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Cloning, sequence analysis and expression profiles of Toll-like receptor 7 from Chinese giant salamander *Andrias davidianus*



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ABSTRACT

The Chinese giant salamander, *Andrias davidianus*, is the largest extant amphibian species in the world, which is of significance due to its specific position in the evolutionary history of vertebrates. Currently, limited information about the innate immune system of this animal is known. In this study, the toll-like receptor 7 (TLR7), designated CgsTLR7, was cloned from Chinese giant salamander, *A. davidianus*. The full-length cDNA of CgsTLR7 is 3747 bp, with an open reading frame of 3150 bp, encoding 1049 amino acids. The TLR family motifs, including the leucinerich repeat (LRR) and Toll/interleukin (IL)-1 receptor (TIR) domain are conserved in CgsTLR7, which includes 19 LRRs and a TIR domain. The predicted amino acid sequence of CgsTLR7 has 71%, 65%, 63% and 55% identity with turtle, chicken, human and fugu TLR7 homologues, respectively. Phylogenetic analysis showed that CgsTLR7 is closest to that of frog TLR7 among the examined species. Quantitative real-time PCR analysis revealed broad expression of CgsTLR7 in tissues from apparently healthy Chinese giant salamanders with the highest expression in the liver and the lowest expression in the intestine. The mRNA expression was up-regulated and reached a peak level in the kidney, liver and spleen at 12 h, 24 h and 48 h after infecting the animals with the giant salamander iridovirus (CSIV), respectively. These results suggest that CgsTLR7 has a conserved gene structure and might play an important role in immune regulation against viral infections in the Chinese giant salamander.

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1. Introduction

The innate immune system, as the first line of defense against the invading microbes, relies on recognizing microbial components through sets of pattern-recognition receptors (PRRs) (Akira et al., 2006). Toll-like receptors (TLRs) act as PRRs which play a key role in sensing pathogen-associated molecular patterns (PAMPs), that lead to stimulation of antimicrobial genes and proinflammatory cytokines, as well as regulating the activation of appropriate immune mechanisms (Armant and Fenton, 2002; Janeway and Medzhitov, 2002; Takeda et al., 2003).

Toll-like receptors are type I transmembrane glycoproteins with an ectodomain of leucine-rich repeat (LRR) motifs, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain (Akira et al., 2006; Palti, 2011). Leucine-rich repeat motifs are anatomically diverse, enabling sensing and responding to a wide range of PAMPs (Medzhitov, 2001; Rebl et al., 2010). The TIRs are evolutionarily conserved and react with adapter proteins to initiate signal transduction

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to downstream cascades (Imler and Hoffmann, 2003; Jault et al., 2004). Upon TLR activation, the TIR domain efficiently recruits several TIR-containing intracellular adapter proteins, including myeloid differentiation factor 88 (MyD88) (Akira et al., 2001) a TIR-domain-containing adapter inducing interferon (IFN) - β (TRIF) (Fitzgerald et al., 2001; Horng et al., 2001). The TLR signaling via MyD88-dependent or independent pathways results in the activation of separate and appropriate innate immune responses (Akria et al., 2006), including the production of pro-inflammatory cytokines, type-I interferons and chemokines.

In mammals, the TLR family consists of 13 members (TLR1-13) (Pifer and Yarovinsky, 2011). Humans have ten TLRs and mice have 12 TLRs. In addition, several other TLRs have been identified in lower vertebrates, including TLR14, TLR15 and TLR23 in chickens (Roach et al., 2005; Yilmaz et al., 2005); TLR18-20 in zebrafish (Meijer et al., 2004) and TLR24 in lamprey (Kasamatsu et al., 2010). The TLR7 homologue has been identified in several vertebrate species, such as humans (Chuang and Ulevitch, 2000), dogs (Okui et al., 2008), the large yellow croaker (Qian et al., 2013), common carp (Tanekhy et al., 2010), rainbow trout (Palti et al., 2010) and Atlantic salmon (Lee et al., 2013). The TLR7 recognizes single stand (ss) RNA in mammals and is activated by ssRNA viruses. Synthetic ssRNA oligonucleotides (ORN) mimic virus RNA and induce the production of cytokines (Heil et al., 2004; Iwasaki

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and Medzhitov, 2004; Judge et al., 2005). The TLR7 also induces production of type I interferon (Crozat and Beutler, 2004), that has a crucial role in the host immune response.

The Chinese giant salamander (*Andrias davidianus*), which belongs to one of the most primitive orders of the urodele amphibians, in the family *cryptobranchidae*, is the largest extant amphibian species in the world (Zhang et al., 2003). In the evolutionary history of the vertebrate immune system, the Chinese giant salamander is considered an ancestral organism, representing the transition from aquatic to terrestrial life, and it is considered to be an important model in scientific research of the phylogeny, biodiversity, and the earth's evolutionary history (Wang et al., 2013; Zhu et al., 2014). Populations of the Chinese giant salamander have declined in the past 30 years, and now this species has been listed in annex I of the convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in the national class Π list of protected animals in China.

The giant salamander iridovirus (GSIV) is an emerging pathogen that has caused high mortality of Chinese giant salamanders. This pathogen causes a disease characterized by subcutaneous hemorrhages, necrotizing limbs and visceral hemorrhages (Meng et al., 2013; Ma et al., 2014). There is limited knowledge on the study of TLR7's expression profile in response to dsDNA. In the study reported herein, a full-length cDNA of CgsTLR7 from Chinese giant salamander was sequenced and characterized. Additionally, the expression profiles of CgsTLR7 among different tissues and expression patterns in response to virus infection were examined in vivo. The results provide important information for understanding the innate immunity of the Chinese giant salamander in response to a virus infection, which may be potentially useful in the development of management strategies for the disease caused by GSIV.

2. Materials and methods

2.1. Cell line, virus, animals

The *epithelioma papulosum cyprini* (EPC) cell line provided by the China Center for Type Culture Collection was grown at 25 °C in Eagle's minimal essential medium (EMEM; Sigma, USA), supplemented with 10% fetal bovine serum (Fijian et al., 1983). The GSIV was isolated from Chinese giant salamander and cultured in EPC cells (Gao et al., 2012; Zhou et al., 2012).

The Chinese giant salamanders (average weight about 70 g) were obtained from the experimental farm of Yangtze River Fisheries Research Institute. The animals were kept in tanks with aerated tap water at 22 °C and fed diced bighead carp for 2 weeks before the experiment. To analyze the tissue distribution of the CgsTLR7 mRNA in healthy animals, tissue specimens including kidney, spleen, skin, brain, muscle, lung, heart, intestine and liver were collected from 3 healthy Chinese giant salamanders after anesthesia with MS-222(0.01%, Sigma). Forty Chinese giant salamanders were divided equally into a test group and a control group. In the test group, each animal was injected intramuscularly with 200 µL of GSIV purified as described previously (Gao et al., 2012), which had a titer of 5×10^7 tissue culture infective dose 50% (TCID₅₀)/ml. In the control group, each animal received an equal volume of Dulbecco's phosphate buffered saline (DPBS) (Sigma) intramuscularly. Spleen, kidney and liver specimens were collected from 3 individuals from each group at 0, 6, 12, 24, 48, 96 h post-injection (hpi) that were stored at -80 °C. These samples were used for RNA extraction.

2.2. Cloning and sequencing of CgsTLR7

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The first strand cDNA was synthesized using PrimeScriptTM 1st Strand cDNA Synthesis Kit

Table 1		
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Primer	sequences	useu m	uns	study.	

Primer name	Sequence(5'-3')	Purpose
P1	GCTCCTTTAACCTGGGTGGGAACCT	CgsTLR7 5' RACE
P2	CTTAGTACACACATGGTCCTTTGGC	Cloning
P3	GTGCCGCCAGATTGAAAGGCTACCG	CgsTLR7 3' RACE
P4	CATTCTATATGGCTCACCAGTGGCT	Cloning
P5	TTCTCCTCCTCTTCCTGCTGTTC	CgsTLR7 full-length
P6	ATACTGTTTGCCTCCTGGGTCTT	Cloning
P7	AAGAGGCTGTGTAAAAGATCGGTTC	CgsTLR7 expression
P8	GCCATGTGAGTGTCTGTTTGTAGTG	Analysis by real-time PCR
P9	TGAACCCAAAAGCCAACCGAGAAAAGAT	β-actin expression
P10	TACGACCAGAGGCATACAGGGACAGGAC	Analysis by real-time PCR

(TaKaRa) and preserved at -20 °C. This sample was used for the quantitative PCR of the gene expression. To obtain the full-length cDNA of TLR7, two pairs of primers labeled P1–P4 (Table 1) for the 5' and 3' RACE-PCR, were designed based on the analysis of Chinese giant salamander transcriptome (data not shown) that was sequenced by our laboratory. The sample for cDNA synthesis was obtained from kidney tissue 12 hpi. The 5' and 3' RACE-PCR was performed using SMARTer RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions. To confirm the integrity of the cDNA sequence, PCR was performed with full-length cloning primers P5 and P6 (Table 1). The PCR product was examined by 1.5% agarose gel electrophoresis and the target DNA fragment was purified using a Silica Bead DNA gel extraction Kit (Fermentas, USA) and ligated to pMD19-T vector (TaKaRa) for cloning and sequencing.

2.3. Sequence analysis

Homologous sequence searches were performed using the BLAST program in the National Center of Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequence and protein analysis were conducted by the ExPASy proteomic tool (http://www.expasy.org/tools/) and the protein domains were predicted using Simple Modular Architectural Research Tool (SMART) program (http://smart.embl-heidelberg.de/). To predict transmembrane domains and signal sequences, the PSORT program (http://www.psort.nibb.ac.jp/form2.html) was used. Both mass and isoelectric point (pI) of the putative protein were calculated using the Prot-Param tool at the ExPASy website (http://www.expasy.org/protparam/) and the molecular tree was constructed by Molecular Evolution Genetics Analysis (MEGA) software version 3.1 using the neighbor-joining method (Kumar et al., 2004). Multiple sequence alignments were analyzed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid sequences of the TLRs in other species were obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/protein).

2.4. Quantitative real-time PCR analysis

In order to test the tissue expression profile of CgsTLR7 among different tissues and expression patterns after infection with GSIV, quantitative real-time PCR (qRT-PCR) was performed with primers P7 and P8 (Table 1). The β -actin of Chinese giant salamander (GenBank accession number: HQ822274) was amplified as an internal control to determine the concentration of each template with the primer set of P9 and P10 (Liu et al., 2014) (Table 1). qRT-PCR was performed on Rotor-Gene 6000 Real-time PCR system (Qiagen, Germany) using a 2 × SYBR real-time PCR premixture (BioTeke, China). The qRT-PCR was conducted in 20 µL volume containing 10 µL of 2 × SYBR real-time PCR premixture, 8 µL of nuclease-free water, 1 µL of cDNA template, and 0.5 µM of each primer. The threshod cycle (CT) value was obtained by the cycling conditions:

	signal peptide transmembrane domain LRR1
AdTLR7	$\underline{MVLCN} \underline{CSSIQWFLLLFLLFLWSKMLAATWYP} KSLPCDVKEIAAE-TLLVECSDRRLTEIPLLIPSNVTNLTLTINHIPNISPRSFLHLPNLVEIDFRCNCVPVRLGPKDHVCTKRLHIENNSFTSLTHLKSLYLD 134$
GgTLR7	$ v \\ Hhartsnallfvllflfpmllsgrwfpktlpcdveafes-tvrvdcsdrrlkevprgipgnatnltltinhiprispasftqlenlveidfrcncvpprlgpkdnvcvtppsiengsfaaltrlkslyld 130$
HsTLR7	MVFPMWTLKRQILILFNIILISKLLGARWFPKTLPCDVTLDVPKNHVIVDCTDKHLTEIPGGIPTNTTNLTLTINHIPDISPASFHRLDHLVEIDFRCNCVPIPLGSKNNMCIKRLQIKPRSFSGLTYLKSLYLD 135
DrTLR7	MTEKTMIIFASFISLLVAAEWYPKSLKCDVSLASNGTEVSVDCTERSLTEVPLGIPTNTTNLTLTINHIPHVMNNSFDNLHNITEIDLRCNCVPVKVGPKDRVCSQSVSIDNGTFWKLKNLKSLYLD 127
	::. : . :: *:**:* *** : : : : : : : :
AdTLR7	${\tt GNQLLEIPWGLSPNLLLLSLEANSIFSISKENLTELENIEFLYMGQNCYYRNPCNVSFYIENDTFLSLRNLSVLSLKANNLSYVPGRFPPrlKelyLynnniqhiqekdfqnlfyleildlsSnCPrcynapfpct 269$
GgTLR7	ANQLSKIPRGLPATLRLLSLEANNIFSIKKNTFSELRNIELLYLGQNCYYRNPCNVSFEIEETAFLNLKNLTVLSLKSNNLTFIPPNLSSTLKELYIYNNRIQEVQEHDLSNLYNLEILDLSGNCPRCYNAPYPCT 265
HsTLR7	
DrTLR7	GNQLSSIPKGLPANIVLLSLEINSIYSILQENLTELTNIRTLYLGQNCYFRNPCNQSYYIEKDAFMLLDKMTLLSLKSNNLSYIPNQLPSSLKELYLYNNNIEKITENDFCNLTELEVLDLSGNCPRCYNAPFPC1 262
	.*** .** **: ***** *.*:** :.:::** **. **: ***** *: *** ::: *: * ::::**** **:::* : *.****:*** *. *.*: ** *::********
AdTLR7	PCPYNAAIAIHANAFHSLINLKILRLRSNSLKSIPYTWFEKTNGLKVLDLSQNFLAKEIGEANFLKCIPNLRELDFSFNFDLQLYPTSMKLSNTFSTLLSLEIFRVKGYVFKELNRNHLLPLLKLKNLTVLDLGT 404
GgTLR7	PCP-NISIKIHSKAFYSLKKLRILRLHSNSLQSIPSSWFKNIKNLKNLDLSQNFLIKEIGDAEFLKLIPSLVELDLSFNFELQWYSPFLNLSKTFSCLSNLETLRIKGYVFKELREENLDPLLNLRNLTVLDLGT 399
HsTLR7	PCKNNSPLQIPVNAFDALTELKVLRLHSNSLQHVPPRWFKNINKLQELDLSQNFLAKEIGDAKFLHFLPSLIQLDLSFNFELQVYRASMNLSQAFSSLKSLKILRIRGYVFKELKSFNLSPLHNLQNLEVLDLGT 405
DrTLR7	PCPNNaPLQIHPNSFKTLRNLKTLRLHSNSLTNIPPEWFQSLADLTLLDLSSNFLAKEITCTSFPSLLPKLEELDLSFNYELQVYPASLSLSESFSQLKSLRVLRIRGYVFQELKLQDIQPLTNLTYLEFLDLGT 392
	** * .: * ::* ::* :*: ****:*** ** :* **:. * ****. *** ***
AdTLR7	NFIKIANLSVFQEFPALQLIDLSVNKISPSSESVLDSCTNSKGSANQYSRSSLQNMHYFRYDENGRSCKSKEKENIPFIPFVNEDCSAFGKTLDLSINNIFFINDLDFKHLSFLKCLNLSGNAISQS 532
GgTLR7	NFIKIADLRVFKKFRSLKIIDLSMNKISPSSGESNFYGFCSDHRITVEQYSRHVLQEMHYFRYDEYGRSCKSKDKEADSYQPLVNGDCMSYGETLDLSRNNIFFVNSIDFQDLSFLKCLNLSGNAISQT 529
HsTLR7	NFIKIANLSMFKQFKRLKVIDLSVNKISPSGDSSEVGFCSNARTSVESYEPQVLEQLHYFRYDKYARSCRFKNKEASFMS-VNESCYKYGQTLDLSKNSIFFVKSSDFQHLSFLKCLNLSGNLISQT 532
DrTLR7	NFIKIAQLSILKNLKNFKIINLSDNKISVPSEGEFSFSNHREAYYGSPMSQGAQYHNGEVKDMHYFLYDEFARSCKYKDKELWIPSP-FNNDCSSFGKTLDISRNNIFFLHS-KFLNLGELRCLNLSGNAMSQS 532
	* *****: ::::: :::::::::::::::::::::::
AdTLR7	LNGSELQYLRQLKYLDFSNNRLDFLYPTAFQELKQLEVLDLSSNKHYFLSEGITHMLNFTHYLPSLNKLLMNWNEISTSTNTAMSSQSLQTLEFKGNRLDVLWRDGNSGYLGFFKNLTNLKKLDISYNSLVFIPP 662
GgTLR7	LNGSEFYYLSGLKYLDFSNNRIDLLYSTAFKELKFLEILDLSNNKHYFLAEGVSHVLSFMKNLAYLKKLMMNENEISTSISTGMESQSLQTLEFRGNRLDIFWSDGKKEYLSFFKNLTNLEQLDISSNMLNFLPP 659
HsTLR7	LNGSEFQPLAELRYLDFSNNRLDLLHSTAFEELHKLEVLDISSNSHYFQSEGITHMLNFTKNLKVLQKLMMNDNDISSSTSRTMESESLRTLEFRGNHLDVLWREGDNRYLQLFKNLLKLEELDISKNSLSFLPS 662
DrTLR7	eq:lingsefvqltnlqyldftdnrldlmypsafqelsnlvvldisknshyfvaeglthmlnftenlsklrklimndnqiststntemksyklehlefkgnrldmlwrdgdtryvnyfknlmslktldisknnlnfipl 660
	*****: * *:****:::::::**:** * :**:* * :**::*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
AdTLR7	GSYEGMPPQLAELYLTNNELKNFNWGKLYLLQKLEVLDLSNNLLTTVPRVLSNCTSTLTKMILRNNRIRRLTEHFLHDAFQLRYLDLSENKLDSITKSSFPEDGIKNLEILLLQGNPFMCNCDAVWFVWWINQTT 792
GgTLR7	$\label{eq:construction} DVFEaMPPELKILNLTSNRLHTFNWGKLHLLTKLITLDLSNNLLTIKKSSFPENIITVPRKLSNCTSTLQELILRNNRITRITKYFLRGAIQLTYLDLSSNKIQINNLRMLLLHNNPFKCNCDAVWFVGWINQTQ~789$
HsTLR7	${\tt GVFD} GWPPNLKNLSLAKNGLKSFSWKKLQCLKNLETLDLSHNQLTTVPERLSNCSRSLKNLILKNNQIRSLTKYFLQDAFQLRYLDLSSNKIQMIQKTSFPENVLNNLKMLLLHHNRFLCTCDAVWFVWWVNHTE~792$
DrTLR7	vv f q g l pv t l k l k l k k k g g l v l k s l l l d t g n l t e v p s c l s ny t k s l q t l v l s k n k i v k l s p n f l k d a f s l k l d l s y n s l q f l d e s s f p e n v l d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l v r w i n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m f v c s c n a t w l n r t s 790 r d h q t l y l n n n m h q t l y l n n n m h q t l y l n n n m h q t l y l n n n m h q t l y l n n n m h q t l y l n n n m h q t l y l n n n m h q t l y l n n n h q t l y l n n n m h q t l y l n n n m h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n n h q t l y l n n
	::.:* * * ::.* *: *.* * * .* ***: * ** ** ** *** :: :: ::* :*:*: **:****:: ::
AdTLR7	transmembrane domain Box 1 Box 2 TYIPRLATDVTCAGPGAHKHQSVIVLDLYTCESDSERVLLHSLSASIILCLMVITVSNHLFFWDMWYGYHFCAARLKGYRRLNSSGKCYDAFIAYITKDVPTAEWVIKELVTNLEDPGGKQYNLCLEERDWVPGQ 922
GgTLR7	VAIPLLATDVTCAGPGAHKGRSLVFLDLNTCELDTSYFIMYALSTSAVLCLMMFAVMSHLYFWDVWYSYHYCTAKLKGYRRIPLPDACYDAFIAYDVTDLAVNEWVMTELVEKLEDQKARQFNLCLEERDWLPGQ 919
HsTLR7	VTIPYLATDVTCVGPGAHKGQSVISLDLYTCELDLTNLILFSLSISVSLFLMVMMTASHLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTKDPAVTEWVLAELVAKLEDPREKH-NLCLEERDWLPGQ 922
DrTLR7	VNIPRLASDVTCASPSAQKGQSVIFLNLQACQHNSLSIILCIFQTTLILTILTILTILTISSHLFLWDVWYIYHFCLAKLKGYRRLSSNSAVYDAFVIYDITDPAVQEWVMQELRVHLEDKGDPR/NLCLEERDWVFGC 920
	* . ** **:*****.*:* :*:: *:*: : .:: : .:: : .:: .:
AdTLR7	TIR domain <u>Box</u> 3 AIFDNLSESIRLSRKTVFVLTNKYAQSGHFKMAFYMAHQWLMDEKMDVIILIFLEKALQNSRYLRLRKRLCKRSVLDWPPSPQAQRYFWICLKNSLQTDTHMAYDKLLKEIV 1049
GgTLR7	PVFDNLSQSIQLSKKTIFVLTNKYIKSGTFKTTFYMAHQRLLDEKIDVIILIFLEKVLQKSRYVQLRKRLCRSSVLEWPTNPRSQPYFWQRLKNAIAMNNTLSYNKLLQETV 1046
HsTLR7	PVLENLSQSIQLSKKTVFVMTDKYAKTENFKIAFYLSHQRLMDEKVDVIILIFLEKPFQKSKFLQLRKRLCGSSVLEWPTNPQAHPYFWQCLKNALATDNHVAYSQVFKETV 1049
DrTLR7	${\tt PLIENLSQSIQLSQRTVFILTERYIRSGSFRTAFYLAHQRLMDERNDVIVLIFLERMPCHSKYLRLRKRLYKKSVLEWPRNPQAQR FWSLRSLMATESQYNTLFQETL 1045$
	****:**:*:*::*::*:: *: :**::** *:**: ***:****: :*:::******

Fig. 1. Alignment of amino acid sequences of TLR7 from different species of vertebrates. The alignment was performed using ClustalW v 2.0 and edited manually; the signal peptide is underlined and transmembrane domain is boxed in black. The leucine-rich repeats (LRRs) are marked with grey shading and the domains are labeled above the alignments. CxRCxxxxPCxxC conserved domain is boxed in dashed. The toll/interleukin (IL) -1 receptor (TIR) domain is underlined with a dotted line. Three active TIR domain motifs are boxed in grey: box1 (YDAFI), box 2 (LC-RD-PG), and box3 (FW). Ad = Chinese giant salamander; Gg = chicken; Hs = human; Dr = zebrafish.

30 s at 95 °C, then 40 cycles at 95 °C for 15 s, 58 °C for 15 s,and 72 °C for 20 s. The data was statistically analyzed using Student's *t*-test.

2.5. Statistical analysis

All qRT-PCR analyses were repeated three times and the relative expression ratio of the target genes were calculated as described by $2^{-\Delta\Delta Ct}$

method (Livak and Schmittgen, 2001) using β -actin as the internal control to the target gene expression (Liu et al., 2014). All data were analyzed using SPSS software and expressed as the mean \pm S.E.M. Student's *t*-test was used to determine the significant difference of gene expression levels in various tissues or in the same tissues at 0 h and later time points post-infection. Values were considered to be significant when P < 0.05.

3. Results

3.1. Characterization of CgsTLR7 cDNA

The CgsTLR7 cDNA sequence with GeneBank accession number of KF573997, is 3747 bp in length, including a 144 bp 5'-untranslated region (UTR), an 3'-UTR of 453 bp, and an open reading frame (ORF) of 3150 bp, encoding a protein of 1049 amino acids. The pI and theoretical molecular weight of CgsTLR7 are 8.07 and 121.23 kDa, respectively. The deduced amino acids of CgsTLR7 exhibit a typical TLR domain architecture, including a signal peptide (residues 1-26) in the extracellular region, a N-terminal leucine-rich repeat (LRR-NT, residues 35-68), 17 LRRs, a C-terminal LRR (LRR-CT, residues 783-834) at the extracellular region, and a Toll/interleukin (IL)-1 receptor (TIR) domain (residues 890-1036) in the intracellular region (Fig. 1). The CgsTLR7 possesses the transmembrane domain in the residues 842-862 by the PSORT program (Fig. 1). Another transmembrane domain (residues 6–26) in the N-terminus of CgsTLR7 is predicted as a hydrophobic signal peptide by the PSORT program. Additionally, four conserved cysteines in the pattern of CxRCxxxxPCxxC (TLR7, residues 259-272) exist in TLR7 (Fig. 1). The CgsTLR7 has three highly conserved regions found in the TIR domain of the TLR family including box 1 (YDAFI), box 2 (LC-RD-PG), and box3 (FW) (Fig. 1).

3.2. Sequence homology and evolutionary analysis

The BLASTp search revealed the identity of the CgsTLR7 with other vertebrate TLR7 molecules (Table 2). The identity of CgsTLR7 to turtle, frog and chicken was 71%, 64% and 65%, respectively. In addition, the identity of CgsTLR7 to zebrafish and fugu was 56% and 55%, respectively.

To reveal the phylogenetic relationship between TLR7 and their counterparts in other vertebrates, an unrooted phylogenetic tree was constructed using MEGA 3.1. The phylogenetic tree analysis also supported the above nomenclature. As shown in Fig. 2, the tree was divided into two branches. One branch was from piscine and the other branch was from mammals, avians, reptiles and amphibians. The sequence of CgsTLR7 is closest to that of frog TLR7 among the examined species (Fig. 2).

3.3. Tissue expression profile of CgsTLR7 and expression modulation of CgsTLR7 in spleen, kidney and liver after injection with GSIV by qRT-PCR

qRT-PCR was used to analyze the expression profile of CgsTLR7 in vivo. As shown in Fig. 3, CgsTLR7 was broadly expressed in the nine tissues that were examined. The lowest expression level of CgsTLR7 was observed in the intestine, which was used to compare the expression level of TLR7 in the other tissues. The expression levels of CgsTLR7 were strongest in the liver and then kidney, moderate in the brain, muscle, spleen and heart, and lowest in the lung and skin.

To further understand the expression changes of CgsTLR7 after virus infection, qRT-PCR was used to detect the expression levels of TLR7 in the spleen, kidney and liver after the animals were injected with GSIV.

Table 2

Sequence identities of the deduced amino acid sequences of CgsTLR7 genes among amphibians, reptiles, fish, chicken and mammal.

TLR7 protein	Accession number	CgsTLR7 (identity, %)
Turtle	XP_005287828	71
Frog	AAI66280	64
Human	NP_0057646	63
Mouse	NM_133211	62
Chicken	CAG15146	65
Carp	BAJ19518	57
Fugu	AAW69375	55
Zebrafish	XP_003199309	56

Note: The closest homolog of CgsTLR7 protein is boxed.

100 – Bison bonasus 79 ovis aries 100 Tursiops truncatus 100 Sus scrofa 99 Equus caballus 100 Homo sapiens Mus musculus 67 Gallus gallus 100 Anas platyrhynchos Alligator sinensis 100 Chrysemys picta bellii 100 Pelodiscus sinensis Andrias davidianu Xenopus tropicalis Cyprimus carpio 100 Danio rerio 100 Oncorhynchus mykiss 100 Pseudosciaena crocea 0.05

Fig. 2. Phylogenetic relationship with the TLR7 gene in different species. Deduced amino acid sequences of TLRs were from GenBank and the tree was constructed with neighbor-joining method in MEGA3.1. The GenBank Accession no. as follows: *Bos Taurus*: ABQ52584; *Ovis aries*: NP_001128531; *Tursiops truncates*: AAW69375; *Sus scrofa*: AFF59211; *Equus caballus*: NP_001041589; *Homo sapiens*: NP_0057646; *Mus musculus*: NM_133211; *Gallus gallus*: CAG15146; Anas platyrhynchoc: AHM88222; Alligator sinensis: XP_00602379; Chrysemys picta bellii: XP_005287828; Pelodicus sinensis: XP_006129906; Xenopus tropicalis: AAI66280; Cyprimus carpio: BAJ19518; Danio rerio: XP_003199309; Oncorhynchus mykiss: ACV41797; Pseudosciaena cricea: KC543351.

As shown in Fig. 4, the expression level of TLR7 reached a peak in the kidney, liver and spleen at 12 h, 24 h and 48 h post-injection, respectively.

4. Discussion

The TLRs play a vital role in the activation of the innate immune system by recognizing PAMPs. In the TLR family, TLR7 is of significant importance. Many TLR molecules have been cloned from vertebrates, but studies on Chinese giant salamander have not been reported to date. In the present study, we cloned, characterized the gene structure, expression profiles of CgsTLR7 and expression patterns in response to GSIV infection in the Chinese giant salamander.

The CgsTLR7 exhibited a typical TLR domain structure including a signal peptide, several LRRs, and a TIR domain in the cytoplasmic region.



Fig. 3. Quantitative PCR analyses of the distribution of the TLR7 gene in different tissues of the Chinese giant salamander. Expression of β -actin was used as an internal control for real-time PCR. Each experiment was performed in triplicate. Deviation bars represent the mean \pm SD (n = 3).The significant differences were indicated with asterisks, *p < 0.05; **p < 0.01. SK = skin; BR = brain; MU = muscle; LU = lung; HE = heart; IN = intestine; LI = liver; SP = spleen; KI = kidney.



Fig. 4. Relative expression of TLR7 at the mRNA level in spleen, kidney and liver of the Chinese giant salamander after 0, 6, 12, 24, 48, 96 h post-infection with GSIV. Deviation bars represent the mean \pm SD (n = 3). The significant differences were indicated with asterisks, *p < 0.05 and **p < 0.01.

The SMART analysis showed that the position of LRRs in CgsTLR7 is conserved among different species but the number of LRRs varied (Fig. 1). There are two transmembrane domains in CgsTLR7, which is the same as the frog (Ishii et al., 2007) and mouse. The CXRCXXXXPCXXC conserved sequence motif, which is essential for nucleic acid binding and pH dependent signal transduction (Lee et al., 2001; Gibbard et al., 2006), was detected between amino acid positions 259 and 272 of CgsTLR7. Three conserved TIR motifs in humans (Slack et al., 2000) were also found in the CgsTLR7 amino acid sequence (Fig. 1). The TIR domain plays important roles in both the intracellular localization and receptor signaling. The high structure conservation of CgsTLR7 suggests that it might possess similar functions compared to other species.

Phylogenetic analysis showed that CgsTLR7 is in the same cluster with avian and reptiles, but different from mammals and fish, which supports the traditional understanding of animal evolution. Additionally, the predicted peptide of CgsTLR7 shares highest identity (71%) with turtle, which is similar with phylogenetic analysis (Fig. 2).

Human TLR7 is expressed predominantly in plasmacytoid dendritic cells (pDCs), B cells and monocytes (Sioud, 2006). CgsTLR7 transcripts were expressed in all the selected tissues in the Chinese giant salamander. The highest expression levels were observed in the liver and kidney. The liver, as one of the important organs, contains abundant multiple subsets of dendritic cell, including myeloid dendritic cells (mDCs) and pDCs (Crispe, 2009), which could express higher level of TLR7 compared to other tissues. Comparison analyses indicated that the expression pattern of the CgsTLR7 was similar to the frog (Ishii et al., 2007). However, the tissue expression pattern of the CgsTLR7 was different from those observed in other vertebrates. In humans, the predominant expression of TLR7 was in the lung, placenta and spleen (Chuang and Ulevitch, 2000). The expression of fugu TLR7 was restricted to the kidney, heart, and gills, but it showed the strongest expression in the kidney and no expression in the spleen (Oshiumi et al., 2003). In common carp, TLR7 was expressed in a variety of tissues, but the expression was more pronounced in the spleen (Tanekhy et al., 2010). The difference expression profile of TLR7 may be due to different species, immunological status, development stage, or genetic background (Renshaw et al., 2002).

In mammals, TLR7 was shown to be activated by synthetic anti-viral imidazoquinoline compounds that were involved in recognizing singlestranded RNA (ssRNA), resulting in the induction of signaling pathways that activate production of various inflammatory cytokines (Qian et al., 2013). In the present study, the expression of CgsTLR7 in the kidney, spleen and liver was up-regulated after injection of GSIV. In the phases after GSIV infection, the temporal expression of CgsTLR7 is highest in kidney, liver and spleen, successively. In kidney and liver, the expression of CgsTLR7 didn't return to control level at 72 h, but continued at a high expression level. In spleen, the CgsTLR7 expression was highest at 48 h and suppressed at 72 h. The different expression profiles in kidney, liver and spleen are also similar to grass carp TLR7 gene after grass carp reovirus (GCRV) infection (Yang et al., 2012). These results suggest that CgsTLR7 is involved in the immune defense against GSIV in vivo.

5. Conclusion

In summary, we cloned and fully characterized the TLR7 gene from Chinese giant salamander. CgsTLR7 were expressed in all the selected tissues with the highest level in liver. The expression of CgsTL7 in liver, kidney and spleen was up-regulated significantly in response to the GSIV infection, suggesting that CgsTLR7 plays an important role in the innate immune response against a viral infection. However, further experiments are needed to explore and enhance our understanding of its functions.

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