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In-depth profiling of miRNA regulation in the body wall of sea cucumber *Apostichopus japonicus* during skin ulceration syndrome progression

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that mediate mRNA degradation or translation repression. Previous study showed that the expression of miRNAs was significantly changed in the body wall of sea cucumber *Apostichopus japonicus* after skin ulceration syndrome (SUS) infection, which is a dynamic process. However, the critical miRNAs from body wall that involved in different infection stages of SUS remain unknown. In this study, four cDNA libraries were constructed with the body wall from healthy and three SUS-infected stages of *A. japonicus*. A total of 248 conserved miRNAs and five novel miRNAs were identified through Illumina HiSeq 2000 platform. Compared to the control, 238 miRNAs showed significant differential expression at three stages of SUS progression. Totally, 3149 miRNA-mRNA pairs were identified by target prediction and 314 miRNA-mRNA pairs showed negative correlation. It is noteworthy that 15 miRNAs and four mRNAs were located at the crucial positions of the network built with the anti-correlated miRNA-mRNA pairs. GO and KEGG enrichment analysis indicated that the predicted targets were involved in many immune-related processes. Deep analysis of miR-31c-5p, miR-29b-3p, NF-kB, mucin 2 and titin showed that they may play important roles in the pathogens attachment and recognition, signaling transduction and lesions repair of *A. japonicus* after SUS infection. These results would be useful for further investigating the potential roles of critical miRNAs and mRNAs in *A. japonicus* immune regulation.

1. Introduction

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into protein products. MicroRNAs (miRNAs) are endogenous small ncRNAs with ~22 nucleotides (nt) in length. They regulate gene expression mainly through binding to the complementary sites on 3′ untranslated region (UTR) of target messenger RNAs (mRNAs), leading to mRNA degradation or translation repression [1]. The binding sites at 2–8 bases from 5′ end of miRNA were highly conserved, which were defined as “miRNA seeds” [2]. Based on the complementary base pairing, miRNAs present vast regulatory potential: a given miRNA has different mRNA targets. Accumulating evidences have shown that miRNAs play important roles in development and progression of human diseases [3,4]. Differential expression of miRNAs is often associated with disease conditions and can be served as diagnostic and prognostic markers [5–7]. Therefore, it is important to explore the dynamic regulatory roles of miRNAs in disease progress.

Apostichopus japonicus is an important economic aquaculture species in China for its nutritive value and medicinal properties. However, epidemic diseases have severely limited the healthy development of *A. japonicus* aquaculture. Skin ulceration syndrome (SUS) was recognized as the most serious disease resulting in high mortality [8]. Pathogens including bacteria, parasite and virus were isolated from the SUS-infected *A. japonicus* [9–12]. However, *V. splendidus* infection has been widely considered as one of the important reasons for SUS outbreak [13,14]. Among the varied classes of immune regulators, miRNAs have effects on the disease phenotypes under pathologic stress [4]. Several studies had reported that miRNAs from different tissues were involved in the immune response in *A. japonicus* after *V. splendidus* infection [15–20]. Among these tissues, body wall as the first line of defense can directly perceive the environmental perturbations and protect sea cucumber from pathogens invasion. Our previous studies showed that the most number of differentially expressed miRNAs were identified from body wall of *A. japonicus* after SUS infection [20]. These miRNAs present spatial and temporal specific expression patterns during the SUS infection, which is a dynamic progress with the expanding of ulcerative and the loss of adhesive ability. In order to explain the regulation mechanism of miRNAs during SUS infection in *A. japonicus*, SUS was

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artificially divided into three stages (stage I, stage II and stage III) [21]. However, no investigations were conducted on the miRNAs regulation during SUS infection progression in *A. japonicus*.

In this study, Illumina HiSeq 2000 platform was employed to identify miRNAs of body wall from healthy and three SUS-infected stages of *A. japonicus*. We constructed four cDNA libraries (HB, SFI, SFII and SFIII) and examined the dynamic expression patterns of miRNAs in the stepwise development of SUS. In addition, target gene prediction, GO and KEGG enrichment analyses were conducted to further elucidate the main functions of the differentially expressed miRNAs. The regulatory network of miRNA-mRNA will provide an overview of the systematic regulation relationship and help us screen the critical molecules functioning during the progression of SUS in sea cucumber.

2. Materials and methods

2.1. Samples collection

The healthy and SUS-infected samples were the same as those had been used for mRNA analysis in our previous study [21]. Healthy *A. japonicus* (10–12 g) collected from Zhuanghe of Liaoning Province, China were acclimated in the laboratory for one week before artificial infection. The seawater used in the experiment was filtered through sand, and then through 300-μm nylon sieves. Twenty-five percent of the seawater in the tank was exchanged daily. The animals were maintained in the seawater at 12 °C, pH 8.1, with salinity of 32 and continuous aeration.

The pathogen *V. splendidus* added to the tank was isolated from SUS-infected *A. japonicus* according the method reported by Deng et al. [22]. The final concentration of the bacteria was maintained at 5×10^9 CFU mL^{-1}. Before samples collection, white skin ulceration was considered to be the most important mark to distinguish diseased and healthy individuals [15,23]. The number and diameter of white skin ulcerations were different at three stages of SUS infection (stage I, stage II and stage III) [21]. In stage I, the animals showed one small white speck of skin ulceration (diameter < 0.2 cm). The animals retained the ability to attach to the surface of the tank and did not eviscerate. In stage II, the animals exhibited 2 to 3 larger white specks (diameter > 0.2 cm). The animals continued to exhibit the ability to attach to the surface of the tank and did not eviscerate. In stage III, the individuals showed several deep and extensive ulcerations, lost the ability to adhere to the tank and eviscerated. The animals cultured under normal conditions without the treatments of cut and bacterial challenge were served as healthy controls. Thirty individuals were selected from each stage of SUS-infected and healthy groups, respectively. All samples were frozen immediately in liquid nitrogen and then stored at −80 °C before RNA isolation.

2.2. Construction and sequencing of small RNA cDNA libraries

The construction of cDNA libraries was performed based on the published paper [20]. Briefly, the total RNA of body wall was extracted using Trizol® reagent (Invitrogen, USA). The integrity and quality of RNA employed for small RNA libraries construction were examined by NanoPhotometer (Implen GmbH, Munich, Germany) and agarose gel electrophoresis. A total of four small RNA cDNA libraries were constructed using TruSeq Small RNA Sample Prep Kit (Illumina, USA) based on the manufacturer's instructions. The cDNA libraries were purified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Finally, ABI StepOnePlus Real-Time PCR System (Life Technologies, USA) was used directly for cluster generation and Illumina Hiseq 2000 platform was used in sequencing analysis.

2.3. Data processing

The clean reads with size larger than 18 nt were used for annotation analysis. Blasting against GenBank and Rfam 11.0 database, the matched and annotated small RNAs including rRNA, tRNA, snRNA, snoRNA and other non-coding RNAs were discarded. The remaining reads were mapped to miRBase 21.0 (http://www.mirbase.org/) for conserved and novel miRNA identification. The perfect matches (allowing for one mismatch) were considered as conserved miRNAs. The reads that did not matched to the miRBase database were aligned with sea urchin genomic sequence (http://www.spbase.org/SpBase/download/) and *A. japonicus* transcriptomes. The matched sequences that could form stable secondary structures predicted by miRDeep2.0 were identified as novel miRNAs [24].

2.4. Differential expression analysis of miRNAs

To obtain the differentially expressed miRNAs, the following method was conducted. At first, the expressions of miRNAs in healthy and SUS-infected groups were normalized by the transcripts per million reads (TPM) (Actual miRNA count/Total count of clean reads *1000000). The miRNAs with TPM ≥1 were considered as reliable expressed ones [25]. When the normalized expression was 0, it would be changed to 0.0005 automatically. Next, fold change and *P*-value were calculated based on the normalized expression. Fold changes between two samples were shown as ratio of TPMs. Poisson statistics was used to get *P*-value [26]. The FDR (False Discovery Rate) was also estimated to correct *P*-value. We set "FDR < 0.01 and the absolute value of $\log_2$Ratio ≥1.0" as the threshold to judge the significance of miRNA expression difference. Based on the K-means method using the Euclidean distance algorithm, the dynamic expression patterns of differentially expressed miRNAs during the three stages of SUS progression were displayed by clustering analysis.

2.5. Prediction and function analysis of target genes

To better understand the functions of miRNAs, putative target genes for each significantly differently expressed miRNAs were predicted using TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/). The overlaps predicted by the two algorithms were retained. The regulatory network between miRNA and its targets was constructed by the software Cytoscape. Enrichment analysis of the predicted target genes was conducted with Gene Ontology (GO) (http://www.geneontology.org/) and KEGG pathway (http://www.genome.jp/kegg/).

2.6. Quantitative real-time PCR

Five nuclear miRNAs were selected for qRT-PCR to confirm the accuracy of high-throughput sequencing. To quantify specific miRNAs by real-time PCR, the total RNAs of body wall from healthy and SUS-infected groups were converted into cDNA by Mir-X miRNA Fist-Strand Synthesis Kit (TaKaRa, Dalian, China). The mix was incubated at 37 °C for 60 min and then at 85 °C for 5 min. Forward primers are highly specific because they were complete mature miRNA sequences (Table 1). The reverse primer was the common primer mRQ 3′ primer contained in the Kit. The U6 RNA gene in the Kit was used for normalization of the expression levels. According to Mir-X miRNA qRT-PCR SYBR Kit (TaKaRa, Dalian, China), 20 μL reaction volume contained 10 μL SYBR Advantage Premix (2×), 0.4 μL ROX Dye (50×), 0.4 μL miRNA specific primer, 0.4 μL mRQ 3′ primer, 1.6 μL cDNA and 7.2 μL ddH_2O. Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System (ABI, Carlsbad, CA, USA) under the following thermal cycling conditions: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 55 °C for 40 s and 72 °C for 25 s. Each reaction was performed in triplicate. The statistical analyses of qRT-PCR were based on five miRNAs expression levels normalized by U6. The threshold cycle (Ct) values generated by qRT-PCR were compared and converted to fold differences by relative quantification method using the Relative