cultured *V. alginolyticus* ATCC17749 was used to challenge the shrimps according to the previous report (Huang et al., 2015).

2.2. Sequencing of small RNAs

The total RNAs were extracted from the hemocytes of the *Vibrio*-infected and uninfected shrimps at 24 h and 48 h post infection by using a miRNA isolation kit (Ambion, USA) in accordance with the manufacturer's protocol. The quantity and purity of total RNAs were quantified by using a NanoDrop ND-1000 spectrophotometer (NanoDrop, 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, USA) with an RNA integrity number (RIN) > 8.0. Subsequently 200 μg RNA sample was separated onto a denaturing 15% polyacrylamide gel. The small RNAs ranging from 16 to 30 nt were excised and dephosphorylated by alkaline phosphatase. After recovery by ethanol precipitation, the small RNAs were ligated sequentially to RNA adapters (5′- ACAGGUUCAGAGUUCUACAGUCCGACGAUC-3′ and 5′-UCGUAUGCCGUCUUCUGCUUG-3′). The reverse transcription and polymerase chain reaction (PCR) amplification were preformed after the ligation. The last products were sequenced on the Genome Analyzer GA-II (Illumina, San Diego, USA) in accordance with the manufacturer's protocol.

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).

2.4. Northern blotting

The total RNA was isolated from the hemocytes of the *Vibrio*-infected and uninfected shrimps at 24 h post infection and then total RNA was quantified by using NanoDrop Spectrophotometer (USA). The next methods are in accordance with the previous report (Huang et al., 2012).

2.5. Prediction of genes targeted by miRNAs

Two computational target prediction algorithms, TargetScan 5.1 (http://www.targetscan.org) and miRanda (http://www.microrna.org), were used to predict the genes targeted by miRNAs. The data-sets used were the assembled EST sequences and the 3′ UTRs of WSSV. TargetScan was used to search for miRNA seed matches (nucleotides 2–8 from the 5′ end of miRNA) in the 3′ UTR sequences. miRanda was used to match the entire miRNA sequences. The miRanda parameters were set as free energy < −20 kcal/mol and score > 50. Finally, the results predicted by the two algorithms were combined and the overlaps were calculated.

2.6. Gene ontology (GO) analysis

The coding sequences of the shrimp 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 shrimp EST sequences. The *P* values were corrected by false discovery rate (FDR).

3. Results and discussion

3.1. Sequence analysis of miRNAs

The recent reports have indicated that shrimp *M. japonicas* employs miRNAs to control the viral infections by WSSV. However, the role of miRNA in response to the bacterial infections has not been reported. In the present investigation, we infected shrimp *M. japonicas* with bacteria *V. alginolyticus* and compared the expression profiles of miRNAs in *Vibrio*-free and *Vibrio*-infected shrimps, and characterized the differentially expressed miRNAs.

Based on the small RNA sequencing, the small RNA sequences of shrimp infected with *V. alginolyticus* were analyzed. The Vibrio-free shrimp were considered as controls for the analysis. The small RNA sequencing generated a total of 7–10 million raw reads. Above 80% raw reads were present at least twice and their lengths were ranged from 16 to 25 nucleotides. After removal of mRNA, rRNA, tRNA, snRNA and snoRNA, the high throughput sequencing generated a total of 2 million sequences. The data analyses showed a low proportion of long RNAs, such as mRNA (2% by kind and 0.4% by count) and rRNA (3% by kind and 0.4% 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 50% miRNAs in control group were of length 19 bp and 35–40% miRNAs in *V. alginolyticus* group were of length 23 bp (Fig. 1)B. Our study provides the first large-scale characterization of shrimp miRNAs in response to *Vibrio* infection. The results show that 50% miRNAs in control group were of length 19 bp while 35–40% miRNAs in *V. alginolyticus* infected group were of length 23 bp. We used small RNA sequencing and identified 55 host miRNAs were differentially expressed in response to *V. alginolyticus* infection. Among these, 15 miRNAs were conserved in other animals and 40 novel miRNAs were identified in *M. japonicas*. Further expression analysis 24 h post infection revealed that 10 miRNAs were up-regulated and 35 miRNAs were down-regulated. While, 16 miRNAs were up-regulated and 30 miRNAs were down-regulated 48 h post infection. Overall the most differentially expressed miRNAs were down-regulated after the infection.

3.2. Host miRNAs involved in V. alginolyticus infection

The expression profiles of miRNAs of control and *Vibrio*-infected shrimp at various times post infection were compared identify and characterize the miRNAs involved during *Vibrio* infection. A *P*-value < 0.01 indicated that differences expression profiles in the miRNA counts were statistically significant. The results showed that