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Understanding microRNAs regulation in heat shock response in the sea cucumber *Apostichopus japonicus*

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ABSTRACT

The sea cucumber *Apostichopus japonicus* is a valuable species in China. The extreme high temperature in the summer often results in high mortality. MicroRNAs (miRNAs) play important post-transcriptional regulatory roles in gene expression and can influence heat shock response (HSR) greatly. In this study, we determined the expression profiles of miRNAs under heat stress (HS) in *A. japonicus* by using high-throughput sequencing technique. Among the differential expression miRNAs, we highlighted 41 differentially expressed miRNAs, many of which were involved in immunity process and disease regulation. Gene ontology and pathway analyses of putative target genes were also carried out. Cell-substrate adherens junction and cell-substrate junction were significantly enriched in GO analysis. Moreover, we made a correlation analysis between remarkable miRNAs and the differentially expressed genes (DEGs) in sea cucumbers under HS. We identified 17 key miRNA-target pairs potentially regulated HSR of sea cucumbers. These results will provide new insights about miRNAs regulation and molecular adaptive mechanisms in sea cucumbers under HS.

1. Introduction

The sea cucumber is popular food in China and other Asia countries because of its famous effects in nourishing and healing [6]. Totally 134 species have been recognized in China, which belong to 57 genera, 15 classes, and 6 orders [12]. *Apostichopus japonicus*, a temperate species mainly distributed in northern China, is one of the most valuable sea cucumbers. Owing to advances in breeding and culture methods, aquaculture of *A. japonicus* is flourishing and expanding industry. According to the statistics data of the Ministry of Agriculture, the yield of *A. japonicus* in China has reached to 204,359 tons in 2016 [18]. However, extreme high temperature in the summer, accompanied with water hypoxia and infectious diseases, results in high mortality of sea cucumbers in the northern China [9] [39]. In recent year, this phenomenon has occurred more frequently, which is becoming a barrier for sustainable development of *A. japonicus* industry. Better understanding the heat shock response (HSR) of *A. japonicus* is useful to determine the relationship between heat stress (HS) and adaption mechanism in this species, but also contributes to breeding new high-temperature tolerant strains.

MicroRNAs (miRNAs) are a class of short (~22 nucleotides [nt]) noncoding RNAs, which could regulate post-transcriptional gene expression through binding to their 3′ untranslated regions (UTRs) of target genes [25]. So far, miRNAs have been proved to play key roles during various processes, such as cell differentiation [25], apoptosis [24], development [27], as well as various stress response [3]. The functions of miRNAs under stress conditions are primarily concentrated on two aspects. Firstly, miRNAs can act as a rapid and reversible mechanism to halt the translation of unnecessary proteins. Many studies reported that miRNAs could provide a mechanism for reversible gene silencing during hibernation and torpor [11] [19]. Secondly, miRNAs can participate cell adaptation for specific needs under stress conditions. For example, the decreasing of *miR*-106 b during hibernation in ground squirrels and bats was associated with the function of alpha subunit of the hypoxia inducible factor (HIF-1α), which was a key factor for stress survival [17] [20].

Recently, roles of miRNAs under HS has drawn increased attention. High-throughput sequencing revealed miRNA profiling under HS in kinds of species, varying from plants, invertebrate to mammals [5] [21,22] [41]. These findings uncovered an additional layer of complexity to the regulation of HS signaling. Further analysis with gene expression identified key pathways participated by these miRNAs. According to a report in *Arabidopsis*, miR160 altered the expression of the heat shock proteins (HSPs), thereby assisting plants to survive HS [16]. In the other hand, the expression of miRNAs was regulated by some functional genes. HSP70 was proved to regulate the abundance of miR-

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23a, which helped to prevent heat-induced apoptosis [23]. Heat shock factor (HSF1), as a post-transcriptional regulator, plays critical roles in HSR of many species [1] [7] [37]. A recent study in *Caenorhabditis elegans* implied that HSF1 controlled gene expression under HS through regulating miRNAs expression [4]. These findings suggested a mechanism for cross-talk between miRNAs and genes expression under HS.

High-throughput sequencing technology of miRNAs is a powerful way to investigate the expression of miRNAs, which has already been applied in the studies of sea cucumbers under hypoxia stress and bacterial challenge [9] [44] [50]. However, the roles of miRNAs under HS has not been discussed yet in this species. In this study, we sought to determine the expression profiles of miRNAs under HS in sea cucumbers by using high-throughput sequencing technique. Gene ontology and pathway analyses of putative target genes were also carried out. Moreover, we made a correlation analysis between remarkable miRNAs and the differentially expressed genes (DEGs) in sea cucumbers under HS. These results will provide new insights about miRNAs regulation and molecular adaptive mechanisms in sea cucumbers under HS.

2. Materials and methods

2.1. Animal and sample treatment

A. japonicus (100–120 g) were collected from an aquaculture farm in Weihai (Shandong, China) in April 2016. The sea cucumbers were transported to our lab in Qingdao and acclimated in tanks for 2 weeks. During acclimation, the temperature and the salinity of sea water was around 16 °C and 30‰ respectively. The sea cucumbers was fed with a regular fodder, and half of water was changed daily [39].

When acclimation finished, three individuals taken from the tank were regarded as the control group. Because our previous studies showed that HSR was significant at 26 °C, the heat stress temperature was also set to 26 °C in this study [36] [47]. The methods of heating and sampling were followed by our previous procedures [39]. In brief, the rate of heating was about 2 °C/h. The moment water temperature reaching 26 °C was regarded as the initial time. Then water temperature was maintained at 26 ± 0.5 °C during the subsequent experiment. Three individuals were taken randomly after 6 h and 48 h stimulation. The intestine tissues of these individuals were immediately sampled and frozen in liquid nitrogen. All the samples were stored at −80 °C.

2.2. RNA extraction and processing

Three biological replicates of intestine tissue in the three groups (the control group, HS 6 h and HS 48 h) were prepared for sequencing. According to the manufacturer's instructions, total RNA of each individual was extracted using Trizol RNA isolation reagent (Invitrogen). The quality and concentration of RNA were measured by NanoDrop 1000 (Thermo Fisher Scientific, USA). RNA molecules ranging from 18 nt to 30 nt were enriched by polyacrylamide gel electrophoresis (PAGE). Adapters were added respectively in 3′- and 5' - end of RNAs.

The ligation products were reverse-transcribed by PCR amplification. The PCR products were enriched to generate the cDNA library. The library products were then sequenced via BGISEQ platform (Beijing Genomics Institute, Shenzhen, China).

2.3. Data processing and analysis

Raw data were cleaned by removing adapter sequences and low-quality reads. Other RNAs, including ribosomal (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) were removed by blasting in the GenBank database (http://blast.ncbi.nlm.nih.gov), the RepeatMasker (www.repeatmasker.org/), and the Rfam database (http://sanger.ac.uk/software/Rfam). Due to the lack of miRNAs information about *A. japonicus* in the miRBase 21.0, the remaining clean reads were aligned to all known precursor and mature miRNAs of all animal species in miRBase 21.0 with ≤2 mismatches. Temporary miRNAs database were constituted of miRNAs with the highest expression for each mature miRNAs family. Clean data were aligned to the above temporary miRNA database, and the expression levels of miRNAs were calculated by summing the read counts which were aligned with the temporary miRNAs database with ≤2 mismatches. The precursor of the identified miRNAs was predicted, and molecules that could not fold into a hairpin structure were regarded as pseudo-miRNA. The potentially novel miRNAs were identified via MIREAP (http://sourceforge.net/projects/mireap/) with stem-loop structure prediction [9].

By comparison of the miRNAs expression among the control group, HS 6 h group and HS 48 h group, the differentially expressed miRNAs were identified and analyzed. This procedure conformed to the BGI standards as follows: (1) the expression of miRNAs in each sample was normalized to determine the transcripts per million (TPM, TPM = actual miRNA count/total count of clean reads · 10^6); (2) The final TPM in each group was averaged by three biological replicates; (3) Fold-change and *P*-values were calculated from the normalized expression based on published studies [9] [27].

2.4. Real-time PCR validation

The total RNA extracted for sequencing was also used for real-time PCR analysis. Total RNA was reverse-transcribed to cDNA using Mir-X miRNA First-strand Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. The reaction was set for 60 min at 37 °C and 5 s at 85 °C. The cDNA was amplified using Mir-X miRNA qRT-PCR SYBR Kit (TaKaRa, Japan) with miRNA-specific forward primers and universal reverse primers. The specific forward primers were listed in Table S1. Small nuclear RNA, RNU6B, was used as a control gene for internal standardization [42] [43].

The $2^{-\triangle\triangle CT}$ method was used to analyze the comparative mRNA expression levels. Homogeneity of variances (F-test) of the data were evaluated first, and homogeneous data were further analyzed by one-way analysis of variance with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All the data were presented as mean ± SD (standard deviation of the mean) ($N = 3$). The level of significance was set with $P \leq 0.05$.

2.5. Target gene prediction and function analysis

The sea cucumber transcriptome was the candidate database to predict the target genes using RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and miRanda (http://www.microrna.org/) [28]. Gene ontology (GO) and kyoto encyclopedia for genes and genomes (KEGG) analyses were carried out to further understand genes biological functions. Target genes of differential expressed miRNAs were mapped to GO terms in the database (http://www.geneontology.org/) and KEGG database (http://www.genome.jp/kegg/) respectively. The calculated *P*-value went through Bonferroni Correction, and corrected *P*-value ≤ 0.05 was regarded as a threshold.

2.6. Correlation analysis of miRNA-target pairs

We had identified differentially expressed genes (DEGs) under heat stress in our previous study [38]. The samples of all three groups in DEGs analysis were in accordance with those in the present study. Therefore, correlation analysis of miRNA-mRNA were carried out in order to identify key miRNA-target pairs. Minimum free energy (MFE) ≤ −25 was set as the threshold for screening miRNA-target pairs. Only the inversely correlated miRNA-target pairs were identified.