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Expression profiles and interaction suggest TBK1 can be regulated by Nrdp1 in response to immune stimulation in large yellow croaker *Larimichthys crocea*

Dong Ling Zhang [a], Da Hui Yu [b], Jian Chen [a], Sigang Fan [b], Zhi Yong Wang [a, *]

[a] Key Laboratory of Healthy Mariculture for The East China Sea, Ministry of Agriculture, Fisheries College, Jimei University, Xiamen 361021, PR China
[b] South China Sea Resource Exploitation and Protection Collaborative Innovation Center (SCS-REPIC), South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, 510300, PR China

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ABSTRACT

TBK1 has been extensively studied in mammals because of its important roles as a molecular bridge, linking the TLRs (TLR3 and TLR4) and RLRs signals to activate transcriptional factors IRF3 and IRF7 for IFN-I production. However, the information on molecular and functional characteristics of TBK1 in teleosts is limited. In this study, the molecular characterization and immune response of TBK1 in *Larimichthys crocea* (named as LcTBK1) as well as its interaction with Nrdp1 were investigated. Sequence analysis demonstrated that LcTBK1 included four functional motifs, the N-terminal protein kinase domain and ATP-binding site, middle ULD and C-terminal coiled-coil domain. The tissue expression profiles indicated that LcTBK1 gene was constitutively expressed in the twelve tissues examined, with high expression in brain. Temporal expression analysis showed that LcTBK1 mRNA was obviously increased in the liver after injection of LPS, Poly I:C and inactive *Vibrio parahaemolyticus*, however, declined at some time points in spleen and head-kidney. Furthermore, we found that LcTBK1 can interact with LcNrdp1, an E3 ubiquitin ligase that involved in immune response to *Cryptocaryon irritans* infection in *L. crocea*. The qPCR showed that LcNrdp1 was also significantly up-regulated in liver, down-regualted at some time points in spleen and head-kidney after LPS, Poly I:C and inactive *V. parahaemolyticus* injection, although the expression patterns of the two genes after the three treatments were different in change magnitude and up-regulation timespan. These results suggested that LcTBK1 was involved in *L. crocea* defense against the pathogen infection and can be regulated by Nrdp1 in PPRs signaling pathway of fishes.

1. Introduction

Large yellow croaker *Larimichthys crocea*, as a marine fish, is mainly distributed along coasts of eastern and southern China. It is one of the most economically important fishes [1]. However, the fish is susceptible to infection of bacteria (*vibrio* etc.), viruses (*iridovirus* etc.) and parasites (*Cryptocaryon irritans* etc.) [2–5]. Yet understanding of its innate immune responses to pathogens is a prerequisite for prevention of diseases.

TANK-binding kinase 1 (TBK1) is an important signaling molecule of innate immune systems. It is involved in both Toll-like receptors (TLRs) and RIG-I like receptors (RLRs) signaling pathways, regulating interferon (IFN)-inducible genes during the immune response to bacterial and viral infections [6–8]. TBK1-deficient cells fail to produce IFN-I in response to viral infection [9,10]. Some viruses have developed defensive mechanisms by antagonizing TBK1 function, *e.g.*, γ134.5 protein of herpes simplex virus type 1 (HSV-1) associates with TBK1 and inhibits TBK1-mediated phosphorylation of IRF3, replicating more efficiently in host cells [11]. So far TBK1 was identified only in several fishes, including zebrafish *Danio rerio* [12], common carp *Cyprinus carpio* [13], Atlantic cod *Gadus morhua* [14], crucian carp *Carassius auratus* [15], and grass carp *Ctenopharyngodon idella* [16]. The information on molecular and functional characteristics of teleosts is limited, and upstream of signaling pathway of TBK1 is still poorly understood either, compared to the investigation in mammals. Thus, a more

* Corresponding author. Fisheries College, Jimei University, Xiamen, Fujian Province, 361021, PR China.
E-mail address: zywang@jmu.edu.cn (Z.Y. Wang).

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comprehensive analysis of TBK1 in fish is necessary to enhance our understanding of its molecular characteristics and roles in immune response.

Neuregulin receptor degradation protein-1 (Nrdp1), as an E3 ubiquitin ligase, is highly conserved in eukaryotes from yeasts to humans [17]. It can inhibit TLR-induced production of pro-inflammatory cytokines, and promote the production of type I interferon in macrophages by LPS stimulation [18]. Nrdp1 transgenic (Nrdp1-TG) mice showed resistance to LPS-induced liver injury and vesicular stomatitis virus [18]. Furthermore, Nrdp1 interacted with transcriptional factor C/EBPβ and enhanced C/EBPβ-triggered transcriptional activation of the Arg1 reporter gene in M2 Macrophage [19]. In addition, Nrdpl regulated the function of CD8+ T cells, and thus maybe involved in the pathogenesis of autoimmune diseases mediated by T cells [20]. In our previous report, Nrdp1 was involved in immune response to *Cryptocaryon irritans* in *L. crocea*. It was reported that Nrdp1 can regulate TLR signaling pathway by directly interacting with TBK1 in mouse macrophages in response to LPS stimulation [19]. So whether it can regulate TBK1 in fishes or not in response to immune stimulations is well worth studying.

In this study, a TBK1 gene in *L. crocea* was firstly identified and characterized, then the expression profiles of TBK1 in response to LPS, Poly I:C and inactive *Vibro parahaemolyticus* were investigated, and finally the interaction between TBK1 and Nrdp1 was verified as well. Based on the interaction, the expression of Nrdp1 was also investigated response to immune stimulation, aiming to understand the immune roles of TBK1 in fish innate immune system as well as its association with Nrdp1.

2. Material and methods

2.1. Fish collection and immune stimulation

Healthy *L. crocea* (average mass: 130 g) were supplied by the Fishery Extension Station of Ningde (Fujian, China). The fish were acclimatized two weeks at salinity 25–26 and temperature 23–26 °C, and fed with a commercial feed. Various tissues, including brain, heart, gill, liver, spleen, kidney, head-kidney, stomach, intestine, skin, muscle and blood cells were collected from five fish for tissue expression profile detection, and immediately preserved in liquid nitrogen for RNA extraction.

For challenging experiments, the fish were intraperitoneally injected with 250 μl formalin-inactivated Gram-negative bacteria suspensions of inactive *V. parahaemolyticus* (10^8 cfu ml^{-1}) (isolated from diseased fish), 250 μl Poly I:C (27472901, GE, 1 mg ml^{-1}) and 250 μl LPS (L2880, Sigma, 1 mg ml^{-1}), respectively [21]. These fish were as test groups, and the fish injected with 250 μl PBS was used as control. Then, liver, spleen and head-kidney of each group were collected at 3 h, 6 h, 12 h, 24 h, 48 h and 72 h after injection. Five fish were sampled from each group at each time point.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated with Trizol reagent (Invitrogen, USA) following the instructions of manufacturer. The RNA was then incubated with RNase-free DNaseI (Promega, USA) at 37 °C for 30 min to remove any contaminating DNA. cDNA was synthesized from 1 μg of the total RNA with PrimeScript Reverse Transcriptase (TaKaRa, Japan) using an oligo (dT) primer, according to the manufacturer's instructions.

2.3. Identification of LcTBK1 cDNA sequence

The full length cDNA sequence of TBK1 was obtained from previous transcriptomic sequences of various tissues from *L. crocea* in our laboratory. Two specific primers TBK1F/R (Table 1) for RT-RCR were designed based on the sequence of TBK1. PCR was carried out using a common PCR program with annealing temperatures 58 °C. The amplified products were purified from 1.2% agarose gel using Purification Kit (TIAGEN, China), cloned into pMD18-T vector (Takara, Japan) and transformed into DH5a cells and sequenced (Invitrogen, USA).

2.4. Amino acid sequence analysis, multiple sequence alignment and phylogenetic tree analysis

Database sequence homology search of the predicted TBK1 amino acid sequence was carried out using BLASTP program. The deduced amino acid sequences were analyzed with EXPASY (http://www.expasy.org/) and the protein domain features were predicted by SMART (http://smart.embl-heidelberg.de/). A multiple sequence alignments were performed using ClustalX 1.83 (http://www.ebi.ac.uk/clustalw/) and a phylogenic tree of TBK1 was reconstructed by MEGA 5.0 program (http://www.megasoftware.net/mega5/mega.html).

2.5. Real-time PCR analysis of LcTBK1 and LcNrdp1 mRNA expression

Tissue expression profile of LcTBK1 in brain, heart, gill, liver, spleen, kidney, head-kidney, stomach, intestine, skin, muscle and blood cell, as well as temporal expression of LcTBK1 and LcNrdp1 in the spleen, head-kidney and liver stimulated with LPS, Poly I:C, inactive *V. parahaemolyticus* (as test group) and PBS (as control group) were detected, respectively, by Quantitative Real-time PCR (qRCR) on ABI 7500 Real-time Detection System (Applied Bio-Systems, USA) using primers TBK1qF/R and Nrdp1qF/R (Table 1). The house-keeping gene β-actin was used as an internal control to verify cDNA template for the corresponding samples [22].

The qPCR was performed with the TaKaRa SYBR PrimeScript™ RT-PCR Kit following the protocol of manufacturer. Each reaction contained the following: 5 μl SYBR Premix Ex Taq, 0.5 μl of each primer, 1 μl diluted cDNA and water to a final volume of 10 μl. Cycling conditions were 2 min at 95 °C, then 40 cycles of 95 °C for 15 s, 60 °C for 20 s. Each experiment was performed in triplicate. Relative gene expression level was analyzed by $2^{-\Delta\Delta Ct}$ method [23]. SPSS software (version 16.0) was used for the significance test between the test group and the control group. Data was expressed as Mean ± SE. $P < 0.05$ was considered statistically significantly different.

Table 1
Primers used for TBK1 cDNA identification, Nrdp1 and TBK1 expression analysis and yeast two hybrid.

Primer	Sequence (5′-3′)	Purpose
TBK1F	ATGCAGAGCACCACCAACTAC	cDNA identification
TBK1R	TCAGCCTCTCAGACCGCC	
Nrdp1qF	CAGGCGGTGGTGGTGATG	mRNA expression
Nrdp1qR	AACCTACTCATCACAGCAAACCC	
TBK1qF	ACACAACAAGTGGGGACGCA	
TBK1qF	GTGAACAGAGACCGAGCCTTG	
β-actin-F	TTATGAAGGCTATGCCCTGCC	
β-actin-R	TGAAGGAGTAGCCACGCTCTGT	
Nrdp1YF	CGGGATCCCGATGGGGTACGACGTTACGAGG	Yeast two hybrid
Nrdp1YR	CGGAATTCCGTTATAAGATCTCCTCCACTCCGT	
TBK1YF	CGGGATCCCGATGCAGAGCACCACCAACTAC	
TBK1YR	CGGAATTCCGTCAGCCTCTCAGACCGCC	
MyD88YF	CGGGATCCCGATGGCGTGTTGCGATAAATC	
MyD88YR	CGGAATTCCGTTATGGCTGTGAGAGAGCTTTG	

2.6. Yeast two-hybrid

Yeast two-hybrid assay was performed to detect the possible interactions between LcTBK1 and LcNrdp1 proteins using Clontech Matchmaker Gold Yeast Two-Hybrid System (Takara, Japan). Briefly, two sets of gene-specific primers TBK1YF/R and Nrdp1YF/R with BamH I and EcoR I restriction sites (Table 1) were designed to amplify the open reading frame (ORF) of the two genes and cloned to pGAKT7 and pGBKT7, respectively. The recombinant plasmid was confirmed by DNA sequencing. The pGAKT7 plasmids were transformed into the yeast strain Y187 and the recombinants were selected with the yeasts growing on plates with synthetically defined medium (SD) that lacks leucine (SD/-Leu). Meanwhile, the pGBKT7 plasmids were transformed into the yeast strain Y2H-GOLD and the recombinants were selected on SD plates lacking tryptophan (SD/-Trp). For self-activation detection, the pGADT7 and pGBDT7 plasmids were spread on the plates with double dropout medium (SD/-Trp/-Leu) and quadruple dropout medium supplemented X-a-Gal and Aureobasidin A (SD/-Ade/-His/-Trp/-Leu/X-a-Gal/AbA), respectively. Positive yeast stains of SD/-Trp and SD/-Leu were hybridized in two YPDA medium and selected on medium SD/-Leu/-Trp. The interactions between LcNrdp1 and LcTBK1 proteins were determined by the hybridized clones growing on quadruple dropout medium SD/-Ade/-His/-Trp/-Leu/X-a-Gal/AbA. The pGBKT7+p53/pGADT7+T and pGBKT7+Lam/pGADT7+T act as positive and negative control, respectively. Meanwhile, interaction between LcNrdp1 and LcMyD88 was tested as described above.

3. Result

3.1. Identification of LcTBK1 cDNA and analysis of putative protein structure

The full-length cDNA sequence of LcTBK1 (KR534322) was identified with 3305 nucleotides (nt), including an open reading frame (ORF) of 2172 nt encoding a polypeptide of 723 amino acids residues, a 5′ untranslated region (UTR) of 77 nt and a 3′ UTR of 1056 nt. Alignment of the predicted protein of TBK1 revealed the presence of a conserved protein kinase domain at position 9-306 aa, an ubiquitin-like domain (ULD) at 297-385 aa and a C terminal coiled-coil region at 678-704 aa (Fig. 1), but no signal peptide cleavage site. The ATP-binding site (LGQGATANV) at N terminal was found in all TBK1 kinases. The calculated molecular mass of deduced TBK1 polypeptide was 8.31 kDa and the isoelectric point was 6.44.

3.2. Phylogenetic analysis of LcTBK1

BlastP search indicated that the deduced amino acid sequence of LcTBK1 ORF was 82–99% identical to that of other fishes. LcTBK1 showed high identity of 98% protein sequence with that from *Oplegnathus fasciatus* (AHX37216), *Paralichthys olivaceus* (AGL54166), and relative low identity of 86% with *D. rerio* (NP_001038213) and 82% with *Astyanax mexicanus* (XP_007256088) and 73% identity with *Homo sapiens* (NP_037386). A Phylogenetic tree was developed based on multiple alignments of TBK1 from various species, including fishes, birds and amphibians (Fig. 2). LcTBK1 was in the same subgroup with other fishes and had the closest phylogenetic relationship with *O. fasciatus*.

3.3. Tissue expression profiles of LcTBK1

To determine LcTBK1 mRNA expression levels in various tissues from *L. crocea*, qPCR was performed. The results showed that LcTBK1 was ubiquitously expressed in the twelve detected tissues, but the expression levels were different among various tissues (Fig. 3). The highest expression level was in brain, followed by muscle, heart and gill ($P < 0.05$). The expression levels of LcTBK1 in the other tissues were not different significantly in quantity except for in brain.

3.4. Expression changes of LcTBK1 gene upon stimulation with LPS, Poly I:C and inactive V. parahaemolyticus

The LcTBK1 expressions in liver, spleen and head-kidney after immune stimulation were shown in Fig. 4. In liver, as shown in Fig. 4 A, LcTBK1 expression levels were significantly up-regulated in the whole time-course after LPS, Poly I:C and inactive *V. parahaemolyticus* stimulations except for 3 h post the injection of Poly I:C. The expression levels reached the peaks at 12 h after three immune stimulations with 2.78, 12.28 and 12.09-fold as much as the control, respectively.

In spleen, as shown in Fig. 4 B, LcTBK1 transcripts declined at the begin, then increased after LPS and Poly I:C stimulation except for 72 h post injection of Poly I:C. The expression level LcTBK1 obviously increased during 72 h of inactive *V. parahaemolyticus* induction, and reached the peak level at 48 h with 6.05-fold as compared with the control.

In head-kidney, as shown in Fig. 4C, LcTBK1 expression decreased to different extent after LPS and Poly I:C stimulation, and it recovered to starting level from 48 h to 72 h after LPS induction. However, LcTBK1 transcripts gradually increased after inactive *V. parahaemolyticus* induction, reaching the peak at 72 h post-induction, with a 6.12-fold increase. Collectively, the mRNA expression of LcTBK1 significantly increased in the three tissues after injection of inactive *V. parahaemolyticus*, however, declined at some time points in spleen and head-kidney after injection of LPS and Poly I:C, especially in head-kidney.

3.5. Yeast two-hybrid

The interaction between LcTBK1 and LcNrdp1 proteins was reflected by the colors of hybridized yeasts with expression plasmids of pGADT7 and pGBDT7 growing on SD/-Ade/-His/-Trp/-Leu/X-a-Gal/AbA plates (Fig. 5). For self-activation detection, pGADT7+LcTBK1 and pGBKT7+LcNrdp1 yeasts grow on the medium neither SD/-Leu/-Trp nor SD/-Ade/-His/-Trp/-Leu/X-a-Gal/AbA, suggesting that LcTBK1 and LcNrdp1 themselves have no transcriptional activity and cannot activate report gene. The yeasts pGBKT7+LcNrdp1/pGAKT7+ LcTBK1 growing on plates of SD/-Leu/-Trp were white, and yeasts growing on plates of SD/-Ade/-His/-Trp/-Leu/X-a-Gal/AbA were blue (Fig. 5). Meanwhile, the yeasts of positive controls were blue and the negative controls could not grow on the medium, respectively. The data demonstrated that LcTBK1 directly interacted with LcNrdp1. Interestingly, we also found that there was interaction between LcNrdp1 and LcMyD88.

3.6. Expression changes of LcNrdp1 gene upon stimulation with LPS, Poly I:C and inactive V. parahaemolyticus

Based on the interaction of LcTBK1 with LcNrdp1, the potential role of LcNrdp1 upon immune response in fish was investigated. A relative quantitative real-time PCR was employed to detect the transcriptional levels in liver, spleen and head-kidney upon stimulation with LPS, Poly I:C and inactive *V. parahaemolyticus*. As shown in Fig. 6, mRNA levels of LcNrdp1 quickly changed by immune stimulation. Meanwhile, no significant changes for the gene expression were detected when injected with PBS as controls.

Expression profiles of LcNrdp1 in liver after immune stimulation

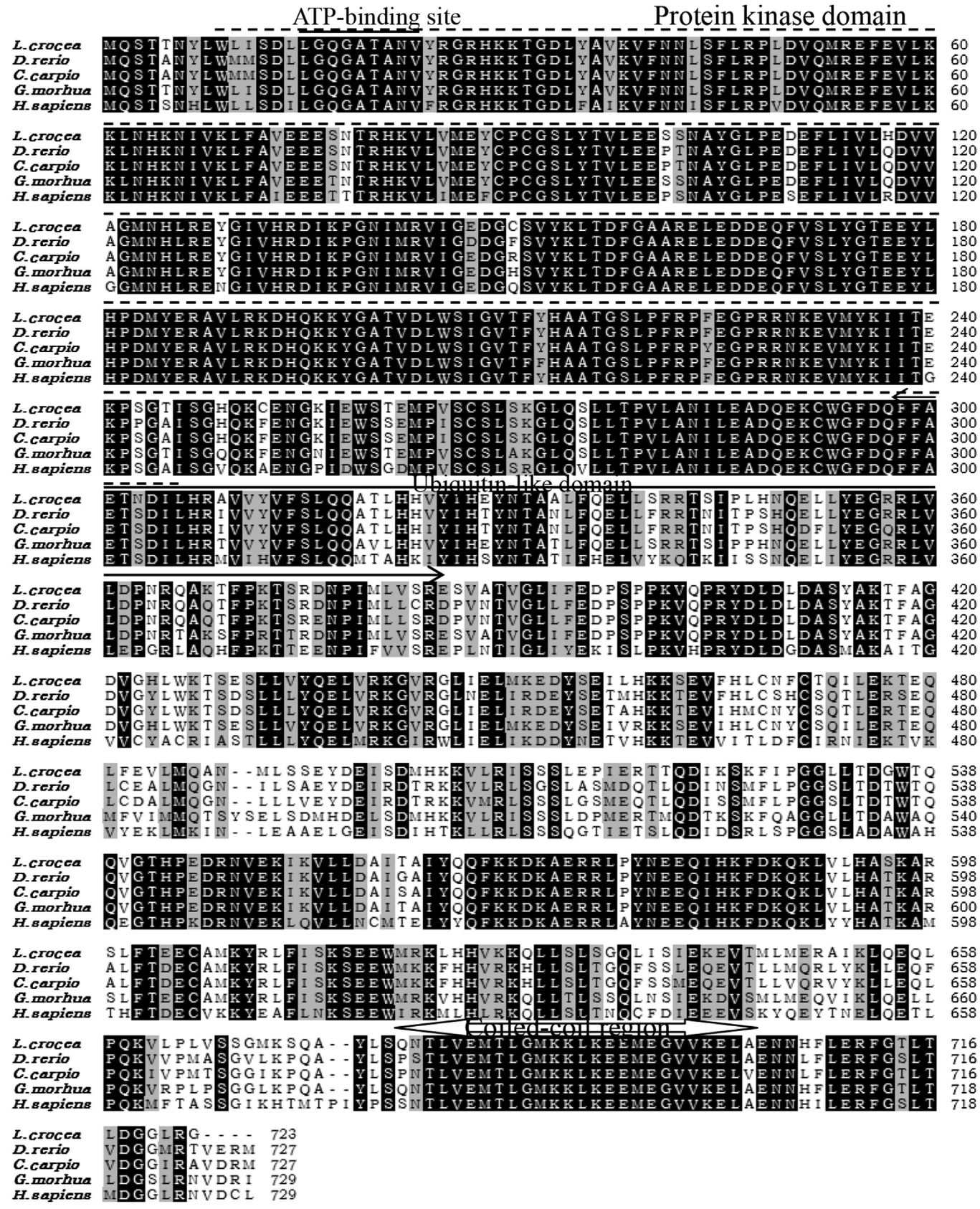

Fig. 1. Multiple alignment of TBK1 amino acid sequences. The comparison includes TBK1 sequences from the teleost species *L. crocea* (GenBank accession: KR534322), *D. rerio* (NP_001038213), *C. carpio* (ADZ55455), *G. morhua* (ADL60136), *H. sapiens* (NP_037386). Identical amino acids are highlighted in black, strongly similar amino acids are printed in white letters with dark gray. ATP-binding site, Protein kinase domain, Ubiquitin-like domain and Coiled-coil region were indicated by ——, - - -, ⇔ and ⟸⟹, respectively.

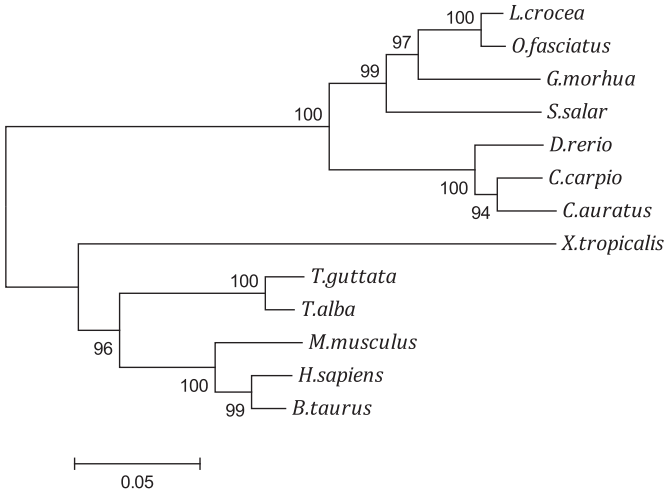

Fig. 2. Phylogenetic trees analysis based on TBK1 amino sequences. GenBank Accession numbers of sequences used are *O. fasciatus* (AHX37216), *S. salar* (NP_001243651), *C. auratus* (AEN04475), *X. tropicalis* (NP_001135652), *T. guttata* (XP_002188051), *T. alba* (XP_009967331), *M. musculus* (NP_062760), *B. taurus* (NP_001179684). GenBank Accession numbers of other animals are as above in Fig. 1. The scale bar is 0.05.

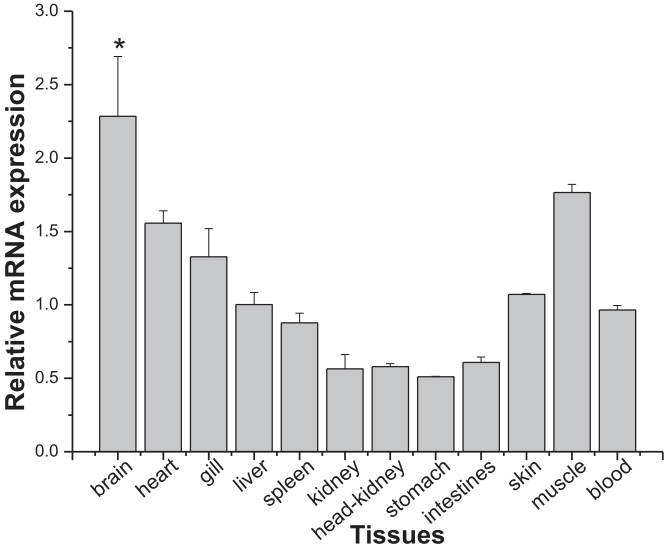

Fig. 3. Expression profiles of LcTBK1 mRNA in various tissues of *L. crocea* by quantitative real-time PCR. The data was normalized against β-actin. Each experiment was performed in triplicate. Data (mean ± SE, n = 5) are indicated with significantly different *.

were shown in Fig. 6 A. Like expression profiles of LcTBK1 in liver after immune stimulation, LcNrdp1 mRNA remained significant increased trends in the whole infection time-course after LPS, Poly I:C and inactive *V. parahaemolyticus* stimulations except for decline at 3 h and 24 h post the injection of Poly I:C ($P < 0.05$). Furthermore, after injection with inactive *V. parahaemolyticus*, LcNrdp1 expression levels increased significantly with 3.98-fold (at 12 h), 7.58-fold (at 24 h), 19.41-fold (at 48 h) and 7.19-fold (at 72 h) as compared with the control ($P < 0.05$).

Expression profiles of LcNrdp1 in spleen after immune stimulation were shown in Fig. 6 B. After injection with LPS, LcNrdp1 mRNA expression was up-regulated at 24 h and 72 h as compared with the control. However, it was down-regulated at 48 h. After injection with Poly I:C, LcNrdp1 expression level significantly increased in the whole time-course with 2.36-fold and 2.08-fold at 12 h and 72 h as compared to the control ($P < 0.05$) except for 3 h and 48 h. In addition, after the stimulation with inactive *V. parahaemolyticus*, LcNrdp1 expression firstly increased at 3 h then declined at 6 h and 48 h, and no obviously change in other time point.

Expression profiles of LcNrdp1 in head-kidney after immune stimulation were shown in Fig. 6C. The expression trend of LcNrdp1 expression was all down-regulated then gradually recovered after inactive *V. parahaemolyticus*, Poly I:C and LPS immune stimulations. And its expression was 1.61-fold and 1.63-fold as much as the control after injection of LPS. In conclusion, expression of LcNrdp1 in liver was up-regulated after inactive *V. parahaemolyticus*, Poly I:C and LPS stimulation, while in spleen and head-kidney, down-regulated in some time point, especially in head-kidney.

4. Discussion

TBK1 has been extensively studied in mammals because of its important roles as a molecular bridge, linking the TLRs (TLR3 and TLR4) and RLRs signals to activate transcriptional factors IRF3 and IRF7 for IFN-I production [24,25]. In the present study, a TBK1 gene was identified and characterized in *L. crocea*. The Blast and ClustalW alignment confirmed that the cloned molecule was a TBK1 homolog sharing highest number of identical amino acids with that in other fishes, even *H. sapiens* (Fig. 1). The protein sequence was conserved among various species, suggesting its important functions in host. Most of the conserved sequences and functional motifs in LcTBK1 were found in the N-terminus of the putative protein from 9 to 306 (protein kinase domain and ATP-binding site), middle ULD from 297 to 385 and C-terminal coiled-coil domain from 678 to 704 (Fig. 1). The kinase domain of TBK1 is essential for its mediated signaling pathway. There is evidence showed that TBK1s, an alternative splicing isoform of TBK1 that lacks the kinase domain, inhibits SeV-induced IFN-β activation [26]. The ULD regulates kinase activity, substrate presentation and downstream signaling pathway. Deletion or mutations of the ULD in TBK1 impaired activation of respective kinases, failed to induce IRF3 phosphorylation and nuclear localization and failed to activate IFN-β promoters [27]. The coiled-coil domain in TBK1 is an adaptor binding domain. Binding of TBK1 with TANK, Sintbad and NAP1 can be selectively abrogated if lacking the coiled-coil domain [28].

LcTBK1 gene was constitutively expressed in various tissues detected (Fig. 3), similar to that in other fishes. However, different expression patterns are observed in different species. TBK1 is found to be predominantly expressed in the liver of *C. carpio* [13], in the spleen of *G. morhua* [14], and in the trunk kidney of *C. idella* [16]. In this study, LcTBK1 was highly expressed in brain, but there were little differences among the other tissues, indicating that LcTBK1 may play multiple roles in normal conditions in *L. crocea*.

In mammals, TBK1 mediates an early cellular response to bacterial infection and can be activated by LPS treatment in primary human macrophages [29,30]. In the present study, the temporal expression of LcTBK1 was significantly induced in examined tissues post LPS, Poly I:C and inactive *V. parahaemolyticus* stimulation, especially with inactive *V. parahaemolyticus* stimulation, the expression with peak reaching 12.09, 6.05 and 6.22-fold at 12 h (in liver), 48 h (in spleen), 72 h (in head-kidney) as much as the control, respectively (Fig. 4), suggesting that bacterial inflammatory reaction might occur at different time in distinctive tissues. However, like Nrdp1 expression, TBK1 expression was inhibited at some time point in head-kidney and spleen post LPS and Poly I:C stimulation, suggesting that the expression of TBK1 was associated with Nrdp1 in response to immune stimulations. Three reports also showed that TBK1 involved in similar immune responses. The significant mRNA change of carp TBK1 was also mainly found in intestine with 11.3-fold as much as the control after SVCV (a

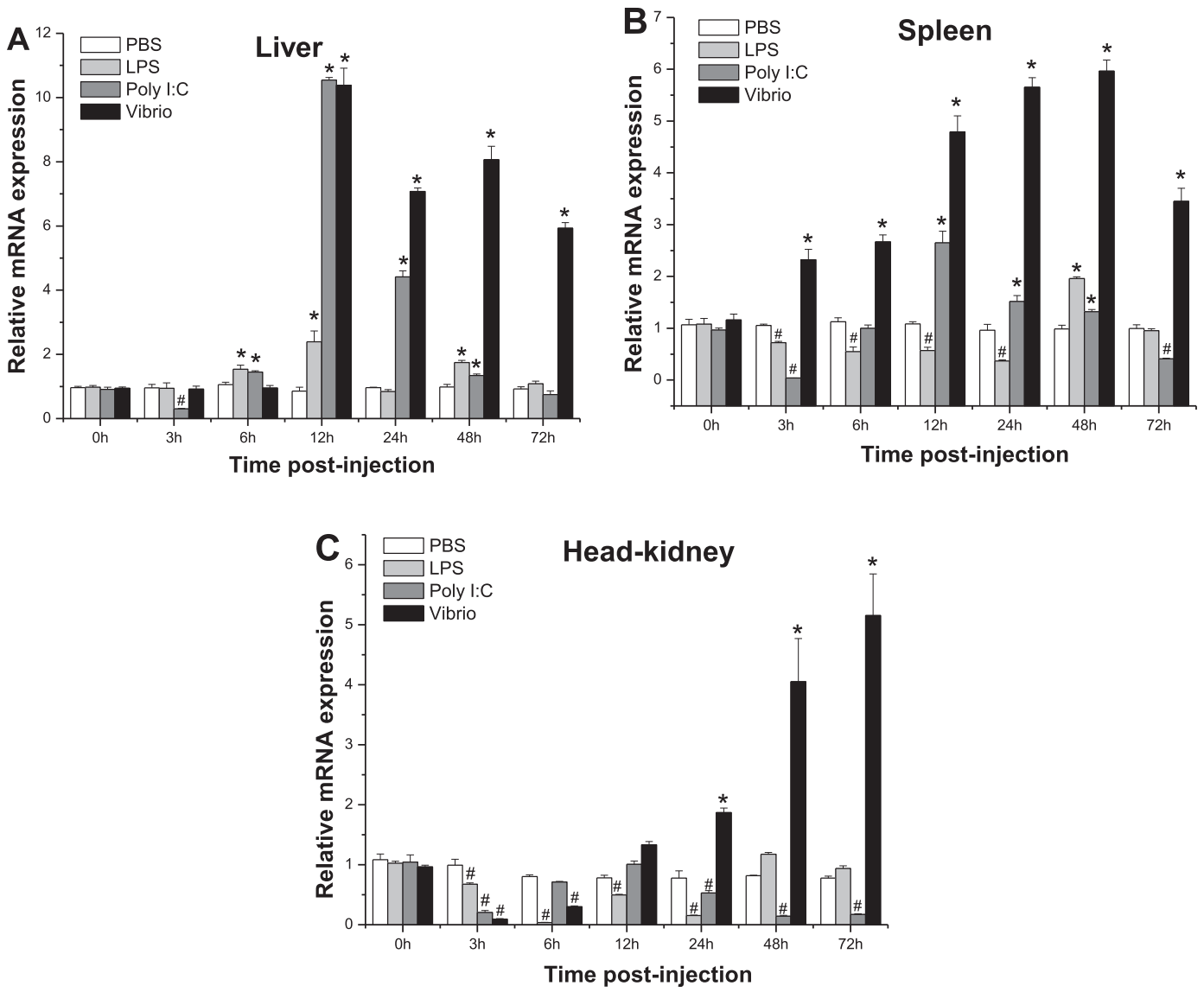

Fig. 4. Transcription expression changes of LcTBK1 in liver (A), spleen (B) and head-kidney (C) after LPS, Poly I:C and inactive *V. parahaemolyticus* stimulation by qPCR. The mRNA expression level was normalized against β-actin. Data were shown as the mean ± SE (n = 5). Each experiment was performed in triplicate. Significant differences of expression among the treatments were indicated with* (up-regulated) and # (down-regulated), $P < 0.05$.

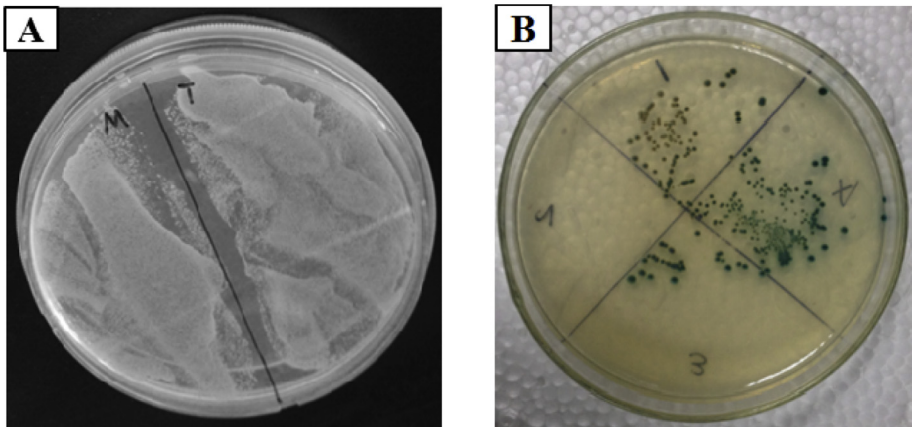

Fig. 5. Yeast two-hybrid assay of the interaction between LcNrdp1 and LcMyD88, LcNrdp1 and LcTBK1. (A) pGBKT7+LcNrdp1/pGAKT7+LcMyD88 (M) and pGBKT7+LcNrdp1/pGAKT7+ LcTBK1(T) were grown on SC-Leu-Trp plate. (B) The positive (1), negative control (2), pGBKT7+LcNrdp1/pGAKT7+LcMyD88 (3) and pGBKT7+LcNrdp1/pGAKT7+LcTBK1 (4) were grown on SD/-Ade/-His/-Leu/-Trp/X-a-Gal/AbA plate.

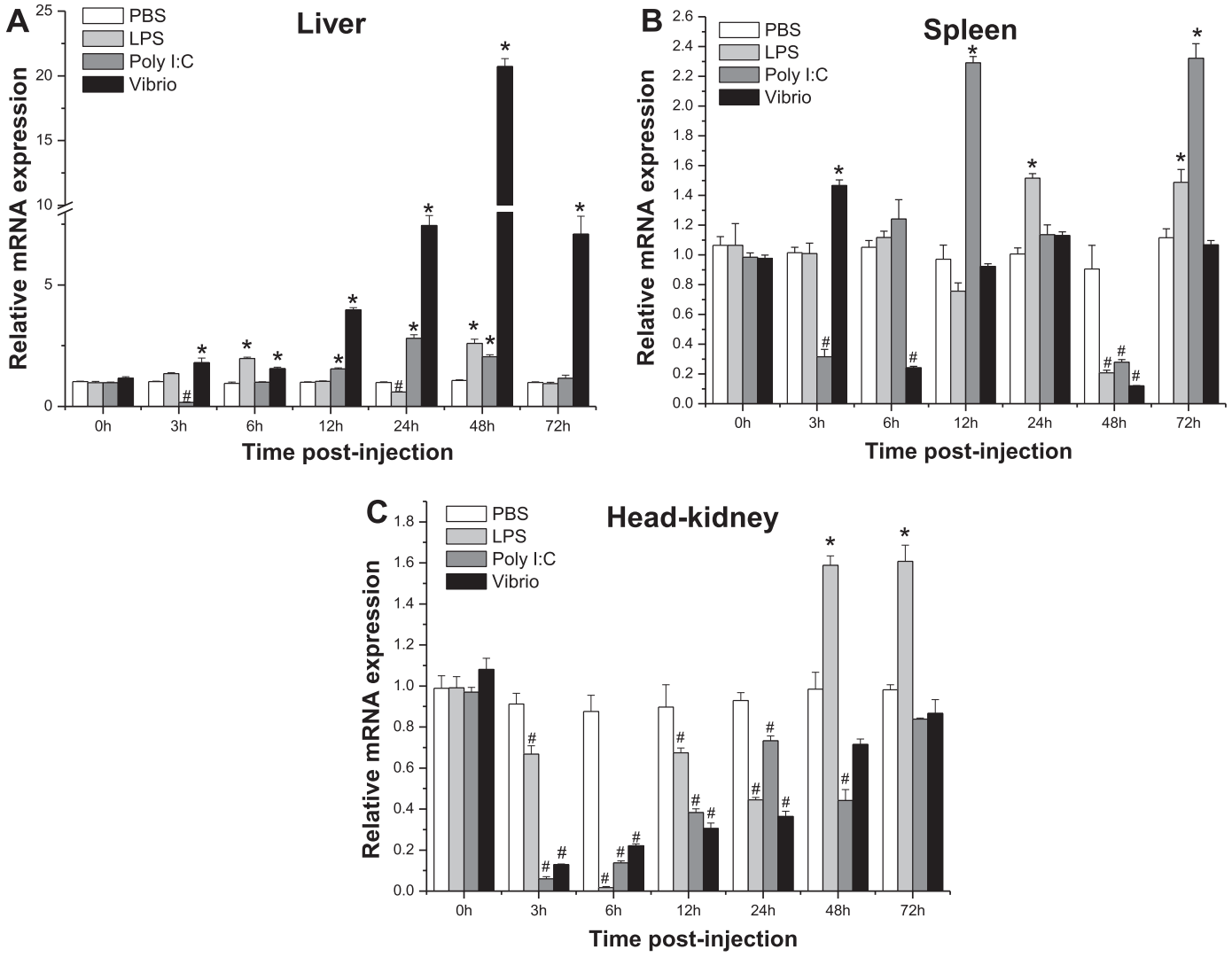

Fig. 6. Analysis of LcNrdp1 expression in liver (A), spleen (B) and head-kidney (C) after immune stimulation by qPCR at 0 h and 3 h, 6 h, 12 h, 24 h, 48 h and 72 h post-injection. β-actin was used as an internal control. The significant difference of LcNrdp1 expression was indicated with * (up-regulated) and # (down-regulated), $P < 0.05$.

negative single-stranded RNA virus) infection, however, declined in spleen [13]. The expression of TBK1 in head-kidney and spleen from *G. morhua* increased at some time-points following PMA, Poly I:C and β-glucan injections, but the expression in the head-kidney cells did not increase after poly I:C stimulation [14]. The transcription expression of TBK1 in blastulae embryonic cells (CABs) and epithelioma papulosum cyprini (EPC) of *C. auratus* was not induced by rIFN treatment or poly(I:C) transfection [15]. However, TBK1 mRNA in *C. idella* was all elevated in spleen, head-kidney and kidney cell post grass carp reovirus, LPS and PGN stimulations [16]. During pathogen invasion, especially when at an excessive concentration, the pathogen would have its own way to fight against the host immune system, *e.g.* the viral microRNA would target host immune system to knock down the immune gene expression [31], and the expressions of MHCII significantly decreased after the pathogen induced in mammals and teleosts [32,33]. Furthermore, it is to be noted that LcTBK1 mRNA was obviously increased in the liver after three stimulations, there are at least two possibilities. One is that pathogen would have its own way to fight against the host immune system, and TBK1 might have different responses in distinctive cell types. The other is that liver is also a crucial immune organ, and many immune-related genes have been significantly up-regulated after the pathogen induced. These results suggested that LcTBK1 was related to fish defense against pathogen infection, but the process is unknown.

Nrdp1 gene, an E3 ubquitin ligase, was identified to interact respectively with TBK1 and MyD88 in *L. crocea* in this study (Fig. 5), suggesting that both TBK1 and MyD88 can be regulated by E3 ubquitin ligase Nrdp1 in fishes. Hawn reported that MyD88-deficient ($MyD88^{-/-}$) mice had dramatically higher bacterial counts in the lungs, with decreased neutrophil counts in the bronchoalveolar lavage fluid as well as absent cytokine and chemokine production at early time points [34]. Feng et al. reported that over-expressed TBK1 in kidney cell of *C. idella* can inhibit the expression of VP4 in reovirus [35]. Thus Nrdp1 can involve immune defense against bacteria and virus in fishes through mediating MyD88 and TBK1. In our previous study, LcNrdp1 gene was obviously up-regulated in skin, gill and spleen, and down-regulated in head-kidney upon *C. irritans* induction [17]. In this investigation, LcNrdp1 was up-regulated in liver and spleen, but down-regulated in head-kidney in most time point after LPS, Poly I:C and inactive *V. parahaemolyticus* stimulation (Fig. 6), similar to its response to *C. irritans* infection [17].

In conclusion, TBK1 was molecularly characterized for this

marine fish for the first time. The expression of LcTBK1 and LcNrdp1 in response to pathogens as well as their interaction suggested that LcTBK1 was involved in innate immunity in fish and probably directly regulated by LcNrdp1.

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