

MOLECULAR CLONING AND EXPRESSION ANALYSIS OF HYPOXIA INDUCIBLE FACTOR 1 α IN TONGUE SOLE, *CYNOGLOSSUS SEMILAEVIS* (ACTINOPTERYGII: PLEURONECTIFORMES: CYNOGLOSSIDAE), SUBJECTED TO ACUTE HYPOXIA

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Qi Z.T., Wang Z.S., Zhang Q.H., Zhao W.H., Qiu M., Peng J.Q. 2013. Molecular cloning and expression analysis of hypoxia inducible factor 1 α in tongue sole, *Cynoglossus semilaevis* (Actinopterygii: Pleuronectiformes: Cynoglossidae), subjected to acute hypoxia. Acta Ichthyol. Piscat. 43 (3): 201–209.

Background. Hypoxia-inducible factor-1 α (HIF-1 α), a subunit of the HIF-1 protein, plays a key role in the regulation of genes involved in hypoxia physiological response. Tongue sole, *Cynoglossus semilaevis* Günther, 1873, a marine teleost, had been proved to be a hypoxia-tolerant species. In this study, the HIF-1 α in tongue sole was cloned and its expression under acute hypoxia was examined to provide further basis for understanding the molecular response of tongue sole under hypoxia.

Materials and methods. The full length of HIF-1 α cDNA sequence was cloned from the liver of tongue sole by RT-PCR and RACE-PCR method. Then, the expression pattern of tongue sole HIF-1 α under acute hypoxic conditions were detected using quantitative real-time PCR method.

Results. The open reading frame of tongue sole HIF-1 α is 2208 bp, encoding 735 amino acids. The amino acid sequence of tongue sole HIF-1 α shared high identities (52.7%–81.8%) with HIF-1 α from other vertebrates, and possessed six typical domains of the HIF-1 family (bHLH, PAS-A, PAS-B, PAC, N-TAD, and C-TAD). In adult fish, HIF-1 α mRNAs were highly expressed in the liver, moderately in the heart, spleen, kidney, stomach, blood and gills, and low in intestine. Under acute hypoxia stress, expression of HIF-1 α mRNAs were significantly up-regulated in many tissues, including the liver, spleen, stomach, blood, heart and gills.

Conclusion. Tongue sole HIF-1 α possessed the similar sequence length, shared higher identities and clustered well with other known HIF-1 α , thus revealing a high degree conservation of HIF-1 α during evolution. Tongue sole HIF-1 α began to be up-regulated from 5 min to 120 min after hypoxia, indicating that it might play a significant role at the early stage of hypoxia.

Keywords: Hypoxic stress, gene expression, quantitative real-time PCR

INTRODUCTION

Hypoxia-inducible factor-1 (HIF-1) is the master regulator of oxygen homeostasis by regulating more than 100 genes expression (Manolescu et al. 2009). HIF-1 is a heterodimer which is composed of two subunits, HIF-1 α and HIF-1 β . The two subunits belong to the helix-loop-helix (Bhlh)-Per-Arnt-Sim (PAS) family, which contain conserved structural domains, such as a bHLH domain and two PAS domains (Kewley et al. 2004). The HIF-1 β subunit, also known as hydrocarbon nuclear translocator (ARNT), is constitutively expressed, and is not affected

by oxygen tension (Wang et al. 1995). HIF-1 α is constitutively transcribed under normoxic conditions, but it is rapidly degraded via an ubiquitin-dependent pathway. However, during hypoxia this pathway is inhibited and HIF-1 α is translocated to the nucleus and dimerizes with HIF-1 β , causing HIF-1 to bind with hypoxia response elements (HREs) of target genes to activate their transcription (Maynard and Ohh 2004).

Currently, HIF-1 has been cloned and characterized in mammals (Gassmann et al. 2002), birds, amphibians, and some fish species (Cao et al. 2008, Kodama et al. 2012).

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However, there are little or limited studies with regard to fish HIF-1, especially when compared with high numbers of fish species worldwide. At the aquatic level, fish are the predominant species and are easily affected by oxygen availability (Wang et al. 2011). As a result, Fish have developed many strategies to adapt to low oxygen stresses during evolution, including decreasing metabolic rates, increasing anaerobic respiration, and increasing hematocrit and hemoglobin O₂ affinities. It has been found that the majority of these strategies are regulated by HIF-1, suggesting the important role it plays in hypoxia adaptation. Recent studies showed that different fish HIF-1 α possessed distinctive expression patterns under normoxic or hypoxic conditions (Soitamo et al. 2001, Law et al. 2006, Rahman and Thomas 2007, Rojas et al. 2007, Shen et al. 2010). Thus, demonstrates that the regulation pathway of HIF-1 in different fish might be different.

Tongue sole, *Cynoglossus semilaevis* Günther, 1873, a marine teleost, is fast becoming an important aquatic species in China and other Asian countries (Shao et al. 2010) from the financial perspective. On this note therefore, our previous studies have found that this species was hypoxia-tolerant and could survive for several days under acute hypoxia stress (dissolved oxygen = 1.0 mg · L⁻¹). Some genes, e.g., transferrin and hemoglobin, were found to be up-regulated when tongue sole were subjected to hypoxia conditions (Wang et al. 2011, 2012). Hence, whether those genes were regulated by HIF-1 remains unclear. In the presently reported study, the HIF-1 α gene of tongue sole was identified and its expression patterns under normoxic and hypoxic conditions were examined. Therefore, the results provide the basis for further understanding the molecular response of tongue sole under hypoxia.

MATERIALS AND METHODS

Fish and acute hypoxia stress. Adult tongue sole, *Cynoglossus semilaevis* (mean length 24 ± 1.01 cm, mean weight 77.44 ± 8.21 g) were purchased from a fish farm at Ganyu county, Lianyungang city of Jiangsu Province and transferred to our laboratory by vehicle equipped with a water recirculation system. The fish were acclimated in 40-L tanks for 7 days before the hypoxia stress.

Then, total of 30 fish were randomly divided into six tanks connected to the same recirculation system. One tank (control group) was maintained under normoxic conditions: dissolved oxygen (DO) = 6.2 mg · L⁻¹. In the other tanks, gaseous nitrogen was bubbled through to induce acute hypoxic conditions (DO = 1.0 mg · L⁻¹). This DO concentration was chosen based on our previous results (Wang et al. 2011, 2012). Tissues from three fish were sampled at 5, 30, 60, 90 and 120 min, and subsequently stored at -80°C until required.

The Institutional Review Board of the Yancheng Institute of technology, China, approved this study as consistent with the country's regulations on experiments on animals.

Total RNA extraction and first-strand cDNA synthesis. Total RNA of different tissues were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) accord-

ing to the manufacturer's instructions. The quantity of RNA was determined by measuring the absorbance at 260 and 280 nm. RNA integrity was determined by electrophoresis. After extraction, total RNA was reverse transcribed into cDNA in an 8- μ L reaction which comprised 1 μ L of CDS, 1 μ L of Smart II oligo (dT) primer, and 1–2 μ g of total RNA. The reaction volume was made up to 8 μ L with DEPC-treated H₂O. The mix was heated at 72°C for 10 min, chilled on ice for 2 min, and then 4 μ L of 5 \times reverse transcription buffer, 2 μ L of 20 mM DDT, 2 μ L of 10 mM dNTPs, 1 μ L of 40 U · μ L⁻¹ RNase inhibitor, 1 μ L of SuperScript™ Reverse Transcriptase (Invitrogen), and DEPC-treated H₂O were added to a final volume of 20 μ L. After incubation at 42°C for 1 h, the reaction was terminated by heating at 72°C for 10 min.

Cloning the tongue sole HIF-1 α gene. Degenerate primers (HIF1DF1 and HIF1DR1) were designed using Primer 5.0 based on the conserved regions of known HIF-1 α sequences from human (*Homo sapiens*) (GenBank Accession No. AF208487), mouse (*Mus musculus*) (NM_01043), rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) (AF304864) and grass carp (*Ctenopharyngodon idella* Valenciennes, 1844) (AY450269). The predicted product size was 1006 bp. Polymerase chain reaction (PCR) amplification was conducted in a total volume of 25 μ L which comprised 2 μ L of cDNA template, 2.5 μ L of 10 \times buffer, 2 μ M MgCl₂, 200 μ M dNTPs, 0.125 U of ExTaq and 0.1 μ M primers. Thermal cycling involved an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, and a final extension step of 72°C for 10 min after the 30th cycle. Amplicons were extracted from agarose gels after electrophoresis and purified using a TaKaRa Agarose Gel Purification Kit (TaKaRa, Japan), then ligated into pMD18-T (TaKaRa) and sequenced on an automatic DNA sequencer (ABI Applied Biosystems Model 377).

Based on the obtained partial sequence, 3' and 5' rapid amplification of cDNA ends (RACE) were conducted using gene-specific primers and adaptor primers (UPM) to obtain the complete sequence of HIF-1 α . For 3' RACE, the first-round PCR was performed using HIF1-3out and UPM as primers as well as subjected to an initial denaturation step of 94°C for 5 min, then 9 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 90 s, followed by 29 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 90 s, with a final extension step of 72°C for 10 min. Products of the first-round PCR were then diluted (1 : 10) and re-amplified by a second-round PCR with HIF1-3in/UPM as primers. For 5' RACE, the first-round primers were HIF1-5out/UPM, and the second-round primers were HIF1-5in/UPM. The resulting products were analyzed as mentioned above. All primers used in our experiments were listed in Table 1.

Sequence analysis. The BLAST program was used to identify homologous sequences in the GenBank database. Deduced amino acids were predicted using software at the ExpASY molecular biology Server (<http://www.expasy.org>).

Signal peptides were identified using the SignalP program (www.dtu.dk/services/SignalP). Multiple sequence alignments were conducted with CLUSTAL W 1.8 and amino acid identities were determined using MegAlign from the DNASTar software suite (Clewley and Arnold 1997). Phylogenetic trees were generated using the neighbor-joining (N-J) method with 1000 bootstraps by MEGA 5.1.2 (Kumar et al. 2008).

Quantitative real-time PCR (qPCR). Following total RNA extraction, samples were digested with RNase-free DNase I (TaKaRa) for 20 min at 37°C to remove genomic DNA. Approximately 2 μ g of RNA was transcribed into cDNA using a PrimeScript® RT reagent kit (TaKaRa) in a total volume of 10 μ L containing 2 μ L of total RNA, 2 μ L of 5 \times PrimeScript™ Buffer 2 μ L, 0.5 μ L of PrimeScript™ RT Enzyme Mix I, 0.5 μ L of Oligo dT Primer (50 μ M), 0.5 μ L of random hexamers (100 μ M), and 4.5 μ L of RNase-free double-distilled H₂O. Reactions were incubated at 30°C for 10 min, 42°C for 60 min, and then 70°C for 15 min. All cDNA samples were stored at -20°C until required.

The partial sequence of tongue sole β -actin and HIF-1 α were obtained and cloned into pMD18-T. The sequences were verified, and plasmid DNA were extracted using a QIAGEN plasmid mini kit (Qiagen, Valencia, CA, USA). Plasmid concentrations were determined using a spectrophotometer and measuring the optical density at 260 nm. All qPCR experiments were performed on an ABI Real-time PCR system (Applied Biosystems 7500). Amplifications were carried out in a final volume of 20 μ L containing 1 μ L of cDNA template, 10 μ L of SYBR premix ExTaq, 0.4 μ L of 50 \times Rox reference dye, 1 μ L of each primer, and 7.1 μ L of double-distilled H₂O. Each sample was analyzed in triplicate using a two-step method, with an initial denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Specificity of the amplification reaction was determined using dissociation curves with the temperature ranging from 60–95°C. Gene expression levels in normal tissue

were measured relative to the expression of the β -actin gene, and fold-changes after hypoxic exposure were calculated by a previously described method (Qi et al. 2011). Results were first analyzed using the ABI Prism 7500 SDS program with final statistical analysis conducted with Microsoft Excel. The significance of the data was evaluated using Student's *t*-test, with a *P*-value less than 0.05 considered statistically significant. All primers used in the qPCR assays were listed in Table 1.

RESULTS

Cloning and characterization of tongue sole HIF-1 α .

Using degenerate primers, a partial sequence of 1002 bp was cloned from liver cDNA. This sequence shared high identities with grass carp HIF-1 α , thereby confirming that the cloned sequence was a partial sequence of tongue sole HIF-1 α . Full-length of tongue sole HIF-1 α cDNA was obtained using 5' and 3' RACE (GenBank Accession No. HQ909440) which was found to be 3460 bp. This sequence contained a 5' untranslated region (5'-UTR) of 243 bp, an open reading frame (ORF) of 2208 bp encoding 735 amino acid (aa) residues, and a 3'-UTR of 1009 bp which includes five mRNA instability elements and a polyA signal sequence.

Sequence alignment showed that tongue sole HIF-1 α shared high identities with HIF-1 α in human (52.7%), frog (*Xenopus tropicalis*) (54.2%), zebrafish (*Danio rerio* Hamilton, 1822) (63.5%), Japanese flounder (*Paralichthys olivaceus*) (80.1%) and seabass (*Lateolabrax japonicus* Linnaeus, 1758) (81.8%). Tongue sole HIF-1 α also contained six conserved domains found in members of the HIF- α family: bHLH; PAS-A; PAS-B; C-terminal of PAS (PAC); N-terminal transactivation domain (N-TAD); and C-TAD. Meanwhile, some important motifs or amino acids needed for HIF-1 α functions were also observed in tongue sole HIF-1 α (Fig. 1). The N-terminal nuclear localization signal (N-NLS) at residues 17–64 and C-NLS (residues 637–642) play an important role in regulating nucleocytoplasmic trafficking

Table 1

Sequences of oligonucleotide primers used in the presently reported study

Primer	Sequence (5' to 3')	Purpose
HIF1DF1	TGA GCT CGG AG(C) CGG (C)AG A(G)A AG	Partial sequence cloning
HIF1DR1	A(G)T G(A)C CAC TGA GC(A) ACA (G)T(A) AGT T	
HIF1-5out	GGT GAG TCT CAT GAT GGA AGC C	RACE PCR
HIF1-5in	TGG GCC AGC TCG TAG AAA ACC T	
HIF1-3out	CTG GCT CCT GCA GCT GGA GAC	
HIF1-3in	CAG AGA TTC AGT TGC TGA ATG	
UPM		
Long	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT	
Short	CTA ATA CGA CTC ACT ATA GGG C	
QHIF1-F1	TGA GCT CGG AGC GCA GAA AG	qPCR
QHIF1-R1	CTG GGT TGA GCA GCT TCC TG	
Qactin-F1	CAG CCA TAC TGT GCC CAT CT	
Qactin-R1	TCC TTG ATG TCA CGC ACG AT	

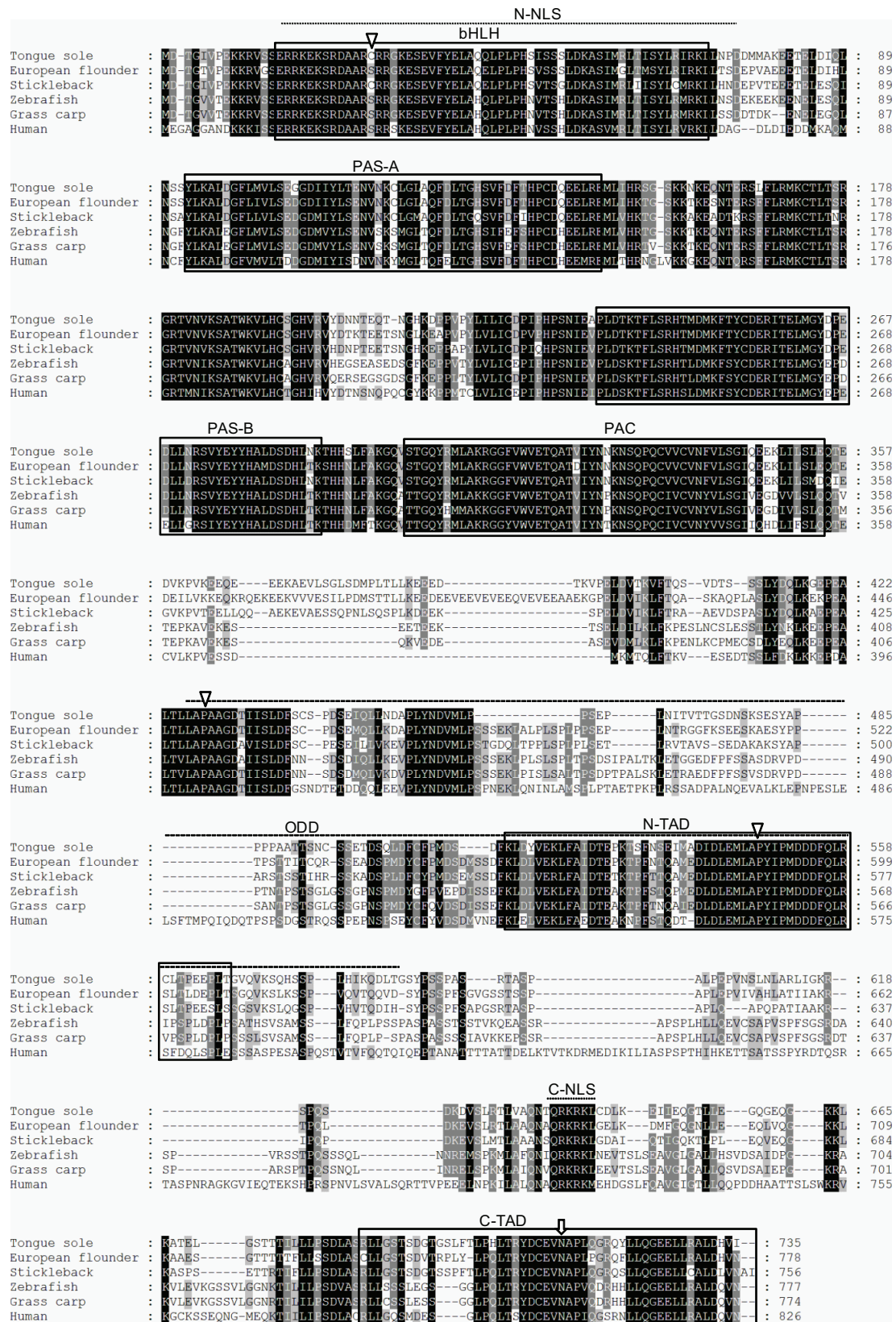


Fig. 1. Alignment of HIF-1 α amino acid sequences from different vertebrate species. Alignment was performed using ClustalW version 2.0 and GeneDoc; Gaps are marked by dashes; The HIF-1 α functional regions (bHLH, PAS-A, PAS-B, PAC, N-TAD, and C-TAD) are boxed; Domains are labelled above the alignments; The two conserved proline (Pro) residues are indicated with the inverted solid triangles; Residue substitutions are marked with an inverted triangle; The white inverted triangle indicates an asparagine residue; Sequences used for this analysis were obtained from NCBI database, and the accession numbers were listed in the caption of Fig. 3.

of the HIF-1 α protein (Luo and Shibuya 2001). Pro-402 mediates the degradation of HIF-1 α under normoxic conditions (Masson et al. 2001), while Pro-564, Tyr-565 and Ile-566 are conserved in the pVHL recognition sequence (PYIXXDDDFXL) within the N-TAD domain, and play an important role in controlling ubiquitin/proteasome degradation of HIF-1 α under normoxic conditions (Pereira et al. 2003). Leu-574 will likely modulates the proteolysis of HIF-1 α by determining the hydroxylation of Pro-564 (Kageyama et al. 2004). Similar to C-TAD in human HIF-1 α , the C-TAD of tongue sole HIF-1 α is leucine-rich and contains Asn-803, Leu-795, Cys-800, Leu-818 and Leu-822, which are required for the interaction of HIF-1 α with CBP/p300 transactivators and play an essential role in activation of the HIF-1 transcriptional pathway (Ruas et al. 2002). Comparison of the different domains in human, zebrafish and tongue sole HIF-1 α showed that the domains shared high identities among each other. The domains in tongue sole HIF-1 α shared higher homologies with those in zebrafish HIF-1 α than in human HIF-1 α (Fig. 2).

Using the grass carp HIF-4 α as an outgroup, a phylogenetic tree was constructed using MEGA 5.1.2 software. The tree was separated into two main clades, one containing HIF-1 α from mammals, amphibians, reptile and birds, and the second one contained fish HIF-1 α . The clade that contained fish HIF-1 α was divided into two clusters. One cluster consisted of HIF-1 α from zebrafish, grass carp and common carp (*Cyprinus carpio* Linnaeus, 1758.), while the second one was further divided into two closely-related sub-clusters with high bootstrap value (99%). The tongue sole HIF-1 α clustered closely with European flounder (*Platichthys flesus* Linnaeus, 1758) HIF-1 α (bootstrap value: 86) in the sub-clusters containing all HIF-1 α of marine teleost (Fig. 3).

Expression of tongue sole HIF-1 α under normoxic conditions. Under normoxic conditions, tongue sole HIF-1 α mRNA was highly expressed in the liver, moderately

expressed in the heart, spleen, kidney, stomach, blood and gills. Low levels of expression were observed for intestinal samples (Fig. 4).

Changes of tongue sole HIF-1 α transcripts under acute hypoxic conditions. Compared with the control group, the expression of tongue sole HIF-1 α mRNA was significantly up-regulated after 30 min of hypoxic stress ($P < 0.05$). Hence, as the time subjected to hypoxic stress was increased, so did the mRNA levels in different tissues. Liver HIF-1 α levels increased 28.99- and 28.78-fold after 90 and 120 min, respectively; while HIF-1 α expression levels in gills increased 17.08- and 19.18-fold after 90 and 120 min, respectively, of hypoxic stress (Fig. 5).

DISCUSSION

In the presently reported study the characterization and expression of HIF-1 α have been studied from a marine teleost—tongue sole, *Cynoglossus semilaevis*. The predicted length of amino acids sequence of tongue sole HIF-1 α was 735 aa which was very similar to HIF-1 α from other vertebrates. The HIF-1 α of tongue sole shared higher identities of amino acids sequence with fish HIF-1 α (63.5%–81.8%) than human (52.7%) and frog (54.2%) HIF-1 α . Similar to other vertebrates HIF-1 α , tongue sole HIF-1 α also contained six highly conserved domains of HIF- α family members (bHLH, PAS-A, PAS-B, PAC, N-TAD, and C-TAD) and some motifs or amino acids involved in modulating the degradation of HIF-1 α under normoxic conditions (e.g., Pro-402 and Leu574) (Masson et al. 2001, Kageyama et al. 2004). Those results suggested a high degree of conservation for HIF-1 α during evolution. Interestingly, there were some substitutions of amino acids observed in tongue sole and other vertebrates HIF- α . It had been speculated that those substitutions might affect protein conformation and could possibly function to counteract effects of low temperature on HIF-1 α conformation in other vertebrate (Morin and Storey 2005, Rytönen et al. 2007, Shen et al. 2010).

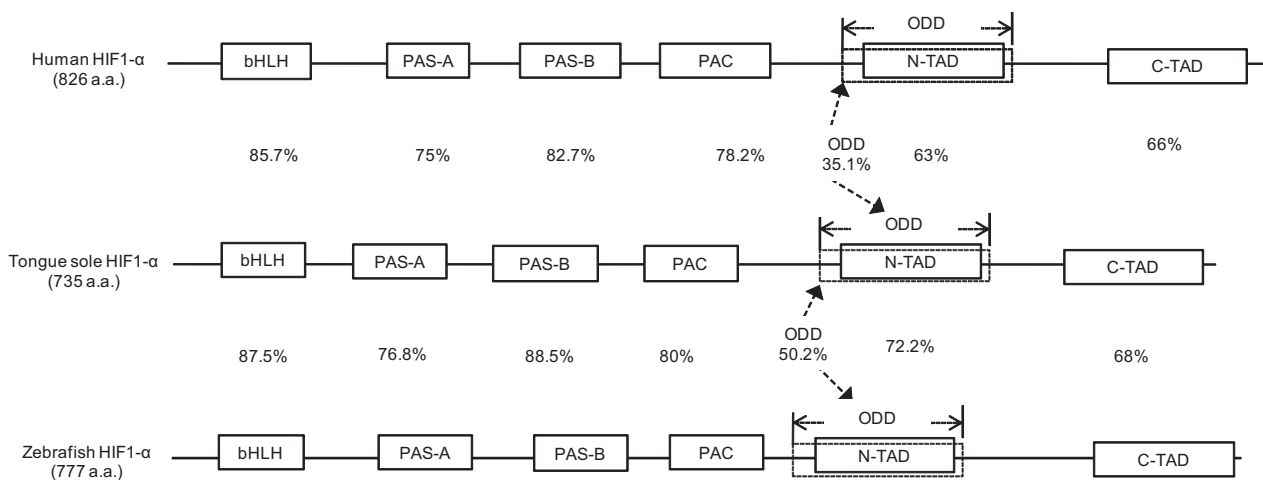


Fig. 2. Comparison of amino acid sequence identities among functional domains of HIF-1 α . The conserved domains include bHLH, PAS-A, PAS-B, PAC, N-TAD, and C-TAD; These are marked with boxes using a solid line; ODD is indicated by a box with a dotted line; Amino acid identities are presented as percentages.

Our phylogenetic tree analysis showed that the HIF-1 α from the evolutionary closely-related species were well clustered together—for example—grass carp and common carp. Thus, it was not surprising that HIF-1 α of two pleuronectiform fish: tongue sole and European flounder were clustered in the same small branch with high bootstrap value (86%). Rytönen et al (2007) found out that European flounder may be considered a hypoxia-tolerant fish. Hence, the combination of our previous studies and current analysis confirmed that the tongue sole was exactly a hypoxia-tolerant species.

Under normoxic conditions, tongue sole HIF-1 α transcription constitute was expressed in all the examined tis-

sues, highly in the liver, moderately in heart, spleen, kidney, stomach, blood and gills and low in intestine. This expression pattern was consistent with the studies in European seabass, Wuchang bream (*Megalobrama amblycephala* Yih, 1955) and Chinese sucker (*Myxocyprinus asiaticus*) (Terova et al. 2008, Shen et al. 2010, Chen et al. 2012). Unlike HIF-1 α in these three teleost, HIF-1 α of grass carp was highly expressed in the eyes and kidneys (Law et al. 2006). HIF-1 α of zebrafish was expressed mainly in the gill, ovary and brain (Rojas et al. 2007), and HIF-1 α of Atlantic croaker, *Micropogonias undulatus* (Linnaeus, 1766), was abundantly expressed in the brain

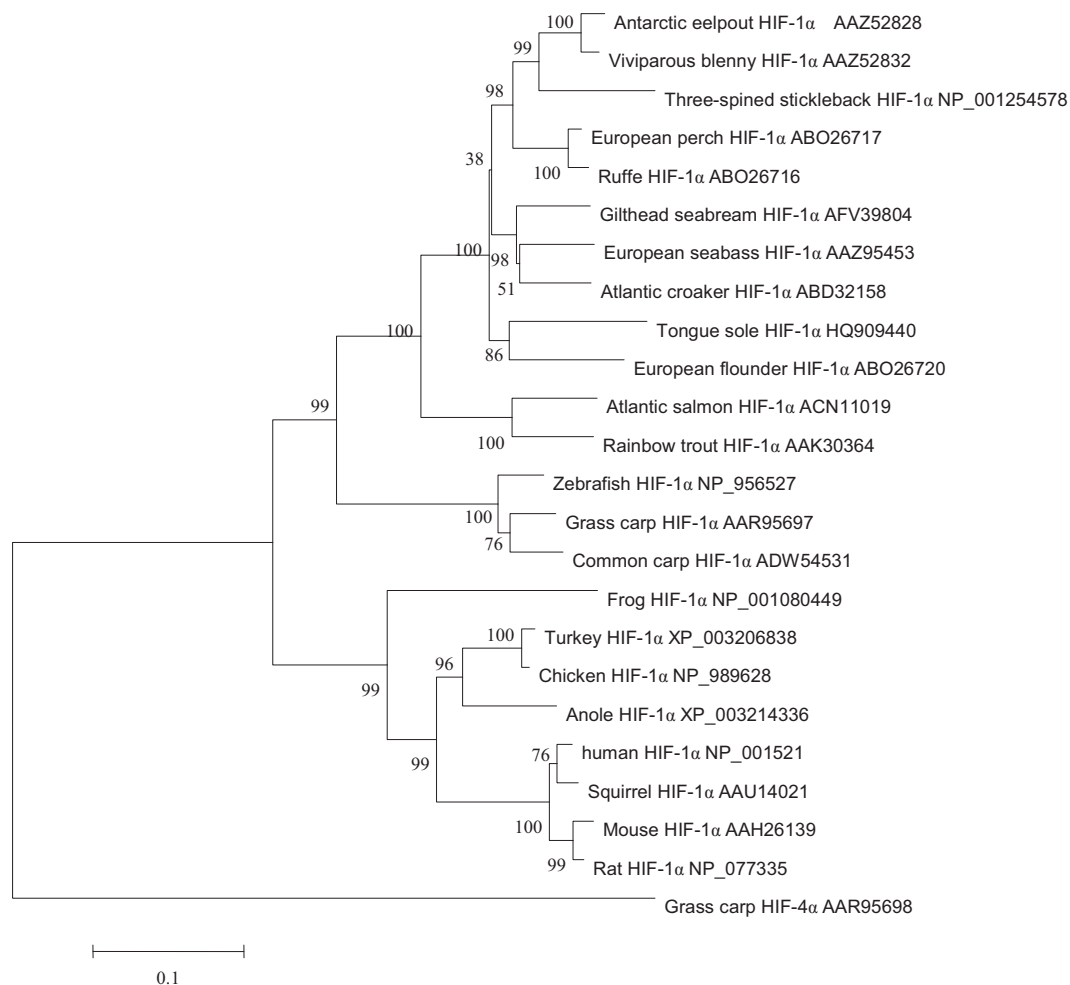


Fig. 3. Phylogenetic tree of HIF-1 α of selected vertebrate species showing the position of HIF-1 α of tongue sole, *Cynoglossus semilaevis*. Bootstrap values were generated by testing the tree 10000 times using MEGA 5.1.2. The GeneBank accession numbers of HIF-1 α sequence used for this analysis were: human *Homo sapiens*, NP_001521; squirrel *Ictidomys tridecemlineatus*, AAU14021; mouse *Mus musculus*, AAH26139; rat *Rattus norvegicus* NP_077335; trukey *Meleagris gallopavo*, XP_003206838; chicken *Gallus gallus*, NP_989628; anole *Anolis carolinensis*, XP_003214336; frog *Xenopus tropicalis*, NP_001080449; common carp *Cyprinus carpio*, ADW54531; grass carp *Ctenopharyngodon idella*, AAR95697; zebrafish *Danio rerio*, NP_956527; rainbow trout *Oncorhynchus mykiss*, AAK30364; atlantic salmon *Salmo salar*, ACN11019; eurpean flounder *Platichthys flesus*, ABO26720; tongue sole *Cynoglossus semilaevis* HQ909440; atlantic croaker *Micropogonias undulatus*, ABD32158; european seabass *Dicentrarchus labrax*, AAZ95453; gilthead seabream *Sparus aurata* AFV39804; ruffe *Gymnocephalus cernuus* ABO26716; european perch *Perca fluviatilis*, ABO26717; three-spined stickleback *Gasterosteus aculeatus*, NP_001254578; viviparous blenny *Zoarces viviparus*, AAZ52832; Antarctic eelpout *Pachycara brachycephalum*, AAZ52828; and HIF-4 α from grass carp was AAR95698. The GeneBank accesssion numbers for the sequeses used for this analysis were also listed next to the vertebrate names.

and gonads (Rahman and Thomas 2007). The distinctive expression of HIF-1 α suggested that this gene might possess different physiological functions in different fish species (Chen et al. 2012).

Under hypoxic conditions, HIF-1 α transcription levels also exerted distinctive patterns. In rainbow trout and Wuchang bream mRNA levels of HIF-1 α remained stable (Soitamo et al. 2001, Shen et al. 2010). However, in grass carp, the mRNA levels of HIF-1 α were increased significantly in the gills and kidneys after 4 h of hypoxia, yet decreased in the brain, heart and liver, and remained unchanged in the eyes (Law et al. 2006). Studies from Atlantic croaker and Chinese sucker also found an increasing pattern for HIF-1 α mRNA when the fish were subjected to short-term or long-term hypoxic stress (Rahman and Thomas 2007, Chen et al. 2012). In the presently reported study, following the short-term hypoxic stress the mRNA expression levels of tongue sole increased significantly in some examined tissues, suggesting that tongue sole HIF-1 α might exert its function at the early stage of hypoxia. Studies in mammals showed that members of the HIF family (HIF-1 α , -2 α and -3 α) might play different roles at different stage of hypoxia (Heidbreder et al. 2003, Ratcliffe, 2007). Whether members of fish HIF family also had distinctive function in different stage of hypoxia needs to be further studied. The significantly increase in the HIF-1 α level also explained why tongue sole was a hypoxia-tolerant species which could survive for a longer period than other fish species under acute hypoxic conditions (Wang et al. 2011, 2012).

In conclusion, we have identified the HIF-1 α isoform in a hypoxia-tolerant marine fish, tongue sole, and analyzed its expression pattern under normoxic and hypoxic stress. Sequence analysis showed that tongue sole HIF-1 α

possessed similar sequence length, shared higher identities and cluster well with other known HIF-1 α , revealing a high degree conservation of HIF-1 α during evolution. Tongue sole HIF-1 α begins up-regulated from 5 min to 120 min after hypoxia, indicating that it might play significant roles at the early stage of hypoxia.

ACKNOWLEDGEMENTS

This work was supported by the grants from the National Natural Science Foundations of China (no. 31302221) and Jiangsu Province (BK2011418) and the Post-doctoral Science Foundation of China (2012M521095 and 2013T60547).

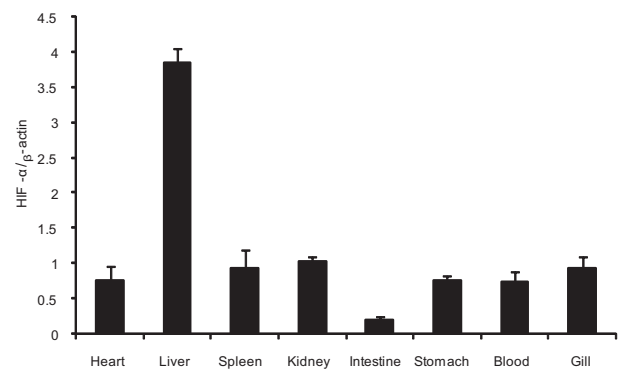


Fig. 4. Tissue distribution of HIF-1 α in healthy tongue sole, *Cynoglossus semilaevis*; Expression of HIF-1 α was measured by normalizing to the expression of β -actin using qPCR method; Each sample was analyzed in triplicate

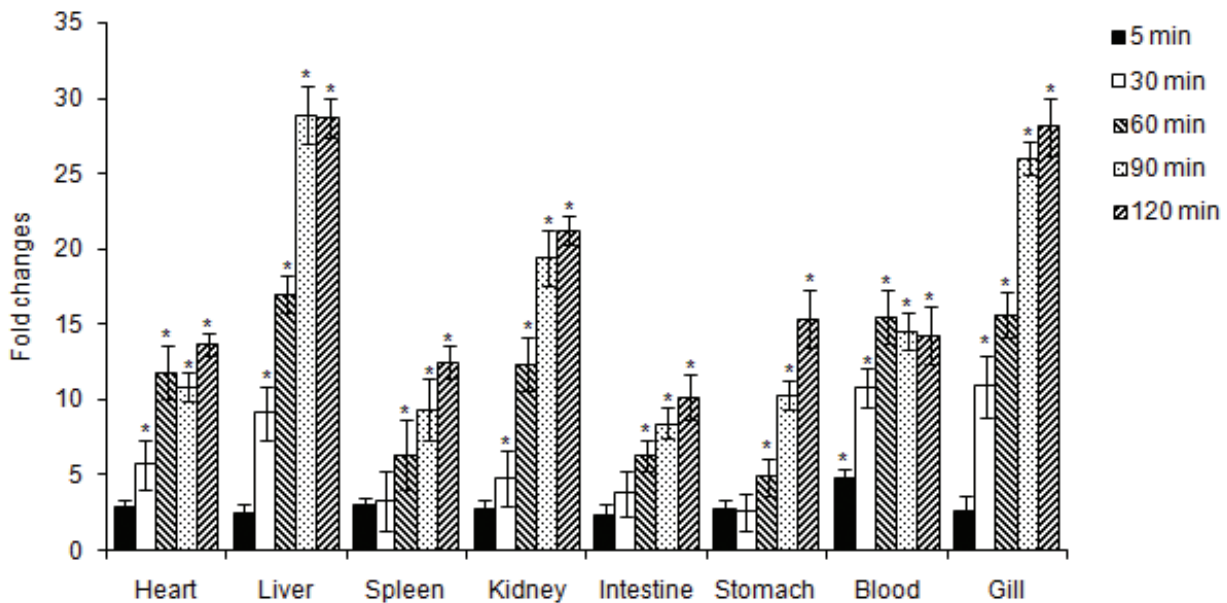


Fig. 5. HIF-1 α expression patterns in different tissues of tongue sole, *Cynoglossus semilaevis*, subjected to acute hypoxia for 5–120 min; The fold-change in HIF-1 α expression was calculated using the formula: fold changes = fold-change in target expression (experimental group/control group)/fold-change in reference gene expression (experimental group/control group); Samples were analyzed in triplicate; * $P < 0.05$

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Received: 14 March 2013

Accepted: 29 June 2013

Published electronically: 30 September 2013