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RESEARCH REPORT

Expression analysis of miR-2005 and its target genes in *Apostichopus japonicus* by *Vibrio splendidus* challenged

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Abstract

MicroRNAs (miRNAs) are important effectors in mediating host-pathogen interaction. MiR-2005 is observed to be involved in immune response processes in *Apostichopus japonicus*. In the present study, the putative target genes of miR-2005 in *A. japonicus* coelomocytes were predicted by bioinformatics analysis of transcriptome database and PCR approaches. A total of 506 potential targets were screened, and 187 targets were annotated. Several immune-related target genes were identified in this study, such as *SLI3*, *CFHR5*, *FGL*, *A2ML*, and *Rab9al*. The expression patterns of miR-2005 and its potential targets were validated by quantitative real-time PCR in *Vibrio splendidus* challenged *A. japonicus*. For further characterization, an overexpression experiment of miR-2005 at cellular levels was applied. Accordingly, significant negative correlation expression profiles were detected between miR-2005 and two candidates targets, suggesting that *SLI3* and *CHRP5* showed high possibility to be the targets of miR-2005 in *A. japonicus*. Altogether, this study will enhance our understanding in the context of miR-2005 modulating the interaction of *A. japonicus* after being challenged by *V. splendidus*.

Key Words: miR-2005; Sea cucumber (*Apostichopus japonicus*); *Vibrio splendidus*; overexpression experiment; spatial expression

Introduction

The sea cucumber, *Apostichopus japonicus* with medicinal effects and rich nutritional value for human consumption, naturally distributed along the coasts of East Asian countries such as China, Japan, Korea and Russia (Sloan *et al*., 1984; Chang *et al*., 2009). Additionally, it is widely cultured as a significant aquaculture species in the above countries of East Asia.

The echinoderm immune system initiates a response when challenged with pathogen-associated molecular patterns (PAMPs), which are recognized through molecules known as pattern recognition receptors (PRRs) (Kawai *et al*., 2010). The combination of PAMPs and PRRs activates many immune factors, such as antimicrobial peptides, lectin, and complement through a series of cascade reactions to kill and eliminate invading pathogenic microorganisms, parasites and apoptotic bodies (Fukuzawa *et al*., 2008). The immune response of *A. japonicus* is shown to be based on coelomocyte activity (chemotaxis and phagocytosis) (He *et al*., 2016), some humoral immune factors (lectins, opsonins and some bactericidal substances) (Mai *et al*., 2009; Wei *et al*., 2015) could also involve in the immune response that resist the invasion of pathogens directly. Within such an intricate immune defense system, there are multiple layers of process for molecular regulation. MicroRNAs (miRNAs) are important effectors in complicated gene expression profiles through sequence-specific regulation, they could prove to be of significant functional importance in intricate molecular regulation. In a previous study, Lv *et al*. (2015) observed that miR-200 could modulate the lipopolysaccharides (LPS) priming and antimicrobial activities via augmenting toll-interacting protein (*AjTollip*). The negative expression profiles between miR-31 and its target Ajp105 were also observed (Lu *et al*., 2015). It's indicated that reactive oxygen species (ROS) accumulation could be stimulated, whether by miR-31 overexpression or Ajp105 silencing. miR-137 and miR-2008 were predicated that could targeted the 3'-UTR of betaine-homocysteine

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S-methyltransferase (*AjBHMT*), which induces respiratory bursts and affects Hcy accumulation in coelomocytes (Zhang *et al*., 2015). In addition, Lv *et al.* (2017b) also found another target of miR-137, and identified the depressed expression profiles of miR-137 and its target gene 14-3-3ζ (*Aj14-3-3ζ*) in both LPS-exposed primary coelomocytes and *Vibrio splendidus*-challenged *A. japonicus* (Lv *et al*., 2017a). Moreover, several putative targets of miR-92a were also identified by Zhang *et al.* (2014). Studies of *Drosophila* and *Euarchonta* (Lu *et al*., 2008; Zhang *et al*., 2008) suggested that gain and loss of miRNAs can be affected by transposition and retrotransposition. Thus, miRNAs have been revealed as major regulators in various hosts-pathogen interaction processes of *A. japonicus* by repressing the transcription and post-transcriptional expression of target genes. However, although miRNA libraries were constructed for understanding the physiological process of *A. japonicus* (Li *et al*., 2012; Chen *et al*., 2013; Chen and Storey, 2014; Wang *et al*., 2014, 2015; Sun *et al*., 2016), the studies on miRNAs and their targets associated with immune response related skin ulceration syndrome of *A. japonicus* are relatively few. Although, miR-2005 is found present only in *Strongylocentrotus purpuratus*, *Lytechinus variegatus* and *Patiria miniata* based on miRBase records, the expression level of miR-2005 in sea cucumber was also discovered by Wang *et al*., (2014) and Zhong *et al*., (2015). Wang *et al*., (2014) indicated that miR-2005 was significantly up-regulated in the tube foot of *A. japonicus*, followed by coelomocytes and respiratory tree. It's suggested that the expression level of miR-2005 was not only highly expressed in the external tissues, but also in the internal environment of *A. japonicus*. Moreover, miR-2005 was also observed to be involved in the immune response process in *A. japonicus* after the LPS injection (Zhong *et al*., 2015). In addition to miR-2005, the expressing levels of miR-2004, miR-133, and miR-137 that was altered along with the expression of *A. japonicus complement C3* changing at different time points after LPS injection was also observed (Zhong *et al*., 2015). According to the evidence resulting from Zhong et al., (2015), it provided further proof for the important regulatory role of miR-2005 in *A. japonicus* immune system. To sum up, miR-2005 may be involved in the development and immunity defense process of *A. japonicus*. However, the knowledge of the regulation mechanism of miR-2005 in immune response is still very vague.

As previously documented, coelomocytes are recognized to be one of the main components in echinoderm animal immune responses. The coelomocytes of *A. japonicus* are proved as an appropriate target to explore immune-related genes and physiological mechanisms in response to pathogenic challenge (Dong *et al*., 2014). *V. splendidus*, as the Gram-negative bacteria, is identified as a major pathogen that can cause skin ulceration disease in *A. japonicus* (Zhang *et al*., 2006). Therefore, *V. splendidus was* chosen as the pathogen to use to understand the regulation mechanism of miR-2005 in immune response by stimulating *A. japonicus* coelomocytes.

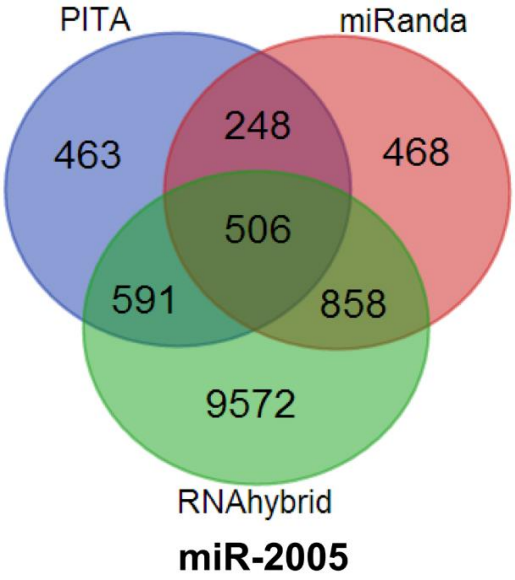

Fig. 1 Predicted target mRNAs of miR-2005.

In this study, we report the identification of target genes of miR-2005 from the transcriptome database, including our transcriptome (unpublished) and that of Sun *et al*., (2013) (accession NO. GSE44995). Then, the relative expression of miR-2005 and its predictive targets were further investigated in *V. splendidus*-challenged *A. japonicus* coelomocytes. Subsequently, overexpression was conducted *in vitro* to obtained the connection of miR-2005 and putative target genes. The present study can strengthen the understanding of the regulatory role of miR-2005 in host–pathogen interactions. Additionally, this study could enhance the knowledge base of *A. japonicus* immunity upon being challenged by pathogens.

Material and methods

Target prediction of miR-2005

The 5'-untranslated regions (5'-UTRs) and 3'-untranslated regions (3'-UTRs) were regarded as the potential binding region between target mRNA and miRNA. The extracted UTRs were obtained by in-house Perl script according to the predicated open reading frame (ORF) as previous study (Zhou *et al*., 2018). The miR-2005 sequences, which were obtained from several previous works (Wang *et al*., 2014; Zhong *et al*., 2015), were used to predicate the target mRNA by using the RNA-Seq sequences containing the extracted UTRs. Three software packages, including RNAhybrid (Rehmsmeier *et al*., 2004), PITA (Kertesz *et al*., 2007) and miRanda-3.3 (Ellegren, 2008), were used to predict the target mRNAs, and the potential target genes were considered by the intersection of all three programs as previously described *(*Zhou *et al*., 2016). The parameters of PITA software were "-l 7-8", and "-sc 140", "-en -17" for miRanda-3.3, while the default parameters were used and filtered by ΔG <= 20 kcal/mol in the RNAhybrid.

Table 1 Primers used in qRT-RCR.

Gene ID	Gene name	Primer Sequence (5'-3')	Application
isotig15273	Serum lectin isoform 3	F: ATTGACAGACACCCTTCCAC R: GACTTCCTGACCTAACATCG	Real-Time PCR
isotig01560	Fibrinogen-like protein	F: GCCAGGATGTTTATGACGCT R: TTGTTACCGAAGCCGTCTCT	Real-Time PCR
isotig15571	Alpha-2-macroglobulin-like	F: CGGAGAGTAGGTCTGATGAT R: GAGTGACAAAGAGGGAGGTT	Real-Time PCR
comp279590_c0	Complement factor H-related protein 5	F: GCCAGTAGTATCCATCATCC R: CGTGCTCCAACAGATTAGTC	Real-Time PCR
comp275604_c0	Rab-9A-like	F: AGCCAGTCCTTCCATACGAT R: CTCTTGAACCTCTCCTGTCCT	Real-Time PCR
	miR-2005	F: AGTCCAATAGGGAGGGCATTGCAGT R: Universal miRNA qPCR Primer	Real-Time PCR
	miR-2005M	AGUCCAAUAGGGAGGGCAUUGCAG GCAAUGCCCUCCCUAUUGGACUUU	miR-2005 mimics
	NCM	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT	Negative control of miRNA mimics
	CYTB	F: TGAGCCGCAACAGTAATC R: AAGGGAAAAGGAAGTGAAAG	Reference gene
	RNU6B	F: ACGCAAATTCGTGAAGCGTT R: Universal miRNA qPCR Primer	Reference gene

Animals and challenge experiment

Healthy sea cucumbers (average weight of 100 g) provided by the Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture (Dalian, Liaoning) were used in this study. For the challenge experiment, the pathogen bacteria *Vibrio splendidus* (D4501) were chosen to cultured and harvested in our laboratory as previously described by (Cheng *et al*., 2017). The *V. splendidus* were cultured at 28 °C overnight with shaking at 200 rpm. Then centrifuged at 4000 rpm for 1 min (4 °C) to harvest the bacteria as previously described by Cheng *et al*., (2017). For bacterial challenge experiment, the overnight cultured *V. splendidus* (D4501) was diluted with the phosphate buffered saline (PBS, 0.1 mM, pH 7.4) to achieve the working solution of *V. splendidus* (D4501) at the concentration of 10^7 CFU/ml. The challenge experiments were divided into a control group and a bacterial challenge group. A total of 60 sea cucumbers were divided into bacterial-challenged group and control group. The bacterial-challenge group (30 individuals) was injected with 100 μl *V. splendidus* (D4501), while control group (30 individuals) was injected only in a tank containing PBS. The celomic fluids from five *A. japonicus* was collected at 0, 4, 8, 12, 24, 48 and 72 h post-injection.

The samples centrifuged directly at 1000 × rpm for 5 min at 4 °C. After centrifugation, the supernatant was discarded, and snap-frozen in liquid nitrogen, then transferred to -80 °C until RNA extraction.

The validation of interaction between miR-2005 and target genes

To evaluate the regulatory relationships between miR-2005 and its predicted targets, the expression level of miR-2005 and five predicted target genes were assessed by qRT-PCR. Total RNAs were extracted from coelomic fluid using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the corresponding cDNAs were synthesized with TransScript miRNA First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Co., Ltd., China). The SYBR® PrimeScriptTM miRNA RT-PCR kit (TaKaRa) was used for miRNA and miRNA quantification in the same samples by using described method with an ABI 7500 Real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The specific primer sets for miR-2005 and potential targets are shown in Table 1. In our study, CYTB and RNU6B (Table 1) were served as a reference gene to normalize targets and miRNA, respectively. In brief, each reaction was performed in a final volume of 16 μl containing 2 μl of the cDNA as in a previous study (Zhou *et al*., 2016a). Three technical replications were performed for each qRT-PCR validation. PCR was conducted as follows: 94 °C for 30 s, 45 cycles of 94 °C for 5 s, and annealing temperature for 32 s.

Primary coelomocytes culture and miR-2005 over-expression in vitro

In this study, coelomocytes were cultured as described by Lu *et al*. (2015) and Shao *et al*. (2015). The *A. japonicus* were dissected and the coelomic

fluids were filtered through a 300 Mesh CellCribble to filter out large tissue debris, and then the coelomic fluids were centrifuged at 900 g 16 °C for 10min with the same volume of anticoagulant solution (0.48 M NaCl, 0.019 M KCl, 0.02 MEGTA, and 0.068 M Tris-HCl, pH 7.6). The isotonic buffer (0.53 M NaCl, 0.001 M EGTA, and 0.01 M Tris-HCl, pH 7.6) was used to wash the harvested cells, and re-suspended in Leibovitz's L-15 cell culture medium (Invitrogen, USA) supplemented with Gentamycin sulfate (100 μg × ml^{-1}), penicillin (100 U ml^{-1}), streptomycin sulfate (100 μg × ml^{-1}), and NaCl (0.39 M) to adjust the osmotic pressure. The coelomic cells that were diluted to 10^6 cells ml^{-1} were the suspended and dispensed into 24-well microplates and incubated at 16 °C for 12 h before the miR-2005 overexpression experiment. The miRNA mimics and negative control of miRNA mimics (NCM) were synthesized at GenePharma and are shown in Table 1. The RNase-free water was used to dissolve miR-2005 mimics and NCM to obtain a working solution of 20 μM. Then 2 μl of miR-2005 mimics and NCM were mixed with an equal volume of HiPerFect transfection reagents (GenePharma, Shanghai), and transfected into primary cultured cells. After 24 h post-transfection, the primary cultured cells were harvested and used for miR-2005 overexpression analysis.

Results

Prediction and analysis putative target genes of miR-2005

By using the query of the obtained UTR reads in our previous study (Zhou *et al*., 2018), the potential target genes of miR-2005 were screened using the program PITA, miRanda-3.3a and RNAhybrid In that order. The Veen plot shows the number of potential target genes that predicted by PITA, miRanda-3.3a and RNAhybrid. As shown in Fig. 1, 506 potential target genes of miR-2005 were predicted. The sequences of predicted target genes and the predicted binding sites for the miR-2005 are listed in Supplementary Table 1 and Supplementary Table 2, respectively. A total of 187 potential target genes were annotated (Supplementary Table 3). From the

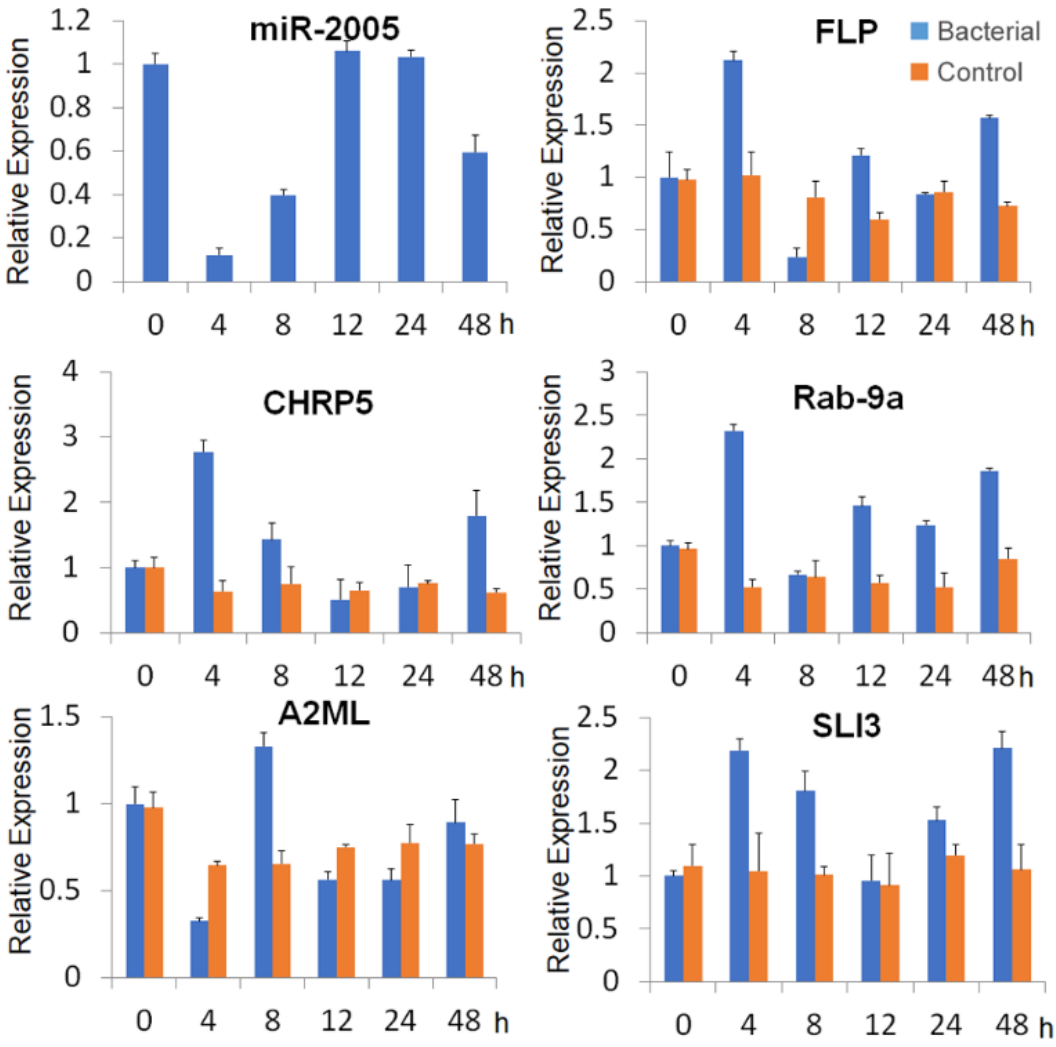

Fig. 2 Time-course expression patterns of miR-2005 and five predict target genes in *A. japonicus* after *V. splendidus* challenged at 0, 4, 8, 12, 24 and 48 h.

187 potential targets, five potential target genes (serum lectin isoform 3, *SLI3*; fibrinogen-like protein, *FGL*; alpha 2-macroglobulin like, *A2ML*; Complement factor H-related protein 5, *CFHR5*; and Rab-9A-like, *Rab9al*) were involved in immune defense against pathogens. Both of SLI and CFHR can possess the functions as opsonization (Boackle *et al*., 2003). FGLA, A2M and Rab9a are also play vital roles in many physiological and biochemical reactions, including blood clotting and regeneration (Lu *et al*., 2002; Wu *et al*., 2014), regulate the prophenoloxidase (proPO) activating system (Jiang *et al*., 2006), biogenesis of lysosome-related organelles and regulates the degradation of cytoplasmic contents in the lysosome (Nottingham *et al*., 2011).

Expression profile of miR-2005 and its target genes in V. splendidus*-challenged* A. japonicus

To further understand the putative role of miR-2005 in *A. japonicus* immune defense *in vitro*, we selected five immune-related targets (*SLI3*, *CFHR5*, *FGL*, *A2ML*, and *Rab9al*) of miR-2005 using qRT-PCR method combined with the result of the bioinformatics analysis result. As shown in Fig. 2, the first drastic cut was found in the expression of miR-2005 at 4 h (0.12-fold), and the second downward trend happened at 48 h. The expression pattern of SLI3, Rab9a and CHRP5 all had two peaks, reached at 4 h and 48 h.

Functional analysis of miR-2005 and its target genes in vitro

The primary coelomocytes of *A. japonicus* were cultured for gain-of-function experiment of miR-2005 (Fig. 3). As shown in Fig.4, the significant increase was obtained in the overexpression of miR-2005. The qRT-PCR results of *SLI3*, *A2ML* and *CFHR5* are also shown in Fig. 4. The overexpression of miR-2005 decreased the mRNA expression levels of *SLI3*, *A2ML* and *CFHR5* obtained in this study, and the downward trend of *SLI3* and *CFHR5* were more significant than *A2ML*.

Discussion

The echinoderm, with no specific immune cells, are mainly rely on the non-specific immune system that composed of cellular immunity and humoral immunity to complete the body's defense response. Many complex molecular regulatory mechanisms are involved in the cellular immunity and humoral immunity of sea cucumber, one of the process is the reprogramming of expression of immune-related genes. The universal transcripts have been demonstrated that are across 70-90 % of mammal genome. However, more than 98 % of total transcripts are represented by non-coding RNAs (ncRNAs) (Tay *et al*., 2014). Accumulative evidence indicated that miRNAs play essential role in multiple regulatory mechanisms of gene-expression to adapt and survive under pathogen infection (Li *et al*., 2012; Wang *et al*., 2014, 2015; Sun *et al*., 2016; Lv *et al*. 2017a,b; Chen *et al*., 2018). Thus, miRNAs have been discovered as major regulators in different hosts-pathogen interaction processes of *A. japonicus* by blocking the expression of target genes.

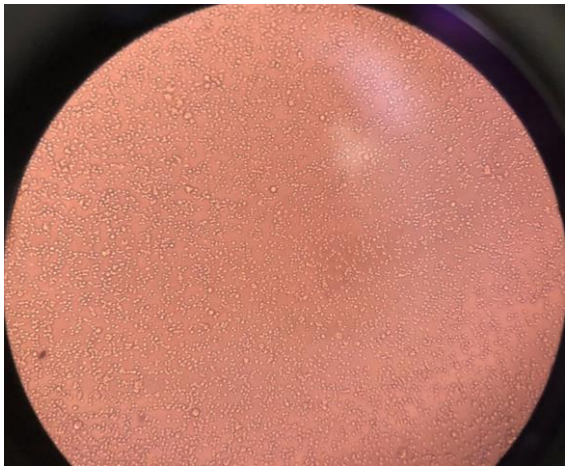

Fig. 3 Primary coelomocytes culture.

In this study, we recognized that miR-2005 played a regulatory role in the interactions between *A. japonicus* and *V. splendidus*. The putative target genes of miR-2005 in *A. japonicus* coelomocytes were predicted by bioinformatics analysis. Among the 506 potential target genes, five immune-related target genes (*SLI3*, *CFHR5*, *FGL*, *A2ML*, and *Rab9al*) of miR-2005 were found and selected to verified their relation expression patterns in *A. japonicus* under *V. splendidus* infection. Two highly confident target genes (*SLI3* and *CFHR5*) are spotted *in vitro* and *in vivo*. Both lectin and complement can be activated by the combination of PAMPs and PRRs. Lectin that exists on the surface of host cells or bacteria are necessary for the specificity recognition between humoral factors and foreign substances. SLI and Ca^{2+} dependent lectin has been found in the sea urchin and sea cucumber, which is able to recognize and attack foreign substances, and similar homologous to vertebrate cell membrane or soluble lectin (Giga *et al*., 1987; Himeshima *et al*., 1994; Matsui *et al*., 1994; Liu *et al*., 2012). After *A. japonicus* infected by *V. splendidus*, the expression pattern of *SLI3* was reached at 4 h and 48 h. Similar expression pattern have been also observed in Liu *et al*. (2012). The expression level of SLI significantly increased in *A. japonicus* after they were infected by *Vibrio* sp. for 3 days (Liu *et al*., 2012). It is suggesting that SLI may play important roles in the immune defense mechanism of sea cucumber against bacterial infection. Moreover, the complement system is also playing a necessary role in the innate defense against common pathogens. As a complement control protein, CFHR is a member of the complement activation family regulators. Five plasma proteins (CFHR1, CFHR2, CFHR3, CFHR4 and CFHR5) that each member binds to the complement component 3b (C3b) can comprise factor H related proteins (Zhou *et al*., 2011; Skerka *et al*., 2013; Józsi *et al*., 2015). In human, CFHR5, composed of nine SCR domains, which is the longest CFHR protein in the CFHR family (Skerka *et al*., 2013). Although, CFHR5 shares high

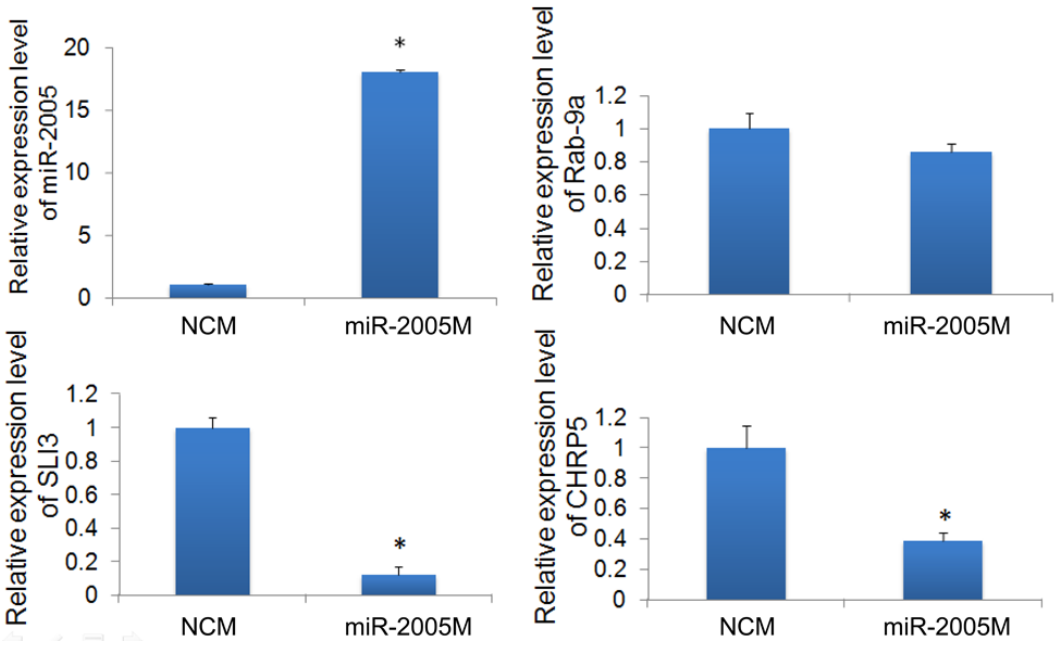

Fig. 4 Relative expression of miR-2005, Rab-9a, SLI3, and CHRP5 in miR-2005 mimics transfected coelomocytes. NCM: Negative control of miRNA mimics; miR-2005M: miR-2005 mimics. * $p < 0.05$.

sequence similarity with factor H. Unlike factor H (FH), CFHR5 can binding to activated C3 and potentially enhance C3 deposition (Zhu *et al.*, 2018). One complement factor H gene and four-factor H-like genes were identified in *Danio rerio* (Sun *et al.*, 2010), which highlights the possibility that all of the five genes were involved in the acute phase due to the fact that they were up-regulated by an LPS challenge (Sun *et al.*, 2010). In our study, the expression pattern of CFHRs was similar to SLI3 that were reached at 4 h and 48 h after *A. japonicus* infected by *V. splendidus*. By using an overexpression experiment of miR-2005 at cellular levels, significant negative correlation expression profiles were detected between miR-2005 and *SLI3* and *CFHR5*. It's suggested that the abnormal expression of mir-2005 may have great effect in *V. splendidus*-challenged *A. japonicus* by targeting *SLI3* and *CFHR5*.

Based on the transcriptome and bioinformatics analysis, a total of 506 target genes of miR-2005 were predicated, and 187 targets were annotated. Significant negative correlation expression profiles were detected between miR-2005 and the two immune-related targets (*SLI3* and *CHRP5*) in *V. splendidus*-challenged *A. japonicus* through qRT-PCR by overexpression experiment of miR-2005. In conclusion, this study will strengthen our understanding of the miR-2005 regulatory role in *A. japonicus* after it is *V. splendidus* challenged.

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Supplementary Table 1 The sequences of target genes of miR-2005

Supplementary Table 2 The predicted binding sites for the miR-2005

Supplementary Table 3 Detailed annotation of miR-2005 target