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Minichromosome maintenance protein 7 regulates phagocytosis in kuruma shrimp *Marsupenaeus japonicas* against white spot syndrome virus

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ABSTRACT

Minichromosome maintenance protein (MCM7) belongs to the MCM protein family and participates in the MCM complex by playing a role in the cell replication cycle and chromosome initiation of eukaryotes. Previously, we found that several genes, including *MCM7*, were over-expressed in *Drosophila melanogaster* after white spot syndrome virus (WSSV) infection. In this study, we aimed to further research the *MCM7* of kuruma shrimp, *Marsupenaeus japonicus* (*mjMCM7*) and determine its role in the innate immune system. To this end, we cloned the entire 2307-bp *mjMCM7* sequence, including a 1974-bp open reading frame (ORF) encoding a 658-aa-long protein. Real-time PCR showed that the gene was primarily expressed in the hemolymph and hepatopancreas and over-expressed in shrimp challenged with WSSV. Gene function study was carried out by knocking down the expression of *MCM7* using small interference RNA (siRNA). The results revealed that *β-actin*, *hemocyanin*, prophenoloxidase (*proPO*) and tumor necrosis factor-α (*TNF-α*) were up-regulated while the cytoskeleton proteins such as *myosin* and *Rho* were significantly down-regulated at 24 h after treatment. The results indicate a possible relationship between *mjMCM7* and the innate immune system, and suggest that *mjMCM7* may play a role in phagocytosis. After WSSV challenge, WSSV copies and mortality count were both higher in the *MCM7*-siRNA-treated groups at 60 h after treatment, and the mortality count approached that of the control groups over time. The phagocytosis rate was significantly lower in the *MCM7*-siRNA-treated group than in the WSSV group. The findings of this study confirm that *mjMCM7* positively regulates phagocytosis and plays an important role against WSSV. These results could help researchers to further understand the function of the MCM7 protein and reveal its potential role in the innate immunity of invertebrates.

1. Introduction

Nucleotide replication is one of the most basic and extremely accurate processes of life in eukaryotes. Many studies have extensively studied nucleotide replication in the budding yeast species *Saccharomyces cerevisiae*. Minichromosome maintenance (MCM) proteins are key elements that function as part of the pre-replication complex to initiate DNA replication in eukaryotes [3,4]. MCM proteins were first found in *S. cerevisiae* [1–3]. The MCM protein complex includes MCM2–7, which form a hexamer [4,5]. Three more members of the MCM family, namely, MCM8 [6,7], MCM9 [8–10] and MCM10 [11–13], have recently been identified. The main function of this complex is to bind to chromatin from the S phase to the G1 phase, ensuring that replication occurs only once per cell cycle, and extends to transcription, chromatin remodeling, and checkpoint responses. MCM proteins are potential diagnostic and prognostic markers for various human tumors [14]. Recent studies have reported a novel function of MCMs in cancer cells, where MCMs were found to be significantly up-regulated and MCM7 was found to serve as an efficient prognostic marker and a potential therapeutic target in human cancer [15–18].

In our previous study, we found a clue between the expression of MCM7 and phagocytosis in *Drosophila melanogaster* using DNA microarray [19]. Here, we aimed to further research MCM7 by studying the role of this molecule in the innate immune system of kuruma shrimp.

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2. Methods and materials

2.1. Shrimp and tissue preparation

Healthy shrimp were obtained from Jinjiang aquatic product market and temporarily kept in a 50 L tank containing sea water with an air pumpat room temperature in our laboratory. The white spot syndrome virus (WSSV) was prepared and used immediately for the challenge according to a previous report [20]. The hemolymph, muscle, gill, heart, hepatopancreas and digestive tract tissues of healthy and pathogen-challenged shrimp were collected for RNA isolation immediately and kept on ice to prevent RNA degradation.

2.2. Rapid amplification of cDNA ends (RACE)

The shrimp were killed by immersion in cold sea water, and healthy muscle tissue was obtained and used for total RNA isolation with the miRNA isolation kit (Ambion by Life Technology, USA), according to the manufacturer's protocol. Special cDNA for RACE was synthesized using the 5′–3′ RACE Kit (Roche) immediately after RNA isolation to avoid degradation, and the resultant cDNA was stored at −20 °C.RACE is an efficient PCR-based approach for the full-length extraction of DNA. Gene-specific primers (GSP) were designed according to the core sequence of *mjMCM7*, which was extracted before amplification of the 3′-end of *mjMCM7* using Primer Premier 5.0. Based on the results of the 3′-RACE, we designed SPs for 5′-RACE using Primer Premier 5.0 software. The primers used in this study are listed in Table 1, and arrows indicate the direction of transcription.

The RACE-amplified gene fragments were divided using 1% agarose gel in TAE buffer, and the specific fragments were purified with DNA Gel Purification Kit (Genery, China) according to the manufacturer's protocol. Briefly, 4 μL of purified product and 1 μL of pMD-19 (Simple) vector (Takara, Japan) was added to 5 μL of Solution 1 (Takara, Japan) and the amplified fragment was transferred into the vector and expressed in *E. coli*. A single herd confirmed by PCR was cultured and sent for sequencing (Biosune, China).

After the RACE products were sequenced, the 5′ sequence, 3′ sequence, and the core sequence were assembled using DNAMan software version 6.0 to obtain the full length of mjMCM7. To confirm the uncertain acid amino within the assembled fragments, a pair of primers, designated MCM7-F and MCM7-R, were designed using the online primer design tool on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.3. Nucleotide sequence and bioinformatics analyses

Similarities in the nucleotide sequences were analyzed by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Protein prediction was performed using the online ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Multiple sequence alignment was performed by DNAMAN 6.0. the phylogenetic tree based on the amino acid sequences was performed by ncbi BLAST tool (Blast Tree View) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The functional domains were predicted using the SMART database (http://smart.embl-heidelberg.de/).

2.4. Expression analysis by real-time PCR

We then assayed the expression levels of *mjMCM7* in multiple organs of both healthy and pathogen-challenged shrimp by real-time quantitative PCR using SYBR green. We also measured variations in expression across different organs over time after pathogen challenge. Briefly, the total RNA was extracted from different organs by EASY spin tissue/cell RNA extraction kit (Aidlab, China) following the manufacturer's protocol. Experiments were performed in triplicate, and at least three shrimp were analyzed for each tissue type. cDNA synthesis was carried out using less than 200 μg of total RNA with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code: FSQ-301; Toyobo, Japan). The synthesized cDNA was stored at −20 °C. RT-qPCR was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method by Office Excel, with *GAPDH* amplification as the internal control. The design and synthesis of the RT-qPCR primers were entrusted to Generay (Shanghai, China), based on the open reading frame (ORF). Table 2 lists the primers that were designed and synthesized by Generay.

2.5. siRNA-mediated silencing of gene expression

We performed an *in vitro* experiment to knock down the expression of *mjMCM7*in order to explore its role in the innate immune system. Briefly, three fragments of short-interfering RNAs (siRNA): siRNA-448 (GCACTGAAGTGAAGCCCAT), siRNA-653 (GGAACACTCTGATCAGGTT) and siRNA-1060

Table 1
Primer sequences and purpose.

Name	Nucleotide Sequence (5′–3′)	Purpose
3′ GSP-1	GGAGCACTGTTGTCTCGTTTCG	First primer for 3′RACE
3′ GSP-2	GCGGATTCTGACAGGACTGCTATT	Nest primer for 3′RACE
5′ GSP-1	TGGGTTGTAGCGTCCATAAGCAGGG	First primer for 5′RACE
5′ GSP-2	GGGCATTTCACCTGTTAGGGGATCTTTC	Nest primer for 5′RACE
5′ GSP-3	GGGAGTCAGACCGACACCACTGGAACCT	Third primer for 5′RACE
MCM7-F	TGAGGTGATGGAGCAACAGAC	FWD primer for full length re-confirming
MCM7-R	TTTGAACTGGGTGGGTGGTT	RVS primer for full length re-confirming
Oligo448-1	GATCACTAATACGACTCACTATAGGGGCACTGAAGTGAAGCCCATTT	448 Oligo DNA-A synthesis
Oligo448-2	AAATGGGCTTCACTTCAGTGCCCCTATAGTGAGTCGTATTAGTGATC	448 Oligo DNA-A synthesis
Oligo448-3	AAGCACTGAAGTGAAGCCCATCCCTATAGTGAGTCGTATTAGTGATC	448 Oligo DNA-B synthesis
Oligo448-4	GATCACTAATACGACTCACTATAGGGATGGGCTTCACTTCAGTGCTT	448 Oligo DNA-B synthesis
Oligo653-1	GATCACTAATACGACTCACTATAGGGGGAACACTCTGATCAGGTTTT	653 Oligo DNA-A synthesis
Oligo653-2	AAAACCTGATCAGAGTGTTCCCCCTATAGTGAGTCGTATTAGTGATC	653 Oligo DNA-A synthesis
Oligo653-3	AAGGAACACTCTGATCAGGTTCCCTATAGTGAGTCGTATTAGTGATC	653 Oligo DNA-B synthesis
Oligo653-4	GATCACTAATACGACTCACTATAGGGAACCTGATCAGAGTGTTCCTT	653 Oligo DNA-B synthesis
Oligo1060-1	GATCACTAATACGACTCACTATAGGGGCATCAACATCTGCCTTATTT	1060 Oligo DNA-A synthesis
Oligo1060-2	AATATTCCGTCTACAACTACGCCCTATAGTGAGTCGTATTAGTGATC	1060 Oligo DNA-A synthesis
Oligo1060-3	AAGCATCAACATCTGCCTTATCCCTATAGTGAGTCGTATTAGTGATC	1060 Oligo DNA-B synthesis
Oligo1060-4	GATCACTAATACGACTCACTATAGGGATAAGGCAGATGTTGATGCTT	1060 Oligo DNA-B synthesis