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A genome-wide survey of expansive NLR-C subfamily in miiuy croaker and characterization of the NLR-B30.2 genes

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ABSTRACT

NOD-like receptors (NLRs) are essential intracellular pattern-recognition receptors that respond to pathogens and regulate innate immunity. NLRs include three distinct subfamilies: NLR-A, NLR-B and NLR-C, thereinto, NLR-C as a large subfamily is unique to bony fish and little research about it has been done. In the current study, we identified the members of NLR-B and NLR-C subfamilies containing 2 and 48 genes respectively in miiuy croaker. Compared with other teleosts except for zebrafish, NLR-C subfamily genes occurred expansion in miiuy croaker. The gene expansions of NLR-C subfamily may illustrate adaptive genome evolution in response to specific aquatic environments. Structural analysis showed that the N-terminus of NLR-C subfamily receptors has different characteristics of the domains including RING domain, FISNA domain or PYRIN domain. Interestingly, the C-terminus of 18 NLR-C subfamily members contains an extra B30.2 domain (named NLR-B30.2 genes) which plays an important role in antiviral immune recognition. Simultaneously, molecular evolutionary analysis indicated that the positively sites in miiuy croaker are mainly located in NACHT domain which was the vital region for signal transduction in immune response. Significantly, pathogens challenge in spleen and macrophages demonstrated that NLR-B30.2 genes exhibited more sensitive response to virus than bacteria, suggesting these genes play enhanced roles in innate antiviral immunity, which may represent a new family used for antiviral infection.

1. Introduction

Vertebrates have an innate immune system to differentiate self from nonself by using diverse pattern recognition receptors (PRRs) which are mainly distributed in extracellular, membrane and cytoplasmic compartments. There are multiple families of PRRs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Hayashi et al., 2010). Nucleotide-binding domain and leucine-rich repeat containing family receptors (NLRs) are a family of intracellular sentinels due to lack of signal peptides and transmembrane domains, involved in both defense against pathogenic microorganisms and cellular damage (Shiau et al., 2013; Wilmanski et al., 2008). NLRs are composed by the presence of characteristic tripartite domains; an N-terminus effector domain that is involved in protein-protein interactions and signal transmission, a central nucleotide oligomerization NACHT (or nucleotide binding oligomerization, NOD) domain and a C-terminal leucine-rich repeat (LRR) domain (Maharana et al., 2013). NLRs are classified into three distinct subfamilies in non-mammalian vertebrates: NLR-A, NLR-B and NLR-C subfamilies. NLR-A subfamilies have been characterized in some fish including channel catfish (Sha et al., 2009; Rajendran et al., 2012; Li et al., 2012) grass carp (Chen et al., 2010), miiuy croaker (Li et al., 2015a, 2015b), Whereas researches on NLR-B and NLR-C subfamily in fish were poor. B30.2 (PRY-SPRY) domain as a characteristic domain of NLR-C subfamily plays an important role in immune recognition. B30.2 domain, the combination of SPRY domain and PRY domain, is maintained as a component of immune defense (Rhodes et al., 2005). Few reports have been conducted on NLR-C subfamily genes expression induced by pathogens in fish (Unajak et al., 2011; Biswas et al., 2016), and the expression in response to bacterial and viral pathogens is still unclear.

Around 20 members of NLRs were identified in mammalian including 22 NLRs in human and 34 in mice that are divided into NOD subfamily, NALPs and divergent members (Laing et al., 2008). Recent studies have reported some members of this family exist in invertebrates and lower vertebrates. NLRs contain more than 200

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members in the purple sea urchin (Hibino et al., 2006) and the lamprey (Smith et al., 2013) genome encodes 34 NLR genes. NLRs as large, complex family, the expansion is not confined to these species and has also occurred in teleost fish. Such as zebrafish and two puffer fish has undergone at least partially separate expansions (Laing et al., 2008). Both of NLRs and TLRs have experienced massive species-specific expansions and domain shuffling that all fits the common phenomenon of parallel evolution (Zhang et al., 2010). Otherwise, NLRs have independently developed in evolution among plant and animal kingdoms and play diverse roles in defense against microbial pathogens (Jacob et al., 2013; Kaparakis et al., 2007). But few studies have been carried out for the evolution of NLRs in fish by molecular evolutionary analysis.

Miiuy croaker, *Miichthys miiuy*, as an economically important high-valued marine fish species, is vulnerable to bacterial and viral invasion, there have been increasingly severe outbreaks of infectious disease (Cheng et al., 2011). Identification and expression analysis of some immune-related genes in miiuy croaker have been carried out and reported (Sun et al., 2012; Xu et al., 2011, 2012; Zhu et al., 2013). Considering the fact that NLRs are implicated in innate recognition, it is worthwhile to explore the genomic structure of NLR family genes and their expression pattern. In the present study, we searched the NLRs in miiuy croaker at whole genome level and analyzed their genomic structure. Expression patterns in spleen of infected fish with *Vibrio anguillarum* and poly (I:C) and in macrophages stimulated with lipopolysaccharide (LPS) and poly (I:C) were both examined. Lastly, molecular evolution was analyzed to verify the positive selection in individual NLR-B30.2 genes. We were the first time to comprehensively research the NLR-C subfamily genes and show the functional responses in tissue and cell to clarify the possible immune recognition in fish that will be beneficial to better understand NLRs and pave the way to elucidate the mechanism by NLR-C genes mediated immune defense against bacterial and viral infection.

2. Materials and methods

2.1. Fish sampling and pathogenic challenge

Healthy miiuy croaker individuals (approximately weight 800 g) were received from Zhoushan Fisheries Research Institute (Zhejiang, China) and maintained in aerated water tanks at 25 °C for about one week to adapt to ambient environment before experimental manipulation. The selected healthy fish with similar size and body weight were injected with 1 ml suspension of *Vibrio anguillarum* (1.5×10^8 CFU/ml) and 1 ml poly (I:C) (2.5 mg/ml) (Xu et al., 2010). Simultaneously, uninfected fish were injected with 1 ml physiological saline and maintained in separate tanks as the control group. The infected and control fish samples were all dissected at 6 h, 12 h, 24 h and 48 h post-injection and the tissue spleen were removed and then kept at −80 °C until RNA isolation (Wang et al., 2015).

Furthermore, total RNA of spleen which was stimulated with LPS, *V. anguillarum*, *Vibrio harveyi*, *Staphylococcus aureus*, and poly (I:C) at 24 h and control group (uninfected fish) was used to conduct mRNA transcriptomes library in our laboratory (unpublished). The library was applied for the deep sequencing by an Illumina platform in the light of the manufacturer's protocol. Subsequently, we analyzed the digital expression patterns of these NLR-B30.2 genes using the deep sequencing data that were compared to those of control group.

2.2. Macrophages isolation and cell-stimulation with LPS and poly (I:C)

Macrophages were isolated from head kidney which was removed from the miiuy croaker aseptically after swabbed with 70% alcohol. Specifically, the removed head kidney was placed in ice-cold L-15 cell culture medium which contains high concentration antibiotics (400 IU/ml penicillin and 400 μg/ml streptomycin). Then the minced samples were filtered through a 100 μm nylon mesh in L-15 medium containing penicillin (100 IU/ml), streptomycin (100 μg/ml), 2% foetal bovine serum (FBS) and heparin (20 U/ml) to obtain macrophages. The macrophages were separated with a 51% Percoll gradient and the cells were resuspended in the cold L-15 medium and used for later experiments. For stimulation experiments, 4×10^7 macrophages were cultured in a 6-well plate with 30 μg/ml of LPS (Sigma, USA) and 10 μg/ml poly (I:C) at 20 °C for 3 h, 12 h and 24 h, respectively. The harvested macrophages were centrifuged at 800 g for 5 min at 4 °C, and stored at −80 °C for RNA extraction.

2.3. RNA isolation and cDNA synthesis

Total RNA of every sample was extracted from immune tissue spleen and macrophages including the control and pathogen-challenged groups using Trizol reagent (Qiagen) according to manufacturer's instructions. The cDNA was obtained utilizing QuantScript RT Kit (TIANGEN) following manufacturer's protocol, and then was stored at −20 °C until later use.

2.4. Database mining

To ensure the entire set of NLR family encoding genes from miiuy croaker, various approaches were used to collect lists of candidate genes. We utilized the closely related species NLR-C genes (Table S1 of Supporting information) as a query set to search the miiuy croaker transcriptome (Che et al., 2014) and whole genome database (Xu et al., 2016) by local BLASTn and tBLASTn programs. To further confirm the accuracy of miiuy croaker NLR-B and NLR-C subfamily sequences, the corresponding identified scaffolds were predicted by softberry software. The cDNA sequences were aligned with the obtained scaffold using MAFFT (Katoh and Standley, 2013).

2.5. Sequences analysis

The potential protein domains of NLR-B and NLR-C subfamily amino acid sequences were predicted by SMART (Letunic et al., 2006). The two subfamily sequences of other species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and Ensemble Genome Browser (http://www.ensembl.org/) database. The identity and similarity of NLR-B30.2 amino acids in miiuy croaker were performed by MatGAT2.01 (Campanella et al., 2003). A multiple alignment based on the deduced amino acid sequences of B30.2 domain were generated by MEGA5 program (Tamura et al., 2011) and DNAman (Woffelman, 1994), meanwhile conservation, quality and consensus were performed by jalview (http://www.jalview.org/). A phylogenetic tree of NLR members was conducted based on amino acid sequences according to the neighbor-joining (NJ) method with bootstrapped 1000 times of MEGA5. Domain architecture of B30.2 domain in miiuy croaker NLR-C subfamily was predicted by SWISS-MODEL Repository (http://swissmodel.expasy.org/repository/) software.