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Short communication

Characterization and evolutionary analysis of duplicated C7 in miiuy croaker

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ABSTRACT

The complement system, as one of the most sophisticated innate immune system, plays an important role in defense against invading microorganisms. The complement component C7 participates in the cytolytic phase of complement activation through a series of polymerization reactions with other terminal complement components. In this study, we derived two C7 genes from the whole genome of miiuy croaker which were the consequence of the fish-specific genome duplication. Our data showed that miiuy croaker C7-1 and C7-2 genes shared same structure domains. The analysis of gene synteny showed that high degree conserved of synteny was retained between miiuy croaker and other teleosts, and miiuy croaker had a relatively closer relationship with fugu. The expression of C7-1 and C7-2 in miiuy croaker healthy tissues revealed that they were ubiquitously expressed in all ten tested tissues. Besides, the immune response of C7-1 and C7-2 were different in spleen with *Vibrio anguillarum*, *Staphylococcus aureus*, poly I:C and LPS at 24 h post-injection, respectively. Furthermore, the expression patterns of C7-1 and C7-2 were different in liver, spleen and kidney after infected with *V. anguillarum* at different time-point. Evolutionary analysis showed that all the ancestral lineages underwent positive selection except for the ancestral lineages of fish C7-2, indicated that the ancestral lineages of fish C7-1 genes undertook more pressures than C7-2 in defense against the invading microorganisms. Meanwhile, a series of maximum likelihood methods were used to explore the evolutionary patterns on extant vertebrates' C7 genes. Three and one positive selection sites were found in extant mammalian C7 genes and fish C7-2 genes, but no positive selection site was found in extant fishes C7-1 genes. The result showed that extant fish C7-2 genes undertook more pressures compared with C7-1. In conclusion, fish C7-1 and C7-2 gene underwent different evolutionary patterns.

1. Introduction

Immune responses are mediated by two general systems: adaptive immunity and innate immunity. Adaptive immunity can date back to the early period of vertebrates evolution, between the divergence of cyclostomes and cartilaginous fish [1]. Innate immunity system is a very old defense mechanism, and it provides the first line of defense before the adaptive immune system comes into play [2]. Fish, the first of true vertebrates, have evolved adaptive immune responses similar to those in higher vertebrates, but the adaptive immunity in fish is inefficient, therefore they should make full use of the well-developed innate immune system to compensate for their rudimentary antibody response [3]. The complement system, one of the most sophisticated innate immune system [4,5], plays a major role during inflammation and infection, as well as a link between innate and adaptive immunity [6]. The complement activated by the classical, lectin or alternative pathway leads to the formation of the membrane attack complex (MAC) on the surface of complement-opsonized cells [7]. The assembly of MAC involves the aggregation of the lytic complement components C5b, C6, C7, C8 and C9 [8]. C5b connects with C6 via a metastable binding site to form a soluble C5b-C6 complex in the vicinity of the activating cell. Subsequent C7 interacts with the C5b-C6 to produce C5b-7, a trimolecular complex that allows insertion of this complex into the target cell membrane [9,10].

Among MAC components, the terminal complement components (TCC) C6, C7, C8α, C8β and C9 belong to the same gene

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family as perforins and they may have emerged through a series of duplications of an ancestral gene [11]. Consequently, they share several common structural motifs such as thrombospondin (TSP), low-density lipoprotein receptors class A (LDLa), epidermal growth factor precursor (EGF) and the MAC/perforin segment (MACPF). These domains are conserved and also present in teleost counterparts [12]. In addition, mammalian C7 possesses complement control protein (CCP) motifs and factor I MAC (FIMAC) modules in the C-terminal domain except for these conserved motifs [13]. As one member of TCC, C7 fulfills a crucial role in the hydrophilic–amphiphilic transition of MAC, so it plays an essential role in the elimination of invading pathogens. In previous studies, the full set of TCC genes had been identified in teleost, amphibian reptilian/aves and mammals except for the avian C9 gene which was not found in the draft genome sequence of chicken [14]. Besides, two C7 genes were identified in rainbow trout [15], although gene duplication was a common evolutionary event, it was the first time to find gene duplication in the C6–C9 gene family.

Miiuy croaker (*Miichthys miiuy*), as one member of family Sciaenidae, mainly distributes from the Western Japan Sea to the East China Sea. Currently, the molecular immunology, genetics and molecular evolutionary of immune genes were studied more in-depth in this species [16–19]. Besides, we have been studying complement system. In our previous studies, the complement component Bf/C2, C3, C4 and C9 genes had been identified and analyzed in miiuy croaker [20–22]. Now we are interested in complement component C7 gene which fulfills a crucial role in the hydrophilic–amphiphilic transition of MAC. In this study, two complement component C7 genes (C7-1 and C7-2) were identified from the whole genome database of miiuy croaker which were in consequence of the fish-specific genome duplication (FSGD or 3R). And we were the first time to analyze the gene synteny of C7-1 and C7-2 in teleost which could provide information for the investigation of the evolutionary relationships and evolutionary mechanism of C7 genes. Furthermore, the expression patterns of C7-1 and C7-2 genes were also analyzed to illuminate the possible role of C7-1 and C7-2 in response to different pathogens infection. Considering the unique status of complement component in evolutionary process of innate and adaptive immunity, we also analyzed the molecular evolution of C7 gene in fishes and mammals.

2. Material and methods

2.1. Sequence analysis and phylogenetic tree construction

In order to identify the C7-1 and C7-2 genes, we used available fish genes as queries to search for the transcriptome [23] and whole genome database of miiuy croaker (unpublished data) by local Blast software. The retrieved reconstructed transcripts were translated using ORF Finder (http://www.ncbi.nlm.nih.gov) and GENSCAN [24]. The predicted open reading frames (ORFs) were verified by BLASTP against NCBI non-redundant protein sequence database. The potential protein domains of amino acid sequences were calculated by SMART program [25]. All sequences from other organisms used in this study were derived from GenBank and Ensemble database (Supplementary table S1). All the gene sequences were aligned under codon model by MUSCLE software for its high accuracy and speed [26]. Phylogenetic tree was constructed with the Bayesian approach by MrBayes v3.2 [27] which was running 5,000,000 generation with 25% of trees burned. For Bayesian inference, the GTR + I + G model was regarded as the best-fit model by Bayesian information criterion (BIC) using jModeltest software [28].

2.2. Molecular evolutionary analysis

The nonsynonymous and synonymous rate ratio ω (dN/dS) stands for the change of selective pressure. The $\omega = 1$, <1, and >1 are indicative of neutral evolution, purifying selection and positive selection, respectively. To investigate the evolutionary process of C7-1 and C7-2 genes, the selective pressure was analyzed by CODEML of PAML software [29] and the Hyphy package of Data Monkey Web Server [30]. All the models of PAML and Hyphy package used in this study were carried out as described by Zhu et al. [31].

2.3. Fish sampling, challenge experiments and RNA extraction

Miiuy croakers were obtained from Zhoushan Fisheries Research Institute (Zhejiang, China). All fishes were held in aerated water tanks to allow for acclimatization and evaluation of overall fish health before using in experiments. Only fishes with similar size and body weight were used. One week later, the challenge experiments of miiuy croaker were intraperitoneally infected with *Vibrio anguillarum*, *Staphylococcus aureus*, poly I:C and LPS. Fish samples were randomly divided into two groups, injection and control groups. In injection group, fishes were injected with 1 ml of *V. anguillarum* (1.5×10^8 CFU/ml), poly I:C (2.5 mg/ml), LPS (1.0 mg/ml) and *S. aureus* (1.5×10^8 CFU/ml), respectively. Meanwhile, the control fishes were injected with 1 ml of physiological water. For injection group with *V. anguillarum*, fish samples were killed at 6 h, 12 h, 24 h, 36 h, 48 h and 72 h post-injection, respectively. While injection group with poly I:C, LPS and *S. aureus*, fish samples were killed at 24 h post-injection, respectively. Three tissues (liver, kidney and spleen) of infection were removed and stored at −80 °C, at the same time ten tissues (liver, kidney, spleen, heart, eye, fin, brain, gill, muscle and intestines) of uninfected were also removed and keep at −80 °C. Total RNA were extracted from the various tissues of adult individuals by Trizol reagent (Qiagen) following the manufacturer's instructions. cDNA was synthesized using QuantScript RT Kit (TIANGEN) according to the manufacturer's protocol.

2.4. miRNA and mRNA transcriptomes and the prediction of target gene

Total RNA of spleen tissue which infected with *V. anguillarum*, poly I:C, LPS and *S. aureus* after 24 h post-injection and the control group were used to prepare the miRNA and mRNA transcriptomes in our Lab (unpublished). These libraries were used for the deep sequencing by an Illumina platform according to the manufacturer's protocol. In order to predict the miRNA target gene of miiuy croaker C7-1 and C7-2, the miRNA target gene database miRGen 3.0 was used to select the corresponding miRNA that exhibited complementarily with mRNA sequencing. We found that miR-27a-3p and miR-1306 were perfect complementary with miiuy croaker C7-1 and C7-2, then we analyzed the expression patterns of these two miRNA using the deep sequencing data.

2.5. Expression analysis of C7-1 and C7-2 genes

Three pairs of primers were used to study the expression patterns of C7-1 and C7-2 genes (Supplementary table S2). Healthy tissues and infected tissues were determined using qRT-PCR. The qRT-PCR was run on a 7300 real-time PCR system (Applied Biosystems, USA) using a RealMaster Mix kit (TIANGEN). The reaction carried out without the template was used as blank control. Cycling conditions were as follows: 15 min at 95 °C, followed by 45 cycles consisting of 15 s at 95 °C and 60 s at 60 °C, dissociation curve analysis was performed after each assay to determine target