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Short communication

Regulation of IL-17 by lncRNA of IRF-2 in the pearl oyster

Xian-De Huang[a,b], Jia-ge Dai[a], Ke-tao Lin[a], Mei Liu[a], Hui-ting Ruan[a], Hua Zhang[b], Wen-guang Liu[b], Mao-Xian He[b,*], Mi Zhao[b,**]

[a] College of Marine Sciences, South China Agricultural University, 483 Wushan Road, Guangzhou, 510642, China
[b] CAS Key Laboratory of Tropical Marine Bio-Resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou, 510301, China

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ABSTRACT

Long noncoding RNAs (lncRNAs), once thought to be nonfunctional, have recently been shown to participate in the multilevel regulation of transcriptional, posttranscriptional and epigenetic modifications and to play important roles in various biological processes, including immune responses. However, the expression and roles of lncRNAs in invertebrates, especially nonmodel organisms, remain poorly understood. In this study, by comparing a transcriptome to the PfIRF-2 genomic structure, we identified lncIRF-2 in the PfIRF-2 genomic intron. The results of the RNA interference (RNAi) and the nucleus grafting experiments indicated that PfIRF-2 might have a negative regulatory effect on lncIRF-2, and PfIRF-2 and lncIRF-2 may have a positive regulatory effect on PfIL-17. Additionally, lncIRF-2, PfIRF-2 and PfIL-17 were involved in responses to the nucleus graft. These results will enhance the knowledge of lncIRF-2, IRF-2, and IL-17 functions in both pearl oysters and other invertebrates.

1. Introduction

Long noncoding RNAs (lncRNAs) were initially deemed nonfunctional "transcriptional noise" [1]. However, emerging evidence suggests that lncRNAs participate in the multilevel regulation of transcriptional, posttranscriptional and epigenetic modifications and play a crucial role in various biological processes such as development, cell differentiation and immune responses [2–5]. There are few structural, functional or mechanistic features common to all mammalian lncRNAs, except for the minimum size limit of 200 nt that excludes most canonical ncRNAs, such as small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and tRNAs. Meanwhile, in contrast with small ncRNAs such as microRNAs and small interfering RNAs, which are highly conserved and participate in many biological processes by silencing target gene expression, lncRNAs are commonly less conserved than coding transcripts [6]. In humans, over 68% (58,648) of 91,013 expressed genes were recently classified as lncRNAs, of which 79% were previously unannotated [7]; the vast majority of lncRNAs remain unknown and have not been explored experimentally for function.

In recent years, lncRNAs have increasingly been reported from invertebrates such as *Caenorhabditis elegans* and Drosophila [8–11], providing a valuable guide and resource for future lncRNA functional explorations in invertebrates. In sea cucumber, large-scale bioinformatic predictions, expression profiling, and the coding-noncoding network construction of lncRNAs have shown the involvement of lncRNAs in the innate immune response and tissue regeneration and have provided an important resource for additional biological research on the regulatory functions of lncRNAs [12]. In *Crassostrea gigas*, lncRNAs associated with shell pigmentation in the mantle were systematically identified and might facilitate the understanding of the molecular regulation of shell color diversity in molluscs [13]. However, the expression and roles of lncRNAs in invertebrates, especially nonmodel organisms, remain poorly understood.

The induction and activation of Interferons (IFNs) are considered to be crucially important to the antiviral innate immunity of vertebrates [14,15]. A number of studies have indicated that the IFN response is subject to regulation by a large number of host- and pathogen-derived lncRNAs [16,17]. The IFN regulatory factor (IRF) family is a group of transcriptional factors that play critical roles in the activation of IFNs and in important physiological and pathological processes such as the immune defense, stress responses, reproduction, development, and carcinogenesis [18–20]. However, it is less clear how lncRNAs participate in the upstream processes of the IFN response, especially those of the IRF family.

* Corresponding author.
** Corresponding author.
E-mail addresses: hmx@scsio.ac.cn (M.-X. He), mi1225zhao@126.com (M. Zhao).

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For a long time, IRF genes have only been known and extensively studied in vertebrates. With the development of high throughput sequencing technology, transcriptomic and genomic databases of invertebrates, especially non-model animals, could elucidate and help characterize the DNA and putative protein sequences of the IRF family [21,22]. Currently, the function of invertebrate IRFs but not the IFN system have been explored in mollusks and shrimp [23–25]. The further exploration of ncRNAs related to the IRF family and their downstream genes will shed more light on the origin and evolution of IFN/IRF-based signal networks.

In our previous study, IRF-2 and Interleukin-17 (IL-17) homologs (PfIRF-2 and PfIL-17) were preliminary characterized from pearl oyster *Pinctada fucata*, which is one of the most important bivalve mollusks for seawater pearl production [25,26]. In this study, by comparing the PfIRF-2 genomic structure to the transcriptome assembled via RNA sequencing (RNA-seq), we identified the lncRNA of the PfIRF-2 gene, termed lncPfIRF-2, and briefly investigated the correlation of lncIRF-2 and PfIRF-2 and their regulation of the downstream gene PfIL-17 using RNA interference (RNAi) and a nucleus grafting experiment.

2. Materials and methods

2.1. Sequence analysis of lncRNA of PfIRF-2

The *Pinctada fucata* genome Ver 2.00 (http://marinegenomics.oist.jp/) was searched by the BlastN program using full-length PfIRF-2 cDNA to obtain the PfIRF-2 genomic sequence [13,27], and the exon/intron structure was analyzed using Spidey, a mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/spidey/). The PfIRF-2 (scaffold376.1, 341933 bp) genomic sequences were aligned to the *P. fucata* unigenes/transcripts of a transcriptome assembled via high-throughput sequencing by our laboratory (Unpublished) by the local BlastN program. After screening out the lower-identity unigenes, an unigene that was completely consistent with the intron sequences of PfIRF-2 was obtained.

2.2. Synthesis of double-stranded RNA (dsRNAs)

The different dsRNAs (treatment: dslncIRF-2 and dsPfIRF-2; control: dsEGFP, enhanced green fluorescence protein gene) were synthesized by in vitro transcription using the T7 RiboMAX™ Express RNAi System (Promega, USA) following the manufacturer's instructions. Briefly, the respective DNA templates for the synthesis of dsRNAs were prepared by PCR using the primers listed in Table S1. After recovery from agarose gel and purification with Gel Extraction Kits (Omega, USA) following the manufacturer's instructions, the PCR products were used as templates to transcribe and synthesize the sense and antisense RNA strands in vitro. Then, equal volumes of complementary RNA reactions were mixed and incubated to anneal the dsRNA. After removed any remaining single-stranded RNA and the template DNA by digestion with DNase, dsRNAs were recovered from the agarose gel, purified as described above, quantified the concentration, and stored at −20 °C until the RNAi experiment.

Pearl oysters (shell length 3–4 cm) were collected from Daya Bay in Shenzhen, Guang-dong Province, China. Pearl oysters were acclimated in indoor cement ponds at ambient seawater temperature for one week before experimentation. For the RNAi experiments, 40 μg (40 μl in volume) of dslncIRF-2, dsPfIRF-2, dsEGFP and PBS were injected into the adductor muscles of four groups of pearl oysters (n = 12). At 48 h after injection, the digestive glands of every 3 pearl oysters were pooled as one sample, and each group had four replicates.

For the analyses of gene expression in different tissues, the digestive gland, gills, mantle, gonad, hemocytes, adductor muscle, heart, intestine and foot were collected and mixed from three pearl oysters. For the hemocytes, the hemolymph was harvested from the pericardial cavity through the adductor muscle using a syringe and immediately centrifuged at 5000 g for 2 min. For the nucleus grafting experiment, "receiving" pearl oysters (shell length 6–7.5 cm) were directly collected from the sea in Xuwen, Zhanjiang, Guang-dong Province, China. The six time points of the nucleus grafting operation experiment were 1, 3, 5, 7, 15 and 30 days after the operation, with day 0 representing the blank group. At each time point, the hemolymph was collected from three pearl oysters and mixed as one hemocyte sample, and each time point had three replicates. All the above samples were stored in Sample Protector (TaKaRa, Japan) until use.

2.3. RT-PCR and quantitative PCR (qPCR) analysis

After the total RNA had been extracted, cDNA was performed using the PrimeScript™ reagent kit with gDNA Eraser (TaKaRa, Japan) following the manufacturer's instructions. RT-PCR was used to determine the gene expression in different tissues. The PCR reactions used the following conditions: denaturation at 94 °C for 3 min, multiple cycles at 94 °C for 35 s, 59 °C for 40 s and 72 °C for 30 s, followed by elongation at 72 °C for 5 min. The number of PCR cycles was 30 for 18S (GI: 62549260) and 40 for lncIRF-2. For the gene expression analysis in the RNAi and nucleus grafting operation experiments, qPCR was conducted with the Master SYBR Green I system (TaKaRa, Japan) and a Roche LightCycler480 instrument (Roche, Switzerland) in accordance with the manufacturer's instructions. The qPCR reactions were performed under the following conditions: one cycle at 94 °C for 3 min and 45 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. In a 384-well plate, each sample was analyzed out with three replicates. Then, the data were analyzed by following the LightCycler480 instrument's operation manual after the qPCR program. The primers for this study are listed in Table S1. The statistical analysis was conducted via Student's t-test in Microsoft EXCEL, and differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1. Sequence analysis of lncIRF-2

By aligning the PfIRF-2 genomic sequence to unigenes/transcripts, lncIRF-2 was obtained, which is located at the first and second exon of the PfIRF-2 genomic sequence, consists of 549 bp (Fig. 1), corresponded to scaffold376.1:33334–33882 in the *Pinctada fucata* genome Ver 2.00 (http://marinegenomics.oist.jp/) and was verified with another genome by Xiao-dong Du et al. [28]. According to the genomic distribution into one or more of the following five categories, sense, antisense, bidirectional, intronic and intergenic [29], lncIRF-2 belonged to intronic lncRNA. Meanwhile, the alignment of lncIRF-2 yielded no matches via the NCBI BlastN search, suggesting that lncIRF-2 was lowly conserved, like most of its lncRNA counterparts that were not conserved [6].

Compared with coding transcripts and other ncRNAs such as microRNAs and small interfering RNAs, lncRNAs typically lack long regions, with high constraints on sequence or secondary structure regions, and their research is more limited due to the requirement of a combination of multiomics especially genomics and transcriptomics. Until recently, although many lncRNA databases were mainly established for vertebrate animals such as fish, only a few lncRNAs have been annotated in species other than humans and mice, and the functions of many lncRNAs remain unknown. In invertebrates, especially nonmodel organisms, studies of lncRNAs have mainly been limited to the prediction, annotation and identification of RNA-sequencing (RNA-seq) data, and few studies have studied the function and characterization of individual lncRNAs [12,13].

3.2. The correlation of lncIRF-2 and PfIRF-2 and their regulation of PfIL-17

Given the correlation between lncRNA and its located gene, RNAi