Journal of **Kim Il Sung** University (Natural Science)
Vol. 3 No. 1
Juche103(2014)

Structural Characteristics and Expression Analysis of Toll-like Receptor 2(Tlr2) Gene from Grass Carp

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Abstract This study reports the cloning of grass carp Tlr2 cDNA, genomic structure of grass carp Tlr2 gene, and its mRNA regulation in bacterial and viral infection. Grass carp Tlr2 gene has several leucine-rich repeats (LRRs) in the N-terminal region and a Toll-interleukin-1 receptor (TIR) domain at the C-terminal end. Structurally, grass carp Tlr2 consists of one exon with an open reading frame of 2 361bp, encoding a protein of 786 amino acid residues. Grass carp Tlr2(gcTlr2) cDNA has a 5′-untranslated region(UTR) of 198bp and a 3′-UTR of 188bp. Grass carp Tlr2 shared highest full-length amino acid sequence similarity with zebrafish(80.8%) and catfish(62.2%). gcTlr2 expression of the anterior kidney was increased 12h after *F. columnare* challenge, and then down-regulated after 36h, although these changes were not statistically significant. The TNF-α was peaked on 48h, but declined on 60h. In the spleen the significant change was observed, that was peaked on 60h. Infection with GCRV also up-regulated gcTlr2 expression in the anterior kidney on the 1st day after post-challenge but this increase was significant (17.5 folds).

Key words grass carp, Tlr2, mRNA, toll-like receptor-2

Introduction

The great leader Comrade **Kim Jong Il** said as follows.

"In addition, we must intensify the study of mathematics, physics, biology and other basic sciences so that they contribute positively to the national economy and the development of science and technology."("**KIM JONG IL** SELECTED WORKS" Vol. 12 P. 202)

Introducing intensive methods into aquaculture, fish production is plagued by a number of diseases including gill-rot disease, red-skin disease by viruses and bacteria. Until now in fish the study on anti-bacterial and viral activation of immunity has not been well done.

Tlr2 receptor is widely known as a receptor recognizing conserved components of Gram-positive bacteria such as lipoteichoic acid (LTA), peptidoglycans (PGN), and lipoproteins [2, 3].

In this study, we report the complete sequence of cDNA of grass carp Tlr2, the grass carp Tlr2 genomic organization and the expression patterns of grass carp Tlr2 mRNA from various healthy tissues for the first time.

Material and Method

Cloning of grass carp Tlr2 cDNA To cloning Tlr2 cDNA of grass carp, degenerate primers for cloning from grass carp Tlr2 were designed, based on the multiple alignments (Table 1). All PCR products were purified by using a gel extraction kit ("Qiagen"), and then were linked into pMD 18-T vector ("TaKaRa") and transformed into *Escherichia coli* DH10B competent cells

("Invitrogen"). Then it was sequenced. The 5′- and 3′-unknown sequences of gcTlr2 were obtained using a BD SMART™ RACE cDNA amplification kit ("BD Clontech"). The full length cDNA sequences were confirmed by sequencing the PCR product.

mRNA regulation after infection of grass carp reovirus(GCRV) or *Flavobacterium columnare* Grass carp was 1-year-old, weighing approximately 27.1～31.8g, and body length 11.3–11.8cm. After *Flavobacterium colnumnare* exposure three individuals of challenge and control were sampled for each group at days 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 of treatment. RNA was extracted from head kidney and spleen using a RN easy kit("Qiagen"). Grass carp was injected intraperitoneally with GCRV, from sample individuals were collected tissues including blood cells, gill, liver, spleen, head kidney, middle kidney, stomach, intestine, muscle, heart, brain, eye, skin and gonad at 0, 1, 2, 3, 4, 5, 6 and 7 days after injection respectively. The non-injected animals were used as blank group. cDNA was reverse transcribed using MMLV reverse transcriptase and random hexamer primers. Expression of gcTlr2 mRNA was assessed in different tissues and infection states using quantitative real-time RT-PCR (qRT-PCR) in an "ABI Prism 7 000 Sequence Detection System" ("Applied Biosystems").

Sequence analysis and phylogeny Sequence homology was obtained using BLAST program, the deduced amino acid sequences were analyzed with the Expasy and the protein domain features were predicted by the simple modular architecture research tool (SMART). Multiple sequence alignments were created using the ClustalW2 and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1.

Nucleotide sequence deposition The gcTlr2 cDNA sequence was submitted to GenBank and has been designated accession number FJ542042.

Table 1. Oligonucleotide primers used to amplify cDNAs for gcTlr2, β-actin and LITAF gene

Primer name	Sequence (5′→3′)	Use
tlr2R1 (forward)	TTBCKCAGYTTGCAGAAGCG	ORF
tlr2F1 (reverse)	AAYAANTTYRTBTGCWCCTG	ORF
gcT2F(forward)	CCGCTTCCATCTTCCAGTC	ORF
gcT2R(reverse)	ATCCCTCTGTAAGGCGGCT	ORF
T2G1(reverse)	CTCCACGGGCCACTCCAGATACG	5′-RACE
T2NG1(reverse)	GACAGCGGAGTCATTGTGCTCATCG	5′-RACE
T2G2(forward)	TCAGCCTTCATTCGCCCTGTGTTTG	3′-RACE
T2NG2(forward)	TCAAGAAGGAGACCATTCCCAAGCG	3′-RACE
T2rt1F(forward)	ACATCTTCTCGGTTGGTCC	Expression
T2rt1R(reverse)	ATTCTGGAGGTCAGCGGTC	Expression
Qb-actinF(forward)	GATGATGAAATTGCCGCACTG	Expression
Qb-actinR(reverse)	ACCAACCATGACACCCTGATGT	Expression
TNFrt2F(forward)	AAACCCACAGAACCCACC	Expression
TNFrt2R(reverse)	CTCCCGAACACCAGACCA	Expression

Results

1. Sequences Analysis of gcTlr2 Genes

Analysis of the domains of the grass carp Tlr2 gene using the SMART program revealed that it encodes two motifs characteristic of TLR genes, several leucine-rich repeats(LRRs) in the N-terminal region and a Toll-interleukin-1 receptor(TIR) domain at the C-terminal end(Fig. 1-a). Structurally, grass carp Tlr2 consists of one exon with an open reading frame of 2 361bp, encoding a protein of 786 amino acid residues (Fig. 1-b).

Grass carp Tlr2 has a 5′-untranslated region (UTR) of 198 bp and a 3′-UTR of 188bp, as determined by comparing the grass carp cDNA and gene sequences. The gene structure of one coding exon in grass carp Tlr2 is shared by zebra-fish, and catfish Tlr2 genes, but not by fugu or Japanese flounder Tlr2 genes which are encoded by 11 and 12 exons respectively[5, 6].

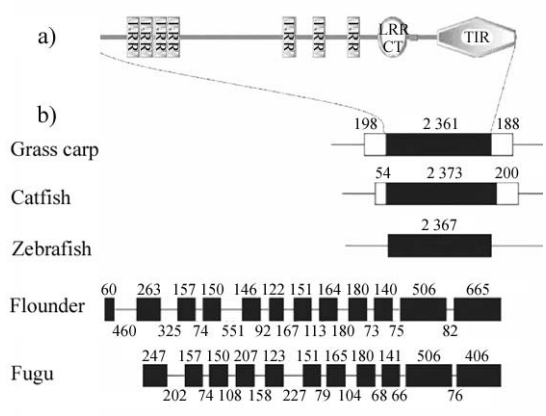

Fig. 1. Schematic diagram of the deduced grass carp Tlr2 protein and gene structure

The Tlr2 gene structure is compared with zebra-fish (*Danio rerio*(NP997977)), catfish (*Ictalurus punctatus*(DQ372072)), fugu (*Takifugu rubripes*(AAW69370)) and japanese flounder (*Paralichthys olivaceus*(BAD01046)) and grass carp (*Ctenopharyngodon idella*(FJ542042)) Tlr2 genes. The coding regions in the exons are shown by black box and sizes (bp) are indicated above the box. The sizes (bp) of the introns are represented in parenthesis.

2. Analysis of the Deduced Amino Acid Sequence of gcTlr2 Protein

A ClustalW-generated multiple sequence alignment using all available Tlr2 sequences from fish species as well as those of other vertebrates indicated high levels of amino acid sequence conservation within the TIR domain(Table 2). Grass carp Tlr2 shared highest full-length amino acid sequence similarity with zebra-fish (80.8 %) and catfish (62.2 %). Phylogenetic and molecular evolutionary analyses result is Fig. 2.

Table 2. Percentages of amino acid identities between grass carp Tlr2 and those from other species using ClustalW

Grass carp	Full sequence/%	TIR domains/%
Zebra-fish	80.8	93.9
Catfish	62.2	86.5
Fugu	43.9	58.1
Flounder	41.2	59.5

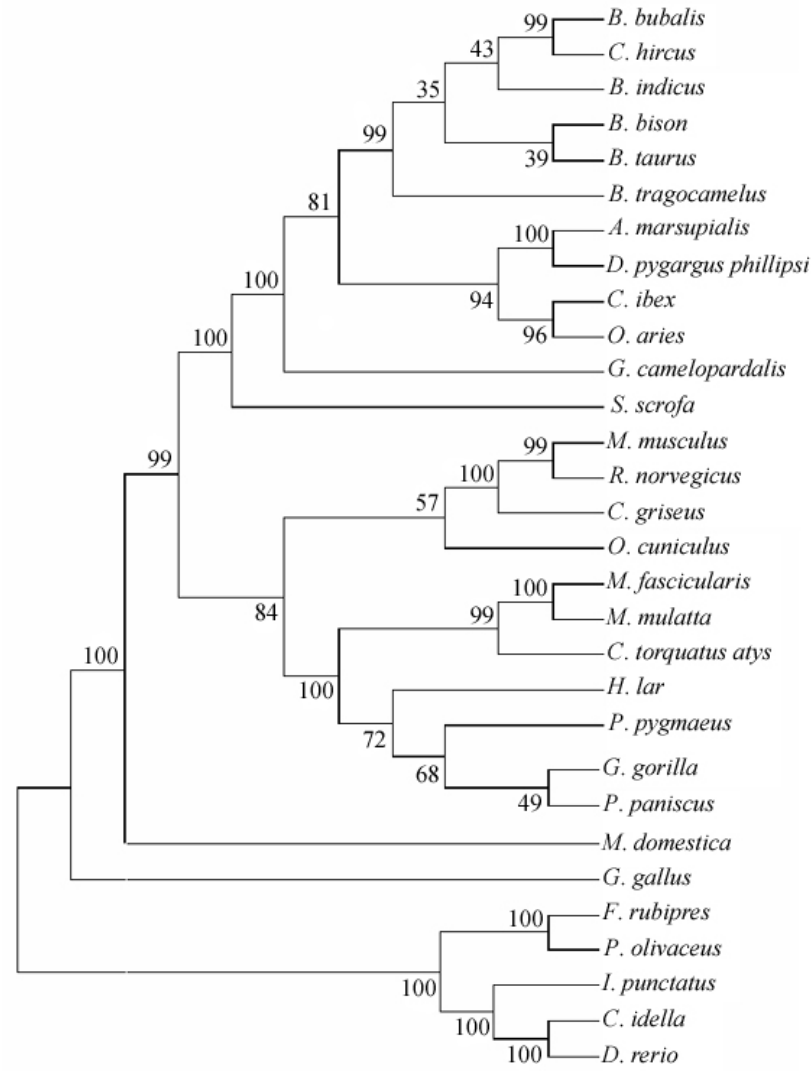

Fig. 2. Phylogenetic relationships of Tlr2 gene

Phylogenetic tree was obtained from a ClustalW alignment and MEGA3.1 Neighbor-Joining of 31 sequences of Tlr2. The bar indicated the distance. GenBank accession numbers for these amino acid sequences are as follows: *C. idella*(FJ542042), *A. marsupialis*(EU580538), *B. bison*(EU580539), *B. indicus*(EU413951), *B. Taurus*(AY634629), *B. tragocamelus*(DQ286731), *B. bubalis*(EU178742), *C. ibex*(EU580540), *C. hircus*(DQ872435), *C. torquatus atys*(EU204932), *C. griseus*(AF113614), *D. pygargus phillipsi*(EU580541), *D. rerio*(NM_212812), *G. gallus*(AB046533), *G. camelopardalis*(EU580542), *G. gorilla*(AB445627), *H. sapiens*(AB445624*), H. lar*(EU488848), *I. punctatus*(DQ372072), *M. fascicularis*(AB445629), *M. mulatto*(NM_001130425), *M. domestica*(XM_001375753), *M. musculus*(AY179346), *O. cuniculus*(NM_001082781), *O. aries*(EU580543), *P. paniscus*(AB445626), *P. pygmaeus*(AB445628), *R. norvegicus*(AY151256), *S. scrofa*(AB208696), *F. rubipres*(AC156432), *P. olivaceus*(AB109395).

3. mRNA Expression Patterns in Grass Carp Tissues

RT-PCR analysis using total RNA from various healthy tissues of grass carp indicated that Tlr2 is ubiquitously expressed albeit at different levels. High levels of Tlr2 expression were detected in anterior kidney, brain, middle kidney, blood, stomach and intestines while only low Tlr2 expression was detected in eye, liver, muscle and spleen (Fig. 3).

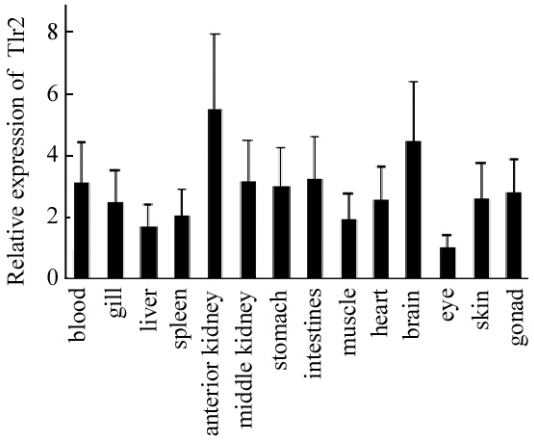

Fig. 3. The tissue expression distribution of Tlr2 in grass carp

We evaluated gcTlr2 regulation after *in vivo F. columnare* challenge, gcTlr2 expression of the anterior kidney was increased 12h after *F. columnare* challenge, and then down-regulated after 36h(Fig. 4-a), although these changes were not statistically significant. The pattern of transcriptional changes for the TNF-α observed, that was peaked on 48h but declined on 60h (Fig. 4-b).

Infection with GCRV also up-regulated gcTlr2 expression in the anterior kidney on the 1st day post-challenge, although this increase was significant(17.5 folds) (Fig. 5). But in the spleen the significant change was observed, that was peaked on 60h. However, immune system stimulation by *Flavobacterium columnare* was evidenced by an elevated expression of the proinflammatory cytokine TNF-alpha.

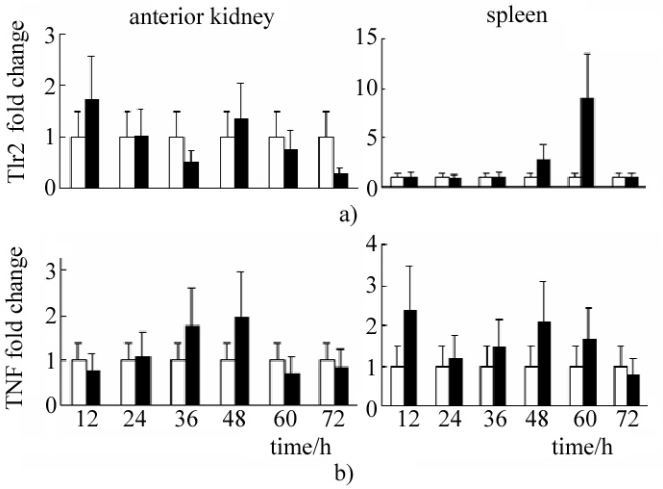

Fig. 4. Tlr2 and TNF-α regulation in grass carp after bacterial (*F. columnare*) challenge

a) *in vivo* assay: gcTlr2 fold change of expression in grass carp 12～72hours after bacterial, challenge. Three individuals were sampled for each group at each time point.

b) TNF-alpha regulation of grass carp after bacterial challenge

□ control, ■ challenged GCRV

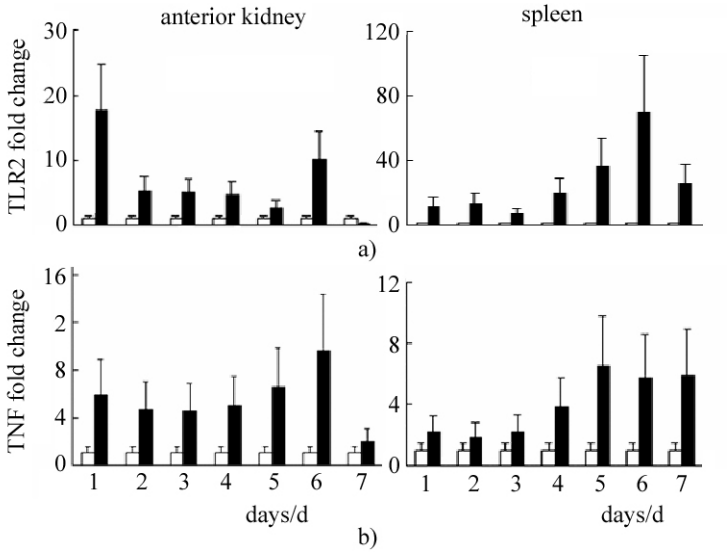

Fig. 5. Tlr2 and TNF-α regulation in grass carp after GCRV challenge
a) *in vivo* assay: gcTlr2 fold change of expression in grass carp 1～7days after GCRV challenge Three individuals were sampled for each group at each time point. b) TNF-alpha regulation of grass carp after GCRV challenge
□ control, ■ challenged GCRV

Discussion

Further work is needed to determine the pathogen specificities of TLRs in fish species. All vertebrate species studied to-date possess a functional, well-conserved Tlr2 gene, indicating strong selective pressure for the gene's ability to recognize important classes of PAMPs. Conservation of microbial PAMPs has likely led to the maintenance of a highly conserved TLR family across vertebrate species. The interspecies conservation of the TLR multi-gene family differs significantly from other immune families of genes under less selective restraints [1, 7, 8].

Much work remains in assessing the potential of TLRs in immune research and their applications in mammalian and fish species. Tlr2 research in mammals continues to uncover polymorphisms associated with infectious and inflammatory diseases [4, 9, 10]. Additionally, understanding the function of fish TLRs and their pathogen specificities may lead to the development of better immunostimulants for use in commercial aquaculture [11]. The future identification and characteristics of additional TLR family members and the components of their signaling pathways [12] in aquaculture species should further our knowledge of the teleost innate immune response.

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