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Identification and expression profile of Id1 in bighead carp in response to microcystin-LR

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ABSTRACT

Microcystin-LR (MCLR) is a widespread cyanotoxin produced in algal blooms, and has potent hepatotoxicity and tumor-promoting activity. We cloned the full-length cDNA of Id1 in bighead carp. The full-length Id1 cDNA was 954 bp and contained a 387 bp ORF. Bighead carp Id1 shared high identity with zebrafish Id1 amino acid sequence, and phylogenetic analysis showed that teleost Id1 evolved closely. Bighead carp Id1 constitutively expressed in all tested tissues in normal. When tested at two different time points post exposure and at 3 different MCLR doses, Id1 expression increased in a time-dependent pattern, and Id1 expression in brain was very sensitive to MCLR exposure. The present study will help us to understand more about the evolution of Id1 molecule and its role in the MCLR induced cell differentiation and cancer promoting in bighead carp.

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1. Introduction

Microcystins (MCs) are a family of cyclic polypeptides produced by different species of cyanobacteria. Their basic structure is a cyclic heptapeptide and the identified structural variations are more than 80 to date. The most extensively studied form is microcystin-LR (MCLR) which contains L-leucine (position 2) and L-arginine (position 4) in the two main variant positions (Butler et al., 2009). MCLR is a potent hepatotoxin in animals, and its target organ is mainly liver. The poisoning toxicity of MCs is a rapid disorganization of the hepatic architecture, leading to massive intrahepatic hemorrhage,

often followed by death of the animals by hypovolemic shock or hepatic insufficiency (Beasley et al., 2000; Li et al., 2005). It also has been recognized to cause stomach and intestinal inflammation, disease of spleen and reproductive system (Liu et al., 2010). Now many pathogenesis and molecular pathways involved in MCLR exposure are investigated, and a lot of genes expression has been found to be regulated by MCLR exposure (Clark et al., 2007), but many genes which take part in withstanding MCLR toxicological effect are still unidentified and their expression change post MCLR exposure are still unknown.

The inhibitor of DNA binding (Id) proteins are members of the basic helix-loop-helix (bHLH) family of transcription

Abbreviations: Id1, inhibitor of DNA binding protein 1; SSH, suppressive subtractive hybridization.

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factors. bHLH proteins are involved in regulating a variety of developmental processes. Id proteins lack the basic DNA-binding region, and work entirely by dimerization with other bHLH proteins. The Id-bHLH heterodimers have the ability to bind to DNA (Wong et al., 2004). During the development process, heterodimers form between tissue-specific and ubiquitous bHLH factors, and function as active transcription factors. The HLH region is found to be necessary and sufficient for Ids' dominant negative function (Pesce and Benezra, 1993).

Id1 is a member of Id protein family, which exist from yeast to human. It functions mainly as a dominant negative inhibitor. Id1 dimerizes with the bHLH transcription factors, and inhibits their transactivation function to regulate cellular process, such as cell differentiation. Other functions of Id1 are regulation of cell cycle progression, apoptosis, and inhibition of cellular senescence. Id1 also is involved in the development and the progression of different cancers. It was found to be up-regulated in a wide range of cancers, and ectopic expression of Id1 in cancer cells induced cancer cell proliferation, angiogenesis and cell survival (Norton, 2000).

The structure of Id1 is similar to proteins with helix-loop-helix motif. In monomer a DNA-binding basic region is followed by two α -helices separated by a variable loop region (Pesce and Benezra, 1993). In dimer the arrangement of two α -helices in the HLH motif helps their interaction extensively with the intervening loop region, and restricts structural flexibility. The two α -helices of the HLH domain primarily contribute to the dimer interactions, while in the loop region a few contacts facilitate spatial apposition of the two helices in the motif. The residues, buried inside of the four-helices bundle of the HLH dimer, form a hydrophobic core. In this core the residues pack against each other through van der Waals interactions to stabilize the dimer (Chavali et al., 2001).

To date, four mammalian Id proteins have been identified, Id1, Id2, Id3 and Id4 (Norton, 2000). The similar genomic structure of the human Id1 and Id3 and murine Id2 and Id4 suggests an evolution from a common ancestral Id gene (Sun et al., 1991). In mammals, Id1, Id2 and Id3 expression overlaps both spatially and temporally during development whereas Id4 has a unique pattern of expression (Jen et al., 1996). Id1 plays a role in cell growth, senescence, and differentiation. It also can be used to mark endothelial progenitor cells which are critical to tumor growth and angiogenesis (Mellick and Plummer, 2010). Id2 has been shown to play pivotal roles in the development of hematopoietic cells, NK cells and the peripheral lymphoid organs, and stimulating cell cycle progression of mammary epithelial cells (Yokota, 2001). Id3 isoform has biological functions distinct from other Ids. The perturbation of Id3 expression has been correlated with a variety of disease states, including cancer, atherosclerosis and autoimmunity. The mature Id3 of human relates with many diverse developmental, physiological and pathophysiological processes, including T- and B-cell development, skeletal muscle differentiation, and vascular smooth muscle cell proliferation (Li et al., 2012). Id4 mostly acts as a tumor suppressor in colorectal, prostate and gastric cancers, whereas in breast and bladder cancer it has oncogenic features (Wu et al., 2005). Two other Id proteins have been described in lower vertebrates, Idx in *Xenopus* (Wilson and Mohun, 1995) and Id6 in zebrafish

(Sawai and Campos-Ortega, 1997). In rainbow trout and other fishes, Id gene family members also have been identified (Gahr et al., 2005). In rainbow trout Id1 and Id2 expression was the highest in the myotome during early development, and diminished as cells matured into myoblasts (Rescan, 1997). And Id1 genes (Id1B and Id1C) highly express in the developing somite with reduced expression during somite maturation (Ralliere et al., 2004).

As a dominant commercial fish in China, bighead carp (*Aristichthys nobilis*) has received extensive research interest for aquaculture production and as a model species for research on toxicology, ecology, physiology, evolutionary genetics and nutrition (Xie, 2003). To better understand the evolution of Id1, as well as its role in the anti-MCLR response of bighead carp, an Id1 cDNA fragment was isolated from a subtracted hepatic cDNA library generated from bighead carp (*A. nobilis*) injected intraperitoneally (i.p.) with 200 μ g/kg bw MCLR (unpublished data) through SSH. The full-length cDNA was obtained using RACE method. Induction of Id1 expression by MCLR was confirmed by semi-quantitative real time PCR (Q-PCR) assays. Then bioinformatics methods were used to analyze the molecular characteristics of bighead carp's Id1, and Q-PCR assays were applied to investigate Id1 expression profiles in bighead carp spatially and temporally post MCLR injection.

2. Materials and methods

2.1. Toxin, bighead carp exposure experiment and sample

MCLR was isolated from surface cyanobacterial blooms (mainly *Microcystis aeruginosa*) collected from Lake Dianchi in China, then purified and quantified using an improved Ramanan method (Li et al., 2009). The product was dissolved in water, and the purity was above 97%.

Healthy bighead carps (weighing 899 ± 251 g) were purchased from a local cauf (Wuhan, China) and acclimated for 14 days prior to experimentation. These carps were reared in a 150 L volume with 6 bighead carps in each group. Water temperature was kept at 25 ± 2 °C, pH was at 7.4 ± 0.9 , dissolved oxygen value was 6.8 ± 0.7 mg/L and light cycle was maintained at 12 h:12 h. The fish were fed with dry commercial feed at a rate of 2% of the body weight per day. No food was supplied 2 days before and during the whole experimentation.

According to the method of Li et al. (2005), three groups (6 carps in each group) were injected intraperitoneally (i.p.) with 50, 200 and 500 μ g MCLR/kg body weight (bw) respectively. No mortality was found during the experimentation. And the another group 6 carps were injected i.p. with the same volume of distilled water as control. In the experiment, sampling time points were at 3 h and 24 h post MCLR injection. Three replicates of tissue were from 3 different carps for each concentration at each sampling time point. Approximately 100 mg of liver, kidney, intestine, brain, heart, muscle, spleen and gill were excised, freed of attached tissue, and respectively stored in 1 ml Trizol (Invitrogen, USA) at -70 °C. Different tissues of randomly selected 3 bighead carps without any treatment were also excised and used to gene expression distribution analysis. Isolation, purification, and quantification

of total RNA and first strand cDNA synthesis were performed according to our previously described protocols (Li et al., 2009).

2.2. RNA extraction, reverse transcription and RACE

Isolation, purification, and quantification of total RNA and first strand cDNA synthesis were performed according to our previously described protocols (Li et al., 2009). The Id1 cDNA fragment was initially isolated from an SSH cDNA library constructed with the mixed livers of bighead carps at different time points post 200 µg MCLR/kg bw injection (unpublished data). The hepatic RNA was used as template to amplify the 5' and 3' ends of Id1 cDNA. All primers used in this study are shown in Table 1. 5' RACE was performed using SMART RACE cDNA Amplification kit (Clontech) according to the manufacturer's instruction. Gene-specific primers of Id1F and Id1R were designed based on the Id1 cDNA fragment. For 5' RACE, the primers UPM and Id1R were used under the conditions of 94 °C denaturation for 5 min, running 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and 72 °C elongation for 7 min. For 3' RACE, the cDNA template was transcribed by AMV Reverse Transcriptase (TaKaRa) with Oligo dT-Adaptor primer (Table 1). PCR was performed with the primers of 3' Adaptor (Table 1) and Id1F under the conditions of 94 °C denaturation for 5 min, running 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, and 72 °C elongation for 7 min.

2.3. Sequence and database analysis

PCR products were purified and ligated into pMD18-T vectors (TaKaRa), and 3 recombinant plasmids randomly selected were sequenced. Sequences were analyzed using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The protein topology prediction was performed using ProtParam at the Expasy Bioinformatics Resource Portal (<http://au.expasy.org/tools/protparam.html>). The helix-loop-helix domain was defined by NCBI conserved domain search (<http://www.ncbi.nlm.nih.gov/structure>). Multiple sequence alignment was carried out using the CLUSTALW 2 program (<http://clustalw.genome.jp/>). Phylogenetic tree was constructed based on the alignment result using the neighbor-joining (NJ) method with 1000 bootstrap in the Mega 5 software package (Kumar et al., 2004).

2.4. Tissue distribution of Id1 mRNA in bighead carp

Tissue distribution of Id1 expression was analysed by reverse transcript PCR (RT-PCR). Total RNAs (10 µg) extracted from various tissues (liver, kidney, intestine, brain, heart, muscle, spleen and gill) of the normal bighead carp were purified, then used to synthesize the first strand of cDNA with AMV Reverse Transcriptase and oligo (dT)₁₈ (TaKaRa). The cDNA was properly diluted and used as template in PCR reactions with primers of Id1F and Id1R. GAPDH was used as internal control. The PCR condition was: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C 15 s, 58 °C 15 s, 72 °C 15 s, followed by 72 °C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The relative

expression was calculated on the densitometry of PCR product on agarose gels.

2.5. Quantification of id-1 mRNA in bighead carp exposed to MCLR

Q-PCR was performed with Chromo 4 TM Continuous Fluorescence Detector from MJ Research using SYBR Green real time PCR Master Mix (TOYOBO). Reaction mixtures (20 µl) were preincubated 5 min at 95 °C, and then amplified by 40 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. Primers Id1F and Id1R were designed for quantitative analysis of Id1 mRNA in bighead carp (Table 1). Specificity of primers was confirmed by analyzing the melting curves and PCR product. The house-keeping gene GAPDH was used as the internal standard. The plasmid that contained Id1 cDNA was used as positive control. Then standard curves were constructed with tenfold serially diluted plasmid. Expression of Id1 following MCLR exposure was rendered as a ratio of target gene Id1 vs. reference gene GAPDH relative to expression in untreated control samples according to the following equation:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_t \text{target}(\text{control-sample})}}{(E_{\text{reference}})^{\Delta C_t \text{reference}(\text{control-sample})}}$$

The data obtained from the Q-PCR analysis were subjected to one-way analysis of variance (one-way ANOVA) using SPSS 13.0 software.

3. Results

3.1. Sequence analysis of bighead carp Id1 cDNA

The full-length of Id1 cDNA was 954 bp and contained a 387 bp ORF coding for a protein of 128 amino acids. Id1 cDNA had a 99 bp 5'-UTR and a 468 bp 3'-UTR including poly (A) (Fig. 1). Except the ORF, no typical signature sequence was present in Id1 cDNA. The deduced Id1 of bighead carp had a molecular weight of 14.19 kD and an isoelectric point of 6.56.

In the secondary structure of the deduced Id1 protein there was a typical helix-loop-helix motif, the residues consisting of the E-box specificity site, the polypeptide binding sites and the nucleic acid binding sites were present. The 3D structure prediction showed that the cloned Id1 had the same structure as a helix-loop-helix domain in specific DNA-binding proteins that act as transcription factors (cd0083 in conserved domain database of NCBI) (Fig. 2).

3.2. Similarity comparison and phylogenetic analysis

An alignment of deduced bighead carp Id1 and other members of Id1 family from different species suggested that the amino acid sequence of Id1 was well conserved. The data of other Id1 amino acid sequences from different animals were obtained from NCBI and worked as references in the alignment. The deduced amino acid sequence of bighead carp Id1 shared 99% identity with zebrafish Id1 sequence; 82–88% identity with the other fish Id1 sequences; 52–55% identity with mammalian Id1 sequences; 41% identity with amphibian frog Id1 sequence and 36% identity with bird chicken Id1 sequence (Table 2).

Table 1 – Primers used for cloning and expression studies.

Primers	Sequence (5'–3')	Application
Id-F1	CTCACGACGCTGAACGCGGAAC	3'RACE and Q-PCR
Id-F2	GGTGGCGCTGAGGTGATGATGG	3'RACE
3' CDS	AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ V N	3'RACE
5' CDS	(T) ₂₅ V N	5'RACE
Id-R1	CAGCGGGATCTTGCACCTGGAG	5'RACE and Q-PCR
Id-R2	CCTCCAACCTTTGCCGCTCTTC	5'RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT (long) CTAATACGACTCACTATAGGGC (short)	5'RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	3'RACE
Id-F	GTGTGGGACCTACCTGCG	RT-PCR
Id-R	GTTAGTCGGTAGCGTGGGCACC	RT-PCR
SMARTIIA oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG	5' and 3'RACE
GAPDH-F	GCCAGTCAGAACATTATCCAGCCT	Internal control
GAPDH-R	GGTCCTCAGTGTATCCAGAATGCC	Internal control

The phylogenetic analysis revealed that Id1 proteins from fish, amphibian, bird and mammals were relatively independent in evolution (Fig. 3). There were two main branches in the NJ tree. Id1 sequences of bighead carp and the other fish clustered closely; and mammalian Id1s clustered together. Id1 sequences from frog and chicken stayed independently and out of the two main branches. In the fish Id1 branch the

relationships between the eight randomly selected fish Id1 were close, though Id1 of bighead carp was closest to that of zebrafish in evolution. In the mammalian Id1 branch eight Id1s were close in the NJ tree, indicating that mammalian Id1s were closely related in evolutionary process. The different degrees of divergence among teleost and mammalian Id1s may reflect their phylogenetic difference.

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ATCGCACTCTCGAAGCTCAAGACCCAAAAGGAGTACGA
GCACATCGCTTCAACTCAAATCAAATCTCTTTGACTTAATCGTCAACTTTTACAGCCAAA
1 M K V V G P T C A L K S G K V G G E D V
100 ATGAAAGTTGTGGGACCTACCTGCGCGCTGAAGAGCGGCAAAGTTGGAGCGGAAGATGTT
21 V R C L S D Q S L A I S K C K I P L L D
160 GTCGCTGCCTCTCCGACCAGAGCCTGGCCATCTCCAAGTGCAAGATCCCGCTGCTGGAC
41 E Q M T M F L Q D M N S C Y S K L K E L
220 GAGCAGATGACCATGTTTCTCCAGGACATGAACAGCTGCTACAGCAAACCTGAAAGAGCTG
61 V P T L P T N K K A S K M E I L Q H V I
280 GTGCCCACGCTACCGACTAACAAGAAAGCCAGCAAGATGGAGATCCTGCAGCACGTTATT
81 D Y I W D L Q V E L E S K K N Q S S A P
340 GATTACATCTGGGACTTGCAGGTAGAGCTGGAGAGCAAGAAGAACCAGAGCTCCGCTCCC
101 R T P L T T L N A E L A S I S V E N G C
400 CGCACTCCCTCACGACGCTGAACGCGGAACTGGCCAGCATCTCGTTGAGAACGCTGT
121 S D D R I M C R *
460 TCAGATGACCGAATCATGTGCGGTTAAAGCTGATGCTCTGGTTTCACATCAACTGATAGA
520 AGCATCGGAAGAAACAACATGGTGGCGCTGAGGTGATGATGGAGAACCGCCGTGACGCTT
580 TGAAGTGTGTTGGGAGAGTCTCATTCTCCTGTTGTAGAGGAAAACAACITTCAGTAGAGA
640 GTCACAAAACCGTGGACATGAGAAGTGTGACGTGCTTTCTCTGAGATCTTCCCAAAC
700 ATACCGATGTGTGTTAGCTGTGGAATCCATGGCGATTATCACTTATAGTTTCATGCATTT
760 TTCTCTCGGTCAAAGTTTCTCCAATGTTTTCTTAACTCTTAGAAAAAATCTATTTTGT
820 TGTAATGTTTTCTCAGATTACTGAGCGAAAGAATGTATTGTATATTACAATGACATATG
880 ATGTGAATGCTTTATTGTGTTTTTACAATGTATATTGTTTTTTTAATTAACATGAA
940 AATGAGAAAAA

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Fig. 1 – The full-length cDNA sequence of the bighead carp Id1 and the deduced amino acids. The start codon and stop codon of the open reading frame are indicated in bold. The residue corresponding to the stop codon is noted by asterisks (*).

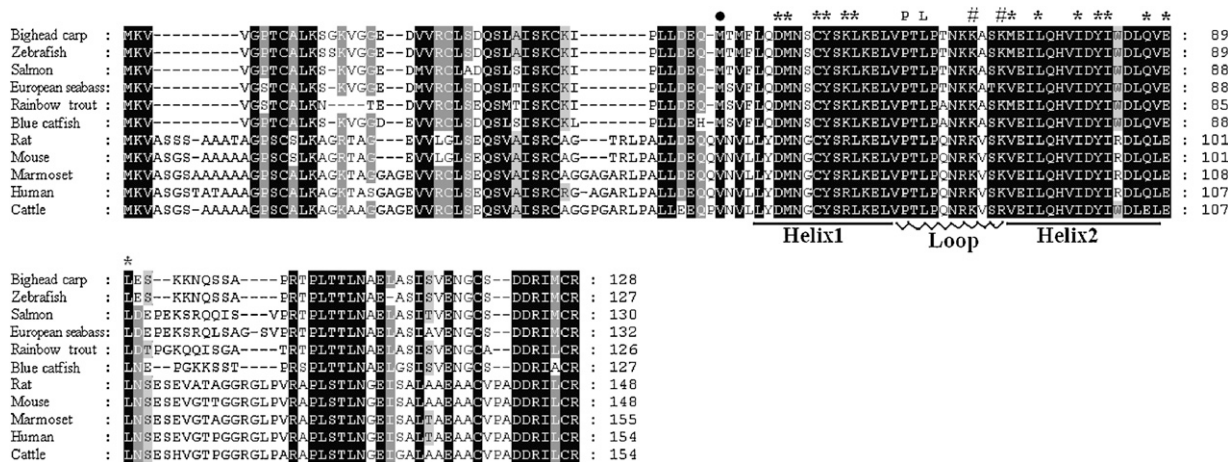


Fig. 2 – Alignment of the full-length amino acid sequences of Id1 from randomly selected species. Correspondences of common names with Latin names and GenBank accession numbers are shown in Table 2. Residues with 100% identity are shown in dark gray boxes, more than 80% identity in gray boxes, and more than 60% identity in light gray boxes. The HLH motif of bighead carp Id1 is shown with words annotation. The E-box/N-box specificity site is marked with black dot (.). The dimerization interface (polypeptide binding site) are noted with #. The DNA binding region (nucleic acid binding site) are marked with asterisks (*). The proline and leucine residues are marked with corresponding letter.

3.3. Tissue distribution of bighead carp Id1 mRNA

The Id1 mRNA distribution was examined using RT-PCR in MCLR uncontaminated fish (Fig. 4A). Id1 mRNA was detected in all the tested tissues, included liver, kidney, intestine, brain, heart, muscle, spleen and gill. Compared to GAPDH expression, the Id1 transcripts of the normal fish were at a moderate expression level (38% to 85%) in these tissues, except a high expression level (150%) in gill (Fig. 4B). No Id1 transcript was detected in blank control (data not shown).

3.4. Expression profiles of Id1 in bighead carp exposed to MCLR

The expression profile of Id1 mRNA was quantified in various tissues of bighead carps injected with 200 µg MCLR/kg bw at

3 h and 24 h post exposure (Fig. 5A). Id1 expression in intestine, spleen and liver did not change, just kept at the normal level, and in gill and kidney there were a little decrease of Id1 expression. While in heart and brain Id1 expression increased by 3-fold ($p < 0.05$) and 6-fold ($p < 0.05$). As time extended to 24 h post MCLR exposure, Id1 expression significantly increased ($p < 0.05$) in intestine, gill and brain by 3.8-fold, 4-fold and 6.2-fold, respectively. No obvious change happened in spleen, heart, kidney and liver. In all, MCLR showed no influence to Id1 expression in spleen, kidney and liver, but induced Id1 expression remarkably in brain.

The expression profile of Id1 in bighead carp injected with 50, 200, or 500 µg/kg bw MCLR at 3 h post injection was shown in Fig. 5B. After exposure to three doses of MCLR, in liver Id1 expression increased with the addition of MCLR dose, Id1 expression did not increase obviously in 50 and 200 µg/kg

Table 2 – Amino acid identity comparison of the bighead carp Id1 protein with other known Id proteins.

Species	Common name	Proteins	Accession no.	Identity	Query coverage
<i>Danio rerio</i>	Zebrafish	Id1	AAH71286.1	99%	100%
<i>Salmo salar</i>	Salmon	Id1	ACH70810.1	88%	100%
<i>Dicentrarchus labrax</i>	European seabass	Id1	CBN81030.1	86%	100%
<i>Osmerus mordax</i>	Rainbow smelt	Id1	ACO09919.1	87%	93%
<i>Esox lucius</i>	Northern pike	Id1	ACO13962.1	86%	92%
<i>Ictalurus furcatus</i>	Channel catfish	Id1	ADO28413.1	84%	100%
<i>Oncorhynchus mykiss</i>	Rainbow trout	Id1	AAAX46287.1	82%	100%
<i>Homo sapiens</i>	Human	Id1	CAA54920.1	55%	96%
<i>Rattus norvegicus</i>	Rat	Id1	NP_036929.2	55%	96%
<i>Callithrix jacchus</i>	Marmoset	Id1	XP_002747402.1	55%	96%
<i>Mus musculus</i>	Mouse	Id1	NP_034625.1	54%	96%
<i>Bos taurus</i>	Cattle	Id1	NP_001091037.1	53%	96%
<i>Canis lupus familiaris</i>	Dog	Id1	XP_852210	53%	70%
<i>Pan troglodytes</i>	Chimpanzee	Id1	XP_0011152873.1	53%	69%
<i>Sus scrofa</i>	Pig	Id1	NP_001231629.1	52%	68%
<i>Xenopus laevis</i>	African clawed frog	Id1	AAB34946.1	41%	59%
<i>Gallus gallus</i>	Chicken	Id1	NP_989921.1	36%	49%

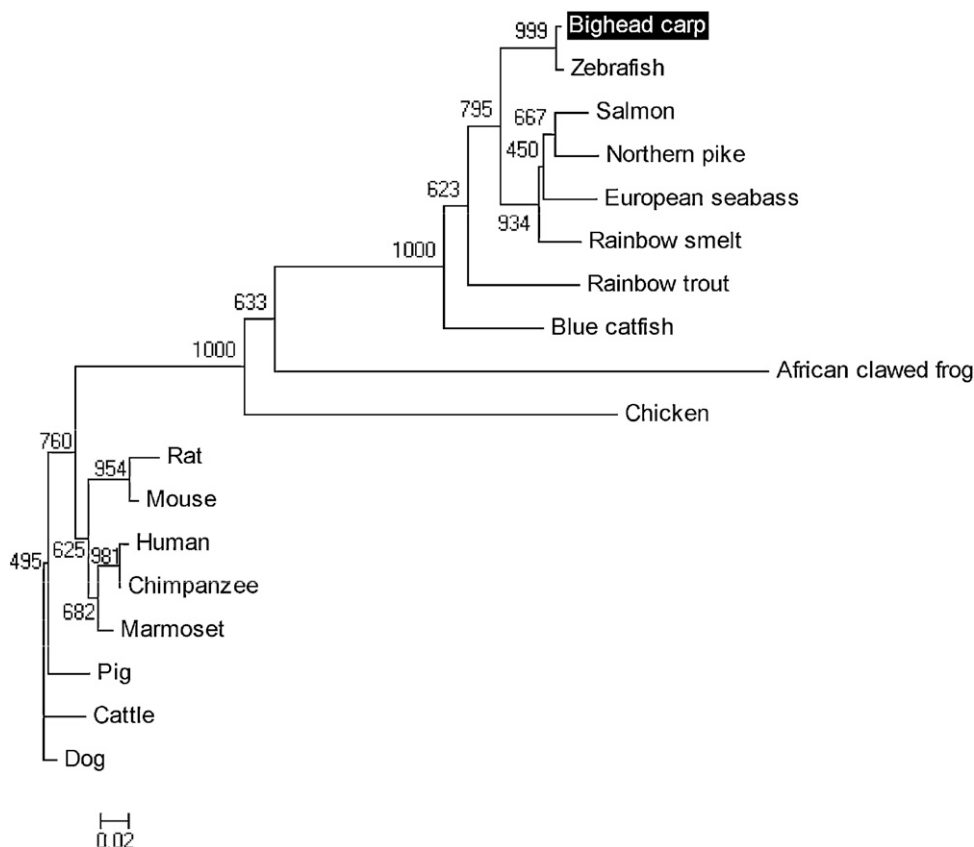


Fig. 3 – Neighbor-joining tree of Id1 of bighead carp and other Id1 proteins from selected species. Bootstrap values are indicated at the nodes. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bar (0.02 mutation per position).

bw groups, and increased by 5-fold ($p < 0.05$) in 500 $\mu\text{g}/\text{kg}$ bw group. While in brain Id1 expression increased dramatically in all the 3 groups, and increased by 7-fold ($p < 0.05$), 28-fold ($p < 0.001$) and 8-fold ($p < 0.05$) in 50, 200, or 500 $\mu\text{g}/\text{kg}$ bw group, respectively. In kidney and gill Id1 expression was just around the normal level under the different doses treatment. In this result, MCLR doses showed no effect to Id1 expression in kidney and gill; Id1 expression increased with MCLR dose addition in liver; and in brain Id1 expression was sensitive to each MCLR dose. This result indicated that MCLR induced Id1 expression with organs specificity.

4. Discussion

In the present study, the full-length Id1 cDNA of bighead carp gene was cloned for the first time. So far many different members of the Id family have been isolated in vertebrates (Sun et al., 1991; Wilson and Mohun, 1995; Sawai and Campos-Ortega, 1997), and the cloned bighead carp Id1 cDNA sequence is similar to other identified Id cDNA sequences, such as Id6 from zebrafish and Xidx from *Xenopus*. There are a short 5'-UTR and a long 3'-UTR in the cDNA sequence, and the full length of ORFs are similar in different species. Bighead carp Id1 shares high sequence identities with members of the vertebrate Id1 family, amino acid residues are strongly conserved

not only within the HLH domain, but also in the other portion of Id1 protein (Fig. 2). All these showed that the cloned Id1 cDNA was correct.

From the alignment analysis we found that the HLH domain was present in the cloned bighead carp Id1, and amino acid residues were strongly conserved in the randomly selected Id1 sequences. The HLH domain of Id1 is necessary and almost sufficient for its activity. The two important residues proline and leucine (shown in Fig. 2) at the N-terminus of the loop are critical for its dominant negative activity, and the loop region is an important structural and functional element of the Id subfamily of HLH proteins (Pesce and Benezra, 1993). The entire HLH domain in all these proteins assumes a similar conformation irrespective of their individual biological effects. The domain controls the affinity of HLH proteins for homo- or heterodimerization, permitting mixing and matching of regulatory factors, and thereby expanding the functional repertoire (Chavali et al., 2001). Recently more evidence has revealed the function of Id1 is related to more cellular activities (Perk et al., 2006; Maw et al., 2010), and all these are depended on Id1 structure.

Based on Id gene structures and homologies from bony fish and human, the notion has been supported by many evidences that Ids originated from an ancestral Id gene, which had conserved its structure through evolution (Deed et al., 1994). In this study phylogenetic analysis suggested that teleost Id1

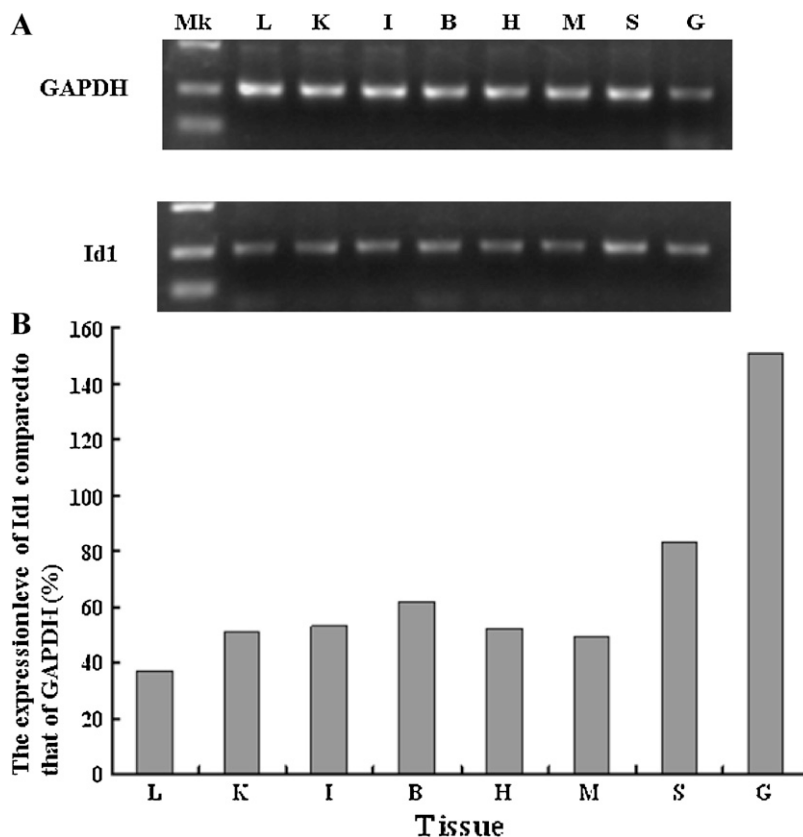


Fig. 4 – Tissues distribution of Id1 mRNA in bighead carp. GAPDH was used as internal control. (A) The results of RT-PCR. (B) The ratio of Id1 expression to GAPDH expression. Mk, marker; L, liver; K, kidney; I, intestine; B, brain; H, heart; M, muscle; S, spleen; G, gill.

evolved closely. The eight fish Id1s are closely related in evolution, though their habitat environment and habits are obviously different. The high sequence similarity and close relationship in evolution with other fish Id1s revealed that bighead carp Id1 evolved conservatively. Eight mammalian Id1s cluster closely in the NJ tree, the mammalian Id1 cluster is not so close to teleost Id1 cluster in evolution. This result showed that in the evolutionary process, though Ids in different species originated from an ancestral Id gene, mammalian Id1 and teleost Id1 evolved diversely after Id gene integrated into mammalian and teleost genome, respectively. Because only an avian Id1 and an amphibian Id1 were selected in NJ tree, it is difficult to deduce the relationship of bird and amphibian Id1 with mammalian and teleost Id1.

As a regulator of the bHLH transcription factors, the Id1 protein has been shown to play an important role in regulating growth and development in a wide variety of tissues. In this study Id1 expression was detected in all tested tissues, this result is similar to reports about the distribution of Id1 expression in other species. As Scott et al. reported in rainbow trout Id1 transcripts were expressed in a variety of tissues and the 4 Id1 paralogues showed similar patterns of expression in adult and developing fish. While in mammals, Id1 expression is highest during early embryonic development in the rapidly proliferating cells and reduces as the cells withdraw from the cell cycle (Wong et al., 2004). The distribution of Id1 expression seems to be closely related to Id1 function, for

Id1 expression has been found to decrease as the cells reach maturation (Yokota, 2001). In two independent experiments, Rescan observed that Id1 expression was limited to the red muscle of growing rainbow trout (Rescan, 1997), while Scott observed that Id1B and Id1C showed higher expression in both red and white muscle in mature rainbow trout. In our study in gill Id1 expression level is much higher than the expression level in the other tissues, perhaps for the function and structure of gill is specific, the cells in gill are proliferating faster than cells in the other tissues (Sollid et al., 2005).

Id1 as a positive regulator of cell growth and its expression may be a key factor required for cell proliferation and cancer promoting (Wong et al., 2004). Id1 can promote cell proliferation and cell cycle progression through inactivation of tumor suppressor and activation of growth promoting pathways in mammalian cells. And in cancer cells Id1 is overexpressed and its expression levels are associated with tumor stage. Etoposide expression of Id1 in cancer cells can induce cell proliferation under sub-optimal conditions and protect the cells against apoptosis. Id1 also promotes invasion of cancer cells through regulating MMP protein as well as invasion (Fong et al., 2003). Animals usually show the dualistic response to MCLR exposure (Gehring, 2004), cell apoptosis is generally reported at high doses of MCLR both in vivo and in vitro, and increased cellular proliferation is observed at lower concentrations. When bighead carps were injected with 200 μ g MCLR/kg bw, Id1 expression increased with time extension of MCLR

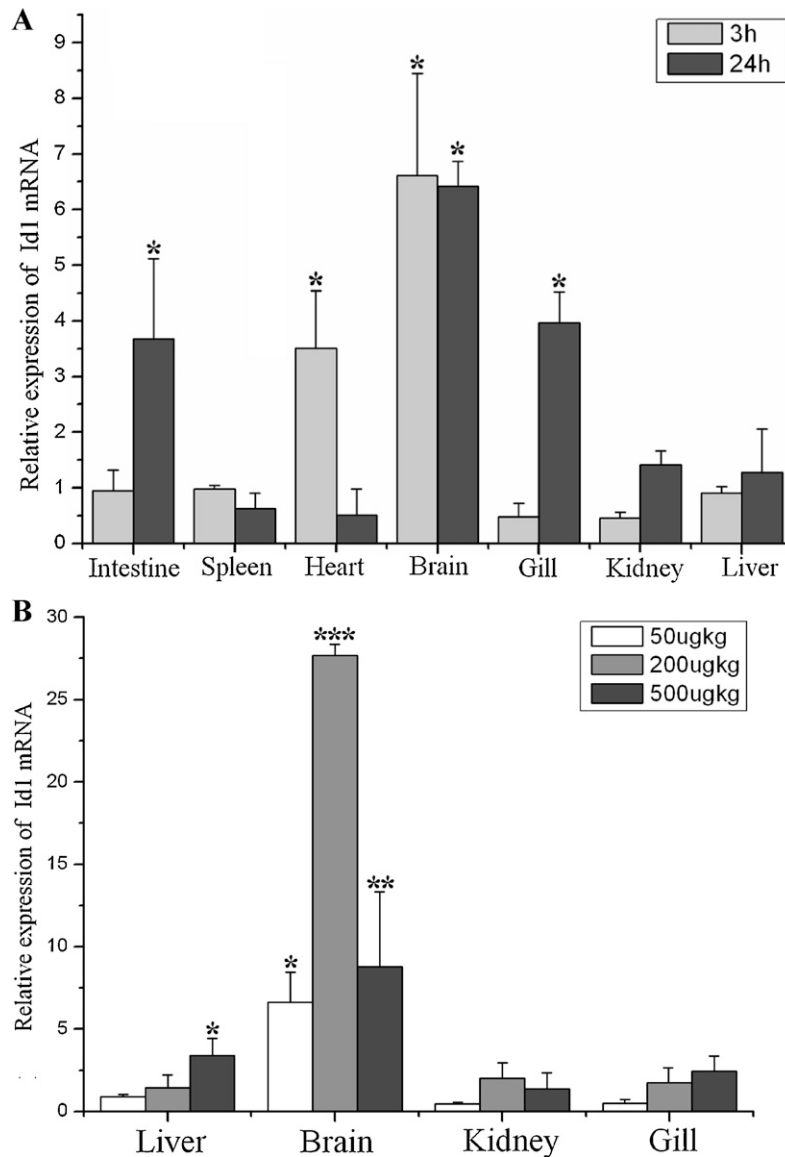


Fig. 5 – Expression profiles of Id1 in bighead carp injected with MCLR. GAPDH was used as internal control. Data are expressed as the mean \pm S.D. ($n = 3$). The significance levels observed are $p < 0.05$ (*), $p < 0.01$ () and $p < 0.001$ (***). (A) Id1 expression profile in all tested tissues at 3 h and 24 h post injection with MCLR 200 $\mu\text{g}/\text{kg}$ bw ($n = 3$). (B) Id1 expression profile in liver, brain, kidney and gill at 3 h post injection with different doses of MCLR ($n = 3$).**

exposure, and this is similar to MCLR toxic effect reported in other published papers (Gehring, 2004). While in heart the upregulation of Id1 expression at 3 h is much more than at 24 h. Maybe at 3 h the dose of MCLR accumulated in heart was low so cells in heart were promoted to proliferation and Id1 expression was at a high level, when at 24 h post MCLR exposure, no MCLR existed in heart according to Lei et al. (2008), so no toxin to promote cell proliferation and Id1 expression decreased when detected at this time.

In this study in brain Id1 expression was more sensitive to MCLR than in any other tissues in all the treatment groups, this perhaps is related to MCLR toxic effect in brain and features of neuro cells. MCLR is a known inhibitor of cellular protein phosphatase (PP) and its neurotoxicity has been investigated (Wang et al., 2010). The results indicated that the chronic

neurotoxicity of MCLR might initiate the serine/threonine protein phosphatase (PP) pathway via an upregulation of PP2C in fish brain, in addition to the reactive oxygen species pathway. Though in the present study high dose MCLR caused the acute toxic effects in bighead carps (Li et al., 2005), neuro cells were influenced adversely by the accumulated toxin in brain. The typical toxicological action of MCLR is to inhibit serine/threonine PP, interrupt signaling transduction, induces oxidative stress and disrupts Na^+/K^+ ATPase pumps. When exposed to MCLR, cells in brain were under many types of stress (Ron de Kloet et al., 2005), and all these lead to cell apoptosis, tumorigenesis and other disease (Wong et al., 2004). In these processes, Id1 played an important role, and the significant upregulation of Id1 expression is reasonable. So it was the whole body co-ordinated system of Id1 expression across

different tissues of bighead carp rather than Id1 expression from a single tissue that helped the fish to partly withstand MCLR induced toxic stress.

5. Conclusion

We cloned the full-length cDNA of Id1 in bighead carp (*A. nobilis*) for the first time. The full-length Id1 cDNA was 954 bp and contained a 387 bp ORF coding for a protein of 128 amino acids. Bighead carp Id1 shared high identity with zebrafish Id1 amino acid sequence, and phylogenetic analysis showed that teleost Id1 evolved closely. Bighead carp Id1 constitutively expressed in all tested tissues (liver, kidney, intestine, brain, heart, muscle, spleen and gill) in normal. When tested at 3 h and 24 h post MCLR exposure and at 3 different MCLR doses, Id1 expression increased in a time-dependent pattern, and Id1 expression in brain was very sensitive to MCLR exposure. The present study will help us to understand more about the evolution of Id1 molecule and its role in the MCLR induced cell differentiation and cancer promoting in bighead carp.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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