Cloning and expressions of chop in loach (Misgurnus anguillicaudatus) and its response to hydrogen peroxide (H$_2$O$_2$) stress

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Abstract C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (chop) which plays an important role in endoplasmic reticulum stress-induced apoptosis was investigated here by RACE and qPCR in an aquaculture animal for the first time. The full-length cDNA sequence of loach (Misgurnus anguillicaudatus) chop was 2533 bp, encoding 266 amino acids. The expression level of loach chop changed during different early life stages, with the highest expression at the 8-cell stage. Among different tissues, loach chop predominantly was expressed in gill, spleen, and gonad. We performed a hydrogen peroxide (H$_2$O$_2$, a common-used disinfectant) stress trial to explore the role of loach chop, with three different concentrations (0 μM, 50 μM, and 100 μM) of H$_2$O$_2$. The 100-μM dose was lethal for half the population but the other concentrations did not result in mortality. The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) in loach gill, liver, and spleen decreased with extended stress time and increased H$_2$O$_2$ concentration. The expression levels of gill chop in loaches from the 100-μM group were significantly higher than those from the other two treatment groups at 12 and 24 h of exposure. atf4 and bax, two pro-apoptotic genes, were significantly upregulated in gills of loaches from the 100-μM group compared to the other two treatment groups 18 h and 24 h after treatment. bcl2, an anti-apoptotic gene, presented an opposite trend. These results indicated a close relationship between H$_2$O$_2$ stress and fish apoptosis with loach chop playing an important role in H$_2$O$_2$ stress response.

Keywords Misgurnus anguillicaudatus · Chop · Gene cloning · Spatiotemporal expressions · Hydrogen peroxide stress

Introduction

C/EBP [CCAAT/enhancer-binding protein]-homologous protein gene (chop), also known as the growth arrest and
DNA damage-inducible gene 153 (GADD153), was considered a master proapoptotic molecule of endoplasmic reticulum (ER) stress-induced apoptosis (Li et al. 2014). Although ubiquitously expressed at very low levels under normal conditions (Ariyama et al. 2008), chop was a highly stress-inducible expressed gene, markedly following the ER stress (Zhang et al. 1999; Cheng et al. 2009; Pirot et al. 2007). Cells responded to ER stress by activating a series of signaling pathways, collectively termed the unfolded protein response (UPR) pathway (Walter and Ron 2011). There were three branches of the UPR pathway: inositol requiring enzym1 (IRE1), prospective evaluation of radial keratotomy (PERK), and activating transcription factor 6 (ATF6). All triggered apoptosis when the ER stress was severe, prolonged, or chronic (Zhang et al. 2016; Deegan et al. 2015; Shang and Lehman 2004). The apoptotic program was activated by the elevated expression of many kinds of transcription factors including chop, X-box binding protein 1 (XBP1), specificity protein 1 (SP1), and SP2 (Rutkowski et al. 2006).

chop was mainly expressed in the PERK pathway. When the organisms were exposed to the ER stress, the expression level of chop was significantly increased (Cao et al. 2012). PERK could upregulate the translations of transcription factor-4 (atf4) (B’chir et al. 2013), by phosphorylating its substrate eIF2α. atf4 was a member of C/EBP family, and it elevated expression of its key downstream target gene chop when stress cannot be alleviated, resulting in the onset of apoptosis (Su and Kilberg 2008). In the PERK branch, chop was suggested to facilitate apoptosis by repressing the transcriptional expression of the antiapoptotic gene B-cell lymphoma-2 (bcl2) in response to ER stress (McCullough et al. 2001). Another widely accepted mechanism of chop-induced apoptosis was the induced overexpression of proapoptotic gene BCL2-associated X (bax), which may function as an executioner in ER stress-mediated apoptosis (Zou et al. 2012). chop-mediated apoptosis enhanced by ER stress played a significant role in the pathophysiology of many mammalian diseases (Gopalan et al. 2013; Oyadomari et al. 2002; Lindholm et al. 2006; Thorp et al. 2009; Gotoh et al. 2010; Lee et al. 2012), affecting the survival of organisms. So far, many studies on functions of chop had been carried out in mammals (Nishimiya et al. 2012; Pereira et al. 2006). However, no information on the cloning and roles of chop in cultured aquatic species was available.

The water environment changes were easy to cause ER stress responses in aquaculture animals (Chen et al. 2015; Zhu et al. 2013; Ji 2014), which had many adverse effects (Cripps and Bergheim 2000), including poor growth, great incidence of disease, increased mortality, and low production (Barton and Iwama 1991). At present, due to the impact of the 2019-nCoV epidemic, hydrogen peroxide (H2O2) was widely used as an environmental disinfectant (Kirst and Juergenlomann 2000). To understand the impact of H2O2 on aquaculture animals, loaches (Misgurnus anguillicaudatus) were used in this study. The loach, belonging to the family Cobitidae, was one of the most commercially cultured species in several Eastern Asian countries including Korea, Japan, and China (Okada et al. 2020). In this study, we first cloned the chop gene in loaches, and then monitored its expression profiles in different tissues and early life stages. In addition, a H2O2 stress experiment was performed to study the stress response in loach in order to explore the role of chop. The results would further our understanding of the H2O2 stress responses and the role of chop in fish.

**Materials and methods**

Fish and sample collections

Loaches were obtained from Baishazhou fish market (Wuhan, China). All loaches were subjected to a ploidy analyzer (Partec, Germany) to determine their ploidy level. Diploid loaches were used and divided into four groups (1 = cloning group, 2 = chop expression group, 3 = RNA extraction group, 4 = H2O2 stress group). The first group (15 g ± 0.2) was used for cloning chop. Three female and three male adult loaches of the second group (15 g ± 0.2) were used for determining expression levels of chop in ten tissues including liver, gill, brain, spleen, heart, intestine, muscle, gonad, kidney, and skin. Each fish served as an experimental unit and n = 6 (3 females +3 males). A pair of parents from the third group (20 g ± 0.2) was used for reproduction, and embryos were collected at different early life stages including oosperm, 2-cell stage, 8-cell stage, 32-cell stage, blastula stage, gastrula stage, neurula stage, tail-bud forming stage, muscle effect stage, heart-beating stage, and hatching stage. All samples were frozen at – 80 °C prior
to RNA extraction. The fourth group (5 g ± 0.2) contained 270 individuals and was used for H$_2$O$_2$ stress experiment.

Cloning the full-length cDNA of chop in loach

Total RNA was isolated from the liver tissues by using RNA isoPlus (TaKaRa, Japan). Quantities and qualities of isolated RNAs were measured by electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Following the method of the SMART™ RACE cDNA amplification kit (Clontech, USA), the RNA obtained was completely reverse-transcribed into cDNA, which was used to clone the gene.

A pair of universal amplified primers was designed (Table S1), depending on the multiple sequence alignments of four species including zebrafish (Danio rerio), common carp (Cyprinus carpio), roughskin sculpin (Trachidermus fasciatus), and yellow catfish (Pseudobagrus fulvidraco). The PCR program was set as follows: initial denaturing for 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 18 s at 72 °C, and extra elongation for 5 min at 72 °C. The PCR products were separated on 2% agarose (Sangon, China) and then purified with a TaKaRa Agarose Gel DNA Purification Kit ver.2.0 (TaKaRa). After purification, the DNA fragments were ligated into PMD19 (TaKaRa) and colonies were randomly screened, and the positive clones sequenced (Escherichia coli was used for cloning) (Invitrogen, China).

Sequence and phylogenetic analysis

Similarity analysis of all the sequences was conducted by BLAST program at the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of loach chop were translated into amino acid sequences by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Multiple alignments of the deduced amino acid sequences were conducted by BioEdit software. Other vertebrate chop amino acid sequences for alignments and constructing phylogenetic tree were obtained from NCBI and the identities of these sequences were blasted by Blastp (NCBI). The phylogenetic tree of amino acid sequence of chop was constructed by MEGA6.0 program using the neighbor-joining method (NJ, bootstrap method: 1000 replications, Arizona State University, USA).

The evolutionary distance between loach chop and orthologous sequences was calculated by using p-distance and gaps were removed by pairwise detection, using default parameters.

H$_2$O$_2$ stress experiment

Two hundred seventy loaches were stocked in nine 30-L white tanks (10 L water volume and 25 ± 0.5 °C water temperature) at a stocking density of 30 fishes/tank. Loaches were respectively treated with 0 μM, 50 μM, and 100 μM H$_2$O$_2$ with triplicate for 24 h (Park et al. 2012). During the experiment, there was no feeding and human interference to prevent introducing other stressors. The number of dead individuals in each tank was recorded every 6 h. The data were input into GraphPad Prism 6.0, and the survival rate curves were generated.

Gill, liver, and spleen tissues were collected from three loaches per tank at 6-, 12-, 18-, and 24-h H$_2$O$_2$ stress exposure. All tissues were frozen in liquid nitrogen and stored at – 80 °C for determining catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activities. In addition, gills were also used for detecting expression levels of chop and its related genes (atf4, bcl2, and bax).

Real-time quantitative PCR

Quantitative PCR (qPCR) was performed using a MiniOpticon real-time detector (Bio-Rad, Hercules, CA, USA). Specific primers of the target genes and reference genes (β-actin and gapdh (glyceraldehyde-3-phosphate dehydrogenase)) are summarized in Table S1. The relative expressions of the target genes were calculated with the comparative Ct method (2\(^{-\Delta\Delta C_{t}}\)). All the procedures were based on the methods from our laboratory described by Cui et al. (2018).

Determination of antioxidant enzyme activities

The activities of SOD, GPX, and CAT were estimated by using the commercially available kits (Nanjing Jiancheng Bioengineering Institute, China). The examinations of the three antioxidant enzyme activities were carried out according to the manuals of the kits.
Statistical analysis

The data were expressed as the means ± SD (standard deviation) or SE (standard error). One-way analysis of variance (ANOVA) was conducted by using Tukey’s post hoc test in SPSS statistical package version 25.0 (SPSS Inc., USA). Alpha of 0.05 was used to determine significance (*P < 0.05, **P < 0.01, ***P < 0.001).

Results

Gene cloning of chop and its sequence analysis in loach

The full-length of loach chop cDNA was 2533 bp, containing 801-bp open reading frame (ORF) encoding 266 amino acids, a 217-bp 5′-untranslated region (UTR) and a 1519-bp 3′-untranslated region.
Deduced amino acid sequence of loach *chop* shared high similarity with those of many other species. The putative results indicated that several completely conserved residues might be functionally important (Fig. 1a). The resulting phylogenetic tree of *chop* amino acid sequences demonstrated that the *chop* in loach was grouped into distinct clade similar to higher vertebrate species, and the deduced amino acid sequence of *chop* in loach displayed high similarity to those in *D. rerio* and *C. carpio* (Fig. 1b).

Spatial and temporal expression profiles of *chop* in loach

The expression levels of loach *chop* in different tissues were measured (Fig. 2a). The highest expression of *chop* was found in gill, the second highest in spleen, and the third highest in gonad. The expression levels of loach *chop* in different early life stages were measured as well (Fig. 2b). The expression of *chop* was highest at the 8-cell stage, followed by muscle effect stage and blastula stage (ANOVA, F (between group df 10, within group df 22) = 459.944, p = 0.000). There were no significant differences in *chop* expression level among oosperm, 2-cell, 32-cell, gastrula, neurula, tail-bud forming, heart-beating, and hatching stages (p = 1). In addition, Tukey’s HSD for multiple comparisons found that *chop* expression was significantly different between 8-cell stage and 32-cell stage (p = 0.000, 95% CI = [lower bound 915.9650, upper bound 1061.0893]).

H$_2$O$_2$ stress experiment

**Survival**

Mortality was first observed at 12 h of H$_2$O$_2$ stress in the 100-μM group. The percentages of death at 12, 18, and 24 h of the 100-μM group were 10%, 25%, and 15%, respectively. After 24 h of H$_2$O$_2$ stress, 50% of mortality was observed in the 100-μM group, while no deaths were numbered in other experimental groups (Fig. 3).

**The antioxidant enzyme activities**

As shown in Fig. 4a, at each time point of H$_2$O$_2$ stress, gill CAT activities of loaches from the 100-μM group...
were significantly lower than those from the 0-μM and 50-μM groups (6 h: ANOVA, $F_{(2, 6)}=93.280, P=0.000$; 12 h: ANOVA, $F_{(2, 6)}=779.163, P=0.000$; 18 h: ANOVA, $F_{(2, 6)}=2339.967, P=0.000$; 24 h: ANOVA, $F_{(2, 6)}=490.376, P=0.000$). Between 12 and 24 h of H$_2$O$_2$ stress, loaches from the 50-μM and 100-μM groups presented significantly lower hepatic CAT activities, compared with the control group (12 h: ANOVA, $F_{(2, 6)}=1987.985, P=0.000$; 18 h: ANOVA, $F_{(2, 6)}=4366.507, P=0.000$; 24 h: ANOVA, $F_{(2, 6)}=3504.436, P=0.000$) (Fig. 4b). Figure 4c shows that spleen CAT activities in the 100-μM group were the lowest. The trends of SOD and GPX activities were similar to the CAT activity trend (Fig. 4d–i).

**Expression levels of chop and its related genes in gill**

Expression levels of *chop* and its related genes *atf4*, *bcl2*, and *bax* in gills of loaches from the 0-μM, 50-μM, and 100-μM groups were analyzed (Fig. 5). The expression levels of *chop* from the 50-μM and 100-μM groups were significantly higher than those from the 0-μM group at 12 (ANOVA, $F_{(2, 6)}=107.228, P=0.000$), 18 (ANOVA, $F_{(2, 6)}=107.228, P=0.000$), and 24 h (ANOVA, $F_{(2, 6)}=191.329, P=0.000$) of H$_2$O$_2$ stress (Fig. 5a). The
expression level of chop from the 100-μM group was the highest at 24 h of H₂O₂ stress, 10 times of that compared to the 0-μM group.

There were no significant differences in the expression levels of atf4 and bax (two proapoptotic genes) among the three groups (0 μM, 50 μM, and 100 μM) at the 6 h (p=0.299 and 0.259) and 12 h (p=0.773 and 0.505) of H₂O₂ stress (Fig. 5b, d). Similar to chop, the expression levels of atf4 and bax from the 100-μM group were the highest at the 24 h of H₂O₂ stress. However, Fig. 5c shows that the expression profile of bcl2 was opposite to those of chop, atf4, and bax.

Discussion

This study cloned the chop gene from an aquaculture animal (namely M. anguillicaudatus) for the first time. The multiple alignments and phylogenetic tree of the deduced amino acid sequences of chop genes showed that the loach chop kept relatively conservative sequences and structural homology compared to other vertebrates. This suggested that chop functions were stable during vertebrate evolution (Luethy et al. 1990; Lee et al. 2011). The expression profiles of loach chop were also determined. The results showed that loach chop was highly expressed during certain embryogenesis periods, consistent with Xenopus (Iijima et al. 2003). In addition, the tissue expression analysis showed that chop was predominantly expressed in the gill and spleen of loach. It had been previously reported that gills and spleens were parts of the immune system, responding to the organism stress (Dautremepuits et al. 2009; Kocabas et al. 2002). The elevated loach chop expression in gills and spleens might indicate its immunological functions related to stress response (Uehara et al. 2014).

To explore the effects of H₂O₂ stress in fish, we treated the loaches with different concentrations of H₂O₂. Our results confirmed that activities of antioxidant enzymes in the loaches had a highly negative
correlation with the prolongation of H₂O₂ stress and the increase of H₂O₂ concentrations, similar to the results of other stress studies. For example, when oxidative stress occurred, the activities of SOD and CAT in gills of zebrafish significantly dropped (Jin et al. 2015). Analogous results were also found in mice liver and spleen (Meng et al. 2004; Matsumoto et al. 2002). In addition, oxidative stress reduced GPX activities in gills and livers in black porgy (Chen et al. 2009), and in spleens of zebrafish (Konomike and Matsuoka 2013). In summary, H₂O₂ appeared to induce stress responses in the loach due to decreased antioxidant enzymes expressions.

In the H₂O₂ stress experiment, we analyzed the expression levels of chop and its related genes atf4, bcl2, and bax in gills of the loach. We showed that the expression level of atf4 in the loach surged under high concentration H₂O₂ stress. The expression of loach chop was significantly upregulated with increased concentrations and the prolongation of H₂O₂ stress. These results were consistent with previous studies (Teijido and Dejean 2010). When the apoptotic pathway was turned on, under the regulation of a series of upstream factors, the mRNA transcription of atf4 increased (Cripps and Bergheim 2000). Afterwards, expression of its key downstream transcriptional target chop elevated (Barton and Iwama 1991), resulting in the repression of the antiapoptotic gene bcl2 and the overexpression of proapoptotic gene bax (Luethy et al. 1990). A reasonable conclusion was that chop might promote apoptosis in the loach under H₂O₂ stress. However, the specific functions of chop warranted further studies.

In conclusion, we identified and characterized full-length cDNA of loach chop for the first time. Expression profiles of chop in different tissues and different early life stages suggested potential immunological functions in loach. The chop expression levels in loaches treated with various concentrations of H₂O₂ indicated that loach chop expression was sensitive to H₂O₂ stress and that the stress induced apoptosis in loach. Finally, this study provided critical information for further investigation of chop functions in fish under H₂O₂ stress.

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Author contribution Xiaojuan Cao and Zhiying Jia designed the experiment. Hui Li, Minxin Kang, Shouxiang Sun, and Jian Gao carried out the experiments. Hui Li and Minxin Kang analyzed the data. The article was written by Hui Li with input from all authors. Xiaojuan Cao contributed to the revision of the manuscript. All authors reviewed and approved the paper.

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Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Ethics approval This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to minimize suffering of the loaches.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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