

# Molecular characterization and expression analysis of three hypoxia-inducible factor alpha subunits, HIF-1 $\alpha$ , -2 $\alpha$ and -3 $\alpha$ in hypoxia-tolerant Indian catfish, *Clarias batrachus* [Linnaeus, 1758]

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**Abstract** The present study aimed at characterization of three HIF- $\alpha$  subunits, HIF-1 $\alpha$  -2 $\alpha$  and -3 $\alpha$  from hypoxia-tolerant *Clarias batrachus*, as well as to elucidate their expression pattern under short and long-term hypoxic conditions and identification of biomarker candidate. The complete cDNAs of HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  were 2,833, 4,270 and 3,256 bp in length, encoding 774, 818 and 628 amino acid residues, respectively. In *C. batrachus*, HIF- $\alpha$  subunits were structurally similar in DNA binding, dimerization, degradation and transcriptional activation domains, but differed in their oxygen-dependent degradation domains. Presence of c-Jun N-terminal kinase binding domain in HIF- $\alpha$  subunits was reported here for the first time in fish. In adult *C. batrachus*, three HIF- $\alpha$  mRNAs were detected in different tissues under normoxic conditions, however HIF-1 $\alpha$  was highly expressed in all the tissues studied, in comparison to HIF-2 $\alpha$  and -3 $\alpha$ . Short-term hypoxia exposure caused significant increase in three HIF- $\alpha$  transcripts in brain, liver and head kidney, while after long-term hypoxia exposure, significant up-regulation of HIF-1 $\alpha$  in spleen and -2 $\alpha$  in muscle was observed and HIF-3 $\alpha$  significantly down-regulated in head kidney. These observations suggest that the differential expression of HIF- $\alpha$  subunits in *C. batrachus* was hypoxic time period dependent and may play specialized roles in adaptive response to hypoxia. HIF-2 $\alpha$ , with its highly elevated expression in muscle tissues, can be a robust biomarker candidate for exposure to hypoxic environment.

**Keywords** *Clarias batrachus* · Hypoxia-inducible factors · Characterization · Expression · Biomarker

## Introduction

In aquatic ecosystems, hypoxic stress is known to cause different physiological (such as erythropoiesis, angiogenesis) and metabolic (glycolysis) changes in organisms, mediated by hypoxia-inducible factors (HIFs) which play a central role in adaptive processes [1]. HIFs are transcription factors that respond to changes in oxygen tension in the cellular environment. Transcriptional activation of HIF-regulated genes are mediated by the stabilization of HIF- $\alpha$  subunit in the cytoplasm, following its translocation to nucleus, where it dimerizes with HIF-1 $\beta$  to form the HIF complex [2]. This complex binds to a hypoxia response element (HRE) sites in the regulatory regions of hypoxia-inducible genes, leading to the activation of many HIF-regulated genes [3].

Three isoforms of the HIF- $\alpha$  subunit (HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$ ) had been reported earlier and widely studied in mammals [4], however, the information regarding fish HIFs is very limited. The HIF-1 $\alpha$  is the most ubiquitously expressed and best characterized in fishes and is recognized as a master regulator of hypoxic signaling [5–7]. HIF-2 $\alpha$  is similar in regulation to HIF-1 $\alpha$ , but its expression is restricted to certain cell types in fish species in response to hypoxia [5, 8, 10], while induction of HIF-3 $\alpha$  is thought to be mediated specifically by HIF-1 $\alpha$  and not by HIF-2 $\alpha$  [10]. In a hypoxia-sensitive fish Chen et al. [11] reported the expression patterns of HIF-3 $\alpha$  along with HIF-1 $\alpha$  and 2 $\alpha$ , during short term hypoxia however; no report was available for the expression patterns of HIF-3 $\alpha$  along with other two subunits in hypoxia-tolerant fish species.

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The Indian catfish *Clarias batrachus* (commonly known as “mangur”), is an air-breathing teleost, endemic to Indian subcontinent [12] and inhabits various habitats of low dissolved oxygen i.e. wetlands, swamps, rivers ponds and tanks, and burrows inside the mudflats during summer periods and thus, well adapted to adverse ecological conditions [13] and is a potential aquaculture species. These features make this fish as an useful model to study the mechanism of hypoxia tolerance as well as identification of candidate for hypoxia biomarkers in fishes. In general, low dissolved oxygen concentration during culture causes stress, decrease respiration and feeding activities. Moreover, fish is not able to assimilate the food consumed and thus resulting in reduced growth rate [14, 15].

Biomarkers are molecular, biochemical, cellular, or physiological responses that are used as indicators of environmental change [16]. Responses at the molecular level tend to be more sensitive and shifts in gene regulation upon exposure to hypoxia may lead to the establishment of transcriptional profiles or identification of genetic biomarkers associated with the extent and duration of hypoxic conditions [17, 18]. However, there is little progress in identifying and applying hypoxia biomarkers to monitoring and management regimens in aquatic systems.

In the present study, three distinct HIF- $\alpha$  genes (HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ ) from hypoxia tolerant *C. batrachus* were characterized and their expression patterns in response to short and long periods of hypoxia exposure were studied. In addition, attempts were made for identification of candidate for hypoxia biomarker in fish. Further, these studies will provide the basis for understanding the molecular responses and protective mechanisms to survive under hypoxic conditions, which will enable sustainable development in the yield and successful management of *C. batrachus* aquaculture.

## Materials and methods

### Animals and hypoxic conditions

For all the experiments, live fishes (30–80 g, 16–20 cm) were collected from commercial catches and brought to laboratory for acclimatization. During transportation, every care was taken not to cause distress to the fish by putting them in sufficient area and volume of water. Fish were acclimatized at normoxia ( $5.00 \pm 0.1$  mg/l, dissolved oxygen); at least for a month in tanks of 100 l capacity filled with 25 l of water at  $22 \pm 3$  °C. They were fed once a day with processed feed of goat liver or flesh and soybean powder. Feeding was stopped 48 h before the start of experiment. The hypoxic treatments to fish were as given in Tripathi et al. [19]. Briefly, fishes were divided into six

batches (three control and three experimental) of three fishes each. First experimental batch was kept in water with the decline in dissolved oxygen (DO) concentration by fish own respiration up to  $0.98 \pm 0.1$  mg/l of DO level, which was referred to as progressive hypoxia (PH). Other two experimental batches were kept at DO  $0.98 \pm 0.1$  mg/l for 1 and 6 h (short-term), which was maintained by aeration. This DO level was initially brought to in the similar way as PH. The corresponding control batches were kept under normoxia for same time periods as that of experimental once. After different treatments, fish were rapidly euthanized with MS222 to ameliorate suffering, prior to sample collection. These experimental fish were weighed and euthanized in a 300-mg/l concentration of tricaine methanesulfonate (MS-222, Sigma). Brain, muscle, liver, spleen and head kidney samples were collected and snap frozen in liquid N<sub>2</sub> until further analysis. The tissue samples were also collected from fishes in natural habitat (exposed to long term hypoxia) during summer periods and control fishes were corresponding lab acclimatized ones. The protocols followed were approved by IAEC.

### Identification of HIF transcript through SSH library construction

Total RNA were extracted from the tissue samples using the Nucleospin RNA II kit (Macherey–Nagel, Germany), according to the manufacturer protocol. cDNA were synthesised from 1  $\mu$ g total RNA using Superscript III first-strand synthesis supermix for qRT-PCR kit (Invitrogen) and SSH library (Super SMART cDNA Subtraction kit, Clontech) was constructed from control and hypoxia challenged fishes. Clones screened from SSH library from *C. batrachus* were sequenced and EST sequences were analyzed for the HIF transcripts expressed under hypoxia exposure.

### Identification of full length cDNA and sequence analysis of *C. batrachus*

Full length cDNAs for HIF-1 $\alpha$ , -2 $\alpha$ , and -3 $\alpha$  were identified from *C. batrachus* and were analyzed using GeneScan tool (<http://genes.mit.edu/GENSCAN.html>) for the identification of translated region. The cDNA were further analyzed for motif in 5' and 3' untranslated region (UTR) by UTRscan-ITB tools ([itbtools.ba.itb.cnr.it/utrscan](http://itbtools.ba.itb.cnr.it/utrscan)). Protein sequence identities were verified using the blastx programme at NCBI database (<http://blast.ncbi.nlm.nih.gov/>). The three HIF- $\alpha$  subunits from *C. batrachus* were aligned together using the ClustalW2 programme (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The deduced amino acid sequences of different HIF- $\alpha$  subunits from fishes (having the three subunits together) and *Homo sapiens* (Table 1)

were obtained from entrez database (<http://www.ncbi.nlm.nih.gov/Entrez/>) and aligned using the ClustalW2 programme. The multiple sequence alignments were adjusted manually to the regions corresponding to different domain sequences. Domain identification of individual subunit was according to *Homo sapiens* HIFs, as present in uniprot database (<http://www.uniprot.org/>). Numbers of serine residues in oxygen dependent and degradation (ODD) domain (identified accordingly Rahman and Thomas [5]; and Chen et al. [11]) were counted in HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  subunits of hypoxia-tolerant and susceptible fish species. The assignment of c-Jun N-terminal kinase (JNK) binding domain (JBD) and LXXLL motif was according to Antoniou et al. [20] and Plevin et al. [21], respectively. While, bipartite type nuclear localization signal (NLS) was assigned according to Luo and Shibuya, [22].

#### Quantitative real-time PCR and statistical analysis

The expression pattern of HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  transcripts in *C. batrachus* were determined by qRT-PCR in all the tissues sampled after short and long-term hypoxia exposure, and their expression was normalized to alpha-tubulin ( $\alpha$ -tub), ribosomal protein L30 (*rpl30*) and elongation factor-1 alpha (*elf1 $\alpha$* ) expression, taken as reference gene under hypoxia (unpublished data). Primers (Table 2) were designed using the PrimerQuestSM (Integrated DNA Technologies). Primer efficiencies (E) were calculated from tenfold dilution series to make standard curves from which E was calculated according to the formula

$E = 10^{-1/\text{slope}}$ . The specificity of the primer sets used was confirmed by the presence of a single band of correct size on gel electrophoresis in addition to the presence of a single peak in the dissociation curve analysis. All qRT-PCR reactions were performed in 20- $\mu$ l total reaction volume (18  $\mu$ l master mix and 2  $\mu$ l undiluted cDNA made from 1  $\mu$ g RNA/PCR product). The master mix contained 7.2  $\mu$ l H<sub>2</sub>O, 0.8  $\mu$ l of each primer (0.4  $\mu$ M final concentration) and 10.0  $\mu$ l of the SYBR Green Mix (Roche Applied Science, Laval, PQ, Canada). The following cycling conditions were used [1]: denaturation, 5 min at 95 °C; [2] amplification repeated 40 times, 10 s at 95 °C, 10 s at 55 °C, and 15 s at 72 °C with ramp rate of 4.4, 2.2, and 4.4 °C/s, respectively; [3] melting curve analysis, 5 s at 95 °C and 1 min at 65 °C with ramp rate of 4.4 and 2.2 °C/s, respectively, then up to 95 °C at a rate of 0.1 °C/s; [4] cooling, 10 s at 40 °C with ramp rate of 2.2 °C/s. For each treatment and control, 3 individuals were analyzed in duplicate and reactions were performed in a Light Cycler 480 (Roche Applied Science) system. Crossing point values (Ct) values were obtained by employing the second derivative maximum method. Crossing point values were compared and converted to fold differences by the relative quantification method using the relative expression software tool (REST) 384 v. 2 [23]. *P* values below 0.05 were considered to be statistically significant.

## Results

### Characterization of *C. batrachus* HIF-1 $\alpha$ , -2 $\alpha$ and -3 $\alpha$ cDNA transcript

The characteristics of the full-length cDNAs of HIF-1 $\alpha$  (Accession No. KC011345), HIF-2 $\alpha$  (Accession No. KC011346) and HIF-3 $\alpha$  (Accession No. KC011347) are given in Table 3. The characterization of HIFs cDNA revealed that the length of open reading frame of HIF-2 $\alpha$  (2,454 bp) was largest in comparison to HIF-1 $\alpha$  (2,322 bp) and HIF-3 $\alpha$  (1,884 bp). The UTRscan analysis revealed that all the three units had known RNA functional elements, K-box and Musashi Binding Element (MBE) motifs, while BRD-box motif was common to HIF-2 $\alpha$  and HIF-3 $\alpha$  (Table 3). However, only one MBE site was identified in HIF-1 $\alpha$  in comparison to four sites identified in HIF-2 $\alpha$  and HIF-3 $\alpha$  (Table 3). The GY-box motif was unique to HIF-3 $\alpha$  subunit.

The three HIF protein forms identified in *C. batrachus* were highly similar in their DNA binding, basic helix-loop-helix (bHLH), Per-ARNT-Sim (PAS)-A and -B and PAS associated C-terminal (PAC) domain, while varying in composition of ODD and N-terminal transactivation (TAD-N) domains (Fig. 1). HIF-3 $\alpha$  had higher similarity

**Table 1** HIF- $\alpha$  protein sequences from different fish species used in the present study for domain identification and sequence alignment analysis

S.No.	HIF- $\alpha$ subunit	Organism	Accession number
1	HIF-1 $\alpha$	<i>Danio rerio</i>	NP_956527.1
2	HIF-1 $\alpha$	<i>Myxocyprinus asiaticus</i>	ADZ23997.1
3	HIF-1 $\alpha$	<i>Megalobrama amblycephala</i>	ADF50043.1
4	HIF-1 $\alpha$	<i>Ctenopharyngodon idella</i>	AAR95697.2
5	HIF-1 $\alpha$	<i>Homo sapiens</i>	NP_001230013.1
6	HIF-2 $\alpha$	<i>Danio rerio</i>	NP_001034895.1
7	HIF-2 $\alpha$	<i>Myxocyprinus asiaticus</i>	ADZ23995.1
8	HIF-2 $\alpha$	<i>Ctenopharyngodon idella</i>	AAT76668.1
9	HIF-2 $\alpha$	<i>Megalobrama amblycephala</i>	ADF50044.1
10	HIF-2 $\alpha$	<i>Homo sapiens</i>	NP_001421.2
11	HIF-4 $\alpha$	<i>Ctenopharyngodon idella</i>	AAR95698.1
12	HIF-3 $\alpha$	<i>Myxocyprinus asiaticus</i>	ADZ23996.1
13	HIF-3 $\alpha$	<i>Danio rerio</i>	AAQ94179.1
14	HIF-3 $\alpha$	<i>Megalobrama amblycephala</i>	ADF50045.1
15	HIF-3 $\alpha$	<i>Homo sapiens</i>	NP_071907.4

**Table 2** Gene-specific primers for RT-PCR analysis of HIF-1 $\alpha$ , -2 $\alpha$ , -3 $\alpha$  and housekeeping genes from *C. batrachus* used in the present study

Gene	Accession number	Primers	Primer efficiency (E)	Amplicon size (bp)
<i>Hif-1<math>\alpha</math></i>	KC011345	F: CAAATGCATGGGACTCACAC R: GCACCATCATCTCCCTGACT	1.96	98
<i>Hif-2<math>\alpha</math></i>	KC011346	F: TCTCTCACTCTCTTTACATTGCCT R: TCAGCACTCCGATCCACATCTACT	1.98	157
<i>Hif-3<math>\alpha</math></i>	KC011347	F: TCTTCATGGAGTTGTGTTCTCAGT R: AACAAAGACATATAGGATTCGACA	1.97	120
<i>rpl30</i>	JK489341	F: TGCACGCTGGCCATCATTGAC R: AAATGGCTCTACTTCTCGCCCT	1.99	85
$\alpha$ - <i>tub</i>	JK488355	F: GTCGAGCAAGGTAACGAACTTCAC R: GTGGATGGAGATACTCACGCAT	1.92	111
<i>elf1a</i>	GT157722.1	F: AATGGTTTAGATCTGCACCTGTTGCC R: ACCTTTATTAATTTCTCAGCAGCTTTCTT	1.99	70

Housekeeping genes: *rpl30* ribosomal protein L30,  $\alpha$ -*tub* alpha tubulin, *elf1a* elongation factor-1alpha

**Table 3** The characteristics of full length cDNA and encoded ORF of three HIF- $\alpha$  subunits of *C. batrachus*

Characteristics	HIF-1 $\alpha$	HIF-2 $\alpha$	HIF-3 $\alpha$
Complete CDS (bp)	2,833	4,270	3,256
5' UTR (bp)	311	204	117
ORF bp (Amino acids)	2,322 (774)	2,454 (818)	1,884 (628)
3' UTR (bp)	200	1,615	1,255
Kozak motif	AGGAATGG	AAAGATGA	CATTATGA
PAS	–	–	AATAAA
Motifs (5'/3')			
K-box	722,729/1067,1074	3951,3958	3167,3174
MBE	3,7	3743,3747/3908,3912/3931, 3935/4179,4183	92,96/2507,2513/2804, 2808/2926,2930
BRD-box	–	1834,1840	2839,2845/2948,2954
GY-box	–	–	442,448

with HIF-1 $\alpha$  (42 %) than HIF-2 $\alpha$  (38 %); however, C-terminal trans-activation (TAD-C) domain was found neither in HIF-3 $\alpha$  protein of *C. batrachus* nor in other vertebrate species (Fig. 2). The deduced amino acid sequences of HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  of *C. batrachus* showed high amino acid identity to other hypoxia tolerant (63–83 %) and susceptible (64–74 %) fish species. In addition, the number of serine residues at the *C. batrachus* ODD domain of HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  was found to be 41, 31 and 12 residues, respectively.

Comparison through multiple sequence alignments of three HIF- $\alpha$  subunits with each other from *C. batrachus* and other fishes showed that there were unique substitutions in various domains of each subunit. Altogether, a total of 16 unique substitutions were found in HIF-2 $\alpha$  as compared to HIF-1 $\alpha$  and -3 $\alpha$ , while 31 in HIF-3 $\alpha$  subunits, as compared to other two subunits, in all domains (Fig. 2, shown with filled triangle and inverted filled triangle). However, only 6 substitutions were found in HIF-1 $\alpha$  subunit. Apart from these substitutions,

one insertion of Glycine (G) was observed in core ODD domain of HIF-2 $\alpha$  subunit of all the fishes examined. Interestingly, HIF- $\alpha$  subunits of *C. batrachus* contain cysteine (C) at position 26 (HIF-1 $\alpha$ ) and 25 (HIF-2 $\alpha$  and HIF-3 $\alpha$ ) in the bHLH domain (Fig. 2, marked as filled circle), which aligned with a serine at similar positions in other fishes (Fig. 2). This finding was further supported by the presence of “C” at similar position in *Ictalurus punctatus* (NM\_001200301), which is also a silurid fish. As identified in other cypriniformes fishes, *C. batrachus* also has conserved aspartic acid (D) and histidine (H) at +5 and +7 amino acid position to asparagine-803 (N-803) (marked as # in Fig. 2), in TAD-C domain of HIF-1 $\alpha$  and -2 $\alpha$ , which was consistent with presence of these residues in *Ictalurus punctatus* (NM\_001200301) also, while, HIF-2 $\alpha$  protein of all fish species have glycine (G) and leucine (L) at similar position.

Presence of JBD, upstream to ODD domain, was observed in the all the three HIF- $\alpha$  subunits, including in *C. batrachus*, and the amino acid composition of JBD was variable in these

	basic helix-loop-helix domain	PAS-A domain	
<i>CbHIF-1α</i>	MDTGVVTEKRRVSSERRKEKSRDAARCRRGKSESEVFYELAQQQLPLPHNVTSHLDKASIMRLIISYLRMRKLLNS ---DEEENESELESQLSNLFYKALD		[97]
<i>CbHIF-3α</i>	MTT---TVAKRPSSEQRKRVLRDAARCRRSQETEVFYELAHSLPLSRRTITSHLDKAGIMRVTLISYLRMRHLLHSSWTKVSETLEEEEEPTDAFYQQALAG		[97]
<i>CbHIF-2α</i>	MTAE--KDKKRSTTERREKSRDAARCRRSKETEVFYELAHLELPLPHSISSHLDKASIMRLAISFLRTCKLLTS---GCNTSETDIERQLDLSLYLKSLEG		[95]
<i>CbHIF-1α</i>	FLMVLSEEDGMVYLSENVCKCMGLTQFDLIGHSIFDFAHPCDLEEVREMMVHRPQKTKVQN - TERSFFLRMKCTLTSGRGTVNIKSATWKVHLHCTGHV		[196]
<i>CbHIF-3α</i>	FILVLSEEGDMVFLSENVNKFIGITQLELLGQCVDYVHPCDQEEELKDLLTKPGLFKKSECTECNFFLRKSTLTSRGTVNIKSATWKVHCTGYM		[197]
<i>CbHIF-2α</i>	FISVVTSDGDMIFLSENINKFMGLTQVELIGHSIFDFTHPCDHEIRENLSLKTGAGNKGKELTTERDFFMRMKCTVTSRGTVNLKSASWKVHLHCTGHL		[197]
		<b>PAS-B domain</b>	
<i>CbHIF-1α</i>	RVQEHSDGSGEGGFKPEPVTVLVLIICEPIPHPSNIEVPLDSKTFLSRHTLDMKFSYC DERITELMGHEPDDLNRSVYEYHALDSDHLNKTTHNLFAKG		[296]
<i>CbHIF-3α</i>	KTLNQ----EGEASSLAVSYMTLLECEPIPHPSVCEVPLDSSTFLTRHTMDLHFSQCDGRVTELVGYHPNDLIGRSAYDFFHALDFDHLTRCLHLVLLSKG		[292]
<i>CbHIF-2α</i>	KMYNSCPTRTLGLKESPLTCVVMLECEVPVPHPSNIDTFFDSKTFLSRHSMDMKFTYCDERVTELIGYNPDDLGRSVYEFYHALDSESVTRSHQNLCFKG		[297]
		<b>PAC Domain</b>	
<i>CbHIF-1α</i>	QATTGQYRMLAKKAGYVWVETQATVIYNPKNSQPQCIVCVNYVLSGIVEEDTVLSLQQTLPKEKTEEKEELQIEHEDSSVMDMMKLFKDKHLSCSVESPK		[396]
<i>CbHIF-3α</i>	QVCTSRYRFLAKNGGFVWVETQATVLYNSKTSQPEAVVCLNFIILSAVEADVVFSIEQTRSDLMQDKLSVIEVEDSD -----DEMSTSSSE--		[378]
<i>CbHIF-2α</i>	QAVSGQYRMLAKYGGFVWVETQGTVIYSSRNSQPQCIVCVNYVLSDIEEKSTVFSKDTESLKLKTHMSSFFSQTGST -----IDPEN--		[379]
		<b>ODD Domain</b>	
<i>CbHIF-1α</i>	SLYDTLKDPEALTVLAPAPGDTFVSLDFDSDSLDQVLKEVPLYNVDMPLPSTSEKLPPLPLSPLAPSDPSPALVKMESGGEEFSSISSTSRTPDTASTPP		[496]
<i>CbHIF-3α</i>	LYHSLKDDPEDLLQAPAPGDTIIPLTG-----HTELFVFPSSPNSVPKCKELCTP-----KLR-----QLLSPIFDSSCP		[446]
<i>CbHIF-2α</i>	-RLIKFKEEPEDLTHLAPTGDGVIPLDFGR-----PSFEEFPVYSKVPVHPSANHSVTDGHNMPKLSANFSIIQASPASSATPVLLSSCSL		[465]
		<b>TAD-N</b>	
<i>CbHIF-1α</i>	S-STSSSEASSPHYFVHSDINFKLDLVEKMFATDTEGNSDFNTD-EMGDLLEMLAPYIPMD-DDFQLH-----IPSP		[567]
<i>CbHIF-3α</i>	T-SPDHSSGD-----EFSMDTGEVEKFFADKPEETQKKTME-DMDDLDDMLAPYISMD-EDFQLT-----FLP		[507]
<i>CbHIF-2α</i>	LNSPDEYKGP-----VDDLKMEITEKLFAMDTQGNKSNYQETDLSDDLLETAPYIPMDGDFQLNPIQOEPRPETVAHGISQYFSFSNIANFFQ		[555]
		<b>Core ODD</b>	
<i>CbHIF-1α</i>	SLNP-----LPSGPQTGSAIKS-----IFQRSPCCPSPTSST---SLKPAPPSPVSPPLHLLQELSSIFGTFPFNEASQTLDNSPE		[639]
<i>CbHIF-3α</i>	KLP-----EADTQT-----PEAQTTASK---KRALDEEDDMPS-----LTSNWEK-----		[544]
<i>CbHIF-2α</i>	PLTPPPGAHFQPDPHSANEQARSSTTVEPWPIFYASPMPLAHYTNPASTPLSSVGGHSHLHWPPDPPINYSNIKGGVIDSLEVKHPQCALQSNHLSLQ		[655]
<i>CbHIF-1α</i>	PGIPLAKRMQVDDDEFASKVLTQVQGRKRKLEITISLSQAVG-----LDALLQS---VDDVGEPEPKRAKVLVLEKGSLLGG-----		[712]
<i>CbHIF-3α</i>	---RRKNCPIDEFLLS-----RSSLMDLQVADG-----LEELDP-----DRWKPSQ---LLTDRDPVLGA-----		[594]
<i>CbHIF-2α</i>	NLRSVENYEPCKAFRDISPARLTVPTTMRKRSFQISMGVSHATTPLDVWKRKMSCAVNLNRMSQSSSALTDEHVHQHKTRYQGNQTALGKKDYTEQC		[755]
		<b>TAD-C</b>	
<i>CbHIF-1α</i>	-----NKTILILPSDLASRLSSSFESSGGPLQLTRYDCEVNAPIQDRHPLLQGEELLRALDQVNGV		[774]
<i>CbHIF-3α</i>	-----TKALCYT-----AALMRNSFSSQ--PPDLMAQLTGTISPLA		[628]
<i>CbHIF-2α</i>	CNYSDYMNVPNTKMQGVASRLLGSPFEMY-CLPELTRYDCEVNAPLQGNLHLLQGSDLLCALDQAT--		[818]

**Fig. 1** Multiple alignment of the deduced amino acid sequences of *C. batrachus* (HIF-1α; -2α; -3α). Domains of HIF-αs are marked on the alignment. The domain characterization of individual subunit was according to *Homo sapiens* HIFs, as present in uniprot database

subunits: HIF-1α (KDEPEALTV); HIF-2α (KEEPEDLTH); HIF-3α (KDDPEDLLQ) (Fig. 2). This is the first study showing the presence of JBD in HIF-α subunits of fishes. Apart from JBD, it was also observed that three LXXLL motifs were present in HIF-3α of *C. batrachus* in PAS-A, -B and ODD domains; however, in other fish species, this motif was found only once in ODD domain (Fig. 2). Also, all the three HIF-α subunits of *C. batrachus* was found to have bipartite type NLS, downstream to ODD domain, similar to those present in other fish species (Fig. 2). However, the length of spacer sequences (X)<sub>n</sub> between two adjacent basic domains (KR-(X)<sub>n</sub>-KR) was 27 amino acids in HIF-1α, 19 in HIF-2α and 16 in HIF-3α of *C. batrachus*, while in other fishes, it was one or two amino acids more.

#### Expression profile of HIF-α isoforms

#### Tissue-specific expression under normoxia

The qRT-PCR results showed that in *C. batrachus*, three HIF-α mRNAs were constitutively expressed in the all the tissues tested (brain, liver, muscle, spleen and head kidney), under normoxic condition. In all tissues transcript level of HIF-1α was highest in comparison to HIF-2α and HIF-3α, which were negligible (Table 4).

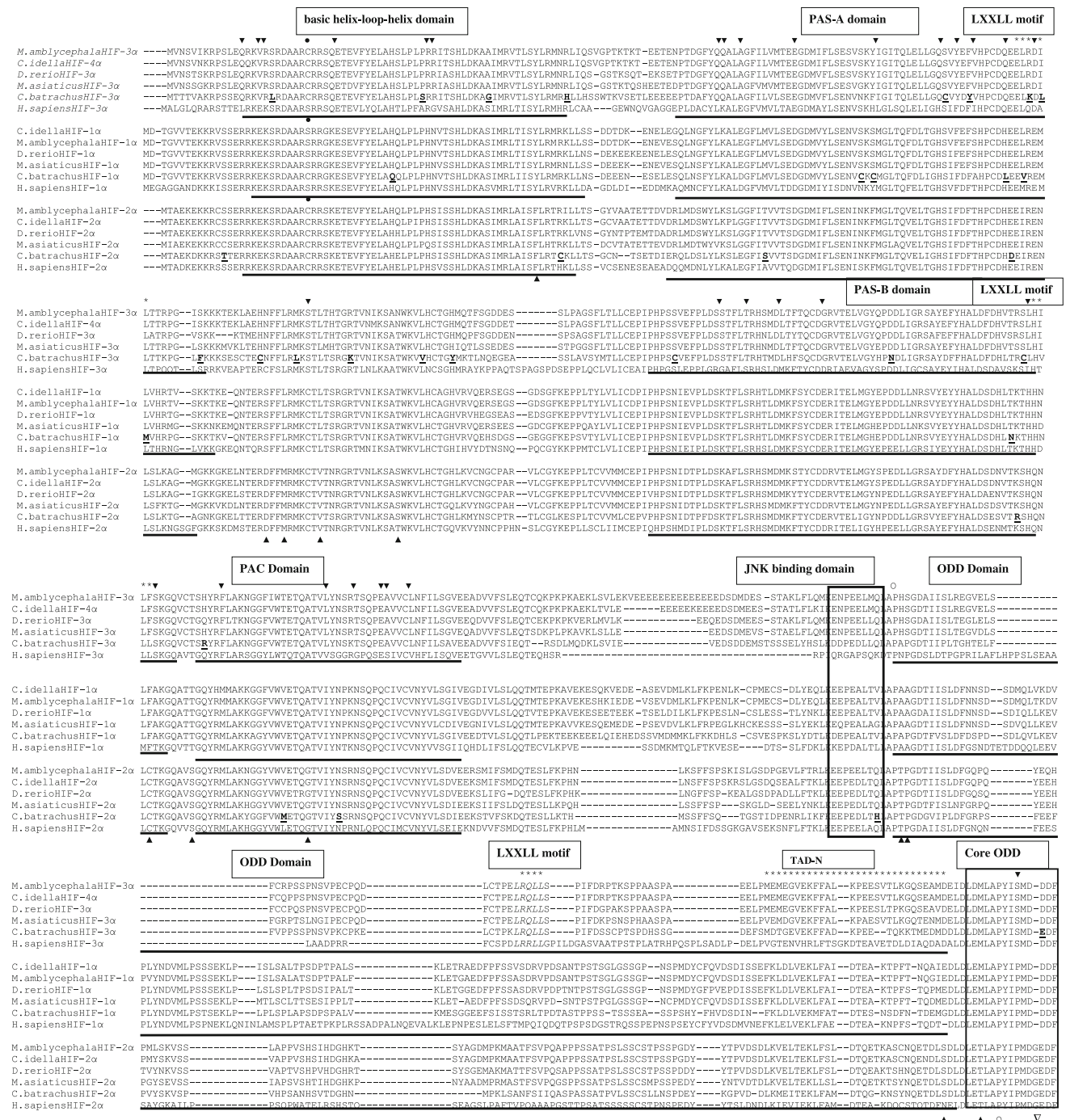
#### After short and long-term hypoxia

During short-term hypoxia exposure, following PH the three HIF-α transcript were significantly up-regulated in brain and head kidney tissues, whereas no significant difference was found in other tissues. After 1 h of hypoxia exposure at 0.98 mg/l DO, HIF-1α was significantly up-regulated in liver; however, it was down-regulated in head kidney tissue, whereas after 6 h of hypoxia exposure at similar DO concentration, HIF-1α and -3α were significantly up-regulated in head kidney tissue only (Fig. 3a–c). However, after long-term (natural) hypoxic conditions, the expression of HIF-1α and -2α was found to be significantly up-regulated in spleen and muscle, respectively, whereas HIF-3α was significantly down-regulated in head kidney in comparison to normoxic conditions (Fig. 3a–c).

#### Discussion

#### Characterization of HIF-α subunits: cDNA and protein

The present study is the first report from a freshwater hypoxia-tolerant fish species *C. batrachus*, on the characterization of three HIF-α subunits (HIF-1α, -2α and -3α) for



**Fig. 2** Multiple alignment of the deduced amino acid sequences of *C. batrachus* (A, HIF-1 $\alpha$ ; B, HIF-2 $\alpha$ ; C, HIF-3 $\alpha$ ) and other hypoxia-tolerant and sensitive fish species. Domains of HIF- $\alpha$ s are marked on the alignment. Unique substitutions among different subunits in different fish species are marked with up and down arrow heads (filled triangle and inverse filled triangle) and substitutions specific to *C. batrachus*, are highlighted in bold and underlined. The two conserved proline residues within the ODD domain are indicated by open circle,

and the asparagine residue (Asn-803 in human) in TAB-D which controlled is indicated by sign #. The filled circle indicates the serine and cysteine residues within bHLH domain, while open triangle indicates the insertion of glycine (G) residue in core ODD domain of HIF-2 $\alpha$ . Blocks represent core ODD, JBD and NLS. The LXXLL motif is highlighted with asterisk. The shown domains were identified according to *Homo sapiens* HIF- $\alpha$  sequence, as presented in uniprot database

their molecular constructions and transcriptional control. The homology of *C. batrachus*, HIF- $\alpha$  proteins to other fish species (60–83 % to HIF-1 $\alpha$ , 56–81 % to HIF-2 $\alpha$  and

55–55 % to HIF-3 $\alpha$ ) showed high amino acid conservation in characterized domains. Unlike highly structured introns, the UTRs of mRNAs are not generally evolved to adopt

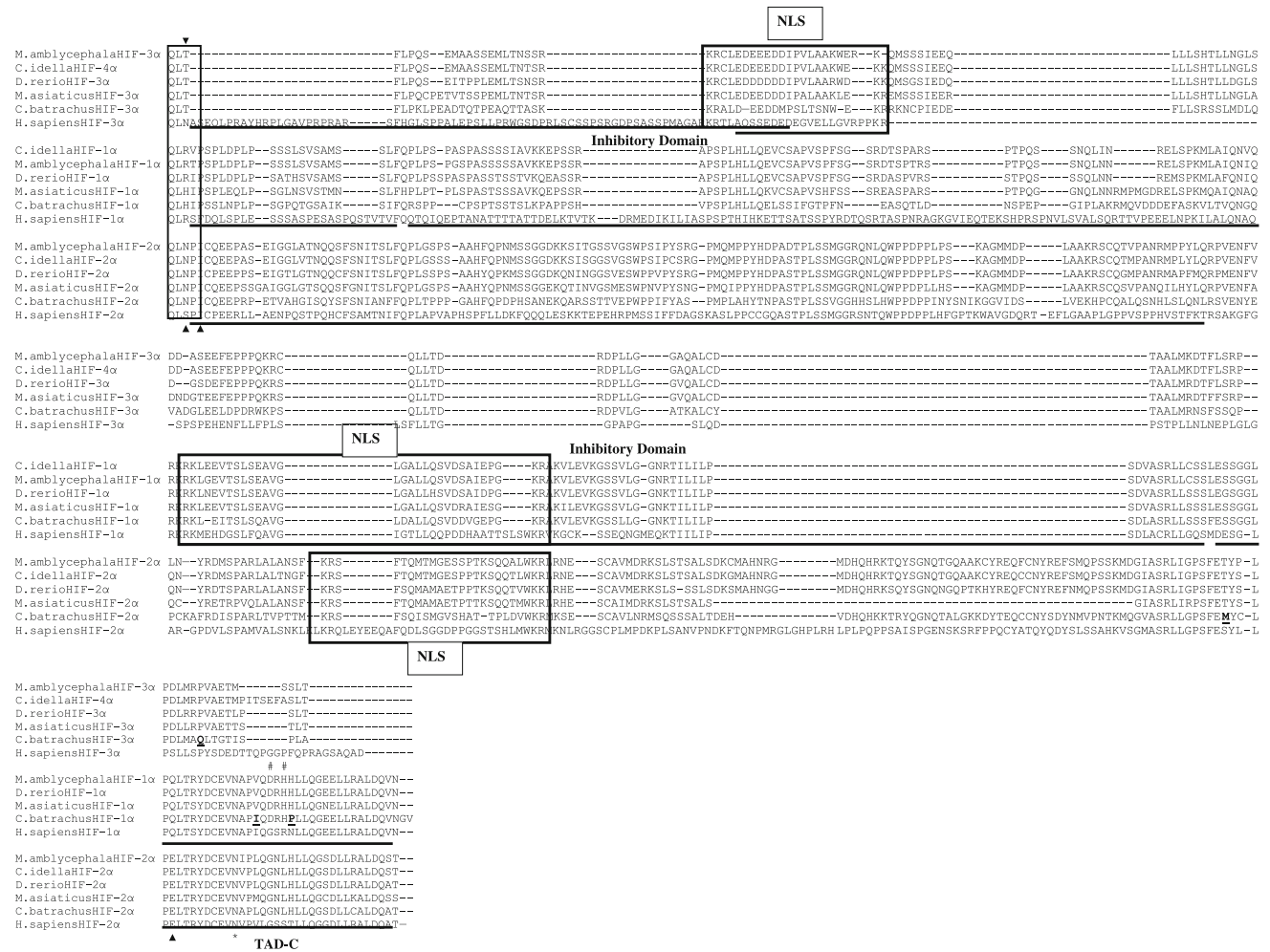


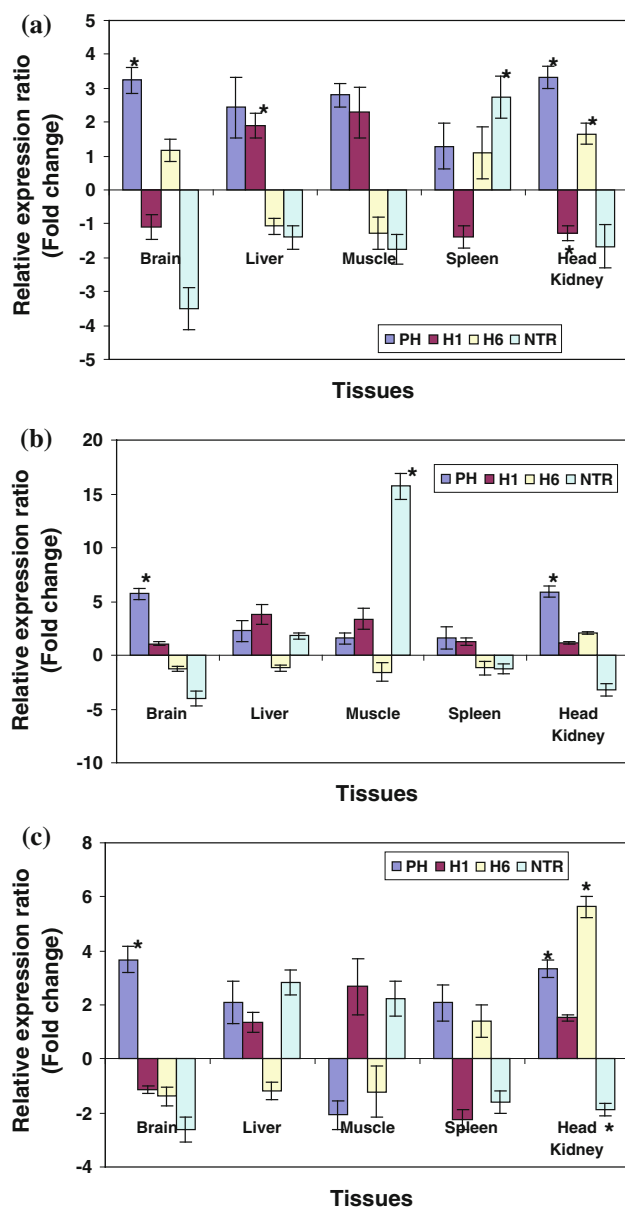
Fig. 2 continued

**Table 4** Expression level (values given for mRNA transcript concentration in ng/μl) of HIF-α subunits in five tissues of *C. batrachus* under normoxia (5.00 ± 0.1 mg/l, dissolved oxygen)

Tissues	HIF-1α	HIF-2α	HIF-3α
Brain	0.0543 ± 0.0069	0.0007 ± 0.00017	0.0006 ± 0.00002
Liver	0.0391 ± 0.0065	0.0004 ± 0.000004	0.0001 ± 0.00001
Muscle	0.0341 ± 0.0148	0.00001 ± 0.000001	0.0001 ± 0.00004
Spleen	0.0168 ± 0.0030	0.0012 ± 0.000354	0.0004 ± 0.00006
Head Kidney	0.0693 ± 0.0041	0.0007 ± 0.000070	0.0005 ± 0.00001

single, well-defined structures; however UTRs are the regulatory elements of genes, acting as controllers of translation and RNA decay, as well as targets for RNA interference (RNAi) [24]. The presence of K-box and MBE motif in all the HIF subunits of *C. batrachus* suggests that these subunits recruit and bind the Musashi protein, an evolutionarily conserved RNA-binding protein, known to have the ability to regulate mRNA translation [25] and provides binding platforms for the 5' seed regions of miRNAs, which negatively regulates their transcription [26]. While, BRD-box and the GY-box, sequence motifs located in the UTRs of many genes are implicated in Notch signaling [26].

When functional domains of HIF-α subunits in *C. batrachus*, were compared, it was observed that these subunits were similar, but distinct in their basic helix-loop-helix-PAS proteins, which have been postulated to activate hypoxia responsive genes [27]. Presence of typical motifs and domains (DNA binding, bHLH, PAS, TAD-N and core ODD) similar to that of other vertebrates, showed that all the three HIF-α subunits are similar in their DNA binding [28], dimerization, degradation and transcriptional activation [29] properties. Additionally, presence of “C” at position 26 and 24 in *C. batrachus* HIF-α subunits showed that their stability and DNA-binding activity are regulated



**Fig. 3** Relative HIF- $\alpha$  mRNA expression in *C. batrachus* following short-term (PH, 1 and 6 h) and long-term (Natural) hypoxia exposure (a–c HIF-1 $\alpha$ , -2 $\alpha$ , and -3 $\alpha$ , respectively). Y-axis represents the mean  $\pm$  SE ( $N = 3$ , in duplicate). PH: up to 0.98 mg/l dissolved oxygen, H1 and H6: hypoxic time period 1 and 6 h at 0.98 mg/l dissolved oxygen, after progressive hypoxia, NTR: long term hypoxia exposure in natural habitat. Asterisk above/below bars represents significant difference ( $p < 0.05$ ) in the expression levels of HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  in comparison to their respective normoxic control groups (refer “Materials and methods” section)

by a redox mechanism, which is very similar to rainbow trout HIF-1 $\alpha$  and mammalian HIF-2 $\alpha$  [30]. Nevertheless, the conservation of core ODD domain sequences in HIF-3 $\alpha$  subunit of *C. batrachus* and other vertebrate species, suggested its degradation mechanism similar to that of its HIF-1 $\alpha$  and -2 $\alpha$  [5]. However, unique substitutions and insertion reported in ODD domain of HIF-2 $\alpha$  and -3 $\alpha$  subunits and

low sequence homology downstream to the core ODD among the three subunits pointed out to the possibility of important functional property differences, perhaps in the sensitivity to induction of activity by hypoxia [31] as observed in mammals [32].

In *C. batrachus* HIF- $\alpha$  protein sequences, clear amino acid signatures that could be associated with its oxygen requirements were not identified. However, in the TAD-C domain of HIF-1 $\alpha$ , two amino acid positions (corresponding to +5 and +7 positions after N-803 in HIF-1 $\alpha$  protein of humans) were specific to, hypoxia-tolerant species and it has been suggested that an aspartic acid (D) and a histidine (H) at these positions, respectively, confers hypoxia dependent regulation via factor inhibiting HIF (FIH) [6]. However, the physiological significance of glycine (G) and leucine (L) at the similar position in HIF-2 $\alpha$  subunit in *C. batrachus* and other fishes is exactly not known. Additionally, it was suggested that in cyprinomorpho fishes, number of serine residues in ODD domain is an indicator of response of fish to hypoxia: hypoxia-sensitive and tolerant fish having less or more than 40 serine residues in this domain, respectively [11]. Similarly, the number of serine residues in ODD domain of *C. batrachus* (41, in this study) and *Ictalurus punctatus* (40, NP\_001187230), indicated the possibility of similar pattern for identifying hypoxia-tolerant fish species from siluriidae also.

Distinguishing characteristic of *C. batrachus* HIF-3 $\alpha$  is the LXXLL motif found immediately upstream of the ODD and in the PAS-A/B domains. The LXXLL motif, present in many transcription factors and cofactors is reported to be widely used between nuclear receptors and co-activators as a protein/protein interaction surface, which can activate or repress transcription [21]. This motif was found in multiple copies in *C. batrachus* HIF-3 $\alpha$ , while, in other fishes and human, present in single copy (as identified in present study). The presence of this motif (in multiple copies) in *C. batrachus* HIF-3 $\alpha$  subunit was suggestive of its potential role in transcriptional regulation. However, LXXLL motif sequence are absent in HIF-1 $\alpha$  and -2 $\alpha$  subunits, indicating that HIF-3 $\alpha$  may bind DNA/promoter sequences or novel interacting protein(s) uncommon to those recognized by HIF-1/2 $\alpha$  [33].

In this study, presence of potential JBD within the N-terminal VHL (Von Hippel-Lindau) recognition sites of all the three HIF- $\alpha$  subunits of *C. batrachus*, was observed and this is the first study characterizing the presence of JBD in HIF- $\alpha$  subunits of fishes. JNK phosphorylates some of its targets through the JBD and there are reports which suggested a role for the JNK in the regulation of HIF-1 $\alpha$  via reactive oxygen intermediates as well as by masking the sites for ubiquitination in the ODD domain [20].

The sequences of NLS are sufficient and necessary for nuclear localization of their respective proteins [34, 35]



while, the translocation of HIF- $\alpha$  subunits to nucleus for the formation of DNA-binding complex with  $\beta$  subunit requires NLS as the targeting signal. This study shows that all the three HIF- $\alpha$  subunits of *C. batrachus* contains bipartite type of NLS, which was consistent with the earlier findings in humans [22]. This may suggest that these subunits have similar mechanism of nuclear transport and depends upon bipartite type of NLS as in humans.

#### Expression of HIF- $\alpha$ subunits: normoxia versus hypoxia

It has been suggested that HIF-1 may be present in normal tissue for a basal induction of genes that are necessary to provide the cellular energy requirements [36]. Accordingly, in this study, it was found that all the three HIF- $\alpha$  subunits were constitutively expressed under normoxic conditions in hypoxia-tolerant *C. batrachus*, however, their expression varies among different tissues. Similar findings were also reported in earlier studies for the expression of HIF- $\alpha$  mRNA in fishes during normoxic conditions; however, their expression was highly variable in different tissues [9, 11]. The higher expression of HIF-1 $\alpha$  than -2 $\alpha$  and -3 $\alpha$  in different tissues of hypoxia-tolerant *C. batrachus* under normoxic conditions might suggest that HIF-1 $\alpha$  has an important role in tissue homeostasis while their distinctive expression pattern implied their possible involvements in different physiological functions.

Present study demonstrated that when *C. batrachus* was exposed to short-term hypoxia, an increase in HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$  transcript levels was observed in brain and head kidney tissues as immediate response. While after long-term hypoxia, marked up-regulation of HIF-1 $\alpha$  and -2 $\alpha$  mRNA in spleen and muscle tissues of *C. batrachus* was indicative of unique molecular mechanisms operating through these two subunits. These observations are in agreement with previous reports in animal species that in response to acute hypoxic exposures, HIF-1 $\alpha$  and -2 $\alpha$  accumulate in rat brain [37, 38]. Further, under short and long-term hypoxic exposure, transcript levels of only one subunit (HIF-1 $\alpha$  or -2 $\alpha$ ) was markedly up-regulated (Wuchang bream [9], Eurasian perch [39] and Sea bass [40]) in hypoxia-sensitive fishes, whereas in present study in *C. batrachus* and earlier reports on hypoxia-tolerant fishes (Atlantic croaker [5] and Grass carp [8]), both the subunits (HIF-1 $\alpha$  and -2 $\alpha$ /-4 $\alpha$ ) were up-regulated. These findings suggest that the expression of HIF-1 $\alpha$  along with -2 $\alpha$  may provide unique mechanism for hypoxia tolerance in fishes. The differential tissue expression patterns of HIF-1 $\alpha$  and -2 $\alpha$  in different fish species indicate that the two  $\alpha$  subunits may have unique and complementary roles in the adaptive response to tissue hypoxia and may play very specialized roles in different fish organs.

The HIF-1 $\alpha$  and -2 $\alpha$  are known to play critical roles in the cellular and systemic adaptation to hypoxia [10], only

one report was available for expression pattern of HIF-3 $\alpha$  in fish species [11]. The down-regulation of HIF-3 $\alpha$  in *C. batrachus* during long-term hypoxia in head kidney tissue was unique finding of this study, as earlier studies reported that its expression was strongly induced by short-term hypoxia in rats as well as in fishes [11, 41], whereas no reports were available during long-term hypoxic conditions. However, in case of cellular hypoxic conditions caused by primary renal carcinomas in humans, it was reported that siRNA mediated knockdown of the variant of endogenous HIF-3 $\alpha$  increases the transcription of hypoxia-inducible genes [42]. This finding was further supported by the fact that variants of HIF-3 $\alpha$  might be a negative regulator of hypoxia-inducible gene expression in the human kidney [43]. However, down-regulated HIF-3 $\alpha$  expression was not accompanied by up-regulation of HIF-1 $\alpha$  in *C. batrachus*, the mechanism of regulation of gene transcription needed to be elucidated, for its regulatory roles during adaptations to long periods of hypoxia.

#### HIF-2 $\alpha$ : as hypoxia biomarker candidate

It is suggested, on the basis of significant up-regulation (over 15 fold change in expression) of HIF-2 $\alpha$  under long-term hypoxia in this study, that HIF-2 $\alpha$  can be a robust hypoxia biomarker candidate from muscle of *C. batrachus*. An ideal biomarker should be easy to assay as non-invasive sampling of the muscle tissues is possible through muscle biopsy technique [44]. Other desirable characteristics of biomarkers are specificity and robustness [17]. Up-regulated gene expression response of HIF-2 $\alpha$  was found specific to the muscle tissue and the differential response was sufficiently robust enough to be easy to measure reproducibly using qRT-PCR approaches. Based on these results and combined with comparison analysis, future work will be necessary to develop distinct patterns of HIF-2 $\alpha$  gene expression in fish muscle for hypoxia biomarker assessment.

#### Conclusion

The present study identified and characterized cDNA and deduced protein of three HIF- $\alpha$  subunits (HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$ ) in a hypoxia-tolerant freshwater Indian catfish, *C. batrachus*. The presence of additional LXXLL motifs in HIF-3 $\alpha$  protein suggests its potential role in transcriptional regulation. In addition, presence of JBD suggested a novel mechanism of JNK dependent stabilization of HIF- $\alpha$  units in fishes. The differential expression of three HIF- $\alpha$  subunits in different tissues under short and long-term hypoxia exposures indicated that HIF-2 $\alpha$  of *C. batrachus* can be a robust biomarker candidate for exposure to hypoxic environment.

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