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The polymorphism and haplotype of *TLR3* gene in grass carp (*Ctenopharyngodon idella*) and their associations with susceptibility/resistance to grass carp reovirus

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ABSTRACT

Toll-like receptors (TLRs) have emerged as crucial sensors of invading microbes through recognition of pathogen-associated molecular patterns (PAMPs) in viruses, bacteria, fungi and protozoa. The polymorphisms in TLRs are closely associated with the resistance to pathogen infections. *TLR3* involved in the recognition of double stranded RNA in humans, mice, pigs and fishes. In present study, the nucleotide sequence polymorphisms of *TLR3* gene in grass carp (*Ctenopharyngodon idella*) (*CiTLR3*) were investigated to explore their association with susceptibility/resistance to grass carp reovirus (GCRV). Twelve single nucleotide polymorphisms (SNPs) and an ins/del mutation were detected in the complete sequence of *CiTLR3*. Ten of them were sited in the non-coding region. The two SNPs in exon were synonymous mutation. The ins/del mutation was coincidental at the start codon. To investigate the association between the polymorphism and the susceptibility/resistance to GCRV, we selected eight SNPs in the non-coding region and analyzed the genotype and allele distribution in susceptible and resistant groups with PCR-RFLP. The statistical results indicated that only −764 G/T was significantly associated with the resistance of grass carp to GCRV both in genotype ($P = 0.040$) and allele ($P = 0.025$). Linkage disequilibrium analysis revealed −543 A/G, −488 G/T, 4116 G/T and 4731 C/T were linkage disequilibrium, and haplotype analysis revealed that haplotype GTTT frequency in susceptible group was significantly higher than that in the resistant group (OR = 2.01, 95% CI 0.996–4.043, $P = 0.049$). To further confirm the correlation, an additional infection experiment was carried out. The mortality in the −764 GG genotype individuals was significantly lower than GT genotype (OR = 0.208, 95% CI 0.067–0.643, $P = 0.011$) and TT genotype (OR = 0.183, 95% CI 0.052–0.648, $P = 0.015$). All the results indicated that haplotype GTTT and genotype −764 TT and −764 GT individuals were susceptible to GCRV while −764 GG was resistant, which could be the optional markers for selective breeding for the GCRV-resistant grass carp in future.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is a crucial aquaculture species in China, but tremendous economical loss is often caused by grass carp reovirus (GCRV), a double stranded RNA (dsRNA) virus [1,2]. To date, preventing this infectious disease is still difficult. The selection of GCRV-resistant strain is the potential solution to this disease. However, the traditional breeding methods are time-consuming and costly, cannot fulfill the urgent requirement. Hence, it is necessary to accelerate and improve selective breeding of resistant strains with molecular biology methods.

Toll-like receptors (TLRs) family is one of the three known innate immune-recognition receptor families (TLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs)), which recognize pathogen associated molecular patterns (PAMPs), and induce anti-microbial immune responses [3,4]. TLRs are type I transmembrane proteins that contain an extracellular N-terminus with leucine-rich repeat (LRR) region, a transmembrane domain and an intracellular C-terminus with a Toll/interleukin-1 receptor (TIR) domain. The cytoplasmatic TIR domain harbors conserved amino acids involving in the signaling as well as in the localization of the TLR, while LRR is involved in pathogen recognition [5–7]. TLRs are essential in the innate immune system by initiating and directing immune responses to pathogens including viruses, bacteria, fungi and protozoans, and control activation of adaptive immune responses [8]. Hence, TLRs are selected as candidate genes for the molecular markers in many investigations [9], and polymorphisms in TLRs are associated with resistance to disease in many species [10–12]. While marker assisted selection in aquaculture is still in the initial stage for the limited number of molecular markers.

Adaptive immune response is slow to develop in fish and the rapid innate immune system is more effective to protect against primary exposure to acute RNA viruses. *Toll-like receptor 3* (*TLR3*), one member

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of TLR family, has been described in a variety of species, which involved in the recognition of dsRNA, produced in the replication of many viruses [13,14]. It has been demonstrated that *TLR3* gene expressions in zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and rare minnow (*Gobiocypris rarus*) were also modulated during viral infection [15–18].

In previous work, we cloned *TLR3* gene in grass carp (*CiTLR3*) and explored its temporal expression after GCRV infection with quantitative real time RT-PCR. The result showed that the expression level of *CiTLR3* after GCRV infection was significantly higher than that in control [1], which implicated that the *CiTLR3* is involved in anti-GCRV defense. In present study, we selected *CiTLR3* as candidate gene for polymorphism detection and association analysis with the resistance of grass carp to GCRV. This research hopefully lays the foundation for selective breeding and sheds new light on disease control in aquaculture.

2. Materials and methods

2.1. Virus challenge and sample preparation

200 grass carp, averaging 10 cm in body length, were collected from three fish farms (Shaanxi, China) and kept in aerated freshwater at 28 °C for one week before processing. All the three farms don't find hemorrhagic disease of grass carp in recent years. The alevin of the three farms were bought from the same fish hatchery. For the viral infection experiment, grass carp were divided into four groups (50 animals in each group). The conditions were the same among tanks and the fish were randomly distributed into different tanks. Three groups were cultured in three aquariums and intraperitoneally injected with 100 μL of GCRV (097 strain, 3.63×10^7 TCID50/ml), suspended in PBS, per gram body weight. The control group was reared in another aquarium and injected with PBS. All the fish were observed every 6 h to survey the mortality and collect samples until the termination of the experiment at 240 h after challenge. Grass carp died in the first 72 h post-challenge were classified as susceptible individuals for their high sensitivity to GCRV and obvious symptoms of hemorrhage disease of grass carp, while the animals that survived over 240 h post-challenge were considered as resistant group, and the rest were discarded for their ambiguity to GCRV. Approximate 1 g caudal fin was cut and kept at −80 °C until DNA isolation. About 100 mg caudal fin was homogenized, and DNA was extracted with traditional phenol-chloroform method and stored at −20 °C.

2.2. Identification of polymorphic loci

The whole sequence of *CiTLR3* gene was scanned with twelve pairs of gene specific primers (Table 1), which were designed based on the *CiTLR3* genomic sequence we have reported in grass carp [1]. The amplicons were from 429 bp to 722 bp. PCR reaction was performed in a Peltier Thermal Cycler (Bio-Rad PTC-200) in 25 μL reaction volume containing 100 ng of DNA template. Annealing temperatures were calculated by primer premier 5.0 software. The PCR products, from ten susceptible individuals and ten resistant individuals, were purified and sequenced with the automated sequencer ABI3730 (Applied Biosystem). The polymorphic loci were detected from the sequence alignment of different individuals using Vector NTI Suite 11.0 (Invitrogen).

2.3. Association analysis between polymorphism and susceptibility/resistance to GCRV

Five single nucleotide polymorphisms (SNPs) in the 5′ UTR and the first intron and three in the 3′ UTR, which could be detected by PCR-RFLP, were selected as candidate loci. To genotype these SNPs, other eight pairs of primers were designed (Table 2). Four of them were introduced restriction enzyme cleavage sites for the following PCR-RFLP. Seven restriction enzymes including *Eco*RI, *Taq*I, *Sty*I, *Hin*dIII, *Msp*I, *Hin*fI, *Dra*I, were selected according to the detected alleles to cleave the PCR products. Five μL PCR product of each susceptible or resistant individual was detected by electrophoresis on 1.0% agarose gel. Another 5 μL product was digested by restriction enzyme at 37 °C (*Taq*I 65 °C) for 12–16 h. The mixture was examined by electrophoresis on 1.0% or 3.0% agarose gel. The results were photographed by quantity one system (Bio-Rad) and stored for statistical analysis. Estimated alleles and genotype frequencies and analyzed their association with susceptibility/resistance to GCRV with SPSS15.0 (SPSS Inc.). To further test the association between the SNPs and susceptibility/resistance to GCRV, we made linkage disequilibrium test according to the genotyping results and the highly linkage disequilibrium loci were selected for haplotype analysis with software SHEsis (http://analysis.bio-x.cn/SHEsisMain.htm). χ^2 test was employed for the significance test. *P* value less than 0.05 was considered statistically significant.

Table 1
Primers used for SNP analyses in *CiTLR3*.

Primer name	Sequence (5′–3′)	Application	Fragment size (bp)
TF1	AACACTTGACCTCGCTGTCG	5′ UTR and intron 1	429
TR1	AGCCCTCCACTCTTTACCAT		
TF2	CTGACCCAACCAGACATAAGA	Intron 1, 5′ UTR and exon 1	722
TR2	GTAAATAAAAAGACACATCAAACT		
TF3	CACTCTACCCCTACCTCTCTTG	Intron 2 and exon 2	552
TR3	AATAGAAACATCGTCCCAACA		
TF4	ATACTTGGACAGAGAGGTGAGAC	Intron 3 and exon 3	657
TR4	GTGTGCTCTGTTATCTCTCGG		
TF5	GAGTCATACATCATCCTTGCCA	Exon 3	747
TR5	AGCATCAAGTGGAATCTCATCTA		
TF6	GTGGTCCAGTGTTTTTCCTCA	Exon 3	654
TR6	ATGGTCTGTCCTTAGCAGTGTG		
TF7	GGATGAAAGCGTTACTGAAGG	Exon 3, intron 4 and exon 4	654
TR7	CCAGTAGAGAACACAGCGAGG		
TF8	AGAACAATCGTGACTCCCTGA	Intron 4 and 3′ UTR	611
TR8	TTGGGCATAAGGAAATAGCAC		
TF9	TTTACAACTCCAAACTAAACACTT	3′ UTR	490
TR9	AAAACATTGACATTTTGACAGTG		
TF10	TGGGAAATAGATGGTCAGAG	Intron 1	712
TR10	GCATGGAAGCAAGTGTAGA		
TF11	TTTCATTTGATGCGACTTG	Intron 2	598
TR11	GCCCAGTTTAGCAGATTTC		
TF12	TTAAGAAGGTAAGAGGAACAGAA	Intron 3	652
TR12	AAGAATGGTGAGAATGGTAGAG		

Table 2
The primers for genotype analyses and the digestion results by PCR-RFLP.

Locus	Primer name	Sequence (5′–3′)	Product (bp)	Restriction enzyme	Fragment (bp)
−764 G/T	TF1	AACACTTGACCTCGCTGTCG	148	*Dra*I	129/19/148
	TR240	TGCATGGGGGAAAATAATTTT			
−613 A/C	TF1	AACACTTGACCTCGCTGTCG	300	*Hin*dIII	279/21/300
	TR246	GCAGTTGACGTACTTAGCAAGC			
−543 A/G	TF10	TGGGAAATAGATGGTCAGAG	712	*Msp*I	596/116/712
	TR10	GCATGGAAGCAAGTGTAGA			
−488 A/G	TF10	TGGGAAATAGATGGTCAGAG	712	EcoRI	542/170/712
	TR10	GCATGGAAGCAAGTGTAGA			
−106 G/T	TF10	TGGGAAATAGATGGTCAGAG	712	*Taq*I	554/158/712
	TR10	GCATGGAAGCAAGTGTAGA			
4116 G/T	TF8	AGAACAATCGTGACTCCCTGA	224	*Hin*fI	199/25/224
	TR248	CACAAAATAAACATTATGGAAGAAT			
4683 G/T	TF9	TTTACAACTCCAAACTAAACACTT	257	*Eco*RI	232/25/257
	TR249	GGAAAATTAATTAATGATAAGAATT			
4731 C/T	TF9	TTTACAACTCCAAACTAAACACTT	387	*Dra*I	280/107/387
	TR257	CTTCTCAGGCTGAAACAATG			

Note: The nucleotides in shadow were changed for restriction enzyme digestion.