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Cloning and analysis of Nile tilapia Toll-like receptors type-3 mRNA

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Abstract

Toll-like receptors (TLRs) are the best understood of the innate immune receptors that detect infections in vertebrates. However, the fish TLRs also exhibit very distinct features and a large diversity, which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. Little is known about the fish immune system structure. Our work was aimed to identify and clone the Nile tilapia TLR-3 as a model of fresh water fish species; we cloned the full-length cDNA sequence of Nile tilapia (Oreochromis niloticus) TLR-3 and according to our knowledge, it is the first report illustrating tilapia TLR-3. The complete cDNA sequence of Nile tilapia TLR-3 was 2736 pair base and it encodes a polypeptide of 912 amino acids. Analysis of the deduced amino acid sequence indicated that Nile tilapia TLR-3 has typical structural features and main component of proteins belonging to the TLR family. Our results illustrate a complete and functional Nile tilapia TLR-3 and it is considered an ortholog of the other vertebrate's receptor.

Key words: *Nile tilapia, single nucleotide polymorphisms, fish, Toll-like receptor 3 – TLR-3, gene expression.*

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Introduction

Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of microbes. They are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals. They activate innate immune responses, protecting the host from infection, by identifying some conserved non-self-molecules. Bacterial lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane of a bacterium, is considered to be the prototypical PAMP. Every TLR receptor is mainly specialized in recognizing one or more patterns where LPS is specifically recognized by TLR-4 [1-8], a bacterial flagellin recognized by TLR-5 [3], lipoteichoic acid from Gram-positive bacteria and peptidoglycan recognized by TLR-2 [1, 9-11], and nucleic acid variants normally associated with viruses, such as double-stranded RNA – dsRNA recognized by TLR-3 [12-14] or un-methylated CpG motifs, recognized by TLR-15, TLR-21 [15-20]. Although the term "PAMP" is relatively new, the concept that molecules derived from microbes must be detected by receptors from multicellular organisms has been held for many decades, and references to an "endotoxin receptor" are found in a lot of older literature. Toll-like receptors are the basic components of the vertebrate pathogen recognition system. Despite the uniform general structure, remarkable variability in domain composition can be found in individual TLRs among species. Toll-like receptors are typical type I transmembrane proteins, and contain three major domains: a tandem repeat leucine-rich repeat (LRR) motif, which identifies PAMPs, a transmembrane region and an

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intracellular Toll/interleukin 1 (IL-1) receptor (TIR) domain, which transmits signals. Knowledge of inter-specific differences is of particular importance to our understanding of selective pressures on TLRs. Toll-like receptors are membrane-bound sensors of the innate immune system, which recognize invariant and distinctive molecular features of invading microbes and are also essential for initiating adaptive immunity in vertebrates. The genetic variation in TLR genes has been directly related to differential pathogen outcomes in humans and livestock. Nonetheless, new insights about the impact of TLR polymorphism on the evolutionary ecology of infectious diseases can be gained through the investigation of additional vertebrate groups not yet investigated in detail. Toll-like receptors are members of the PRRs, which detect PAMPs and have a role in initiating the innate as well as adaptive immune defense [1, 12-15, 18, 21-26]. They play a vital role in host immune responses through the recognition of LPS, lipopeptides, flagellins, dsRNA or CpG DNA motifs [16]. The TLR system is a part of ancient machinery that is evolutionary conserved with homologs present in insects, nematodes, plants, fish, mammals and birds [27]. A range of TLR genes has been identified in non-mammalian vertebrates including birds and fish [12]. The numbers of TLR genes vary among various organisms. Thirteen TLRs (TLR 1-13) have been identified in mammals, and functionally these receptors recognize and respond to a wide range of exogenous as well as endogenous ligands. Of the 13 mammalian TLRs, TLR-11, TLR-12, and TLR-13 were identified only in the murine genome. In teleost fish, orthologs of TLR 1-5, 7-9 have been identified, while various reports indicated that TLR-6 and TLR-10 do not exist in teleost fish [28, 29]. In addition to the orthologs of TLRs in mammals, 'fish-specific' TLRs have been reported including TLR-18, TLR-19, TLR-20, TLR-21, TLR-22, and TLR-23 [17, 28, 30, 31]. However, all these fish TLRs and their signaling cascade factors represent high structural similarity to the mammalian TLR system. Currently, most TLRs are characterized only in a limited number of model species, including Salmon fish, according to our knowledge, there is almost no data about the Nile tilapia TLRs, which is considered one of the most common farm fish and has a great economic importance all over the world. Here we want to describe the TLRs in the Nile tilapia. The research dedicated to the description of the enormous diversity of molecules involved in pathogen recognition is of vital importance in human and veterinary medicine. It is equally important to the evolutionary biology of host-parasite interactions. Much effort has been devoted to the characterization of immune system components in human and mouse models, while much less is currently known about the architecture of the immune system in other species [32]. Information concerning any one of the wealth of living species may bring new insights into the principles of the vertebrate immune function. Aiming to describe general patterns of immune system evolution in terrestrial vertebrates, the investigation of the fish clade may be particularly useful. Fish form a well-diversified taxon with origin distinct to mammals but with physiology comparable to them. However, in contrast to mammals, our knowledge of the molecular structure of the fish immune system is limited [8, 31]. Considering these reasons, new models are required to verify the universal validity of the results obtained in human and mice, so we chose Nile tilapia as a representative of the freshwater fish [28, 33-36].

Material and methods

Samples

Kidney, brain, spleen, intestine, muscle, liver, gills and heart and skin samples were collected from the Nile tilapia fish. The Nile tilapia fish was brought as a live mature fish from common farm and kept under inspection for 5 days to be sure that it is free from any clinical infection, then the samples were collected and stored in –80°C.

Primer design

We downloaded the complete TLR-3 mRNA sequences of the rainbow trout (Oncorhynchus mykiss): AAX68425; Takifugu rubripes (Fugu rubripes): AAW69373; Larimichthys crocea (large yellow croaker): ADW79423; Paralichthys olivaceus (Japanese flounder): BAM11216; Epinephelus coioides (orange-spotted grouper): AEX01718 from the GenBank. The sequence was aligned using the ClustalW application (primer premier 5.1 software) where we designed degenerative primers match with the alignment of the sequences to the clone short sequence, then we got the full sequence by RACE system SMARTer RACE cDNA Amplification kit (Clontech, CA, USA) following the manufacturer's instructions, to get the full length for both 3'end and 5'end direction, all the primers were designed in our lab (unpublished data).

Molecular cloning of Nile tilapia TLR-3

Total RNA from fish kidney, brain, spleen, skin, intestine, muscle, liver, gills, heart and skin were extracted by TRIZOL (Invitrogen, USA) according to the manufacturer's protocol. Both the quantity and quality of total RNA were assessed at OD260 and OD280 using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA samples were used for synthesis from the cDNA library in 10 µl reaction mixture using BioRT cDNA first strand synthesis kit (Hangzhou Boiler, China) according to the manufacturer's instructions. Briefly, oligo dT primer (0.5 µg) was used to reverse transcribe 1 µg of respective RNA in the presence of dNTP's (250 µM), reverse transcriptase buffer (50 mM Tris-HCl, 100 mM KCl, 4.0 mM DTT and 10 mM $MgCl_2$), AMV Reverse Transcriptase (5 units/µg) and RNasin Ribonuclease inhibitor (40 unit/µl) at 42°C for 45 min following inactivation at 95°C for 5 min.

The PCR was performed to amplify the target gene using specific primers, where 25 µl PCR mixtures contained 50 pmol for each forward and reverse primer, 2 µl template cDNA, 200 µM each of dNTP mix and 2.5 U Ex Taq polymerase (Takara Bio, Dalian, China) in 1× Ex buffer. Amplification conditions were as follows: initial denaturation at 94˚C for 5 min, 35 cycles at 94˚C for 35 s, annealing at 56-60˚C for 35 s, and extension at 72˚C for 2 min, followed by the final extension at 72˚C for 10 min. PCR amplicons were verified by 1.2% agarose gel electrophoresis in TBE buffer at 70 mA for 45 min and products visualized by staining with ethidium bromide, we checked the band and got the images with GelDoc™ XR+ system (Bio-RAD, USA). We used SMARTer RACE cDNA amplification kits (Clontech, USA) according to the manufacturer's instructions to get the full length toward the 3'end and 5'end.

Sequence analysis

The sequence of the Nile tilapia TLR-3 mRNA was blast in the GenBank using nucleotide blast and the translated amino acids were also blast by protein blast to check whether the new sequence is related to any other cloned gene. The sequence of Nile tilapia TLR-3 was compared with the known TLR-3 mRNA sequences from different species which were downloaded from the GenBank and aligned by CUSTALW, MEGA 5 software [37]. A phylogenetic tree was constructed from the amino acid alignments using two methods: (A) the neighbour-joining method with options of pairwise deletion, poisson correction and different evolutionary rates with a gamma parameter of 1 and (B) the maximum parsimony method using the close neighbour-interchange search method with random additive trees (10 replicates), all the phylogenetic analyses were made by MEGA5 software [37] and the reliability of branching was tested by 1000 bootstraps. The extracellular, transmembrane, and cytoplasmic domains of these protein sequences were predicted with the analysis tools provided at the websites (http://smart.embl-heidelberg.de and http://split.pmfst.hr).

Results

Nile tilapia TLR-3

The complete mRNA sequence of Nile tilapia TLR-3 was deposited in the NCBI GenBank database under accession no. JQ809460. Where it consists of 2736 nucleotides and the consensus cDNA sequence showed 79% identity with Larimichthys crocea and 78% identity with Epinephelus coioides, while it showed 72% identity with Takifugu rubripes, which confirmed that the new sequence is probably homolog to fish TLR-3. The predicted protein encoded by Nile tilapia TLR3 mRNA sequence is composed of 912 amino acids where it begins with (ATG) which is similar to the other fish TLR-3 sequence. The Nile tilapia TLR-3 domain structure has been estimated using SMART web tool, where Nile tilapia TLR-3 started by signal peptide (20 amino acids from 1-20), then 16 LRR domains (residues 21-639) and one C-terminal LRR domain (LRR-CT, residues 652-705) in the extracellular region, and a TIR domain (residues 764-907) in the cytoplasmic region as shown in Table 1. The encoded amino acids vary among different available amino acids where it has 20 different amino acids; the highest amino acid encoded is Leucine while the lowest is Tryptophan, we made a chart explaining the amount and ratio of the encoded amino acid in the Nile tilapia TLR-3 as shown in Fig. 1.

Table 1. Transmembrane structure of Nile tilapia TLR-3 showing the size and position of its motifs done by SMART analysis web based application

Name	Start	End	*P*-value
Signal peptide	1	20	N/A
LRRNT	21	61	7.91
LRR	104	127	28.4
LRR	128	150	274
LRR	151	183	238
LRR	175	198	25.4
LRR	280	301	251
LRR_TYP	304	327	0.00014
LRR	358	381	3.24
LRR	435	462	195
LRR_TYP	512	535	0.0311
LRR	536	559	1.31
LRR	569	591	162
LRR_TYP	592	615	0.0012
LRR	616	639	1.07
LRRCT	652	705	0.000074
Transmembrane region	714	736	1390
TIR	764	907	4.52e-12

Similarity with other TLRs

We used two methods to construct a phylogenetic tree (neighbour-joining and maximum parsimony) based on the amino acid of TLR-3, which was downloaded from the GenBank. The phylogenetic analysis had been performed using the translated Nile tilapia amino acid sequence with almost all the known amino acid sequences found in the GenBank, both phylogenetic methods provided almost the same results, where the phylogenetic analysis showed that Nile tilapia TLR-3 is closely related to Larimichthys crocea TLR-3, Epinephelus coioides TLR-3 and Takifugu rubripes TLR-3. The structure of Nile tilapia TLR-3 amino acids is in general sim-

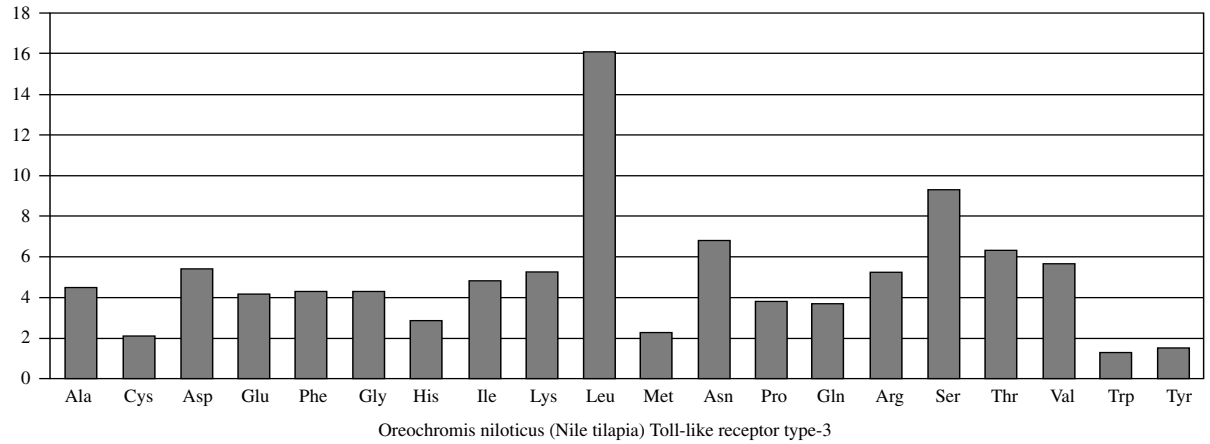

Fig. 1. Nile tilapia TLR-3 amino acid composition and percentage

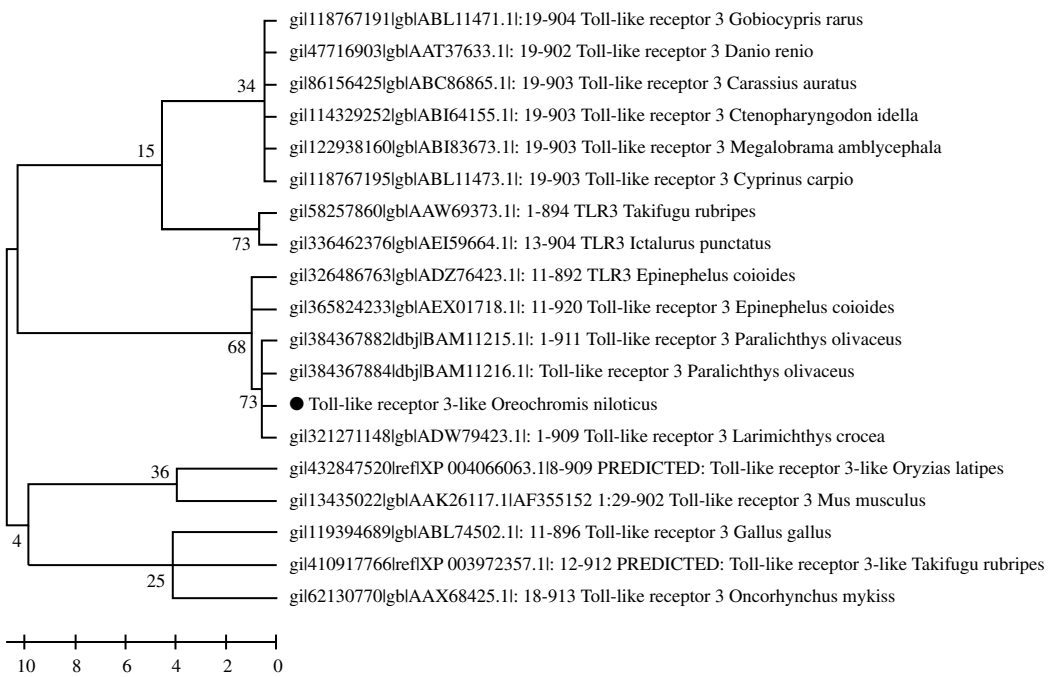

Fig. 2. Phylogenetic tree of Nile tilapia TLR-3 against the available TLR-3 sequence in the GenBank

ilar to other identified TLR-3 sequence with 36-48% identity to different mammalians, 71% identity to rainbow trout (Oncorhynchus mykiss), Epinephelus coioides (orange-spotted grouper), Paralichthys olivaceus (Japanese flounder) and Larimichthys crocea (large yellow croaker) TLR-3 while 68% to Takifugu rubripes (Fugu rubripes) TLR-3 and 39% with chicken as shown in Fig. 2.

Expression pattern of Nile tilapia TLR-3

The transcription of Nile tilapia TLR-3 was highly expressed in kidney, brain, spleen, intestine, muscle, liver, gills, heart and skin (Fig. 3), semiquantitative PCR following the reverse transcription showed differences in the expression level among the tested tissue, where the expression was the highest in the spleen, muscle and liver, also showed a moderate expression level in the kidney and intestine.

Discussion

This is the first study to characterize the Nile tilapia TLR-3 as most studies focus on non-fish vertebrates. Our results provided Nile tilapia TLR-3, which is considered

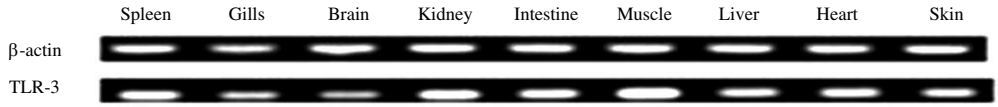

Fig. 3. Tissue-specific expression of Nile tilapia TLR-3 mRNA. Total RNAs were extracted in various tissues from three healthy fish; cDNAs were equally mixed from three healthy fish in corresponding tissues

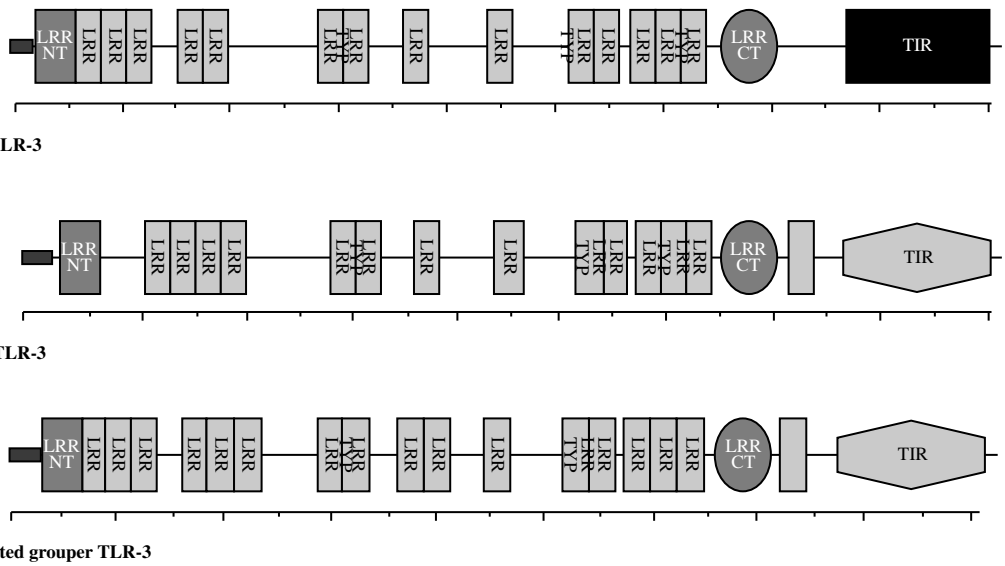

Fig. 4. The transmembrane structure of TLR-3 in rain trout, Nile tilapia, and orange-spotted grouper

a homologe for rainbow trout, orange-spotted grouper, zebra fish and other vertebrates TLR-3; the structure analysis showed that the Nile tilapia receptor is very close to rainbow trout, orange-spotted grouper with 71% identity and more closer to rain trout than to zebra fish.

The transmembrane structure analysis for the Nile tilapia receptor showed that rainbow trout and orange-spotted grouper have one motif which is absent in tilapia while tilapia has one motif plus zebra fish, and with rainbow trout, orange-spotted grouper sharing the same signal peptide at the beginning of the gene (Fig. 3). The Nile tilapia TLR domain has 143 amino acids, while rainbow trout has 136 amino acids, and, it may be due to the unequal force distribution during the evolution, also the duck showed a unique LRR domain at position 128-150 which is absent in rainbow trout and orange-spotted grouper as shown in Fig. 4. This may have a role in the difference of the immune response against pathogen between the Nile tilapia and other fish species. The expression of Nile tilapia TLR-3 varies between different tilapia organs as in Fig. 3 where it is highly expressed in kidney, brain, spleen, intestine, muscle, liver, gills, heart and skin.

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The authors declare no conflict of interests.

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