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Research Article

Characterization, Expression, and Ligand Binding of LGP2 and MDA5 in Largemouth Black Bass *Micropterus salmoides* (Lacepède, 1802)

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Melanoma differentiation-associated gene 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2) are family members of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which play important roles in the immune response against pathogens invasion. In the present study, MDA5 and LGP2 genes were identified in largemouth bass (*Micropterus salmoides*), a fish species with a great economic value. The two proteins contained similar conserved domains and motifs as their counterparts of other vertebrates, including the DExDc domain (the DEAD/DEAH box helicases domain), HELICc domain (helicases superfamily domain), and regulatory domain (RD). Real-time qPCR revealed that the two genes were constitutively expressed in tissues of healthy fish and could be induced in the spleen by polyinosinic and polycytidylic acid (polyI:C) challenge *in vivo*. Also, selective pressure analysis revealed that the negative selection had roles in the evolutions of the two genes. Furthermore, the dsRNA binding mechanism of msLGP2 and msMDA5 were analyzed by the molecular docking strategy. The amino acids of msLGP2 involved in dsRNA binding were V604, N663, L682, and L684, which were located in the regulatory domain (RD) of msLGP2. The amino acids of msMDA5 involved in dsRNA binding were G429, H434, L842, and L845, which were located in the DExDc domain and the RD domain of msMDA5. These results indicated that fish LGP2 and MDA5 might share similar functions and ligand binding mechanism as their mammalian counterparts.

1. Introduction

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are important host pattern recognition receptors (PRRs) which are involved in sensing RNA of a replicating virus in the cytoplasm [1]. Mammalian RLR family consists of three members, namely, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [2]. RIG-I and MDA5 share similar structures. Both of them contain two N-terminal caspase activation and recruitment domains (CARDs) that act as signaling domains, a central DExD/H RNA helicase domain, also known as "Walker motif," that is involved in ATP hydrolysis and RNA binding, and a C-terminal domain (CTD) that aids in ligands recognition [3]. LGP2 lacks the two CARDs but possesses the DExD/H RNA helicase domain [4].

It has been found that mammalian RIG-I can recognize ssRNA with 5′-phosphates (5′ ppp-ssRNA), short dsRNA (<1 kb) and poly (dA-dT) DNA [5, 6]. MDA5 is a sensor of long dsRNA (>3 kb) including synthetic analogs (e.g., polyinosinic: polycytidylic acid (polyI:C)) [7]. After binding their ligands, RIG-I or MDA5 will bind the adaptor molecule, mitochondrial antiviral signaling adaptor (MAVS), and trigger the expressions of interferons (IFNs) and other proinflammatory cytokines to

eliminate the virus [8]. LGP2 can also recognize dsRNAs and 5′ ppp-ssRNA but is unable to interact with MAVS due to the lack of the CARD domain [9]. LGP2 was initially identified as a feedback inhibitor of RIG-I/MDA5-trigged IFN signaling [10]. Recent report found that fish and human LGP2s switched regulatory roles from a positive one to a negative one in increasing concentrations of poly (I: C)-triggered IFN response [11].

Until now, RLRs had been characterized in several fish species [1, 12], with surprisingly finding that RIG-I was absent in some fish species, such as large yellow croaker, mandarin fish [13], and snakehead [12]. Fish RLRs shared similar structures and functions as mammalian RLRs, which could be induced by dsRNA, polyI:C, or viruses (e.g., grass carp reovirus (GCRV) and viral hemorrhagic septicemia virus (VHSV)) [14–16]. It had been confirmed that dsRNA was the ligand of fish RIG-I and MDA5 [17], whilst the ligand of fish LGP2 remains unclear. In addition, teleost RLRs could be induced by LPS or bacterial infections. *Edwardsiella ictaluri* induced the expressions of RIG-1, MDA5, and LGP2 in the liver of channel catfish (*Ictalurus punctatus*) [18]. LPS exposure upregulated the expressions of RIG-1, MDA5, and LGP2 in primary trunk kidney cells of grass carp (*Ctenopharyngodon idella*) [14]. These results indicate that fish RLRs were involved in the immune defense against bacterial and viruses.

Largemouth black bass (*Micropterus salmoides*) (Lacepède, 1802) has become an economically important fish species due to its fast growth and flavor [19]. However, studies on largemouth bass RLRs have not been reported. In the present study, the sequences and structures of largemouth bass LGP2 and MDA5 (msLGP2 and msMDA5) were analyzed, and their expressions in normal tissues and the spleen following polyI:C challenge were detected. Furthermore, the polyI:C binding ability of msLGP2 and msMDA5 were evaluated. Also, the evolutionary process of LGP2 and MDA5 was analyzed based on selective pressure. Our results provide the basis for understanding the immune functions of fish RLRs.

2. Materials and Methods

2.1. Fish. This study was strictly carried out in accordance with the regulations for the Administration of Laboratory Animals in Jiangsu Province, P. R. China. Fish was anaesthetized with ethyl-3-amino-benzoate methanesulfonic acid (50 mg/L, MS-222) (Sigma, USA) for tissue collection [20].

Largemouth black bass (50.0 ± 5.0 g; 17.5 ± 1.2 cm) were provided by Sheyang Kangyu Aquatic Technology Co. Ltd. (Yancheng, Jiangsu Province, China). The fish were acclimatized to the experimental conditions for at least two weeks. The fish were reared in the indoor circular plastic tanks (volume: 100 L) and fed with a commercial diet (45.97% crude protein, 10.42% crude lipid, and 7.79% fiber) twice daily (8:00 and 17:00). During the experimental period, water temperature and pH were measured daily and the values were, respectively, controlled as 22 ± 1°C, 7.0–7.5. Dissolved oxygen (DO) was controlled over 5 mg/L and total ammonia nitrogen was below 0.5 mg/L.

2.2. RNA Extraction, cDNA Synthesis, and Gene Cloning. The total RNA of each tissue was extracted using the Trizol reagent (Sangon Biotech, China) according to the manufacturer's instruction. Then, the first strand cDNA was synthesized using SMART™ RACE cDNA amplication kit (Clontech, USA), following the manufacturer's protocol.

The partial sequences of msLGP2 and msMDA5 were obtained by searching the splenic transcriptome database that we constructed previously using local BLAST software [21]. The reverse transcription polymerase chain reaction (RT-PCR) was performed on T100 Thermal Cycler (Bio-Rad, USA) with the spleen cDNA as a template and specific primers (MDA5-F1/MDA5R1; LGP2-F1/LGP2-R1) that were designed according to the obtained sequences to validate the correction of the sequences. PCR was carried out in the 25 μL reaction system as follows: 12.5 μL of 2 × EasyTaq® PCR SuperMix (TransGen Biotech, China), 0.1 μM of each primer, 1 μL of cDNA template, and 11.3 μL of nuclear-free water. PCR amplification was conducted under the conditions of an initial denaturation step at 94°C for 5 min, followed 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and finally, an extension step at 72°C for 10 min. The PCR products with the expected size of 893 bp of msMDA5 and 886 bp of msLGP2 were ligated into the pMD18-T vector (TaKaRa, Japan) and were verified by sequencing. Next, the full cDNA sequences of largemouth bass LGP2 and MDA5 were obtained using SMART™ RACE cDNA amplication kit (TaKaRa, Japan), following the manufacturer's instructions. The amplified cDNA fragments were assembled to get the full cDNA sequences of msLGP2 and msMDA5 by using the SeqMan program in the DNASTAR software (Madison, WI, USA) [22]. All primers used for gene clone are listed in Table S1.

2.3. Tissue Distributions of msLGP2 and msMDA5. Seven tissues including the brain, gill, head kidney (HK), intestine, liver, skin, and spleen were, respectively, sampled from three healthy largemouth bass (50.0 ± 5.0 g; 17.5 ± 1.2 cm) that were fasted for 3 days. The total RNA of each tissue was extracted using the Trizol regent (Sangon Biotech, China). The cDNA used for quantitative real-time PCR (qPCR) were synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Japan), following the manufacturer's instruction. Expression levels of msLGP2 and msMDA5 in each tissue were detected using the qPCR method. All primers used for gene expression analysis are listed in Table S1.

2.4. Expressions of msLGP2 and msMDA5 in the Spleen following PolyI:C Challenge. A total number of 60 largemouth bass (50.0 ± 5.0 g; 17.5 ± 1.2 cm) were randomly divided into two groups (30 fish per group): the polyI:C challenge group in which fish were intraperitoneally (i.p.) injected with 1 mg/mL polyI:C (Sigma-Aldrich, USA) (100 μg per 100 g fish) [23] and the control group in which fish were i.p. injected with the same amount of sterilized phosphate-buffered saline (PBS). During the challenge period, fish were fed twice daily. At 0, 6, 12, 24, 48, and 72 h postinjection (HPI), the

spleen of three fish in each group was sampled. The transcript changes of msLGP2 and msMDA5 were detected using the qPCR method. All primers used for gene expression analysis are listed in Table S1.

2.5. qPCR. The qPCR was performed on the CFX96 Touch™ real-time PCR detection system (Bio-Rad, USA) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan). The reaction volume and reaction conditions were done as in the previous study [23]. The relative expressions of msLGP2 and msMDA5 in normal tissues and spleens following polyI:C challenge were analyzed using the $2^{-\Delta\Delta CT}$ method [24] with β-actin as the reference gene. The primers for qPCR were LGP2-F/LGP2-R, MDA5-F/MDA5-R, and β-actin-F/β-actin-R (Table S1). All data were presented as the mean ± standard error (SE) and analyzed using SPSS statistics package 24 (SPSS, USA) as described in the previous studies [23, 25].

2.6. Evolutionary Analysis of LGP2 and MDA5. A total number of 24 LGP2 sequences and 23 MDA5 sequences from different vertebrate species were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov) and used for evolutionary analysis. The sequence alignment was analyzed using Clustal O [26]. The phylogenic analysis was carried out using MEGA 11 software using the neighbor-joining method and the Jones–Taylor–Thornton (JTT) model, and the bootstrap value was set as 10,000 replicates [27]. The selective pressure analysis was performed using Datamonkey 2.0 program [28]. In brief, the nonsynonymous to synonymous nucleotide substitution rate (ω) was calculated for all selected nucleotide acid sequences. The site-wise synonymous was estimated using the FEL (fixed effects likelihood) and SLAC (single likelihood ancestor counting) models. The nonsynonymous/synonymous (dN/dS) rate ratio >1 or <1 represents positive selection or negative selection from the abovementioned two models, respectively. Positive and negative selected sites were detected and selected from both two analyzed models according to P value <0.05.

2.7. Molecular Docking of msLGP2 and msMDA5 with PolyI:C. The 3D structures of msLGP2 and msMDA5 were constructed using the SWISS-MODEL server [29]. The quality and quantity of 3D structures were verified using PROCHECK [30], ProQ [31], ProSA [32], and MolProbity [33]. The molecular docking of msLGP2 and msMDA5 with polyI:C was carried out using AutoDock software [34]. In brief, the PDB files of msLGP2 and msMDA5, obtained from SWISS-MODEL server, were imported and set as receptor proteins, whereas the PDB file of PolyI:C was set as ligand molecule for molecular docking. The number of GA runs was set to 50 to obtain the optimal conformation and assess the docking binding energy and binding sites. The optimal conformation result file was exported and imported into PyMOL software [35], and the root mean square deviation (RMSD) value was calculated to see if the docking result was stable [36]. The 2D structure of polyI:C (CID: 135478809) was obtained from the PubChem database [37], and its 3D coordinates were generated subjecting to chirality, full charge, and energy minimization in the PRODRG2 server [38].

3. Results and Discussion

LGP2 and MDA5, belonging to the RLRs family, play crucial roles in the immune response against virus invasion. In the present study, LGP2 and MDA5 were first identified in largemouth black bass. Then, their expressions in normal tissues and the spleen following polyI:C stimulation were investigated. Our results provided the basis for studying the function and mechanism of the RLRs signaling pathway.

3.1. Sequence Features of msLGP2 and msMDA5. The full cDNA sequences of msLGP2 and msMDA5 were obtained by RT-PCR and RACE-PCR. The sequence features of the two genes are listed in Table S2. In brief, the cDNA sequence of msLGP2 was 2,326 bp in length, containing 45 bp of the 5′-untranslated region (UTR), 151 bp of 3′-UTR, and 2,130 bp of the open reading frame (ORF) which encodes 709 amino acids. The cDNA sequence of msMDA5 was 3,368 bp in length, containing 50 bp of 5′- UTR, 351 bp of 3′-UTR, and 2,967 bp of ORF encoding 988 amino acids. Both proteins shared similar amino acids length as their fish counterparts [12, 13]. Also, the two proteins shared higher sequence identities with their counterparts of other fish species than with mammalian homologous (Tables S3 and S4). msLGP2 had 61.4%–83.4% sequence identities with fish LGP2, and whist had 44.7%–49.5% sequence identities with LGP2 of other vertebrates (Table S3). Similar to their mammalian counterparts, the two proteins contained some conserved domains, including the DExDc domain (the DEAD/DEAH box helicases domain), HELICc domain (helicases superfamily domain), and regulatory domain (RD) (Figures S1 and S2). Among these domains, the DExDc domain contained an ATP-binding site, an RNA unwinding motif, and a putative Mg^{2+} binding motif, which are involved in dsRNA binding and unwinding [39]. The HELICc domain took part in the cleavage of unmethylated double-stranded foreign DNA and the self-DNA defense from damage [40]. The RD domain was involved in detecting and binding both ssRNA and dsRNA with 5′-triphosphate containing RNA [41]. These domains were important for the antiviral functions of the two proteins. In addition, these conserved domains, six conserved helicases motifs (motif I–VI), were found in the two proteins, with motif I–III located in the DExDc domain and motif IV–VI located in the HELICc domain, which are involved in interacting with dsRNA [42]. Besides, two more caspase activation and recruitment domains (CARDs) were identified in msMDA5 (Figure S2), which are involved in MAVS activation [43]. These conserved domains or motifs found in msLGP2 and msMDA5 indicated that the two proteins had similar functions as their counterparts in other animals. Furthermore, phylogenetic tree analysis showed that the two proteins of the largemouth black bass were well clustered with their fish counterparts, supported by high bootstrap values (100%) (Figure 1). These analyses confirmed

that the two genes we cloned were exactly the homologous of other animals.

3.2. Expressions of msLGP2 and msMDA5 in Normal Tissues. The expressions of msLGP2 and msMDA5 in normal tissues of the largemouth black bass were detected using qPCR. Results showed that the two genes were constitutively expressed in all selected tissues, including the brain, gill, head kidney, intestine, liver, skin, and spleen (Figure 2(a)). Similar results were also observed in Japanese flounder [1], snakehead (*Channa argus*) [12], mandarin fish [13], zebrafish [15], and grass carp [14, 16], indicating that many tissues were involved in the LGP2- or MDA5-mediated immune reactions. However, their expressions in different tissues were varied. MsLGP2 was highly expressed in the gill, intestine, liver, and spleen and lowly in the brain, HK, and skin (Figure 2(a)). In contrast, msMDA5 was highly expressed in the brain, liver, and skin, moderately in the gill, intestine, and spleen, and lowly in the HK (Figure 2(a)). The spleen is important lymphoid organs of fish [44], and high expressions of msLGP2 and msMDA5 in the spleen indicated that both genes had important roles in the immune reaction of largemouth black bass. Among these tissues, the gill, intestine, and skin are important mucosal immune organs of fish [45]. The high expression of msLGP2 and msMDA5 in these tissues indicates their roles in the mucosal immunity of largemouth black bass. Also, expressions of the same gene from different fish species were distinct. Snakehead LGP2 was mainly expressed in the intestine, liver, stomach, heart, and blood [12], and miiuy croaker (*Miichthys miiuy*) LGP2 was highly expressed in the kidney [46], whilst Indian major carp (*Labeo rohita*) LGP2 was highly expressed in the blood and liver [47]. Similarly, common carp (*Cyprinus carpio*) MDA5 was highly expressed in gills and the spleen [48], while snakehead MDA5 was mainly found in the intestine, liver, stomach, heart, and blood [12]. Thus, the expressions of fish LGP2 and MDA5 might be species specific and tissue specific.

3.3. Expressions of msLGP2 and msMDA5 in the Spleen following PolyI:C Challenge. PolyI:C is a synthetic analog of dsRNA virus which can induce the fish RLRs signaling pathway [11]. The spleen is an important immune organ of fish [49]. As we found that msLGP2 and msMDA5 were highly expressed in the spleen of healthy fish (Figure 2(a)), we further analyzed the expressions of msLGP2 and msMDA5 in the spleen following polyI:C challenge to study the potential antiviral functions of these genes. Results showed that the expressions of the two genes were similar in the spleen following polyI:C challenge. The expression of msLGP2 in the spleen was upregulated from 6 h to 48 h post polyI:C challenge (Figure 2(b)) and that of msMDA5 was significantly induced by polyI:C from 6 h to 72 h, when compared with that with PBS injection (Figure 2(c)). Our results were in line with the previous studies. Snakehead LGP2 and MDA5 were induced in the primary cells isolated from the spleen at 3 h and 6 h post polyI:C challenge [12]. Common carp MDA5 was induced in the spleen from 3 h to 120 h post polyI:C challenge [48]. Also, the Indian major carp LGP2 was induced in *L. rohita* gill (LRG) cell at 4 h after polyI:C stimulation [3]. Similarly, Japanese flounder LGP2 was upregulated in leukocytes from 6 h to 48 h post polyI:C stimulation [1]. In addition, virus infection could regulate the expressions of LGP2 and MDA5. For example, Japanese flounder LGP2 could be induced from 3 h to 6 h after viral hemorrhagic septicemia virus (VHSV) infection [1]. These results indicated that fish LGP2 and MDA5 could be induced by virus, similar to mammalian LG2 and MDA5.

3.4. Selective Pressure Analysis of msLGP2 and msMDA5. To better understand the evolution of LGP2 and MDA5 genes, the selective pressure analysis of the two genes were analyzed with two site models, the FEL and SLAC models. The values of d_N/d_S for the two genes were lower than 1, indicating that both of them were under negative selection pressure. Furthermore, amino acids under positive and negative selection pressures were obtained (Table 1). Only one positive selection site was identified in msLGP2 (Table 1), which is located in the RD domain of msLGP2 (Figure S1). Similarly, a previous study also identified one positive selection site in miiuy croaker LGP2 [46]. Four positive selection sites were found in msMD5 (Table 1), among which two sites (W73 and V572) were, respectively, located in the CARD1 and DExDc domains of msMDA5 (Figure S2). The positive pressure selection in those sites may have significant effects on the functions msLGP2 and msMDA5 [50].

3.5. Molecular Docking of msLGP2 and msMDA5 with PolyI:C. We further analyzed the potential binding mechanism of msLGP2 and msMDA5 with polyI:C using the molecular docking strategy. Results showed that both msLGP2 and msMDA5 could bind with polyI:C (Figures 2(a) and 2(b)). The amino acids of msLGP2 involved in binding with polyI:C were V604, N663, L682, and L684 (Figures 3(a) and 3(c)). All these four binding sites were located in the RD domain of msLGP2 (Figure S1). The amino acids of msMDA5 involved in binding with polyI:C were G429, H434, L842, and L845 (Figures 3(b) and 3(d)), among which G429 and H434 were located in the DExDc domain, and L842 and L845 were located in the RD domain of msMDA5 (Figure S2). It had been found that both the RD domain and the DExDc domain of mammalian LGP2 and MDA5 play roles in dsRNA binding [31, 39]. Our molecular docking results confirmed that these domains in fish LGP2 and MDA5 are also involved in dsRNA binding, indicating that fish LGP2 and MDA5 may share similar ligand binding mechanism as their mammalian counterparts.

In conclusion, two RLRs, LGP2 and MDA5, were identified in largemouth black bass. These two RLRs were constitutively expressed in all selected tissues and could be induced in the spleen by polyI:C challenge. Also, the negative selection had important roles in the evolutions of the two genes. Furthermore, the molecular docking strategy revealed that msLGP2 and msMDA5 might share similar ligand binding mechanism as their mammalian counterparts. These results provided the basis for understanding

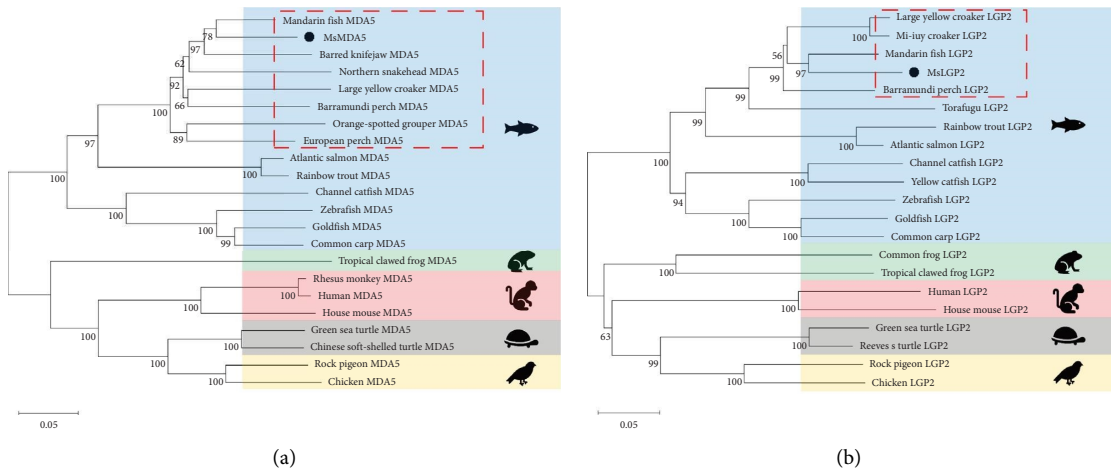

FIGURE 1: Phylogenetic analysis of vertebrates' LGP2 (a) and MDA5 (b) amino acids. Neighbor-joining (NJ) trees were constructed by protein sequences. All GenBank accession numbers of MDA5, LGP2, and MAVS sequences are given in Tables S3 and S4.

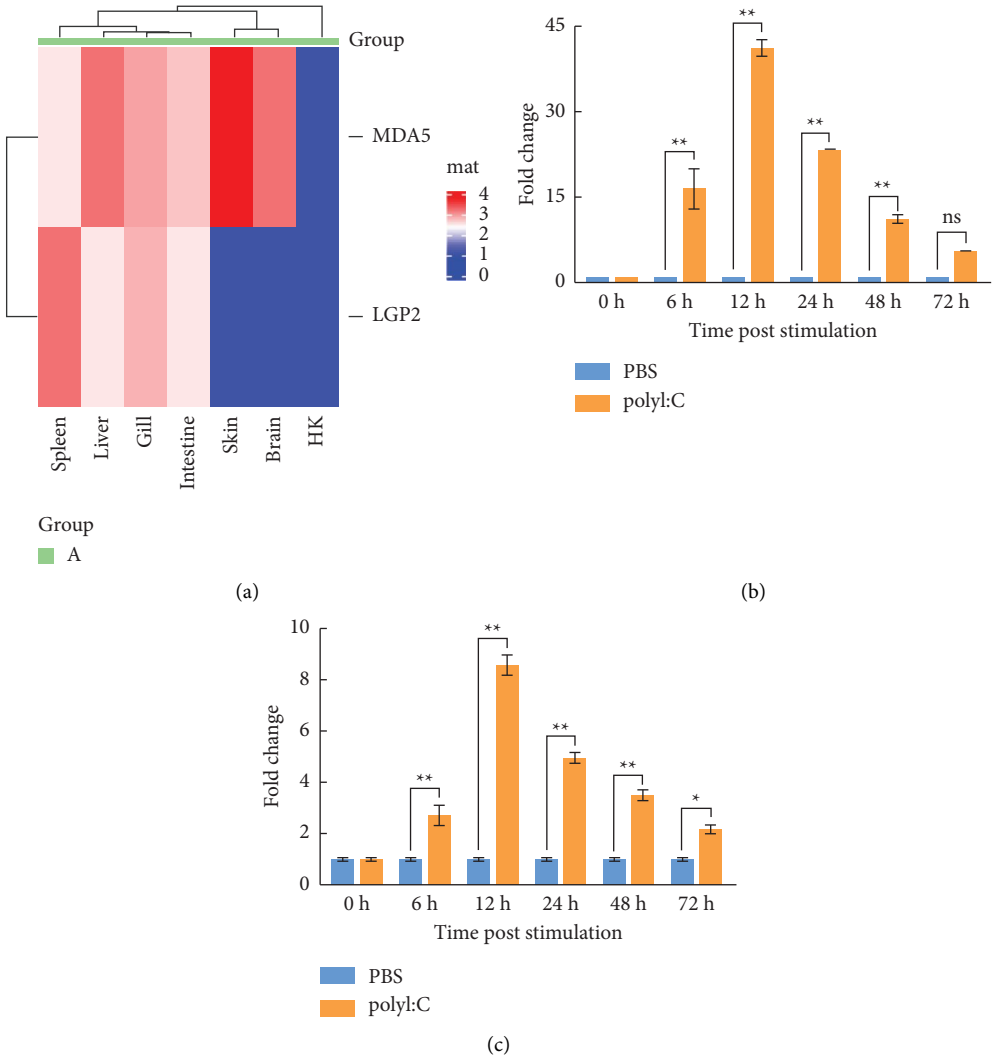

FIGURE 2: Expression analysis of msLGP2 and msMDA5 genes. (a) Expressions of msLGP2 and msMDA5 genes in normal tissues of largemouth bass. Different tissues of fish ($n = 3$) were collected to detect gene expression by qPCR, and the levels of gene expression were normalized against that of β-actin. (b) Expressions of msLGP2 in the spleen after polyI:C challenge. (c) Expressions of msMDA5 in the spleen after polyI:C challenge. The fish for polyI:C challenge were i.p. injected with polyI:C (100 μg per 100 g fish) and fish for control were i.p. injected with the same amount of sterilized PBS. At 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h postinjection (hpi), the spleen were sampled and the gene expressions were detected by qPCR. Data were expressed as mean ± SE, with * and ** indicating $P < 0.05$ and $P < 0.01$, respectively.

Table 1: Selective pressure analysis of msLGP2 and msMDA5.

Genes	d_N/d_S (FEL model)	d_N/d_S (SLAC model)	Sites ($d_N/d_S < 1$)	Sites ($d_N/d_S > 1$)
LGP2	0.1849	0.2109	31, 40, 92, 150, 183, 246, 282, 341, 414, 436, 479, 562, 605, 643, 652, 700	672
MDA5	0.2336	0.2651	61, 95, 319, 358, 472, 522, 611, 642, 765, 803, 813, 927, 969, 991, 1027	73, 251, 262, 572

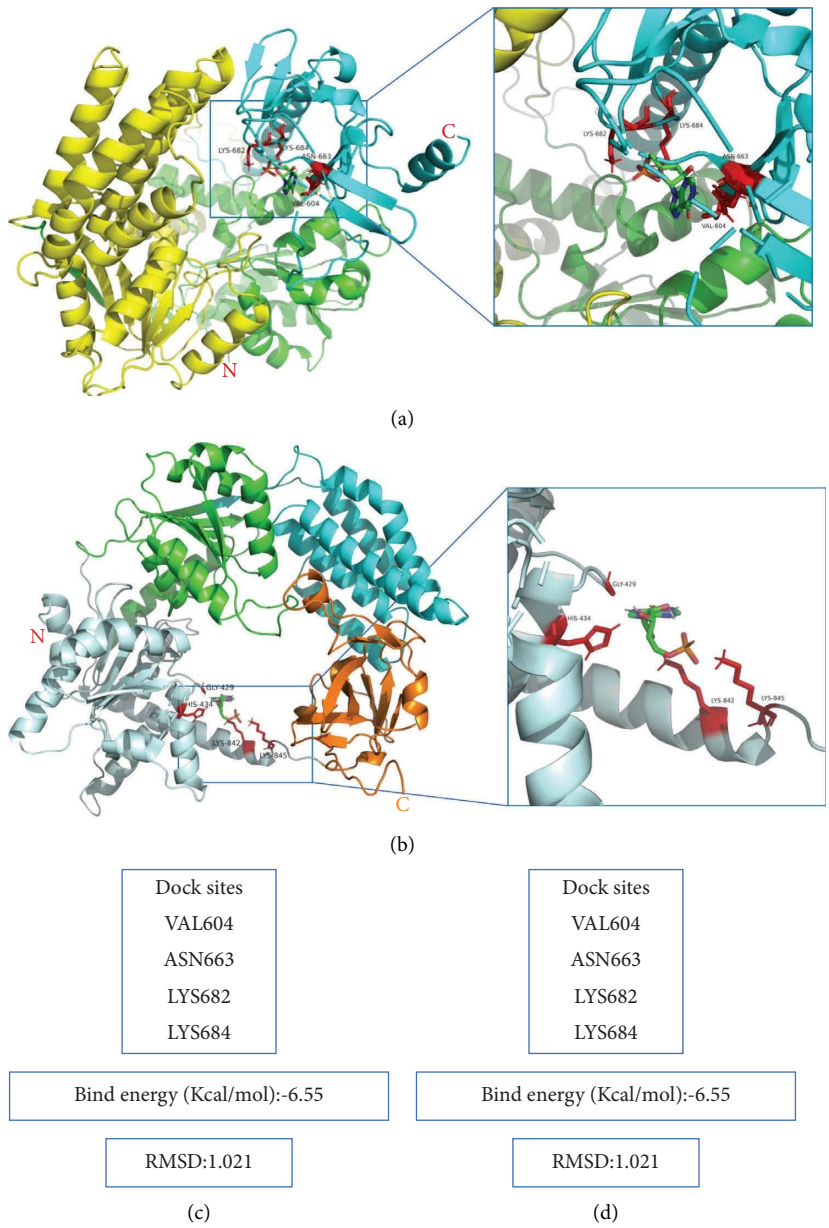

Figure 3: Molecular docking results of msLGP2 (a) and msMDA5 (b) with polyI:C. The molecular docking was carried out using AutoDock software. The binding energy and RMSD (c, d) were calculated to evaluate the stability of the receptor-ligand complex.

the immune functions and mechanism of the two RLRs in fish.

Data Availability

The data used to support the findings of this study are available on request from the corresponding author.

Ethical Approval

This study was conducted in accordance with the regulations for the Administration of Laboratory Animals in Jiangsu Province, P. R. China. For the tissue collection, the fish were treated with ethyl-3-aminobenzoate methanesulfonate (MS-222, Sigma, USA).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Xiangyu Pi and Yang Xu contributed equally.

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Supplementary Materials

Table S1: primers used in the study. Table S2: sequence features of largemouth bass LGP2 and MDA5. Table S3: sequence identity between largemouth bass LGP2 and vertebrates' LGP2. Table S4: sequence identity between largemouth bass MDA5 and vertebrates' MDA5. Figure S1: sequence alignment vertebrates' LGP2. Three conserved domains, the DExDc domain, HELICc domain, and RD domain, respectively, were marked by the yellow line, blue line, and green line under the sequences. Six conserved helicases motifs (motif I–VI) were marked by red boxes. Figure S2: sequence alignment vertebrates' MDA5. The four conserved domains, the CARD domain, DExDc domain, HELICc domain, and RD domain, respectively, were marked by the red line, yellow line, blue line, and green line under the sequences. Six conserved helicases motifs (motif I–VI) were marked by red boxes. (*Supplementary Materials*)

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