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Identification and characterization of miR-31 potential targets by RNA-seq

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ABSTRACT

In our previous work, miR-31 displayed differential significant expression in *Apostichopus japonicus* sea cucumber with skin ulcer syndrome and modulated coelomocytes ROS production by targeting p105. To identify other promising targets ofmiR-31, 4 transcriptome libraries of coelomocytes, as well as 2 control libraries, were constructed frommiR-31 mimics (31 M) or AMO-miR-31 (31I) and injected into a sea cucumber at 12 and 24 h. A total of207,977 unigenes with an average length of 363 bp were assembled, in which17,204 distinct sequences (8.27% of the unigenes) were successfully matched with annotated protein sequences. Fragments per kilobase of transcript per million fragments mapped analysis indicated that 1325 unigenes displayed up-regulated expression profiles in the 31I-12 group and were depressed in the 31M – 12 group compared with the control group. A total of 1470 unigenes showed down-regulated expressions in 31I-12 and were induced in 31 M-12. Similarly, 2079 and 2098 unigenes were detected at 24 h post-injection. Among these unigenes, 36 unigenes (depressed expression in the 31 M group and induced in the 31I group) showed consistent expression patterns at 2 examined time points and were considered promising targets of miR-31. qPCR analysis confirmed that all 4 unigenes showed opposite expression profiles to miR-31 in cultured coelomocytes. Our present work provided a fast and feasible method of identifying miR-31 targets by transcriptome analysis. The results of this study would enhance our present understanding ofmiR-31 function insea cucumber immune regulation.

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1. Introduction

Apostichopus japonicus sea cucumber is one of the most important aquaculture animals in China, with a production value of more than40 billion RMB in 2014 (China Fishery Statistical Yearbook, 2015). However, various diseases occur in cultured *A. japonicus* populations, thus causing catastrophic losses to species aquaculture. Skin ulceration syndrome (SUS) is considered to be the most severe disease of *A. japonicus* because of its highly infectious nature and lethality [1]. To establish an efficient disease control strategy, efforts have been conducted such as pathogen identification and intervention [2–4], improvement of culture conditions [5], and development of disease-resistant species. The latter approach has proven to be a new, promising way for disease control. Many immune-related molecules, including Toll-like receptor [6] and its cascades [7,8], lectin [9], and LBP/BPI [10], have been identified and characterized in this important economic animal. Expression analysis further validated that these molecules can be induced towards pathogen infection or PAMP exposure. However, the intrinsic mechanism of these molecules is largely unknown in this non-model organism.

microRNA (miRNA), which is a small non-coding RNA, is considered a critical regulator of gene expression in many pathogenic or physiological processes at transcriptional or translational levels [11]. We have successfully identified 8 differential-expressed miRNAs from SUS-diseased sea cucumber by deep sequencing; miR-31 and miR-2008 displayed the highest expression change [12]. In humans, Valastyan and Weinberg [13] identified miR-31 as a crucial overseer of several normal and diseased phenotypes. Various targets of miR-31 were also identified and characterized in

Abbreviation: list: miR-31, microRNA 31; LPS, Lipopolysaccharide; SUS, Skin ulceration syndrome; PAMP, Pathogen associated molecular pattern; AMO-miR-31, Antisense microRNA-31 oligonucleotides; NCM, Negative control of miR-31 mimics; 31 M, miR-31 mimics; NCI, Negative control for AMO-miR-31; 31M-12, miR-31 mimics injection for 12 h; 31I-12, AMO-miR-31 injection for 12 h.

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different diseases, including hypoxia inducible factor I [14] and E-selectin [15], thus further supporting the regulatory roles of miR-31 in the host immune response and host–pathogen interactions. In our previous work, we confirmed that miR-31 could modulate respiratory burst by targeting Ajp105 during sea cucumber pathological development [16]. Given that a miRNA might have multiple different mRNA targets [17], identifying the other targets and potential regulatory molecules of miR-31 were the most important means to investigate its functional ways. In the present study, six transcriptomes were constructed and sequenced after miR-31 mimics (31 M) or AMO-miR-31(31I) injection. Different unigenes with the opposite expression profiles between mimics and inhibitor groups were further enriched. Some candidates were further evaluated by qPCR. We hope to provide a fast and feasible method to identify miR-31 targets by transcriptome analysis.

2. Material and method

2.1. Sea cucumber

Healthy adult sea cucumbers (*A. japonicus*, 95–110 g) were obtained from Dalian Pacific Aquaculture Company in May 2015 and were acclimatized in Dalian Ocean University. All experiments were performed in the Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture.

2.2. miR-31 mimics or AMO-miR-31 injection in vivo

The modified31Mor 31Ifor *in vivo* assay, as well as the negative control (NC), were synthesized in GenePharma (Shanghai, China). The sequence information can be found in our previous work [16]. These miRNAs were then dissolved into RNase-free water to obtain a working solution of 20 μM. We mixed 10 μL of each modified mimics or inhibitorc with 10 μL of transfection reagent and 80 μL of PBS to serve as the working solution. A total of 15 sea cucumbers were injected with 100 μL of each mixture or the NC, respectively. After 12 and 24 h, the treated and control coelomocytes were collected for expression and RNA-seq analysis. These sampling time points were determined on our previous works [16] [18] [19]. To minimize the individual's deviation, the coelomocytes from five individuals were mixed together and considered one experimental sample. Three independent samples were employed for miR-31 expression levels assay.

2.3. Assay miRNA-31overexpression or inhibitory efficiency

The total RNA from each group was extracted using RNAiso plus reagent (Takara, Japan) according to the manufacturer's instructions. The SYBR green qRT-PCR assay was used for miRNA quantification analysis. Approximately 500 ng of RNA-containing miRNAs was polyadenylated using poly(A) polymerase and converted to cDNA by reverse transcription via the miScript Reverse Transcription Kit (Qiagen, Germany). qRT-PCR was performed using the miScript SYBR Green PCR Kit (Qiagen, Germany) and the provided miScript Universal primer and miRNA-specific forward primers [12] on a Rotor-Gene Q 6000 Real-time PCR cycle. RNU6 B was served as internal control. Each reaction was performed in a final volume of 20 μL containing 2 μL cDNA, 1 μL of each 10 μM primer, 6 μL RNase-free water, and 10 μL SYBR Green PCR Master mix (Qiagen, Germany). The amplification profile included initial denaturation at 94 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 70 °C for 30 s, in which fluorescence was acquired. Immediately following amplification, a melting curve analysis was performed. Each sample was run in triplicate.

2.4. Normalized cDNA library construction and sequencing

Three treated samples at 12 or 24 h, as well as the control, were mixed together and employed for RNA-seq analysis. Experimental protocols for the cDNA normalization sequence were performed according to the manufacturer's technical instructions. The total RNA was isolated from coelomocytes with TRIZOL reagent (Invitrogen, Grand Island, NY), and the RNA was purified by the oligo (dT) magnetic beads. The purified RNA was quantified on an Agilent 2100 RNA Nano 6000 Assay. Equal amounts of high-quality mRNA samples from each group were first fragmented into small pieces with an additional fragmentation buffer before cDNA synthesis (Invitrogen, CA, USA). The double stranded cDNA was subsequently purified for dA tailing, adaptor ligation, and sequential DNA fragment enrichment. Finally, the DNA was enriched by PCR with 15 cycles of amplification according to the TruSeq protocol. The quantities of libraries were investigated by Qubit2.0 and then diluted to the final concentration of 1 ng/μL. RNA-Seq was performed in Illumina HiSeq 2500 with 100 base pair length reads.

2.5. Data processing and assembly

After the initial image was taken, the data were transformed into sequence data. These raw reads were then cleaned by removing the adaptor sequences and any ambiguous or low-quality reads. *De novo* transcriptome assembly was conducted with the short-read assembly program Trinity [17]. First, short reads were assembled into high-coverage contigs that could not be extended farther in either direction in a k-mer-based approach for fast and efficient transcript assembly. Thereafter, the related contigs were clustered, and a de Bruijn graph for each cluster was constructed. Finally, in the context of the corresponding de Bruijn graph and all plausible transcripts, alternatively spliced isoforms and transcripts were derived.

The BLASTX analysis of unigenes longer than 200 bp was conducted against the Uniprot database (www.uniprot.org), the non-redundant database (www.ncbi.nlm.nih.gov/protein), theclusters of orthologous groups (COG) database (www.ncbi.nlm.nih.gov/COG/), and the KEGG database (http://www.genome.jp/kegg/). The E-valuewas 0.001. The best BLASThit from all BLAST results was parsed for a homology-based functional annotation. Gene ontology (GO) annotation was performed on the basis of annotation information from Uniprot by using Blast2GO (http://www.balst2go.org/). The classification of GO functions was conducted using R software.

2.6. Differentially expressed genes in miR-31 mimics or AMO-miR-31groups

Gene expression levels were calculated using the fragments per kilobase of transcript per million fragments mapped method. The calculation of unigene expression levels and the identification of unigenes that were differentially expressed between the libraries were performed by Cufflinks [19] on the basis of different normalization patterns. The normalization pattern of the unigene expression level calculation was a total-hits-norm as well as a normalization pattern of differentially expressed unigenes, whereas the calculation followed a compatible-hits-norm [20]. For the significance analysis of differentially expressed unigenes, the false discovery rate method was used to determine the q-value threshold in multiple tests [21]. The significance of differences in gene expression was screened using the threshold of $q < 0.05$. Thereafter, the differentially expressed genes across the samples were further annotated by GO and KEGG pathway analysis by a hypergeometric test.

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