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Pathogen recognition receptors in channel catfish: II. Identification, phylogeny and expression of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)

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ABSTRACT

Vertebrates including teleost fish have evolved an array of pathogen recognition receptors (PRRs) for detecting and responding to various pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs), nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), and the retinoic acid inducible gene I (RIG-I) like receptors (RLRs). As a part of the series of studies targeted to characterize catfish PRRs, we described 22 NLR receptors in the sister contribution. Here in this study, we focused on cytosolic PRRs recognizing nucleotide pathogen-associated molecular patterns (PAMPs) of invading viruses, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLR receptors). Three RLRs with DExD/H domain containing RNA helicases, retinoic acid inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), were identified from channel catfish, *Ictalurus punctatus*. The catfish RIG-I encodes 937 amino acids that contains two CARDs, a DExDc, a HELICc and a RD domains. MDA5 encodes 1005 amino acids with all the domains identified for RIG-I. LGP2 encodes 677 amino acids that contain other domains but not the CARD domain at the *N*-terminus. Phylogenetic analyses of the three genes of catfish showed close clustering with their counterparts from other teleost fish. All the genes were found to be constitutively expressed in various tissues of catfish with minor variations. Channel catfish ovarian cells when infected with channel catfish virus showed significant increase in the transcript abundance of all the three genes. Further, RLR genes showed significant increases in expression in the liver tissue collected at different time-points after bacterial infection as well. The results indicate that the catfish RLRs may play important roles in antiviral and anti-bacterial immune responses.

1. Introduction

Pathogen recognition receptors (PRRs) play a crucial role in pathogen surveillance in all eukaryotic organisms. PRRs recognize the conserved molecular signatures associated with pathogens termed pathogen-associated molecular pattern (PAMPs), that include proteins, lipids and nucleotides, and result in activation of host innate immune response (Pichlmair and Reise Sousa, 2007). After sensing the PAMPs, host innate immune cells initiate a broad spectrum of defense mechanisms that result in the development of inflammation and host resistance to infection (Akira et al., 2006). PRRs comprise an array of sensors and are found in the extracellular space, membrane-associated variant cell types or in the cytosol. Three major groups of PRRs have been identified: toll-like receptors (TLRs), nucleotide oligomerization domain (NOD) containing protein-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Akira et al., 2006; Meylan et al., 2006; Franchi et al., 2010; Chen et al., 2009; Hansen et al., 2011).

RLRs are key cytosolic pattern recognition receptors for detecting nucleotide PAMPs of invading viruses and they are crucial for the RNA virus-triggered interferon response (Takeuchi and Akira, 2008; Zou et al., 2009). Similar to Dicer of the RNAi pathway, RLRs belong to the phylogenetically conserved DExD/H-box family of helicases (Lu et al., 2009). Three genes encode RIG-I-like receptors: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), which share a common functional RNA helicase domain near the *C*-terminus (HELICc) specifically binding to the RNA molecules with viral origin and a *C*-terminal regulatory domain (RD) (Yoneyama et al., 2004, 2005; Holm et al., 2007; Venkataraman et al.,

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2007; Yoneyama and Fujita, 2007, 2008; Zou et al., 2009). The structure of RIG-I and MDA5 are very similar, as the *N*-terminal region of RIG I and MDA5 are characterized by the presence of two tandem arranged caspase activation and recruitment domains (CARDs) involved in protein–protein interactions. This activation triggers the interferon response via activation of interferon regulatory factor 3 and NFkB (Yoneyama et al., 2004; Holm et al., 2007). On the contrary, LGP2 does not possess a CARD domain at the *N*-terminal.

Each RLR plays a different role in recognizing viral PAMPs in the cytoplasm (Takahasi et al., 2009). RIG-I is a key mediator of antiviral immunity by inducing interferon (IFN) production after recognizing both RNA and DNA viruses (Ablasser et al., 2009; Choi et al., 2009; Gack et al., 2010; Pothlichet et al., 2009; Rehwinkel et al., 2010). However, among the PRRs, RIG-I is known to have a key role in recognizing RNA viruses (Baum et al., 2010). Recently it has been reported that RIG-I has broad regulatory functions that include its role in antibacterial responses, apart from its previously described antiviral roles (Kong et al., 2009). RIG-I is capable of discriminating host RNA from viral RNA based on the chemical nature of the 5′ end of RNA (Hornung et al., 2006). Further, RIG-I can recognize short double-stranded RNA in contrast to MDA5 which recognizes long dsRNA (Takahasi et al., 2009). The ability to discriminate and bind viral originated nucleic acids is provided by the specific amino acid differences in the RD domains of the RLRs (Kato et al., 2008; Yoneyama and Fujita, 2008; Takeuchi and Akira, 2008; Pichlmair et al., 2009).

MDA5 was initially discovered by Kang et al., 2002 as an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties in human melanoma cells. Later, it has been reported that MDA5 plays a crucial role in intracellular signal transduction pathway that could lead to the activation of the IFN-β promoter and could mediate type I IFN responses against nucleic acid PAMPs (Andrejeva et al., 2004; Gitlin et al., 2006; Kato et al., 2006). Although MDA5 is known to recognize long dsRNA (Takahasi et al., 2009), it has been reported later that it is not simply long molecules of dsRNA that are required for the activation of MDA5 but that higher-order RNA structures generated during virus infection are also required (Pichlmair et al., 2009).

The third RLR, LGP2, is a regulatory protein (Komuro et al., 2008) and structurally similar to the other two RLRs except for the lack of a CARD domain (Yoneyama et al., 2004, 2005; Rothenfusser et al., 2005; Holm et al., 2007). It has been shown to interfere with the binding process of RIG-I/MDA5 to viral RNAs (Saito et al., 2007) and negatively regulate RLR signaling (Komuro et al., 2008). However, recently, it has been suggested that LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses and can potentiate IFN production during viral infection (Satoh et al., 2010).

Most of the studies on RLRs have been focused on mammals and only a little is known about such molecules in other organisms. However, a survey of purple sea urchin genome has revealed multiple putative RIG I-like homologues (Hibino et al., 2006). Recently, RIG-like homologues have been identified *in silico* in the nucleotide databases of many invertebrates and vertebrates including teleost fish (Zou et al., 2009). In addition, lately, RLRs have been reported in many teleosts species such as zebrafish, Atlantic salmon, grass carp and Japanese flounder and fathead minnow-derived EPC cell line (Biacchesi et al., 2009; Lauksund et al., 2009; Huang et al., 2010; Ohtani et al., 2010, 2011; Su et al., 2010; Yang et al., 2011).

Channel catfish, *Ictalurus punctatus*, is the major aquaculture species in the United States, and its immune system has been well characterized (Vallejo et al., 1992; Wilson et al.,1990, 1997; Clem et al., 1990, 1996; Miller et al., 1994, 1998; Khayat et al., 2001; Bengtén et al., 2002; Barker et al., 2000, 2002; Zhou et al., 2001, 2003; Shen et al., 2002, 2003; Shen, 2004; Quiniou et al., 2005; Ghaffari and Lobb, 1993; Magor et al., 1994; Hogan et al., 1999; Godwin et al., 2000; Antao et al., 2001; Hawke et al., 2001; Ventura-Holman and Lobb, 2002; Shen, 2004). A number of immune genes/innate PRRs such as chemokines (Peatman et al., 2006; Peatman and Liu, 2007; Bao et al., 2006) anti-microbial peptides (Bao et al., 2005, 2006; Xu et al., 2007), several TLRs (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2006, 2007a,b), a number of lectin family of proteins (Takano et al., 2008; Zhang et al., 2011) and a few NLRs have been characterized in catfish (Sha et al., 2009). In a sister contribution, we reported a complete set of NLR receptors from channel catfish (Rajendran et al., 2012), and here we focus on RLRs. The objectives of the present study were to identify RLR genes in catfish through *in silico* analysis of the transcripts generated through RNA-seq, validate of the transcripts through in silico analysis with the catfish genome database (Lu et al., 2011, and unpublished genome sequences), elucidate the phylogenetic relationships of the genes, determine tissue expression patterns, and analyze expression profiles of the RLR genes after infection with channel catfish virus (CCV) or bacterial infection with *Edwardsiella ictaluri*.

2. Materials and Methods

2.1. Database mining and sequence analysis

To identify the RLR genes, RNA-seq and the whole genome database of catfish, *I. punctatus*, were searched using available zebrafish (*Daniorerio*) and human RLRs as queries. The RNA-seq database was generated from the transcriptome assembly of expressed short reads of a doubled haploid channel catfish (Liu et al., 2011, 2012). The quality of three cDNA sequences obtained from RNA-seq database was confirmed by comparison with the preliminary catfish whole genome assembly (unpublished data) which was also originated from sequencing a doubled haploid channel catfish. The retrieved reconstructed transcripts were translated using ORF finder (http://www.ncbi.nlm.nih.gov) and GENSCAN (Burge and Karlin, 1997). The predicted ORFs were verified by BLASTP against NCBI non-redundant protein sequence database. The RLR genes from other organisms were retrieved from the NCBI database for analysis. The cDNA sequences in this work were obtained and submitted to GenBank as we have done for NLRs of catfish (Rajendran et al., 2012). The Simple Modular Architecture Research Tool (SMART) was used to predict the conserved domains based on sequence homology and further confirmed by conserved domain prediction from BLAST. The full-length amino acid sequences as well as the partial sequence coding for the conserved domains were used in the phylogenetic analysis. Multiple protein sequence alignment was done using the ClustalW program. Neighbor-joining and maximum parsimony analyses were conducted using MEGA version 4 (Tamura et al., 2007).

2.2. Expression analysis of catfish PRRs

Reverse-transcriptase PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were employed to study the mRNA expression of selected RLR genes. To study the normal expression of these genes in healthy fish, blood, skin, muscle, gill, heart, liver, spleen, intestine, head kidney, trunk kidney and brain were collected from five individual fish and pooled. Three such pools were used in the present study. The tissues were snap-frozen in liquid nitrogen and immediately subjected to RNA extraction using RNeasy Minikit (Qiagen, USA) following manufacturer's protocol. The extracted total RNA was quantified using UV-spectrophotometer and an aliquot (1 μg) of RNA was treated with 1 unit of RNase-free DNAse (Qiagen) prior to reverse transcription. Uniform quantity of