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Molecular cloning and functional characterisation of *NLRX1* in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The nucleotide-binding domain and leucine-rich-repeat-containing (NLR) proteins regulate innate immunity. Although the positive regulatory impact of NLRs is clear, their inhibitory roles are not well defined. In the present study, the NLR family gene *NLRX1* from grass carp (*Ctenopharyngodon idella*) was cloned and characterised. *NLRX1* was widely expressed in all tissues examined, albeit at varying levels. After exposure to the grass carp reovirus (GCRV), *NLRX1* mRNA expression levels were altered in immune organs, and dramatically altered in liver. Subcellular localisation indicated that NLRX1 protein co-localised with the mitochondria in the transfected cells. Additionally, the bimolecular fluorescence complementation (BiFC) system was introduced to detect the interaction between tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) and NLRX1. Moreover, deficient of NLRX1 in CIK cells with small interference RNA (siRNA) promoted polyinosinic:polycytidylic acid (poly (I:C))-induced *IFN*-related genes production, including *IRF3*, *IRF7*, and *IFN-I*, which reveals that NLRX1 is a negative regulator of IFN. Taken together, our results demonstrate that *NLRX1* gene plays an important role in innate immune regulation and provide new insights into understanding the functional characteristics of the NLRX1 in teleosts.

1. Introduction

The grass carp (*Ctenopharyngodon idella*) is one of the most famous aquaculture species worldwide, accounting for 13% of global freshwater aquaculture production [1,2]. However, outbreaks of hemorrhagic disease caused by the grass carp reovirus (GCRV) resulted in huge economic losses to grass carp aquaculture and leaded to about 80% of yearling fish death [3,4]. GCRV, belonging to the family *Reoviridae*, genus *Aquareovirus*, is a double-stranded RNA (dsRNA) virus reported mainly in China and with more than 10 strains of GCRV have been isolated [5,6]. Currently, the mechanism underlying hemorrhagic disease is still largely unclear, therefore, understanding the mechanism underlying the immune response is important for defense against GCRV. In *vitro*, the grass carp kidney cell line (CIK cells) constantly served as infection model to study infection mechanism, fish antiviral innate immune system screening antiviral drugs and developing vaccine [7,8].

The nucleotide-binding domain and leucine-rich-repeat-containing (NLR, also known as NOD-like receptor) proteins regulate innate immune in mammal and are indispensable for cellular responses to pathogens [9]. The proteins in NLR family are also involved in inflammasome-mediated responses to both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns [10]. However, several NLR proteins, including NLRX1, NLRC3, NLRC5 and NLRP12, act as negative regulators of innate immune with the ability to check type I interferon (IFN-I) responses or NF-κB-induced pro-inflammatory cytokines [11–15]. NLRX1 is unique among the NLR proteins in terms of its localisation in the mitochondria and interaction with the mitochondrial antiviral signaling (MAVS) protein to disrupt virus-induced RLH-MAVS interactions [9]. NLRX1 negatively regulates lipopolysaccharide-induced activation of NF-κB, interacting with tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) in unstimulated cells and being recruited to the NEMO-IKK signaling complex following lipopolysaccharide stimulation via its leucine-rich-repeat domain [12]. Deletion or functional knockdown of NLRX1 results in heightening interferon responses to the synthetic RNA duplex and Toll-like receptor 3 (TLR3) against polyinosinic:polycytidylic acid (poly (I:C)) or RNA viruses, as well as increased inflammatory responses [9,12,and14]]. NLRX1$^{-/-}$ mice exhibited increased expression of antiviral signaling molecules interferon-β (IFN-β), activator of

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transcription 2 (STAT2), oligoadenylate synthetase 1 (OAS1), nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), signal transducer, activator of transcription 3 (STAT3), and interleukin 6 (IL-6) following influenza virus infection. Consistent with increased inflammation, NLRX1$^{-/-}$ mice exhibited marked morbidity and histopathology [16,17]. Evidence supports that NLRX1 functions as a checkpoint inhibitor of early innate immune responses against both DNA viruses and RNA viruses.

However, not all studies have shown that NLRX1 exerts negative regulatory effects on innate immune responses to viruses. NLRX1 promotes immediate Interferon regulatory factor 1 (IRF1)-directed antiviral responses by limiting dsRNA-activated translational inhibition [15]. Overexpression of NLRX1 enhances NF-κB signaling by amplifying the production of reactive oxygen species in response to several stimulation, rather than inhibiting such signaling [18]. Compared to the wild-type mice, although inflammatory responses to infection with influenza virus are enhanced in the lungs of NLRX1$^{-/-}$ mice [16], their macrophage-mediated interferon responses are impaired, secondary to enhanced apoptosis [19]. Thus, it is important to determine whether and how NLRX1 controls these signaling during immunoregulation.

In the present study, the *NLRX1* gene from grass carp was cloned and characterised. Expression profiles in different tissues and in response to GCRV infection were examined in *vivo*. Additionally, the subcellular localisation of NLRX1 protein was investigated. Moreover, deficient of *NLRX1* in CIK cells with small interference RNA (siRNA) was performed to investigate the possible roles of *NLRX1*. These findings provide new insights for understanding the functions of *NLRX1* gene in teleosts.

2. Materials and methods

2.1. Cells, plasmid, fish, and GCRV challenge

The CIK cells used in the study were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum at 28 °C in a humidified atmosphere with 5% CO_2. The pMN155 and pMC156 plasmids used in the study were a kind gift from Professor Zongqiang Cui, Wuhan Institute of Virology, Chinese Academy of Sciences. Four-month-old healthy grass carps (weight, about 10 g; average length, 7 cm) were obtained from the GuanQiao Experimental Station, Institute of Hydrobiology, Chinese Academic of Sciences, and acclimatized in aerated freshwater at 28 °C for 1 week before the viral challenge experiment. The fish were fed twice a day with a commercial feed, and used for further experiments after no abnormal symptoms were observed.

The GCRV preparation and GCRV challenge experiment were carried out as described previously [20,21]. Briefly, dead fish with typical symptoms of hemorrhagic diseases were homogenized together with an equal volume of 0.75% saline. The mixture was centrifuged, and then, the supernatant was filtered through a 0.22-μm Millex filter (Millipore, USA). Grass carps were intraperitoneally injected with 200 μl of GCRV solution. All the injected fish were fed with the commercial feed twice a day and monitored carefully every day. The temperature was maintained at 28 °C throughout the experiment.

Three uninfected fish were selected, and samples of the middle kidney, head kidney, liver, intestine, spleen, and gill were collected, and RNA was extracted for full-length cDNA cloning. In addition, these tissues were also sampled from three infected fish at different days after GCRV infection (1, 2, 3, 4, 5, 6, and 8 days). RNA was extracted from these tissues to analyse the expression pattern of *NLRX1* and *IFN*-related genes after GCRV infection. All the samples were homogenized in TRIzol reagent (Invitrogen, USA) and stored at −80 °C prior to RNA extraction.

Table 1
Primers used in this study.

Primers	Sequences (5′—3′)	Purpose
3 NLRX1-F	GGTCCTCGGGTGAAGGAGGGC	3′ RACE
5 NLRX1-R	CCACAGATGCCATCTGATCGG	5′ RACE
NLRX1-F	CCAGCCTCTAGATTAGGGG	cDNA cloning
NLRX1-R	AAGGCTGACTAAACTG	
qNLRX1-F	TGAACAACTGCTGGTCTTCCTG	qRT-PCR
qNLRX1-R	ATAAGCGCAGAGTTTGCCTCAC	
qIL-6-F	CAGCAGAATGGGGGAGTTATC	
qIL-6-R	CTCGCAGAGTCTTGACATCCTT	
qIRF3-F	TCCAGGCCAAGCATACGAA	
qIRF3-R	CCATTTGCAACAGCCATCAT	
qIRF7-F	CGCCTGTGTTCGTCACTCGT	
qIRF7-R	GGTGGTTGGAAAGCGTATTGG	
qIFN-I-F	AAGCAACGAGTCTTTGAGCCT	
qIFN-I -R	GCGTCCTGGAAATGACACCT	
qβ-actin-F	AGCCATCCTTCTTGGGTATG	
qβ-actin-R	GGTGGGGCGATGATCTTGAT	
F	GCGACCAAUCGGCACAAAUTT	siRNA
R	AUUUGUGCCGAUUGGUCGCTT	

Table 2
Primers used in this study.

Primers	Sequences (5′—3′)	Purpose
L-NLRX1-F	ATAGAATTCTATGTGGAGATTTGGAAGACC	subcellular localisation
L-NLRX1-R	TATGGATCCTTTTGTACCGGTTCCTGTTCC	
LN- NLRX1-F	ATAGAATTCTATGTGGAGATTTGGAAGACC	
LN- NLRX1-R	TATGGATCCGAGCATCTCGGCCGCCTTGT	
LC- NLRX1-F	ATAGAATTCTCTTCTTCATTACGGGAACC	
LC- NLRX1-R	TATGGATCCTTTTGTACCGGTTCCTGTTCC	
L-TRAF6-F	ATAGAATTCTATGGCTTGCAGCGACATGGAGAAG	
L-TRAF6-R	TATGGTACCAAGTGAAGGTTCTGGGCCCCG	
BF- NLRX1-F	CTCAAGCTT*ACTCCCGCCACCTCCACTCC* *CGCCACCTCC*ATGTGGAGATTTGGAAGACC	BiFC Analysis
BF- NLRX1-R	GACGGATCCCGTTTTGTACCGGTTCCTGTTCC	
BF-TRAF6-F	ACCAGATCTATGGCTTGCAGCGACATGGAG	
BF-TRAF6-R	AATGAATTCGA*ACTCCCGCCACCTCCACTC* *CCGCCACCTCC*AAGTGAAGGTTCTGGGCCC	

2.2. Cloning the full-length cDNA of NLRX1

Total RNA was extracted from healthy samples by using TRIzol reagent, according to the manufacturer's instructions, and first-strand cDNA synthesis was performed using DNase I (Promega, USA) with total RNA as the template and random nonamer primers as the control for reverse transcriptase (Toyobo, Japan). Incomplete but specific fragments of *NLRX1* were obtained by blasting the sequences of the zebrafish (*Danio rerio*) *NLRX1* genes with draft genome of grass carp [2]. The 5′ Full RACE Kit and 3′ Full RACE Kit (TaKaRa, Japan) were used to obtain the 5′ and 3′ untranslated regions (UTRs) of the *NLRX1* gene. The primers for RACE were listed in Table 1. The full-length cDNA sequences were amplified using PCR with primers (Table 1) within the 5′- and 3′-UTRs. The PCR products were purified, ligated into pMD18-T vectors (TaKaRa, Japan), and transformed into competent *Escherichia coli* DH5α cells (TransGen, China). Five positive colonies were selected and sequenced by a commercial company (Tsing Ke, China).

2.3. Sequence analysis

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for the gene sequences in other species. Amino acid sequences of NLRX1 proteins were predicted using open reading frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/), and multiple sequence alignments were performed using ClustalW 2.1 (http://www.ebi.ac.uk/tools/clustalw2.1). Pfam (http://pfam.xfam.org/) was used to predict