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Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*

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ABSTRACT

As fundamental immunologic mechanism, the innate immunity system is more important than the specific immunity system in teleost fishes during pathogens infection. Antimicrobial peptides are integral parts of the innate immune system, and play significant roles against pathogens infection. NK-lysin, the compounds of the natural killer cells and cytotoxic T cells, are potent and effective antimicrobial peptides widely distributed in animals. In this study, we reported the sequence characteristics, expression profiles and antibacterial activities of a NK-lysin gene (Lc-NK-lysin) from a commercially important marine fish, the large yellow croaker (*Larimichthys crocea*). The open reading frame of Lc-NK-lysin cDNA sequence was 447 bp in length, coding 148 amino acids. The genomic DNA of Lc-NK-lysin has the common features of NK-lysin family, consisting of five exons and four introns, and in its deduced mature peptide, there are six well-conserved cysteine residues and a Saposin B domain. Lc-NK-lysin was expressed in all tested tissues (skin, muscle, gill, brain, head kidney, heart, liver, spleen, stomach and intestine) with different expression patterns. In pathogens infection the expression profiles of Lc-NK-lysin varied significantly in gill, head kidney, spleen and liver, indicating its role in immune response. Two peptides (Lc-NK-lysin-1 and Lc-NK-lysin-2) divided from the core region of the Lc-NK-lysin mature polypeptide were chemically synthesized and their antibacterial activities were examined; the potential function on the inhibition of bacteria propagation was revealed. Our results suggested that Lc-NK-lysin is a typical member of the NK-lysin family and as an immune-related gene it involves in the immune response when pathogens invasion.

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1. Introduction

Compare to higher vertebrates, the innate immune system in teleosts plays a foundational and primary role in immune response because their adaptive immunity was immature and not effective during infections by a wide range of pathogens [1]. The antimicrobial peptides (AMPs) are short polypeptides with multiply biological functions and widely present in almost all organisms. AMPs are regarded as noteworthy candidates to prevent and treat diseases in teleosts, especially to antibiotic-resistant strains and drug-resistant pathogens [2].

NK-lysin is a novel and potent AMP with notable activities to against pathogens such as bacteria, fungi, viruses, parasites, and tumor cells, and harmless to the red blood cells [3–5]. As the homologue of granulysin, NK-lysin is also the member of the Saposin-like protein family, which released from the natural killer cells and cytotoxic T lymphocytes [4,6–9]. NK-lysins were characterized by the surfactant-associated protein B (Saposin B) domain as well as six similarly located cysteine residues, and the features were found in different species, from teleosts to mammals [10–13]. Because of its notable activities, NK-lysin may become a hopeful candidate to combat with those drug-resistant pathogens in teleosts.

NK-lysin was initially founded in the upper small intestine from porcine [7], and had been identified in several higher vertebrates such as bovine [14], equine [15], chicken [16] and water buffalo [17]. In teleosts, NK-lysin was found in channel catfish (*Ictalurus punctatus*) [10], Japanese flounder (*Paralichthys olivaceus*) [11], half-smooth tongue sole (*Cynoglossus semilaevis*) [13] and zebrafish (*Danio rerio*) [18]. Even though NK-lysin has been identified and

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described in teleosts, the studies on its immune responses and antibacterial activities were still insufficient.

In this study, we described a NK-lysin gene (Lc-NK-lysin) isolated from the large yellow croaker (*Larimichthys crocea*), a commercially important marine fish in China. The gene structure, expression patterns and antibacterial activities of Lc-NK-lysin were investigated. Because of the serious diseases caused by the outbreak of ciliate protozoan *Cryptocaryon irritans* in the large yellow croaker, this study may give an insight into developing a new measure to prevent the diseases infection.

2. Materials and methods

2.1. Experimental sampling and database built

Tissues from the large yellow croaker were collected in June 2012 from Ningde (Fujian Province, China) according to the method described previously for the following tests [19]. Briefly, 100 healthy individuals of the large yellow croaker (average body weight of 85.5 ± 15.1 g and average body length of 19.2 ± 1.3 cm) were purchased and acclimatized for two weeks before sampling. Subsequently, five individuals were randomly captured, and ten tissues (skin, muscle, gill, brain, head kidney, heart, liver, spleen, stomach and intestine) were collected and saved in RNAfixer (BioTeke) immediately. Meanwhile, another 60 healthy individuals were randomly selected for the artificial infection assays. *Cryptocaryon irritans* was originally separated from the gill and skin of the infected individuals of the large yellow croaker that cultured in enclosure culture area of Ningde during outbreak of *C. irritans* in May 2011. After identification, the trophonts of *C. irritans* were propagation according to the standard method [20]. The 60 healthy individuals were immersed into 500 L seawater with *C. irritans* pathogen suspension, with the concentration about 26,665 theronts for every individual. All the 60 individuals were transferred into fresh seawater without *C. irritans* pathogen after 4 h challenge. The third to fourth day after challenge, all the infected individuals were transferred into another cement pool to avoid reinfection by *C. irritans*. The fifth to seventh day post infection, the trophonts had fall off from the infected individuals and the skin hyperaemia and fester were the common symptoms, indicated the hosts were infected by bacteria in seawater spontaneously.

Seven time points (6 h, 24 h, 48 h, 72 h, 96 h, 120 h and 168 h post *C. irritans* infection) were selected to collect tissues. In each time point, 5 individuals were randomly captured and then the four tissues (gill, head kidney, spleen and liver) were sampled.

To examine the discrepant gene expression pattern of the large yellow croaker, the liver samples from both healthy and 3 d post infected individuals were preserved and performed the transcriptome sequencing analysis (Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China). As a result, the liver transcriptome database was constructed, and a NK-lysin gene sequence was found based on the gene annotation results. This NK-lysin gene was named as Lc-NK-lysin.

2.2. cDNA and DNA cloning of Lc-NK-lysin

Based on the initial sequence of Lc-NK-lysin gene, specific primers were designed to amplify partial sequence. The PCR conditions were as follows: initial denaturation for 5 min at 94 °C, followed by the step: 94 °C for 45 s, 52 °C for 45 s 72 °C for 1 min, cycled 30 times in total, and finally extension for 10 min at 72 °C. Subsequently, the PCR products were separated by 1% agarose gel (BIOWSET) in TBE electrophoretic buffer solution. The fragment was subsequently purified and connected with pMD19-T vectors (TaKaRa) before cloned into Trans1-T1 phage resistant chemically competent cell (TransGen). Individual colonies were sequenced (Sangon Biotech Co., Ltd., Shanghai, China). According to the verified sequence, specific primers were designed to extend both 3′ and 5′ untranslated region (UTR) of the sequence using rapid amplification of cDNA ends (RACE) method. Corresponding genomic fragment was eventually amplified to distinguish the exons and introns distribution of Lc-NK-lysin gene. All the primers used in gene cloning were synthesized (BGI, Beijing, China) and the detailed sequences were listed in Table 1.

2.3. Molecular characterization analysis

The nucleic acid sequences from vector were identified and removed using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Sequence similarity analysis was performed using Basic Local Alignment Search Tool (BLAST) grogram (http://blast.ncbi.nlm.nih.gov/Blast.cgi). An open reading frame (ORF) search tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict the ORF region of Lc-NK-lysin gene and deduced its amino acid composition. To determine the location and length of introns in its DNA sequence, the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi) was used to perform the mRNA and genomic sequences alignment. Signal peptide cleavage sites in Lc-NK-lysin amino acid sequence was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Secondary structure and specific domains of the Lc-NK-lysin polypeptide were predicted using the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) and Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/), respectively. Multiple sequences alignment and analysis were performed using BioEdit software (version 7.09). The phylogenetic tree was constructed with MEGA software (version 6.05) using the Neighbor-Joining method, and bootstrapped 10,000 times.

2.4. Tissues expression profiles and inducible expression profiles of the Lc-NK-lysin gene

In order to study the tissue expression profiles of Lc-NK-lysin gene under normal physiological condition, the ten tissues (see Section 2.1) were tested. The tissue expression and pathogen-induced expression of the Lc-NK-lysin gene were quantitative analyzed using an Applied Biosystems 7500-FAST quantitative PCR instrument in 96-well formats. Based on the result of Lc-NK-lysin mRNA and genomic alignment, specific primers were designed, and the β-actin gene was tested and selected as reference (Table 1). Both of the primer sequences were across the exon-intron boundaries in order to avoid the genomic DNA interference. The reagents used in RNA extraction, first-strand cDNA synthesis and real-time quantitative PCR amplification were produced by TaKaRa Co., Ltd. (Japan). The response procedures were as follows: 95 °C for 5 s, 55 °C for 30 s and 72 °C for 33 s, 40 cycles in total. Template-free reactions were used as blank control. Each sample measurement repeated three times.

Serial 5-fold dilutions method was used to generate the standard curve of target and reference genes. The experimental data processed using the $2^{-\Delta\Delta Ct}$ method [22]. Specificity analysis of the PCR products was performed according to the melting curve examined results. The significance testing method used in statistical processing was one-way ANOVA, as significance at p value less than 0.05, and data were expressed as the means ± SD.

2.5. Antibacterial activity of the synthetic Lc-NK-lysin peptides

2.5.1. Polypeptides identification and synthetic

Based on the predicted secondary structure, two sequences

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