Developmental and Comparative Immunology 33 (2009) 991–999

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish

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ARTICLE INFO

Article history:
Received 30 January 2009
Received in revised form 3 April 2009
Accepted 27 April 2009
Available online 14 May 2009

Keywords:
NLR
Receptor
Catfish
Fish
Ictalurus punctatus
Immunity
Disease resistance
NACHT
NOD
CARD
Pathogen recognition

ABSTRACT

The NLRs (nucleotide-binding domain and leucine-rich repeat containing family receptors) are a recently identified family of pattern recognition receptors in vertebrates. Several subfamilies of NLRs have been characterized in human, mouse, and zebrafish, but studies of NLRs in other species, especially teleost species, have been lacking. Here we report characterization of five NLRs from channel catfish: NOD1, NOD2, NLRC3, NLRC5, and NLRX1. Structural analysis indicated that the genes were organized in a similar fashion as in the mammals and in zebrafish. Phylogenetic analysis suggested that they were orthologous to the NOD-like subfamily of NLRs. All five NOD-like genes exist as a single copy gene in the catfish genome. Hybridization of gene-specific probes allowed mapping of three NLR genes to the catfish physical map, laying a foundation for genome characterization and for establishing orthologies with NLR genes from other species. These genes are widely expressed in various tissues and leukocyte cell lines. While the majority of the NLR genes appeared to be constitutively expressed, NOD1 was induced after infection with a bacterial pathogen, *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), suggesting its involvement in immunity against the intracellular pathogen.

1. Introduction

In lower vertebrates, the innate immune response is the primary line of defense against many acute infections of pathogens [1]. The innate immune system provides an immediate and rapid response to infection by recognition of foreign molecules that have invaded the host. Specifically, pattern recognition receptors (PRRs) are proficient at detecting exogenous microbial components. These microbial components, termed pathogen-associated molecular patterns (PAMPs), are typically conserved molecular signatures crucial to the survival of the pathogen. Microbial PAMPs include flagellin, lipopolysaccharides, lipoproteins, peptidoglycan, and nucleic acids. Once a PAMP is detected by the host innate immune system, a vigorous response to eliminate the pathogen ensues. This response involves induction of inflammation and further immune signaling cascades including phagocyte recruitment, production of secretory anti-microbial peptides, and stimulation of the adaptive immune system, along with other co-regulatory physiological responses [2,3].

PRRs can be found in the extracellular space, membrane-associated to various host cell types, or in the cytosol. Of the PRRs that have been identified to-date, the Toll-like receptors were the earliest characterized and have since become the focus of many studies. These receptors have been demonstrated to recognize various PAMPs of bacteria, viruses, fungi, and parasites at the cell membrane or the endosomal region [2]. The Toll-like receptors, however, could not account for all host pattern recognition due to

Abbreviations: BIR, baculovirus inhibitor of apoptosis protein repeat; CARD, caspase recruitment domain; CIITA, class IImajor histocompatibility complex, transactivator; HET-E, bacterial nucleotide triphosphatase protein; IPAF, ice protease-activating factor; LRR, leucine-rich repeat; NACHT, NAIPCIITA, HET-E, and TP-1 proteins; NAIP, neuronal apoptosis inhibitory protein; NALP, NACHT domain-leucine-rich repeat-, and PYD-containing protein; NLR, nucleotide-binding domain and leucine-rich repeat containing family receptor; NLRC3, NLR familyCARD domain containing 3; NLRC5, NLR familyCARD domain containing 5; NLRX1, NLR family member X1; NOD, nucleotide-binding oligomerization domain; NOD1, nucleotide-binding oligomerization domain containing 1; NOD2, nucleotide-binding oligomerization domain containing 2; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; PYD, pyrin; TP-1, telomerase-associated protein.

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doi:10.1016/j.dci.2009.04.004

their incapability to recognize some intracellular pathogens. This led to studies suggestive of other PRRs involved in recognition of intracellular pathogens in early host defense [4–6]. The nucleotide-binding domain and leucine-rich repeat containing family receptors (NLRs) were established as a set of proteins capable of inducing inflammation after infection with an invasive form of *Shigella flexneri* in human epithelial cells [4,6]. The NLRs, best characterized in mammals, have since been categorized as cytosolic surveillance molecules in both Gram-positive and Gram-negative intracellular bacterial infections. The NLRs have neither signal peptides nor transmembrane domains, indicative of their localization to the cytosol. Recent studies have also demonstrated NLR responsiveness to extracellular pathogens, which occurs once the specific immunogen is internalized by the host cell and reaches the cytosol [7].

The NLR family was found to share a distinct structural motif similarity to the disease resistance superfamily of proteins (R-proteins) in plants [8]. The NLR and R-proteins share not only structural similarities but also some functional and regulatory similarities, suggesting a common evolution route in the NLR pathway [9]. The typical characteristics of the NLR family include a structure with three domains: (1) an N-terminal effector binding domain, (2) a central NACHT domain (named after NAIP, CIITA, HET-E, and TP-1 proteins), and (3) a leucine-rich repeat (LRR) C-terminus. The C-terminal LRR domain possibly serves as the pattern recognition or ligand-binding site. The centralized NACHT domain is responsible for oligomerization and autoactivation of the molecule. The N-terminus is responsible for protein–protein interaction, signal transduction, and initiation of immune cascades [10]. The NLRs contain either a caspase recruitment domain (CARD), a baculovirus inhibitor of apoptosis protein repeat (BIR) domain, or a pyrin (PYD) domain at the N-terminus.

The presence of the different N-terminal domains divides the NLRs into subfamilies: the NODs (nucleotide-binding oligomerization domain) and IPAF (ICE protease-activating factor) (CARD), the NAIPs (neuronal apoptosis inhibitory proteins) (BIR), and the NALPs (NACHT domain-, leucine-rich repeat-, and PYD-containing proteins) (PYD) [7,10]. An examination of the zebrafish genome suggests that most of the innate components in mammals possess orthologs in fish [11]. This was demonstrated for the NLRs recently, where zebrafish may contain three subfamilies of NLRs: one that resembles mammalian NODs, one that resembles mammalian NALPs, and one unique subfamily of genes with portions that resemble both mammalian NOD3 and NALPs [12]. Obviously, studies in other fishes are needed to elucidate the identity and functions of this family of receptors, particularly considering whole genome duplication events that have occurred during teleost evolution, and various levels of gene duplication resulting from random gene loss after whole genome duplication.

Channel catfish (*Ictalurus punctatus*) is the predominant aquaculture species in the United States (USDA-NASS, 2008; http://www.nass.usda.gov/QuickStats/index2.jsp). The catfish aquaculture industry continues to suffer serious economic losses due to diseases, including enteric septicemia of catfish (ESC) caused by the Gram-negative intracellular bacterium *Edwardsiella ictaluri*. The closely related blue catfish (*I. furcatus*) possesses several superior performance traits over channel catfish, including disease resistance to ESC [13–15]. This makes blue catfish and channel catfish excellent candidates for the investigation of the teleost immune system. The catfish immune system is one of the best characterized from fish species, and it is the only fish species where clonal functionally distinct lymphocyte lines have been established [16–21]. Further, catfish represent a diverse and ancient lineage of fishes [22], and are a natural choice for the study for evolutionary and comparative genomics as well as genome duplication.

A number of innate immune genes have been characterized in catfish including a large number of chemokines [13,23–29] and anti-microbial peptides [23,30–33]. Currently, a few innate PRRs in catfish have been characterized including several Toll-like receptors [34–37], and a few members of the lectin family of proteins [38,39]. However, research on intracellular PRRs in the catfish has been lacking. Here we report the identification and characterization of catfish NLRs homologous to the NOD zebrafish/mammalian subfamily and investigate their expression after the bacterial infection.

2. Materials and methods

2.1. Sequencing and identification of NOD-like genes

Catfish ESTs were downloaded from GenBank dbEST to search for NLR sequences. Sequences were clustered using CAP3 [40] and putative identities assigned using BLASTX against the non-redundant databases at the NCBI. Additional EST sequences recently sequenced from the Joint Genome Institute (clones CBPO016765 and CBPO016765 for NOD1; CBPO1077 and CBPO831 for NOD4; CBZC4133, CBZC23554, and CBCZ12005 for NOD5) were identified using BLAST and included in the CAP3 clustering. NOD-like sequences were identified and partial sequences for NOD1, NLRC5, and NLRX1 homologues were found. In zebrafish, NOD1-5 receptors are referred to as NLR subfamily A: NLR-A1 to NLR-A5 [12]. The NOD1-5 subfamily in mammals has many names in the literature; however, a standard nomenclature was recently recommended by the HUGO Gene Nomenclature Committee (HGNC) for humans and mouse [41]. We will use this nomenclature to refer the catfish homologues for this study. The NOD1-5 subfamily of NLRs in catfish will be named as NOD1, NOD2, NLRC3, NLRC5, and NLRX1, respectively, for standardization (Table 1).

After clustering and BLAST analysis, degenerate primers designed from NLR alignments as well as primers designed from zebrafish sequences were used to amplify NLRs that were absent from the EST clusters (NOD2 and NLRC3, Supplemental Table 1). Total RNA was extracted from a healthy adult catfish liver and spleen using TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's recommendations. The RNA was pooled (250 ng

Table 1
Nomenclature of the NOD subfamily of NLRs.

Common name	Zebrafish subfamily designation	Zebrafish symbol designation	Catfish subfamily designation	Catfish symbol	HGNC approved human subfamily	HGNC approved human symbol	HGNC human domain organization[a]	Human orthologous sequence
NOD1	NLR-A	NLR-A1	NLRC	NOD1	NLRC	NOD1	C-N-L	NP_006083
NOD2	NLR-A	NLR-A2	NLRC	NOD2	NLRC	NOD2	C-C-N-L	NP_071445
NOD3	NLR-A	NLR-A3	NLRC	NLRC3	NLRC	NLRC3	C-N-L	NP_849172
NOD4	NLR-A	NLR-A4	NLRC	NLRC5	NLRC	NLRC5	C-N-L	NP_115582
NOD5	NLR-A	NLR-A5	NLRX	NLRX1	NLRX	NLRX1	X-N-L	NP_078894

[a] The following domain abbreviations are used: C = CARD; N = NACHT; L = LRR, leucine-rich repeat; X = unknown effector domain.

from each tissue) and cDNA synthesized using the SuperScript® First-Strand Synthesis System (Invitrogen). The cDNA was used as templates for PCR with primers designed using zebrafish sequences (Supplemental Table 1). The PCR fragments were cloned using pGEM-T easy vector (Promega, Madison, WI) per the manufacturer's protocol and sequenced using an ABI 3130*XL* sequencer (Applied Biosciences, Foster City, CA). Partial NOD2 and NLRC3 genes were identified using this method. Next, rapid amplification of cDNA ends (RACE) was performed on all 5 NODs to obtain more sequences from both the upstream and downstream regions (primers shown in Supplemental Table 1). RACE was performed using the SMART RACE cDNA amplification kit (Clontech/BD biosciences, Mountain View, CA) per the manufacturer's instructions.

2.2. Sequence analyses

All NOD1-5 protein sequences in HomoloGene (Supplemental Table 2) were accessed from GenBank and used in the sequence analysis along with the sequences of catfish NODs described here (Table 2). Translated catfish NOD protein sequences were used in searches for conserved domains and genetic architecture. This was performed using the Simple Molecular Architecture Research Tool (SMART) web server [42]. Both human and zebrafish NOD1-5 homologues were included for comparison to the catfish NODs. Two zebrafish NOD2 homologues were included, one from GenBank and one from UniProt, as the HomoloGene sequence for this gene appeared truncated.

2.3. Phylogenetic analysis

Translated catfish NOD protein sequences were subjected to phylogenetic analysis. A total of 40 sequences were used for the construction of a phylogenetic tree: the 5 catfish NODs described here, all complete HomoloGenes available in GenBank, along with the zebrafish NOD2 ortholog from UniProt (Supplemental Table 2). The complete amino acid sequences from the public databases were used in this phylogenetic analysis. Protein sequences were aligned using ClustalW. A phylogenetic tree was constructed using MEGA4 [43]. The tree was created using the minimum evolution method. Poisson correction was used with complete deletion of gaps and/or missing data. The quality of the phylogenetic tree was evaluated with 10,000 bootstrap replicates.

2.4. Determination of genomic copy numbers

To determine the genomic copy numbers of each NOD gene in catfish, Southern blot analysis [44] was conducted for each of the five catfish NOD genes. The oligos used to generate gene-specific probes and the probe segments amplified are shown in Supplemental Table 1. Briefly, genomic DNA was extracted from whole blood of three individual adult catfish using the Gentra Puregene blood kit (Gentra-Puregene/Qiagen) according to the manufacturer's protocol. The genomic DNA (10 μg) was digested with restriction endonucleases, *Eco*RI, *Hin*dIII, and *Pst*I (New England Biolabs, Ipswich, MA) and electrophoresed on a 1% agarose gel along with a lambda DNA-*Hin*dIII digested molecular weight ladder (New England Biolabs). After gel electrophoresis, DNA was transferred to a positively charged nylon membrane, cross-linked to the membrane under UV light, pre-hybridized for 2 h with salmon sperm DNA, and hybridized with a ^{32}P-dCTP labeled probe at 65 °C overnight. The membrane was washed twice (2× SSC, 0.1% SDS buffer) and exposed to X-ray film overnight at −80 °C. The membrane was stripped and reused for each of the other NODs. Southern hybridizations were performed with duplicate membranes to corroborate results.

2.5. Mapping of NOD genes to the catfish physical map

The catfish NOD genes were mapped to the BAC contig-based physical map [45] using filter hybridizations. High-density filters of a channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), and screened using radioactively labeled cDNA probes. Each set of filters contained a 10X-genome coverage of the channel catfish BAC clones from BAC library CHORI 212 [46]. Hybridization was performed at 65 °C for 18 h in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). Filters were washed and exposed to X-ray film at −80 °C for 2 days. To assess the filters for false-positives, BAC clones from the filter hybridizations were screened by dot-blotting. Briefly, positive BAC clones from the CHORI 212 library were isolated and cultured [47], and bacterial cultures were spotted on five nylon membranes by pipetting. Each membrane was hybridized separately with the five NOD probes as described above.

2.6. Gene expression analyses

Both reverse transcription PCR (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) were performed to study NOD gene expression. For both methods, total RNA was isolated from each cell line or tissue using the TRIzol reagent (Invitrogen) according to the provided protocol from the manufacturer. After extraction, total RNA was checked for quality and quantity using denaturing agarose gel electrophoresis containing formaldehyde and a spectrophotometer. Total RNA [500 ng/tissue or cell line] was used for the synthesis of first-strand cDNA using the SuperScript® First-Strand Synthesis System (Invitrogen) per the manufacturer's protocol for use in RT-PCR or adjusted to 100 ng and used directly for each qRT-PCR reaction. Primers were designed by using the FastPCR software [48] (Supplemental Table 1).

For RT-PCR, gene-specific primers [5 pmol] and β-actin primers [1 pmol] were both included in each reaction. Each RT-PCR reaction mixture also contained: 1.5 μl 10× buffer, 1.2 μl $MgCl_2$ [25 mM], 0.3 μl dNTP mix [10 mM], 0.15 μl of JumpStart™ *Taq* polymerase (Sigma), and PCR-grade water up to 15 μl total volume. A 32-cycle PCR reaction was used for each RT-PCR, with denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. For RT-PCR, three analyses were performed. RT-PCR products were analyzed on a 1.5% agarose gel. First, expression was tested on a total of 13 different healthy adult catfish tissues. Tissues included blood, brain, gill, head kidney,

Table 2
A summary of cDNAs of the NOD subfamily of NLRs from channel catfish (*Ictalurus punctatus*).

Gene	Nucleotides sequenced	Complete cDNA	ORF (bp)	5′-UTR (bp)	3′-UTR (bp)	Accession numbers
NOD1	4290	Complete	2841	372	1077	FJ004844
NOD2	1705	Partial	N/D	N/D	120	FJ004845
NLRC3 (NOD3)	2624	Partial	N/D	N/D	221	FJ004846
NLRC5 (NOD4)	5970	Complete	5181	73	716	FJ004847
NLRX1 (NOD5)	4007	Complete	3006	153	848	FJ004848

trunk kidney, heart, intestine, liver, muscle, skin, spleen, stomach, and ovary. Second, expression was tested on catfish RNA isolated from 6 different leukocyte cell lines. Cell lines included cytotoxic T-cell line TS32.15, cytotoxic T-cell line TS32.17, T-cell line 28.3, macrophage cell line 42TA, B-cell line 3B11, and B-cell line 1G8. Third, expression was tested on tissues before and after challenge with the bacterium *E. ictaluri*. Healthy catfish head kidney, spleen, and intestine tissues were used along with the same tissues from infected fish. The tissues of infected fish were harvested from catfish at 4 h, 1 day, and 3 days post-infection after a 2 h submersion challenge with *E. ictaluri*. Submersion challenge was performed as previously described [49].

For qRT-PCR, three replicates were performed on each control and treatment RNA. Triplicate qRT-PCR reactions were also performed on each sample using the β-actin as an internal control. For this analysis, healthy catfish spleen and intestine tissues were used along with those from infected fish. A one-step qRT-PCR was performed using a LightCycler 1.0 real-time cycler (Roche Applied Science, Indianapolis, IN) and the Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications [49]. Briefly, all reactions were carried out in a 10-μl total reaction volume (9 μl master mix and 1 μl of 100 ng RNA template). The master mix contained 4.3 μl H_2O, 0.6 μl $Mn[OAc]_2$, 0.3 μl of each primer (0.1 μg/μl), and 3.5 μl of the SYBR Green mix. The same cycling parameters were used for all tested genes and time-points: (a) reverse transcription, 20 min at 61 °C; (b) denaturation, 30 s at 95 °C; and (c) amplification at 50 cycles: 5 s at 95 °C, 5 s at 52 °C, 20 s at 72 °C. Since non-specific DNA labeling was used (SYBR Green), melting curve analyses were performed for each reaction to confirm amplification of a single amplicon. Crossing point (Ct) values were automatically generated, and each sample was compared to its control by relative quantification using the Relative Expression Software Tool (REST v.2005) [50] assuming 100% PCR efficiencies. REST software normalized the data and generated up- or down-regulated expression values at a significance level of $p < 0.05$.

3. Results

3.1. Identification of the catfish NOD cDNAs

NOD1, NLRC5, and NLRX1 partial sequences were initially identified from catfish ESTs ([51]; Z. Liu, unpublished data). Complete cDNAs of these genes were then obtained by RACE. NOD2- and NLRC3-related sequences could not be found from the ESTs. Therefore, zebrafish corresponding cDNA sequences were used to design primers to amplify these cDNAs in catfish. After PCR, the amplicons were cloned and sequenced. Similar comparison using BLAST indicated the amplified segments were partial cDNA sequences of NOD2 and NLRC3. 5′- and 3′-RACE were then used to obtain the complete cDNAs of these two genes. Sequence analysis revealed that the 3′-RACE led to the cloning of the entire downstream portions of the two cDNAs; however, repeated 5′-RACE failed to obtain the complete cDNA from the 5′-end (Table 2), presumably because of strong secondary structures.

3.2. Sequence of the catfish NOD cDNAs

As summarized in Table 2, complete cDNAs were sequenced for NOD1, NLRC5, and NLRX1; partial cDNAs were sequenced for NOD2 and NLRC3. The cDNA sequences have been deposited into GenBank with consecutive accession numbers of FJ004844–FJ004848, for NOD1, NOD2, NLRC3, NLRC5, and NLRX1, respectively. The cDNA of NOD1 is 4290 bp long with 372 bp of 5′-untranslated region (UTR), 1077 bp of 3′-UTR, and an open reading frame of 2841 bp encoding 946 amino acids. The 1705-bp partial cDNA sequence of NOD2 included partial coding sequence and a 120-bp 3′-UTR, but lacked the upstream coding sequence of approximately 150 amino acids assuming that the length of the catfish gene is similar to those from other vertebrate species. This cloned catfish NOD2 segment lacks the 2xCARD domain and portion of the NACHT domain from the cDNA sequence. The 2624-bp partial cDNA sequence of NLRC3 included part of the coding region and a 221-bp 3′-UTR, but lacked the upstream coding

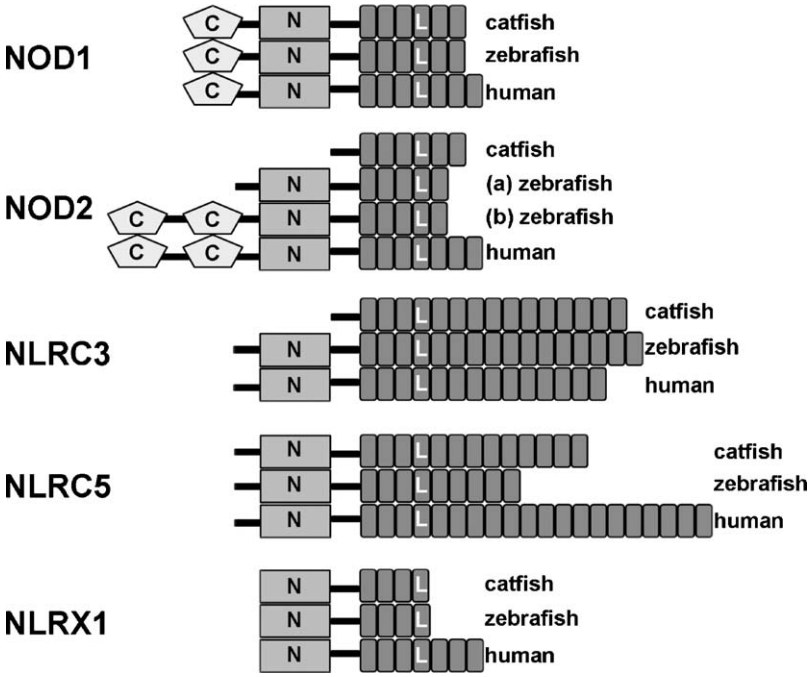

Fig. 1. A schematic presentation of organizations of the NLR genes. The NLR gene sequences of catfish, zebrafish, and human were analyzed using the SMART web server with the NOD domains indicated. Two zebrafish NOD2 homologues were used in the comparison. The accession numbers of these sequences are summarized in Supplemental Table 2. Symbols used are: C = CARD domain; N = NACHT domain; L = LRR or leucine-rich repeat region. Each box represents the number of LRRs detected in each gene.

sequence of approximately 300 amino acids assuming that the length of the catfish gene is similar to those from other vertebrate species. This catfish NLRC3 segment lacks the 5′ effector domain and a portion of the NACHT domain from the cDNA sequence. The complete cDNA sequence of NLRC5 (NOD4) was 5970 bp long including a 73-bp 5′-UTR, a 5181-bp ORF, and a 716-bp 3′-UTR. The complete cDNA sequence of NLRX1 (NOD5) was 4007 bp long including a 153-bp 5′-UTR, a 3006-bp ORF, and an 848-bp 3′-UTR (Table 2).

3.3. Structure of the catfish NOD receptors

The NOD subfamily in catfish appears to be well conserved through vertebrate evolution. Analysis using SMART revealed that the catfish cDNAs had very similar domains and architectures as the zebrafish and human counterparts, with a few exceptions (Fig. 1). For NOD1, the complete cDNA is characterized in catfish, and the domains and organizations are the same as those of the human and zebrafish genes. The human NOD1 contains an additional LRR. For NOD2, the catfish cDNA was only partially characterized, but has the LRR region and a partial region up to the NACHT domain. In zebrafish, the HomoloGene listed in GenBank was truncated at the 5′-end from the reference sequence provided by UniProt (Supplemental Table 2), evident after a pairwise alignment (using bl2seq in GenBank) of the two genes (data not shown). Therefore we included both genes in our SMART analysis, and we observed that the HomoloGene is indeed truncated at the 5′-end, missing the 2xCARD domain structure (Fig. 1, zebrafish (a)). The zebrafish NOD2 (b) and the human ortholog contain the expected CARD–CARD–NACHT–LRR domain as listed by HUGO (Table 1). The catfish NOD2 is partial, missing the CARD and NACHT domains. For NLRC3, the zebrafish and human genes share structural similarity in their NACHT–LRR domains, however, the expected CARD domain was not detected at the 5′-end of the genes. In catfish, the LRR regions are well conserved, but the CARD–NACHT domains are absent from this partial sequence. For NLRC5, the complete cDNA in catfish was obtained. Gene structures are again well conserved, but no CARD domain was detected using the domain searches based on sequence homology. The human sequence appears to contain a much longer LRR region. In NLRX1, the complete catfish cDNA is characterized. Its gene structure is similar to that of zebrafish, but with fewer LRR than in the human gene (Fig. 1).

3.4. Phylogenetic analysis

A phylogenetic tree was constructed using the mammalian, zebrafish, and the catfish NODs. In all cases, the catfish NODs fell within their corresponding clades, confirming their identities as suggested by similarity comparisons (Fig. 2). Overall, NLRX1 is the most conserved among the NODs analyzed, followed by NOD1 and NOD2. In all cases, the catfish NODs fell within the same clades with the zebrafish NODs, compatible with their closely related phylogenetic relationship. There is another sequence in the GenBank similar to zebrafish NLRC3 (XP_687605). However, this zebrafish sequence did not fall into the same clade with catfish NLRC3, but rather was more divergent from the catfish NLRC3 than the mammalian NLRC3 (data not shown). BLAST searches allowed the identification of a NLRC3 sequence from zebrafish (XP_001920433) that fell into the teleost clade, suggesting that it was the orthologous sequence for NLRC3 (Fig. 2).

3.5. Genomic copies of NOD genes

Gene copy numbers for each of the catfish NOD genes were determined using Southern blot analyses. All five catfish NODs have a single copy in the catfish genome (Fig. 3). All Southern blots contained a single hybridization band, with the exception of NOD2 and NLRC3, which contain two bands when *Pst*I was used. However, both contain one *Pst*I restriction site within the probes, and therefore, two bands were expected for a single copy gene.

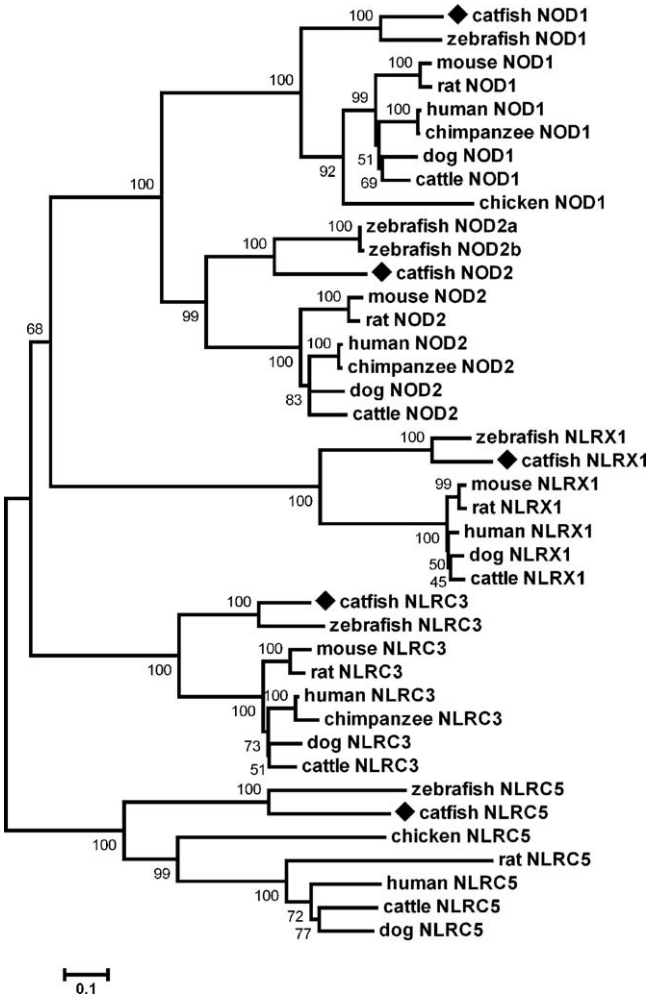

Fig. 2. Phylogenetic analysis of the catfish NLR genes. All NLR-related sequences were downloaded from GenBank and aligned using ClustalW software and a minimum evolution tree was created using the MEGA4 software. The consensus tree is shown with 10,000 replicates from the bootstrap test. Numbers next to branches are percentages of bootstrap values. The genes used in the analysis are listed in Supplemental Table 2.

3.6. Mapping of NOD genes to the catfish physical map

NOD gene-specific probes were used to map the catfish NLR genes to the catfish physical map [45]. The hybridization results are summarized in Supplemental Table 3. NOD1 probes hybridized to 8 BAC clones, but these clones were unmapped to the physical map. NOD2 probes hybridized to 14 BAC clones, of which eight fell into three contigs: contig 247 (6 clones), contig 1185, and contig 1378. It is interesting that NLRC3 probes hybridized to 11 BAC clones, of which six fell into six separate contigs. Similarly, NLRC5 probes hybridized to 20 BAC clones, of which six could be mapped to contigs: three clones to contig 557, and one each to contig 622, contig 3333, and contig 3442. NLRX1 probes hybridized to 9 BAC clones; however, these BAC clones were unmapped to the physical map (Supplemental Table 3). As Southern blot analysis indicated the presence of a single copy for all the five NLR genes in the catfish genome, the multiple contigs detected by a single probe were

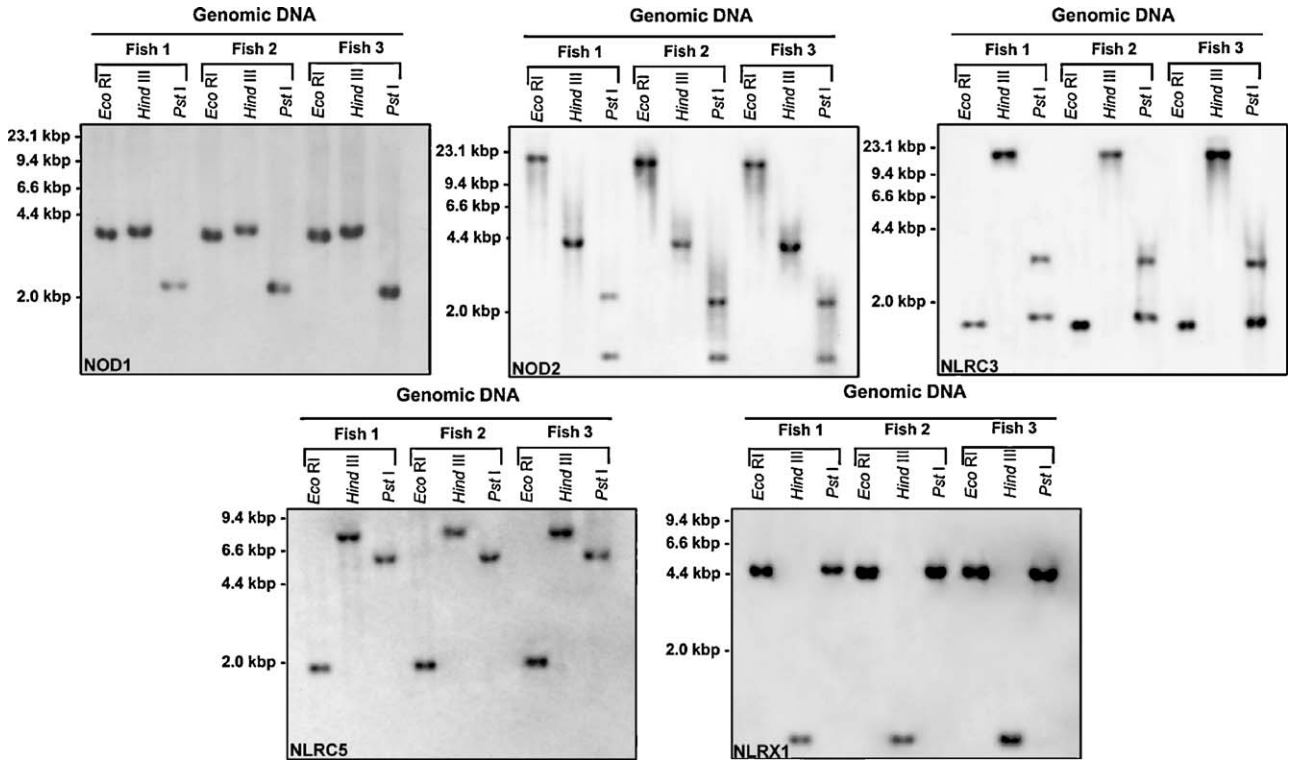

Fig. 3. Southern blot analysis of the catfish NLR genes for the determination of genomic copy numbers. Southern hybridizations on all five channel catfish NLR genes were conducted as detailed in Section 2. DNA from three individual catfish was digested with *Eco*RI, *Hind*III, and *Pst*I as specified in the figure, transferred to nylon membrane, and hybridized to gene-specific probes. Hybridization signals were detected by exposure of the blots to X-ray films. Molecular weight (kbp) standards are indicated on the left margin of each blot. Genes are specified at the lower left corner of each blot.

likely divided because of the use of high stringency during contig construction [45]. For instance, contig 557 and contig 3442, both positive for NLRC5, merged together to a single contig at 1e-15. These results should provide genomic information for mapping of these genes on the genetic linkage map in relation to their functions in immunity and disease defense. Furthermore, such studies should facilitate the merging of contigs, improving the existing physical map [45].

3.7. Expression of catfish NODs

Tissue expression patterns of the five catfish NOD genes were determined using RT-PCR with 13 healthy catfish tissues (Fig. 4). NOD1 was expressed highly in blood, brain, gill, head kidney, trunk kidney, and intestines, but at lower levels in heart, liver, muscle, skin, spleen, stomach, and ovary. NOD2 was constitutively expressed in all tissue. Tissue expression preferences were observed with NLRC3, NLRC5, and NLRX1. NLRC3 was expressed most highly in head kidney, trunk kidney, intestine, and spleen, all important tissues for immune functions. NLRC5 and NLRX1 were expressed in almost all tissues, but the levels of expression varied greatly (Fig. 4). NLRC5 was expressed at relatively low levels in the liver and muscle, and at extremely low levels in the ovary (Fig. 4).

Expression of the five NOD genes was examined in catfish leukocyte cell lines. As shown in Fig. 5, the five catfish NOD genes were expressed highly in all the tested leukocyte cell lines including cytotoxic T-cell lines, macrophage cell line, and B-cell lines.

3.8. Expression of catfish NODs after bacterial infection

In order to determine the expression of the five catfish NOD genes after bacterial challenge with *E. ictaluri*, RT-PCR and qRT-PCR were conducted. For the most part, all the NOD genes but NOD1 exhibited similar expression levels before and after infection, in the tested tissues of head kidney, intestine, and spleen (Fig. 6). For NOD1, bacterial infection induced an elevated expression in the intestine, but not in the head kidney or spleen (Fig. 6). These results were confirmed by real-time RT-PCR (Fig. 7). Only NOD1 from the intestine had significant ($p < 0.05$) differential expression after bacterial infection (Fig. 7). The immediate NOD1 gene response at 4 h post-infection was not significant ($p = 0.469$), but the response

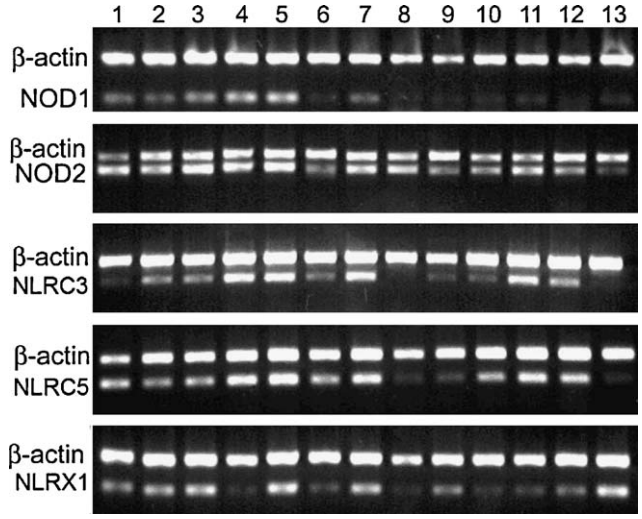

Fig. 4. Tissue expression analysis of NOD1 (top panel), NOD2 (second panel), NLRC3 (third panel), NLRC5 (fourth panel), and NLRX1 (bottom panel) using RT-PCR. Total RNA samples from 13 healthy channel catfish tissues were used in the RT-PCR reactions: 1, blood; 2, brain; 3, gill; 4, head kidney; 5, trunk kidney; 6, heart; 7, intestine; 8, liver; 9, muscle; 10, skin; 11, spleen; 12, stomach; and 13, ovary. Gene names are indicated on the left margin of the gels and β-actin was used as an internal control.

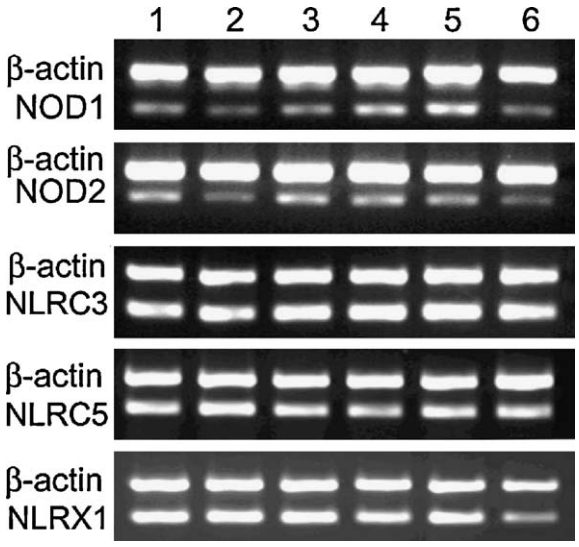

Fig. 5. Analysis of expression in leukocyte cell lines of NOD1 (top panel), NOD2 (second panel), NLRC3 (third panel), NLRC5 (fourth panel), and NLRX1 (bottom panel) using RT-PCR. Total RNA samples from six leukocyte cell lines were used in the RT-PCR reactions: 1, cytotoxic T-cell line TS32.15; 2, cytotoxic T-cell line TS32.17; 3, T-cell line 28.3; 4, macrophage cell line 42TA; 5, B-cell line 3B11; and 6, B-cell line 1G8. Gene names are indicated on the left margin of the gels and β-actin was used as an internal control.

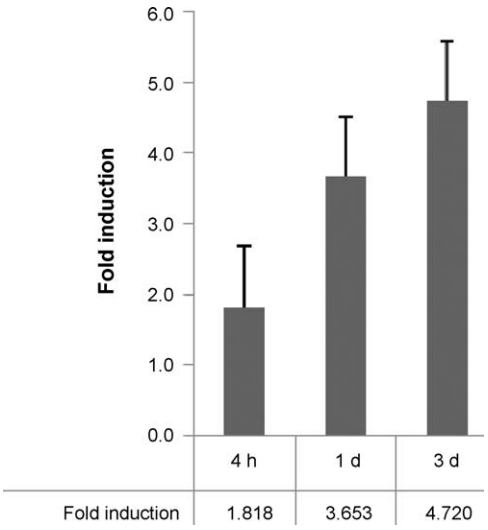

Fig. 7. Results of quantitative real-time PCR on NOD1 in the catfish intestine. See Section 2 for details of bacterial infection and analysis of expression using real-time PCR. Values given are normalized expression levels. Expression was significantly ($p < 0.05$) up-regulated at 1 day and 3 days post-challenge.

at 1 day and 3 days post-infection was statistically significant. From the RT-PCR images (Fig. 6), the other possible candidate for differential gene expression was NLRX1 in the spleen. This gene appeared to be up-regulated after ESC infection, but the difference was not statistically significant with real-time RT-PCR analysis ($p = 0.2$ or greater, data not shown).

4. Discussion

The importance of NLR-mediated innate immune responses has only recently begun to be understood in mammalian systems. While the better-characterized Toll-like receptors detect PAMPs at the cell surface or within endosomes, NLRs trigger inflammatory responses through cytosolic recognition of microbial components. Several genomic surveys have demonstrated the presence of NLR-like molecules in the genomes of model fish species [11,12], but almost nothing is known about the homeostatic or pathogen-induced expression patterns of fish NLR transcripts. In this study, therefore, we have identified, cloned, sequenced, and characterized the expression of the transcripts of the NOD subfamily of the NLRs from catfish: NOD1, NOD2, NLRC3, NLRC5, and NLRX1.

Sequencing and phylogenetic analysis of the five catfish NLR transcripts revealed high levels of sequence and structural identity (Figs. 1 and 2). For the NOD1, NLRC5, and NLRX1 transcripts, for which complete ORFs could be obtained, structural organization differed only in the number of LRR present. Sequence identity between catfish and zebrafish NODs ranged from 45% to 81%, with

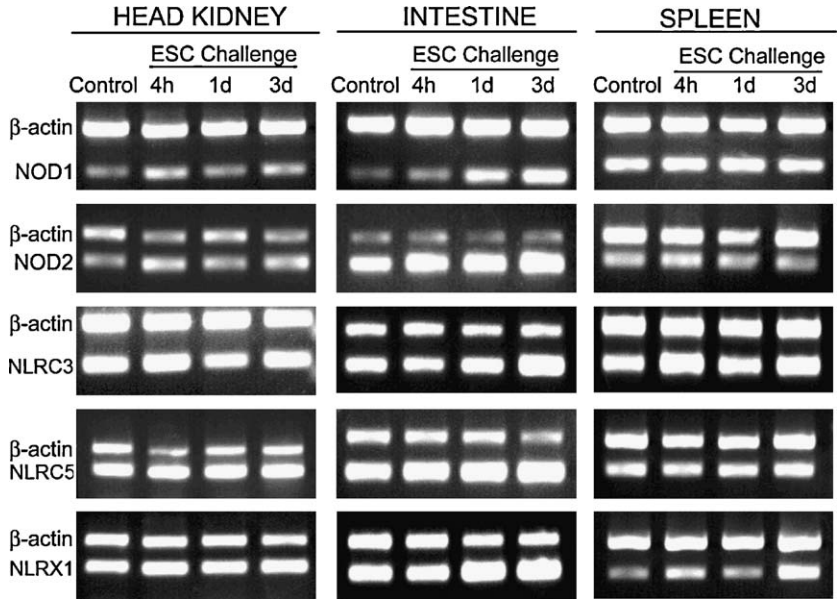

Fig. 6. Analysis of expression of NOD1 (top panel), NOD2 (second from the top panel), NLRC3 (third from the top panel), NLRC5 (fourth from the top panel), and NLRX1 (bottom panel) after infection of the channel catfish with *Edwardsiella ictaluri* (ESC) using RT-PCR. Total RNA samples from head kidney, intestine, and spleen were used in the RT-PCR reactions. Tissues were collected for RNA preparation at 0 h (control), 4 h, 24 h (1 day), and 72 h (3 days) after challenge. Gene names are indicated on the left margin of the gels and β-actin was used as an internal control.

highest sequence identity present in NOD2 and lowest sequence identity in NLRC5. Sequence identity between catfish and human NODs ranged from 26% to 51%, with highest sequence identity present in NOD1 and lowest sequence identity in NLRC5. Each of the five catfish NOD members was clearly orthologous to NLR genes present in both the mammalian and zebrafish genomes (Fig. 2) allowing us to adapt the established nomenclature for naming the catfish genes. The low levels of sequence divergence observed between mammalian and teleost NOD equivalents indicates the importance of these genes in pathogen recognition and increases the likelihood that they may recognize the same microbial ligands (see below). Similarly, while other subfamilies of NLRs appear to have expanded dramatically during teleost evolution [12], the NOD subfamily appears to be maintained as single copy genes in the catfish genome (Fig. 3). This fact may again be indicative of conservation of function – recognition of evolutionarily – stable pathogen-associated molecular patterns.

Only one report to-date has described the homeostatic expression patterns of teleost NODs. Laing et al. [12] reported the expression of NOD orthologues in zebrafish intestine, liver, and spleen. Here, we examined the expression levels of the five catfish NOD transcripts in a large panel of tissues from naive catfish (Fig. 4) as well as in a number of catfish leukocyte cell lines (Fig. 5). In general, catfish NODs appeared to be widely expressed. Catfish NOD2 was notable for its ubiquitous expression in all tested tissues, whereas NOD1 exhibited the most restricted expression profile. Expression patterns and levels varied widely, indicating that immunosurveillance functions may be spatially partitioned among the family members.

Expression of teleost NLR family members has not been previously reported in leukocytes. Interestingly, the five catfish NOD transcripts were strongly expressed in all tested catfish leukocyte cell lines, including macrophages, cytotoxic T cells, T cells, and B cells. Strong leukocyte-based expression undoubtedly contributes to the expression patterns observed in the panel of complex catfish tissues, especially in the lymphoid and lamina propria rich organs. NOD1 and NOD2 expression in macrophages has been widely studied in mammals [52], but there are also reports of NOD expression in T and B cells [53]. Little is known, however, about the role of NODs in mammalian lymphocytes. Fritz et al. [54] reported that NOD1 expression in T and B cells had no effect on peptidoglycan-sensing in mice. Given the unique phagocytic capabilities of teleost B lymphocytes [55], it would be of great interest to study the ancestral roles of the NOD genes in teleost B cells.

We next asked whether expression of catfish NODs is induced following infection with a Gram-negative intracellular bacterium, *E. ictaluri*. We examined expression patterns in head kidney, intestine, and spleen at three time-points following infection (Fig. 6). Notably, upregulation of gene expression was only observed for NOD1 in intestine. NOD1 expression levels rose steadily above those of the control, and at 3 days post-infection expression was up-regulated approximately 5-fold. In mammals, NOD1 recognizes peptidoglycan-related molecules containing the amino acid meso-diaminopimelic acid (meso-DAP) that is produced predominantly by Gram-negative bacteria. NOD1 is one of the best characterized NLRs in the mammalian system, and numerous studies have focused on its role in detection of invasive bacteria in intestinal cells [52,56–57]. Intestinal epithelial cells and lamina propria-associated macrophages are constantly exposed to microbial ligands produced by commensal flora present in the gut. These cells express low levels of Toll-like receptors and are effectively desensitized to Toll-like receptor ligand-stimulated signaling in order to prevent inappropriate immune system activation. NOD1 (and NOD2) appear to play a unique role in initially sensing pathogenic and/or intracellular bacteria and signaling to induce primary immune responses. These signals, in turn, resensitize intestinal Toll-like receptors to further guard against bacterial invasion. It is presently unknown whether catfish NOD1 is mediating a similar process in the intestine. However, it has recently been reported that Toll-like receptor 4, the mammalian lipopolysaccharide receptor, when present in fish, does not recognize lipopolysaccharides and, in fact, negatively regulates inflammatory responses [58]. In light of these findings, further work is warranted to study the potential interplay between teleost NOD1 and Toll-like receptors in the intestine under both homeostatic conditions and following infection with Gram-negative bacteria.

Acknowledgements

This project was supported by a grant from USDA NRI Animal Genome Basic Genome Reagents and Tools Program (USDA/NRICGP award # 2006-35616-16685), and partially by a grant from National Natural Science Foundation of China (Grant number 30871941) supporting the senior author (Z.S.). We would like to thank Dr. Melanie Wilson of the University of Mississippi Medical School for providing RNA from the leukocyte cell lines used in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2009.04.004.

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