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Effect of acute ammonia exposure on expression of GH/IGF axis genes GHR1, GHR2 and IGF-1 in pufferfish (*Takifugu obscurus*)

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Abstract Waterborne ammonia has become a persistent pollutant of aquatic habitats. The exposure to ammonia stress can reduce growth in a wide range of aquatic organisms. To assess the effect of ammonia exposure on the growth hormone/insulin-like growth factors (GH/IGF) axis, we identified and characterized GHR1, GHR2 and IGF-1 from pufferfish. Comparative analysis showed that these genes shared high identity and similarity with corresponding genes in other fish species. The transcripts of these genes were widely expressed in all tested tissues. The highest level of GHR1 mRNA was found in the brain, whereas GHR2 and IGF-1 mRNA levels were the highest in the liver. Following acute ammonia exposure (100 mg/L total ammonia–nitrogen), GHR2 expression in the liver did not change at 6 h and then significantly decreased at 12, 24 and 48 h, whereas GHR1 and IGF-1 expressions were significantly down-regulated at 6, 12, 24 and 48 h, respectively. These results indicated that ammonia stress decreased the expression of GH/IGF axis genes, which might have negative effect on the growth and development of pufferfish.

Keywords Pufferfish · Environmental stress · Cloning · Real-time PCR

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Introduction

The growth hormone/insulin-like growth factors (GH/IGF) axis has been considered as an important neuroendocrine parameter regulating the growth and development in fish (Berryman et al. 2008). In addition, GH/IGF axis is also involved in other biological actions of fish such as metabolism, osmoregulation, reproduction, behavior and immunity (Walock et al. 2014). The GH/IGF axis includes growth hormone (GH), growth hormone-binding proteins (GHBP), growth hormone receptors (GHR), insulin-like growth factor (IGF), insulin-like growth

factor receptors (IGFR) and insulin-like growth factor-binding protein (IGFBP; Deane and Woo 2009). GH is a pituitary hormone which is regulated by both endocrine and environmental factors (Nakano et al. 2013; Klein and Sheridan 2008). The physiological actions of GH are triggered by binding to GHR on target tissues. Associations of GH with the GHR activate a post-receptor signaling system that stimulates the transcription of target genes such as IGF-1 (Zhong et al. 2012). IGF-1 then can act on target tissues to stimulate cell proliferation, differentiation and ultimately body growth (Pierce et al. 2005). Additionally, IGF-1 may exert significant negative feedback on pituitary GH secretion.

GHR is one of the key factors in the control of GH/IGF axis in teleosts. It is a single transmembrane receptor which belongs to the cytokine class I receptor superfamily (Prinzio et al. 2010). Members of this superfamily have many characters, including a single transmembrane domain, an extracellular N-terminal domain that links to several cellular effector pathways, and an intracellular domain containing a conserved Y/FGEFS motif (Kelly et al. 1991; Very et al. 2005). The GHR cDNA was firstly isolated from rabbit and human liver tissue (Leung et al. 1987). Several GHR mRNAs of teleost fish have been reported, such as goldfish (*Carassius auratus*; Lee et al. 2001), turbot (*Scophthalmus maximusi*; Calduch-Giner et al. 2001), rainbow trout (*Oncorhynchus mykiss*; Reindl and Sheridan 2012) and orange-spotted grouper (*Epinephelus coioides*; Li et al. 2007). These sequences show that fish GHRs share various similar characteristics with the mammalian GHRs, suggesting that the signal transduction and internalization mechanisms of GHR are conserved between fish and mammals. When the amino acid sequences of various fish GHRs are aligned, they can be clustered into two clades: GHR1 and GHR2 (Prinzio et al. 2010; Ma et al. 2007). Li et al. (2007) reported that two GHRs of orange-spotted grouper (*E. coioides*) had different structural features and tissues expression patterns. Pierce et al. (2012) suggested that two types of tilapia GHRs gene expression were differentially regulated by physiological levels of GH and insulin in tilapia primary hepatocytes.

IGF-1, a peptide hormone, is highly conserved among mammalian species (Cueni et al. 1998). The mature IGF-1 is composed of 70 amino acids, which contains domains A and B separated by C domain and D domain (Escobar et al. 2011). IGF-1 produced in the liver is the principal source of endocrine IGF-1. In addition, IGF-1 can be expressed in several other organs where it most likely acts in an autocrine/paracrine manner (Escobar et al. 2011). IGF-1 is a key molecule that regulates numerous cellular processes including cell proliferation, differentiation, survival and migration (Reindl and Sheridan 2012). The biological actions of IGF-1 are mainly mediated through type 1 (IGF-1) receptor on the cell surface, leading to tyrosine phosphorylation of the beta-domain of the receptor and subsequent signaling cascades such as the mitogen-activated protein kinase (MAPK), phosphoinositol-3-kinase and protein kinase B (Akt) signaling pathways (Lee et al. 2001; Aksakal et al. 2010).

GH/IGF axis is influenced by not only nutritional elements but also environmental factors such as ammonia (El-Shebly and Gad 2011). Sinha et al. (2012a, b) reported that growth-regulating hormone and receptor genes such as GH, IGF-1 and GHR were affected by ammonia exposure. Sinha et al. (2012a, b) reported that GHR and IGF-1 mRNA levels were decreased following 10–21 days of high environmental ammonia. Those facts suggested that the effects of environmental stressors such as ammonia may affect the important growth functions and GH/IGF axis (Baldisserotto et al. 2014).

Ammonia is one of the main environmental factors in fish culture especially in recirculation systems. It had been reported that elevated concentration of ammonia in pond water can influence the growth, survival, physiological functions and immune defenses in the teleost fish (Sun et al. 2011). The river pufferfish (*s*), widely distributed in the Sea of Japan, the East China Sea and the Yellow Sea, is an anadromous fish. They usually grow in the sea before sexually mature and then return to fresh water to spawn. In the natural environment, they must confront with drastic changes especially diverse pollutants during their spawning movement from the ocean to the river. Therefore, it has been suggested that pufferfish is a suitable model fish species for the study of stress responses (Kim et al. 2010). Because of the relatively small size and simple organization of its genome, *Takifugu* is used as model organism to understand peculiar physiology, morphology and genomics (Aparicio et al. 2002). However, in recent years, the production of wild pufferfish has sharply declined because of overfishing and due possibly to water pollution.

In this study, we cloned and characterized the full length of pfGHR1 (GHR1 of pufferfish), pfGHR2 (GHR2 of pufferfish) and pfIGF-1(IGF-1 of pufferfish). Furthermore, the effects of ammonia stress on the expression of GH/IGF axis genes were investigated. The aims of the present study were to further elucidate the regulation mechanism of GH/IGF axis response to this.

Materials and methods

Animals

Pufferfish (with average body weight 25.5 ± 1.8 g) were supplied by a fish farm in Panyu (Guangdong, China), and acclimated for 2 weeks in 250-L cycling-filtered plastic tanks containing continuously circulating aerated water at 25 ± 1 °C (pH 7.5; 6.2 mg/L dissolved oxygen). During the acclimation period, the commercial fish diet (42 % protein, 8.0 % fat, 5.0 % fiber and 15 % ash, supplied by Haida Group Foods, Guangdong, China) was employed to feed twice a day until 24 h before the experimental treatments.

Ammonia challenge experiments

In order to assess the effect of ammonia exposure on mRNA expression of GH/IGF axis genes, twelve plastic tanks with 100 L water were prepared, and about 15 individuals were placed in each tank. In our previous experiments, the 96-h LC_{50} for pufferfish was 183 mg/L total ammonia–nitrogen under the same conditions of pH. Based on the LC_{50}, we intentionally chose a high concentration of ammonia (55 % 96-h LC_{50}) in order to assess the effect of acute ammonia exposure on expression of GH/IGF axis genes. Two ammonia levels (0.2 mg/L control and 100 mg/L treatment) were taken in the exposure experiment. Ammonia test solutions were prepared by dissolving ammonium chloride (NH_4Cl) in aerated water until the desired concentration was attained. During the exposure experiment, the pH values of water aquaria were registered every 12 h using a pH meter. The total ammonia–nitrogen levels were measured by nesslerization and adjusted by adding NH_4Cl solution every 12 h. After exposure for 0, 3, 6, 12, 24, 48 and 72 h, six fish from each group were randomly sampled and dissected after anesthesia in MS-222. The liver tissue was collected from each fish and immediately frozen in liquid nitrogen before storage at −80 °C.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the liver tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and then dissolved in DEPC-treated water. The quantity of isolated RNA was later determined by measuring their absorbance at 260 and 280 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA), and its integrity was tested by electrophoresis in 1.2 % agarose gel. Single-stranded cDNA was synthesized from 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. The cDNA templates were then stored at −80 °C for later analysis.

Gene cloning and sequence analysis

The degenerate primers (Table 1) for pfGHR1, pfGHR2 and pfIGF-1 were designed using the conserved domains after multiple alignments of full-length cDNA sequences found in other species. The polymerase chain reaction (PCR) was carried out in a 25 μL final volume in Mastercycler gradient (Eppendorf, Germany). PCR was carried out using PCR program as follows: 4 min denaturing step at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and final elongation step at 72 °C for 10 min. The amplified products were purified from 1.2 % agarose gel using TIANgel Midi Purification Kit (TIAGEN, China), ligated into pMD18-T vector (Takara, Japan) and transformed into DH5a competent cells. Finally, recombinant plasmids were isolated and sequenced by Invitrogen Corporation.

To obtain the full-length cDNA sequences of pfGHR1, pfGHR2 and pfIGF-1, the rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. The forward and reverse primers were designed based on the partial cDNA sequence obtained above (Table 1). The nested PCR program for 5′- and 3′RACE were as follows: 94 °C, for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at

Table 1 The sequences of primers in this experiment

Gene	Primer name	Primer sequence (from 5′ to 3′)	Purpose	Amplification efficiency
GHR1	GHR1-F	CCTTCCTGGGGCCCTCAG	CDS	0.96
	GHR1-R	GTGTCGGGATTAAACGTC	CDS	
	GHR1-3-1	TCTCTCACCCTGGCACTGG	3′RACE	
	GHR1-3-2	ATCTTAATTTCATCCTGAGT	3′RACE	
	GHR1-5-1	GAGCCTGACTCCCTCTTCAGGTA	5′RACE	
	GHR1-5-2	GGAGGGGCTTATGTTCAGCAGA	5′RACE	
	GHR1-RT-F	TAAACTGGGAGCCCCCGCCCTCT	RT-PCR	
	GHR1-RT-R	CGGTGCGTAGCTGGGCAGGCTAC	RT-PCR	
GHR2	GHR2-F	CCTGGACATTGCTCAACCAGA	CDS	0.98
	GHR2-R	CGTAGTACCGTCATTATACCG	CDS	
	GHR2-3-1	TTCCAGTGAACCAGAGG	3′RACE	
	GHR2-3-2	AATCAGCAGCAGCAAAGG	3′RACE	
	GHR2-5-1	GGGTAGAGGTGTAGTCGACT	5′RACE	
	GHR2-5-2	GTGGCTCTCAAAACGTCGC	5′RACE	
	GHR2-RT-F	ACCTTGTGAAAAGCACCTCTC	RT-PCR	
	GHR2-RT-R	GACAGGTTCCAAAGTCAG	RT-PCR	
IGF-1	IGF-1-F	TCAGTGGCATTTATGTGAT	CDS	0.94
	IGF-1-R	TGGGCGTAACGGAGCTG	CDS	
	IGF-1-3-1	GAGAGAGGCTTTTATTT	3′RACE	
	IGF-1-3-2	CACCTAAGGCCGGTGGCAC	3′RACE	
	IGF-1-3-3	GTCCATCTATTCGGAGTC	3′RACE	
	IGF-1-5-1	CTCTATGTCGTGTAACGTGAGA	5′RACE	
	IGF-1-5-2	GAGGTGTATGTTTGACGTC	5′RACE	
	IGF-RT-F	CGACACGCTGCAGTTTG	RT-PCR	
	IGF-RT-R	TGATACAGGTCTATTTCCA	RT-PCR	
β-Actin	β-Actin-F	CATCACCATCGGCAACGAGAGG	RT-PCR	
	β-Actin-R	CGTCGCACTTCATGATGCTGTTG	RT-PCR	

72 °C for 7 min. The nested PCR products were then cloned and sequenced as described above.

Sequence analysis

Analysis on sequence identities and deduced amino acid homology were performed using the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The open reading frame (ORF) was predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf). Translation and protein analysis was performed by ExPASy tools (http://www.expasy.org/tools). Protein structure was predicted by Prosite Server (http://www.expasy.org/tools/scanprosite). Multiple sequence alignment was created using the ClustalX. The phylogenetic tree was constructed based on using Molecular Evolution Genetics Analysis (MEGA) software version 4.1 by the neighbor-joining method and 1,000 replications of bootstrap.

Expression of pfGHR1, pfGHR2 and pfIGF-1 mRNA in different tissues

To further investigate the functions of pfGHR1, pfGHR2 and pfIGF-1, the quantitative RT-PCR was studied to quantify the basal expression level of pfGHR1, pfGHR2 and pfIGF-1 in different tissues. Tissues (brain, gill, heart, spleen, kidney, liver, muscle and blood) were carefully collected separately from

six fish. Total RNA extraction, DNase I treatment, and cDNA synthesis were conducted according to the method as described above.

Real-time PCR (RT-PCR)

The expression of mRNA levels of pfGHR1, pfGHR2 and pfIGF-1 mRNA in the liver tissue under ammonia exposure was measured by quantitative real-time PCR. Information on gene-specific primers used for the real-time PCR was given in Table 1. The β-actin gene was used as a housekeeping gene, and it was amplified using β-actin-F and β-actin-R gene-specific primers. Real-time PCR was amplified in an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, Dalian, China) following the manufacturer's recommendations. The reaction mixtures were of 20 μL, containing 2 μL cDNA sample, 0.4 μL ROX, 10 μL 2× SYBR Premix Ex Taq, 0.4 μL each of the 10 mM forward and reverse primers, and 6.8 μL dH_2O. The real-time PCR conditions were as follows: 94 °C for 10 min, then 45 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by 10 min at 72 °C. All samples were run in triplicate, and each assay was repeated three times. After finishing the program, the threshold cycle (Ct) values were obtained from each sample. Relative gene expression levels were evaluated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data were expressed as mean ± standard deviation. Significant differences were evaluated by a one-way ANOVA followed by Duncan's multiple range tests. Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). p value <0.05 was considered to be statistically significant.

Results

Identification and characterization of pfGHR1, pfGHR2 and pfIGF-1 genes

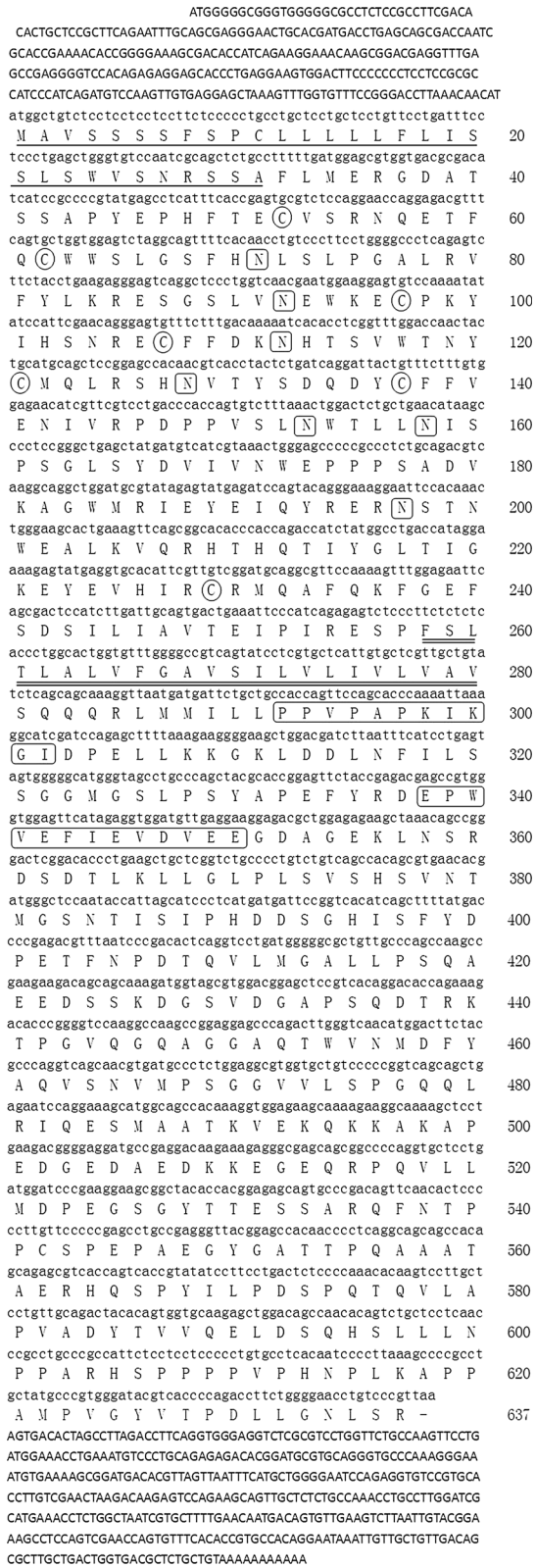
To obtain the full-length sequences of pfGHR1, pfGHR2 and pfIGF-1, the degenerated primers were used to amplify the conserved region and then 5′- and 3′RACE analyses were performed. The full-length pfGHR1 cDNA was 2,608 bp with an ORF of 1,914 bp encoding a putative protein of 637 amino acids, a 5′-untranslated region of 284 bp and a 3′-untranslated region of 410 bp (Fig. 1). The calculated molecular mass of pfGHR1 was 70.5 kDa with a predicted isoelectric point (PI) of 4.95. The deduced amino acid sequence of pfGHR1 was composed of a signal peptide (23 aa), an extracellular domain (234 aa), a single transmembrane region (23 aa) and an intracellular domain (357 aa). The complete cDNA sequence for pfGHR2 had 1,886 bp with an ORF of 1,695 bp encoding for 565 amino acids, while the 5′region was 57 bp and the 3′UTR was 134 bp (Fig. 2). The amino acid sequences of pfGHR2 had a predicted molecular weight of 62.2 kDa and an isoelectric point of 4.97. The pfGHR2 peptide contained a putative signal peptide of 23 amino acids, a 223-amino acid extracellular domain, a single transmembrane domain of 22 amino acids and a 297-amino acid intracellular domain. Both pfGHR1 and pfGHR2 had several characteristics of GHR including N-linked glycosylation sites, cysteine residues, GHR ligand-binding motif FGEFS, and the box 1 and box 2 regions.

The full-length sequence of pfIGF-1 was 2,630 bp, containing a 549-bp ORF that encoded a 182-amino acid protein, a 229-bp 5′UTR and a 1,852-bp 3′UTR. The amino acid sequence had a predicted molecular weight of 19.9 kDa and an isoelectric point of 9.16. The deduced protein consisted of a signal peptide sequence of 44 amino acids, a 66-amino acid mature polypeptide (including B, C, A and D domains) and a 72-amino acid E domain (Fig. 3).

Phylogenetic analysis of pfGHR1, pfGHR2 and pfIGF-1

To understand the evolutionary relationship, a phylogenetic tree was constructed based on the deduced amino acid sequences of pfGHRs with other vertebrates. As shown in phylogenetic analysis (Fig. 4a), all teleost fish formed a clade, which was distinct from that of other vertebrates. Fish GHRs were mainly divided into two groups, GHR1 and GHR2.

On the other hand, to determine the evolutionary position of pfIGF-1, the amino acid sequences of IGF-1 proteins from different species were selected for phylogenetic analysis. As shown in Fig. 4b, the inferred phylogeny of the IGF-1 gene family showed



◀**Fig. 1** Nucleotide sequence and deduced amino acid sequence of GHR1 cDNA. The signal peptide is *underlined*. Conserved cysteine residues are denoted by a *circle* around the amino acid. Potential N-linked glycosylation sites are denoted by a *square* around the amino acid. Transmembrane region is *double underlined*. Conserved box 1 and box 2 are *squared* in an *open rectangle*

two distinct clusters: one for mammals and another for fishes. In the fish cluster, pfIGF-1 shared the closest relationship with *Lateolabrax japonicas*.

Tissue distribution of pfGHR1, pfGHR2 and pfIGF-1

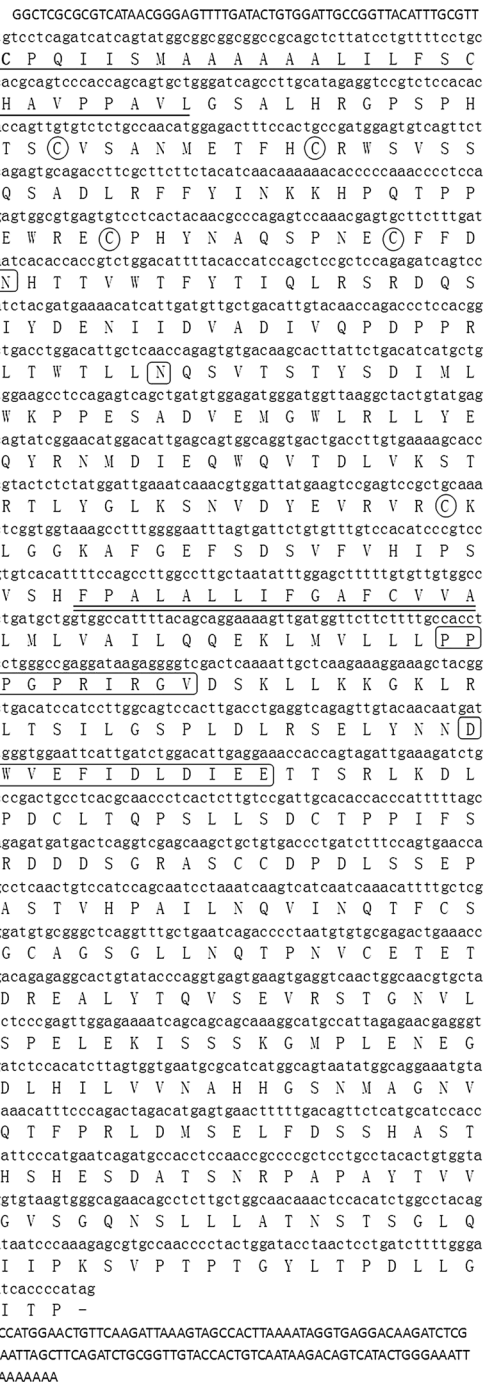
RT-PCR was undertaken to examine the expression of pfGHR1, pfGHR2 and pfIGF-1 mRNAs in various tissues of pufferfish. As shown in Fig. 5, pfGHR1, pfGHR2 and pfIGF-1 transcripts were widely expressed in all selected tissues (brain, gill, heart, kidney, spleen, liver, muscle and blood). The highest level of pfGHR1 mRNA was found in the brain, while pfGHR2 mRNA level was the highest in the liver. There was a high-level expression of pfIGF-1 in the liver, blood and muscle, while a low-level expression in spleen, heart, gill, brain and kidney. The highest level of pfIGF-1 expression was detected in the liver.

pfGHR1,pfGHR2 and pfIGF-1 expression in response to exposure to ammonia stress

The effects of ammonia stress on the transcription of pfGHR1, pfGHR1 and pfIGF-1 in the liver are shown in Fig. 6. pfGHR1 and pfIGF-1 mRNA levels in the liver significantly decreased at 6, 12, 24 and 48 h post-stress, respectively (Fig. 6a, c). However, at 6 h post-stress, there was no significant difference in pfGHR2 mRNA level between control and stressed fish (Fig. 6b). After 12 h post-stress, pfGHR2 mRNA level then began to significantly decrease.

Discussion

The GH/IGF axis is associated not only with growth, but also with tissue differentiation, metabolism, reproduction, osmoregulation and immune response (Walock et al. 2014). GHRs and IGF-1 are known to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E N H T T V W T F Y T I Q L R S R D Q S 120
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TATTTTATACACTGCACCATTCCATACTGGGAGGAATTCTTTGTTAATGCAATGTAAAAGACTAG
CTTAGCTGCTGAGACACAAACAAGAGCTTAATGTCACCTCCATATGGGGGGGCTGCAGCAG
GGGGCTCCAGGTGGGACAAATCCATCTGGGTTGTAGCCAATCAGAGAGCTGCAAGCTGGC
TTTGCAAACTAATGGTCCACCTTGTTCCTTGAGGTTTGTGGAGGTATCAGCCCTTTTGACCCC
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AAAAATTAACTCGTTTTTATGCTTTATATCCCCTTCATTGACTGAGTGCAGCATCGGTCCATCTAT
TCGGAGTCCCTTAAAGGTTTGAGTCCAACTCAAATCCCCCGTGAGCTTGAGGAAGTGTGGGG
GCCCTGAGGTCTAAGTCTAAAAGCCATAGACTTCTCCAACGACATATCAGAGCCGATTAGAAG
GAACAGCAAGTCCCGCAGAACAACGCTTTCTCCTGCTTTCCTAAACAGGGAGTCCAATAAACC
AGAAACTAGGCTGAATTATTGCATTTGGCCAGGCAGCACATGGTACGTGTGCGGTTGTCAGAA
GTGGGTCAGTCAGCGGACTCAAAGTGCATTTGGCTGGAGGGGTAGAGACAAATCAAACAGTC
CCAATCAGGCAAGATGTTTGTCGTCTGCATCGTTTTCACATGGCACCACCGCGGCCAAATTAGA
TTTTTGTCAACAGTCCAGTGGCCGGTGATTGAGCTCAGAGTTTGAGGGCTATGTTCAAGGTTGG
CCACTAGTTGTGAAAGTTTAGCCACAGCAAGTCAACAAGCGCTTTACAGGCCACCCATTGGCGA
GTATCAGCTCGGGTTAAACGTTGCTATCACGGCCACAATAACATTGTCCGCGTAGATATCAGCTTT
AATGACCTCGGTTTGTTTTTATCTGGTCAGTTTAAGATGCCCAAGATTGCTCCTACATCTGGTGGC
TTCACCACGCCATTACGGTTCGTGGCGCGTGGTCCGAAGACCACCCAAAACACATGCGGACTC
CGCGCTGCCGTCAATGCCCTCGACCAAAATGGCAGGTCCCTGCGGAGACCCGAAATAGGGCAT
GCATGTAACAGTACACAAGCACACATTTGTGTAACCGCGAGCAAGGATCAAGCACACATCAACA
GTTCTGAGAAGCCCTGGCATGAGGGCTGGCAGTCACGACTGTTTGTCCTGGCTTCCGTGTTGA
TTTACAATCATTTTATCCCACGATCATCCCGAGACATCTCATCTCTATCTCTGCCTCTTCTCTGTG
TATAACCAAACAATATTCCCATTTATGTTAACCTTTGCATTACTATCATTCATTGTACACGATGGTTT
CATATAGCCAAACTCTATTAAGAATCCTATCCACTGTATAATGGTGCTATTTTGTAGTTTGTTACTTG
CAAGCGATGGCTAAGATGGACAGATGGCAGGGCTTACATTGGAGGTCTTCCCTTTTGCACAGCC
TTGTGGCCACCATTTTAATAGTATTTTTATTTGTAAGCTGTTTCAGTAACATAGTGTACAGTAAGTG
AGTATCTGTGTGAAATCTGTGAATAAATGGAGTTTAACAAAAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 2 Nucleotide sequence and deduced amino acid sequence of GHR2 cDNA. The signal peptide is *underlined*. Conserved cysteine residues are denoted by a *circle* around the amino acid. Potential N-linked glycosylation sites are denoted by a *square* around the amino acid. Transmembrane region is *double underlined*. Conserved box 1 and box 2 are *squared* in an *open rectangle*

Fig. 3 Nucleotide sequence and deduced amino acid sequence of IGF-1 cDNA. The signal peptide is *underlined*. The beginning of each structural domain is designated with the *triangle symbol* and is labeled

be the key factors in the control of GH/IGF axis in teleosts. In the present study, the full-length cDNA sequence and characterization of GHR1, GHR2 and IGF-1 gene from pufferfish were reported. The deduced amino acid sequence of these genes displayed high identity and similarity with corresponding genes in other vertebrate species, suggesting that these genes might play a similar function in fish and mammals.

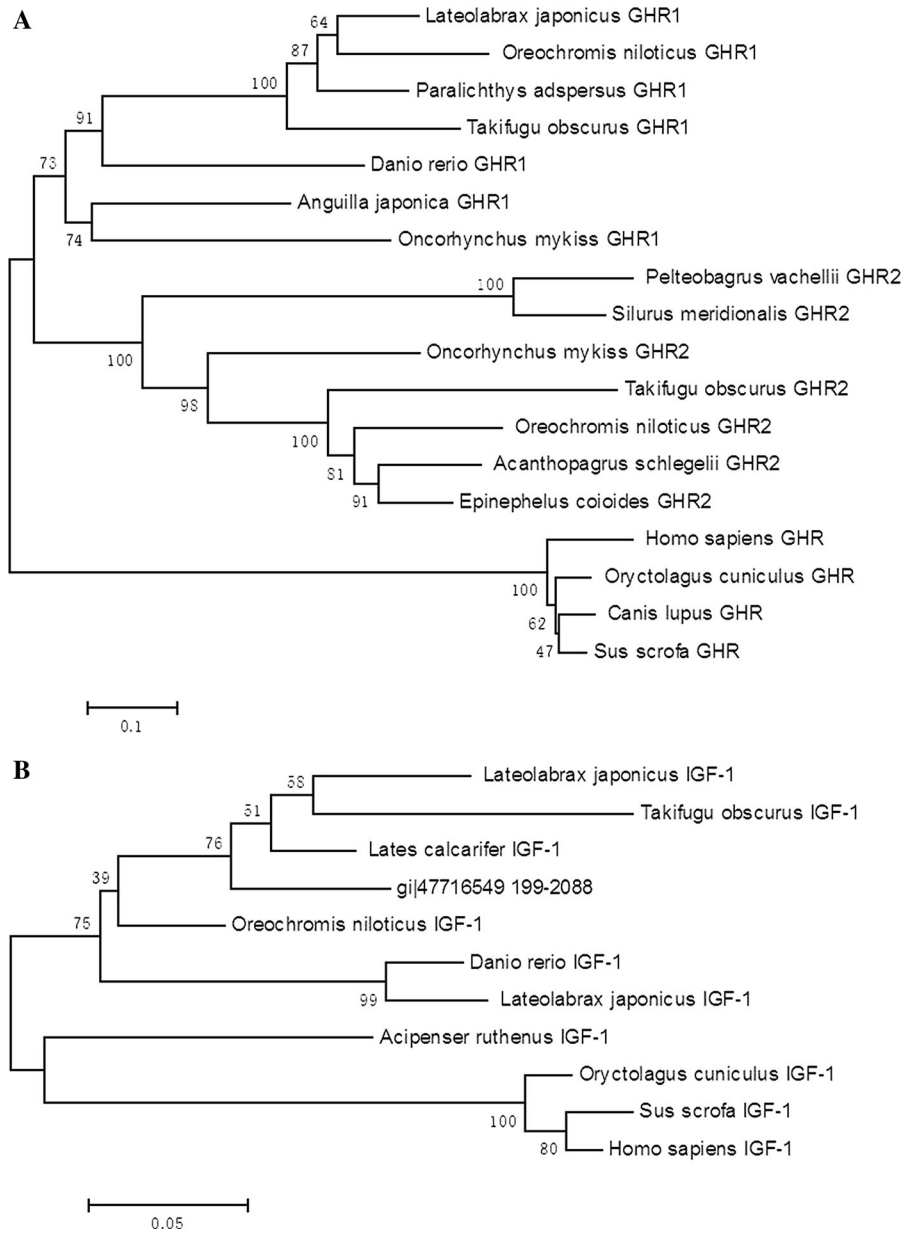
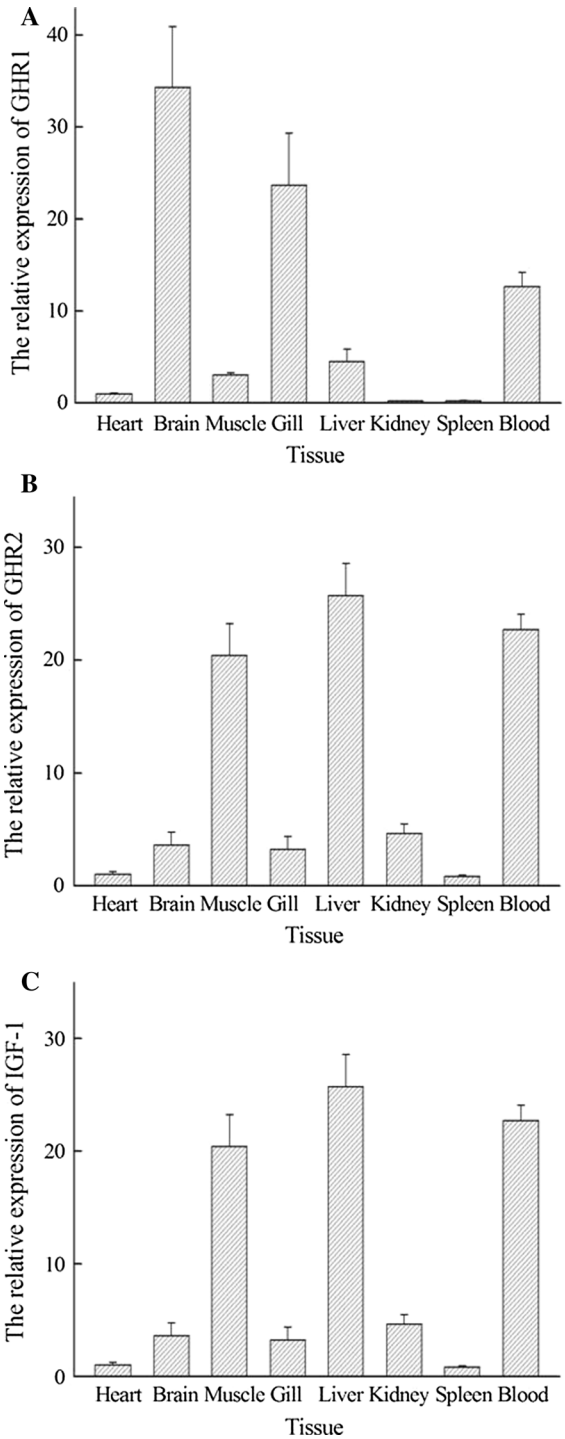


Fig. 4 Phylogenetic analysis of pfGHRs with other members of the GHRs (**a**). Phylogenetic analysis of pfIGF-1 with other members of the IGF-1 (**b**). The phylogenetic tree was constructed using MEGA software 4.1 by the neighbor-joining method and 1,000 replications of bootstrap

GHR belongs to the class I cytokine receptor superfamily that has highly conserved regions (Prinzio et al. 2010). The pfGHR1 contained seven conserved extracellular cysteine residues, which formed three internal disulfide bonds and provided an unpaired cysteine to form disulfide linkage during GH-induced GH receptor dimerization (Li et al. 2007). On the other hand, pfGHR2 lacked one pair of extracellular cysteine residues. In the orange-spotted grouper GHR2, only five conserved cysteine residues were present (Li et al. 2007). With the advent of teleosts, GHR1 retained the ancestral conditions with some slight variability in the last position, whereas GHR2 had between 4 and five cysteine residues (Walock et al. 2014). Potential N-linked glycosylate sites in the extracellular domain may be involved in cell surface targeting (Walock et al. 2014). The deduced amino acid sequences of pfGHRs showed that pfGHR1 had six potential N-glycosylation sites and pfGHR2 had two potential N-glycosylation sites in the extracellular



◀**Fig. 5** Tissue-specific mRNA expression of GHR1 (**a**), GHR2 (**b**) and IGF-1(**c**) determined by quantitative real-time PCR. The relative GHR1, GHR2 and IGF-1 mRNA expression of each tissue was calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as a reference gene. The relative mRNA level was compared with heart expression. Data are presented as mean ± SD ($N = 3$) and are derived from six different individuals

domain. These potential N-glycosylation sites were well conserved through species. Conserved areas in the intracellular domain were the proline-rich box 1 and box 2, which were important for signal transduction (Ozaki et al. 2006). The box 1 region is a site for JAK2 binding, while the box 2 region is involved in the proliferative response of the receptor. The box 1 sequence is generally conserved as PPVPVP in mammalian. However, pfGHRs was changed to PPVPAP or PPVPGP in this study. The differences in sequences probably reflect subtleties to accommodate the specific intracellular signaling proteins in fish (Lee et al. 2001). In fact, minor conservative changes have also been seen within this region in the other fish GHRs (Calduch-Giner et al. 2001; Pierce et al. 2012). Box 2 region (WVEFI) is highly conserved among vertebrates. These results suggested that pfGHRs could have a post-receptor signaling mechanism similar to mammalian GHRs. The phylogenetic relationships of pfGHRs compared with other vertebrate GHRs are shown in Fig. 4a. GHRs in teleost fish were segregated into two groups: GHR1 and GHR2. The pfGHR1 and pfGHR2 belonged to type I and type II GHR clade, respectively. The emergence of two GHR types in pufferfish may be the result of whole genome duplication.

The predicted amino acid sequences of pfIGF-1 showed high sequence identity with other teleosts. The cDNA sequence of pfIGF-1 was predicted to encode a prepropeptide of 182 amino acid residues, which contained a signal peptide and B, C, A, D and E domains. During posttranslational processing, the signal peptide and the E domain were cleaved from the prepropeptide to produce mature IGF-1 (Ma et al. 2011). The homologies of B and A domains between the different species are the greatest conservation. The highly identical B and A domains are important in maintaining the tertiary structure and involved in the binding with its receptors and insulin-like growth factor-binding protein (IGFBP; Escobar et al. 2011; Ma et al. 2011).

pfGHR1 and pfGHR2 mRNAs were constitutively expressed in all examined tissues. Such wide distribution was consistent with the other fish (Calduch-Giner et al. 2001; Li et al. 2007). However, the two

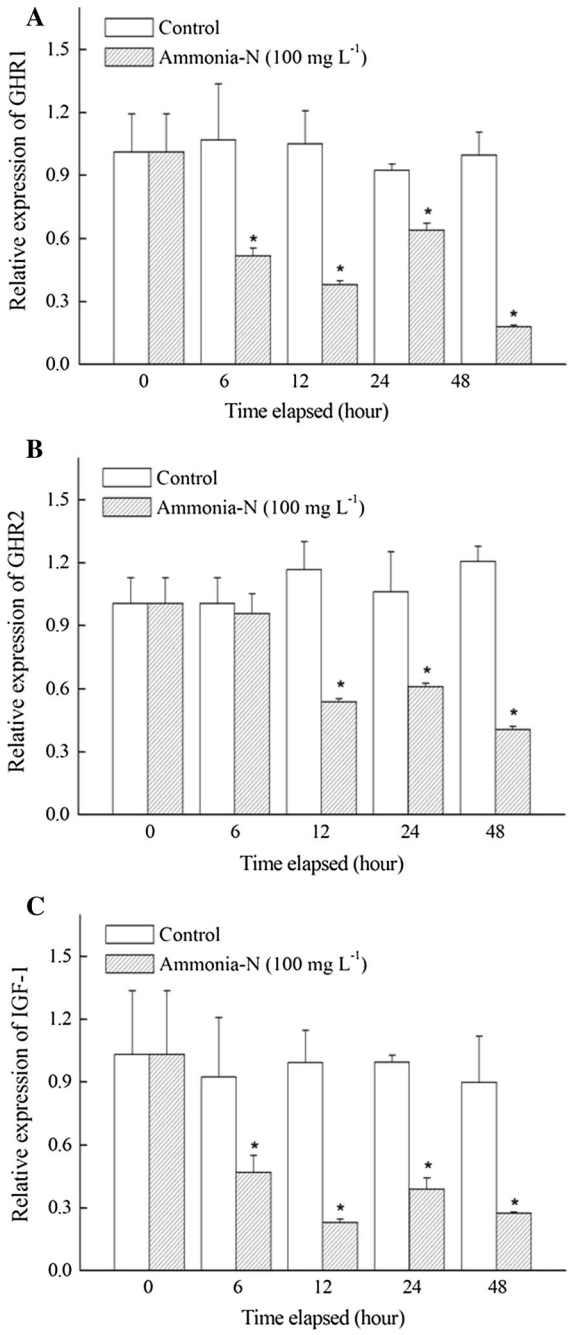


Fig. 6 Effects of ammonia exposure on expression of GHR1 (**a**), GHR2 (**b**) and IGF-1 (**c**) in the liver of pufferfish. The relative expression of the transcript from qRT-PCR was calculated based on the standard curve and normalized to the β-actin mRNA level. Data are presented as the mean ± SD. *Asterisks* indicate statistical significance at the level of $p < 0.05$ relative to appropriate control

pfGHR mRNAs were differentially expressed in different tissues. pfGHR1 transcripts were present at the highest level in the brain, suggesting pfGHR1 played an important and extensive role for GH in the central nervous system, while pfGHR2 mRNA level was the highest in the liver. The liver is the most important tissue for binding of GHR with GH, and signal transduction might explain the high abundance of pfGHR2 in the liver (Li et al. 2007). In rainbow trout, GHR1 was more abundant in the brain than GHR2, while GHR2 was more abundant in the spleen and pancreas than GHR1 (Reindl and Sheridan 2012). In tilapia, GHR1 expression was higher in the fat, liver and muscle, while GHR2 expression was higher in the muscle, heart, testis and liver (Ma et al. 2007). The differential expression of pfGHR mRNAs suggested that they may possess some differences in their signal transduction mechanisms.

Real-time PCR analysis revealed the highest amount of pfIGF-1 transcripts was in the liver, which was consistent with findings in other teleosts (Zhong et al. 2012; Cueni et al. 1998; Escobar et al. 2011). The liver is the primary site for IGF transcription in fish and other vertebrates. Therefore, the high expression of pfIGF-1 in pufferfish liver is not surprising. Besides, the pfIGF-1 mRNA expression was also found in a wide range of tissues in this study.

There are a lot of environmental factors affecting living systems including ammonia. Ammonia is toxic to fish because of many reasons. High ammonia levels may interfere with the endocrine system of animals, thus affecting growth and reproduction (Elango et al. 2006; Lopata et al. 2006). This phenomenon might be related to GH/IGF axis. In this study, pfGHRs and pfIGF-1 mRNA levels in the liver were down-regulated in response to ammonia stress. So far, there have been a few researches regarding the effect of external factors on the expression levels of GH/IGF in fish. Aksakal et al. (2010) demonstrated that IGF-1, IGF-2 and GH-1 mRNA levels in rainbow trout muscles significantly decreased with increasing deltamethrin concentration. Very et al. (2008) found that somatostatins implantation significantly reduced IGF-1 mRNA levels of rainbow trout. All these results indicated that acute physiological stress could strongly affect the GH/IGF axis at multiple levels.

Cortisol is a key component of the stress response, which modulates a variety of biological processes (Soaresa et al. 2014). In the primary response to chemical or physical stressors, cortisol synthesis increases in interrenal tissues and enters circulation (Contreras et al. 2014). Although not measured in this study, plasma cortisol levels were shown to increase after exposure to high ammonia in fish (Baldisserotto et al. 2014; Sinha et al. 2012a, b). Cortisol may reduce fish growth by directly altering GH secretion or antagonizing GH action (Beauloye et al. 1999). In this study, we found that transcripts of pfGHRs were depressed in the liver after acute ammonia exposure. A previous study in juvenile gilthead sea bream showed that GHR2 mRNA was depressed after acute confinement (Saera-Vila et al. 2005). However, the regulation mechanism of GHR in response to environmental stressors in fish is not clear. Several hormones (GH and cortisol) are known to involve in regulating GHR expression in various species (Pierce et al. 2012). In mammals, increased cortisol levels induced the down-regulation of GHR gene expressions (Loeb 1976). In addition, GHR expression in fish tissue was reported to be affected by cortisol (Klein and Sheridan 2008). Small et al. (2006) found that feeding cortisol to channel catfish reduced hepatic GHR-II mRNA levels. These findings suggest that levels of hepatic GHR mRNA may be correlated to plasma cortisol levels induced by environmental stressors. Then, the reduction in the transcription of pfGHR mRNAs could decline the binding capacity of GH to the hepatic GH receptors and reduce circulating IGF-1.

It is well known that IGF-1 mRNA levels might be influenced by many different environmental factors and physiological conditions (Nakano et al. 2013; Hanson et al. 2014). In this study, exposure to ammonia suppressed pfIGF-1 transcripts in a manner similar to that observed with pfGHR mRNAs. Sinha et al. (2012a, b) reported that a slight increase in circulating cortisol level induced by ammonia stress may suppress hepatic IGF-1 expression in common carp.

In conclusion, we identified, sequenced and characterized the GHR1, GHR2 and IGF-1 genes from pufferfish. Comparative analysis showed that these genes shared high identity and similarity with corresponding genes in other fish species. Real-time PCR results showed that pfGHR1, pfGHR2 and pfIGF-1 transcripts were expressed in a wide range of tissues. However, two pfGHRs had different tissue expression patterns. Moreover, pfGHRs and pfIGF-1 mRNA levels in liver were down-regulated in response to ammonia stress. These results indicated that ammonia stress decreased the expression of GH/IGF axis genes, which might have negative effect on the growth and development of pufferfish.

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