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An oyster species-specific miRNA scaffold42648_5080 modulates haemocyte migration by targeting integrin pathway

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ABSTRACT

miRNAs are important gene regulators at post-transcriptional level and can modulate diverse biological processes, including immune response. Dozens of species-specific miRNAs have been identified in oyster *Crassostrea gigas* while their functions remain largely unknown. In the present study, an oyster species-specific miRNA scaffold42648_5080 was found responsive to LPS stimulation and might target a total of 31 oyster genes possibly involved in cell communication, cellular localization and cellular response to stimulus. Besides, in gain-of-function assay of scaffold42648_5080 *in vivo*, the phagocytosis (30.90% in miRNA group *verse* 23.20% in miRNA control group), apoptosis (3.10% in miRNA group *verse* 5.30% in miRNA control group) and migration rate (13.88% in miRNA group *verse* 21.03% in miRNA control group) of oyster haemocytes were found significantly altered after the injection of scaffold42648_5080 mimics. Among the target genes, integrin-linked kinase (CgILK) was considered crucial in cell migration and its interaction with scaffold42648_5080 was then verified both *in vitro* and *in vivo*. Consequently, a significant decrease of relative luciferase ratio was observed in CgILK 3′-UTR luciferase reporter assay after transfection of scaffold42648_5080 mimics (0.70-fold of that in blank group, $p < 0.01$). Meanwhile, when scaffold42648_5080 was overexpressed *in vivo* (5.41-fold of miRNA control group, $p < 0.01$), the expression of CgILK declined significantly to 0.25-fold of miRNA control group ($p < 0.01$). Comparatively, a significant decrease of the haemocyte migration rate (19.76% *verse* 34.82% in siEGFP control group, $p < 0.01$) was observed after knock-down of CgILK *in vivo*. The present study, as far as we know, for the first time revealed the immunomodulation role of an oyster species-specific miRNA, which might provide new insights into miRNA-mediated adaptation mechanism of oysters.

1. Introduction

miRNAs are an important class of gene regulators at post-transcriptional level [1]. They are about 22 nt in length with a 7 nt seed region at 5′ terminus and could be transcribed by RNA polymerase II from either intragenic or intergenic regions [2]. It has been found that mature miRNAs in the cytoplasm could be incorporated in RNA-induced silencing complex (RISC) before binding to target genes and could lead to mRNA degradation or translational repression afterward [3]. Almost all biological processes, including cell differentiation, pattern formation, tissue development and cellular metabolism, could be modulated by miRNAs [4].

The earliest animal miRNAs, as known, are found in Porifera [5] and more than 28,000 miRNAs have been identified to date in over 200 species [6]. It is suggested that animal miRNAs are continuously being expanded through evolutionary [1], while they have also undergone constantly shifting under the opposing influences of gain and loss [7]. Researchers have estimated that new emerged miRNAs would be kept in genome steadily if they were integrated into gene regulation network while organisms at terminal nodes in a phylogeny tend to have more new emerged and lineage-specific miRNAs [8], which might dedicate crucially during the host adaptation against environment changes [7].

The response mechanism against pathogen invasion is crucial

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for the organism's survival and adaptation in its ecological niche, and it relies greatly on the well orchestration of pathogen recognition, immune signal transduction, immunocytes migration and synthesis of immune effectors [9,10]. Previous studies have demonstrated that all these immune events could be vigorously modulated by miRNAs [11]. For example, it was found that multiple miRNAs could be rapidly induced after pathogen challenge and diverse immune-related genes including pathogen recognition receptors, signal transducers and cytokines could be regulated afterward [12,13]. Meanwhile, dysregulation of certain miRNAs might also result in disequilibrium of host immune system and serious diseases [14]. The orchestration of immune responsive miRNAs is therefore decisive in the well balance of immune system as well as the homeostasis of host during biotic stress [15]. However, majority of studies about immunomodulation role of miRNAs were conducted in mammals and less is known in invertebrates [16–19].

Oyster *Crassostrea gigas* is a worldwide bivalve with great economic value and inhabits at the pathogen rich intertidal region. Hence, a robust immune system is vital for the thriving of oysters [20,21]. With the release of detailed genome information, oyster *C. gigas* is now considered as an ideal model in investigating invertebrate immune system [22]. It was found that massive of immune-related genes were expanded in oyster genome, providing powerful armory against invaded pathogens [23]. Recently, 55 immune responsive miRNAs were also identified in *Vibrio splendidus* challenged oysters and 11 of them (including scaffold42648_5080) were oyster species-specific, indicating the complicated immunomodulation network of oysters [18]. Moreover, scaffold42648_5080 were also found expressed abundantly in haemocytes with the highest expression level among all oyster species-specific miRNAs, suggesting its unique role in oyster [18]. The purpose of this study, therefore, was to (1) draw the phylogeny of scaffold42648_5080, (2) survey and verify its immune-related target genes and (3) investigate its potential regulation on cellular immune response by interaction with its target genes, hopefully providing new hints for the species-specific miRNA-mediated immunomodulation roles in oysters.

2. Methods and materials

2.1. Sequence alignment of scaffold42648_5080 and prediction of its target genes

Mature and stem-loop sequence of scaffold42648_5080 were subjected to blastn program at miRBase website [6] with default settings to find homologues of scaffold42648_5080. The stem-loop sequence was also subjected to a local blastn program using all identified oyster miRNAs in search of potential homologues. Mature or stem-loop sequences of matched miRNAs were subsequently aligned using ClusterX to demonstrate sequence similarity.

The target genes of scaffold42648_5080 were predicted globally by miRanda software (http://www.microrna.org/microrna/home.do, parameter of miRanda: -sc 160, -en −23) with oyster 3′-UTR sequences obtained according to the genome information. The target genes were subsequently annotated by WEGO [24] for gene ontology analysis.

2.2. Oyster preparation, primary cell culture and lipopolysaccharide (LPS) challenge

Oysters *C. gigas* employed in the present study were obtained locally from an aquaculture farm and were all sawed at the shell where was close to the adductor muscle to make a narrow notch for later injection [16]. The oysters were then returned to aseptic seawater with aeration and cultured for two weeks before subjected to any trial.

The *in vitro* culture of primary oyster haemocytes was conducted using methods described previously with some modification [25]. Briefly, oyster haemocytes collected from posterior adductor muscle sinus with acid citrate-dextrose anticoagulant agent (22 g L^{-1} sodium citrate, 8 g L^{-1} citric acid, 24.5 g L^{-1} glucose, pH 7.4) at ratio of 8:1 [26] were first seeded into a 6-well plate at 5×10^6 cells per well and then cultured with modified Leibovitz's L-15 medium (L-15 medium, Gibco) at 18 °C. Then the primary cells were challenged with 2 μg mL^{-1} lipopolysaccharide (LPS, Sigma) and sampled at 0, 1, 3, 6 and 9 h later for subsequent miRNA and mRNA extraction. Expression pattern of scaffold42648_5080 and CgILK were subsequently analyzed by quantitative real-time (qRT)-PCR. All trials were conducted with three biological replicates.

2.3. Gain-of-function assay of scaffold42846_5080 in vitro and in vivo

The scaffold42648_5080 mimics and inhibitors were synthesized by GenePharma according to the sequence of scaffold42648_5080. Negative control mimics and inhibitors designed by GenePharma were also prepared for subsequent assay. All RNA sequences were listed on Table 1.

The *in vitro* gain-of-function assay of scaffold42648_5080 was conducted in primary haemocytes using Neon™ Transfection System (Invitrogen). Briefly, a total of 1×10^6 oyster haemocytes were first washed in phosphate buffered saline without Ca^{2+} and Mg^{2+} and resuspended in Resuspension Buffer from the Neon™ Kit at a final density of 1×10^7 mL^{-1}. Then the scaffold42848_5080 mimics (miRNA group) or miRNA control mimics (miRNA control group) was added into the resuspension mix at a final concentration of 10 nM and subjected to the Neon™ device and Neon™ Pipette Station for electroporation (parameter: voltage, 1400 V; width, 20 ms; pulses, 1 pulse). Transfected haemocytes were then seeded into 6-well plate and cultured for 24 h using modified L-15 medium [25] before harvested for qRT-PCR analysis of scaffold42648_5080 and CgILK.

For *in vivo* gain-of-function assay of scaffold42648_5080, a total of 270 oysters from three groups designated as SW, miRNA and miRNA control were injected with 100 μL sterile seawater, 2.5 nmol scaffold42648_5080 mimics (in 100 μL sterile seawater) and 2.5 nmol control mimics (in 100 μL sterile seawater), respectively. Haemocytes from five oysters in each group were randomly collected at 24 h for subsequent qRT-PCR analysis of scaffold42648_5080. Another five oysters from each group were also sampled for haemocytes to investigate expression changes of CgILK. Haemocytes from the remaining oysters were sampled with anticoagulant agent for detection of haemocyte phagocytosis, apoptosis and migration rate.

Three parallel replicates were applied in all trials.

2.4. The cloning and sequence analysis of CgILK full-length cDNA

Nucleic acids sequence of CgILK (EKC33674) was retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/gene). A pair of gene specific primers was designed to clone the full-length cDNA of CgILK. The cDNA and amino acid sequence were analyzed subsequently by DNAman software. Homologous sequences of CgILK were obtained using NCBI blastp program with default settings and listed in Table 2. Deduced protein domains of CgILK were predicted by SMART (http://smart.embl-heidelberg.de/). Phylogenetic tree of selected ILKs was constructed by MEGA program with neighbor-joining algorithm and the reliability of the branching was tested using bootstrap resampling (1000 times).