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MDA5 and LGP2 acts as a key regulator though activating NF-κB and IRF3 in RLRs signaling of mandarinfish

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ABSTRACT

RIG-I-like receptors (RLRs), as key cytoplasmic sensors of viral pathogen-associated molecular patterns, can recognise viral RNA and enhance the antiviral response. Some investigations have focused on the roles of RLRs in the innate immune response in grass carp, large yellow croaker, and rainbow trout. However, little is known about the function of RLRs in mandarinfish (*Siniperca chuatsi*), an important economic fish in Perciformes. Here, we functionally characterized the RLRs involved in the immune responses of mandarinfish (*Siniperca chuatsi*), by evaluating three RLRs, namely, *RIG-I*, *MDA5*, and *LGP2*. The results revealed that *MDA5* and *LGP2* were present in mandarinfish, whereas *RIG-I* was absent. The *MDA5* and *LGP2* cDNA sequences contained 2976 and 2046 bp and encoded 991 and 681 amino acids, respectively. Multiple sequence alignments showed that *MDA5* and *LGP2* of mandarinfish were clustered together with their homologs from other teleost fishes and shared high similarities with those from other vertebrates, and *RIG-I* of mandarinfish was absent. Moreover, quantitative real-time PCR (qPCR) analysis suggested that *MDA5* and *LGP2* were constitutively expressed in all tissues tested, and *MDA5* mRNA expression was relatively high in the gill, and spleen, whereas *LGP2* mRNA expression was high in the liver, gill, and head kidney. After stimulation with lipopolysaccharide or poly I:C, the expression of *MDA5* and *LGP2* was upregulated in spleen, gill and head kidney, but the pattern was not exactly the same, *MDA5* transcripts generally increased and then declined with the prolonged infection, while *LGP2* transcripts went up continuously, which showed that mandarinfish MDA5 and LGP2 may play independent roles in antiviral response. Besides, it is further revealed that the *MDA5* could activate NF-κB and IRF3 to inducing the production of IFN-β by constructing tet-on stable strain of 293T cell, however over-expression of LGP2 resulted in decreased NF-κB, IRF3 and IFN-β production in cells challenged with LPS and polyI:C Taken together, our results demonstrated that *MDA5* and *LGP2*, as a positive and negative regulator, respectively, played an important role in modulating antibacterial andantiviral immune responses though activating NF-κB and IRF3 in RLRs signaling of mandarinfish.

1. Introduction

In host innate immunity, pattern recognition receptors (PRRs) are crucial to protect against pathogen invasion. PRRs recognise conserved pathogen-associated molecular pattern motifs, including proteins, lipids, and nucleotides, and then initiate the first line of defence against various pathogens [1,2]. Among these PRRs, cytosolic RIG-I-like receptors (RLRs), including retinoic acid-inducible gene I (*RIG-I*), melanoma differentiation associated gene 5 (*MDA5*), and laboratory of genetics and physiology 2 (*LGP2*), are involved in the sensing of RNA virus invasion in the cytoplasm [3,4]. RIG-I and MDA5 share homologous core structural domains, including two N-terminal caspase recruitment domains (CARDs), a DExD/H box RNA helicase domain, and a C-terminal regulatory domain. Although the domain structure and amino acid sequence are quite similar, RIG-I and MDA5 recognise different types of nonself RNAs and viruses. RIG-I has been shown to detect the majority of RNA viruses through the recognition of uncapped 5′-triphosphate on viral genomic RNA [5], whereas MDA5 recognises long double-stranded RNA (dsRNA), including the synthetic dsRNA mimic, poly I:C [6]. The N-terminal CARDs of RIG-I and MDA5 are responsible for triggering the CARD of the RLR adaptor protein to activate the production of type I interferons (IFNs) [7–9].

In contrast, LGP2 does not possess an N-terminal CARD domain, and the role of LGP2 is still controversial. Some studies have shown that LGP2 can negatively regulate RIG-I/MDA5-mediated signaling pathways by isolating dsRNA and binding with RIG-I, thereby inhibiting the

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Table 1
Primers used in the study.

Primer name	Primer sequence (5′→3′)	Annealing temperature (°C)	Application
RIG-I-F	TGCCCAGATGCTGCTGGA	65	RT-PCR
RIG-I-R	TGAGCCGTCATCACCACGATA		
MDA5-F	CTAGCTAGCATGACATCGGACAACGATGA	60	RT-PCR
MDA5-R	CCGGAATTCCGTAGTTGTCATTGATTCTG		
LGP2-F	CTAGCTAGCATGGCAGATTTTGAACTGTA	60	RT-PCR
LGP2-R	CCGGAATTCATCAAAGATGTCGGGGAAGT		
TRE3Gv-MDA5-F	TATACTTGGATCACCGGTGGCCACCATGACATCGGACAACGATGA	62	RT-PCR
TRE3Gv-MDA5-R	TACCCGGTAGAATTGGATCCTCACTTGTCATCGTCATCCTTG		
TRE3Gv-LGP2-F	TTATACTTGGATCACCGGTGCCACCATGGCAGATTTTGAACTGTATGCA	62	RT-PCR
TRE3Gv-LGP2-R	TACCCGGTAGAATTGGATCCTCACTTGTCATCGTCATCCTTG		
MDA5 5′ Router	CATTCTGGTCGGCTTC	60	5′ RACE-PCR
MDA5 5′ Inner	AAATAATCCAGAACCCGC		
MDA5 3′ Router	GCAAAGACTGTGGGCAGAGATGGG	60	3′ RACE-PCR
MDA5 3′ Inner	CTCGCCGTCAAGTTTTCTGCCTTC		
LGP2 5′ Router	CCACCACCTTAGCCTT	60	5′ RACE-PCR
LGP2 5′ Inner	GTGTGGTCTCCAGGTGTT		
LGP2 3′ Router	GCCCTGCTGCCCAACATAGCCATT	60	3′ RACE-PCR
LGP2 3′ Inner	TGGCAGGATGACTGTAAAGAAGTGGAA		
qMDA5-F	TTGCTGCCAGTTGGGTGT	60	qRT-PCR
qMDA5-R	CCTAAAGTTTTCAATGAGACGC		
qLGP2-F	GGAATGGGGATTTGAGATGAA	60	qRT-PCR
qLGP2-R	CACTTCTTTACAGTCATCCTGCCAT		
β-actin-F	AGAGGGAAATCGTGCGTGAC	60	qRT-PCR
β-actin-R	ATACCGAGGAAGGAAGGCTG		

expression of type I IFN [10,11]. Moreover, overexpression of MDA5 inhibits iridovirus replication, whereas LGP2 negatively regulates the IFN-dependent immune response [12,13]. Additionally, studies have also shown that LGP2 acts as an upstream regulator of the RIG-I/MDA5 signaling pathway in antiviral infection [14], and Shen et al. reported that LGP2 may function as a positive regulator of *MDA5* in the large yellow croaker [15].

Interestingly, although *MDA5* and *LGP2* gene homologs have been found in many teleost fishes, clear *RIG-I* homologs have only been identified in catfish, salmon and carp, suggesting that the *RIG-I* homolog has been lost from some fish genomes or has been differentiated into an unidentified new gene [16]. Besides, some studies also found that *RIG-I* is absent in the tree shrew genome, although *MDA5* and *LGP2* are both present. And loss of *RIG-I* is accompanied by a functional substitute of MDA5 involving LGP2 in tree shrews [13]. Although there were few evidences that indicated the presence of RIG-I only in Cypriniformes, Siluriformes and Salmoniformes. In large yellow croaker, the RIG-I gene was likely to be lost in this species and the other two genes of RLRs (MDA5 and LGP2) played an essential function in the innate antiviral immunity. In this sense, whether RIG-I-1 is missing or not, the animal body can perform its immune function normally.

The mandarinfish, as a teleost fish, is one of the most precious aquaculture species in China. In this study, we conducted molecular cloning and characterization of mandarinfish *RLR* genes, investigated their expression levels challenged with lipopolysaccharide (LPS) and poly I:C *in vivo*, and analyzed whether overexpression of MDA5 and LGP2 induced more production of IFN-β, nuclear factor (NF)-κB, and IFN regulatory factor 3 (IRF3) during LPS and Poly I:C treatment. Our data revealed the functions of MDA5 and LGP2 in antibacterial and antiviral infections, providing new insights into our understanding of the innate immunity mechanisms of mandarinfish.

2. Materials and methods

2.1. Cells, tissues, and reagents

HEK293T cells were maintained at 37 °C and 5.0% CO_2 in complete growth medium (Dulbecco's modified Eagle medium [Gibco, CA, USA] containing 10% foetal bovine serum (Gibco, CA, USA). Poly I:C and LPS were purchased from Invivogen (San Diego, CA, USA) and Sigma (St. Louis, MO, USA), respectively. Tetracycline was purchased from Meilunbio (Dalian, China). A total of thirty-six mandarinfishes with 40–60 g in body weight were kept in aquarias with a flowthrough water system and aerated freshwater at 29 °C. Tissues, including the heart, liver, spleen, gill, head kidney, muscle, and intestine, were collected from healthy mandarinfish. Additionally, mandarinfish were divided into two groups: 18 fish were injected with 50 μg/ml of poly I:C, and the other 18 fish were treated with 10 μg/ml of LPS [17]. All fish were injected intramuscularly. At 0, 2, 4, 8, 16, and 24 h after injection, the spleen, gill, and head kidney tissues were collected. Tissue samples were harvested, immediately snap-frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA was extracted from all tissues with TRIzol (Invitrogen, CA, USA).

2.2. Mapping reads

To annotate the mandarin fish transcriptome, we performed BLASTx alignment (e-value $< 10^{-5}$) between All-unigene and protein databases, such as European Bioinformatics Institute (EBI), National Center for Biotechnology Information (NCBI), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO) databases. Functional annotation by GO terms (http://www.geneontology.org) was carried out using Blast2GO V.x.x software (BioBam, ESP). After obtaining the GO annotation for each unigene, we applied Web Gene Ontology Annotation Plot software (WEGO) [18] to conduct GO functional classification for all unigenes, and these data have been read in the EBI ArrayExpress database (accession number: E-MTAB-1365) [19].

2.3. Cloning of RLR cDNA

Total RNA of *Siniperca chuatsi*, *Cyprinus carpio*, *Carassius auratus*, and *Ctenopharyngodon idella* from the spleen was isolated with TRIzol (Invitrogen, USA). cDNA was synthesised using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TAKARA, Dalian, China) with 5 μg of total RNA. Based on the conserved sequences of *Cyprinus carpio*, *Carassius auratus*, and *Ctenopharyngodon idella*, the primer of RIG-I was designed (Table 1). And the partial coding sequences (CDSs) of *MDA5* and *LGP2* were obtained by annotating unigenes, and rapid amplification of cDNA ends (RACE)-ready first-strand cDNA was synthesised using a Takara RACE cDNA amplification kit (Takara, Dalian, China) in accordance

with the manufacturer's protocol. RACE primers were designed according to the manufacturer's protocol (Table 1). Subsequently, RACE was performed on the 5′ and 3′ ends of the *MDA5* and *LGP2* cDNA. The sequences of *MDA5* and *LGP2* were submitted to GenBank under accession numbers MF574069 and KY974319, respectively. All primer sequences are shown in Table 1.

2.4. Sequence alignment and homology analysis

Multiple sequence alignments were carried out using AlignIR V2.0 (LI-COR Biotechnology, USA), and then a phylogenetic tree was constructed based on the deduced amino acid sequences using the Neighbour-Joining (NJ) algorithm within MEGA 6.0 Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was performed to construct multiple sequence alignments of the amino acid sequences of MDA5 and LGP2 proteins. The conserved domains of MDA5 and LGP2 were predicted using the NCBI Conserved Domains Database Tools (CDD Tools; https://www.ncbi.nlm.nih.gov/Structure/cdd/).

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted from different tissues using TRIzol (Invitrogen, CA, USA), and 1 μg total RNA was used with FastQuant RT Kit(With gDNase)(Tiangen, Beijing, China) during reverse transcription. The process included an initial phase at 42 °C for 3 min, then incubation at 42 °C 15 min, and incubation at 95 °C for 3 min. The cDNA was stored at −80 °C. The primers are listed in Table 1. QuantStudio 5 (Applied Biosystems, Thermo Fisher Scientific) was used to perform the assays, and quantitative polymerase chain reaction was performed using SYBR Green Master Mix (Vazyme, Nanjing, China) to determine the patterns of *MDA5* and *LGP2* mRNA expression. The following reaction conditions were applied: one cycle at 95 °C for 5 min; 30 cycles of 95 °C for 10 s and 60 °C for 30 s; and a final cycle of 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The relative mRNA expression was calculated using the $2^{-\triangle\triangle CT}$ method [20], and the *β*-actin gene was used as an internal control.

2.6. Plasmid construction

The CDS of *MDA5* and *LGP2* were ligated to the NheI and EcoRI sites of the pcDNA3.1$^+$ vector (Invitrogen, CA, USA) to construct the plasmids pcDNA3.1$^+$-*MDA5* and pcDNA3.1$^+$-*LGP2*. The primers are listed in Table 1. And the lentiviral vector of TRE3Gv-MDA5 and TRE3Gv-LGP2 were constructed with the EcoRI and BamHI sites of the TRE3Gv-MCS-PGK-Puro vector using ClonExpress II One Step Cloning Kit according the according to the manufacturer's instructions (Vazyme, Nanjing, China). All the plasmids used in this study were verified by DNA sequencing at Sangon Biotech Co., Ltd. (Shanghai, China).

2.7. Lentivirus infection of 293T cells to construct tet-on stable strain

293T cells were co-transfected with packaging mix and TRE3Gv-MDA5 or TRE3Gv-LGP2 vector, and the virus was packaged, then the virus stock was collected, concentrated by ultrafiltration, and the titer was determined. Subsequently, the target cells are infected with the packaged helper lentivirus to obtain Tet3G-Neo 293 Cell Line (NEO total lethal concentration 800 μg/ml, maintaining concentration of 400 μg/ml). Then the Tet3G-Neo 293 Cell Line was infected with the packaged lentivirus to obtain tet-on stable strain of MDA5 and LGP2 cultured with 10 ng/ml tetracycline, respectively (puro total lethal concentration 1.5 μg/ml, maintaining a concentration of 0.75 μg/ml).

2.8. Western blotting analysis

MDA5 293T cells and LGP2 293T cells were seeded in 6-well plates and cultured with 10 ng/ml tetracycline, after seventy-two hours, the cells were treated with LPS (1 mg/mL) or poly I:C (1mglmL) and then collected 10 h later. The cells were collected with lysis buffer using total protein extraction kit (including Protease Inhibitor Cocktail) (Thermo Scientific, MA, USA). Cell lysates were denaturated under 95 °C for 5 min before separation by SDS-PAGE, and transferred to a nitrocellulose membrane. Western blotting was performed using rabbit anti- IFN-β (Abcam, Cambridge, England) at 1:500, anti-IRF3 or p-NF-κB P65 (CST, Massachusetts, USA) at 1:1000 and anti-GAPDH (Abcam, Cambridge, England) antibody at 1:10000 and goat anti-mouse secondary antibody or goat anti-rabbit secondary antibody at 1:5000 (Thermo Scientific, MA, USA). Proteins were visualized by chemiluminescence using the ECL kit (GE Healthcare).

2.9. Statistical analyses

Data from all experiments are expressed as means ± standard errors. A database was established in Excel 2003, and the data were statistically analyzed with SPSS 13.0 using the one-way analysis of variance (ANOVA) with the Duncan method. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Read assembly and functional annotation

To annotate these unigenes of mandarinfish, we searched the reference sequences using BLASTX against protein databases using a cut-off E-value of 10^{-5} and performed a de novo assembly into the unigene; the reads were assembled into 64,999 unigenes. We found that the *RIG-I* gene was absent, whereas the *LGP2* and *MDA5* genes were present.

3.2. cDNA cloning and sequence analysis of mandarinfish RLRs

To further confirm whether *RIG-I* was absent in mandarinfish, RT-PCR was performed with different kinds of fishes. The result showed that *RIG-I* transcript was missing in mandarinfish, but was present in *Cyprinus carpio*, *Carassius auratus*, *Ctenopharyngodon idella* (Fig. S1). Using RACE technology, the full-length sequence of *MDA5* was obtained. The sequence included a 39-bp 5′ untranslated region (UTR), a 2976-bp open reading frame (ORF), and a 420-bp 3′ UTR, encoding 991 amino acids. The *LGP2* cDNA was cloned with a 41-bp 5′ UTR, a 2046-bp ORF encoding 681 amino acids, and a 711-bp 3' UTR. The primers used are shown in Table 1. The sequences of *MDA5* and *LGP2* were submitted to GenBank (accession numbers: MF574069 and KY974319, respectively). Furthermore, using the NCBI Conserved Domains Database Tools (CDD Tools) to predict the conserved domains of MDA5 and LGP2, we found that MDA5 contained two tandem CARDs (residues 6–97 and 106–197), one DEAD-like C-terminal helicase domain (DEXDc; residues 289–815), and a C-terminal regulatory domain (residues 854–969; Fig. 1A), whereas LGP2 included a DEXDc (residues 4–192) and a C-terminal regulatory domain (residues 362–474; Fig. 1B). Comparisons of the CDSs and amino acid sequences of MDA5 and LGP2 with those of MDA5 and LGP2 from other species, including multiple alignments and amino acid sequences, are shown in Fig. 1A and B, respectively.

3.3. Phylogenetic analysis

To evaluate the molecular evolutionary relationships between MDA5 and LGP2 in other vertebrates, a phylogenetic tree was constructed based on the amino acid sequences of mammalian, avian, and fish RLRs. The inferred phylogenies of RIG-I, MDA5 and LGP2 are presented in three distinct clusters (Fig. 2). Mandarinfish MDA5 shared high protein sequence similarity with seabass and sea perch, but had a distant evolutionary relationship with mammals and birds, illustrating the closer relationships between mandarinfish MDA5 and those of other

Fig. 1. (A) Multiple alignments of MDA5 protein sequences from mandarinfish (ATQ36109.1), zebrafish (NP_001295492.1), grass carp (AFC88291.1), grouper (AEX01716.1), and mice (ABO33314.1). (B) Multiple alignments of LGP2 protein sequences from mandarinfish (KY974319), seabass (AOV82294.1), sea perch (ALE66118.1), grass carp (AFQ93565.1), and humans (NP_077024.2). CARD: tandem caspase-associated and recruitment domain; DEXDc: DEAD-like C-terminal helicase domain; RD: C-terminal regulatory domain. Asterisks (*) represent identical amino acids, and the symbols "." and ":" denote conservative substitutions.

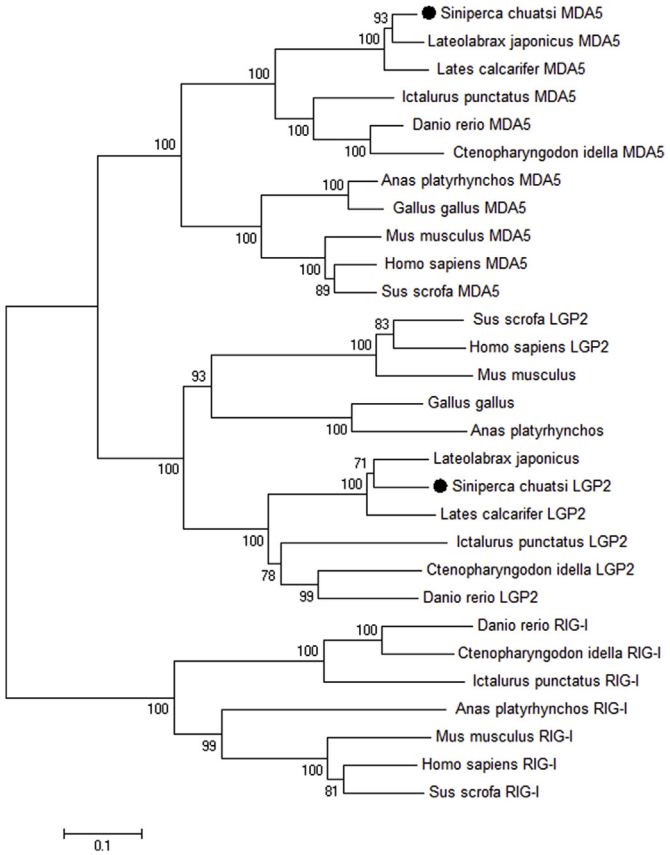

Fig. 2. Phylogenetic analysis of avian, mammalian, and fish MDA5 and LGP2 was carried out. The tree was constructed by the neighbour-joining tree method using amino acid sequences aligned with MEGA6. The bar indicates the bootstrap value (%).

fish species. Mandarinfish LGP2 was most closely related to those of seabass, sea perch, grouper, channel catfish, and grass carp, in a branch separate from that of mammals and birds. Furthermore, the protein sequence of RIG-I were absent in mandarinfish, seabass, sea perch and duck.

3.4. Tissue expression profiles of MDA5 and LGP2

To determine the tissue expression levels of *MDA5* and *LGP2*, qRT-PCR was performed. mRNA expression of *MDA5* and *LGP2* was detected in all seven tissues collected. We found that *MDA5* mRNA was highly expressed in the gill, spleen, head kidney, and muscle, with particularly high expression in the gill. Additionally, *LGP2* mRNA expression was observed in the liver, gill, and head kidney, with generally higher expression compared with the other four tissues. In contrast, relatively low expression of *MDA5* and *LGP2* was detected in the heart and intestine (Fig. 3).

3.5. Expression of MDA5 and LGP2 in tissues after LPS and poly I:C treatment

To further determine the effects of LPS and poly I:C treatment on *MDA5* and *LGP2* expression *in vivo*, different tissues from treated and untreated groups were collected. The mRNA levels of *MDA5* and *LGP2* in different tissues were detected by qRT-PCR at various time points after injection. The relative transcript levels of *MDA5* and *LGP2* in all the tested tissues peaked at 16 h.p.i following LPS stimulation, with the exception that *LGP2* mRNA showed an increasing trend after 16 h.p.i in the spleen (Fig. 4A and B). Following poly I:C injection, the expression levels of *MDA5* and *LGP2* in the spleen got peaked at 8 h.p.i and the *MDA5* and *LGP2* mRNA showed an increased trend in gill. In contrast, in the head kidney, the difference in *MDA5* mRNA was not significant, and *LGP2* mRNA showed a decreasing trend after 8 h.p.i (Fig. 4C and D). Additionally, the peaked time of *MDA5* and *LGP2* mRNA expression was earlier during poly I:C stimulation than LPS treatment.

3.6. Functional analysis of the induction of IFN-β, NF-κB, IRF3 by MDA5 and LGP2 following LPS and poly I:C treatment

To characterise the roles of MDA5 and LGP2 in type I IFN signaling, we constructed the MDA5 tet-on 293T cell and LGP2 tet-on 293T cell, respectively, and the key NF-κB and IRF3 were chosen to evaluate induction of IFN-β. The results showed that overexpression of *MDA5* significantly induced the expression of IFN-β in poly I:C and LPS-treated cells. Additionally, after LPS stimulation, overexpression of *MDA5* significantly increased the production of IFN-β (Fig. 5A). Moreover, *MDA5* also modulated NF-κB and IRF3 production after poly I:Cand LPS stimulation (Fig. 5B and C). When LGP2 was overexpressed, the content of IFN-β, NF-κB and IRF3 were significantly upregulated, however, during poly I:C and LPS treatment, the production of IFN-β, NF-κB and IRF3 showed a decreased trend. Taken together, these data demonstrated that mandarinfish *MDA5* and *LGP2* played an essential role in modulating antiviral and antibacterial immune responses though activating NF-κB and IRF3 in RLRs signaling (Fig. 6).

4. Discussion

RLR proteins play key roles in the innate immune response against viral infection by recognising viral RNA molecules [8,21]. Unlike *MDA5* and *LGP2*, which appear to be common to all teleosts, the *RIG-I* gene is absent in some fish species, including the green spotted puffer, Japanese flounder, medaka, Nile tilapia, orange-spotted grouper, stickleback, and large yellow croaker, which all belong to the superorder Acanthopterygii [15,22]. Genome assembly of unigenes showed that there were no sequences with potential *RIG-I* orthology in the draft genome of mandarinfish. Additionally, We found the *RIG-I* gene was apparently not present in mandarinfish by PCR amplification, which might be either gene loss or divergence into an unrecognisable gene [16]. However, further studies are needed to determine the exact reason for the absence of the *RIG-I* gene in this fish species.

Our phylogenetic construction and amino acid homology analysis revealed that mandarinfish MDA5 and LGP2 shared high similarities with MDA5 and LGP2 proteins from other teleost fishes, but low similarities with those of mammals and birds, besides, the protein sequence of RIG-I were absent in mandarinfish, seabass, and sea perch. Then, we isolated and identified the *MDA5* and *LGP2* genes and characterized their gene structures. The conserved domains of mandarinfish predicted by the CCD tool showed that MDA5 had two CARD domains, a DEXDc, and C-terminal regulatory domain; however, LGP2 lacked the N-terminal CARD domain, which had a structure similar to those of grass carp [23,24], large yellow croaker [15], and grouper [12,13]. Taken together, our results showed that the *MDA5* and *LGP2* genes were evolutionarily conserved not only in protein sequences but also in functionally significant domains, indicating similar functions of MDA5-and LGP2-like teleostean counterparts.

Tissue expression profile analysis showed that *MDA5* and *LGP2* genes were widely expressed in all collected tissues. Additionally, *MDA5* mRNA was expressed at higher levels in the spleen, gill, and muscle, whereas the expression of *LGP2* was high in the liver, gill, and head kidney. Similarly, the *MDA5* gene was also highly expressed in the spleen and gill in channel catfish [25], flounder [26] and grass carp [23]. In contrast, *MDA5* is deficient in the muscle in channel catfish [25] but shows relatively high expression in the muscles of catfish. *LGP2* is highly expressed in the head kidney, and gill in flounder [27] and large yellow croaker [15]. The expression of *MDA5* and *LGP2* in various tissues in the mandarinfish indicated that these genes may have

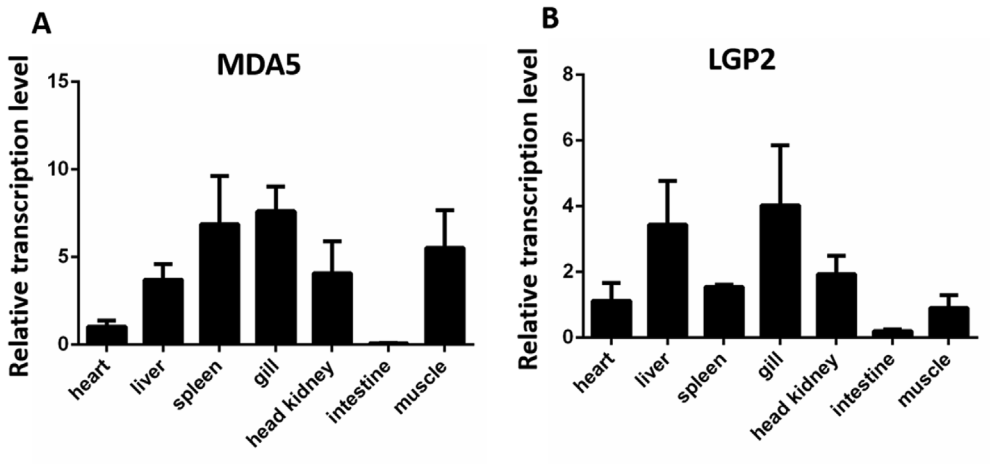

Fig. 3. mRNA expression of *MDA5* (A) and *LGP2* (B)in various tissues. *MDA5* and *LGP2* levels were determined using qRT-PCR and normalised to the expression of β-actin. The heart, liver, spleen, gill, head kidney, muscle, and intestine were examined.

roles in the innate immune system and that many organs have the potential to be involved in the RLR-mediated response to viral infection.

Several reports have demonstrated that *MDA5* exerts antiviral effects against various viruses. In grass carp, the expression of *MDA5* mRNA was significantly upregulated in the spleen and liver after grass carp reovirus injection [23]. Further, in channel catfish, the level of *MDA5* expression was upregulated until 4 days after infection with the gram-negative bacterium *Edwardsiella ictaluri* [25]. Unfortunately, there is no evidence suggesting that MDA5 can directly sense bacterial PAMPs. A proper explanation is that LPS is sensed by other PRRs such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [28], and the signaling cross talk could enhance the expression of MDA5 by responsing to LPS stimulation [29]. A significant observation in this study was that the expression of the *MDA5* gene was upregulated *in vivo* following stimulation with LPS or poly I:C, suggesting the involvement of *MDA5* in immune function and antibacterial and antiviral responses.

Similar to *MDA5*, *LGP2* was also demonstrated to function as cytosolic RNA sensors in mammalian cells. In olive founder and rainbow trout, expression of the LGP2 gene is dramatically induced by poly I:C stimulation and viral infection, and*LGP2*-knockout mice produce higher amounts of type I IFNs than wild-type mice after stimulation with poly

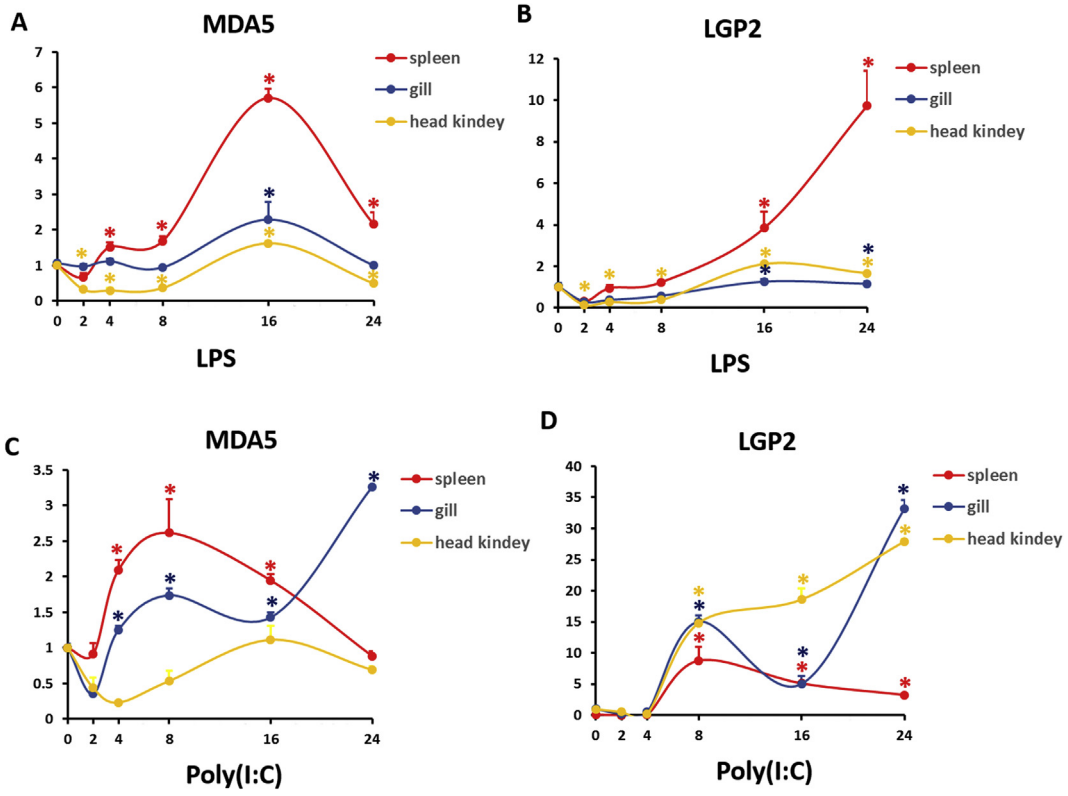

Fig. 4. mRNA expression levels of *MDA5* and *LGP2 in vivo* following LPS or poly I:C injection. Fish were injected intraperitoneally with LPS or poly I:C, and the spleen, gill, and head kidney were collected at 0, 2, 4, 8, 16, and 24 h.p.i. Gene expression was determined using real-time RT-PCR and was normalised to the expression of β-actin. Relative transcription levels were obtained by comparing the normalised expression levels between different time points. Vertical bars represent the means ± standard errors (each time point used three fish). X-axis represented each time point, y-axis represented the fold change in expression level. Asterisks represent significant differences relative to controls ($P < 0.05$).

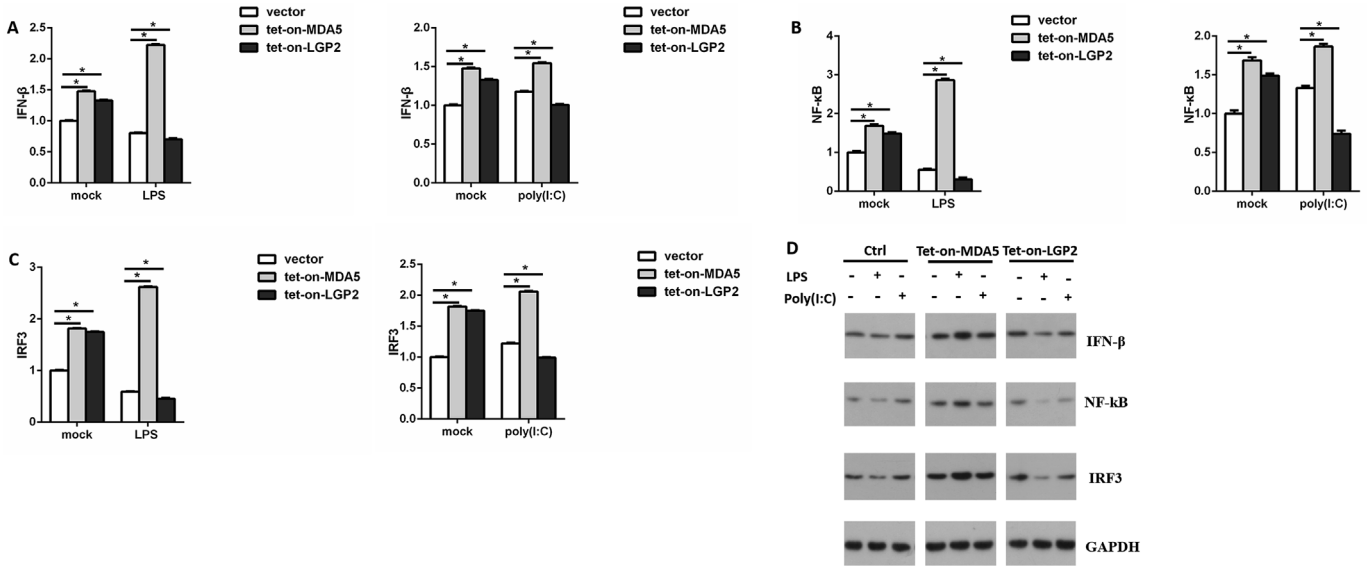

Fig. 5. Characterization of the effects of *MDA5* and *LGP2* on induction of IFN-β, NF-κB and IRF3. MDA5 293T cells and LGP2 293T cells were seeded in 6 well plates and treated with 10 ng/ml tetracycline, respectively, after seventy-two hours culturing, the cells were stimulated with poly I:C (1 mg/mL) or LPS (1 mg/mL). Ten hours later, the cells were treated with RIPA to carry out the western blot analysis. Gray value detection of western blot (A–C) were repeated at least three times, and rubber map of western blot was used (D). The data shown are means ± standard errors (n = 3) from one representative experiment. Significance was analyzed with One -Way ANOVA with the Duncan method (*$P < 0.05$).

I:C infection [30]. Similarly, in channel catfish [25], the levels of *LGP2* were increased following treatment with the gram-negative bacterium *Edwardsiella ictaluri*, and a regulatory role of *LGP2* in response to intracellular bacterial pathogens has been demonstrated in mice [33]. However, there is conflicting evidence, for example, *LGP2* has been shown to strongly induce type I IFN gene expression in response to poly I:C transfection [27,31,32]. This data suggests that *LGP2* antiviral function may differ in some species of teleosts, therefore we must be cautious when drawing conclusions.

In the present study, we demonstrated a dramatic induction of mandarinfish *LGP2* expression *in vivo*, during LPS treatment, the expression of *LGP2* peaked at 16 h.p.i in the gill and head kidney and tended to increase in the spleen. LGP2 mRNA was also upregulated at 8 h in the spleen and gill after poly I:C stimulation (Fig. 4). The LGP2 gene was similarly induced by poly I:C transfection in olive founder HINAE cells [32]. Because the mandarinfish genome lacks the RIG-I gene, the role of LGP2 and MDA5 gene expression is critically important. Accordingly, the transcriptional regulation of MDA5 and LGP2 expression, which is dramatically upregulated, could play a key role in the immune response. Similar cases were also reported in Acanthopterygii fish species such as fugu, medaka and stickleback without RIG-I gene [16]. Besides, the peak time of *MDA5* and *LGP2* mRNAs was

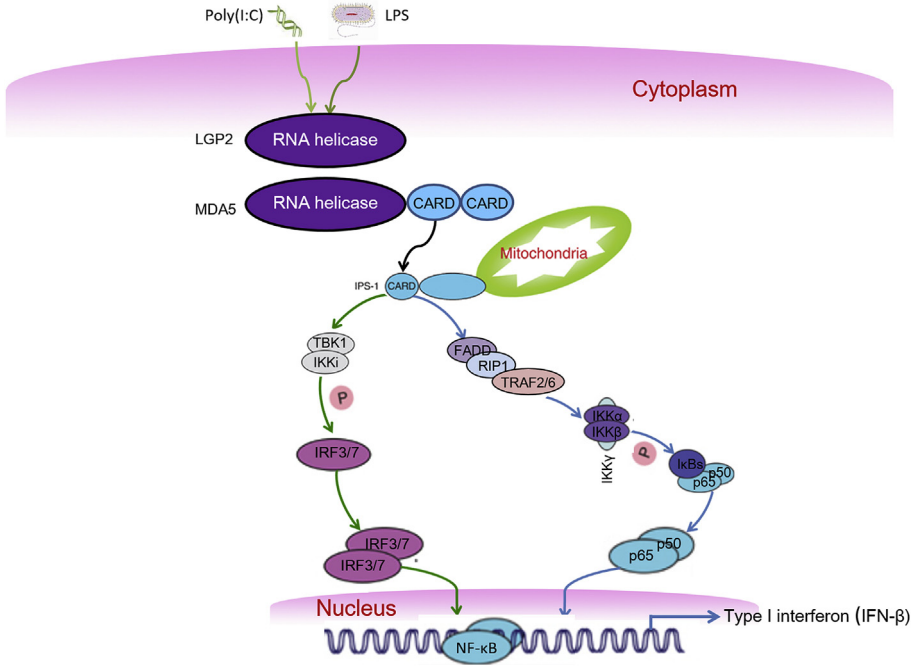

Fig. 6. The RLRs Signal transduction of mandarinfish. When poly(I:C) and lipopolysaccharide stimulated, the pathway of NF-κB and IRF3 may affect the production of IFN-β.

earlier following poly I:C stimulation than LPS treatment, suggesting that *MDA5* and *LGP2* could more easily recognise long dsRNA during the immune response. Notably, in this study, the expression of *LGP2* mRNA showed more higher than MDA5 mRNA after poly I:C stimulation, which may be that the affinity of MDA5 for dsRNA such as poly I:C was low compared than LGP2 and it is possible that LGP2 binds poly I:C and helps to recruit MDA5 [34]. These data demonstrate that RIG-I like receptor (*MDA5* and *LGP2*) are important sensors in recognising both bacteria and double stranded RNA viruses in fish.

To better understand the immune mechanisms in RLRs signaling of Mandarinfish here, the MDA5 and LGP2 acts as a key regulator though activating NF-κB and IRF3 in RLRs signaling of mandarinfish. RLR induced IFN response by regulating IFN regulatory factor 3/7 and NF-κB signaling pathway. It was reported that NF-κB was severely impaired in tree shrew cells when overexpressing MDA5 [13]and LGP2 knockout mice showed a higher level of IFNs than the wild type animals and were more resistant to vesicular stomatitis virus infection [35]. In our study, our results showed that overexpression of *MDA5* significantly increased the production of NF-κB, IRF3 and IFN-β during viral and bacterial induction, however, overexpression of *LGP2* significantly decreased the transcription of NF-κB, IRF3 and IFN-β, which showed a negative regulatory in antiviral response. The regulatory effect of *LGP2* on interferon response is obviously different from another member of RLR family, *MDA5*, suggesting that *LGP2* might play a negative feedback of interferon response in mandarinfish, which is similar to grouper [36] and rainbow trout [31]. This may be because that *LGP2* lacks CARD domains at the N terminal region but possesses other domains of *RIG-I*, and is believed to sequester the viral RNA PAMPs from the *RIG-I* and *MDA5* molecules, hence acting as a negative regulator to control excessive production of IFNs keeping the IFN level in check when viral infection diminishes. Therefore, we tentatively put forward the MDA5 and LGP2 induced and inhibited the production of IFN-β though activating NF-κB and IRF3 in RLRs signaling of Mandarinfish respectively.

5. Conclusion

In this study, we performed genome assembly into unigenes and demonstrated that there were no potential *RIG-I* orthologues in the draft genome of mandarinfish. We then sequenced and characterized the RLR genes *MDA5* and *LGP2* in mandarinfish. Sequence analyses suggested that *MDA5* and *LGP2* were evolutionarily conserved with other teleosts. Transcriptional analyses showed the MDA5 mRNA expression was relatively high in the gill, and spleen, whereas LGP2 mRNA expression was high in the liver, gill, and head kidney. Morever, the *MDA5* and *LGP2* genes were rapidly and significantly upregulated *in vivo* after poly I:C and LPS challenge in three tissues. Finally, the overexpression of *MDA5* enhanced the production of IFN-β though activatingNF-κB and IRF3, but *LGP2* inhibited the IFN-β in the same manner. In summary, we have identified the function of *MDA5* and *LGP2* in antiviral and antibacterial infective effect, which offered novel evidence for understanding of the innate immunity mechanisms in mandarinfish.

Conflicts of interest

The authors have declared that no competing interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.054.

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