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Identification of *cda* gene in bighead carp and its expression in response to microcystin-LR

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ABSTRACT

Microcystin-LR (MCLR) is a widespread cyanotoxin, which can influence genes transcription and cause nucleic acid damage in different organisms. To identify MCLR induced transcriptionally changed hepatic genes in bighead carp by subtractive suppression hybridization, we obtained the cDNA fragment of *cda*. Then we cloned its full-length cDNA, which encodes a cytidine deaminase (CDA). 3D structure prediction showed that the 3D structure and amino acid residues related to function sites of bighead carp CDA were highly conserved. Bighead carp CDA shared high identities with other CDA sequences, and evolved closely to non-mammalian CDAs. Bighead carp expressed *cda* in all tested tissues under normal situation, and changed its expression profile in a time inversely dependent and dose dependent manner to MCLR, so as to protect itself from MCLR induced toxic damage. These indicated that *cda* might be involved in anti-MCLR response, especially in the regulation of cytidine and dexocytidine metabolism pathway.

1. Introduction

The *cda* gene encodes a cytidine deaminase (CDA, EC 3.5.4.5), which is also known as cytidine nucleoside deaminase, that catalyzes the irreversible hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. A variety of deaminases has been evolved in nature, to facilitate the deamination of purines and pyrimidines (Navaratnam and Sarwar, 2006). The cytidine deaminase is one type of several deaminases responsible for maintaining the cellular pyrimidine pool, and is important for the pyrimidine salvage pathway from bacteria to human. It also enables organisms to utilize exogenous pyrimidine bases and nucleosides economically, which are not intermediate products in *de novo* pyrimidine synthesis (Carter, 1995). In human, the expression of *cda* differs among tissues (Watanabe and Uchida, 1996). The clinical interest in this enzyme is due to its capability to deaminate several anti-tumoral and anti-viral cytosine nucleoside analogs, leading to reduced sensitivity to cytosine nucleoside analogs used in the treatment of certain childhood leukemia and their pharmacological inactivation (Laliberte et al.,1992; Eliopoulos et al., 1998; De Clercq, 2001).

CDAs include a homotetrameric form (T-CDA) and a homodimeric form (D-CDA) (Johansson et al., 2002). T-CDA is widely distributed in the three domains of life (Vincenzetti et al., 1996), and consists of four identical subunits. In each T-CDA subunit, there is an active site and a zinc ion coordinating with three negatively charged residues. D-CDA, only found in some species of proteobacteria and *Arabidopsis thaliana* (Faivre-Nitschke et al., 1999), consists of two symmetrical monomers. Each monomer contains a small N-terminal domain, a catalytic domain combined with a zinc ion, and a domain that is structurally similar to the catalytic domain (Johansson et al., 2004).

CDAs from various organisms such as *E. coli*, *Bacillus* sp. and human have been characterized and studied for nearly 4 decades (Ashley and Bartlett, 1984; Laliberte and Momparler, 1994; Vincenzetti et al., 1999). Secondary and tertiary structures of CDA from *Bacillus* species and *E. coli* have been investigated by X-ray analysis (Johansson et al., 2002). At present, *cda* homologs have been cloned in different species such as human, chimpanzee, dog, cow, mouse, rat, chicken, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Magnaporthe grise* and *Neurospora crassa*, and CDA is well conserved in its sequence and structure (Johansson et al., 2002). The *cda* gene have also been identified and characterized from different kinds of fish such as zebrafish (*Danio rerio*) (Strausberg et al., 2002), Atlantic salmon (*Salmo salar*) (Leong et al., 2010), orange-spotted grouper (*Epinephelus coioides*) and rainbow smelt (*Osmerus mordax*) (from NCBI database). However, to date, there is no report for *cda* gene clone and identification in bighead carp.

Abbreviations: MCLR, Microcystin-LR; bw, Body weight; CDA, Cytidine deaminase; SSH, Suppression subtractive hybridization

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Bighead carp (*Aristichthys nobilis*) not only is a commercially important fish in China, also consumes great quantities of toxic cyanobacteria, which may contain high concentrations of microcystins (MCs). As a freshwater phytoplanktivorous fish, compared with mammals, bighead carp is more resistant to the toxic effects of microcystin-LR (MCLR), one of the most common and toxic microcystins (He et al., 1997; Xie et al., 2004). Now bighead carps have received extensive research interest for aquaculture production and as a model species for research on toxicology, ecology, physiology, evolutionary genetics and nutrition (Xie, 2003).

During the past decades, the frequent occurrence of toxic cyanobacterial blooms in freshwaters has received great public attention (Chen et al., 2009). Among the various cyanotoxins, MCs are the most widespread groups. Now more than 80 kinds of MCs have been identified, and MCLR is the most common and toxic one (Fastner et al., 2002). MCs have a significant adverse impact on both aquatic organisms and human (Chen et al., 2009; Li et al., 2009a). So far, most studies have documented toxic effects of MCLR on transcription and translation of known genes (Li et al., 2009a; Takumi et al., 2010). Li et al. (2009a) found that when male Wistar rats were injected i.v. with MCLR, the transcription of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in liver, kidney and testis were upregulated and no change happened in translational level. Takumi et al. (2010) reported that MCLR induced the phosphorylation and accumulation of p53, and activated Akt signaling through the phosphorylation of Akt and glycogen synthase kinase 3β in HEK293 cells. While few research was done to find unidentified genes whose expressions were influenced by MCLR. Some published papers have shown that one of MCLR toxic effects was induction oxidative DNA damage and chromosome breakage (Rao and Bhattacharya, 1996; Zegura et al., 2003). CDA is a deaminase, which takes part in maintaining the cellular pyrimidine pool and the pyrimidine salvage pathway. The relationship between *cda* gene and MCLR is interesting.

To identify MCLR induced transcriptionally changed hepatic genes in bighead carp, two hepatic cDNA libraries were constructed by forward and reverse subtractive suppression hybridization. In total 367 clones expression changed in liver of bighead carp injected intraperitoneally with 200 μg MCLR/kg bw, and the clone inserted into cDNA fragment of *cda* was isolated (unpublished data). To better understand the evolution of *cda* gene, as well as its role in the anti-MCLR response of bighead carp, we obtained the full-length cDNA using RACE method. By quantitative PCR (Q-PCR) assays, we confirmed that *cda* expression was induced in bighead carp liver by MCLR. The molecular characteristics of bighead carp's CDA and gene expression profiles were further analyzed.

2. Materials and methods

2.1. Fish preparation

Healthy bighead carps with weight of 899 ± 251 g were purchased from a local fish hatchery (Wuhan, China) with no MCLR contamination. Bighead carps were acclimated for 14 days prior to experimentation in a 150 l volume and 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 bighead carps were fed with dry commercial feed at a rate of 2% of the body weight per day. No food was fed to the bighead carps 2 days before and during the course of the experiment.

2.2. Toxin

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

2.3. Experimental set up

Three groups (6 carps in each group) were injected intraperitoneally along the ventral midline into the peritoneum using syringes with 50, 200 and 500 μg MCLR/kg body weight (bw) respectively, according to the method of Li et al. (2005). No mortality was found during the experimental period. And in the other 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 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. All of the experimental researches on bighead carp were performed with the approval of the animal ethics committee in the Institute of Hydrobiology, Chinese Academy of Sciences (Study ID# Y11309-1-201).

2.4. RNA extraction

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

2.5. Q-PCR and RT-PCR

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. The plasmid that contained *cda* cDNA was used as positive control. Then standard curves were constructed with tenfold serially diluted plasmid. Primers CDA-F1 and CDA-R1 were designed for Q-PCR (Table 1). Specificity of primers was confirmed by analyzing the melting curves. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Expression of *cda* following exposure was rendered as a ratio of target gene *cda* vs. reference gene GAPDH relative to expression in unexposure control samples according to the following equation:

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

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

RT-PCR was performed according to the following protocol. Total RNA (2 μg) from the various tissues of untreated bighead carps was applied as template to synthesize the first strand of cDNA using AMV Reverse Transcriptase (TaKaRa, Japan) and oligo $(dT)_{18}$ (TaKaRa, Japan). GAPDH was used as the internal standard. The cDNA was diluted and used as template in PCR reactions with primers of CDA-F and CDA-R. The PCR reaction was carried out as follows: one initial step of denaturation at 94 °C for 5 min; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and a last extension step at 72 °C for 10 min. The PCR products were electrophoresed on agarose gels stained with ethidium bromide. Negative controls for each experimental group were performed by PCR without template.

2.6. Bighead carp cda cDNA fragments and rapid amplification of cDNA end (RACE)

The bighead carp *cda* cDNA fragment was initially isolated from an SSH cDNA library constructed with the mixed liver tissues of bighead carps at different time points post 200 μg/kg bw MCLR injection (unpublished data). The hepatic RNA was used as template to amplify the cDNA 5′ and 3′ ends of *cda*. All primers used in this study were listed in Table 1. RACE was performed using SMART RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's instruction. Gene specific primers CDA-F1, CDA-F2, CDA-R1 and CDA-R2 were designed based on bighead carp *cda* cDNA fragments. Briefly, the primers, UPM (long and short), 5′CDS, CDA-R1 and CDA-R2 were applied for 5′ RACE under the following conditions: 94 °C denaturation for 5 min; then 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C; and a last extension step at 72 °C elongation for 10 min. For 3′ RACE, the cDNA template was transcribed by AMV Reverse Transcriptase and oligo $(dT)_{18}$. PCR was performed with the primers of 3′CDS, NUP, CDA-F1 and CDA-F2 under the same conditions as 5′ RACE PCR conditions.

2.7. Sequence and data analysis

PCR products were ligated into pMD18-T vector (TaKaRa, Japan) and at least 3 recombinant plasmids were sequenced. Sequences were analyzed based on nucleotide and protein databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The catalytic motif, zinc binding sites and active sites were identified by NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/structure).

Table 1
Primers used for cloning and expression studies.

Primers	Sequence (5′-3′)	Application
CDA-F1	GCACTCCTGACACATGACGGGACGG	3′RACE and Q-PCR
CDA-F2	GGGACTGTGCGCTGAGAGAACTGCC	3′RACE
3' CDS	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N	3′RACE
5' CDS	$(T)_{25}$V N	5′RACE
CDA-R1	CCAAACTCCCTCATGAACTGCCTGCAGC	5′RACE and Q-PCR
CDA-R2	CTCCACAGGGGGAAATGAAGTGCTCACA	5′RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT (long) CTAATACGACTCACTATAGGGC (short)	5′RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	3′RACE
CDA-F	GGGAGACCAAAAGATGAGACATGAG	RT-PCR
CDA-R	GTCCTCGGGACCAAAAGATGCAG	RT-PCR
SMARTIIA oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG	5′ and 3′RACE
GAPDH-F	GCCAGTCAGAACATTATCCCAGCCT	Internal control
GAPDH-R	GGTCCTCAGTGTATCCCAGAATGCC	Internal control

Fig. 1. The full-length cDNA sequence of bighead carp *cda* gene and the deduced amino acids. The TATA box is underlined. 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 (*). The poly (A) signal is shaded. The conserved active site motif $CAEX_{30}PCGGC$ is shown in box.

The protein topology prediction was performed using ProtParam at the ExPASy Bioinformatics Resource Portal (http://au.expasy.org/tools/protparam.html). Multiple sequence alignment was carried out using the CLUSTALW2.0 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the sequence identities were calculated using GeneDoc (http://www.psc.edu/biomed/). Phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap in the Mega 5 software package (Kumar et al., 2004).

3. Results

3.1. cDNA sequence and protein analysis of bighead carp cytidine deaminase

The full-length of *cda* cDNA was 541 bp and contained a 480 bp ORF that encoded a 159 amino acids protein, and had a 43 bp 5'–UTR and a 31 bp 3' –UTR including poly (A). The polyadenylation signal AAGAAA was 24 bp upstream of poly (A) tail. The conserved signature motif $CAEX_{30}PCGGC$ of (H/C)(A/V)E($X_{24\text{-}30}$)(PCXXC) was shown in box (Fig. 1). The TATA box was present within the 5'–UTR sequence, and ahead of the translation start codon.

The deduced CDA of bighead carp had a molecular weight of 17,745.3 Da and an isoelectric point of 6.42, based on the online software ProtParam prediction. The 3D structure prediction revealed that the cloned *cda* gene encoding a zinc ion depending homotetrameric form cytidine deaminase, with 54% identity with chain A of crystal structure of cytidine deaminase from *Bacillus Subtilis* in complex with the inhibitor tetrahydrodeoxyuridine (Johansson et al., 2002). There were four identical subunits in CDA, and each subunit was composed of a mixed β-sheets (β1-5) with one α-helix (α1) on one side and five α-helices (α2-6) on the other side. Three parts were present, including the active site at residues 42, 44, 60, 62, 71–73 and 105, 108; the catalytic motif at residues 71–73, 104, 105 and 108; the zinc ion binding sites at residues 71, 73, 105 and 108 (Fig. 2).

According to the high similarity of CDA amino acid sequences and structure between the bighead carp and *Bacillus subtilis*, the function of individual amino acid residues of bighead carp CDA could be easily deduced (Costanzi et al., 2006). Residues Cys71, Cys105 and Cys108 served as ligands for the zinc ion, and Glu73 functioned as proton donor and acceptor in catalysis. The conserved residues Phe42, Asn60, Glu62, Ala72 and Phe143 mediated substrate binding, while Ser40, Arg109, Gln110, Glu114 and Leu139 were engaged in tetramer interactions. The conserved glycine residues (Gly45, Gly53, Gly58, Gly105) were pivotal for the geometry of the surrounding residues, just as the Gly106 between the zinc coordinating residues Cys105 and Cys108. Pro38 was present where the monomer chain made a turn, and Pro140 was at the end of α-helix α5. The two conserved alanine residues (Ala31 and Ala45) were both present in the middle of secondary structural elements (α1 and β1) with their side chains pointed into the hydrophobic core of the protein.

3.2. Similarity comparison and phylogenetic analysis

An alignment of deduced bighead carp CDA and other 16 members of CDA family from different species suggested that the amino acid sequence of CDA was well conserved. The deduced amino acid sequence of bighead carp CDA shared 91% identity with zebrafish CDA sequence, 70–76% identity with snail and other fish CDA sequences; 50% and 54% identity with *Aspergillus* and *Bacillus* CDA sequences respectively, 70–72% identity with xenopus, chicken and mammals CDA sequences, respectively (Table 2).

Our phylogenetic analysis showed that CDA sequences clustered in two main branches, with CDAs of European seabass, *Aspergillus* and Atlantic salmon were out of the two main branches. In one main group, noted as cluster I, bighead carp CDA had the closest relationship to zebrafish CDA. The two fish CDAs, bighead carp and zebrafish, were closer to that of xenopus and chicken than those of channel catfish, snail and *Bacillus* in

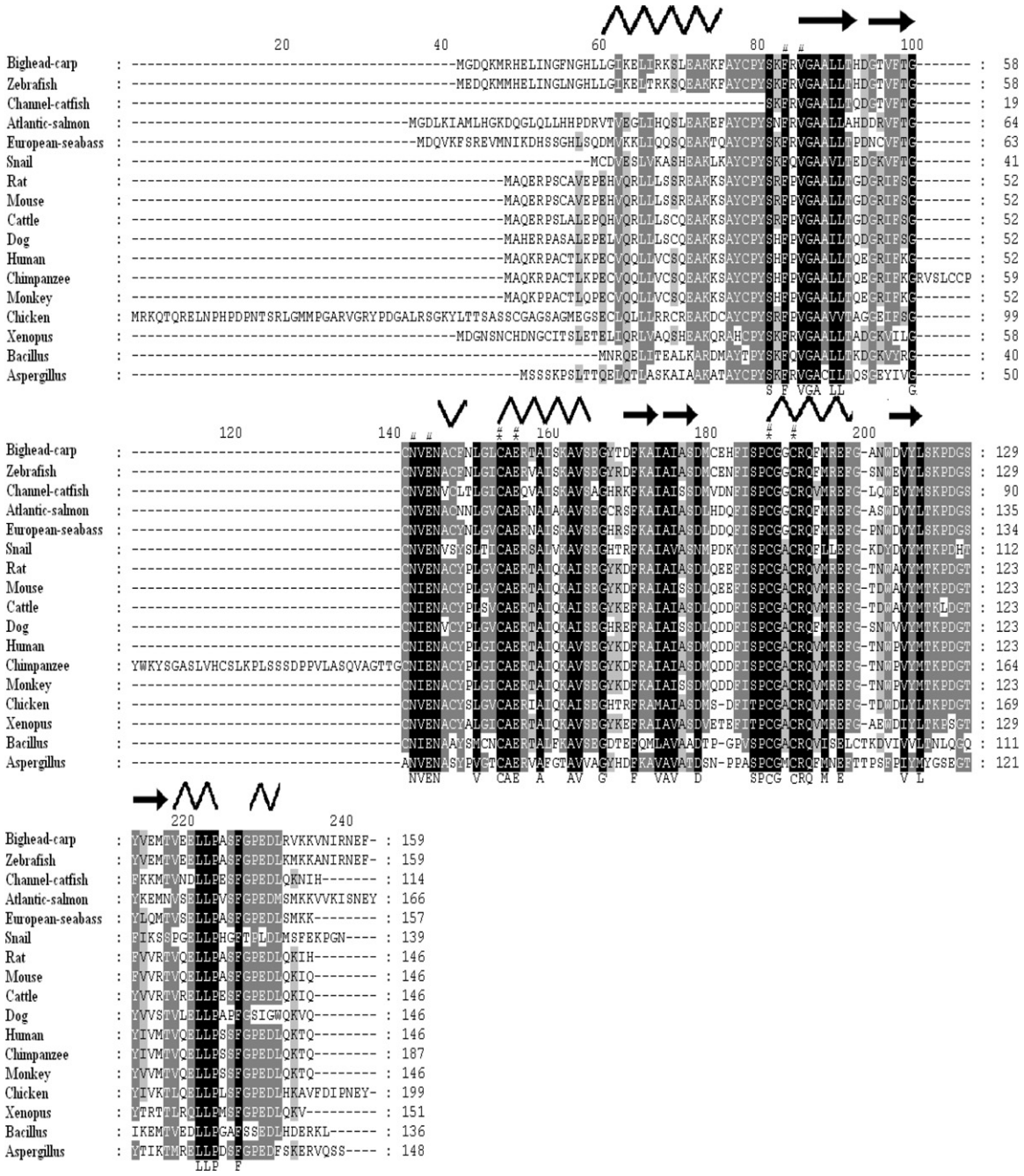

Fig. 2. Alignment of the full-length amino acid sequences of CDA from selected species. Correspondences of common names with Latin names and GenBank accession numbers are shown in Table 2. Residues with 100% identity are shown in dark gray boxes, more than 80% identity in gray boxes and more than 60% identity in light gray boxes. The residues with 100% sequence identity are also displayed below the aligned sequences. The secondary structure elements from bighead carp CDA are shown above the sequences with a twisted rod for α-helix and an arrow for β-sheet. The three zinc ligands are marked with asterisks (*). The active site residues are noted with mark #.

evolutionary distance. In the other big branch, marked as cluster II (Fig. 3), CDAs of rat, mouse, cattle, dog, human, monkey and chimpanzee were closely related to each other with little diversification compared to the non-mammals branch. Unexpectedly, European seabass CDA was more closely related to mammalian CDAs than to CDAs of bighead carp and other fish.

3.3. Expression profile of cda in tissues of bighead carp without any treatment

Expression of the *cda* mRNA was examined by semi-quantitative reverse transcript PCR in bighead carps without any treatment. GAPDH mRNA was used as the internal control, which had been reported to be constantly expressed in all tissues and not affected by MCs (Chen et al., 2005; Hudder et al., 2007). Normalized with GAPDH mRNA expression level, the *cda* gene transcription under normal condition was detected in almost all tested tissues (liver, kidney, intestine, brain, heart, muscle, spleen and gill), as shown in Fig. 4. Compared to the expression level of GAPDH, the strong expression was detected in liver, kidney and spleen; the moderate expression was detected in intestine, brain, heart and muscle; and a very weak expression was found in gill. Distilled water was applied as the template in negative control, and no *cda* transcript was detected (data not shown).

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

Species	Common name	Proteins	Accession no.	Identity	Query coverage	E-value
Danio rerio	Zebrafish	CDA	NP991242.1	91%	100%	3*e*-82
Salmo salar	Atlantic salmon	CDA	NP001140065.1	76%	87%	2*e*-56
Ictalurus punctatus	Channel catfish	CDA	ABC75569.1	75%	69%	2*e*-44
Rattus norvegicus	Rat	CDA	NP001102158.1	72%	81%	2*e*-51
Macaca mulatta	Monkey	CDA	XP001096632.1	72%	81%	7*e*-52
Homo sapiens	Human	CDA	NP001776.1	71%	81%	1*e*-51
Pan troglodytes	Chimpanzee	CDA	XP_001161389.1	71%	85%	1*e*-51
Dicentrarchus labrax	european seabass	CDA	CBN80897.1	70%	100%	2*e*-59
Mus musculus	mouse	CDA	NP082452.1	70%	81%	1*e*-50
Xenopus tropicalis	Xenopus	CDA	NP001017217.1	68%	81%	8*e*-49
Bos taurus	Cattle	CDA	NP001180039.1	67%	81%	6*e*-47
Gallus gallus	Chicken	CDA	NP001139516.1	65%	80%	1*e*-45
Canis familiaris	Dog	CDA	XP544519.1	66%	77%	3*e*-45
Biomphalaria glabrata	Snail	CDA	AAZ39529.1	60%	80%	7*e*-41
Aspergillus oryzae RIB40	*Aspergillus*	CDA	XP003191056.1	50%	84%	9*e*-31
Bacillus subtilis subsp. natto BEST195	*Bacillus*	CDA	BAI86051	54%	79%	4*e*-30

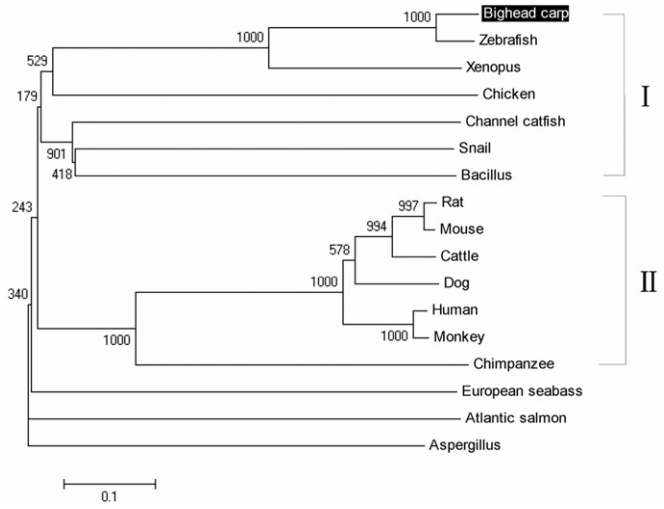

Fig. 3. Neighbor-joining tree of CDAs from bighead carp and other selected species. Bootstrap values are indicated at the nodes. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bar (0.1 mutation per position).

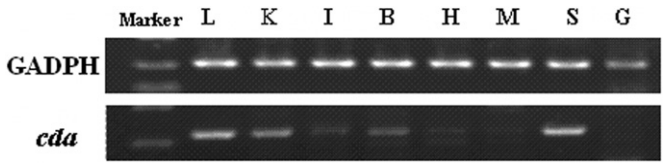

Fig. 4. Expression analysis of bighead carp *cda* by reverse transcript PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. L: Liver; K: kidney; I: intestine; B: brain; H: heart; M: muscle; S: spleen and G: gill.

3.4. Expression profiles of cda mRNA in bighead carp exposed to MCLR

The expression profile of *cda* mRNA was quantified in various tissues of bighead carps injected with 200 μg MCLR per kg bw at 3 h and 24 h post exposure (Fig. 5a). In intestine, *cda* expression increased to about 2.6 fold at 3 h, but then decreased to almost no expression at 24 h. In spleen, *cda* mRNA expression was increased by 6 fold at 3 h ($p < 0.05$) post injection and but was returned to control levels by 24 h post-injection. In heart, *cda* expression was not significantly different from control levels at 3 h post injection, but increased by 3.7 fold at 24 h ($p < 0.05$) post-injection. In brain, *cda* expression enhanced to 15 fold greater at 3 h ($p < 0.001$), but only increased to 5.4 fold at 24 h ($p < 0.05$). In gill, *cda* expression was approximately 8 fold greater at 3 h ($p < 0.01$) then returned to normal expression level at 24 h. In kidney and liver, there was no obvious increase or decrease in *cda* mRNA expression at 3 h and 24 h post-injection. This result showed that MCLR induced *cda* expression in a time inversely dependent pattern in the acute toxic reaction.

The expression profile of *cda* in bighead carp injected with 50, 200 or 500 μg/kg bw MCLR at 3 h post injection was shown in Fig. 5b. In liver, there was no notable expression change in the 50 and 200 μg/kg bw groups, but there was a significantly up-regulation in the 500 μg/kg bw group. In brain, *cda* expression increased to about 14 fold ($p < 0.01$) in the 50 μg/kg bw group, and increased to 50 fold ($p < 0.001$) in the 200 μg/kg bw group, then reached to about 7 fold ($p < 0.01$) in the 500 μg/kg bw group. In kidney, there was no obvious induction of *cda* expression at doses of 50 and 200 μg/kg bw, but increased to about 3.5 fold ($p < 0.05$) at 500 μg/kg bw. In gill, *cda* expression was significantly induced to 8 fold ($p < 0.01$), 7.5 fold ($p < 0.01$) and 15 fold ($p < 0.01$) at the doses of 50, 200 and 500 μg/kg bw. This result indicated that MCLR induced *cda* expression in a dose dependent pattern.

4. Discussion

In the present study, we cloned the full-length cDNA of bighead carp *cda* gene for the first time. A TATA box, a conserved poly (A) signal sequence and a poly (A) tail in the cloned sequence were typical features of an eukaryotic cDNA. Analysis of the deduced amino acid sequence showed that the CDA shared the highest identity with fish and other vertebrates CDA sequences, and the conserved signature motif (H/C)(A/V)E(X24-30)(PCXXC) present in all cytidine deaminases was also found. All these strongly supported that this transcript sequence obtained from bighead carp was a *cda* homolog (Michael, 2006).

In this study, the 3D structure prediction and the alignment of CDA sequences showed that bighead carp *cda* encoded a homo-tetrameric form cytidine deaminase and conserved in evolution. It might be one of the ancestral members of the cytidine deaminase superfamily in nature, which act only on free bases (nucleosides or nucleotides) and cannot deaminate polynucleotide substrates (Navaratnam and Sarwar, 2006). The conserved structure is favorable to the CDA stable function in different species. In cytoplasm CDAs bind to bases at the exact position and catalyze hydrolytic deamination of cytidine and deoxycytidine into uridine

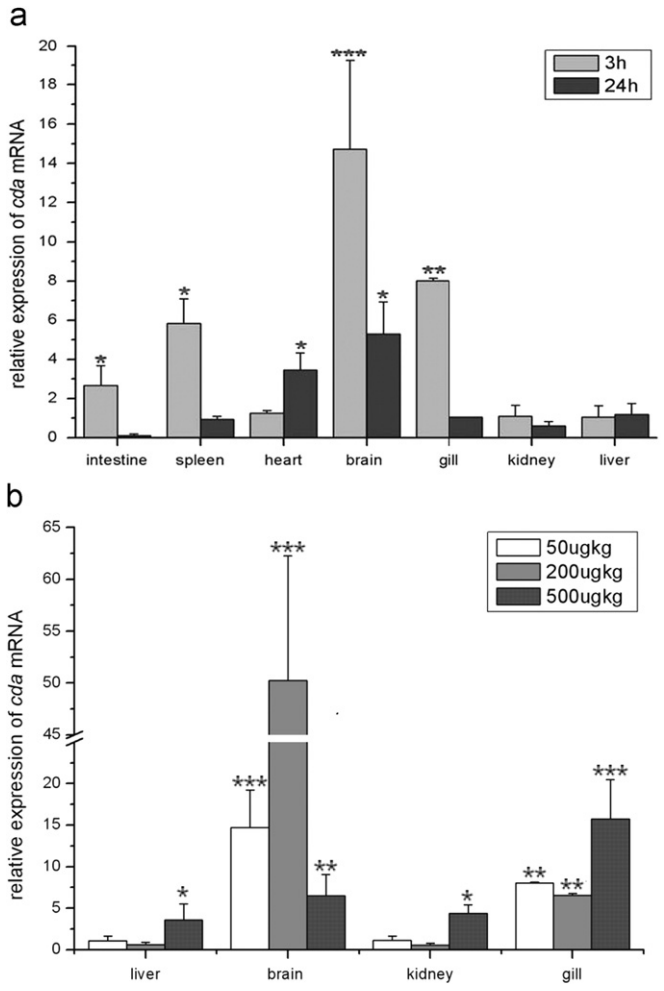

Fig. 5. Expression profiles of *cda* in bighead carp injected with MCLR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Data are expressed as the mean ± S.D. ($n=3$). The significance levels observed are $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). (A) *cda* expression profile in all tested tissues at 3 h and 24 h post injection with MCLR 200 μg/kg bw ($n=3$). (B) *cda* expression profiles in liver, brain, kidney and gill at 3 h post injection with 50, 200, 500 μg/kg bw MCLR ($n=3$).

and deoxyuridine, respectively (Betts et al., 1994). Although CDA sequences displayed high interspecies conservation, the N-terminal and C-terminal sequences varied in amino acid number and composition. While the CDA function is conserved in different species, it was likely that both ends had no important function in the protein, for they mutated to different phenotype in the process of species adaptation to different living environmental conditions and had no adverse effect on CDA function (Yang et al., 2008).

A phylogenetic tree was constructed to further analyze the evolutionary relationship of the CDA proteins. There was an evolutionary diversification among the selected CDAs, and the evolution of CDAs and species was independent (Armin et al., 2007). Bighead carp CDA showed the closest relationship and shared the highest sequence identity with zebrafish CDA. The close relationship of bighead carp CDA and zebrafish CDA in evolution may be that both fishes are classified into cyprinidae family and live in warm freshwater environment. Bighead carp CDA was not so close to the CDAs from other 3 fishes, even far from the CDAs of European seabass and Atlantic salmon in evolution. Though Channel catfish also thrives in fresh waters, the CDA of Channel catfish was not so close to and not so far from bighead carp CDA in the phylogenetic tree. European seabass is a primarily ocean-going fish, and Atlantic salmon undergoes two different growth process in salt waters and fresh waters respectively. The dramatic separation of fish CDAs in the phylogenetic tree perhaps is caused by the ecological distribution of fish CDAs. Different survival environment of fish led to CDAs evolutionary diversification. Although *Bacillus* and *Aspergillus* were microbes, bighead carp CDA was closer to the prokaryote *Bacillus* and far away from the eukaryote *Aspergillus* in evolution. In the tree mammalian CDAs were closely related to each other and significantly different with those of non-mammalian CDAs. CDAs of European seabass, *Aspergillus* and Atlantic salmon stayed outside of the two main branches, indicating that their CDAs evolved in different ways. From the CDA evolutionary tree and the sequences alignment, we concluded that CDAs' evolution was not so strictly coordinated with species evolution, though their functions and sequences were well conserved (Davis, 2002).

In this study, bighead carp *cda* mRNA was detected in all the tested tissues under normal condition, which was in accordance with the CDA tissues distribution in human (Watanabe and Uchida, 1996). CDA is responsible for maintaining the cellular pyrimidine pool and for the pyrimidine salvage pathway in various cell types (Marilia, 2004). The different *cda* expression level in tissues perhaps was related to the active level of pyrimidine metabolism. In human high CDA activity was reported in liver and spleen, and moderate in lung, kidney, large intestine mucosa and colon mucosa (Ho, 1973). In bighead carp liver and spleen, where *cda* expression was higher than other tissues, more CDAs may be were required to keep the balance of cellular pyrimidine deamination and synthesis. While in heart, brain and muscle the *cda* expression was lower, probably only a few CDAs were needed to satisfy the cellular pyrimidine metabolism.

In this study bighead carp *cda* seemed to be an MCLR inducible gene, because *cda* mRNA expression increased post MCLR exposure. There were three different expression regulation patterns in tested tissues after bighead carps were injected into 200 μg MCLR per kg bw. The different *cda* expression response to the same MCLR dose in each tissue probably was the result of both a toxicokinetic (uptake and distribution into the single tissues) related response and a tissue specific (causing different *cda* expression by same cellular MCLR concentration) response. According to Li et al. (2007), in bighead carp tissues the distribution and depuration of MCs changed temporarily, and the change pattern was similar in two MCs treated groups. In both groups, the highest contents of MCs were found in liver, followed by kidney and intestine, and small amounts of MCs were detected in muscle and spleen at different time points post MCs injection. MCs content in each tissue were correspondingly higher in high dose group. The low injection dose led to low content MCLR accumulation in tissues correspondingly. So the short time reactions would not appear in the lower dosed animals, but *cda* expression would also changed in some tested tissues, though *cda* expression was not tested in bighead carps exposed to 50 μg MCLR per kg bw. After MCs were injected into fish abdomen, it at first entered into the intestine, then was transported rapidly through blood system to various organs, and subsequently resulted in different levels of MCs in tissues (liver > kidney > intestine > muscle > spleen). In this study under the treatment of 200 μg MCLR per kg bw, in bighead carp liver the highest MCLR accumulated at 3 h post injection, and at 24 h little MCLR existed. While 500 μg/kg bw was so high that MCLR led severe damage in bighead carp liver. So we identified hepatic genes under 200 μg MCLR per kg bw treatment, and compared changes of genes transcription at different MCLR doses.

In our study, at 3 h post exposure in liver and heart *cda* expression did not increase, though at this time much MCLR was accumulated in these two tissues. While no MCLR

accumulated in intestine, spleen, brain and gill, *cda* expression increased obviously. At 24 h post exposure, no MCLR existed in the tested tissues, and *cda* expression decreased compared to the expression level at 3 h. This phenomenon probably was closely related with CDA function and cellular metabolism activities. Because at 3 h post exposure, in liver and heart MCLR dose perhaps was too high to be tolerated by cells in these organs, so a part of cells were killed and dying, and some cellular metabolism activities were inhibited. At this time *cda* expression did not increase when the samples of tissues were tested. At the same time in the other tested tissues MCLR was little, so cells in these tissues were still vibrant and different enzymes were sensitive to MCLR toxicity, leading to cellular metabolism change. Many reports have showed that one of MCLR toxic effects was induction oxidative DNA damage and chromosome breakage (Rao and Bhattacharya, 1996; Zegura et al., 2003). Nucleotides are the components of DNA and substrates of the enzyme CDA. When MCLR induced cytoplasmic nucleotides metabolism change, more CDAs than normal were required to keep the balance of pyrimidine metabolism and to maintain cellular functions in these tissues, so *cda* expression increased. When exposure time extended to 24 h, some MCLR was transformed to other non-toxic metabolites and some was excreted to the outside environment, little MCLR remained in tissues and cells worked as normal, so *cda* expression decreased and returned to basal levels.

When exposed to 3 different doses MCLR, the increase pattern of *cda* expression was similar in liver, kidney and gill, *cda* expression was highest in 500 μg/kg bw group. It seemed reasonable that the increase of *cda* expression followed the addition of MCLR dose at 3 h post exposure. When bighead carps were injected into more MCLR at the same environmental condition, more MCLR was accumulated in tissues and entered into cells, leading to more serious breakage in cells, then more CDAs were needed to maintain the cellular pyrimidine pool, and help to keep the balance of cellular metabolism. While in brain the highest expression level was in 200 μg/kg bw group. This was may be because 500 μg/kg bw MCLR was so much that many nerve cells were killed by MCLR and were dying, and MCLR cannot regulate *cda* expression to remain cellular functions in brain. The increase of *cda* expression in brain was more sensitive to MCLR than in other 3 tested tissues, perhaps because nerve cells are more sensitive to MCLR toxic effect, such as oxidative stress and inhibition of phosphatase activity (Wang et al., 2010). *cda* expression in brain at 3 h post 200 μg/kg bw MCLR exposure was different in Fig. 5a and Fig. 5b. The difference might have been caused by two independent RT-PCR operation and the followed calculation on Ct value, or samples were from different brain parts. Though the data were different, it also showed the similar regulation pattern that *cda* expression was upregulated at this time point and under this dose treatment. In all it was the whole body co-ordinated system of *cda* gene expression across different tissues of bighead carp rather than *cda* expression from a single tissue that helped the fish to partly withstand MCLR induced toxic stress.

5. Conclusion

We cloned a *cda* homolog in bighead carp (*Aristichthys nobilis*), whose transcription increased post MCLR exposure. The full-length cDNA of *cda* was 541 bp and the deduced ORF encoded a homotetrameric form cytidine deaminase. 3D structure prediction showed that four identical subunits existed in this cytidine deaminase, and each subunit was composed of five β-sheets and six α-helices. The amino acid residues related to catalytic sites, active sites and zinc binding sites were highly conserved. The cloned CDA shared high identities with other CDA sequences, and evolved closely to zebrafish and the other non-mammalian CDAs through phylogenetic analysis. Bighead carp *cda* expressed in all tested tissues (liver, kidney, intestine, brain, heart, muscle, spleen and gill) in normal condition. The bighead carp *cda* changed its expression profile in a time inversely dependent and dose dependent manner to MCLR in most tested tissues, so as to protect itself from MCLR induced toxic damage. It was the whole body co-ordinated system of *cda* gene expression across different tissues of bighead carp rather than *cda* expression from a single tissue that helped the fish to partly withstand MCLR induced toxic stress.

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