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Molecular characterization of caspase-8-like and its expression induced by microcystin-LR in grass carp (*Ctenopharygodon idella*)

LiLi Wei[a,*], Li He[a], Jianping Fu[b], Yi Liu[b], Jiming Ruan[a], Lin Liu[a], Qiwang Zhong[c,**]

[a] *College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, Jiangxi Province, 330045, PR China*
[b] *College of Life Sciences, Jiangxi Normal University, Nanchang, Jiangxi Province, 330022, PR China*
[c] *College of Bioscience and Bioengineering, Jiangxi Agricultural University, Nanchang, Jiangxi Province, 330045, PR China*

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ABSTRACT

Caspase-8, an initiator caspase, plays a vital role in apoptosis. In this study, caspase-8-like (named as Cicaspase-8-like), a homologue of caspase-8, was identified in grass carp (*Ctenopharygodon idella*). The full-length cDNA sequence of CiCaspase-8-like was 1409 bp and contained a 162 bp 5′-UTR, a 239 bp 3′-UTR and a 1008 bp coding sequence. The putative amino acids sequence was 335 residues long, including a large subunit (P20) and a small subunit (P10), but lacking conserved death effector domains. A histidine active site DHSQMDAFVCCVLSHG and a cysteine active-site motif KPKLFFIQACQG were found in P20. Phylogenetic analysis showed that Cicaspase-8-like clustered with the caspase-8 and caspase-8-like of other fish and grouped closely with *Carassius auratus* caspase-8-like. Quantitative real-time PCR revealed that the Cicaspase-8-like mRNA were expressed constitutively in all tested tissues from healthy grass carp, with high expression level in the blood, spleen, liver and gill, indicating its role in immune reaction. The expression of Cicaspase-8-like mRNA was decreased significantly in the liver because of the stress caused by microcystin-LR (MC-LR) (75 and 100 μg MC-LR/kg BW) at 24 h and 96 h post injection ($P < 0.05$), but it was increased significantly in grass carp treated with 25 μg MC-LR/kg BW at 24 h ($P < 0.05$) post injection. Cleaved fragments of Cicaspase-8-like were observed using western blot analysis, and the expression of Cicaspase-8-like protein was increased after MC-LR treatments. Moreover, the expression of both caspase-9 and caspase-3 mRNA increased significantly after treatment with the three doses of MC-LR. TUNEL assay results showed remarkable changes in apoptosis after the MC-LR treatment. These results suggest that Cicaspase-8-like is an important caspase and plays an essential role in MC-LR-induced apoptosis.

1. Introduction

There are two types of apoptosis pathways, the intrinsic and extrinsic pathways, and both kinds of apoptosis are processes that rely on the activation of members of the aspartate-specific cysteine protease (caspase) family [1]. Caspases are cysteinyl aspartate proteases, and can be classified into three groups according to their functions: initiator apoptotic caspases, effector apoptotic caspases, and inflammatory caspases [2]. As a member of the initiator caspase, caspase-8 is well characterized as a proximal enzyme involved in extrinsic apoptosis [3–5]. Extrinsic apoptosis relies on the formation of a death-inducing signaling complex, which always includes Fas-associated via death domain and caspase-8 [1]. The activated caspase-8 can trigger apoptosis by activation of caspase-3 [6]. It is reported that the B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins play a prominent role in the regulation of apoptosis [7]. However, caspase-8 can cleave Bcl-2 homology 3 interacting domain death agonist (BID), and the cleaved BID (cBID) then activates the effector proteins Bcl-2 associated X protein (BAX) and Bcl-2 antagonist killer (BAK) and enables a crosstalk to the intrinsic apoptotic pathway [8,9]. Therefore, caspase-8 plays a critical role in the caspase-dependent apoptotic pathway.

Excessive growth of cyanobacteria can result in the accumulation of cyanobacterial toxins in many eutrophic-hypertrophic lakes, ponds and rivers. The toxins could be classified into five functional groups, namely, hepatotoxins, neurotoxins, cyanotoxins, dermatotoxins, and irritant toxins. Among the hepatotoxins, microcysitins (MCs) are cyclic heptapeptides, and more than 100 different variants of MCs produced by different genera of cyanobacteria have been reported to exist in nature water [10]. On the basis of acute toxicity, microcysitin-LR (MC-LR), a kind of MCs, is the most potent hepatotoxin [11]. Previous

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[*] Corresponding author.
[**] Corresponding author.
E-mail addresses: hbliliwei@163.com (L. Wei), zhongqw2000@163.com (Q. Zhong).

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Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5′-3′)	Application
MF1	CAAAGTTGAAGTGCGGGA	middle fragment
MR1	TCAGGAGACTTGCTTCATG	middle fragment
MF2	ATGGATGAAACTGAAACTG	middle fragment
MR2	ATTCCCAGAACAGAGCCT	middle fragment
RC3-1	CTCTGCAAGCAGCTGGAAGATGGC	3′RACE
RC3-2	TCCAAGGAACAAGGACATAAACTCAA	3′ RACE
Oligo (dT)-adaptor	GGCCACGCGTCGACTAGTACT16	3′ RACE
AP	GGCCACGCGTCGACTAGTAC	3′ and 5′ RACE
Oligo (dG)-adaptor	GGCCACGCGTCGACTAGTACG10	5′ RACE
RC5-1	CCTCCTAACAGCTTTC	5′ RACE
RC5-2	CATCTGATGCTTTGCCCT	5′ RACE
RC5-3	TTGCCCATTCTTGGTAGC	5′ RACE
ORF-F	ATGGATGAAACTGAAACTG	ORF
ORF-R	TCAGGAGACTTGCTTCATG	ORF
Cicaspase8-like-F	ATCTGGTTGAAATCCGTGAA	Quantitative PCR
Cicaspase8-like-R	TCCATCTGATGCCCATACAC	Quantitative PCR
caspase9-F	CTGTGGCGGAGGTGAGAA	Quantitative PCR
caspase9-R	GTGCTGGAGGACATGGGAAT	Quantitative PCR
caspase3-F	GCTGTGCTTCATTTGTTTG	Quantitative PCR
caspase3-R	TCTGAGATGTTATGGCTGTC	Quantitative PCR
β-actin-F	CATCACCATCGGCAACGAGAGG	Quantitative PCR
β-actin-R	CGTCGCACTTCATGATGCTGTTG	Quantitative PCR
Cicas8-like-4T-F	GTTCCGCGTGGATCCCCGGAAATGGATGAAACTGAAACTGTCA	expression primers
Cicas8-like-4T-R	TCAGTCACGATGCGGCCGCTCTTATTGAGAGTGATCCCTCTCTGC	expression primers

studies have shown that MC-LR acts mostly on hepatocytes because of specific transport to liver cells by the bile acid transport systems [12] and causes changes such as overphosphorylation of liver enzymes, liver necrosis, and even intrahepatic bleeding [13]. Many studies showed that MC-LR induced different apoptotic pathways in various cell types depending on the exposure conditions [14,15]. MC-LR either triggers apoptosis through the intrinsic pathway [16,17] or a caspase-independent pathway [18]. Overall, it suggests that MC-LR can induce apoptosis, and caspase family proteases might be involved in the regulation of apoptotic processes in most cases.

Although apoptosis has been widely studied in mammals, only a few caspase molecules have been cloned and characterized in fish. To date, caspase-8 gene has been characterized only in sea bass (*Dicentrarchus labrax*), zebrafish (*Danio rerio*), purse red common carp (*Cyprinus carpio*), big-belly seahorse (*Hippocampus abdominalis*) and blunt snout bream (*Megalobrama amblycephala*) [19–23]. Recently, a homologue of caspase-8, caspase-8-like, was characterized in Hong Kong oyster (*Crassostrea hongkongensis*) and Pacific oyster (*Crassostrea gigas*) [24,25]. However, there is no report in grass carp (*Ctenopharyngodon idella*). Grass carp is an important economic fish species in China, with the highest production among all cultivated freshwater aquatic species. However, recently, grass carp cultivation has suffered drastically from hepatobiliary syndrome due to the eutrophication of water bodies. Using RNA-seq, we had found that MC-LR could induce differential expression of the caspase-8-like gene in grass carp [26]. In this study, we reported the caspase-8-like (named as Cicaspase-8-like) cDNA sequence of grass carp, and investigated the mRNA and protein expressions of Cicaspase-8-like induced by MC-LR in grass carp in vivo. In addition, the expression of other caspase genes (caspase-9 and caspase-3) mRNA and the apoptosis in liver of grass carp were also analyzed. The work presented here would provide more information to understand the caspase family and the mechanism of MC-LR-induced apoptosis in teleost fish.

2. Materials and methods

2.1. Experimental fish, MC-LR challenge, and tissue collection

Healthy juvenile grass carp (~22 g) were purchased from an aquaculture farm in Nanchang, Jiangxi Province, China, and cultured in plastic tanks at 22 ± 1 °C for three weeks before the experiment. To detect the tissue distribution of the Cicaspase-8-like gene, the gill, heart, liver, intestine, muscle, skin, kidney, spleen, head kidney, and blood were sampled from three healthy grass carp. To investigate the expression of Cicaspase-8-like mRNA and protein induced by MC-LR, 120 healthy grass carp were divided randomly and evenly into three experimental groups and one control group. Grass carp from three experimental groups were injected intraperitoneally with 25, 75 and 100 μg/kg body weight (BW) of MC-LR, respectively, and the fish from control group were injected with equal amounts of physiological saline (0.80%). At 24 and 96 h post injection, fish were anesthetized with 100 mM MS-222 (Sigma, St. Louis, MO, USA), and the livers from six grass carp in each group were sampled and frozen immediately in liquid nitrogen.

2.2. RNA extraction and cDNA synthesis

Total RNAs of the grass carp samples were extracted using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The cDNA for rapid amplification of cDNA ends (RACE) PCR was prepared using the Smart™ RACE cDNA Amplification Kit (Clontech, USA) and reverse-transcribed for quantitative real-time PCR (qRT-PCR) with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas Life Sciences, Hanover, MD, USA).

2.3. cDNA cloning

A partial sequence of caspase-8-like was obtained using Illumina HiSeq™ 2500 sequencing analysis [26]. Two pairs of primers were designed to amplify and verify Cicaspase-8-like sequence from the RNA-seq. Then, the primers for 3′ and 5′ RACE were designed based on the verified cDNA sequence (Table 1). For the 3′ end, the first-round PCR primers were RC3-1 and oligo (dT)-adaptor, whereas the second-round primers were RC3-2 and anchor primer (AP). For the 5′ end, the first-round and second-round primers were RC5-1 and RC5-2, respectively, and oligo (dG)-adaptor, whereas the third-round primers were RC5-3 and AP. The resulting PCR products from 3′ RACE and 5′ RACE were cloned into the PMD18-T simple vector and sequenced, respectively. All the sequences were assembled using DNAMAN 6.0 software for the full-length cDNA of Cicaspase-8-like. To confirm the integrity of the cDNA

Table 2
Percent identity and similarity of Cicaspase-8-like to other known caspase-8 and caspase-8-like sequences.

Species	Gene name, GenBank accession number	Identity (%)	Similarity (%)
Carassius auratus	caspase-8-like isoform X1, XP_026115009.1	71	80
Ctenopharyngodon idella	caspase-8, ALB35227.1	68	80
Cyprinus carpio	caspase-8, AGQ03809.1	63	78
Danio rerio	caspase-8, NP_571585.2	61	75
Astyanax mexicanus	caspase-8-like, XP_007230922.2	54	66
Oncorhynchus kisutch	caspase-8-like, XP_020319149.1	54	67
Oncorhynchus mykiss	caspase 8, NP_001268251.1	54	67
Scleropages formosus	caspase-8-like, KPP75551.1	53	70
Lepisosteus oculatus	caspase-8, XP_015214951.1	48	61
Oryzias melastigma	caspase-8-like, XP_024129567.1	46	64
Tinamus guttatus	caspase-8-like, XP_010222877.1	45	62
Apteryx australis mantelli	caspase-8, XP_013807775.1	44	60
Homo sapiens	caspase-8 isoform X4, XP_011510271.1	41	58

sequence, a set of primers, open reading frame (ORF)-F/ORF-R, were used to amplify the ORF of Cicaspase-8-like sequence.

2.4. Sequence analysis

The amino acid sequence of Cicaspase-8-like was deduced using the Expasy translation tool (http://ca.expasy.org/tools). Putative signal peptide prediction was performed using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Topological structure of the transmembrane protein was analyzed using the TMHMM server online tool (http://www.cbs.dtu.dk/services/TMHMM/). The protein domains were predicted using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The basic local alignment search tool of NCBI was used to identify similar sequences (Table 2). Multiple amino acid sequence alignments of the caspase-8-like or caspase-8 were performed using the CLUSTAL program. A phylogenetic tree was constructed using the neighbor-joining method in Mega 6.0 software (http://www.megasoftware.net/mega.html), and the reliability of the trees was assessed by bootstrapping, with 1000 bootstrap replications.

2.5. Quantitative analysis

qRT-PCR was performed on CFX96 Touch™ Real-Time PCR Detection System, according to the manufacturer's instructions. Gene-specific primers for Cicaspase-8-like, caspase-3 and caspase-9 (Table 1) were used for qRT-PCR. The 20 μl reaction systems containing 10 μl of GoTaq® qPCR Master Mix, 2 μl of cDNA template diluted for 10 times, 0.8 μl of each forward and reverse primer (10 μM), and 6.4 μl of sterile distilled water. The amplification protocol was as follows: initial 5 min denaturation and enzyme activation at 95 °C, 40 amplification cycles at 95 °C for 15 s and 58 °C for 15 s. Each PCR assay was performed in triplicate, and β-actin was used to normalize the data for differences between samples. The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Expression and purification of Cicaspase-8-like recombinant protein

The expression primers were designed and used to amplify amino acids 1–160 of Cicaspase-8-like (Table 1). After PCR amplification, the purified products were digested and ligated into the pGEX-4T-1 expression vector (Novagen, USA) and then transformed into DH5α-competent cells. The pGEX-4T-1-Cicaspase-8-like construct was confirmed by DNA sequencing, then transformed into *Escherichia coli* BL21strain for protein expression. The fusion protein was induced by isopropyl-beta-D-1-thiogalactopyranoside (IPTG) and analyzed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the polyclonal antibodies were prepared as previously described in other study [21].

2.7. Western blotting

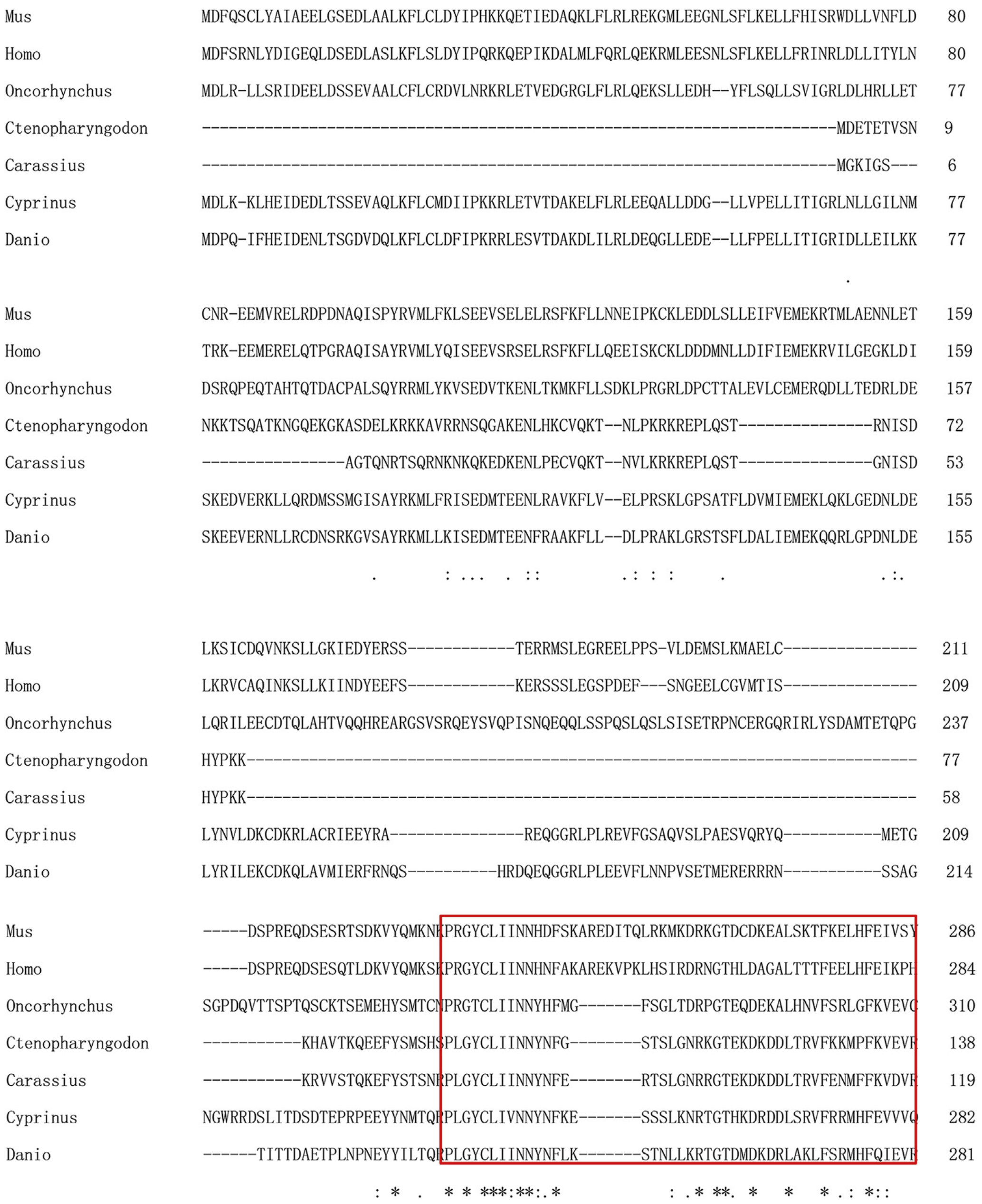
The protein of grass carp livers from experimental and control groups were extracted and the assay of western blotting was analyzed as previously described in common carp [21]. Briefly, samples, equivalent to 10 mg of the total protein, was run on 12% SDS-PAGE gel and subsequently transferred to a 0.45 μM polyvinylidene-fluoride membranes (PVDF) (Millipore, USA) by using standard procedures, and then analyzed by western blotting with Cicaspase-8-like polyclonal antibody diluted 1000-fold in blocking buffer. The immunoblots were detected with 1:2000 diluted goat anti-rabbit IgG conjugated with peroxidase (Beyotime, Shanghai, China). The protein bands were detected using the Pierce Plus enhanced chemiluminescence Western blotting substrate (ThermoFisher Scientific, USA) and x-ray film. Densitometric analysis of the protein bands was performed using a Gel-Pro Analyzer (Media Cybernetics, USA). Besides, mouse anti-GAPDH monoclonal antibody (Proteintech Group Inc., USA) diluted 1:10,000 was used as a control in this experiment. The proteins were quantified using Bio-Rad Quantity One software, and the data are presented as mean ± SD of independent experiments performed in triplicate.

2.8. Detection of MC-LR-induced apoptosis in the liver by terminal deoxynucleotidyl transferase-mediated dUPT-biotin nick end labeling (TUNEL) assay

TUNEL assay was utilized to directly demonstrate MC-LR-induced apoptosis and morphological changes in the liver. Firstly, the liver tissues from experimental and control groups were dissected at 24 and 96 h post injection, washed in cold PBS (0.1 mol/L, pH 7.2) and fixed with 4% paraformaldehyde (#G1101, Servicebio, Wuhan, China) at 4 °C for more than 24 h. Then, the tissues were embedded in paraffin wax, sectioned (4 μm), and mounted on glass slides. The Step TUNEL Apoptosis Assay Kit (#C1086, Beyotime) was used, according to the manufacturer's protocol, and the slides were observed using a fluorescence microscope (Nikon, Japan).

2.9. Statistical analyses

The statistical analyses were performed using SPSS software (version 17.0). The expression of Cicaspase-8-like, caspase-9 and caspase-3 mRNA and protein induced by MC-LR were analyzed with factorial analysis of variance (ANOVA) model, represented by two independent variables (time and dose) and their interactions. The analyses in each time were tested using Tukey's HSD post hoc test. Cicaspase-8-like mRNA expression level in the different tissues were analyzed using one-way ANOVA. P value < 0.05 was considered statistically significant, and all results were expressed as mean ± SD values.



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Mus                MDFQSCLYAIAEELGSEDLAALKFLCLDYIPHKKQETIEDAQKLFLRLREKGMLEEGNLSFLKELLFHISRWDLLVNFLD   80
Homo               MDFSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDALMLFQRLQEKRMLEESNLSFLKELLFRINRLDLLITYLN   80
Oncorhynchus       MDLR-LLSRIDEELDSSEVAALCFLCRDVLNRKRLETVEDGRGLFLRLQEKSLLEDH--YFLSQLLSVIGRLDLHRLLET   77
Ctenopharyngodon   -----------------------------------------------------------------------MDETETVSN   9
Carassius          -----------------------------------------------------------------------MGKIGS---   6
Cyprinus           MDLK-KLHEIDEDLTSSEVAQLKFLCMDIIPKKRLETVTDAKELFLRLEEQALLDDG--LLVPELLITIGRLNLLGILNM   77
Danio              MDPQ-IFHEIDENLTSGDVDQLKFLCLDFIPKRRLESVTDAKDLILRLDEQGLLEDE--LLFPELLITIGRIDLLEILKK   77
                                                                                           .

Mus                CNR-EEMVRELRDPDNAQISPYRVMLFKLSEEVSELELRSFKFLLNNEIPKCKLEDDLSLLEIFVEMEKRTMLAENNLET   159
Homo               TRK-EEMERELQTPGRAQISAYRVMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNLLDIFIEMEKRVILGEGKLDI   159
Oncorhynchus       DSRQPEQTAHTQTDACPALSQYRRMLYKVSEDVTKENLTKMKFLLSDKLPRGRLDPCTTALEVLCEMERQDLLTEDRLDE   157
Ctenopharyngodon   NKKTSQATKNGQEKGKASDELKRKKAVRRNSQGAKENLHKCVQKT--NLPKRKREPLQST---------------RNISD   72
Carassius          ----------------AGTQNRTSQRNKNKQKEDKENLPECVQKT--NVLKRKREPLQST---------------GNISD   53
Cyprinus           SKEDVERKLLQRDMSSMGISAYRKMLFRISEDMTEENLRAVKFLV--ELPRSKLGPSATFLDVMIEMEKLQKLGEDNLDE   155
Danio              SKEEVERNLLRCDNSRKGVSAYRKMLLKISEDMTEENFRAAKFLL--DLPRAKLGRSTSFLDALIEMEKQQRLGPDNLDE   155
                                      .       : ...  . ::         .: : :     .                 .:.

Mus                LKSICDQVNKSLLGKIEDYERSS------------TERRMSLEGREELPPS-VLDEMSLKMAELC---------------   211
Homo               LKRVCAQINKSLLKIINDYEEFS------------KERSSSLEGSPDEF---SNGEELCGVMTIS---------------   209
Oncorhynchus       LQRILEECDTQLAHTVQQHREARGSVSRQEYSVQPISNQEQQLSSPQSLQSLSISETRPNCERGQRIRLYSDAMTETQPG   237
Ctenopharyngodon   HYPKK---------------------------------------------------------------------------   77
Carassius          HYPKK---------------------------------------------------------------------------   58
Cyprinus           LYNVLDKCDKRLACRIEEYRA---------------REQGGRLPLREVFGSAQVSLPAESVQRYQ-----------METG   209
Danio              LYRILEKCDKQLAVMIERFRNQS----------HRDQEQGGRLPLEEVFLNNPVSETMERERRRN-----------SSAG   214

Mus                -----DSPREQDSESRTSDKVYQMKNKPRGYCLIINNHDFSKAREDITQLRKMKDRKGTDCDKEALSKTFKELHFEIVSY   286
Homo               -----DSPREQDSESQTLDKVYQMKSKPRGYCLIINNHNFAKAREKVPKLHSIRDRNGTHLDAGALTTTFEELHFEIKPH   284
Oncorhynchus       SGPDQVTTSPTQSCKTSEMEHYSMTCNPRGTCLIINNYHFMG-------FSGLTDRPGTEQDEKALHNVFSRLGFKVEVQ   310
Ctenopharyngodon   -----------KHAVTKQEEFYSMSHSPLGYCLIINNYNFG--------STSLGNRKGTEKDKDDLTRVFKKMPFKVEVR   138
Carassius          -----------KRVVSTQKEFYSTSNRPLGYCLIINNYNFE--------RTSLGNRRGTEKDKDDLTRVFENMFFKVDVR   119
Cyprinus           NGWRRDSLITDSDTEPRPEEYYNMTQRPLGYCLIVNNYNFKE-------SSSLKNRTGTHKDRDDLSRVFRRMHFEVVVQ   282
Danio              ------TITTDAETPLNPNEYYILTQRPLGYCLIINNYNFLK-------STNLLKRTGTDMDKDRLAKLFSRMHFQIEVR   281
                                       : *  .  * * ***:**:.*          : .* **.  *    *    * .: *::
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Fig. 1. The protein domain features of Cicaspase-8-like and alignment of its amino acid sequence with those of other vertebrates.
The same amino acids are marked by asterisks, whereas those with high and low similarity are indicated by semicolons and dots, respectively. The P20 large subunit is marked by a red box, and the P10 small subunit is marked by a blue box. The cysteine active sites (KPKLFFIQACQG) and caspase family histidine (DHSQMDAFVCCVLSHG) are shaded. The residues putatively involved in catalysis are indicated with filled triangles. GenBank accession numbers of the caspase-8 or caspase-8-like amino acid sequences are as follows: *Carassius auratus*, XP_026115010.1; *Cyprinus carpio*, AGQ03809.10; *Oncorhynchus mykiss*, NP_001268251.1; *Danio rerio*, NP_571585.2; *Mus musculus*, NP_033942.1; *Homo sapiens*, NP_203519.1.

3. Results

3.1. Characterization of the Cicaspase-8-like full-length cDNA

The full-length Cicaspase-8-like mRNA is 1409 nt and contains an ORF of 1008 nt, which has been deposited in GenBank (accession number MG797686). It contains a 162-bp 5′ untranslated region (UTR) and a 239-bp 3′ terminal UTR with a canonical polyadenylation signal sequence (AATAAA) and a poly (A) tail. The putative Cicaspase-8-like is predicted to be a peptide of 335 amino acids, with a calculated molecular weight of 38 kDa and isoelectric point of 9.11. ScanProsite analysis of the deduced amino acid sequence showed that it contained caspase-family P20 large subunits (amino acid position: 94–216) and P10 small subunits (amino acid position: 244–331) (Fig. 1). The caspase family signature histidine active site DHSQMDAFVCCVLSHG and a cysteine active-site motif KPKLFFIQACQG which is characteristic of caspase-8, were found in the P20 domain (Fig. 1). However, no death effector domain (DED) was detected in Cicaspase-8-like.

3.2. Homologous and phylogenetic characteristics of Cicaspase-8-like

The deduced amino acid sequence of Cicaspase-8-like had the highest amino acid identity and similarity to *Carassius auratus* caspase-8-like (71% and 80%, respectively), followed by other fish, bird, and mammal caspase-8 or caspase-8-like (Table 2). To further understand the evolutionary relationships of Cicaspase-8-like with teleost and other

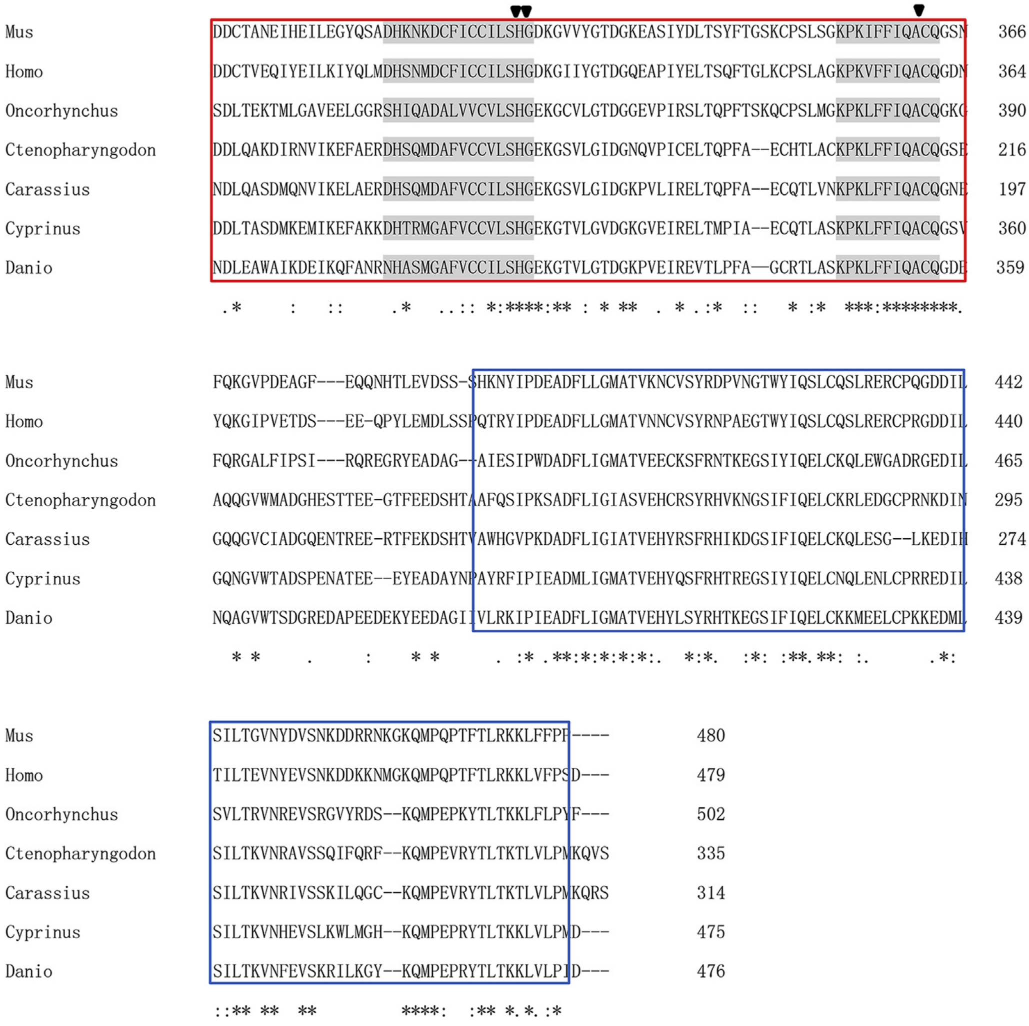

Fig. 1. (*continued*)

vertebrates, a phylogenetic tree was established with multiple caspase-8 and caspase-8-like amino acid sequences using the neighbor-joining method (Fig. 2). As shown in Fig. 2, the phylogenetic tree was divided into four main clusters, namely, mammals, birds, shellfish and fish. The putative Cicaspase-8-like sequence was located within the fish group and close to *Carassius auratus* caspase-8-like.

3.3. Distribution of Cicaspase-8-like in healthy grass carp

The Cicaspase-8-like mRNA expression in different tissues was examined using qRT-PCR. Cicaspase-8-like showed constitutive expression in almost all the examined tissues (Fig. 3). The highest level of Cicaspase-8-like was observed in blood, about 19.42-fold higher than that in head kidney, followed by liver (11.52-fold), spleen (9.67-fold), gill (8.08-fold), heart (5.30-fold), muscle (4.80-fold) and intestine (3.89-fold).

3.4. Effects of MC-LR on the expression of Cicaspase-8-like mRNA in grass carp liver

The expression of Cicaspase-8-like mRNA in grass carp livers was analyzed using qRT-PCR at 24 and 96 h post injection with different doses of MC-LR (25, 75, and 100 μg/kg BW). The results showed that the expression of Cicaspase-8-like mRNA were changed significantly at the different time points ($F = 6.029$, $P = 0.017$) and affected by three doses of MC-LR ($F = 40.804$, $P = 0.000$). Significant interaction between exposure time and dose was observed in the liver ($F = 7.578$, $P = 0.000$). Low dose of MC-LR (25 μg MC-LR/kg BW) increased significantly ($P < 0.05$) the expression of Cicaspase-8-like mRNA at 24 h post injection, but not significant ($P > 0.05$) at 96 h post injection. On the contrary, High doses of MC-LR (75 and 100 μg MC-LR/kg BW) reduced significantly ($P < 0.05$) the expression of Cicaspase-8-like mRNA at both 24 h and 96 h post injection (Fig. 4).

3.5. Effects of MC-LR on the expression of Cicaspase-8-like protein in grass carp liver

To analyze whether the activation of Cicaspase-8-like is involved in inducing apoptosis in the liver of grass carp treated with MC-LR, the expression plasmid pGEX-4T-1-Cicaspase-8-like was transformed into the *E. coli* BL21 strain. The pGEX-4T-1-Cicaspase-8-like fusion proteins, with a molecular weight of about 45 kDa (Fig. 5), were then used to immunize the rabbits to gain polyclonal antibodies for western blotting.

The proteins induced in the liver by 25, 75, and 100 μg MC-LR/kg BW for 24 and 96 h were detected with western blotting analysis (Fig. 6A) and quantified using Bio-Rad Quantity One software. The relative expression of Cicaspase-8-like protein is shown in Fig. 6B. The results showed that cleaved fragments were detected, except in grass carp treated with 75 μg MC-LR/kg BW for 24 h and 25 μg MC-LR/kg BW for 96 h. The expression of pro-Cicaspase-8-like was increased in three experimental groups after treatment for 24 and 96 h, but no statistically significant differences were found ($P > 0.05$).

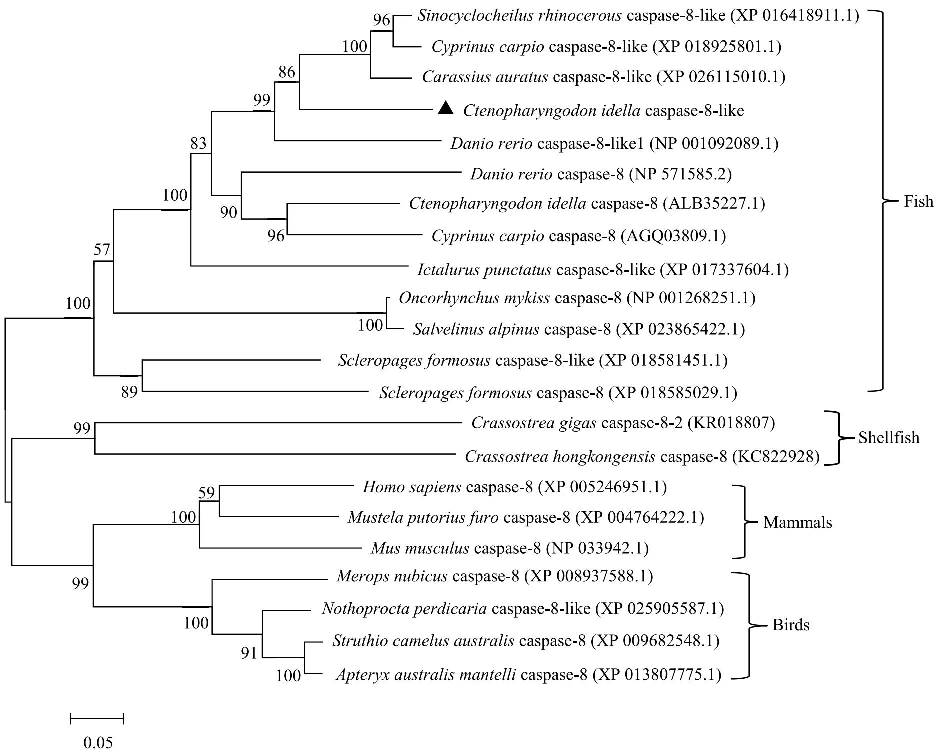

Fig. 2. Phylogenetic analysis of the relationship between Cicaspase-8-like and other known caspase-8 family members.
The tree was constructed using the neighbor-joining method in MEGA 6.0. The numbers at the nodes represent bootstrap values based on 1000 replicates. The scale represents Nei's genetic distance. The accession number for each sequence is provided after the species name and molecular type. Cicaspase-8-like is indicated with a triangle.

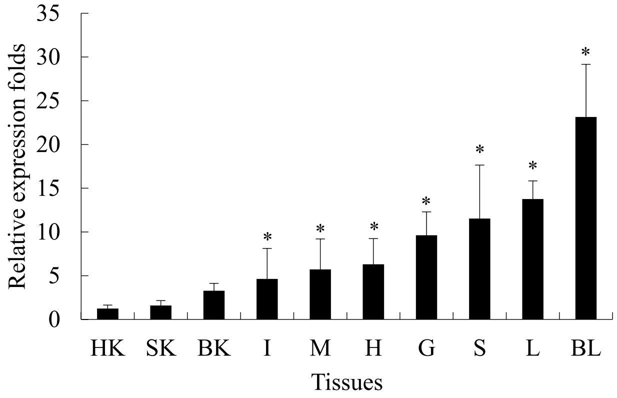

Fig. 3. Distribution of Cicaspase-8-like in different tissues of grass carp.
Note: qRT-PCR was performed to detect the expression of Cicaspase-8-like mRNA in different tissues, namely, head kidney (HK), skin (SK), body kidney (BK), intestine (I), muscle (M), heart (H), gill (G), spleen (S), liver (L), and blood (BL). β-actin was used as the positive control to normalize the samples. Data are presented as mean ± SD (n = 3) according to the head kidney expression, error bars indicate standard deviation. Statistical significance was analyzed using one-way ANOVA ($^{*}P < 0.05$).

3.6. Effects of MC-LR on the expression of other caspase genes in grass carp liver

The expression of caspase-9 mRNA was induced significantly ($P < 0.05$) in all the treated grass carp (Fig. 7A). The expression of caspase-3 mRNA was promoted significantly ($P < 0.05$) in all treated grass carp, but no statistically significant differences were found in grass carp treated with 75 and 100 μg MC-LR/kg BW at 24 h post injection (Fig. 7B). The factorial ANOVA results showed interactive effects between the dose and exposure time for the expression of caspase-9 and caspase-3 mRNA, and the mRNA expression of the two caspases were changed significantly ($P < 0.05$) at the different time points and doses.

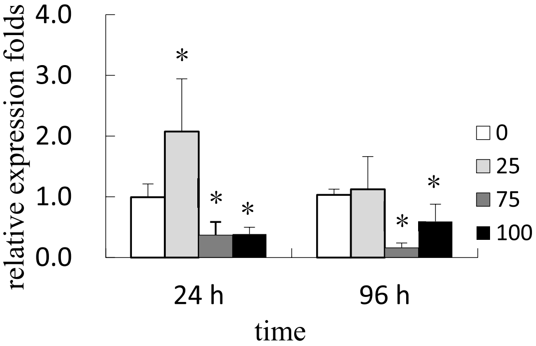

Fig. 4. The expression of Cicaspase-8-like mRNA in the liver of grass carp after injected with MC-LR.
Note: The data are expressed as mean ± S.D (n = 6). Statistical significance was analyzed using factorial ANOVA, and statistically significant differences were accepted at $P < 0.05$. The column bars with asterisks indicate that the value for the treated group is significantly different from that for the control at $P < 0.05$.

3.7. Detection of MC-LR-induced apoptosis in grass carp liver by TUNEL assay

In the TUNEL assay, green fluorescence indicated the apoptotic cells. The apoptotic cells were visually identified in 10 randomly selected fields and photographed at a magnification of × 400. No obvious apoptotic cells were detected in the control group, whereas a large number of apoptotic cells were observed in groups treated with 75 and 100 μg MC-LR/kg BW at 24 and 96 h post injection. The apoptosis induced by MC-LR could be clearly observed in Fig. 8.

4. Discussion

In this study, a homologue of caspase-8 (Cicaspase-8-like) was cloned from grass carp. Cicaspase-8-like had the caspase family signature with the typical P20 and P10 domains, and the conservative

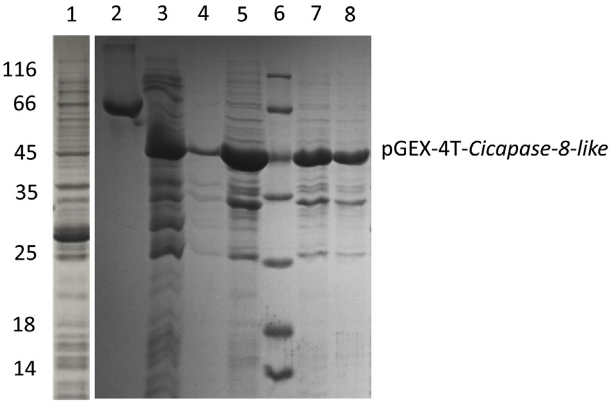

Fig. 5. SDS-PAGE analysis of recombinant Cicaspase-8-like.
Lane 1: Empty vector of pGEX-4T; Lane 2: 0.4 mg/L BSA; Lane 3: supernatant of recombinant pGEX-4T-Cicaspase-8-like; Lane 4: supernatant of recombinant pGEX-4T-Cicaspase-8-like (inclusion body was dissolved by 2 M urea); Lane 5: precipitate with double dilution (precipitate was dissolved by 8 M urea); Lane 6: protein molecular standard; Lane 7: precipitate with five-fold dilution (precipitate was dissolved by 8 M urea); Lane 8: precipitate with 10-fold dilution (precipitate was dissolved by 8 M urea).

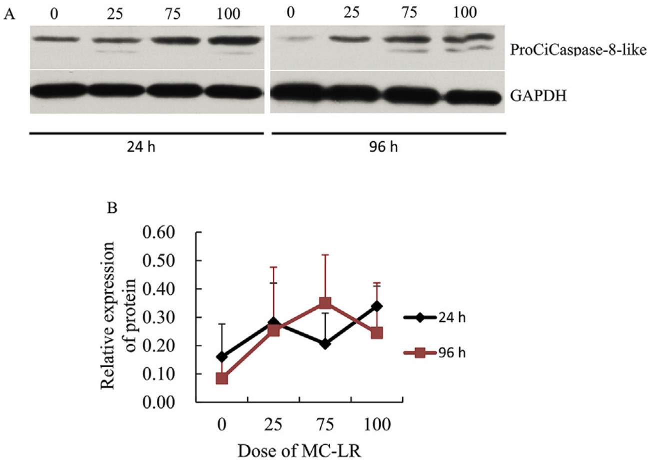

Fig. 6. The expression of Cicaspase-8-like protein in the liver of grass carp after injected with MC-LR.
Note: (A) Western blotting results for Cicaspase-8-like induced in grass carp liver by different doses of MC-LR. (B) Relative quantification analysis of Cicaspase-8-like. The proteins were quantified using Bio-Rad Quantity One software, and GAPDH was used as the internal control. The data are presented as mean ± SD (n = 3)of independent experiments performed in triplicate.

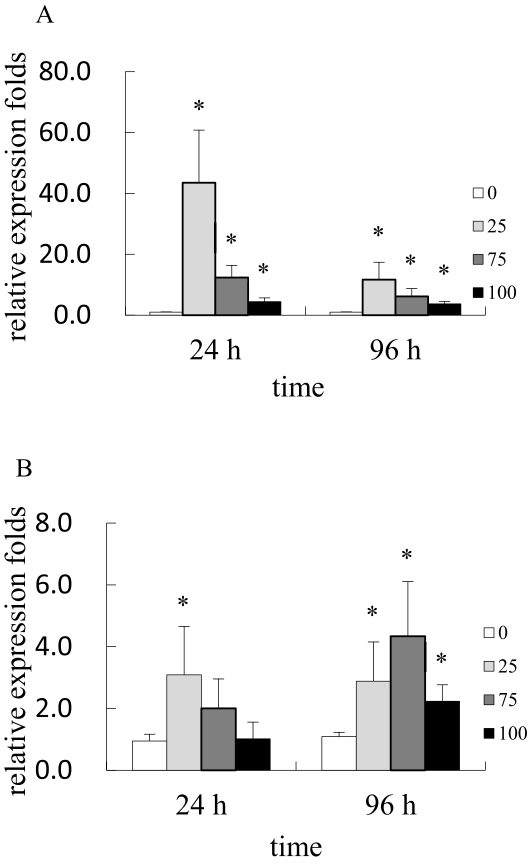

Fig. 7. The expression of the other caspase genes in the liver of grass carp after injected with MC-LR.
A shows the mRNA level of caspase-9 in the liver of grass carp after injected with MC-LR. B shows the mRNA level of caspase-3 in the liver of grass carp after injected with MC-LR. The data are expressed as mean ± S.D (n = 6). Statistical significance was analyzed using factorial ANOVA, and statistically significant differences were accepted at $P < 0.05$. Column bars with asterisks indicate that the value for the treated group is significantly different from that for the control at $P < 0.05$.

active-site pentapeptide QACQG (Fig. 2). In humans, mature caspase-8 is generated from procaspase-8 by cleaving the sites “VETDS” and “LEMD” between the P20 and P10 domains [5]. However, these cleavage sites were not predicted in the sequence of Cicaspase-8-like. In addition, homology analysis showed that Cicaspase-8-like shared a higher similarity with previously reported other species caspase-8 or caspase-8-like sequences. The phylogenetic analysis indicated that Cicaspase-8-like was grouped in the cluster of fish caspase-8 or caspase-8-like, suggesting that it might be a homologue of the fish caspase-8 family. In vertebrates, caspase-8 has two DED motifs in the prodomain, and these motifs are responsible for the auto-activation of inactive proenzymes [27]. However, the DED motif was not found in Cicaspase-8-like, which is similar to colored abalone (*Haliotis diversicolor*) caspase [28]. A novel marine shrimp (*Marsupenaeus japonicas*) caspase without DED was also cloned [29]. DED can form intracellular filaments and function as a crucial nexus to transmit the extrinsic death signal to downstream effectors through the cleavage of caspase-8 in mammals [30]. Huang et al. [28] strengthened the argument that the DED motif evolved later after analyzing the putative prodomian of *Haliotis diversicolor* caspase. Thus, the lack of DED in Cicaspase-8-like indicates that it may play novel roles, and identification of these potential functions is important for further research on this protein.

Previous studies have shown that caspase-8, are widely distributed in several fish species. For example, the expression level of caspase-8 mRNA was high in the liver of the blunt snout bream *Megalobrama amblycephala* [23], however, the lowest expression of caspase-8 mRNA was found in the liver of the big-belly seahorse *Hippocampus abdominalis* [22]. In the present study, Cicaspase-8-like was expressed in all examined tissues, with higher expression level in the blood and spleen, which is similar to the expression pattern of caspase-8 in human [31]. These findings suggest that Cicaspase-8-like may have important roles in taking part in the innate immune response.

Recently, gene expression alterations have been increasingly used in toxicological experiments [32,33], and many studies have shown that MC-LR can activate caspases. It has been reported that the expression of zebrafish caspase-8 mRNA increased significantly in a dose-dependent manner [34], while the expression of Cicaspase-8-like mRNA was increased significantly just at 24 h after treatment with 25 μg MC-LR/kg BW. Recent research demonstrated that the expression of caspase-9 and caspase-3 mRNA was elevated significantly in fish after MC-LR treatment. For example, the expression of caspase-3 mRNA in the livers of

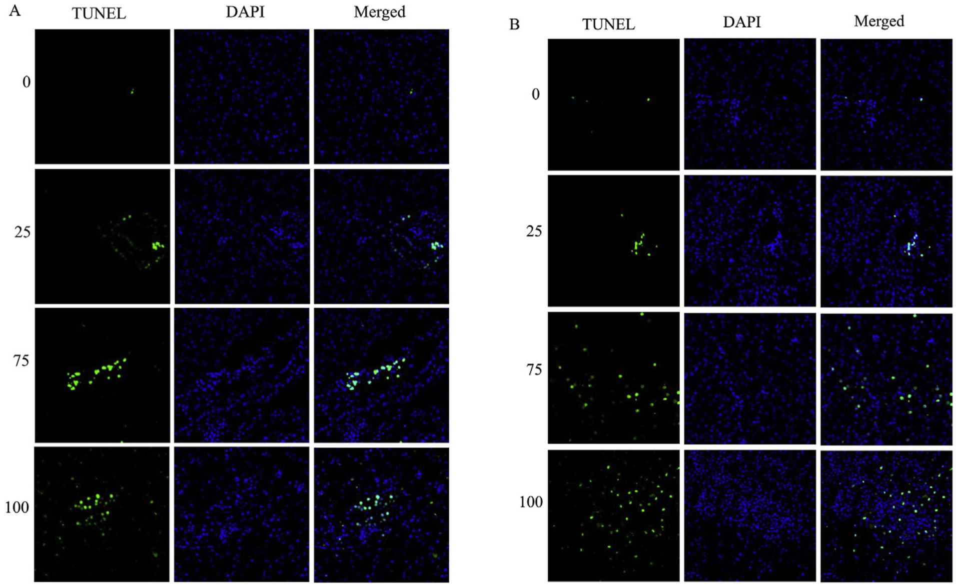

Fig. 8. Detection of apoptosis in grass carp liver treated with MC-LR by TUNEL assay.
A shows the TUNEL assay results for grass carp treated with different doses of MC-LR for 24 h. B shows the TUNEL assay results for grass carp treated with different doses of MC-LR for 96 h. Apoptosis was determined using the TUNEL assay and DAPI staining in the liver of grass carp treated with MC-LR. Green fluorescence indicates TUNEL-positive cells and the cell nuclei stained with DAPI is blue. 0, 25, 75 and 100 represents the dose of MC-LR, respectively.

zebra fish exposed to 50 μg MC-LR/kg was up-regulated at 12 °C and 32 °C compared with that in the control group [35]. Zeng et al. [36] reported that the expression of caspase-9 and caspase-3 mRNA increased greatly in zebrafish embryos treated with 5 mg/L MC-LR. Li et al. [37] found that expression of caspase-9 and caspase-3 mRNA improved in the Sertoli cells of rats after MC-LR treatment for 24 h. In this study, the expression of caspase-9 and caspase-3 mRNA was increased significantly in grass carp when treated with three different doses of MC-LR, which was consistent with the findings of the above-mentioned reports. The caspase family is the final executor of apoptosis, especially, caspase-3 is a key proteolytic enzyme required to cleave the substrate downstream of the apoptotic pathway [38], and it is activated during the final step of apoptosis [39]. These results suggested that apoptosis can be induced by MC-LR.

The activation of Cicaspase-8-like was supported by the observation that the cleaved form was detected in the liver tissue of grass carp after stimulation with MC-LR, and the protein expression of Cicaspase-8-like in three treatment groups increased compared with control group. Caspases play crucial roles in mediating various apoptotic responses, and the cascade led by caspase-8 is involved in death receptor-mediated apoptosis. Active caspase-8 activates BID and then triggers the mitochondrial pathway or directly activates caspase-3, committing the cell to apoptosis [40]. Although Cicaspase-8-like is a homologue of caspase-8, it has no DED motif, which cannot reveal whether MC-LR-induced liver cell apoptosis in grass carp is related to the death receptor pathway. Overall, the results of our study suggested that Cicaspase-8-like plays important roles in the response to MC-LR challenge.

The TUNEL assay is a widely used staining method used for detecting apoptotic cells in tissue sections [36]. In the present study, we performed the TUNEL assay and found TUNEL staining was nearly undetectable in the liver of the control grass carp, whereas a high level of TUNEL-positive cells were observed in the livers of grass carp treated with 25, 75, and 100 μg MC-LR/kg BW at 24 and 96 h post injection. The results of TUNEL further suggested that MC-LR might induce the apoptosis in the liver of grass carp.

5. Conclusions

In conclusion, Cicaspase-8-like, a caspase-8 homologue, was identified in grass carp. The sequence analysis showed that Cicaspase-8-like had characteristics of the caspase family but without two DED motifs. Expression analyses revealed that Cicaspase-8-like was ubiquitously expressed in all tested tissues of healthy grass carp, with the highest expression in the blood, spleen, liver and gill. The TUNEL assay results, altered expression of caspase-9 and caspase-3 mRNA, and changes in the mRNA and protein expression of Cicaspase-8-like showed that MC-LR could induce apoptosis and Cicaspase-8-like might play an indispensable role in the response to MC-LR challenge.

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