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The role of oncoprotein NM23 gene from *Exopalaemon carinicauda* is response to pathogens challenge and ammonia-N stress

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ABSTRACT

Oncoprotein NM23, as a family of genes encoding the nucleoside diphosphate (NDP) kinase, plays important roles in bioenergetics, DNA replication, differentiation and tumor metastasis. In this study, a full-length cDNA of NM23 (designated EcNM23) was cloned from *Exopalaemon carinicauda* by using rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of EcNM23 was 755 bp, which contains an open reading frame (ORF) of 518 bp, encoding a 175 amino-acid polypeptide with the predicted molecular weight of 19.60 kDa and estimated isoelectric point of 7.67. The deduced amino acid sequence of EcNM23 shared high identity (86%–93%) with that of other crustaceans. a NDP kinase super family signature was identified in *E. carinicauda* EcNM23. Quantitative real-time RT-qPCR analysis indicated that EcNM23 was expressed in all the examined tissues with the high expression level in hemocytes and ovary. The EcNM23 expression in immune-related tissues changed rapidly and reached peak at different time after pathogens (*Vibrio parahaemolyticus* and WSSV) challenge and ammonia-N stress treatment. The results suggested that EcNM23 might be associated with the immune defenses to pathogens infection and ammonia-N stress in *E. carinicauda*.

1. Introduction

The ridgetail white prawn *Exopalaemon carinicauda* is an economically important shrimp species naturally distributed in the coasts of the Yellow Sea and the Bohai Sea, China, which contributes to one third of the gross output of the polyculture ponds in eastern China [1]. Due to its commercial value, “milky shrimp” disease caused by *Hematodinium* [2], immune gene discovery by expressed sequence tags (ESTs) [3] and transcriptome analysis [4], and identification of immune-related genes such as heat shock protein (HSP90) [5], selenium dependent glutathione peroxidase (GPx) [6] and calreticulin (CRT) [7] have been studied in *E. carinicauda*. However, with the development of intensive culture and the ecologic environmental deterioration, various diseases caused by pathogens and environmental stresses have blossomed within booming in cultured shrimp populations, causing economic losses to commercial shrimp aquaculture [6,7]. Previous studies have demonstrated that the suboptimal environmental conditions could affect the immunity of *E. carinicauda*, for example, pH and ammonia stress could cause affect the immune response of HSP90 [5], pathogens challenge could induce the immune-related genes such as GPx [6] and CRT [7]. Therefore, better understanding of the innate immune abilities and immune defense mechanisms of shrimp will be beneficial to the development of health management in shrimp aquaculture.

Biosynthesis of non-adenine nucleoside triphosphates is critical for bioenergetics, DNA replication, sugar and lipid biosynthesis, and signal transduction pathways, and this function is achieved by nucleoside diphosphate (NDP) kinase (EC 2.7.4.6) [8,9]. Oncoprotein NM23 is a functional NDP kinase, which catalyzes phosphoryl transfer c-phosphate from nucleoside triphosphate (NTP) to diphosphate (NDP) [10,11], and involved in several cellular functions, such as the control of transcription, DNA repair and cellular proliferation, differentiation, apoptosis, and invasion suppression [12,13]. NM23 was initially identified as a potential tumor

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metastasis inhibitor, with subsequent studies showing that it belongs to a structurally large and functionally conserved gene family [14]. The NM23 family of proteins can be separated into two groups based on their sequence homology, such as group I (NM23-H1 to H4) and group II (NM23-H5 to H8) [15]. The NM23 genes of group I possesses the classic enzymatic activity of NDP kinase, however, only one product of the group II genes, NM23-H6, has been demonstrated to catalyze the NDP kinase reaction [16]. In crustaceans, only one isoform of NDP kinase has been reported [11,12,17], and has documented functions in various stress and immune response, for example, NM23 transcripts were increased significantly in *Eriocheir sinensis* after *Vibrio anguillarum* challenge [12], and up-regulated upon WSSV challenge in *Litopenaeus vannamei* [8,17].

Previous studies have demonstrated that the suboptimal environmental conditions, such as ammonia nitrogen (ammonia-N) stress could affect the immunity [18,19], growth and molting [20], oxygen consumption and ammonia excretion of crustaceans [21]. *Vibrio parahaemolyticus* and WSSV caused the most serious disease leading to major losses in the shrimp aquaculture industry around the world [7,22]. For example, acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), which causative agents was *V. parahaemolyticus*, has caused large scale losses in farmed shrimp production [22–24], influenced the antioxidative status and caused oxidative stress and tissue damage via confusion of antioxidant enzymes [25], and induced the expression level of c-Fos and c-Jun gene in shrimps [26]. So far there are still no effective preventive and therapeutic measures against AHPND/EMS diseases. Shrimps lack an adaptive immune system and their defense mechanisms mainly rely on innate immune responses for protecting them against invaders and the environmental stresses. Nucleoside kinases are antiviral targets of several pathogens, because they can process the activation of nucleotide or nucleoside analogues [8]. However, none is known about the potential role of EcNM23 in the ridgetail white prawn *E. carinicauda* against pathogens challenge (such as *V. parahaemolyticus* and WSSV) and ammonia-N stress.

The aim of this study was to clone the full-length cDNA of NM23 from hemocytes of *E. carinicauda*, compare its sequence with other known NM23s, investigate the expression pattern of EcNM23 in various tissues of *E. carinicauda*, and evaluate its expression in immune-related tissues of *E. carinicauda* after pathogens (*V. parahaemolyticus* and WSSV) challenge and ammonia-N stress. These results will be essential to understand the role of EcNM23 in immune response against pathogens challenge and ammonia-N stress in *E. carinicauda*.

2. Materials and methods

2.1. Animals materials

Healthy adult *E. carinicauda*, averaging weight 1.33 ± 0.32 g, were collected from a commercial farm in Qingdao, China. They were cultured in 200 L polyvinyl chloride polymer (PVC) tanks with filtered aerated seawater (salinity 30‰, pH 8.2) at 22 ± 0.5 °C for 7 days before processing. There were 100 shrimps in each group. The shrimps were fed daily with a ration of 10% of body weight, and two-thirds of the water in each group was renewed once daily.

2.2. RNA extraction and cDNA synthesis

Hemocytes were collected with a syringe which contained an equal volume of anti-coagulant buffer (1.59 g sodium citrate, 3.92 g sodium chloride, 4.56 g glucose, 0.66 g EDTA-2Na, 200 mL ddH_2O) [27], and centrifuged at 800 g, 4 °C for 15 min. Total RNA was extracted from hemocytes using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA samples were analyzed in 1.0% agarose electrophoresis and quantitated at 260 nm, all OD_{260}/OD_{280} were between 1.8 and 2.0. The 3′ and 5′ ends RACE cDNA template were synthesized using SMART™ cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

2.3. Cloning the full-length cDNA of EcNM23

An EST sequence corresponding to NM23 was obtained from *E. carinicauda* hemocytes cDNA library of our laboratory (GenBank accession no. JK996340), and has been reported by Duan et al. (2013) [3]. BLAST analysis showed that it has high identities with NM23s of other crustaceans. According to the EST sequence, a gene specific primer F1 was designed for 3′ RACE, and primer R1 was designed for 5′ RACE (Table 1) and its 3′ and 5′ ends were obtained using SMART RACE cDNA Amplification Kit (Clontech, USA). For 3′ RACE, the PCR reaction was performed using the primer F1 and the anchor primer UPM (Table 1). The 50 μL PCR reactions contained the cDNA 2.5 μL of template, 10× Advantage 2 PCR buffer 5 μL, dNTP Mix (10 μmol/L) 1 μL, 50× Advantage 2 Polymerase Mix 1 μL, primer UPM (10 μmol/L) 5 μL, primer F1 (10 μmol/L) 1 μL, PCR-Grade water 34.5 μL. The PCR reaction conditions were 5 cycles of 94 °C for 30 s, 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. For 5′ RACE, the PCR reaction was performed using the primer R1 and the anchor primer UPM (Table 1). The PCR reaction systems and conditions were the same as described above.

The PCR fragments were subjected to electrophoresis on 1.5% agarose gel to determine length differences, and the target band was purified by PCR purification kit (Promega, USA). The purified products were cloned into PMD18-T vector, following the instructions provided by the manufacturer (TaKaRa, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

2.4. Sequence analysis

The nucleotide and deduced amino acid sequences of EcNM23 cDNA were analyzed and compared using the BLAST search programs (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP/). The multiple sequence alignment of NM23 amino acid sequences was performed using the programs of Vector NTI advance 10.3 (Invitrogen). A phylogenetic NJ tree of NM23s was constructed by the MEGA 4.0 software [28].

Table 1
Primer sequences used in this study.

Primer name	Sequence (5′–3′)
EcNM23	
F1 (forward)	GAACGCACTTTCATCGCCGT
R1 (reverse)	ACAGCTACACAGTCAGTCCT
F2 (forward)	CCTTTCTACCCAGGACTTTGC
R2 (reverse)	CCATCATTACACGGGCTGTT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC
18S rRNA	
18S-HF	TATACGCTAGTGGAGCTGGAA
18S-HR	GGGGAGGTAGTGACGAAAAAT
WSSV	
F3 (forward)	ACAATGGTCCCGTCCTCATC
R3 (reverse)	TGCCTTGCCGGAAATTAGTG
Probe (T)	TET-CAGAAGCCATGAAGAATGCCGTCTATCAC-TAMRA