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Short communication

Molecular characterization, expression and functional analysis of NOD1, NOD2 and NLRC3 in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The nucleotide-binding oligomerization domain proteins NOD1, NOD2 and NLRC3 are cytoplasmic pattern recognition receptors (PRRs) of the Nod-like receptor (NLR) family. In the present study, the Nile tilapia (*Oreochromis niloticus*) *NOD1* (*ntNOD1*), *NOD2* (*ntNOD2*) and *NLRC3* (*ntNLRC3*) genes were cloned and characterized. The full-length *ntNOD1*, *ntNOD2* and *ntNLRC3* genes were 3924, 3886 and 4574 bp, encoding 941, 986 and 1130 amino acids, respectively. The three Nod-like receptors have a NACHT domain and a C-terminal leucine-rich repeat (LRR) domain. In addition, *ntNOD1* and *ntNOD2* have a N-terminal CARD domain (*ntNOD2* has two). Phylogenetic analysis showed that the three NLRs are highly conserved. Tissue expression analysis of the three receptors revealed that the highest mRNA and protein levels of *ntNOD1*, *ntNOD2* and *ntNLRC3* were in the spleen. The expression patterns of NLRs during embryonic development showed that the expression levels of *ntNOD2* and *ntNLRC3* significantly increased from 2 to 8 days post-fertilization (dpf). The expression levels of *ntNOD1* significantly increased from 2 to 6 dpf, decreased at 7 dpf and then increased at 8 dpf. Upon stimulation with an intraperitoneal injection of *Streptococcus agalactiae*, expression levels of the *ntNOD1*, *ntNOD2* and *ntNLRC3* mRNA and protein were clearly altered in the blood, spleen, kidney, intestine and gill. Furthermore, after cotransfection with an NF-κB reporter plasmid, NF-κB activation in ntNOD1-overexpressing 293T cells significantly increased compared with that in control cells, before or after i-EDPA-stimulation. By contrast, compared with control, ntNOD2 and ntNLRC3 had no effect on NF-κB activation in 293T cells, when their potential ligands were not stimulated. However, after MDP-stimulation, ntNOD2 and ntNLRC3 overexpression increased NF-κB activation in 293T cells. NOD1 and NLRC3 were uniformly distributed throughout the cytoplasm in 293T cells, whereas NOD2 was distributed throughout the cytoplasm and nucleus. Our results indicate that the three Nod-like receptors are functionally conserved and may play pivotal roles in defense against pathogens such as *Streptococcus agalactiae*.

1. Introduction

The innate immune system is the first line of defense in the fish immune response. In this system, the recognition of microbial pathogens mediated by pattern recognition receptors (PRRs) is critical to the initiation of innate immune responses. The PRRs recognize microbial pathogens through sensing the conserved molecular structure of a pathogen, known as pathogen-associated molecular patterns (PAMPs), and induce subsequent host immunity through multiple signaling pathways that contribute to the eradication of the pathogen [1]. PAMPs include lipopolysaccharides (LPS), polyinosinic: polycytidylin acid (Poly: IC), lipopeptide, peptidoglycan (PGN), flagellin, dsRNA, ssRNA and CpG DNA, among others. The PRRs contain four major classes, including Toll-like receptors (TLRs), Nod-like receptors (NLRs), retinoid acid-inducible gene-1 (RIG-1)-like receptors (RLRs) and C-type lectin receptors (CLRs). The TLRs are the best-known group of innate immune receptors whose function has been reasonably well characterized in different infectious diseases. In contrast, NLRs and RLRs are intracellular cytosolic sensors [2,3] that are not well characterized, especially in lower vertebrates.

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Table 1
Primers that were used in this study.

Primer name	Sequence (5′-3′)	Amplicon length (bp) and application usage
Nod1-sf	TACCACAGAGAGCTGCTCGTGA	Amplification of ntNOD1 partial sequence
Nod1-sr	GTGGAACCGCAGCCGCTTCTCT	Amplification of ntNOD1 partial sequence
Nod1-up1	AACTTTTCTGTGGGCTGGAAT	Amplification of upstream of ntNOD1
Nod1-down1	TGGCAGTTTGCTGAACAATCT	Amplification of upstream of ntNOD1
Nod1-up2	GCCCTCACTCACAACAAAGGA	Amplification of downstream of ntNOD1
Nod1-down2	TGTCTGAAGAAAGCAGCGAGG	Amplification of downstream of ntNOD1
Nod2-sf1	CATCTGGAGAGAGTTCTGGATA	Amplification of partial cDNA fragment of ntNOD2
Nod2-sr1	TAATTTGTTGTTGAAGAGCGCA	Amplification of partial cDNA fragment of ntNOD2
Nod-3-1	TGCAATTCCCTTTACCTCAGA	Amplification of downstream partial sequence of ntNOD2 cDNA
Nod-3-2	TTGAGCTGCATTTATTCAATC	Amplification of downstream partial sequence of ntNOD2 cDNA
NOD2-5-3	CAGCTAGAAAGAGCCCACATGT	5′RACE of NOD2
NOD2-5-4	TTGGTCGTTGAGCCAGAAGAGT	5′RACE of NOD2
NLRC3-sf	ACCGAAGCAAACGCATTACGT	Amplification of partial cDNA fragment of ntNLRC3
NLRC3-sr	ATTGCACCAGCGATAGCTTGA	Amplification of partial cDNA fragment of ntNLRC3
NLRC3-sf2	TCTGAGGGAGAACTCCATTGG	Amplification of downstream partial sequence of ntNLRC3 cDNA
NLRC3-sr2	GGTACCGTTTCAATTAAGTCC	Amplification of downstream partial sequence of ntNLRC3 cDNA
NLRC3-5-1	TCTCCAGCAGCAGACTTTAGA	Amplification of upstream partial sequence of ntNLRC3 cDNA
NLRC3-5-3	TTAATCTGATCCTGCAATCGG	Amplification of upstream partial sequence of ntNLRC3 cDNA
NOD1-sense2	TGGATAACAACAACATCAGCG	Real-time PCR
NOD1-antise2	TACCAATCTTGAGAACTCGCA	Real-time PCR
NOD2-sense1	AAGCAGTGAGCCCATTGTTAA	Real-time PCR
NOD2-antise1	GTTCGCCTGCATTGACTCTAA	Real-time PCR
NLRC-sf2	CCTCTCTGAACGCCTCAACAG	Real-time PCR
NLRC-sr2	GAGCCGATGTTGTTATTCCGG	Real-time PCR
EF-sense	GGAAATCCGTCGTGGATACG	Real-time PCR
EF-antise	AAACTTGGGGCTGTCCTCAA	Real-time PCR
ntNOD1bf	GCCACCATGGATCAGATAAAAGAAGC	Eukaryotic expression
ntNOD1br	CGTGGAACCGCAGCCGCTTCT	Eukaryotic expression
ntNOD2bf	GCCACCATGCTTGCCGAGGAACTAGT	Eukaryotic expression
ntNOD2br	CGAAGATCAGTCTTGATTCCA	Eukaryotic expression
ntNLRC3 bf	GCCACCATGGAGAGGACACATTACGA	Eukaryotic expression
ntNLRC3 br	CGATGTCCACCACGCAGCCGG	Eukaryotic expression
NOD1-ORF1	GAGGTGACTGCTGTCCTGTAA	Amplification of full-length of NOD1
NOD1-ORF2	TCTGTGCATGCGTCATGTGAG	Amplification of full-length of NOD1
NOD2-ORF1	CATGGGCACTGATATGGACAA	Amplification of full-length of NOD2
NOD2-ORF2	TCTTTTGCCAGTGCAATACAA	Amplification of full-length of NOD2
NLRC3-ORF1	GCGGCAGACATATATCAGAGT	Amplification of full-length of NLRC3
NLRC3-ORF2	TCTCTCTGGTGGGCTAGATGT	Amplification of full-length of NLRC3

NLRs play a pivotal role in microbial sensing and lead to the initiation of antimicrobial and antiviral immune responses [4]. A characteristic feature of the NLR family is its three structural domains: a N-terminal protein-protein binding or effector domain, a central nucleotide oligomerization (NACHT) domain and a C-terminal leucine-rich repeat (LRR) domain [5–9]. Based on its N-terminal effector, a unified standard nomenclature for the NLR gene family was recently proposed. In mammals, NLRs are divided into five main subfamilies: NLRA/CIITA, NLRB/NAIP, NLRC, NLRP and NLRX/NLRX1 [10,11]. NOD1, NOD2, and NOD3/NLRC3 belong to the NLRC subfamily.

To date, several Nod-like receptors have been identified in different bony fishes, including the NLR-A, NLR-B and NLR-C families in zebrafish [12], NLRC3 in Japanese flounder, Asian seabass, turbot [13–16], NOD1 in olive flounder [17], two NOD2 in trout [18], NOD1 in miiuy croaker [19], NOD1 and NOD2 in rohu [20,21], NOD1 and NOD2 in grass carp and twenty members of Nod-like receptors in catfish [8,22], NOD1 and NOD2 in orange-spotted grouper [23], NOD1 and NOD2 in mrigal [24], NOD1, NOD2 and Nlrx1 in goldfish [25], NOD1 and NOD2 in miiuy croaker [26], NLRC1-13 in Japanese pufferfish [27], and the NLRC family in miiuy croaker [28].

NOD1 and NOD2 are differentially expressed in all tissues, with notable differences in different fish. For example, NOD1 and NOD2 were highly expressed in the spleen of goldfish [25] and the liver of the miiuy croaker [26], while the highest mRNA levels of NLRC3 were detected in head kidney macrophages (HKMs) of Japanese flounder [14]. The injection of *Vibrio anguillarum* and PolyI:C in miiuy croaker resulted in the up-regulation of NOD1 and NOD2 expression [26]. Treatment of goldfish macrophages with LPS, Poly I:C, MDP, PGN, heat-killed *Aeromonas salmonicida* or *Mycobacterium marinum* differentially altered the expression of the Nod-like receptors NOD1, NOD2 and Nlrx1 [25]. Stimulation by formalin-killed *Edwardsiella tarda*, *Streptococcus iniae*, and lipopolysaccharide (LPS) resulted in increased NLRC expression in Japanese flounder a few hours after stimulation, suggesting that this novel protein is involved in the immediate response against both Gram-positive and Gram-negative bacteria [13]. Similarly, Japanese flounder NLRC3 mRNA expression was up-regulated in response to LPS and *E. tarda* immune challenges [14]. To date, relatively few studies have identified and characterized the ligands of NLRs in bony fish.

In mammals, NOD1 senses the presence of bacterial pathogens through the recognition of peptidoglycan (PGN) molecules that contain meso-diaminopimelic acid (meso-DAP) [29]. NOD2 detects muramyl dipeptide (MDP) in both Gram-positive and Gram-negative bacterial peptidoglycan; both receptors trigger the immune response via the activation of NF-κB [30–32]. NOD1 and NOD2 are very important intracellular pattern recognition receptors in recognizing bacteria or viruses [33]. NLRC3 expression primarily occurs in lymphocytes and is attributed to inhibiting T-cell activity [12,34]. In contrast to most NLRs, which activate innate immunity, mouse NLRC3 inhibits Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-κB [35]. Recent studies have also indicated that mammalian NLRC3 negatively regulates STING-dependent innate immune activation in response to cytosolic DNA, cyclic di-GMP, and DNA viruses and reduces the production of IFN and other cytokines [36]. In zebrafish and rohu, NOD1 recognizes i-EDPA and NOD2 recognizes MDP [37–40]. However, in tilapia, this NLR function remains unknown.

Tilapia is one of the most important freshwater aquaculture species