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Characterization of NLR-A subfamily members in miiuy croaker and comparative genomics revealed NLRX1 underwent duplication and lose in actinopterygii

Jinrui Li [a], Lingcong Kong [c], Yunhang Gao [c], Changwen Wu [b, **], Tianjun Xu [a, *]

[a] Laboratory of Fish Biogenetics & Immune Evolution, College of Marine Science, Zhejiang Ocean University, Zhoushan, 316022, China
[b] National Engineering Research Center of Marine Facilities Aquaculture, Zhejiang Ocean University, Zhoushan, 316022, China
[c] College of Animal Science and Veterinary Medicine, Jilin Agriculture University, Changchun, 130118, China

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ABSTRACT

The NOD-like receptors (NLRs, nucleotide-binding domain and leucine-rich repeat containing receptors) are a recently identified family of intracellular pathogen recognition receptors in vertebrates. Several subfamilies of NLRs have been characterized in mammals and implicated in immunity and apoptosis, but studies of NLRs in teleost species have been lacking. Here we analyzed three NLR-A subfamily members from miiuy croaker: NLRC3, NLRC5, and NLRX1. Structural analysis showed that miiuy croaker NLR-A subfamily members own the feature of 5′UTR intron which may influence their role in enhancing translation level. Comparative analysis revealed NLRX1 duplicated into NLRX1a and NLRX1b, then NLRX1a was lost in actinopterygii and NLRX1b formed NLRX1 that now we called. Simultaneously, molecular evolutionary analysis indicated that the ancestral lineages of NLRX1 in tetrapod and actinopterygii under positive selection pressure. The positively sites in actinopterygii are mainly located in NACHT domain which was the critical region for signal transduction, suggesting that the evolution of NLRX1 gene in the ancestor of actinopterygii is beneficial in immune response. Pathogens challenge demonstrated that the expressions of NLRC3 and NLRC5 in miiuy croaker were induced not only by *Vibrio anguillarum* but also by poly (I:C), whereas NLRX1 exhibited more sensitive response to bacteria than virus.

1. Introduction

The innate immune system in vertebrates has diverse pattern-recognition receptors (PRRs) which are mainly distributed in extracellular, membrane and cytoplasmic compartments to recognize pathogen-associated molecular patterns (PAMPs) and trigger inflammatory pathway [1]. Both the major extracellular PRRs such as complements and major membrane-associated PRRs like Toll-like receptors (TLRs) are proficient at detecting extracellular PAMPs, while NOD-like receptors as a group of intracellular proteins, are responsible for the recognition of intracellular PAMPs or other cytosolic danger signals [2,3]. The NOD-like receptors contain three domains: the C-terminal leucine-rich repeat (LRR) domain, the central nucleotide oligomerization (NACHT) domain and the N-terminus which includes the pyrin domain (PYD), caspase recruitment domain (CARD) or baculovirus inhibitor of apoptosis repeat domain (BIR) [4].

To date, at least 22 members of NLRs have been identified in human genome including 5 NLR-A subfamily members: NOD1, NOD2, NLRC3 (NOD3), NLRC5 (NOD4), NLRX1 (NOD5) and 14 NALPs, along with 3 divergent members [5]. NLRC5 contains the largest number of LRRs as the largest member of NLR family with 204 kDa molecular weight in human [6]. Recent work reported that NLRC5 is a potent negative regulator of NF-κB and type I interferon signaling pathways [7] while another study defines a positive function for NLRC5 in anti-viral innate immune responses [8]. The function of NLRC5 remains to be further clarified in both the innate and adaptive immune responses [9]. NLRX1 was demonstrated to prevent mitochondrial anti-viral immunity by inducing reactive oxygen species production [10,11]. In animals, NLRs play a crucial role in innate immunity against pathogenic microbes, but very few

* Corresponding author.
** Corresponding author.
E-mail addresses: wucwzjou@163.com (C. Wu), tianjunxu@163.com (T. Xu).

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studies have been carried out for NLRC3, NLRC5 and NLRX1 in fish and the expression in response to various pathogens is still unclear. Only one report described five NLR-A subfamily genes expression induced by intracellular bacterial and virus pathogens in fish [12].

NLRs have developed evolutionarily conserved proteins and play important roles in defense against microbial pathogens among members of the animal kingdoms [13]. Several studies have been reported concerning the origin and evolution of NLRs [14]. Comparative genome-wide analyses revealed plant and animal NLRs have independently arisen in evolution [15]. Furthermore, both of NLR and TLR families have underwent large expansions and extensive domain recombination in various lineages, indicating a consequence of convergent evolution is a common phenomenon in the evolution of innate immunity [16]. However, so far, no studies have been conducted on the evolution of NLRs in vertebrates by comparative analysis of gene synteny and molecular evolutionary analysis.

The diseases caused by bacteria and virus are serious in aquaculture of miiuy croaker (*miichthys miiuy*) resulting in economic damages [17]. Identification of miiuy croaker immune-related genes and researches on better understanding of the defense mechanism has been studied and reported [18–23]. Researches on intracellular PRRs in fish were poor and the immune responses to bacteria and virus are still unclear. We have characterized NOD1 and NOD2 in miiuy croaker and the expression analysis demonstrated that NOD1 and NOD2 play an important role in the innate immune response against pathogens [24]. In the present study, we identified NLRC3, NLRC5 and NLRX1 in miiuy croaker to examine expression patterns in tissues and immune responses to *Vibrio anguillarum* and poly (I:C) infection. Otherwise we conducted an overview of genomic organizations and domains of five NLR-A subfamily members in miiuy croaker and proposed the evolution mechanism of NLRX1 in vertebrates using comparative genomics with other species. Meanwhile, molecular evolution was analyzed to test the positive selection in individual NLRC3, NLRC5 and NLRX1. We were the first to comprehensively analyze five NLR-A subfamily genes in fish, being beneficial for comprehensive understanding NLRs family.

2. Materials and methods

2.1. Identification of NLRC3, NLRC5 and NLRX1 in miiuy croaker

To obtain the NLRC3, NLRC5 and NLRX1 cDNA and gene sequences from miiuy croaker (we named the related gene as mmNLRC3 in this study), some closely related species from GenBank (Table S1) were used to conduct a query set to search the miiuy croaker transcriptome [25] and whole genome database (unpublished data) using local BLASTn and tBLASTn programs. The cDNA sequences were aligned with the obtained corresponding scaffold to determine the genomic structure of the three NLR-A subfamily members by MAFFT [26]. The nucleotide sequences of NLR-A subfamily members in miiuy croaker have been deposited in GenBank database and the other sequences in vertebrates used in this study were all listed in Table S1.

2.2. Sample preparation and pathogenic challenge

Healthy miiuy croakers (mean weight 800 g) were collected from Marine Fish Market (Zhoushan, China) and reared in aerated water tanks with a flow-through seawater supply at ambient temperature 25 °C for about one week before experimentation. After one week of acclimatizing, only the determined healthy fish with similar size and body weight were used for further experiment. The selected fish were stimulated by intraperitoneal injection of 1 ml *V. anguillarum* (1.2×10^8 CFU/ml) and 1 ml ploy (I:C) (2.5 mg/ml), respectively and the control group was injected with physiological saline. The injected fish samples were killed at 6 h, 12 h, 24 h and three tissues (liver, head kidney and spleen) were removed and stored at −80 °C until RNA isolation. Total 13 healthy tissues (liver, spleen, head kidney, intestine, blood, skin, eye, gill, brain, heart, muscle, stomach and gonad) of the other selected healthy fish were also sampled. Total RNA was conducted using Trizol reagent (Qiagen) following instructions of the manufacturer and cDNA was synthesized utilizing QuantScript RT Kit (TIANGEN) according to manufacturer's descriptions.

2.3. Sequences analysis and phylogenetic tree construction

SMART program [27] and Compute pI/Mw tool (http://web.expasy.org/compute_pi/) were used to predict the potential protein domains of amino acid sequences and compute the theoretical pI (isoelectric point) and Mw (molecular weight). We obtained all the other species NLR-A subfamily sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and Ensemble Genome Browser (http://www.ensembl.org/) database. MEGA5 program [28] and DNAman [29] were used for the multiple alignments based on the deduced amino acid sequences of NACHT domain. The phylogenetic tree of five NLR-A subfamily members in several species was conducted by the neighbor-joining (NJ) method with bootstrapped 1000 times performing MEGA5. Synteny maps for the genomic neighborhoods surrounding NLRC3, NLRC5 and NLRX1 in other species were obtained from Genomicus [30] and Ensembl Genome Browser. Using these genes as queries, we identified the presence of adjacent genes in miiuy croaker NLRC3, NLRC5 and NLRX1 genomic regions using BLAST programs.

2.4. Molecular evolutionary analysis

We analyzed the selective pressures imposed on ancestral branches of tetrapod and actinopterygii in NLRC3, NLRC5 and NLRX1 genes using the maximum likelihood (ML) methods in CODEML program of PAML 4 [31]. Firstly, the one ratio model which assumes all branches have only one ratio was used to determine the selective pressures. Then compared with the one-ratio model, the free-ratio model which allowed the varied ω ratio on each branch, was utilized to check whether this model more suitable for the data by the likelihood ratio test (LRT). Finally, branch-site model was mainly designed to detect the interested foreground lineages. The site model including six site models (M0, M1a, M2a, M3, M7 and M8) to test the possible positive selection sites imposed on the tetrapod and actinopterygii, therein the Bayes empirical Bayes (BEB) in the models M2a and M8 to analyze the Bayesian posterior probability (BPP) of the codon sites under a positive selection [32]. Twice the differences of log-likelihood values (2ΔlnL) can evaluate significant different between models using the likelihood ratio test (LRT).

2.5. Gene expression analyses

Four pairs of primers (NLRC3-RT-F/R, NLRC5-RT-F/R, NLRX1-RT-F/R, β-actin-RT-F/R) were designed to study the expression level and β-actin was used as internal control (Table S2). We conducted PCR reaction by Veriti Thermal Cycler (Applied Biosystems, USA) to test the expression levels in thirteen healthy tissues of miiuy croaker as semi-quantitative analysis and then the PCR products were analyzed in a 2% agarose gel.

qRT-PCR was used to determine the expression pattern of NLRC3, NLRC5 and NLRX1 genes in injected miiuy croaker three immune tissues (liver, spleen, head kidney) by 7300 Real time PCR