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Short communication

Identification of a novel miR-146a from *Pinctada martensii* involved in the regulation of the inflammatory response

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ABSTRACT

Increasing evidence demonstrated that microRNAs (miRNAs) play critical roles in innate immunity in vertebrates and invertebrates. MiR-146a/b is reported as a key regulator of the immune response through mediating Toll-like receptor and cytokine signalling. In this study, a novel miR-146a was identified and characterised from *Pinctada martensii* (designated as pm-miR-146a), and its roles in modulating the inflammatory response after LPS stimulation were also investigated. Pm-miR-146a ubiquitously expressed in all examined tissues, with the highest level in the mantle and lowest expression in the haemolymph. Pm-miR-146a increased at 24 h after lipoplysaccharide injection, in union with up-regulated NF-κB ($P < 0.05$). The over-expression of pm-miR-146a in vivo could significantly inhibit the expression of macrophage migration inhibitory factor (MIF), the potential target gene predicted by miRanda, while enforcing pm-miR-146a involved in the down-regulation of NF-κB. Thus, we propose that pm-miR-146a plays a role of negative feedback regulation to the NF-κB signal by repressing the expression of the pro-inflammatory cytokine MIF. These findings revealed that miR-146a represents a critical role in inflammatory response and offers new evidence for miRNAs in the innate immunity of molluscs.

1. Introduction

MicroRNAs (miRNAs) are important endogenous small non-coding RNAs that mediate the negative post-transcriptional regulation of target gene expression [1]. Increasing evidence implicated that miRNAs are involved in controlling diverse biological processes, including cell differentiation, proliferation, growth and apoptosis [2]. In the immune response, miRNAs contribute to the differentiation of T-lymphoid lineage, signal transduction and secretion of pro-inflammatory factor [3–5].

In molluscs, 19 miRNAs were reported to be involved in the proliferation and activation of macrophages, inflammation, apoptosis and oxidative damage in the flat oyster (*Ostrea edulis*) [6]. MiRNAs correlated with bacterial challenge and heat stress were also found in oyster *Crassostrea gigas* [7,8]. In a previous study, a total of 258 miRNAs were harvested in pearl oyster *Pinctada martensii* by Illumina sequencing and bioinformatic prediction; several immune-related miRNAs, such as miR-100 and miR-184, were also identified [9–11]. The miR-146 family, including miR-146a and miR-146b, has been extensively investigated as regulators of the immune response in vertebrates [12,13]. In mammals, the TLR4 signalling pathway can activate the expression of miR-146 mediated by NF-κB. Further studies revealed that the induction of miR146a/b can negatively regulate the expression of genes in the signalling cascade downstream of TLR4, including TNF receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), leading to a model of negative feedback loop to protect from an excessive TLR4 response [14–16]. In the present study, a novel mir-146 generated from the 5′ stem was found in pearl oyster through bioinformatic analysis based on the transcriptome and genome database of *P. martensii* (pm-miR-146a). Its roles in the immune responses of *P. martensii* were further determined. Present data could help further understand the intricate and extensive roles of the miR-146 family in molluscs.

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2. Material and methods

2.1. Experimental animals

Adult pearl oysters (about two years of age) were obtained from Liusha Gang, Zhanjiang, Guangdong Province, China. The samples were cultured at 25 °C-27 °C in tanks with recirculating seawater for 2 days before the experiment. Different tissues including adductor muscle (A), mantle (M), gill (Gi), gonad (Go), foot (F) and haemolymph (B) from three pearl oysters were acquired and immediately stored in liquid nitrogen until usage. For the immune challenge, 100 μL of 10 μg/mL lipopolysaccharide (LPS; Sigma, USA) was injected into the adductor muscle of pearl oysters in the challenged group, along with 100 μL of PBS in the control group. Haemocytes collected from the adductor muscle of pearl oysters at 0, 2, 4, 8, 12, 24 and 36 h post-injection were immersed in Trizol reagent for RNA separation (Invitrogen, USA).

2.2. Identification of miR-146a by bioinformatic method

All of the miR-146 genes were collected from miRBase (http://www.mirbase.org/). The homologous fragments were identified using Blastn in comparison with the transcriptome data of pearl sac, and the number of mismatches was limited (no more than 4). The candidate precursors were then tested for the proper stem-loop structure remodelling by Mfold (http://unafold.rna.albany.edu/?q=mfold). The selected precursor of miR-146 should be located on the genome of pearl oyster *Pinctada fucata* (http://marinegenomics.oist.jp/genomes/downloads?project_id=20).

2.3. Over-expression of miR-146a in vivo

Twelve pearl oysters were distributed into two groups, namely, the experimental group injected with the mimics of pm-miR-146a and negative control group (N.C.). The mimics of pm-miR-146a and negative control were purchased from Shanghai GenePharma Co.,Ltd., and the sequences are shown in Table 1. The mimics of both groups were diluted to 15 μg/100 μL using PBS. Subsequently, 100 μL solutions with pm-miR-146a mimics and N.C. mimics were respectively injected into the muscles of *P. martensii.*

2.4. RNA extraction and cDNA synthesis

Total RNA was prepared using Trizol reagent according to the manufacturer's instructions. RNA quantity was detected by NanoDropND1000 spectrophotometer (Thermo Scientific, USA), and RNA integrity was determined by 1.0% agarose gel. Templates for mRNA amplification were prepared using random primers and specific primer (Table 1) with M-MLV reverse transcriptase (Promega, USA).

2.5. Quantitative real-time PCR (qRT-PCR) assay

The qRT-PCR assay was performed with Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) according to the manufacturer's protocol. The fluorescence was detected by the Applied Biosystems 7500/7500 Fast Real-time system (Applied Biosystems, Foster City, CA, USA). To detect the expression pattern in different tissues, the fold expression was calculated by the $2^{-\triangle\triangle ct}$ method using U6 as a reference gene. The expression level of adductor muscle served as a control in individuals. Data are presented as the mean ± SD for three replicates of qRT-PCR reactions. To calculate the expression level after LPS stimulation, the fold expression of NF-κB and pm-miR-146a was calculated by the $2^{-\triangle\triangle ct}$ method using GAPDH and U6 as reference genes, respectively. PBS served as controls for LPS stimulation. To analyse the expression changes between pm-miR-146a mimics and N.C. mimics, the expression levels of MIF, NF-κB and pm-miR-146a were calculated by the $2^{-\triangle ct}$ method using GAPDH and U6 as reference genes, respectively. Data are presented as the mean ± SD for three replicates of qRT-PCR reactions.

2.6. Identification of pm-miR-146a target

Target analysis between pm-miR-146a and the 3′-UTR region of immunity-related genes collected from the NCBI database was performed using miRanda. The parameters of the miRanda algorithm were restricted with score ≥100 and free energy ≤ −10 kcal/mol.

2.7. Statistical analysis

The data were analysed using one-way ANOVA in SPSS 19.0 (IBM, USA). A P-value less than 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Identification of novel pm-miR-146a

By comparing the sequences of pm-miR-146 downloaded from miRbase with the transcriptome of pearl sac from *P. martensii,* we found one homologous fragment (designated as pm-miR-146a) similar to mature pm-miR-146a identified from *Gorilla gorilla*

Table 1
The primer sequences for RT-PCR and mimics sequences of pm-miR-146a and N.C.

Primer name	Sequences
Pm-miR-146a(A)	CAGTGCGTGTCGTGGAGTC
Pm-miR-146a(S)	CAAGAACTGAATTCCTATGGATG
miR-146a(RT)	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACATCCATAGG
U6(RT)	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAAAATATGG
U6(S)	ATTGGAACGATACAGAGAAGATTAG
U6(A)	TGCGTGTCGTGGAGTC
MIF(S)	TCACGCCGACAAAATAGCAAC
MIF(A)	CCTCCGAAGCCAACATCCTC
NF-κB(S)	GGGGATTCAACTTGGATTCTT
NF-κB(A)	AGGTGTATGGGCTTCTGCTTT
Mimics-miR-146a(S)	CAAGAACUGAAUUCCUAUGGAU
Mimics-miR-146a(A)	AUCCAUAGGAAUUCAGUUCUUG
N.C.(S)	UUCUCCGAACGUGUCACGUTT
N.C.(A)	ACGUGACCACGUUCGGAGAATT