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The amphioxus *ERK2* gene is involved in innate immune response to LPS stimulation

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ABSTRACT

The *ERK2* gene is a member of the MAPK family, which plays very important roles in responses to external environmental pressures. However, the *ERK2* has yet not been identified in amphioxus to date. To further illuminate the function and evolutionary mechanism of the *ERK2* gene, in this present study, we have cloned the full length of the *ERK2* gene of *Branchiostoma belcheri* (designed as *AmphiERK2*), which is highly homologous to these vertebrate *ERK2* genes. The AmphiERK2 protein contains the conserved S_TKc domain and the TEY motif, and its 3D structure is also highly similar to human ERK2 protein. Taken together, our results indicate that the *AmphiERK2* gene belongs to a member of the *ERK2* gene family. We further use qRT-PCR technology to detect an ubiquitous expression of *AmphiERK2* gene in all five investigated tissues (muscle, notochord, gill, hepatic caecum and intestine), and the expression level of *AmphiERK2* in both notochord and muscle is significantly higher than the other three tissues. Meanwhile our results also demonstrate that LPS stimulation can induce the up-regulation expression of *AmphiERK2* gene and significantly increase the phosphorylation level of AmphiERK2 protein, which seems to imply that the AmphiERK2 may be involved in amphioxus innate immune responses. Overall, our findings provide an important insight into amphioxus innate immune function and evolution of the *ERK2* gene family.

1. Introduction

Mitogen-activated protein kinases (MAPKs) play very vital roles in regulating intracellular biological processes including inflammation, proliferation, differentiation, apoptosis and immune response [1]. In mammalian cells, MAPKs are divided into three major subfamilies, i.e. extracellular signal-regulated kinases (ERKs), c-JUN amino-terminal kinases (JNKs) and p38 MAP kinase [2].

The ERK subfamily of MAPKs can be activated by phosphorylation in the threonine and tyrosine residues in the activation loop to regulate cell proliferation and survival [3]. Especially, the protein kinases ERK1 and ERK2 have been extensively studied. ERK1 and ERK2 as the regulator kinases are found to participate in the Ras-Raf-MEK-ERK signal transduction cascade [4]. Notably particularly, ERK1 and ERK2 proteins are encoded by different genes [5], but they have very high identity in amino acid sequences and share the similar substrates and functions [6]. However, some recent studies have revealed that ERK1 and ERK2 can perform various functions [7,8]. Moreover, ERK1 and ERK2 are also reported to exert redundant functions in mouse development [9,10]. At present it is yet not clear whether ERK1 and ERK2 carry out specific functions or act redundant functions in animal evolution, Therefore, in order to shed more light on this issue, ERK1 and ERK2 from more animal species need to be further studied.

The *ERK2* gene is firstly cloned from rat fibroblast cell line [11]. The ERK2 protein contains highly conserved serine/threonine kinases (S_TK) domain, which is activated via phosphorylation on a threonine and a tyrosine residue within the specific Thr-Glu-Tyr (TEY) motif in the phosphorylation lip by mitogen-activated protein kinase/ERK kinase (MEK) 1/2 [12]. To date, the ERK2 homologs have been identified in many species, such as human [13], rat [14], mouse [15], zebrafish [16] and frog [17]. In mammals, the structure, function and regulation of ERK2 have been in-depth studied. The ERK2 could be activated by diversified stimuli and environmental stresses involved in cell death, immune responses as well as many other physiological responses. In

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zebrafish, the ERK2 is involved in the induced expression of Hsp70 in response to heat stress [18]. The ERK could be activated by high temperature stress in *Drosophila melanogaster* [19]. In chicken heterophils, ERK1/2 could be significantly up-regulated after flagellin (FLG) and lipopolysaccharide (LPS) stimuli [20]. In Chinese mitten crab, the expression of *E. sinensis* ERK2 (EsERK2) is also sensitive to the extracellular stimuli [21]. During pathogen-caused inflammation, the ERK2 from *Epinephelus coioides* plays a crucial role in specific tissues [22].

Although the functions of ERK2 involved in innate immune response to pathogenic infection or pathogen-derived substances have been studied in many species, there is still limited knowledge about the functions of ERK2 in amphioxus. Amphioxus belong to the chordate phylum, the subphylum cephalochordata, as a species that is transferred from invertebrate to vertebrate in evolutionary history [23]. And Amphioxus are both structurally and genetically related to vertebrate ancestors [24]. Although amphioxus does not have an adaptive immune system, it has a complex innate immune system. Therefore, studying the function and evolution of the *ERK2* gene in amphioxus not only helps us to understand how the ERK2 is involved in the innate immune response of amphioxus, but also has important scientific significance for tracing the evolution and origin of the innate immune response in animals.

2. Materials and methods

2.1. Cultivation and immune stimulation of amphioxus

These matured adults of amphioxus (*Branchiostoma belcheri*) were captured in Zhanjiang (Guangdong province, China), and cultured at 24–25 °C in a tank filled with air-pumped circulating artificial seawater, as well as supplied daily with Chlorella. The experiment and control groups were established by cavum injection with 15 μl of LPS (dissolved in PBS suspension 1 mg/ml) and PBS, respectively. Then both the experiment and control groups were cultured at 28 °C, and twelve samples (6 LPS stimulated amphioxus and 6 PBS stimulated amphioxus) were separately collected at different time points (2 h, 4 h, 6 h, 8 h, 12 h and 24 h). All collected samples were frozen by liquid nitrogen for RNA extraction.

2.2. Clone of AmphiERK2 gene

A mix of total RNA was extracted from the whole amphioxus using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The first strand of cDNA was synthesized with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV; TaKaRa, Dalian, China). According to the expressed sequence tag (EST) of *Branchiostoma belcheri* (http://genome.bucm.edu.cn/lancelet), specific primers were designed to amplify the full-length ORF. All the primers used are listed in Table 1. And the amplified fragments were subcloned into pMD® 19-T Simple Vector and sequenced.

Table 1
Primers used in this study.

Primer name	Sequence (5'-3')
For ORF cloning	
AmphiERK2-ORF2-F	ATGGCGGAGGGCGCAGGAAAACC
AmphiERK2-ORF2-R	GGTCACTTGTTTTGGTGGCTGAAAAACT
For RT-PCR	
AmphiERK2-F	GAGAATGTGATTGGCATTGAGAAC
AmphiERK2-R	TTGGTAGAGGAAGTAGCAGACG
Amphiβ-actin-F	TCTGGCATCATACCTTCTACAA
Amphiβ-actin-R	TCTGTGTCATCTTTTCCCTGTT

2.3. Sequence comparison and three dimensional structure analysis of AmphiERK2 protein

The predicted AmphiERK2 protein sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and these ERK2 protein sequences of other species were identified with BLASTP searches using the AmphiERK2 protein sequence at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The AmphiERK2 protein domain was predicted by the simple modular architecture research tool (SMART) version 4.0 program (http://www.smart.emblheidelberg.de/) [25]. The similarity and identity of the AmphiERK2 with other known ERK2 protein sequences were calculated using the MatGAT program with default parameters [26]. Multiple alignment of ERK2 was carried out with the GenDoc program. The three dimensional structure of ERK2 protein was constructed by the online prediction software SWISS-MODEL. The structure chart of the ERK2 protein was displayed on the visualization software Pymol-0_97- binwin32 program.

2.4. The qRT-PCR of AmphiERK2 mRNA for spatial express pattern and LPS stimuli analysis

For the spatial distribution analysis of the *AmphiERK2* in different tissues, total RNA was extracted from muscles, gills, intestine, notochord and hepatic cecum of fifteen healthy amphioxus (*B. belcheri*). In the temporal expression analysis, total RNA was extracted from the whole amphioxus collected at different time points after injection. All RNA was isolated by the Trizol reagent (Invitrogen, Carlsbad, USA), and then the cDNA was synthesized using PrimeScript RT reagent kit (TaKaRa, Dalian, China). Two gene-specific primers of *AmphiERK2* were designed to amplify a product of 1077 bp (Table 1). As an internal control, the *β-actin* gene of amphioxus was used for normalizing the expression level of *AmphiERK2* with the specific primers (Table 1). The qRT-PCR was performed in a total volume of 15 μl containing 7.5 μl of 2 × SYBR Green Real time PCR MasterMix, 1.5 μl of cDNA, 0.3 μl of each primer and 5.4 μl of double-distilled water. The reaction conditions were applied: 95 °C for 10 min, followed by 40 cycles (95 °C for 10 s and 62 °C for 30 s). The assays were performed on StepOnePlus™ Real-Time PCR System (ABI, Switzerland). All samples were analyzed in three duplications, the results were shown in terms of relative mRNA level as mean ± SE (n = 3). Data were quantified using the $2^{-\Delta\Delta Ct}$ method based on Ct values of *AmphiERK2* and *β-actin*, and the changes of gene expression level between the control group and the treatment group were analyzed using the two tailed Student's t-test ($P < 0.05$, considered as statistical significant).

2.5. Western blot analysis

Total cellular protein of amphioxus was homogenized on ice in RIPA lysis buffer containing protease inhibitors after injection (i.e. 0.5, 1, 3, 6 h). The samples were loaded onto 10% SDS polyacrylamide gels and transferred electrically to PVDF membranes. The membranes were washed with TBS buffer (20 mM Tris and 150 mM NaCl), and then blocked in TBS buffer with 5% (w/v) non-fat milk. After blocking, the membranes were washed three times in TBST buffer (20 mM Tris and 150 mM NaCl, 0.05% (V/V) tween 20), and then were incubated overnight at 4 °C with primary antibody diluted in TBST with 5%BSA. Blot was probed with the following rabbit polyclonal antibodies: anti-α-tubulin antibody (Bioworld, bs1699; 1:5000) and anti-phosphorylated ERK2 antibody (Bioworld, bs4759; 1:700). After three times washes with TBST, the membranes were incubated for 1 h with secondary antibody diluted in TBST with 5% (w/v) non-fat milk. The membranes were washed three times in TBST and detected. The detection was normalized using α-tubulin as internal control.

2.6. Sequence alignment and phylogenetic analysis of the ERK2 protein family

Sequence alignment was performed for homologous proteins of ERK2 using Clustalx program with default parameters. Phylogenetic analysis was conducted using Neighbor joining (NJ) based on amino acids alignment with MEGA 7.0 package. The *Caenorhabditis elegans* was used as an outgroup to root the tree. Data were analyzed using poisson model, with gaps removed by complete deletion. Phylogeny support was verified with the bootstrap consensus tree inferred from 1000 replicates.

3. Results

3.1. Cloning and characterization of AmphiERK2

The complete ORF sequence of *AmphiERK2* and deduced AmphiERK2 protein sequence have been identified. Sequence analysis revealed that the open reading frame (ORF) of *AmphiERK2* is 1077 bp, encoding a putative protein with 358 amino acids. The molecular weight of AmphiERK2 protein was 41.3 kDa and the theoretical PI was 6.5. Both ORF sequence and deduced protein sequence of *AmphiERK2* have been submitted to GenBank (ID: MH411240).

From homology analysis of AmphiERK2 protein sequence with other known ERK2s, we found their strong conservation in the S-TKc domain (Fig. 1). The AmphiERK2 protein has the highest identity (80.2%) to *Mus musculus* ERK2, and shared about 87.5–90.2% similarity to the other known ERK2 homologues (*Homo sapiens*, *Mus musculus, Xenopus tropicalis and Danio rerio*) (Table 2). Through sequence alignment and tertiary structure prediction, we found AmphiERK2 have a small amino-terminal lobe and large carboxyterminal lobe that contain several conserved α-helices and β-strands (Fig. 1 and Fig. 2). The N-terminal contains a five-stranded antiparalled β-sheet. The C-terminal is mainly α-helical with six conserved segments (αD-αI) and four short conserved -strands (β6-β9). Conserved glycine-rich loop (GxGxxG) located between the β1 and β2 strands and a salt-bridge between the β3-lysine and the αC-glutamate were found in the N-terminal. The catalytic loop located near the β6-and β7-strands and the activation segments begins with DFG and ends with APE were found in the C-terminal. The specific Thr-Glu-Tyr (TEY) motif in the phosphorylation lip was contained in the activation segment.

3.2. Phylogenetic analysis of AmphiERK2

To analyze the evolutionary dynamics of ERK1/2 family genes, ERK1 and ERK2 protein sequences from different species were used to construct a phylogenetic tree using the neighbour-joining method (Fig. 3). The evolutionary relationships of ERK1/2 family were shown in Fig. 3. The ERK1/2 family was mainly classified into three clades when *Caenorhabditis elegans* as an outgroup. Clade I included ERK2 of *Branchiostoma belcheri*, which is a cephalochordate; Clade II included ERK2 of *Ciona intestinalis*, which is a urochordate; and Clade III included the vertebrate, and may be further classified into two groups: all vertebrate ERK1 were clustered in one group, and the same for ERK2. As expected, the AmphiERK2 protein was located at the bottom of the chordate. The high bootstrap values supported the precision of topology.

3.3. Expression of AmphiERK2 gene in different tissues

Quantitative RT-PCR was employed to quantify the expression pattern of *AmphiERK2* gene in five tissues including gill, hepatic cecum, intestine, muscle and notochord. As shown in Fig. 4, the result indicated that the *AmphiERK2* gene was constitutively expressed in all detected tissues in *Branchiostoma belcheri*. However, the expression levels were different. The expression level was significantly higher in notochord, followed by muscle, gill and hepatic cecum, while the lower expression level was detected in intestine.

3.4. Expression of AmphiERK2 gene at different time points after LPS stimulation

To explore the expression pattern of *AmphiERK2*, quantitative RT-PCR method was used to measure the *AmphiERK2* gene expression at different time points (2, 4, 6, 8, 12 and 24) after LPS stimulation. As showed in Fig. 5, the relative expression of *AmphiERK2* mRNA was up-regulated at 24 h after injection, which was 1.98-fold higher than that in the control group ($P < 0.05$).

3.5. Activation of AmphiERK2 protein upon LPS stimulation

To test whether AmphiERK2 protein is activated in response to inflammatory infection, we examined the phosphorylation-ERK2 (p-ERK2) in amphioxus upon LPS stimulation. As Fig. 6 shown, the p-ERK2 was detected at 0.5 h, 1 h, 3 h and 6 h after injected by LPS and the samples injected by PBS were as the control. It is obvious that the phosphorylation level of AmphiERK2 prorein was significantly increased at 0.5 h after LPS treatment. The activation of AmphiERK2 is in a faster and more transient manner than the expression of *AmphiERK2* upon LPS stimulation.

4. Discussion

4.1. AmphiERK2 is a new member of ERK2 family

In this study, the AmphiERK2 has been identified and further characterized to be a new member of ERK2 family for the following reasons. First, the *AmphiERK2* gene shows highly homologous with these identified ERK2 family members (Table 2). Second, the AmphiERK2 protein contains the conservative ERK-specific dual-phosphorylation TEY motif and S_TKc domain, which are the characteristic representative conservative structure in MAPK family [27]. Third, the 3D structure of AmphipERK2 protein is also similar to that of human ERK2 (Fig. 2). The AmphipERK2 has some common conservative structures and activation sequence segments with human ERK2, implying that AmphiERK2 might have the conservative catalytic function of ERK1/2 family. Fourth, the phylogenetic analysis shows that the AmphiERK2 is located at the base of ERK1/2 of urochordate and vertebrate (Fig. 3), which is in general accordance with the classical taxonomy. This result implies that AmphiERK2 might be the ancestral genes of vertebrates. Taken together, our results strongly urge that AmphiERK2 is a new member of ERK2 family. In addition, we also performed the transcriptome and genome wide search for the *ERK1* gene, and found no *ERK1* gene in *Branchiostoma belcheri*. Thus we speculate that the ERK1 may not exist in amphioxus, which is consistent with the sister species *Ciona intestinalis* [28].

4.2. Spatial expression pattern of ERK2

Previous study has shown that *ERK2* is mainly expressed in the skin and brain in large yellow croaker [29], whereas the *ERK2* is primarily expressed in hepatopancreas and muscle in Chinese mitten crab [21], which seems to indicate that the spatial expression patterns of *ERK2* are different in different species. Our present results show that the *AmphiERK2* is ubiquitously expressed in all examined tissues (Fig. 4), suggesting that AmphiERK2 might play very important roles in a variety of physiological processes of amphioxus. Furthermore, our results demonstrate that the relatively higher expression of *AmphiERK2* is in notochord and muscle, which is inconsistent with the two previous reports [21,29]. The cause may be due to their direct contact with different aquatic organisms and external environment, respectively.

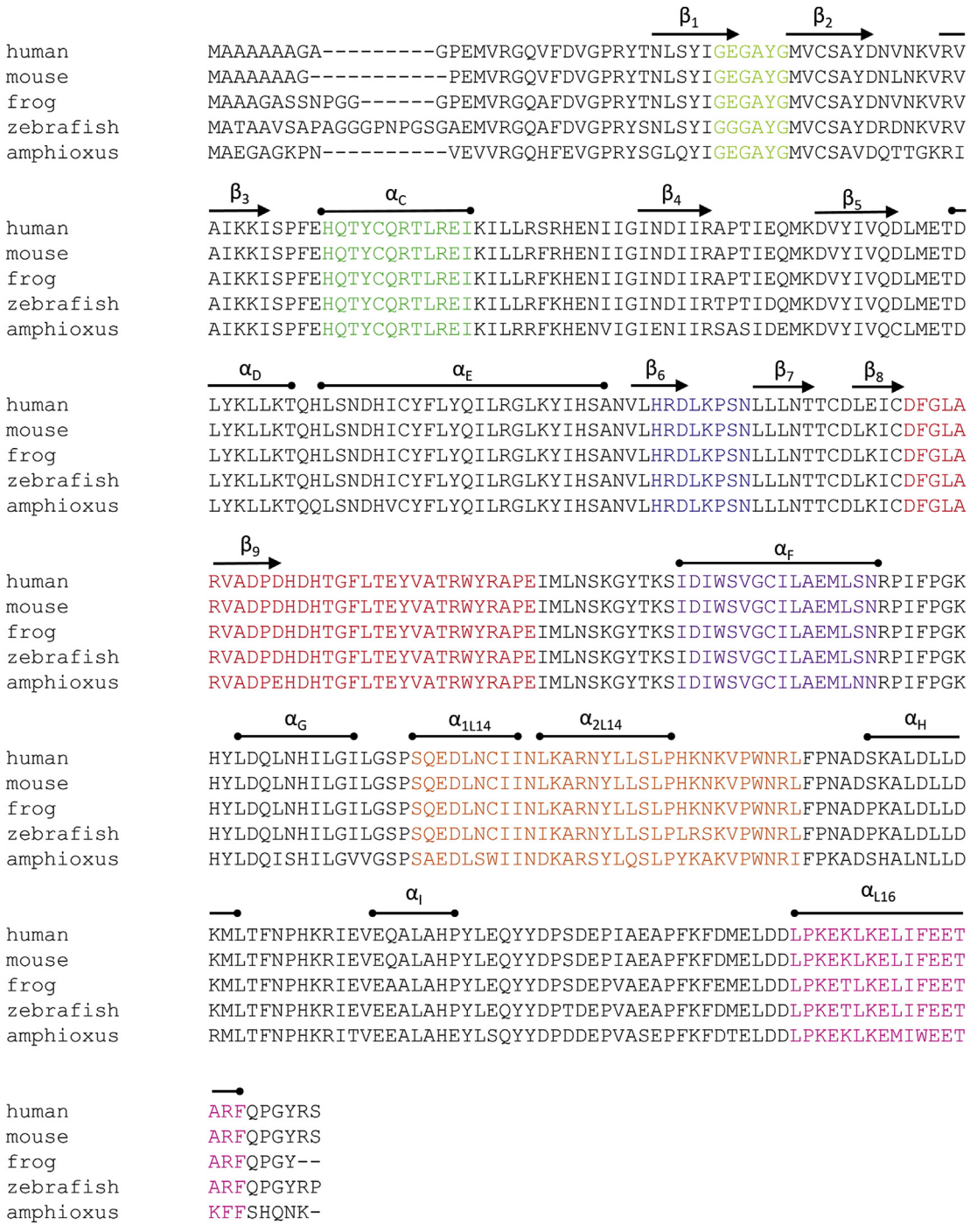

Fig. 1. The sequence alignment of AmphiERK2 with other known ERK2 proteins. The location of α-helices (straight lines) and β-strands (arrows) are noted. The color code correspond to the three dimensional structure of AmphiERK2 protein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Homology analysis of AmphiERK2 amino acid sequence with other known ERK2 amino acid sequences.

Species	Accession number	Amino acids	Similarity (%)	Identity (%)
Homo sapiens	AAH99905.1	360	89.2	79.4
Mus musculus	NP_036079.1	358	90.2	80.2
Xenopus tropicalis	NP_001017127.1	361	89.2	79.3
Danio rerio	AAH65868.1	369	87.5	79.1

4.3. The AmphiERK2 is involved in innate immune responses to LPS stimulation

The ERK2 is related protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade, which is involved in innate immune responses by converting extracellular stimuli into a wide range of cellular responses [30,31]. To further understand the possible biological function of AmphiERK2, in this work, the mRNA expression and phosphorylation level of AmphiERK2 is further examined at different time-points after LPS stimulation in the whole amphioxus. Previous studies have indicated that LPS stimulation

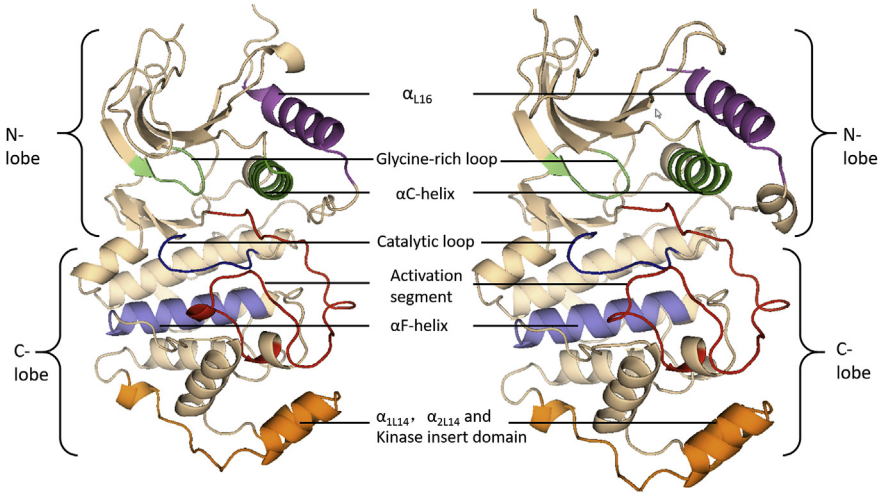

Fig. 2. The three dimensional structure of AmphiERK2 protein. (A) The 3D structure of Human ERK2 protein. **(B)** The 3D structure of AmphiERK2 protein. The tertiary structure of ERK2 proteins were constructed using the online prediction software SWISS-MODEL. Pymol-0_97-bin-win32 program as a visualization software was used to display the structure charts.

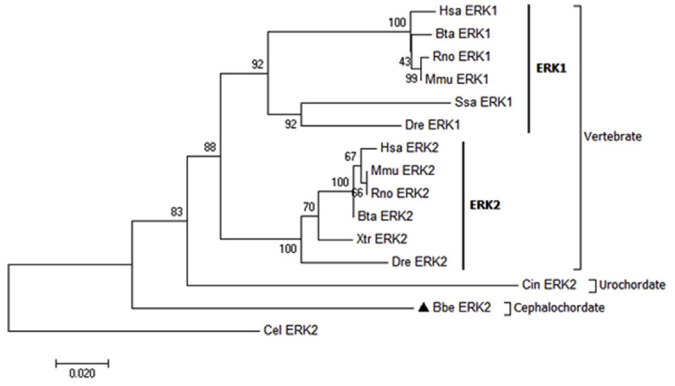

Fig. 3. Phylogenetic tree of ERK1/2 family proteins including AmphiERK2. The tree was deduced by neighbor-joining analysis based on the alignment of amino acid sequence of ERK1/2 proteins, which was carried out by MEGA7.0 program. Bootstrap values (1000 replicates) are indicated at the nodes.

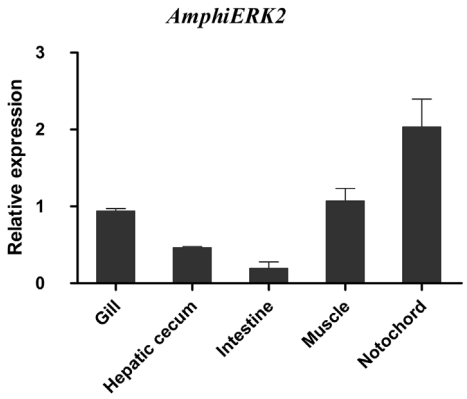

Fig. 4. *AmphiERK2* gene expression in different tissues. Real-time RT-PCR was carried out with mRNA from intestine, gill, notochord, muscle and hepatic cecum of healthy *B. belcheri.* Each sample was run in triplicate. In this experiment, the β-actin gene of *B. belcheri* was used as internal control to calibrate the cDNA template for all sample. Vertical bars are the means ± SE (n = 3).

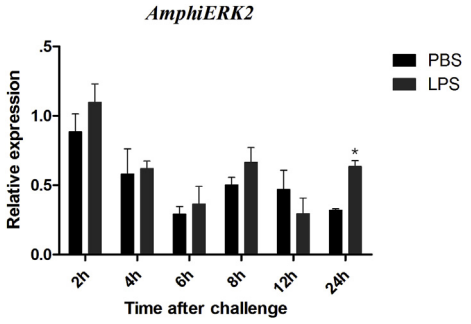

Fig. 5. *AmphiERK2* expression analysis after LPS stimuli. *AmphiERK2* mRNA expression in LPS infection samples was normalized to that in the PBS group. The amphioxus *β-actin* gene was used as a control to calibrate the cDNA template for all samples. Vertical bars represented the means ± SE (n = 3).

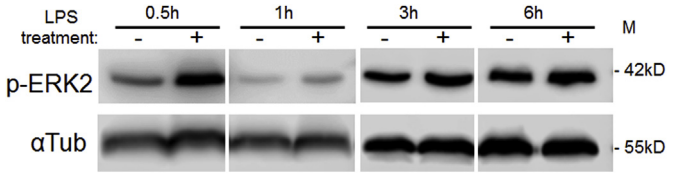

Fig. 6. Expression of p-ERK2 protein after LPS stimuli. p-ERK2 were detected at 0.5 h, 1 h after injected by LPS and the samples injected by PBS were as the control. The blots were detected with α-tubulin as the control.

could significantly up-regulate the phosphorylation level of ERK2 protein in human [32] and mouse [33]. Our present results show that the p-ERK2 is obviously increased at 0.5 h, but decreased at 1 h in amphioxus after LPS stimulation. By contrast, the mRNA expression of *AmphiERK2* is up-regulated at 24 h after LPS stimulation. This above result may imply that the activation of AmphipERK2 protein in a faster and more transient manner than the mRNA expression of *AmphiERK2* upon LPS stimulation (Figs. 5 and 6). Taken together, we suggest that the expression of AmphiERK2 in response to LPS stimuli might be in a time-dependent way, i.e. firstly amphioxus responds to pathogen infection by phosphorylation of ERK2, then amphioxus fights against pathogens by transcription up-regulation to increase the amount of AmphipERK2 protein to avoid the initial activation of AmphipERK2 is not sufficient to eliminate the pathogens. Therefore, our present results have revealed that AmphiERK2 might be involved in innate immune response of amphioxus in a time-dependent way.

In conclusion, in this work, we have identified the *ERK2* gene of *Branchiostoma belcheri* (designed as *AmphiERK2*), and revealed its innate immune function and evolutionary dynamic. Our results not only enrich the *ERK2* gene resources, but also provide valuable clues for further studying the function and evolution of the *ERK2* gene family.

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