Fish & Shellfish Immunology 58 (2016) 193–202

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

The evolution and functional characterization of miiuy croaker cytosolic gene LGP2 involved in immune response

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ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form
5 September 2016
Accepted 12 September 2016
Available online 13 September 2016

Keywords:
LGP2
Miiuy croaker
Expression
Molecular evolution
Immune response
Cellular localization

ABSTRACT

The laboratory of genetics and physiology 2 (LGP2) is a member of retinoic acid-inducible gene I (RIG-I)-like receptors (RLR receptors), which may participate in the immune regulation process. The role of LGP2 on modulating signaling was ambiguous, some researchers suggested that the regulation mechanism of LGP2 to melanoma differentiation-associated gene 5 (MDA5) and retinoic acid inducible gene-I (RIG-I) were different. In this study, the bioinformatics and functions of LGP2 from miiuy croaker (mmLGP2) were characterized. Comparative genomic analysis showed that the evolution of LGP2 in mammals was more conserved than it in fish. LGP2 contains three structural domains: ResIII, HelicaseC and RD, and ResIII structural domain of LGP2 was extremely conservative. The mmLGP2 was ubiquitously expressed in the tested miiuy croaker tissues and the expressions were significantly upregulated after stimulation with poly(I:C), indicating that LGP2 might participate in the immune response, especially antiviral immunity. Furthermore, immunofluorescence of miiuy croaker LGP2 presents in the cytoplasm in Hela cells. The overexpression of mmLGP2 can activate ISRE, but cannot activate NF-κB luciferase reporter, implying that mmLGP2 might act as a positive regulator in immune responses through activating ISRE to induce the expression of IFN. The research of mmLGP2 will enrich the information of fish LGP2, and the functional experiments will be helpful for the future research about fish immune systems.

1. Introduction

Vertebrates are threatened by microorganisms and virus invasion constantly, they have evolved immune systems to eliminate infective pathogens [1]. Generally, the immune system can be divided into two branches: innate and acquired immunity. Based on the existing research results, the innate immune system is the first line of host defense against invading pathogens, recognizing microorganisms via germline-encoded pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) [2]. These PRRs have been identified in many species, including humans, rodents, birds, and teleost fishes [3].

RLRs as a type of intracellular PRR result in the development of inflammation and host resistance to infection [4]. The members of RLRs: RIG-I, MDA5, and LGP2 act as sensors of viral replication, which activated downstream interferon-regulatory factors (IRFs) and proinflammatory cytokine genes by interacting with a mitochondrion-anchored adaptor molecule, IPS-1 [5,6]. The LGP2 gene lack a caspase recruitment domain (CARD) comparing with the other members of RLR family, therefore, LGP2 can't directly induce cellular response, but it plays critical role in regulating cellular immune response [7]. The LGP2-deficient mice also had the capacity to resist the lethal infection with a RIG-I-dependent virus, and produced more IFN, which were performed by Venkataraman and colleagues, revealed that LGP2 may inhibit responses mediated by RIG-I [8,9]. However, further study revealed that LGP2 is required for IFN production to resist viruses recognized by MDA5 [10]. The immune-regulatory effects of LGP2 to immune response mediated by RIG-I was negative regulation, and positively regulate immune response mediated by MDA5 [9]. In addition, RIG-I gene absent in most fish, and the LGP2 gene and MDA5 gene were proposed to function separately in fish, that different from the functions of LGP2 in mammals [11].

The functions of LGP2 on antiviral immunity are variety and uncertainty, so more researches about LGP2 should be performed in fish. In this study, the characterization of sequences and tertiary structure of mmLGP2 were analyzed for better understanding the characteristic of LGP2. The molecular evolution and positive

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http://dx.doi.org/10.1016/j.fsi.2016.09.025
1050-4648/

selection pressure on LGP2 was performed for analyzing the evolution of LGP2 in different species. Furthermore, the location of miiuy croaker LGP2 was showed by immunofluorescence. Meanwhile, the functional experiments of mmLGP2 were performed in vivo and in vitro to detect the role of LGP2 in immunity. The researches about LGP2 will provide more information for functional study in the future.

2. Materials and methods

2.1. Samples

We acquired healthy miiuy croakers from Zhoushan Fisheries Research Institute (Zhejiang, China), then placed in aerated water tanks at ambient temperature (25 °C). Acclimatizing one week, only healthy miiuy croaker (general appearance and level of activity) were performed the challenge experiments as previously described [12,13]. Miiuy croaker were randomly divided into six groups, experimental group injected with 1 ml lipopolysaccharides (LPS) (1.0 mg/ml), 1 ml *V. anguillarum* (1.5×10^8 CFU/ml), 1 ml *V. harveyi* (1.5×10^8 CFU/ml), 1 ml *Staphylococcus aureus* (1.5×10^8 CFU/ml) and 1 ml poly(I:C) (2.5 mg/ml), respectively, and control group injected with PBS. Each group of twelve fish, tissues (liver, spleen and kidney) from three fish of each kind of infected fish were removed at each sampling point (6 h, 12 h, 24 h, 36 h), respectively. Twelve tissues (spleen, liver, kidney, gill, heart, intestine, brain, blood, skin, muscle, eye and fin) were removed from healthy fish. The obtained tissues were kept at −80 °C for future use.

2.2. Isolation of miiuy croaker macrophage and Poly(I:C) exposure

Isolation of macrophage from miiuy croaker was as follow. Head kidney (HK) were minced thoroughly and filtered by a 100-μm pore size cell strainer in L-15 medium with penicillin (400 IU/ml), streptomycin (400 μg/ml), 2% fetal bovine serum (FBS) and heparin (20 U/ml), then the suspension was placed on a 51% Percoll gradient and centrifuged at 400 g for 40 min at 4 °C. Subsequently, the cells at the interface were collected carefully and washed twice with L-15 medium. For the Poly(I:C) exposure, macrophage were seeded in 6-well plates at a density of 4×10^7 cells per well in L-15 containing 0.1% FBS and cultured at 26 °C, 4% CO_2. The next day, the culture medium was replaced with fresh complete L-15 medium supplemented with 5% FBS containing Poly(I:C) (10 μg/ml), then the cells were harvested at 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h, respectively.

2.3. RNA isolation, cDNA synthesis and sequencing

Total RNA was extracted from different tissues of individuals and cells using Trizol reagent (Qiagen) and the cDNA template was reverse-transcribed utilizing QuantScript RT Kit (TIANGEN) according to the manufacturer's instructions. An Illumina HiSeq™ 2000 sequencing system was used to sequence the cDNA libraries at the Beijing Genomics Institute (BGI) [14].

2.4. Sequence analysis

Gene sequences of LGP2 from different species in NCBI database or Ensemble database were used to query the miiuy croaker transcriptome and whole genome database by the BLASTP and TBLASTN programs [14–16]. The complete cDNA sequence of mmLGP2 and the full length of LGP2 gene were successful identified. MEGA5 performed MUSCLE software to alignment the LGP2 sequences of miiuy croaker and other species [17]. To ensure introns and exons of LGP2 gene, we modified multi-sequences alignment of LGP2 sequences manually to reduce the error information and manually sequences in miiuy croaker based on the principle of GT-AG. The conserved structure domains were predicted by a Pfam HMM [7]. The domain architecture of three domains (ResIII, HelicaseC and RD) of LGP2 from miiuy croaker, human and stickleback were predicted by SWISS-MODEL Repository software (http://swissmodel.expasy.org/repository/). Gene synteny were analyzed in order to better understanding the evolution of LGP2. The genes surrounding LGP2 were confirmed in ensemble database, genomics and Map Viewer in NCBI.

2.5. Molecular evolution of LGP2 analysis

We chose the representative species to structure phylogenetic tree of LGP2 by the maximum likelihood (ML) method with MEGA5 package, then the phylogenetic tree was used to test whether the ancestral branches of evolutionary trees under different selection pressure get the corresponding adaptive generation [18]. The ML methods in CODEML program of PAML v4 were used to analyze all the ancestral branches in LGP2 gene [19]. We used one-ratio model and free ratio model to identify the selective pressures in LGP2 gene. Finally, we detect the interested foreground lineages by using the branch-site model.

Individual site-domains in the site model of CODEML program were implemented to account for the different function and structural constraint experienced of the subsets of mammalian and teleost LGP2 sequences [19]. Meanwhile, the Hyphy package in the Data Monkey Web Server (http://www.datamonkey.org) was also implemented to detect the candidates for positive selection [20]. In CODEML, six site models were used on the mammalian and teleost LGP2 sequence subsets to test the possible positively selected site. The twice differences of log-likelihood values (2ΔlnL) between each two nested models were calculated following a chi-squared distribution with degrees of freedom [21]. Using the Bayes empirical Bayes (BEB) in the case of models M2a and M8 calculate the Bayesian posterior probability (BPP) of the codon sites under positive selection was calculated [22].

2.6. qRT-PCR analysis

Twelve tissues (spleen, liver, kidney, gill, heart, intestine, brain, blood, skin, muscle, eye and fin) from normal miiuy croaker, three poly(I:C) infected tissues (liver, spleen and kidney) and macrophage with different time points were used to analyze the expression pattern. Two pairs of gene-specific primers (LGP2-RT-F/R and β-actin-RT-F/R) were designed and used in the study of the mmLGP2 gene expressions. A 7300 qRT-PCR system (Applied Biosystems, USA) was used to analyze the expression pattern. Each sample was performed in triplicate for PCR amplification with the cycling conditions: 10 s at 95 °C, followed by 40 cycles consisting of 5 s at 95 °C, then 34 s at 60 °C. The relative expression levels of LGP2 were normalized against β-actin according to the $2^{-\triangle\triangle CT}$ method [23]. And comparisons between groups were analyzed by one-way ANOVA using a Duncan test for identification of the statistically distinct groups.

2.7. Expression plasmids construction

The full length CDS region of mmLGP2 was amplified by PCR with primers containing HA tag, Kpn I and Xba I restriction enzyme cutting sites. The product was digested and then ligated into the pCDNA3.1 vector which was digested by corresponding enzymes. The recombinant plasmid was confirmed by double enzyme digestion and sequencing. The Endotoxin-Free Plasmid DNA Miniprep Kit (Tiangen) for cell transfection was used to extract the