Fish & Shellfish Immunology 29 (2010) 388–398

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Identification and characterization of common carp (*Cyprinus carpio* L.) granzyme A/K, a cytotoxic cell granule-associated serine protease

Rong Huang [a,c,1], Shan Zhong [a,c,1], Hong Liu [b], Renqiu Kong [a], Yaping Wang [a], Wei Hu [a], Qionglin Guo [a,*]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[b] Key Laboratory of Aquatic Animal Diseases, Shenzhen Exit & Entry Inspection and Quarantine Bureau, Shenzhen 518001, China
[c] Graduate School of Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 30 December 2009
Received in revised form
5 April 2010
Accepted 17 April 2010
Available online 6 May 2010

Keywords:
Granzyme A/K
RACE
Molecular cloning
Viral infection
Common carp

ABSTRACT

Granzyme (Gzm) is an important member of serine protease family, and key component in the specific and non-specific cell-mediated cytotoxicity. Partial GzmA/K cDNA sequence of common carp (*Cyprinus carpio* L.) was isolated from thymus cDNA library by the method of suppression subtractive hybridization (SSH). Subsequently, the full length cDNA of carp GzmA/K was obtained by means of 3′ RACE and 5′ RACE, respectively. The full length cDNA of carp GzmA/K was 1053 bp, consisting of a 5′-terminal untranslated region (UTR) of 65 bp, a 3′-terminal UTR of 214 bp, and an open reading frame of 774 bp. Amino acid sequence analysis indicated the existence of a signal peptide, eight consensus cysteine residues, one conserved IIGG motif and three conserved residues as central elements of the GzmA/K active site. Carp GzmA/K shared 36% and 39% amino acid identity to human GzmA and K, respectively, and was phylogenetically related to the granzyme A and K subgroups. Then, a genomic DNA, which covers the promoter region and entire coding region of carp GzmA/K, was obtained by PCR. In the 5.4 k-long genomic sequence, five exons and four introns were identified. Real-time RT-PCR analysis showed that carp GzmA/K transcript was predominantly detected in the immune-related tissues, and after SVCV infection, was up-regulated in most immune-related tissues in a time-dependent manner. Real-time RT-PCR results also showed that carp GzmA/K transcript was up-regulated in thymus tissue of GH transgenic carp. These results will help to understand the molecular characterization and the potential role of teleost GzmA/K, a cytotoxic cell granule-associated serine protease.

1. Introduction

Cytotoxic T (Tc) cells and natural killer (NK) cells, both key components of the host immune system, play an important role in the surveillance and destruction of foreign or infected tissues and cells. Although NK cells are able to kill cells in an apparently non-MHC restricted manner without need for prior sensitization (do not recognize presented antigens) [1,2], both cytotoxic effector cells act through either the death receptor pathway (i.e., Fas/FasL) and/or the granule secretion pathway (reviewed in Ref. [3]). The cytotoxic cell granule secretion pathway is critical for eliminating tumor and viral infected cells through a process in which the pore-forming protein, perforin (PFP), delivers the granzymes (Gzms), into cells targeted for destruction (reviewed in Ref. [4]). In other words, both granule-associated PFP and Gzm are key components in the specific and non-specific cell-mediated cytotoxicity (CMC).

Gzms belongs to a family of serine proteases (SPs) localized in the electron dense cytoplasmic granules of activated NK and Tc cells [5]. Gzm loci have been placed into three groups by their chromosomal location and structural similarities. They have three primary specificities (aspase, chymase, tryptase metase) referring to the type of activities of the proteases encoded for in their genes (reviewed in Ref. [6]). To date, several Gzms have been identified in humans (GzmA, B, H, K and M) and in mice (GzmA–G, K, M–N) (reviewed in Ref. [3]). Expression of the Gzms was originally viewed as limited to NK and Tc cells. *Ex vivo*-derived Tc cells, mainly produce GzmA, B and K, whereas *in vitro* propagated Tc cells variably express all Gzms. Transcripts or proteins of GzmA and B are present in pro-thymocytes and T regulatory cells. Non-lymphoid cells can contain Gzms, often without PFP, such as mast cells, macrophages, dendritic cells (GzmB), and brain cells (GzmK). The functions for Gzms in these cells are unknown (reviewed in Ref. [4]).

* Corresponding author. Tel.: +86 27 68780003; fax: +86 27 68780123.
E-mail address: qlguo@ihb.ac.cn (Q. Guo).
[1] These authors contributed equally to this work.

1050-4648/$ – see front matter
doi:10.1016/j.fsi.2010.04.002

Gzms probably possess both extracellular (e.g. extracellular matrix, [ECM] proteolysis) and intercellular activities. Intercellular activity includes apoptosis, cytokine induction and cleavage of viral proteins. Gzms are not entirely devoted to the cytotoxic activities but, in addition, possess non-cytotoxic activities that might include the cleavage of ECM and viral proteins, and the induction of pro-inflammatory cytokines [7–10]. Although the cytotoxic potential role of the Gzms remains pre-eminent, based on the experiment evidence, their potential non-cytotoxic roles could contribute to the control of pathogens and tumors (reviewed in Ref. [4]). The alternative antigen usually used to monitor activated T cells is IFN-γ which is released by both Th and Tc cells, whereas antigen-dependent release of Gzm from T cells indicates the presence of functional T-cell mediated killing [11].

GzmK was originally discovered in granules of human lymphokine-stimulated killer cells as a second tryptase among all the Gzms [5]. In evolutionary terms, GzmK and GzmA are the only two tryptases among all the Gzms, and are the most closely related Gzms. They both are encoded by single copy gene, closely linked together on the same chromosome both in human and mice, respectively [5,12–14]. GzmK, like GzmA and GzmB, is synthesized as a zymogen precursor, containing a signal and a short propeptide preceding the mature polypeptide chain [15]. GzmK and GzmA differ from GzmB by their substrate specificity [5]. The study showed that GzmA-deficient CTLs still contain 20% tryptase activity and their cytolytic activity is just slightly reduced [16], suggesting that GzmK might be a potent Gzm to rescue the GzmA activity. Although it is less abundant than GzmA and GzmB in human lymphokine-activated killer (LAK) cells, GzmK expresses at high levels in $CD56^{high}$ NK cells, memory CD8+ T cells and CD56+ T cells [17]. A recent report showed that circulating levels of GzmK are significantly elevated in virus-infected patients [18]. It suggests GzmK may play an important role in NK/Tc-mediated viral clearance. Although much work on GzmK has been done in human and mice, it is unclear how GzmK functions in cytolytic lymphocyte-mediated cytotoxicity [19].

Fish are the most primitive vertebrates that possess an adaptive immune system with lymphocyte subsets [20]. Specific CMC against virus-infected cells has been reported in ginbuna crucian carp (*Carassius auratus langsdorfii*) and rainbow trout (*Oncorhynchus mykiss*), suggesting that teleosts have virus-specific cytotoxic T-lymphocytes (CTLs, Tc cells) with functions and features similar to those of mammals. At present, CD8 gene expression is the best tool available to monitor Tc cells in teleosts, such as in rainbow trout, ginbuna crucian carp and common carp (*Cyprinus carpio*) [21–23]. Specific CMC is executed by Tc cells, while non-specific CMC reactions are mainly executed by NK cells, but also by non-specific cytotoxic cells (NCCs). In teleost, channel catfish NK-like cells freshly isolated from PBL kill allogeneic [24] but also virus-infected cells [25]. These studies from channel catfish NK-like cell clones, on whose cells TCR and NCC receptor protein1 is absent, indicated that this population of cytotoxic effector cells is heterogeneous with respect to target cell specificity and their cell surface antigen expression patterns comparing to Tc cells [24,25]. It has been demonstrated that fish NK cells are involved in the killing of virus-infected MHC class I mismatching target cells (review in Ref. [26]).

Based on the molecular structure and phylogenetic analysis, Praveen et al. identified a cDNA that codes for a Gzm-like homologue, channel catfish (*Ictalurus punctatus*) granzyme-1 (CFGR-1), from non-specific cytotoxic cells (NCCs) in 2004 [6]. Subsequently, Atlantic cod (*Gadus morhua*) GzmA/K, Gzm-like I and channel catfish Gzm-like I-III sequences were also identified [27]. Although the granule content of teleost NK and Tc cells have not been formally proven, Zhou et al. and Shen et al. demonstrated that clonal catfish NK and Tc cells contain granules and kill allogeneic targets by an apoptotic mechanism that involves DNA fragmentation. Moreover, the killing can be completely inhibited by chelating extracellular calcium [28,29]. These findings strongly suggest that the killing is mediated exclusively by the secretory perforin/granzyme mechanism. Here, this paper reported the structure and organization of carp GzmA/K gene, compared its amino sequence with other known Gzms, analyzes its expression characterization in GH transgenic and viral infected carp. The results obtained in the present study will help to understand the molecular characterization and potential role of teleost GzmA/K, a cytotoxic cell granule-associated serine protease.

2. Materials and methods

2.1. Fish, total RNA and partial GzmA/K cDNA sequence of carp

Common carp (*C. carpio* L.) were supplied by the Henan Institute of Aquaculture, Henan, China. Total RNAs were extracted from the various tissues of carp using Trizol Reagent (Invitrogen) following the user's manual. The quality and concentration of total RNA were analyzed by agarose gel electrophoresis and optical density reading at 260 and 280 nm. The thymus tissues of transgenic carp and control carp were used as tester sample and driver sample, respectively. Total RNAs were extracted from two samples, and then mRNAs were isolated from total RNAs using the PolyATract Isolation System (Promega), respectively. SSH library was performed between mRNAs from tester and driver using the PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's instruction. The partial cDNA sequence of carp GzmA/K was isolated from the thymus subtractive cDNA library by SSH [30].

2.2. Viral infection of carp

Spring viraemia of carp virus (SVCV) strain SVC-10/3 (supplied by Office International des Epizooties Reference Laboratory) was propagated in EPC cells [31] at 15–18 °C. Cells were grown in TC199 containing 10% fetal calf serum (FCS) and standard concentration of antibiotics. The virus titers, given as tissue culture infective dose (TCID50/0.1 ml), were calculated by the method of Reed and Muench [32]. Nine-month-old carp at an average weight of 150 g were raised in clean tanks at 15 °C. This temperature is optimal for SVCV infectivity [33]. Fifty carp were randomly divided into two groups, control and SVCV infection. In the SVCV-infection group, each fish was intraperitoneally injected with 250–300 μL SVCV for a dosage of approximately 10^7 $TCID_{50}$ kg^{-1} body weight, while fish in the control group were injected same amount of saline. RT-PCR and nested PCR reaction were used to confirm whether the experiment carp were infected by SVCV [34]. At 5 days post-infection, RT-PCR and nested PCR reactions showed that the experimental carp were infected by SVCV (Figure not shown). The various tissue samples of control (At 1 d after injection, 3 control carp as a group, $n = 3$) and SVCV-infected carp (At 1 d, 3 d, 5 d, 7 d, 10 d after infection, 3 infected carp as a group, $n = 3$) were immediately removed and frozen in liquid nitrogen, or stored at −80 °C until RNA isolation.

2.3. 3′ RACE

The cDNA template was transcribed by AMV reverse transcriptase (TaKaRa) with Oligo dT adapter (Table 1). Sense primer GzmF (Table 1) was designed according to the partial cDNA sequence of GzmA/K. After reverse transcription, PCR was performed with the primers (GzmF and 3′ Adapter) (Table 1) under the condition of

Table 1
Primers used for cloning and expression studies.

Primer	Sequence (5′–3′)	Application
Gzm F	GGCACCTGTTGGGGAGATTC	3′ RACE
Gzm R	CCTCCTGCACACAGCATGTC	5′ RACE
Gzm-Fl	TCTGAGGTAACACAATGACTTTGG	genomic PCR
Gzm-Rl	ATGTGTGAAAGATAATCCAGTATTGCT	genomic PCR
Gzm-R2	GCATCCATCTTGCTGCCTGTTTTCTCACC	genomic walking (first round PCR)
Gzm-R3	CCAAAGTCATTGTGTTACCTCAGACATCC	genomic walking (second round PCR)
EGzm-Fl	GTGTTGGCATCGTCAGTTACG	RT-PCR primer used in expression study
EGzm-Rl	AGTACCCCAACCTGTCACG	RT-PCR primer used in expression study
β-actin-Fl	GATGATGAAATTGCCGCACTG	RT-PCR control used in expression study
β-actin-Rl	ACCAACCATGACACCCTGATGT	RT-PCR control used in expression study
5′CDS	$(T)_{25}$ VN-3′ N = A, C, G, or T, V = A, G, or C	5′RACE PCR
BD SMART oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG	5′RACE PCR
5′UPM	CTAATACGACTCACTATAGGGCAAGCAGTG-GTATCAACGCAGAGT CTAATACGACTCACTATAGGGC	5′RACE PCR
3′ Adapter	GGCCACGCGTCGACTAGTAC	3′ RACE PCR adaptor
Oligo dT adapter	GGCCACGCGTCGACTAGTACT_{17}	first strand cDNA synthesis
AP1	GTAATACGACTCACTATAGGGC	genomic walking adaptor primer 1
AP2	ACTATAGGGCACGCGTGGT	genomic walking adaptor primer 2

94 °C denaturation for 2 min, running 30 cycles of 94 °C 30 s; 56 °C 30 s; 72 °C 30 s and 72 °C elongation for 6 min.

2.4. 5′ RACE

5′ RACE was performed using the BD SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instruction. The primer of 5′ CDS, BD SMART oligo, UPM and a Gene-Specific Primer GzmR (Table 1) were used for the 5′ sequence of GzmA/K. The PCR condition was: 94 °C denaturation for 2 min, 30 cycles of 94 °C 30 s; 56 °C 30 s; 72 °C 45 s, and 72 °C elongation for 6 min.

2.5. Cloning of the genomic sequence and promoter region

Genomic DNA was purified from the muscle of carp by the phenol chloroform method. Based on the resultant GzmA/K cDNA sequence, primers (Gzm-F1, Gzm-R1) (Table 1) were designed at beginning and end of cDNA to get the full length sequence of the corresponding DNA. The 5′ flanking region of GzmA/K was obtained using a gene walking approach by constructing genomic libraries with a Genome Walker™ Universal Kit (Clontech). A pair of primers (Gzm-R2 and Gzm-R3) (Table 1) were designed from the 5′ end of carp GzmA/K cDNA, priming upstream amplification through two rounds of PCR when used with the adaptor primers AP1 and AP2 (Table 1). The cycling protocol utilized two step and touchdown method for long-distance PCR. The genomic cloning was as follows: 94 °C 2 min, followed by 5 cycles of 94 °C 30 s, 72 °C 6 min with the annealing temperature lowing down 1 °C for each cycle, and 32 cycles of 94 °C 30 s, 68 °C 6 min, and 68 °C elongation for 10 min. For promoter cloning, the primary PCR was as follows: 94 °C 2 min, followed by 6 cycles of 94 °C 30 s, 72 °C 3 min with the annealing temperature lowing down 1 °C for each cycle, and 30 cycles of 94 °C 30 s, 67 °C 3 min, and 67 °C elongation for 10 min. The secondary PCR was carried out with 1 μL of the first round PCR mixture: 20 cycles of 94 °C 30 s, 67 °C 3 min, and 67 °C elongation for 10 min.

2.6. Cloning and sequencing

PCR products were separated by agarose gel electrophoresis, and the incised gels were purified using the Gel Extraction kit (OMEGA). The extracted products were ligated into pMD18-T vector (TaKaRa) and used to transform competent E. coli DH5α cells. Positive colonies were screened by the method of PCR. The recombinant plasmids were sequenced by the dideoxy chain termination method with M13 universal primers. The sequences were automatically collected on the ABI PRISM 3730 Genetic Analyzer.

2.7. Computer-aided sequence analysis of cloned GzmA/K DNAs

Sequences were analyzed for similarity with other known sequences by BLAST program and the multiple sequence alignments were generated using CLUSTAL W program. The signal sequence of carp GzmA/K was predicted by the SignalP 3.0 Server [35]. The protein family signature was identified by InterPro [36] program. The glycosylation site was predicted by the NetNGlyc 1.0 Server [37]. The phylogenetic tree was constructed based on the full length amino acid sequences of partial known Gzms using neighbor-joining algorithm within MEGA version 3.1 [38].

2.8. Quantification of GzmA/K mRNA in the SVCV-infected and control carp

Expression of GzmA/K gene was determined by real-time RT-PCR. 1 μg total RNAs isolated from various tissues of carp were, respectively, used to synthesize the first strand cDNAs with oligo dT primer. The first strand cDNAs were used as PCR amplification template with the primers of EGzm-F1, EGzm-R1 (Table 1) which could specifically amplify a part of GzmA/K cDNA. Serial 2-fold dilutions of the first strand cDNA, e.g. ranging from 2^5 down to 2^0 input cDNA copies, are used as a standard curve in each PCR run. The reaction carried out without template was used as blanks. Real-time RT-PCR was conducted on a Chromo 4 Real-Time Detection System (MJ Research, USA). The expected amplified fragment size of GzmA/K was 173 bp. Each examined tissue sample had three replicates. The GzmA/K reaction was performed with the condition of 94 °C denaturation for 4 min, followed by 40 cycles of 94 °C 20 s; 57 °C 20 s; 72 °C 15 s and 72 °C elongation for 3 min. The primers for β-actin cDNA amplification were β-actin-F1 and β-actin-R1 (Table 1), and the expected amplified fragment from cDNA was 135 bp. The reaction was performed with the condition of 94 °C denaturation for 4 min, followed by 40 cycles of 94 °C 20 s; 58 °C 20 s; 72 °C 15 s and 72 °C elongation for 3 min.

2.9. GzmA/K expression in thymus tissue of GH transgenic carp

1 μg total RNAs isolated from the thymus tissues of transgenic carp and control carp at the age of four-month (GH transgenic and control carp were supplied by the Laboratory of Fish Gene

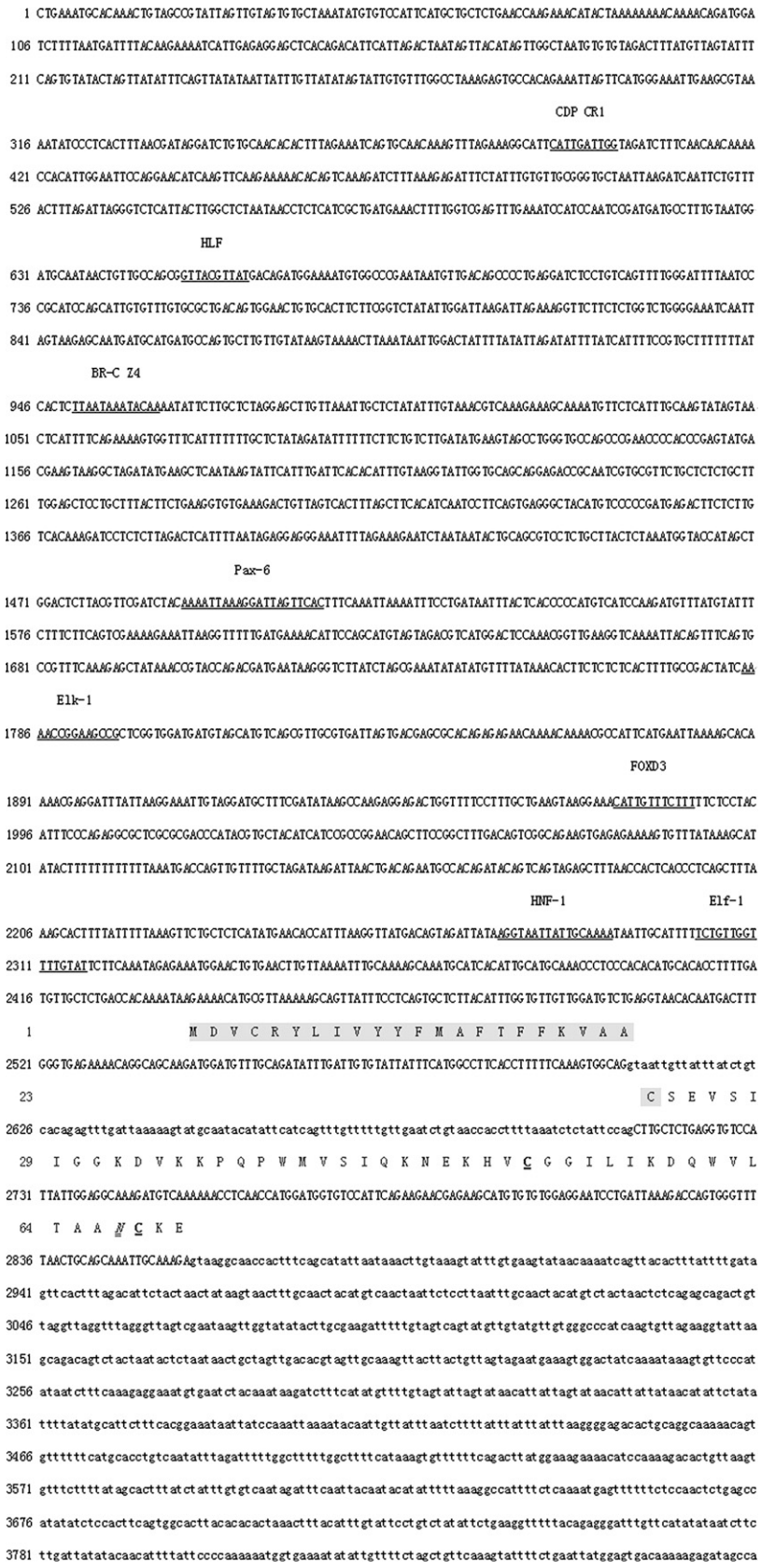

Fig. 1. Genomic DNA sequence and deduced amino acid sequence of the open reading frame (above) of carp GzmA/K gene. Exons are in upper case and introns in lower case. Nucleotides are numbered from the first base at the 5′ end. Amino acids are numbered from the initiating start codon methionine. The signal sequence of carp GzmA/K is shadowed. Three conserved residues at the catalytic site are shown in the italic letters with double underline. Eight cysteine residues (Cys-52, Cys-68, Cys-141, Cys-173, Cys-189, Cys-200, Cys-210 and Cys-224) are shown in bold letters with bold underline. A putative glycosylation site is boxed, The potential regulatory elements, the non-conventional poly (A) signal, and the poly (A) addition site are underlined.

Fig. 1. (*continued*).

Engineering, Institute of Hydrobiology, Chinese Academy of Sciences) were respectively used to synthesize the first strand cDNAs with oligo dT primer. The first strand cDNAs were used as PCR amplification template with the primers of EGzm-F1 and EGzm-R1. Amplification of β-actin (β-actin-F1 and β-actin-R1) was as the internal reference. The real-time RT-PCR was performed according to above procedure.

3. Results

3.1. Sequence analysis of carp GzmA/K gene

The full length cDNA of carp GzmA/K was 1053 bp with a 774 bp open reading frame coding for a protein of 257 amino acids. The 5′ untranslated region was located at 65 bp upstream of the putative start codon (ATG). The 3′ untranslated region of 214 nucleotides ended at a poly (A) tail. A possible poly (A) signal sequence for polyadenylation, AATAAA, was located at 15 bp upstream of the poly (A) tail (Fig. 1).

ProtParam program of EXPASy Molecular Biology Server was used to analyze the basic physical and chemical characteristics of the deduced protein GzmA/K. The molecular weight of GzmA/K was 28,597.5 Da and its isoelectric point was 9.51; it was comprised of 21 negatively charged residues (Asp and Glu) and 40 positively charged residues (Arg and Lys). The Lys (13.2%) and Val (8.9%) contents were high while that of Trp (1.9%) and His (1.2%) was low.

The analysis by comparison to other known Gzms suggested that the translated cDNA sequence was a member of the Gzm family of serine proteases. The carp GzmA/K cDNA sequence was submitted to Genbank (accession number: GU362096). As shown Fig. 1, amino acid sequence analysis indicated the existence of a signal peptide (amino acids 1–23) at N-terminal end, eight highly consensus cysteine (Cys-52, Cys-68, Cys-141, Cys-173, Cys-189, Cys-200, Cys-210 and Cys-224) residues and three conserved residues as central elements of the active site (Asn-67, Asp-111, Ser-204). Of these, eight cysteine residues were high conserved in all Gzms and predicted to form four disulfide bonds (Cys-52 with Cys-68, Cys-141 with Cys-210, Cys-173 with Cys-189, Cys-200 and Cys-224) using DISULFIND web serve [39]. Among the three active sites, the Asn-67 was special for carp GzmA/K, while the Asp-111 and Ser-204 were completely identical in the other compared sequences. Carp GzmA/K contained the highly conserved IIGG (Ile-Ile-Gly-Gly) motif at the N-terminus of the active enzyme. In addition, there was a single putative glycosylation site (NSTS) in amino acid sequence of carp GzmA/K (Fig. 1).

The three-dimensional structure of carp GzmA/K was modeled using SWISS-MODEL in the first approach mode accessible via the Internet (http://www.expasy.org/swissmod) [40]. The templates used for modeling were based on known Gzms crystal structures. The overall structure of the predicted model for carp GzmA/K was similar to that of other Gzms, especially human GzmA, K and zebrafish Gzm (Fig. 2).

A 5.4 k genomic DNA sequence covering the entire coding region of carp GzmA/K was amplified using long-PCR and gene specific primers (Table 1), and compared with the cDNA sequence to clarify the exon/intron organization. As shown in Fig. 3, the carp GzmA/K gene was composed of five exons and four introns with the exon/intron-junctions at the same positions as human GzmA and K gene, suggesting that the entire gene structures of GzmA and K have been maintained throughout evolution. The locations of splice donor/acceptor sites in all introns follow the consensus ‘ GT/AG’ rule. The carp GzmA/K had a similar length to the zebrafish counterpart and much shorter than human GzmA and K (Fig. 3). A number of transcription regulatory elements were predicted in the 2.4 kb promoter regions of the carp GzmA/K gene using the program

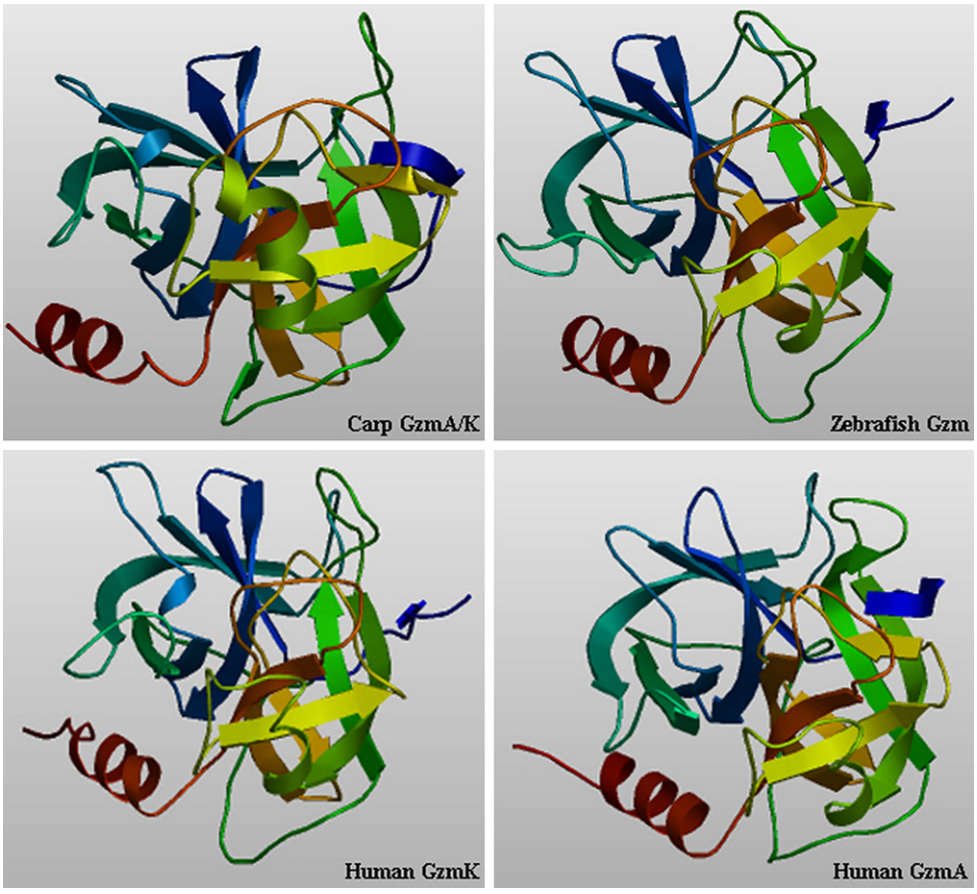

Fig. 2. Predicted three-dimensional models for carp GzmA/K, zebrafish Gzm, human GzmK and A: A comparison of four granzyme structures among three species.

Match™ [41]. It had several potential recognition sequences for transcription factors including CDP CR1, HLF, BR-C Z4, Pax-6, Elk-1, FOXD3, HNF-1, Elf-1 (Fig. 1). The genomic sequence of carp GzmA/K was submitted to Genbank (accession number: GU369939).

3.2. Similarity comparison and phylogenetic analysis

Deduced amino acid sequence of carp GzmA/K shared 70% identity to zebrafish (*Danio rerio*) Gzm, 53%, 38%, 41%, 40%,40% identity to catfish (*I. punctatus*) Gzm, cod (*G. morhua*) GzmA/K, Halibut (*Paralichthys olivaceus*) Gzm III-1, salmon (*Salmo salar*) GzmA and frog (*Xenopus laevis*) AK, 40%, 39%, 38%, 37%, 36% identity to horse (*Quus caballus*), human (*Homo sapiens*), rat (*Rattus norvegicus*), cattle (*Bos taurus*), dog (*Canis familiaris*) GzmKs and 36% identity to human GzmA. While, amino acid sequence of zebrafish Gzm shared 40% identity to human GzmK and 39% identity to human GzmA.

To further analyze the relationship of carp GzmA/K with the GzmA, K, B, C and K subfamilies, a phylogenetic tree was constructed using the neighbor-joining method. This suggests that though the carp GzmA/K we cloned is closer to mammalian GzmA and K (Figs. 2 and 4), it does not clearly fall into the GzmA or K subgroup in their phylogenetic analysis. As shown in Fig. 5, carp GzmA/K forms a separate cluster with previously reported fish and amphllian GzmA/K on the GzmA and K branches. In addition, mammalian GzmB, C, and M form different clusters from the carp GzmA/K.

3.3. GzmA/K expression in thymus tissue of GH transgenic carp

By real-time RT-PCR, the GzmA/K mRNA level in thymus tissues of transgenic and control carp was normalized with β-actin shown in Fig. 6. The sizes of PCR products of β-actin and GzmA/K fragment were the same as expected. Expression level of GzmA/K in thymus of transgenic carp was significantly increased (4.1-fold) ($p < 0.05$) compared to control carp according to the real-time RT-PCR result (Fig. 6).

3.4. Tissue distribution of carp GzmA/K mRNA

The carp GzmA/K mRNA distribution was examined by using real-time RT-PCR (Fig. 7). Normalization with β-actin, the GzmA/K gene was examined in ten tissues from three healthy carp. The carp GzmA/K gene was detectable in all examined tissues, and was predominantly detected in spleen, gills, head kidney and thymus, followed by kidney, peripheral blood and intestine, lower

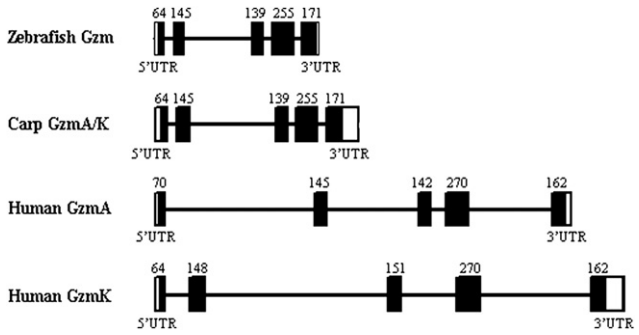

Fig. 3. Schematic representations of the carp GzmA/K, zebrafish Gzm, human GzmA and K genes. Exons are numbered (top) and indicated with black boxes. White boxes represent 5′ and 3′ untranslated exons.

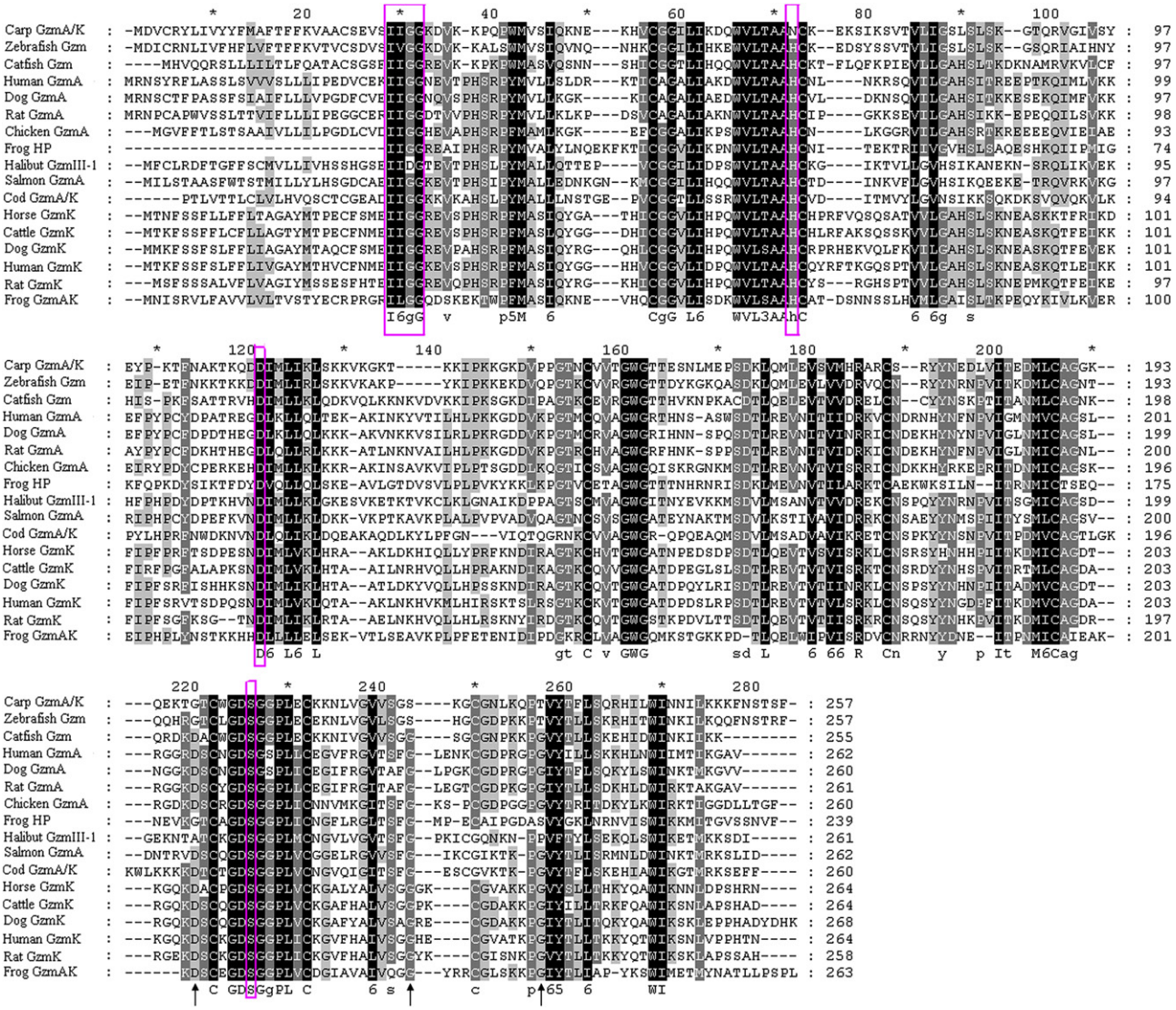

Fig. 4. Multiple alignment of the deduced amino acid sequences of carp GzmA/K with other known Gzms. GenBank accession numbers for the sequences used are listed in Fig. 4 legend. Black shaded sequences indicate positions where all the sequences share the same amino acid residue, gray shaded sequence indicates conserved amino acid substitutions; Light gray shaded sequences indicate semi-conserved amino acid substitutions, and dashes indicate gaps. Several important domains are shown in boxes. Arrows indicate three key residues within the S1 pocket.

expression level was detected in skin, liver and muscle. No GzmA/K transcript was detected in negative control.

3.5. Quantification of GzmA/K mRNA in the viral infected carp

Some symptoms were found in the SVCV-infected carp, such as a hyperaemia in skin and caudal/ventral fin, a marked inflammation in gills and intestine. The inducible expression of carp GzmA/K in various tissues at different time points after SVCV infection was shown in Fig. 7. After infection, in thymus, the GzmA/K mRNA was elevated at 1 d, the highest expression occurred at 10 d (2.6-fold) ($p < 0.05$); in spleen, the GzmA/K mRNA was induced at 1 d (2-fold), then decreased significantly; in both head kidney and kidney, the change of GzmA/K mRNAs was similar and up-regulated at 3 d (1.9-fold and 2-fold, respectively) ($p < 0.05$); in peripheral blood and intestine, the highest expression of carp GzmA/K occurred simultaneously at 10 d (4.8-fold and 3.2-fold, respectively) ($p < 0.01$); in skin, carp GzmA/K was significantly up-regulated at 3 d and 7 d (3.6-fold and 2.8-fold). Noteworthily, in liver, the change for the GzmA/K mRNA was induced at 1 d and significantly up-regulated at 5 d (12.2-fold), and remained high level at 7–10 d (11-fold) ($p < 0.01$); in gills, carp GzmA/K mRNA was down regulated from 1 d to 7 d, while the highest expression occurred at 10 d (2-fold) ($p < 0.05$).

4. Discussion

In the present work, we described for the first time the molecular characterization of a member of the Gzm family in a cyprinid fish, common carp, and its expression profiles induced by SVCV. The full length cDNA of carp GzmA/K was 1053 bp with a 774 bp open reading frame coding for a protein of 257 amino acids. The 5′ untranslated region was located at 65 bp upstream of the putative start codon (ATG). The 3′ untranslated region of 214 nucleotides ended at a poly (A) tail. These sequence characteristics all indicated a full length cDNA being obtained. The deduced amino acid sequence analysis showed that this Gzm cDNA had highly conserved structure characteristics, shared the 39% and 36% identity to human GzmK and A

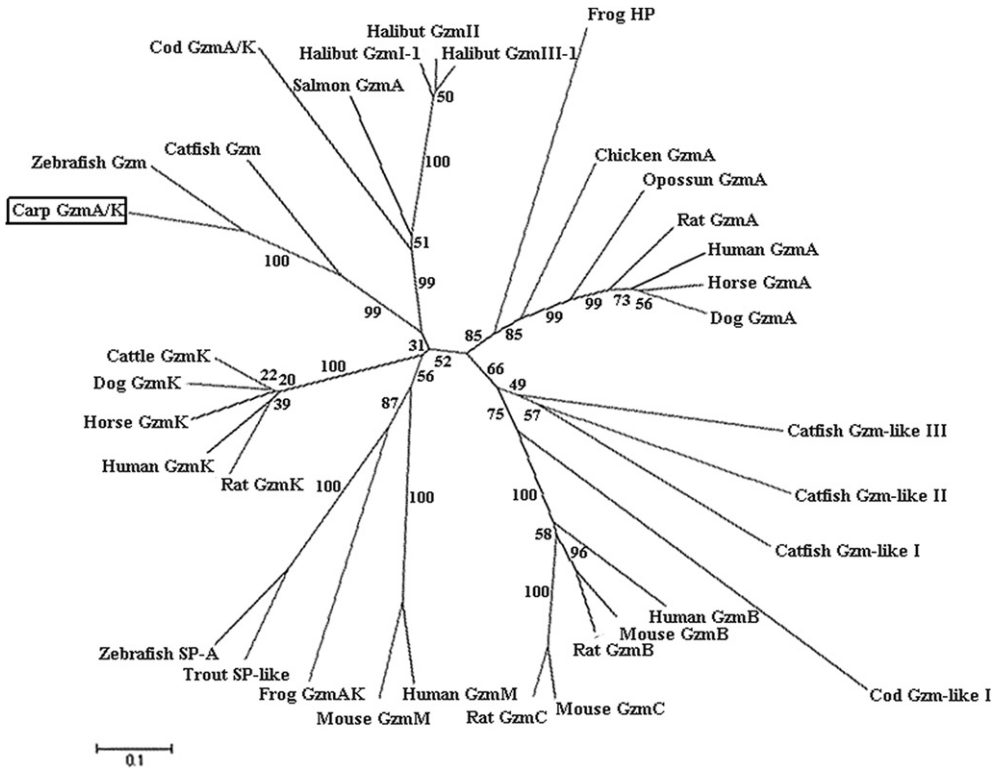

Fig. 5. Phylogenetic tree produced using the neighbor-joining algorithm. The GenBank accession numbers used are as follows: Cattle (*Bos taurus*) GzmK, XP_607307; Dog (*Canis familiaris*) GzmK, XP_546318; Horse (*Equus caballus*) GzmK, XP_001497064; Human (*Homo sapiens*) GzmK, NP_002095; Rat (*Rattus norvegicus*) GzmK, NP_058815; Frog (*Xenopus laevis*) GzmAK, NP_001088093; Catfish (*Ictalurus punctatus*) Gzm, AAQ54830; Zebrafish (*Danio rerio*) Gzm, XP_001335166; Cod (*Gadus morhua*) GzmA/K, AAV73910; Halibut (*Paralichthys olivaceus*) III-1, BAD91572; Salmon (*Salmo salar*) GzmA, ACI68491; Frog GzmHP, CAD66429; Chicken (Gallus gallus) GzmA, NP_989788; Opossum (Monodelphis domestica) GzmA, XP_001380969; Rat GzmA, EDM10367; Human GzmA, NP_006135; Horse GzmA, XP_001494094; Dog GzmA, XP_544335; Human GzmM, NP_005308; Mouse GzmM, NP_032530; Human GzmB, A61021; Mouse GzmB, NP_038570; Rat GzmB, NP_612526; Mouse GzmC, NP_034501; Rat GzmC, NP_599159; Trout SP-like, AAC17927; Catfish Gzm-like I, AY942183; Catfish Gzm-like II, AY942182; Catfish Gzm-like III, AY942184; Cod Gzm-like I, AAV73911; Zebrafish SP-A, AAH78380; Halibut Gzm I-1, BAD91570; Halibut Gzm II, BAD91569.

respectively, and was phylogenetically closer to the granzyme A and K subfamilies described previously, which suggests that like cod GzmA/K [27], this sequence obtained from common carp is as mammalian GzmA and K homologue, and is named as carp GzmA/K. Moreover, carp GzmA/K is also closely related to the CFGR-1 and cod GzmA/K in the phylogenetic tree (Fig. 5). Our result supports that teleost GzmA/K, a common ancestral gene, separated into GzmA and K after the separation of lobe limbed vertebrates from the ray-finned fish [27]. And in the phylogenetic tree, we found that teleost Gzms was mainly closer to the mammal granzyme A, K and B subfamilies. Whether this suggests that teleost GzmA/K like and GzmB like are two older ancestral gene, this remains to be more elucidated.

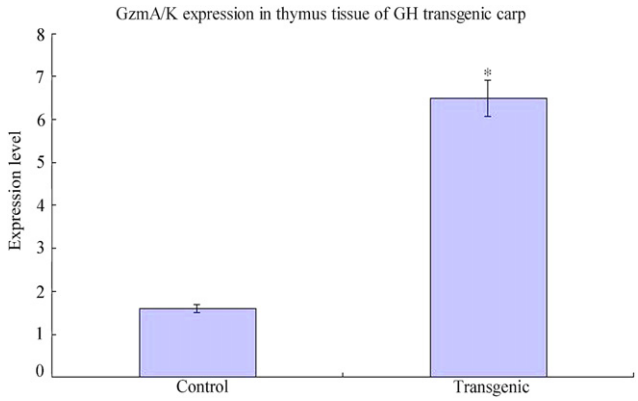

Fig. 6. Analysis of GzmA/K gene expression in thymus tissue of GH transgenic carp and control carp. β-actin was as the internal reference. Control indicates the thymus tissue of control carp. Transgenic indicates the thymus tissue of transgenic carp. $^{*}p < 0.05$, $n = 3$.

Similar to other known GzmA and Ks (Fig. 4), carp GzmA/K contained a signal sequence of 23 amino acids, which is sorted to the secretory pathway, and eight cysteine residues that are conserved in all known GzmKs and are expected to form four cystein bridges [27]. All known Gzms has a triad of key residues (His, Asp, Ser) at the catalytic site which is a characteristic for chymotrypsin [42]. Carp GzmA/K also contained three conserved residues (Asn-67, Asp-111, Ser-204) at the catalytic site which is required to form a scaffold that achieves the precise three-dimensional orientation [43]. Among the three active sites, the Asn-67 was special for carp GzmA/K, while the Asp-111 and Ser-204 were completely identical to the other compared sequences. To date, this difference has been only found in carp GzmA/K, and may be considered due to evolutionary pressure. Like all other known Gzm, carp GzmA/K had a highly conserved Ile-Ile-Gly-Gly sequence at the amino acid terminus of the active enzyme. For most Gzms the IIGG is preceded by a charged dipeptide that is cleaved off by dipeptidyl peptidase I to form the active enzyme [44,45]. As shown in Fig. 4, like CFGR-1 and cod GzmA/K, the carp GzmA/K is produced as propeptide (tetrapeptide), the Gzm signature motif. The leader sequence is a necessary requirement for the translated message of these enzymes to be compartmentalized to the rough endoplasmic reticulum and their final destination in the exocytic granules (reviewed in Ref. [6]). In addition, one potential glycosylation site

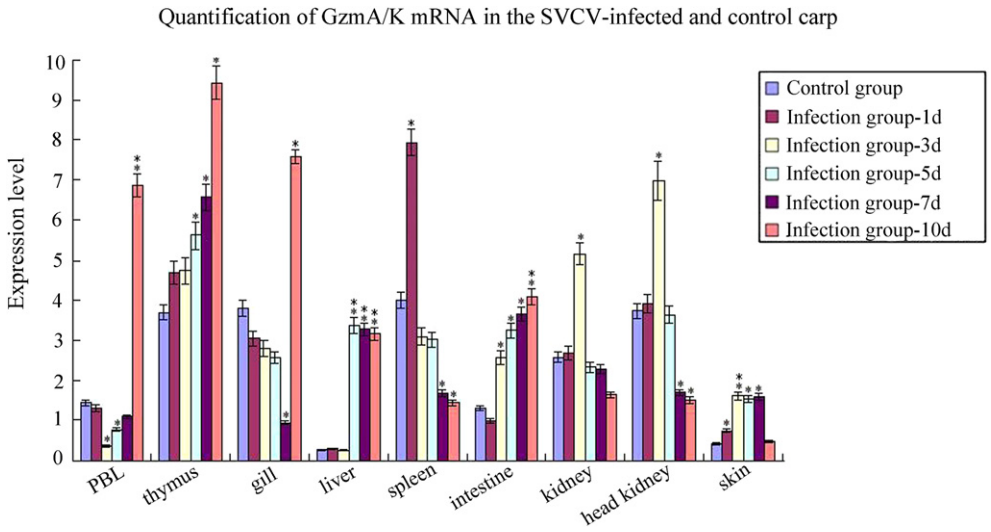

Fig. 7. Analysis of tissue expression of carp GzmA/K gene in control and infection groups by real-time RT-PCR. The mRNA level of carp GzmA/K is expressed as a ratio to β-actin mRNA levels. 1d, 3d, 5d, 7d, 10d indicate the infection time. $^{*}p < 0.05$, $n = 3$.

was found at C-terminal of carp GzmA/K, which is different from CFGR-1 and cod GzmA/K in which glycosylation site was found at N-terminal. Although the actual glycosylation status of these fish granzymes is not presently known, in mammals, it is important to target these enzymes to secretory granules [6]. This high degree of sequence conservation through evolution suggests an important and basic function for GzmA/K.

The structure and mutagenesis studies have confirmed that there are three key positions within the S1 pocket (aa 189, 216 and 226) that determine the cleavage specificity of Gzms [46,47]. To further compare carp GzmA/K with other species Gzms, we analyzed these S1 pocket sequences, the specificity-conferring triplets. As shown in Fig. 4, catfish CFGR-1, cod GzmA/K and all mammalian GzmK and A hold the specificity-conferring triplet D-G-G and presumably display trypsin-like specificity [27]. However, notably, the specificity-conferring triplet D-G-G was substituted by G-S-T within the S1 pocket in carp GzmA/K and zebrafish Gzm. It is difficult to predict substrate specificities for the carp GzmA/K, since the combinations of aa in the three crucial positions did not resemble any of the previously described GzmA and Ks in Fig. 4 for which the specificity is known.

Analysis of the transcription factor binding sites in carp GzmA/K gene promoter suggests that at least eight potential transcription factors could regulate GzmA/K gene transcription (Fig. 1). Among these factors, Elk-1 can activate the transcription of TNF-α. Noteworthily, BR-C Z4, FOXD3, Elk-1 and HNF-1 have been found in human GzmK promoter, and BR-C Z4, HLF and CDP CR1 have been found in human GzmA promoter. While, CDP CR1, BR-C Z4, Pax-6, Elk-1, FOXD3 and HNF-1 have also been reported in carp NKEF-B promoter [48]. This seems that carp NKEF-B and GzmA/K gene may be regulated by the same system and cooperate to play their role in carp immune system.

In mammals, the Gzms are expressed in Tc and NK cells (including γδ T cells) and involved in the specific and non-specific CMC reactions (reviewed in Ref. [3,4]). Little is known about the tissue expression characterization of fish Gzm. Praveen et al. found that three tissues (head kidney, spleen, liver) enriched with NCCs, and the NCCs purified from three tissues had a higher level of CFGR-1 mRNA by RT-PCR, indicating that NCCs and head kidney, spleen, liver are the main source of CFGR-1 in catfish [6]. Interestingly, Northern blot analysis showed a higher expression level of cod GzmA/K in spleen than in head kidney and thymus. Thereby, Wernersson et al. predicted that the cod GzmA/K was primarily expressed in mature T cell and/or NK cell populations, since the head kidney (bone marrow equivalent in fish) and thymus represent the primary lymphoid organs of bony fish [27]. By real-time RT-PCR, our results showed that carp GzmA/K was predominantly detected in spleen, gills, head kidney and thymus (the highest in spleen). Furthermore, as shown in Fig. 7, carp GzmA/K mRNA level between these organs had no great difference. It is reported that the transcripts of some mammalian Gzms (GzmA and B) are present in pro-thymocytes [4], thus, we suggest that carp GzmA/K could be expressed in both mature and immature T cell and/or NK cell populations.

As a thymopoietic factor, GH plays an important role in lymphopoiesis and haematopoiesis, Thereby, GH has aroused extensive attention. Our studies indicated that the transgene for “all-fish” GH in common carp effectively promotes thymus development and thymocyte proliferation, and retards thymus degeneration [49]. Similarly, in fish, GH was shown to promote haematopoiesis, and enhance phagocytosis, NK cell activity and antibody production (reviewed in Ref. [50]). In the present study, we investigated the expression of GzmA/K gene in GH-transgenic carp, and found that carp GzmA/K was significantly up-regulated in thymus tissue compared to common carp by real-time RT-PCR (Fig. 6). The result probably implies that the number and functional killing of Tc and/or NK cells in this primary lymphoid organ of fish may be influenced by GH, and also further suggests that these increased Tc/NK cells probably include both mature and immature Tc/NK cell populations.

There is increasing evidence for the involvement of Gzms in infections by various viruses. Based on research on their biological role in cell death, all Gzms, in the presence of PFP, participate in the NK/Tc cell mediated cytolytic processes (review in Ref. [3]). Also, it has been reported that mammalian GzmK is higher expression level in CD56+NK cells and CD8+T (Tc) cells [17]. Since in contrast to the detection of IFN-γ, Gzms can act as a specific marker for the presence of function T-cell mediated killing [11], and little is known about the expression changes of fish Gzm in viral infection, in this study, the expression analysis of carp GzmA/K with SVCV infection was performed by real-time PCR. Our studies revealed that carp GzmA/K is a inducible gene with virus. As shown Fig. 7, the carp GzmA/K in thymus and head kidney (primary lymphoid organs) was up-regulated by SVCV infection, and among the immune-related organs, the up-regulated expression of carp GzmA/K in

peripheral blood was the most significant (4.8-fold), probably because of a great NK/TC cell increase. Noteworthily, the carp GzmA/K in intestine and gills (where there were marked inflammatory) was significantly up-regulated (3.2-fold and 2-fold, respectively), suggesting that in these important sites entry of virus, carp GzmA/K may play an important role in NK/Tc-mediated cytolysis in viral clearance. Interestingly, the highest level (12.2 fold) of carp GzmA/K mRNA was found in liver comparing to the uninfected carp. Since the macrophages can contain Gzm (such as GzmB) (reviewed in Ref. [4]), we predict that it may be related to macrophage increase in hepatic sinusoid, although the functions for Gzms in non-lymphoid cells are unknown (reviewed in Ref. [4]). It is reported that some cytokine (IL-12 and IFN-α/β) transcriptions of carp were up-regulated upon SVCV infection [23]. It is now becoming clear that GzmA and K might also have extracellular non-cytotoxic activities (including the cleavage of ECM and viral proteins, and the induction of pro-inflammatory cytokines) [7–10]. Meantime, the Gzm (such as GzmA) might also influence the innate response leading to the production of type I IFN (reviewed in Ref. [4]). Thus, we suggest that in addition to intercellular cytotoxic activities, the potential non-cytotoxic roles of GzmA/K could also contribute to the anti-viral defense in carp.

In conclusion, the result obtained in the present study broadens our knowledge on the granzyme superfamily in fish. It provides a new molecular marker for the presence of function Tc and NK cell mediated killing, which can help us for analyzing the relationship between the molecular basis of Tc and NK cells and anti-viral defense of fish. Moreover, the result also implies that the cell-death pathway induced by granzymes, with recently identified Japanese flounder (*P. olivaceus*) perforin [51], like mammals, may play an important role in the immune system of fish.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 30671599, 30871912), the National Basic Research Program of China (Grant No. 2009CB118704), and State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences (Grant No. 2010FB05). We would like to thank Prof. Xudong Xu (Institute of Hydrobiology, Chinese Academy of Sciences) and Prof. Yulin Jiang (Key Laboratory of Aquatic Animal Diseases, Shenzhen Exit & Entry Inspection and Quarantine Bureau) for technical supports and valuable advices.

References

[1] Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer 1975;16:230–9.
[2] Herberman RB, Ortaldo JR. Natural killer cells: their roles in defenses against disease. Science 1981;214:24–30.
[3] Pardo J, Aguilo JI, Anel A, Martin P, Joeckel L, Borner C, et al. The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation. Microbe Infect 2009;11:452–9.
[4] Froelich CJ, Pardo J, Simon MM. Granule-associated serine proteases: granzymes might not just be killer proteases. Trends Immunol 2009;30:117–23.
[5] Hink-Schauer C, Estbéanez-Perpiñá E, Wilharm E, Fuentes-Prior P, Klinker W, Bode W, et al. The 2.2-Å crystal structure of human pro-granzyme K reveals a rigid zymogen with unusual features. J Biol Chem 2002;277:50923–33.
[6] Praveen k, Evans DL, Jaso-Friedmann L. Evidence for the existence of granzyme-like serine proteases in teleost cytotoxic cells. J Mol Evol 2004;58:449–59.
[7] Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol 2008;26:389–420.
[8] Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, et al. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity 2008;29:720–33.
[9] Buzza MS, Zamurs L, Sun J, Bird CH, Ian Smith A, Trapani JA, et al. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem 2005;280:23549–58.
[10] Kramer M, Simon MM. Are proteinases functional molecules of T cells? Immunol Today 1987;8:140–3.
[11] Waterhouse NJ, Sedelies KA, Clarke CJ. Granzyme B: the chalk-mark of a cytotoxic lymphocyte. J Transl Med 2004;2:36.
[12] Denizot F, Brunet JF, Roustan P, Harper K, Suzan M, Luciani MF, et al. Novel structures CTLA-2α and CTLA-2β expressed in mouse activated T cells and mast cells and homologous to cysteine proteinase proregions. Eur J Immunol 1989;19:631–5.
[13] Fink TM, Lichter P, Wekerle H, Zimmer M, Jenne DE. The human granzyme A (HFSP, CTLA3) gene Maps to 5q11-q12 and Defines a New locus of the serine protease superfamily. Genomics 1993;18:401–3.
[14] Baker E, Sayers TJ, Sutherland GR, Smyth MJ. The genes encoding NK cell granule serine proteases, human tryptase-2 (TRYP2) and human granzyme A (HFSP), both map to chromosome 5q11-q12 and define a new locus for cytotoxic lymphocyte granule tryptases. Immunogenetics 1994;40:235–7.
[15] Sayers TJ, Lloyd AR, McVicar DW, O'Connor MD, Kelly JM, Carter CR, et al. Cloning and expression of a second human natural killer cell granule tryptase, HNK-Tryp-2/granzyme 3. J Leukoc Biol 1996;59:763–8.
[16] Davis JE, Smyth MJ, Trapani JA. Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. Eur J Immunol 2001;31:39–47.
[17] Bratke K, Kuepper M, Bade B, Virchow Jr JC, Luttmann W. Differential expression of human granzymes A, B, and K in natural killer cells and during CD8 + T cell differentiation in peripheral blood. Eur J Immunol 2005;35:2608–16.
[18] Bade B, Lohrmann J, ten Brinke A, Wolbink AM, Wolbink GJ, ten Berge IJM, et al. Detection of soluble human granzyme K in vitro and in vivo. Eur J Immunol 2005;35:2940–8.
[19] Guo Y, Chen J, Zhao T, Fan Z. Granzyme K degrades the redox/DNA repair enzyme Ape1 to trigger oxidative stress of target cells leading to cytotoxicity. Mol Immunol 2008;45:2225–35.
[20] Miller NW, Sizemore R, Clem LW. Phylogeny of lympnocyte heterogeneity: the cellular requirement for in vitro antibody responses of channel catfish leukocytes. J Immunol 1985;134:2884–8.
[21] Utke K, Bergmann S, Lorenzen N, Köllner B, Ototake M, Fischer U. Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. Fish Shellfish Immunol 2007;22:182–96.
[22] Somamoto T, Yoshiura Y, Sato A, Nakao M, Nakanishi T, Okamoto N, et al. Expression profiles of TCRβ and CD8α mRNA correlate with virus-specific cell-mediated cytotoxic activity in ginbuna crucian carp. Virology 2006;348 (2):370–7.
[23] Forlenza M, de Carvalho Dias JD, Veselý T, Pokorová D, Savelkoul HF, Wiegertjes GF. Transcription of signal-3 cytokines, IL-12 and IFN alpha beta, coincides with the timing of CD8 alpha beta up-regulation during viral infection of common carp (*Cyprinus carpio* L. Mol Immunol 2008;45:1531–47.
[24] Yoshida SH, Stuge TB, Miller NW, Clem LW. Phylogeny of lymphocyte heterogeneity: cytotoxic activity of channel catfish peripheral blood leukocytes directed against allgeneic targets. Dev Comp Immunol 1995;19:71–7.
[25] Honan RJ, Stuge TB, Clem LW, Miller NW, Chinchar VG. Anti-viral cytotoxic cells in the channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 1996;20: 117–27.
[26] Fischer U, Utke K, Somamoto T, Köllner B, Ototake M, Nakanishi T. Cytotoxicity activities of fish leucocytes. Fish Shellfish Immunol 2006;22:209–26.
[27] Wernersson S, Reimer JM, Poorafshar M, Karlson U, Wermenstam N, Bengtén E, et al. Granzyme-like sequences in bony fish shed light on the emergence of hematopoietic serine proteases during vertebrate evolution. Dev Comp Immunol 2006;30:901–18.
[28] Zhou H, Stuge TB, Miller NW, Bengten E, Naftel JP, Bernanke JM, et al. Heterogeneity of channel catfish CTL with respect to target recognition and cytotoxic mechanisms employed. J Immunol 2001;167:1325–32.
[29] Shen LL, Stuge TB, Bengten E, Wilson M, Chinchar VG, Naftel JP, et al. Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 2004;28:139–52.
[30] Sun XF, Huang R, Xiao MY, Hu W, Wang YP, Guo QL. Identification of thymus development relative genes in "all fish" GH transgenic common carp (*Cyprinus carpio* L.) by suppression subtractive hybridization. High Tech Lett 2007a;17: 1184–91 [abstract in English].
[31] Fijan N, Sulimanovic D, Bearzotti M, Muzinic D, Zwillenberg LO, Chilmonczyk S. Some properties of the epithelioma papulosum cyprinid (EPC) cell line from carp (*Cyprinus carpio*). Ann Virol (Institue Pasteur) 1983;134:207–20.
[32] Reed RJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493–503.
[33] Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR. Spring viremia of carp (SVC). Dis Aquat Org 2002;52:261–72.
[34] Koutna M, Vesely T, Psikal I, Hulova J. Identification of spring viraemia of carpvirus (SVCV) by combined RT-PCR and nested PCR. Dis Aquat Org 2003;55:229–35.
[35] Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: signalP 3.0. J Mol Biol 2004;340:783–95.
[36] Apweiler R, Attwood TK, Bairoch A, Bateman A, Birney E, Biswas M, et al. The InterPro database, an integrated documentation resource for protein familes, domains and functional sites. Nucleic Acids Res 2001;29:37–40.
[37] Gupta R, Jung E, Brunak S. Prediction of N-glycosylation sites in human proteins. http://www.cbs.dtu.dk/services/NetNGlyc/2004, in preparation.

[38] Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17:1244–5.
[39] Ceroni A, Passerini A, Vullo A, Frasconi P. DISULFIND: a disulfide bonding state and cysteine connectivity prediction server. Nucleic Acids Res 2006;34: W177–81.
[40] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201.
[41] Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, et al. TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic Acids Res 2003;31:374–8.
[42] Smyth MJ, O'Connor MD, Trapani JA. Granzymes: a variety of serine protease specificities encoded by genetically distinct subfamilies. J Leukoc Biol 1996;60:555–62.
[43] Branden C, Tooze J. Introduction to protein structure. New York Garland Publishing; 1991. pp. 231–46.
[44] Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol 2001;2:3014.1–7.
[45] Pham CT, Ley TJ. Dipeptidyl peptidase I is required for the processing and activation of granzymes A and B in vivo. Proc Natl Acad Sci USA 1999;96: 8627–32.
[46] Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. Protein Sci 1995;4:337–60.
[47] Wouters MA, Liu K, Riek P, Husain A. A despecialization step underlying evolution of a family of serine proteases. Mol Cell 2003;12:343–54.
[48] Huang R, Gao LY, Wang YP, Hu W, Guo QL. Structure, organization and expression of common carp (*Cyprinus carpio* L.) NKEF-B gene. Fish Shellfish Immunol 2009;26(2):220–9.
[49] Guo QL, Wang YP, Jia WZ, Zuo ZY. Transgene for growth hormone in common carp (*Cyprinus carpio* L.) promotes thymus development. Chin Sci Bull 2003; 48:1764–70.
[50] Harris J, Bird DJ. Modulation of the fish immune system by hormones. Vet Immunol Immunopathol 2000;77:163–76.
[51] Hwang JY, Ohira T, Hirono I, Aoki T. A pore-forming protein, perforin, from a non-mammalian organism, Japanese flounder, *Paralichuthy olivaceus*. Immunogenetics 2004;56:360–7.