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Molecular characterization and function analysis of three RIG-I-like receptor signaling pathway genes (MDA5, LGP2 and MAVS) in *Oreochromis niloticus*

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ABSTRACT

The recognition of microbial pathogens, which is mediated by pattern recognition receptors (PRRs), is critical to the initiation of innate immune responses. In the present study, we isolated the full-length cDNA and genomic DNA sequences of the MDA5, LGP2 and MAVS genes in Nile tilapia, termed OnMDA5, OnLGP2 and OnMAVS. The OnMDA5 gene encodes 974 amino acids and contains two caspase-associated recruitment domains (CARDs), a DExDc domain (DExD/H box-containing domain), a HELICc (helicase superfamily C-terminal) domain and a C-terminal regulatory domain (RD). The OnLGP2 gene encodes 679 amino acids and contains a DExDc, a HELICc and an RD. The OnMAVS gene encodes 556 amino acids and contains a CARD, a proline-rich domain, a transmembrane helix domain and a putative TRAF2-binding motif (269PVQDT273). Phylogenetic analyses showed that all three genes from Nile tilapia were clustered together with their counterparts from other teleost fishes. Real-time PCR analyses showed that all three genes were constitutively expressed in all examined tissues in Nile tilapia. OnMDA5 presented the highest expression level in the blood and the lowest expression level in the liver, while OnMAVS presented the highest expression level in the kidney. The highest expression level of OnLGP2 was detected in the liver. An examination of the expression patterns of these RIG-I-like receptors (RLRs) during embryonic development showed that the highest expression levels of OnMDA5 occurred at 2 days postfertilization (dpf), and the expression significantly decreased from 3 to 8 dpf. The expression levels of OnLGP2 significantly increased from 4 to 8 dpf. The expression levels of OnMAVS mRNA were stable from 2 to 8 dpf. Upon stimulation by intraperitoneal injection of *Streptococcus agalactiae*, the expression levels of OnMDA5 were first downregulated and then upregulated in the blood, gill and spleen. In the intestine and kidney, the expression of OnMDA5 was first upregulated, then downregulated, and then upregulated again. The expression of OnLGP2 was upregulated in the kidney and intestine, and the expression of OnMAVS was upregulated in the spleen. Overexpression of OnMAVS increased NF-κB activation in 293 T cells ($p < 0.05$), and after co-transfection with OnMDA5, the OnMAVS-dependent NF-κB activation was slightly increased ($p > 0.05$), after cotransfection with OnLGP2, the OnMAVS-dependent NF-κB activation was significantly decreased ($p < 0.05$). These findings suggest that, although the deduced protein structure of OnMDA5 is evolutionarily conserved with the structures of other RLR members, its signal transduction function is markedly different. The results also suggest that OnLGP2 has a negative regulatory effect on the OnMAVS gene. OnMDA5 and OnMAVS were uniformly distributed throughout the cytoplasm in 293 T cells, whereas OnLGP2 was distributed throughout the cytoplasm and nucleus. These results are helpful for clarifying the innate immune response against bacterial infection in Nile tilapia.

1. Introduction

Innate immunity plays an important role in the immune defenses of fish. The recognition of microbial pathogens, mediated by pattern recognition receptors (PRRs), is critical to the initiation of innate immune responses. PRRs recognize conserved microbial components known as

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pathogen-associated molecular patterns (PAMPs), which include proteins, lipids and nucleotides, and induce subsequent host immunity through multiple signaling pathways that contribute to the eradication of the pathogen [1]. To date, three classes of PRRs have been identified: toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) [2–6]. Recent research has shown that RLRs recognize not only viruses but also bacteria [7–9].

The RIG-I-like receptor (RLR) family is comprised of three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [10–12]. RLRs harbor a central DExD/H box helicase domain and a C-terminal regulatory domain (RD). RIG-I and MDA5 also contain two N-terminal caspase recruitment domains (CARDs), whereas LGP2 does not [13–15]. The N-terminal CARD domains are required for downstream signaling; they interact with and recruit the adaptor protein MAVS (mitochondrial antiviral signaling protein), which also has a CARD domain in its N-terminal region. MAVS is located on the outer membrane of the mitochondria and on the membranes of peroxisomes [16,17]; its activation starts a downstream signaling cascade that activates different transcription factors like NF-κB and IRFs and finally results in the production of type I IFNs and proinflammatory cytokines [12,18]. RIG-I and MDA5 have been well studied and have been found to play different roles in the recognition of RNA viruses. RIG-I recognizes relatively short double-stranded (ds) RNAs (up to 1 kb), and the presence of a 5' triphosphate end greatly potentiates its type I IFN-inducing activity [19–21]. On the other hand, MDA5 detects long dsRNAs (more than 2 kb), such as polyinosinic-polycytidylic acid (poly I: C) [22]. LGP2 is thought to have less function in virus recognition and signal transduction and possibly acts as a regulator of the RIG-I and MDA5 signaling pathways [14]. MDA5 and LGP2 can be found in vertebrate species from fish to mammals; however, a functional RIG-I gene appears to be lost in some fish species [6,23].

Teleostean RLRs and MAVS have been identified in many fish species, such as grass carp, *Ctenopharyngodon idella* (RIG-I, MDA5, LGP2 and MAVS) [24–27]; channel catfish, *Ictalurus punctatus* (RIG-I, MDA5 and LGP2) [28]; Japanese flounder, *Paralichthys olivaceus* (MDA5, LGP2 and MAVS) [29–31]; large yellow croaker, *Larimichthys crocea* (MDA5, LGP2 and MAVS) [32]; rainbow trout, *Oncorhynchus mykiss* (MDA5 and LGP2) [33]; rock bream, *Oplegnathus fasciatus* (MAVS) [34]; black carp, *Mylopharyngodon piceus* (MAVS); and Atlantic salmon, *Salmo salar* (MAVS) [35,36]. MDA5 genes have also been identified in zebrafish (*Danio rerio*) [37], green chromide (*Etroplus suratensis*) [38], sea perch (*Lateolabrax japonicus*) [39], orange-spotted grouper (*Epinephelus coioides*) [40], and Asian seabass (*Lates calcarifer*) (MDA5) [41]. In addition, LGP2 genes have been reported in sea perch (*Lateolabrax japonicas*) [42], black carp (*Mylopharyngodon piceus*) [43], olive flounder (*Paralichthys olivaceus*) [44], and grouper (*Epinephelus* spp.) [45]. Results from these studies have strongly confirmed that RLRs and the adaptor protein MAVS play important roles in innate immune responses to viral infection in teleost fishes.

Previous studies have shown that RLRs recognize not only viruses but also bacteria. For example, CiIPS-1 not only performs important functions in antiviral immune responses but also participates in viral/bacterial PAMP-triggered immune responses, which are tightly controlled to prevent harmful effects resulting from excessive activation [7]. Common carp (*Cyprinus carpio*) MDA5 may be involved in both antiviral and antibacterial innate immune processes [8]. LGP2 can act as both an antiviral and an antibacterial cytosolic receptor and may play a significant role in embryonic and larval development in marine euryhaline teleosts like Asian seabass [9]. However, in tilapia, the potential functions of RLRs remain unknown.

In addition, the detailed roles of signaling pathways involving RLR-family proteins remain elusive. For instance, although RIG-I and MDA5 appear to be well conserved among vertebrates and can activate fish antiviral responses, LGP2 appears to be an activator of the antiviral system in rainbow trout, which is different from its role in mammals as the suppressor of RIG-I− and MDA-5-activated IFN responses [46]. Furthermore, a recent review proposes LGP2 synergy with MDA5 in RLR-mediated RNA recognition and antiviral signaling [47]. The antiviral signaling mediated by black carp MDA5 is positively regulated by LGP2 [48]. Further studies are needed to clarify the specific mechanisms involved in these pathways in teleost fish.

Tilapia are one of the most important freshwater aquaculture species in the world. However, tilapia farming has been severely threatened since 2009 due to *Streptococcus agalactiae* infection [49]. An efficient way to prevent the disease has not yet been found. Understanding the innate immune responses elicited by various PRRs will potentially help to develop effective measures to control *Streptococcus* infection. In this study, we report on the cloning and characterization of Nile tilapia MDA5, LGP2 and MAVS genes. We explored the expression patterns of these genes in different tissues as well as their expression profiles during bacterial infection. We also investigated the expression profiles of the three receptors during embryonic development. Eukaryotic expression vectors of the three receptors were constructed and were cotransfected into 293 T cells with NF-κB reporter plasmids. Using dual-luciferase reporter assays, we revealed the effect of overexpression of the three receptors on NF-κB activation in HEK 293 cells. This study will provide useful information for understanding the innate immune mechanisms in tilapia and will provide a basis for the selection of disease resistance-related markers for breeding Nile tilapia.

2. Materials and methods

2.1. Experimental infection and sample collection

Tilapia (*O. niloticus*) (body weight: 60–80 g; body length: 16–20 cm) were collected from the Gaoyao fish farm of the Pearl River Fisheries Research Institute (Guangzhou, China). Healthy fish were kept in a 500-L tank at 30–31 °C for two weeks. The fish were fed twice daily with a commercial diet (~30% crude protein) at a rate of 3% of their body weight per day. Water quality parameters, including dissolved oxygen, pH, and ammonia concentrations, were monitored during the experiment. Six healthy individuals were anesthetized with MS222 (Sigma, Missouri, Saint Louis, USA) (dose: 30 mg L^{-1}) and dissected, and 11 types of tissues, including the kidney, liver, gill, brain, intestine, muscle, heart, stomach, skin, blood, and spleen, were harvested for RNA extraction. Tilapia fin samples were collected for genomic DNA extraction.

For the gene expression analysis during embryonic development, approximately 80 tilapia embryos were randomly sampled at 2, 3, 4, 5, 6, 7 and 8 days postfertilization (dpf). RNA was extracted from three samples (approximately 25 embryos per sample) at each time point.

To determine the challenge concentrations, different concentrations of *S. agalactiae* were tested in a prechallenge experiment. Twenty fish were used in each group (one control group and three challenge groups). *S. agalactiae* were cultured at 37 °C for 15 h until the density reached 3×10^8 CFU mL^{-1}. The bacterial suspension was then diluted to 10^6, 10^7 and 10^8 CFU mL^{-1} in phosphate-buffered saline (PBS, pH 7.2). Then, 200 μL of the bacterial suspension was injected into each individual. Fourteen and eleven individuals died in the 10^8 CFU mL^{-1} group and 10^7 CFU mL^{-1} group, respectively. Six individuals died in the 10^6 CFU mL^{-1} group. Therefore, we used 10^7 CFU mL^{-1} in the challenge experiment. Approximately 120 individuals in the treatment groups were each intraperitoneally injected with 200 μL of bacterial suspension (10^7 CFU mL^{-1}), while 40 individuals in the control group were intraperitoneally injected with the same volume of PBS. Four individuals were randomly sampled at 8 h postinfection (hpi) and at 1, 3, 6 and 9 days postinfection (dpi). Tissues from the tilapia, including the intestine, gills, spleen, kidney and blood, were collected for total RNA extraction to analyze the temporal gene expression profiles after the