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SNP detection of *TLR8* gene, association study with susceptibility/resistance to GCRV and regulation on mRNA expression in grass carp, *Ctenopharyngodon idella*

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ABSTRACT

Toll-like receptor 8 (TLR8), a prototypical intracellular member of TLR family, is generally linked closely to antiviral innate immune through recognizing viral nucleic acid. In this study, 5′-flanking region of *Ctenopharyngodon idella TLR8* (*CiTLR8*), 671 bp in length, was amplified and eight SNPs containing one SNP in the intron, three SNPs in the coding region (CDS) and four SNPs in the 3′-untranslated region (UTR) were identified and characterized. Of which 4062 A/T was significantly associated with the susceptibility/resistance to GCRV both in genotype and allele ($P < 0.05$), while 4168 C/T was extremely significantly associated with that ($P < 0.01$) according to the case (susceptibility)–control (resistance) analysis. Following the verification experiment, further analyses of mRNA expression, linkage disequilibrium (LD), haplotype and microRNA (miRNA) target site indicated that 4062 A/T and 4168 C/T in 3′-UTR might affect the miRNA regulation, while the exertion of antiviral effects of 4062 A/T might rely on its interaction with other SNPs. Additionally, the high-density of SNPs in 3′-UTR might reflect the specific biological functions of 3′-UTR. And also, the mutation of 747 A/G in intron changing the potential transcriptional factor-binding sites (TFBS) nearby might affect the expression of *CiTLR8* transcriptionally or post-transcriptionally. Moreover, as predicted, the A/G transition of the only non-synonymous SNP (3846 A/G) in CDS causing threonine/alanine variation, could shorten the length of the α-helix and ultimately affect the integrity of the Toll-IL-1 receptor (TIR) domain. The functional mechanism of 3846 A/G might also involve a threonine phosphorylation signaling. This study may broaden the knowledge of *TLR* polymorphisms, lay the foundation for further functional research of *CiTLR8* and provide potential markers as well as theoretical basis for resistance molecular breeding of grass carp against GCRV.

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1. Introduction

Pattern recognition receptors (PRRs), an essential piece of the innate response, are known to recognize the pathogen associated molecular patterns (PAMPs) [1], and trigger a series of signaling programs to eliminate the pathogenic microbes [2]. To date, four major families of PRRs, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs), have been identified in the innate immune response [3]. The family of TLRs is the best understood of the innate immune receptors that detect infections [4]. TLRs, belonging to type I transmembrane proteins, was characterized by extracellular leucine-rich repeats (LRRs) which mediate the recognition of PAMPs, a membrane spanning segment and intracellular Toll-IL-1 receptor (TIR) domain that initiates downstream signaling pathways through interacting with TIR adaptor proteins [2,5,6]. Of the intracellular TLRs, TLR7 and TLR8, with phylogenetic and structural similarities [7], are intrinsically capable to recognize nucleic acids of pathogenic microbes, single-stranded RNA (ssRNA) and short double-stranded RNA (dsRNA) of viruses mainly [8]. The functional difference between human TLR7 and TLR8 in antiviral responses has been demonstrated in innate immune cells [9]. Furthermore, mouse TLR8 has been proved to possess a cross-talk with TLR7, which regulates TLR7 expression and may also have a pivotal role in the development of systemic autoimmunity [10,11]. Whatever, it is confirmed that TLR8, with a crucial role in recognizing virus and triggering efficient antiviral immune responses, is worthy of in-depth study for its underlying antiviral regulation mechanisms.

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In this regard, *TLR8* gene has aroused the attention of many researchers and has been identified and described in various species [4]. A few reports have linked TLR8 to antiviral immunity of fish, such as TLR8 is associated with the resistance against infectious pancreatic necrosis virus (IPNV) in Atlantic salmon (*Salmo salar*) [12]. As to *Ctenopharyngodon idella* TLR8 (CiTLR8), its pivotal role in anti-GCRV (grass carp reovirus) immune response is initially confirmed by the observation of *CiTLR8* mRNA expression *in vitro* and *in vivo* post-GCRV infection [13]. On the other hand, as more and more immune-related genes in fishes are characterized, it might be a scientific and effective way against disease to target their resistance-related factors especially polymorphisms, which could also be utilized in selective breeding [14]. Furthermore, as an abundant genetic polymorphism, single nucleotide polymorphisms (SNPs), which have been detected in the resistance-related genes of various fishes such as *Parallchthys olivaceus* [15], *Cyprinus carpio* [16] and *C. idella* [17], has greater potential application on marker-assisted selection (MAS). But till now, with respect to *TLR8* gene, no research probing into the polymorphisms and their correlation with viral infection diseases in fish has been reported. Hence, it is seemingly indispensable to detect the *TLR8* SNPs of grass carp, a crucial species of freshwater aquaculture in China whose production is severely impeded by a dsRNA virus named grass carp reovirus (GCRV) [18].

The purpose of this study is to identify the SNPs and the potential haplotypes constructions of *CiTLR8*, and analyze the association with susceptibility/resistance to GCRV and regulation on mRNA expression. This may not only lay a foundation for the mutation and regulation researches of *CiTLR8*, but also provide potential markers, contributing to selective breeding and the improvement of disease control strategies of grass carp.

2. Materials and methods

2.1. Animal, virus infection and sample collection

220 grass carp, averaging approximately 10 cm in body length, were collected by random sampling without distinguishing gender from three fish farms (Hengkou, Ankang and Zhouzhi fish farms in Shaanxi, China), where no outbreaks of GCRV were found in recent years and the fry were bought from the same fish hatchery. One week prior to GCRV infection, all the fish were transferred to the quarantine facilities with aerated freshwater at 28 °C for adaptation.

In the challenge experiment, all individuals randomly divided into four groups of 55 animals were separately fostered in aquariums, supplied with aerated and filtrated water at 28 °C. The method of preparing the GCRV solution for challenge experiment has been described in detail in our previous research [19]. Individuals in three random groups were intraperitoneally injected with 100 μL of GCRV solution (097 strain, 3.63×10^7 $TCID_{50}$/mL), suspended in PBS, per gram body weight. Meantime, the fourth group was injected with PBS as control. All the fish were maintained in the same condition, and symptoms of hemorrhagic disease, activity and mortality of grass carp were observed and recorded daily. The dead and survived fish were collected until the termination of the experiment at 7 days post-challenge. Approximate 1.0 g dorsal muscle tissues were scissored and preserved at −80 °C for DNA extraction. About 200 mg muscle per individual was homogenized and genomic DNA was extracted with traditional phenol–chloroform method. After being estimated the content spectrophotometrically, all DNA samples were stored at −20 °C for subsequent analyses.

2.2. Cloning of the 5′-flanking sequence and feature analyses of CiTLR8

The 5′-flanking region of *CiTLR8* was PCR-amplified from genomic DNA using GenomeWalker™ Universal Kit (Clontech) according to the manufacturer's instructions. Two gene-specific downstream primers, ER732 and ER733, were designed for this process (Table S1). Based on the full-length sequence of *CiTLR8*, structural characteristics including simple sequence repeat (SSR) in intron and exon–intron architectures were detected and analyzed.

2.3. SNP discovery, genotyping

Thirteen pairs of gene specific primers scanning the complete *CiTLR8* genomic sequence were designed in virtue of Primer Premier 5.0 software for SNPs detection (Table S1). PCR was performed in 25 μL of reaction volume, containing 50–100 ng genomic DNA, 10 μM of each primer, buffer (including 1.5 μM $MgCl_2$), 200 μM dNTPs, and 2 U of Taq DNA polymerase (MBI). PCR reactions were carried out using a PCR thermal cycler system (Bio-Rad, USA). An initial denaturation for 5 min at 94 °C; 35 cycles of 94 °C for 30 s; annealing at annealing temperature for 30 s; primer extension at 72 °C for 45 s. The final extension was performed at 72 °C for 5 min. The PCR products of 24 random individuals (12 susceptible fish and 12 resistant fish), were purified and commercially sequenced. Based on the obtained sequence, SNPs were detected by manual arrangement and sequence alignment using MegAlign.

As sequencing errors were unavoidable, those SNPs should be confirmed using PCR-RFLP. In this procedure, twelve susceptible and twelve resistant DNA samples were used as templates for PCR. 3 μL PCR products were electrophoresed on a 1.0% agarose gel for quality measuring. Another 5 μL PCR products were digested with specific restriction enzyme at the corresponding incubation temperature for 4–5 h, and then, the mixtures were examined by electrophoresis on a 1.0% or 2.0% agarose gel. After that, the agarose gel was photographed by quantity one system (Bio-Rad). To this end, all the 220 individuals were genotyped based on each site that showed polymorphism. And, according to the genotyped data, the Hardy–Weinberg equilibrium (HWE) test based on likelihood ratio for different site-population combinations and the number of observed and effective alleles had also been assayed with the goodness-of-fit χ^2 test for each site. Primers and restriction enzymes for the verification and genotyping were shown in the Table S2.

2.4. Association analysis between SNPs and susceptibility/resistance to GCRV

Estimating frequencies of allele and genotype, and their associations with the susceptibility/resistance to GCRV infection were analyzed. As a rule to check whether observed frequencies of genotype and allele between parents and their off-spring were in equilibrium in a population, the HWE test had also been assayed for the sites chosen to make the susceptibility/resistance-related analysis. Above analyses were in virtue of SHEsis software [20]. Besides, the linkage disequilibrium (LD) structure measured by D′, 95% confidence interval and r^2 was performed with Haploview 4.2 software. Since SNPs within haplotype blocks may be in LD [21], the SNP pairs in highly linkage disequilibrium were selected for haplotype analyses. The model was similar to that of single SNP site association analysis, except that the interaction between the two SNPs was included as a fixed effect. The *P* value less than 0.05 and less than 0.01 were considered statistically significant and extremely significant, respectively. In addition, linkage disequilibrium generates as a result of genetic mutation or recombinant and

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