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Co-expression of *march5b* and *tlr7* in large yellow croaker *Larimichthys crocea* in response to *Cryptocaryon irritans* infection

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In this study, molecular characteristics of *march5b* and co-expression of *march5b* and *tlr7* in response to the infection of *Cryptocaryon irritans* in the large yellow croaker *Larimichthys crocea* were investigated. The full-length complementary (c)DNA of *march5b* was 1314 bp, including an open reading frame of 846 bp encoding a polypeptide of 281 amino acids, and the full-length genomic sequence was composed of 23 577 nucleotides, including six exons and five introns. The putative March5b protein contained a RINGv motif and four transmembrane domains. The *march5b* transcripts were broadly distributed in all detected tissues, with a strong expression in blood, brain and gills, and a weak expression in kidney by quantitative PCR analysis. The expression of *march5b* and *tlr7* in the skin, gills, spleen and head kidney changed in the same manner at most time points post-primary infection with *C. irritans*. Significant increase was observed in the skin with *march5b* at days 2 and 3 by 26·10 and 6·88 fold, respectively, and with *tlr7* at day 3 by 57·68 fold, when compared with the control. Their expressions, however, were decreased in the gills, especially at day 3 (*march5b* by 8·9%, *tlr7* by 22·06%). In the spleen and head kidney, *march5b* and *tlr7* transcripts were up-regulated early, then noticeably declined at day 3. These results suggested that *march5b* and *tlr7* are co-expressed in response to parasite infection and March5b probably catalyses ubiquitination of some proteins of TLR7 signalling pathway.

Key words: E3 ubiquitin ligase; marine fish; parasite; toll-like receptor; ubiquitination.

INTRODUCTION

The membrane-associated Ring-ch (March) family is a Ring finger protein family of E3 ubiquitin ligases, consisting of 11 members in mammals. Yet, the precise physiological functions of the March proteins are still unknown. Many studies show that March members are associated with immune defences. It has been reported that March1 and March8 down-regulate cell-surface immune regulatory molecules, including major histocompatibility complex II (MhcII) and cluster of differentiation (Cd86) (Ohmura-Hoshino *et al.*, 2006; Thibodeau *et al.*, 2008); March4 and

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March9 are involved in MhcI ubiquitination, leading to MhcI removal from the cell surface and degradation in lysosomes (Bartee *et al.*, 2004; Wang *et al.*, 2008). Among others, mammalian March5 positively regulates Tlr7 signalling pathway by catalysing the K63-linked poly-ubiquitination of tumor necrosis factor (TNF) receptor-associated factor (TRAF) family member-associated NF-kB activator (Tank) and impairing the inhibition of Tank to Traf6 (Shi *et al.*, 2011). Whether March5 in fishes regulates Tlr7 or not remains unknown. Duplicated *march5* (*march5a* and *march5b*) have been identified in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792), and *march5a* is involved in immune defences (Rebl *et al.*, 2011). Therefore, it is necessary to perform more comprehensive analysis of *march5* in fishes to enhance the understanding of its molecular characteristics and roles in immune defences.

Toll-like receptors (TLR) are a major class of pattern recognition receptors (PRR). Until now, at least 13 TLRs have been identified from mammals and 17 TLRs from fishes (Rebl *et al.*, 2009). Of the TLRs, TLR7 is located in the endosomal compartments of some immune and non-immune cells, and can recognize ssRNA and parasite RNA (Heil *et al.*, 2004; Blasius & Beutler, 2010; Yarovinsky, 2014). Thus, it is possibly an important immune gene against parasites. In order to explore its roles in immune defences, the expression analysis of *tlr7* has been performed in Atlantic salmon *Salmo salar* L. 1758, grass carp *Ctenopharyngodon idella* (Valenciennes 1844), common carp *Cyprinus carpio* L. 1758, catfish *Ictalurus punctatus* (Rafinesque 1818) and zebrafish *Danio rerio* (Hamilton 1822) in response to bacterial, virus, vaccine and cytokine stimulation (Meijer *et al.*, 2004; Tanekhy *et al.*, 2010; Yang *et al.*, 2012; Lee *et al.*, 2013; Zhang *et al.*, 2013). Information of fish *tlr7* in response to parasites infection, however, remains unknown.

Large yellow croaker *Larimichthys crocea* (Richardson 1846) is a commercially important fish species, cultured mainly in Fujian and Zhejiang Provinces of China. It has suffered from serious diseases, especially infection by parasite *Cryptocaryon irritans*. *Cryptocaryon irritans* is a common obligate ciliate protozoan which infects marine fishes. So in this study, what and how *march5b* and *tlr7* in *L. crocea* respond to infection with *C. irritans* were investigated. Finally, their possible involvement in ubiquitination was analysed.

MATERIALS AND METHODS

FISH, CHALLENGE AND SAMPLING

Healthy *L. crocea* (average mass: 130 g) were obtained from a mariculture farm in Ningde, Fujian, China. The fish were acclimatized 10 days at salinity 25–26 and temperature 23–26° C, and fed with a commercial feed. Samples of blood, gill, skin, muscle, liver, heart, spleen, stomach, intestine, kidney, head kidney and brain tissues were collected from five fish, and preserved in liquid nitrogen for RNA extraction.

The *L. crocea* was infected with *C. irritans* according to a previously described procedure (Niu *et al.*, 2013; Zhang *et al.*, 2015). Fish immune stimulation was performed by infection with 25 000 *C. irritans* theronts per fish for 6 h. Subsequently, the fish were transferred into a new cement pool containing fresh seawater. To avoid the auto-reinfection, the fish were transferred into a new pool at day 3 post-infection. At 6, 12 h, 1, 2, 3 and 5 day post-primary infection, the skin, gills, spleen and head kidney samples were collected. The control group was uninfected with *C. irritans*, and the procedures of treatment and sample collection

TABLE I. Primers used in this study of *Larimichthys crocea*

Primers	Sequence(5′-3′)	Purpose
*march5*QF	CGACCCACTGTTCCTGCTCA	mRNA expression
*march5*QR	TGCTGGGATACGAGGAACTGG	
*tlr7*QF	GCTTCCTCGCCCTGTCCAT	mRNA expression
*tlr7*QR	GCACATTTCCTTCATCACCCACT	
β-actin-F	TTATGAAGGCTATGCCCTGCC	mRNA expression
β-actin-R	TGAAGGAGTAGCCACGCTCTGT	

were the same as for the test group. Five fish were sampled from each group at each time point.

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was extracted with Trizol reagent (Invitrogen; www.lifetechnologies.com/no/en/home/brands/invitrogen.html) following the instructions of the manufacturer. The RNA was then incubated with RNase-free DNaseI (Promega; www.promega.com.cn) at 37° C for 30 min to remove any genomic DNA. First strand complementary (c)DNA was synthesized from an aliquot (1 μg) of the total RNA with PrimeScript Reverse Transcriptase (TakaRa; www.clontech.com/takara). The cDNA was used as the template for gene expression analysis.

IDENTIFICATION OF THE cDNA AND DNA SEQUENCE OF *MARCH5B*

The cDNA of *march5b* sequence was obtained through previous transcriptome sequences of various tissues from *L. crocea* in the laboratory. Basic local-alignment search tool (BLAST; Kent, 2002) was used to map cDNA sequence of *march5b* to the genomic sequence of *L. crocea* (unpubl. data), and the length of intron was set to be not more than 750 000 bp. Then, the genomic structure and potential splicing sites of *march5* were visualized according to the start and end mapping positions of cDNA sequence fragments on the genomic sequence, as well as according to the value of sequence identity. If there was more than one BLAST result in cDNA mapping, the results based on the E-values were sorted and the lowest one for genomic sequence was selected. Alternative splicing site analysis was performed as described above. March5b protein structure analysis was performed with the simple modular-architecture research tool (SMART) programme (http://smart.embl-heidelberg.de/). The multiple sequence alignment was conducted with ClustalX 1.83 (www.ebi.ac.uk/Tools/msa/clustalw2).

QUANTITATIVE REAL-TIME PCR

To detect the expressions of *march5b* and *tlr7*, primers *march5*QF/R, *tlr7*QF/R and *β-actin*F/R were designed with the Primer 5.0 software, and *β-actin* was used as internal control (Table I). Real-time PCR was performed on an ABI 7500 Real-time Detection System (Applied BioSystems; www.lifetechnologies.com/no/en/home/brands/applied-biosystems.html) using SYBR Green I Real-time PCR Master Mix (TOYOBO; www.bio-toyobo.cn/index.php). The PCR conditions were as follows: 95° C for 1 min, followed by 40 cycles at 95° C for 10 s, 60° C for 20 s and 72° C for 32 s. The specificity of the PCR products was confirmed by melting curve analysis and sequencing. Each sample was amplified in triplicate. The relative messenger (m)RNA expression levels of *march5b* and *tlr7* compared with the reference gene were calculated with $2^{-\Delta\Delta CT}$ method. SPSS 16.0 (www-01.ibm.com) was used for the significance

test between the test group and the control group. Data were expressed as mean ± S.E. $P < 0{\cdot}05$ was considered statistically significantly different.

RESULTS

cDNA AND GENOMIC SEQUENCE ANALYSIS OF *MARCH5B*

The full-length cDNA sequence of *march5b* (KM577603) was 1314 bp, including an open reading frame (ORF) of 846 bp encoding a polypeptide of 281 amino acid residues, a 5′ untranslated region (UTR) of 378 bp and 3′ UTR of 90 bp. Prediction of protein domains by the SMART programme revealed that March5b consisted of a RingV motif at position 16–72 amino acids, and four transmembrane (TM) domains at positions 100–122 , 142–164, 212–231 and 241–259 amino acids.

As expected, the amino-acid residues were well conserved among March5 proteins in vertebrates as illustrated by the amino acid sequence alignment. March5b amino acid sequences from *L. crocea* contained seven cysteine residues, as well as one histidine residue at position 46 corresponding to the consensus pattern C3HC3. Moreover, species-specific deletions or insertions were present in March5b proteins, *e*.g. a missing chain of six amino acid residues after position 188 within *L. crocea* March5b protein.

The full-length of the genomic sequence of *march5b* gene (exons and introns) was 23 577 bp (KM669657). Comparison of the cDNA and genomic DNA sequences revealed that *march5b* gene included six exons and five introns, and no alternative splicing sites were found. The first exon was in the 5′ UTR, the second exon encoded the RingV domain, and the other four exons consisted of the TM domains. The genomic structure of *march5b* was similar to those of *D. rerio* and humans *Homo sapien*s. The lengths of the exons 2, 3, and 5 were entirely conserved.

TISSUE EXPRESSION PROFILES OF *MARCH5B*

Quantitative reverse transcription (qRT)-PCR showed that *march5b* mRNA was broadly expressed in 12 different tissues with various expression levels, as shown in Fig. 1. The highest *march5b* mRNA concentration was present in blood, followed by brain and gills, and the lowest mRNA copy number was detected in kidney. Moderate expression of *march5b* was detected in the spleen and liver.

EXPRESSION MODULATION OF *MARCH5B* AND *TLR7* IN RESPONSE TO *C. IRRITANS* INFECTION

To explore responses of host to parasites infection, *march5b* and *tlr7* mRNA expression levels were examined in local infection organs (skin and gills) and systemic immune organs (spleen and head kidney). In the skin, *march5b* and *tlr7* were both up-regulated from 6 h to 3 days, but down-regulated at day 5 post-primary infection as compared with the control [Fig. 2(a), (b)]. As shown in Fig. 2(a), *march5b* was significantly up-regulated at days 2 and 3 (26·10 and 6·88 fold, respectively), and *tlr7* at day 3 (57·68 fold) post-primary infection [Fig. 2(b)]. In the gills, *march5b* expression fluctuated slightly post-infection, and significant down-regulation was observed at 12 h and 3 day (66·51 and 8·90% of the control, respectively, $P < 0{\cdot}05$) post-primary infection [Fig. 2(c)]. Expression level of *tlr7* significantly decreased to

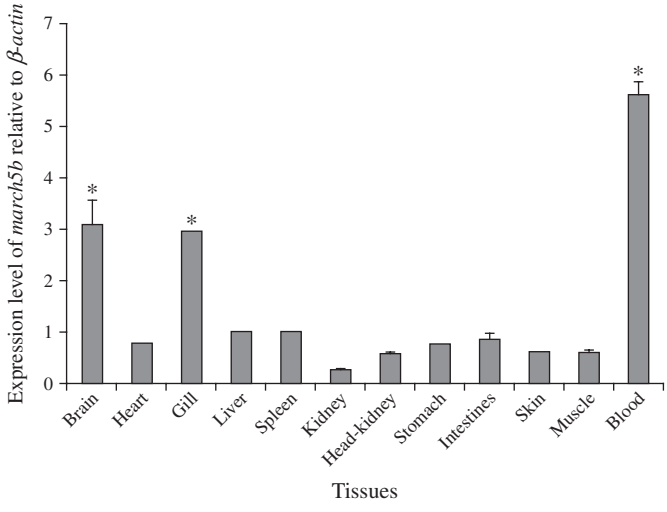

FIG. 1. Expression profiles of *march5b* in various tissues of healthy *Larimichthys crocea* by real-time PCR. The mRNA expression level was normalized against *β-actin*. Each experiment was performed in triplicate. Data (mean ± S.E. $n = 5$) are indicated when significantly different (*, $P < 0{\cdot}05$).

47·04, 19·19 and 22·06% as much as those of the control group at 12 h, 1 day and 3 days after *C. irritans* infection [Fig. 2(d)]. In the spleen, *march5b* transcripts were up-regulated from 6 h to 2 days except the slight down-regulation at 1 day post-primary infection. Then, *march5b* transcripts sharply declined at 3 day (21·28% of the control, $P < 0{\cdot}05$) and 5 day [Fig. 2(e)] post-primary infection. Expression level of *tlr7* early increased at 6 h (3·86 fold, $P < 0{\cdot}05$) and 12 h, then gradually decreased from 1 day to 3 days (32·22% of the control, $P < 0{\cdot}05$). Eventually, *tlr7* expression slightly increased at 5 days as compared with the control [Fig. 2(f)]. In the head kidney, *march5b* transcripts were significantly up-regulated at 6 h (4·63-fold) and 2 days (2·05 fold) and down-regulated at 3 days (16·37% of the control, $P < 0{\cdot}05$) [Fig. 2(g)]. Expression level of *tlr7* significantly increased at 6 h, 1 day, 2 days and 5 days (5·74, 1·42, 1·78 and 1·79 fold, respectively) and decreased at 12 h and 3 days (31·84 and 22·29% of the control, $P < 0{\cdot}05$, respectively) post-primary infection [Fig. 2(h)]. In conclusion, *march5b* expression levels increased at early stage and then decreased from days 3 to 5 post-primary infection, and *tlr7* expression level also significantly increased early and then decreased at day 3, but increased at day 5 in the spleen and head kidney.

ASSOCIATION OF *MARCH5B* AND *TLR7* EXPRESSION POST-INFECTION

To analyse the expression change relationship between *march5b* and *tlr7* in response to *C. irritans* infection, the expression change folds over the control was compared at the same time point, especially at day 3, the most seriously infected period (Fig. 3). Expression level of *march5b* followed the change trend as that of *tlr7* in gills, spleen and head kidney [Fig. 3(b)–(d)] as compared with the control group. Moreover, significant down-regulation was observed at day 3. In the skin, both *march5b* and *tlr7* expressions were up-regulated post *C. irritans* infection at most time points, with the exception of

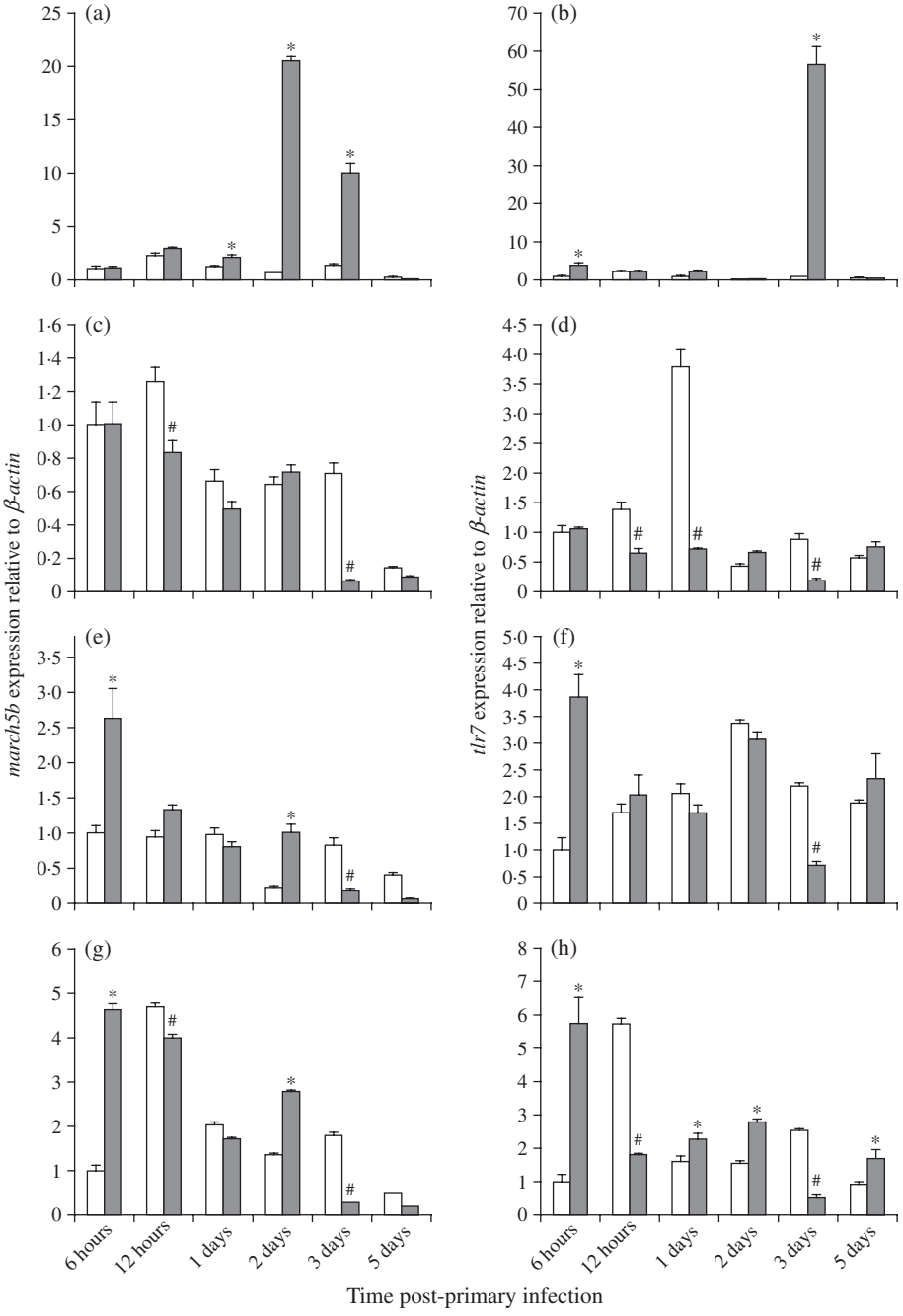

FIG. 2. Expression modulation of (a, c, e, g) *march5b* and (b, d, f, h) *tlr7* in *Larimichthys crocea* (a, b) skin, (c, d) gills, (e, f) spleen and (g, h) head kidney infected by *Cryptocaryon irritans* with real-time PCR at various time points post-challenge. The mRNA expression level was normalized against *β-actin*. Data are shown as the mean ± S.E. ($n = 5$). Each experiment was performed in triplicate. Significant differences of expression between control group (□) and *C. irritans* infection group (■) at each time point are indicated (*, significant increase, $P < 0{\cdot}05$; #, significant decrease, $P < 0{\cdot}05$).

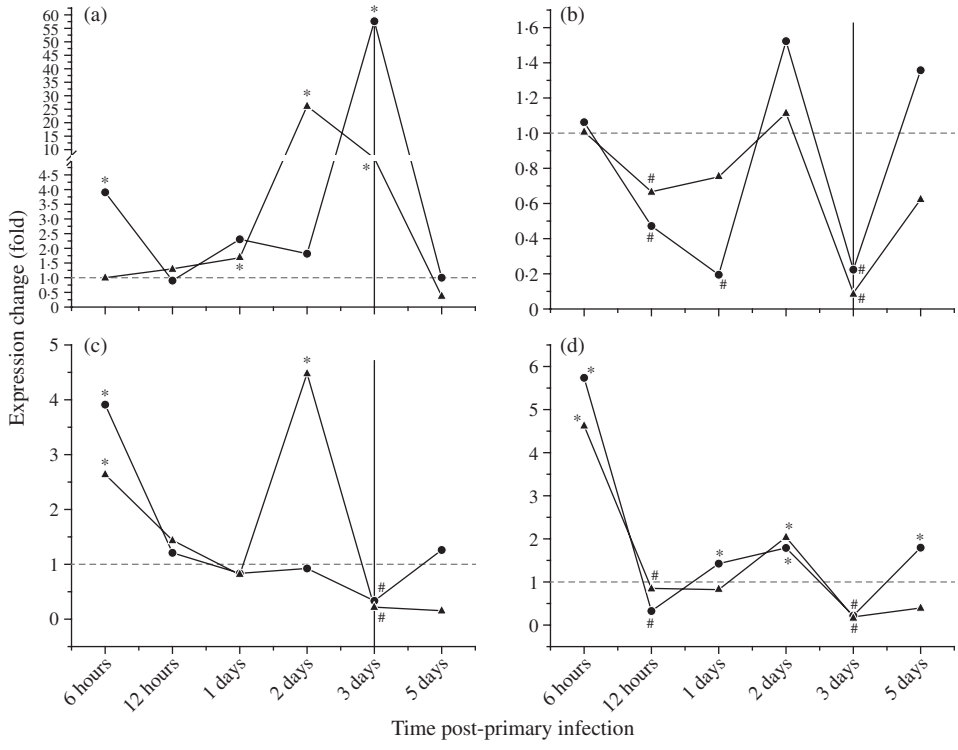

FIG. 3. Expression change (fold relative to base line, - - - -) of *march5b* (–▲–) and *tlr7* (–●–) in the (a) skin, (b) gills, (c) spleen and (d) head kidney of *Larimichthys crocea* infected with *Cryptocaryon irritans*. The mRNA expression level was first normalized by *β-actin*, and then the fold was calculated of the test group's gene expression to its control at the same time point to indicate the fold change (*, significant increase, $P < 0{\cdot}05$; #, significant decrease, $P < 0{\cdot}05$).

day 5 when a slight down-regulation was observed in *march5b* expression [Figs 2(a), (b) and 3(a)], whilst *march5b* and *tlr7* expressions were noticeably up-regulated in 3 day infected fish.

DISCUSSION

The proteins of March family generally have two (March1, 2, 3, 4, 8, 9, 11), four (March5) or 12 (March6) TM domains, but March7 and March10 have no TM domains predicted. In this study, March5b contained four TM domains and a Ring domain that is essential for ubiquitin transfer activity (Karbowski *et al.*, 2007). The gene structure and protein sequence of March5b were similar to those of other fishes and humans. Yet, fishes possessed a fish-specific variant March5a which was less identical to March5b and was similar to that found in *L. crocea* (unpubl. data). These observations showed that March5b in fishes should have similar function of that in humans as they have similar structure, although the full function of March5 was yet unknown in mammals.

The *march5b* transcripts were broadly expressed in all detected tissues from *L. crocea* under normal conditions. The tissue expression profile of *march5b* was quite different

from that of *O. mykiss* in which *march5b* was predominant in heart, head kidney, gills and spleen (Rebl *et al.*, 2011), while *march5b* was highly expressed in blood, brain and gills in this study (Fig. 1). These varied expression profiles were perhaps due to species difference, immunological status, developmental stage and genetic background divergence.

Expression changes of mitochondrial genes after pathogen challenges were reported in many studies (Zhong *et al.*, 2008; Scott, 2009; Onoguchi *et al.*, 2010). In this study, mitochondrial *march5b* mRNA copy number changes have been detected in the local infection skin and gills as well as in immunocompetent organs spleen and head kidney from *L. crocea* post *C. irritans* infection (Fig. 2). The transcripts of *march5b*, however, were generally up-regulated in the skin but down-regulated in the gills, although both are the first defence lines of antipathogen. After examining the clinical signs of the *L. crocea* exposure to *C. irritans*, many *C. irritans* were found in the skin but little in the gills. This was perhaps the reason that caused the different expression levels. The similar situation was found in orange-spotted grouper *Epinephelus coioides* (Hamilton 1822), in which MyD88 was up-regulated in the skin and spleen but down-regulated in the gills and head kidney at most time points after *C. irritans* infection (Li *et al.*, 2011). In the spleen and head kidney from *L. crocea*, *march5b* expression increased from 6 h to 2 days, but noticeably decreased at day 3 (21·28% in the spleen and 16·37% in the head kidney), and the expression was also emerged in the gills at day 3 (8·90%) as compared with the control, suggesting that the pathogen would have its own way to fight against the host immune system. For instance, the viral microRNA can target host immune system by knocking down the immune gene expression (Stern-Ginossar *et al.*, 2007); the expression of *mhcIIb* gene in *E. coioides* was sharply down-regulated in the head kidney, spleen and gills from day 1 to day 3 post *C. irritans* infection (Lu *et al.*, 2012).

Recent studies have shown that Tlr7 is a critical innate immune receptor involved in recognition of *Trypanosoma cruzi* (Chagas 1909) RNA (Caetano *et al.*, 2011), which has glycosylphosphatidylinositol (GPI) anchors that are recognized by Tlr2/4 (Campos *et al.*, 2001; Oliveira *et al.*, 2004). *Cryptocaryon irritans* also contains these molecules (Hatanaka *et al.*, 2007, 2008), so it should be recognized by host TLR genes. In this study, *tlr7* was up-regulated in the skin (57·68-fold to the control at 3 days), but significantly down-regulated in the gills (47·04, 19·19 and 22·06% of the control at 12 h, 1 day and 3 days), similar to *march5b*. In the spleen and head kidney, *tlr7* expression level increased early (3·86 and 5·74 fold at 6 h, respectively), then gradually decreased (especially at day 3, 32·22 and 22·29% of the control, respectively), eventually increased at 5 days. According to the present results, it appeared that *tlr7* was involved in the host response to *C. irritans*, but the compounds of *C. irritans* that were sensed by *L. crocea* TLRs remain unknown. In addition, Qian *et al.* (2013) reported that the expression levels of *tlr7* in *L. crocea* were up-regulated in spleen and head kidney during 72 h of poly (I:C) induction, yet down-regulated after inactivated bacterial vaccine stimulation. The responses of *tlr7* to *C. irritans*, poly (I:C) and bacterial vaccine were different, suggesting that the host has different ways to fight against various pathogens.

Ubiquitination plays central roles in regulating protein stability and activity. Proteasome may up-regulate or down-regulate expression of protein by degrading different loci of protein molecules, and consequently adjusting the immune function of organism (Lehner *et al.*, 2005; Ishido *et al.*, 2009). Shi *et al.* (2011) previously reported that human MARCH5 catalyses the K63-linked poly-ubiquitination of TANK, and

this modification releases the inhibitory effects of TANK on TRAF6. Consequently, ectopic expression or knockdown of March5 enhances or impairs nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-mediated pro-inflammatory gene expression in response to TLR7 activation. In this investigation, *march5b* and *tlr7* displayed the same trends of expression changes in all the detected tissues at most time points. Significant increase was observed at 3 days in the skin, while decreased at 3 days in the gills, spleen and head kidney (Fig. 3). These data suggested that March5b catalyses ubiquitination of some proteins of Tlr7 signalling pathway in fishes. Yet, the mechanisms need to be further studied.

In summary, the full-length cDNA and genomic sequences of *march5b* were cloned *in silico* in *L. crocea*. The *march5b* transcripts were broadly expressed in most detected tissues with the highest level in the blood cells and the lowest in the kidney. Moreover, the expression of *march5b* and *tlr7* in the skin, gills, spleen and head kidney changed significantly in response to *C. irritans* infection, indicating that both *march5b* and *tlr7* play important roles in fishes in response to parasite infection. In addition, *march5b* and *tlr7* expression changes followed similar trend at most time points after *C. irritans* infection, suggesting that March5b was probably involved in ubiquitination of some proteins of Tlr7 signalling pathway in teleosts.

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