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Immune gene discovery in the crucian carp *Carassius auratus*

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ABSTRACT

The crucian carp *Carassius auratus* (Cyprinidae) is one of the important fish species in aquaculture. Although the crucian carp has several economic benefits, their immune system and gene information have not been investigated in depth as yet. Here, we performed the transcriptome analysis of *C. auratus* using the pyrosequencing method and selected several immune-related genes. Of unigenes obtained in this species, we identified a number of immune system-related genes (e.g. adhesive protein, antimicrobial protein, apoptosis- and cell cycle-related protein, cellular defense effector, immune regulator, pattern recognition protein, protease, protease inhibitor, reduction/oxidation-related protein, signal transduction-related protein and stress protein) that are potentially useful for studies on fish immunity. To be of public and practical use, we designed primer pairs of each gene from the crucian carp for real-time RT-PCR application and tested the amplicon identity of entire gene sets with the total RNA sample. For comparative analysis, we measured tissue-preferential transcript profiles of selected genes. This study will be helpful to extend our knowledge on the immune system of the crucian carp in comparative aspects and to develop the crucian carp as a potential model organism for aquatic quality monitoring in fish farming.

1. Introduction

The crucian carp *Carassius auratus* is a member of the family Cyprinidae (Teleostei). These fishes are world-wide distributed in freshwater systems such as river, lake, and pond. Previously, *C. auratus* was highlighted as one of most important commercial species in aquaculture including several marine fish and crustaceans as their large production, for their excellent growth traits, good taste, and suitability in culture systems (FAO; Food and Agriculture Organization of the United Nations). Although the crucian carp is highly susceptible to bacterial and pathogen challenges, exploration of immunity at the molecular level is dominated by several aquaculture model animals by diverse experimental approaches with little attention to the crucian carp *C. auratus*. To date, in crucian carp, several immunity-relevant factors such as interferon regulatory factor 7 [63], *CD8α* [46], interferon-stimulated gene ISG15 [64], *GATA3* [49], MHC class I and β-2 microglobulin [52], and *IRF9* [45] were cloned and characterized but have not been reported as yet for the identification of extensive immune genes and their employment for immunity. Therefore, investigation of key immune components and characterization of gene/protein expression of immunity-relevant genes would have priority in the immune research of crucian carp.

Molecular genomic and proteomic approaches are able to provide a better understanding on the intracellular mechanism on the immune system [14,47]. Recently, several Next Generation Sequencing (NGS) technologies enable us to obtain the massive complementary DNA (cDNA) or genomic DNA (gDNA) information, and thus to date the extensive sequence information of important immune-relevant genes has been identified in several fish species and employed for further understanding of immune system [2,14,47,55]. On the usefulness of the crucian carp as a potential model species for aquaculture monitoring, we sequenced transcriptomes for the gene/protein information-based application to

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detect early molecular biomarker genes of the disease induction or pollution in crucian carp.

In this study, we obtained expressed cDNA information by pyrosequencing in the crucian carp, *C. auratus*. Our results are the first report on the extensive identification of various immune mechanism related genes in this species. To employ transcriptional profiling for aquaculture monitoring, we designed primer sets for entire immune-relevant genes of the crucian carp and tested amplicon identity for public and practical use in immunity research. Finally, we supposed that these results would be useful to obtain a better understanding of the mode of action of disease induction by pathogens in the crucian carp, *C. auratus*.

2. Materials and methods

2.1. Fish

The crucian carp *C. auratus* were reared and maintained at the National Institute of Environmental Research (Incheon, South Korea). The fish were maintained at 25 °C with 12 h/12 h light/darkness. The automated water changing system was set for constant flow-through and water quality (pH, salinity, and temperature) was recorded using various instruments (5.71 ± 0.19 mg O_2/L). Experimental fish were anaesthetized on ice and sacrificed by decapitation.

2.2. Total RNA extraction

The main objective of this study was the mining of expressed transcripts that provide a potentially immune-relevant gene pool of the crucian carp for public use in diverse immune research. Therefore, to enrich transcriptome information, we sampled different total RNA pools from immunized or non-immunized tissues. In particular tissues collected from brain, eye, gill, heart, head kidney, intestine, kidney, liver, muscle, skin, spleen, and stomach of 10 fish at the adult stage (≈8 month) of both sexes. Each pooled tissues were homogenized in 3 volumes of TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH) with a tissue grinder and stored at −80 °C until use. Total RNAs were isolated from each pooled tissues according to the manufacturer's instructions. DNA digestion was performed using DNase I (Sigma, St. Louis, Mo). After DNase treatment, the total RNAs were purified using RNeasy Mini Spin Column (Qiagen), and were quantified by absorption of light at 230, 260, and 280 nm (A230/260, A260/280) using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience). To check the genomic DNA contamination, we loaded the total RNAs in a 1% agarose gel which contained ethidium bromide (EtBr) and visualized on a UV transilluminator (Wealtec Corp., Sparks, NV). Also, to verify the total RNA quality, we loaded the total RNAs in a 1% formaldehyde/agarose gel with EtBr staining and checked the *18/28S* ribosomal RNAs integrity. After RNA quality was determined, single-strand cDNA was synthesized from total RNA using an oligo$(dT)_{20}$ primer for reverse transcription (SuperScript™ III RT kit, Invitrogen, Carlsbad, CA).

2.3. cDNA library preparation

cDNA library was constructed according to the manufacturer's instructions (Roche Applied Science, Genome Sequencer 20™ System). Briefly, cDNA was fractionated into smaller fragments (300–500 base pairs) that were subsequently blunt-ended using Fragment End Polishing kit supplied by manufacture (Roche Applied Science). Short Adaptors (Two adaptors, A and B, were provided in the GS-20 Library Preparation Kit) were then ligated onto the ends of the fragments. These adaptors were provided priming for both amplification and sequencing of the sample-library fragments. Adaptor B contained a 5′-biotin tag that enables immobilization of the library onto streptavidin-coated beads. After fill-in reaction for nicks, the non-biotinylated strand was used to isolate a single-stranded template DNA (sstDNA) library. The sstDNA library was assessed for its quality and the optimal amount (DNA copies per bead) needed for emulsion PCR (emPCR) was determined by titration.

2.4. Pyrosequencing

Expressed cDNA sequencing was performed with GS 20 sequencer. Constructed sstDNA library was clonally amplified via emPCR, thereby generating millions of copies of templates per bead. The DNA beads were then distributed into picolitre-sized wells on a fiber-optic slide (PicoTiter-Plate™) along with a mixture of smaller beads coated with the enzymes required for the pyrosequencing reaction including the firefly enzyme luciferase. The four DNA nucleotides were then flushed sequentially over the plate. Light signals released upon base incorporation were captured by a CCD camera, and the sequence of bases incorporated per well was stored as a read.

2.5. Assembly, gene annotation, and GO analysis

For assembly, we used the Newbler Assembler software (454 Life Sciences, Roche Diagnostics company) in the assembly stage 1, and the assembled EST translations were aligned with the GenBank NR (non-redundant; including all GenBank, EMBL, DDBJ, and PDB sequence except EST, STS, GSS, or HTGS) amino acid sequence database using BLASTx. The aligned data were arranged according to read length, gene annotation, GenBank number, *E*-value, species, and its accession number. All the bacterial clones were eliminated from the annotated genes. The Gene ontology (GO) functional annotations were assigned by Blast2GO automated sequence annotation tool ([11]; http://www.blast2go.org). Three main categories for biological process, cellular component, and molecular function were obtained by using default parameters. Assembly, gene annotation, and GO analysis were performed at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University (Seoul, South Korea).

2.6. Immune-relevant gene mining

In each section of GO terms, immune-relevant gene mining was performed manually within the expected value threshold of 1.00E-05 that has potential immune-relevant function. Selected genes were arranged with read length, gene annotation, GenBank number, *E*-value, and species with its accession number.

2.7. Primer design and real-time RT-PCR

Primers were designed after comparing exon/intron boundary to genomic DNA using GENRUNNER software (Hastings Software, Inc. N. Y. USA) and confirmed by Primer 3 program (Whitehead Institute/MIT center for Genome Research). To determine the amplicon identity, all the PCR products were cloned into pCR2.1 TA vector, and sequenced with an ABI 3700 DNA analyzer (Bionics Co., Seoul, South Korea). To analyze transcriptional expression patterns in different tissues of the crucian carp, several immune-relevant genes were collected from the assembled EST database, and their transcript abundances were analyzed with real-time RT-PCR. Template cDNA was made by pooled total RNA of each tissue from 10 adult fish of both sexes. Each reaction included 1 μl of cDNA and 0.2 μM primer (real-time RT-F/R or *18S rRNA* RT-F/R). Optimized

conditions were transferred according to the following CFX96™ real-time PCR protocol (Bio-Rad, Hercules, CA, USA). Reaction conditions were as follows; 95 °C/3 min; 40 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/30 s. To confirm the proper amplification of specific products, cycles were continued to check the melting curve under the following conditions; 95 °C/1 min, 55 °C/1 min, and 80 cycles of 55 °C/10 s with 0.5 °C increase per cycle. SYBR® Green (Molecular Probes Inc., Invitrogen) was used to detect specific amplified products. Amplification and detection of SYBR® Green-labeled products were performed using CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). Data from each experiment were expressed relative to expression levels of the *18S rRNA* gene to normalize the expression levels between samples. All the experiments were done in triplicate. Data were collected as threshold cycle (C_T) values (PCR cycle number where fluorescence was detected above a threshold and decreased linearly with increasing input target quantity), and used to calculate ΔC_T values of each sample. The fold change in the relative gene expression was calculated by the $2^{-\Delta\Delta C}$t method [27].

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). Significant differences were analyzed using one-way and/or multiple-comparison ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered significant. SPSS ver. 17.0 (SPSS Inc., Chicago IL, USA) software package was used for statistical analysis.

3. Results and discussion

3.1. Overview of the crucian carp ESTs

Overall pyrosequencing result on transcriptome of the crucian carp was summarized in Table 1. As raw read data, we obtained 221,542 reads, and all the reads were trimmed and cleaned with several bioinformatics tools. Repetitive and low quality reads were filtered out from the entire raw read set for non-redundant consensus. After the cleaning process, 19,492 contigs and 58,743 singletons were aligned, respectively. After all the transcripts were compared with the GenBank NR amino acid sequence database using BLASTx, those assembled transcripts with *E*-values ≤1.00E-05 were used to obtain reliable annotation results in this study. The average read length of those was 106 bp in contig and 84 bp in singleton reads, respectively. The largest contig length was 1012 bp and the largest singleton length was 191 bp, respectively. Regarding sequencing quality and average read lengths of views in using the GS-20 platform, overall pyrosequencing results of the crucian carp were similar to previous results using the bacterium *Mycoplasma genitalium* (110 bp) [31], the hermaphroditic fish *Kryptolebias marmoratus* transcripts (116–129 bp) [36], and the rockshell *Thais clavigera* (114 bp) [37] that were sequenced by the GS-20 pyrosequencing method. To increase valid nomenclatures, entire reads were sorted out with the cut-off value more than 200 bp with *E*-value ≤1.00E-05. Subsequently, selected reads were analyzing their Gene Ontology (GO) to find specific gene function of their own as well as their immune-relevant role.

Table 1
Overview of pyrosequencing and assembly result.

# EST sequences	221,542
Sequence cleaning process[a]	
# valid reads	203,281
# trimmed reads of valid process	5947
# trashed reads[b]	12,314
Lucy cleaning process	
# valid reads	193,553
# trashed reads	9728
Assembly Stage[c]	
# contigs (2+)[d]	19,492
average read length (bp)	106
largest read length (bp)	1012
# singletons[e]	58,743
average read length (bp)	84
largest read length (bp)	191
# transcripts[f]	78,235
# hits[g]	10,532
% with hits[h]	13

[a] Minimum length: 60 base pair.
[b] Trimmed reads composed of short reads (7341), low quality reads (1657), short quality reads (3241), and dust reads (75).
[c] Assembly was performed using the Assembly Software (Newbler Assembler Software) of the Genome Sequencer 20 Software Version 1.0.53.
[d] Number of contigs with 2 or more sequences.
[e] Number of contigs with 1 sequence.
[f] Total number of transcripts including singletons.
[g] Number of transcripts that have a BLASTX hit.
[h] Percent of assembled transcripts that have a BLASTX hit.

3.2. Annotation and functional classification for immune-relevant gene mining

Of the 78,235 contig and singleton cDNA fragments, 10,532 reads (13%) were aligned with valid similarities in the NCBI database (Table 1). In 10,532 reads, 7625 genes (72%) were matched with those of fish species. Those genes were classified according to their functions into each ontology class such as molecular function, cellular process, or biological process in Blast2GO analysis. Briefly, the vast majority of genes were involved in "binding" (49%), and "catalytic activity" subcategories (28%) in the molecular function category (Fig. 1A). In the biological process class, most genes were categorized in "cellular processes" (15%) and "metabolic processes" (15%) (Fig. 1B). The "immune system process" subcategory was occupied by 3% of the biological process category. In the cellular component class, most of genes were related to "cell" (22%) and "cell part" (22%) and followed by the "organelle" (16%) subcategory (Fig. 1C). Based on functional analysis, we identified immune-relevant genes from each subcategory as many of them were incorporated into different categories for coordination of diverse functions with other genes. Finally, 77 immune system-related sequences corresponding to adhesive protein, antimicrobial protein, apoptosis- and cell cycle-related protein, cellular defense effector, immune regulator, protease and protease inhibitor, reduction/oxidation-related protein, signal transduction-related protein, stress protein, and acute phase response/inflammation-related genes were identified in the EST database for further work (Table 2). Taken together, pyrosequencing-based GO profiling is of value in the identification of immune-relevant genes, indicating that this kind of approach would be available to better understand the functional role of certain gene sets or signaling pathways.

3.3. Immune-relevant genes in the crucian carp

To provide public and practical use of immune-relevant genes, we designed primer pairs for the entire immune gene set identified in the crucian carp (Table 3) and tested their amplicon identity with electrophoresis (Fig. 2). Subsequently, we measured tissue-preferential transcript profiles of selected genes that were confirmed in other teleosts to validate the usefulness of gene-specific applications for further immune-relevant work in the crucian carp.

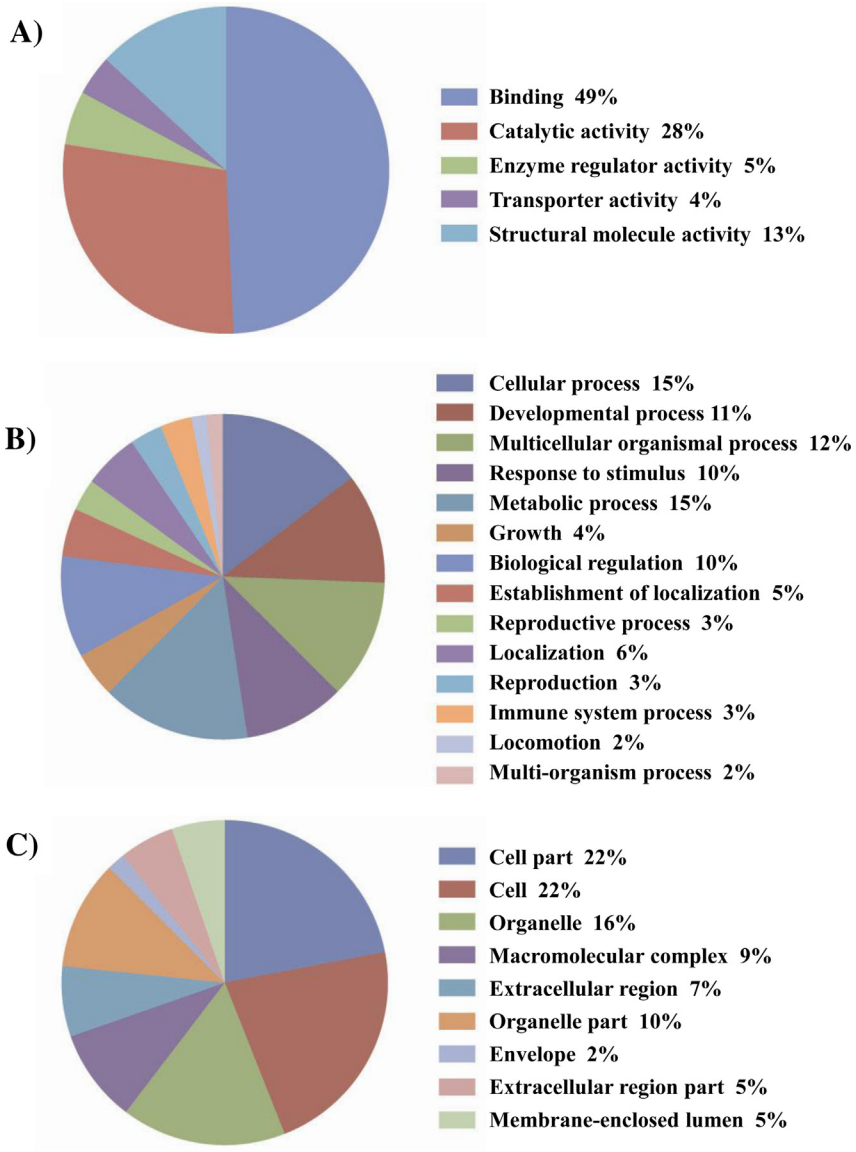

Fig. 1. Categories of gene ontology for the crucian carp ESTs. (A) molecular function, (B) biological process, (C) cellular components.

3.3.1. *C-type lectin*

Lectin is a large glycoprotein family that is capable of binding sugar moieties of foreign pathogens with a carbohydrate recognition domain (CRD) [12]. Lectins are classified according to their secondary and/or tertiary structure as calnexin, C-, L-, P-, I-, R-, and S-type lectins. Of lectins, the C-type lectins as a large group of lectins are pattern-recognition proteins which are functionally important for pathogen recognition and immune regulation in animals [58]. In the crucian carp, the highest mRNA expression level of C-type lectin (*Ca-CTL*) was observed in liver, while moderate expression level in intestine, spleen, and muscle tissues (Fig. 3). Previously, Kondo et al. [21] reported that C-type lectin transcript was only observed in liver tissues of the Japanese flounder, *Paralichthys olivaceus*. In the orange-spotted grouper *Epinephelus coioides*, C-type lectin transcript was abundant in liver and skin [53]. Also, transcriptional abundance of C-type lectin was highest in liver but was lowest in kidney [62]. On the contrary, dissimilar transcriptional profiles were observed in several teleosts [42,60]. Specific immune-relative function of C-type lectin is still unclear in different tissues of teleosts as yet, although some hepatitis C viral glycoproteins are known to invade liver cells for infection via attachment to C-type lectins in vertebrates [17,35]. Moreover, an appropriate gene family backbone is not available, as each lectin family is composed of large complex subfamilies. In the case of C-type lectin, amino acid similarity is not high within teleosts (identity: <81% in BLASTx). For example, Ca-CTL amino acid showed a moderate similarity to Japanese flounder (*P. olivaceus*, 41%) and orange-spotted grouper (*E. coioides*, 46%). Based on various isotypes of C-type lectin and their different transcriptional expressions in tissues, each C-type lectin would have potentially specialized immune-related roles in different tissues, and subsequent comparative phylogenic and functional studies will be highlighted in elucidating the role of teleosts' C-type lectin family.

3.3.2. *CC chemokine*

Chemokines are a large family of cytokines that are classified according to structural characteristics and conserved cysteine residues [65]. Chemokines play a pivotal role in both, the non-specific

Table 2
Immune-relevant genes annotated in the EST library from *C. auratus*.

Putative function	GenBank	Species (Accession no.)	*E*-value
Adhesive protein			
Coagulation factor II	JX477179	*Danio rerio* (NP_998555)	0
Coagulation factor VII	JX477180	*Ictalurus furcatus* (ADO28333)	4E-30
Coagulation factor X	JX477181	*Danio rerio* (NP_958870)	5E-125
Coagulation factor V	JX477182	*Danio rerio* (AAN71005)	2E-176
C-type lectin	JX477183	*Ctenopharyngodon idella* (ACS16044)	1E-140
Fibrinogen, α chain	JX477186	*Danio rerio* (NP_001181918)	3E-67
Fibrinogen, β polypeptide	JX477184	*Danio rerio* (NP_997939)	2E-168
Fibrinogen, γ polypeptide	JX477185	*Hypophthalmichthys molitrix* (ADF97606)	4E-77
F11 receptor	JX477187	*Danio rerio* (NP_001076451)	9E-30
Intelectin 3	JX477188	*Ctenopharyngodon idella* (AAY43357)	1E-53
CD59 glycoprotein	JX477189	*Danio rerio* (XP_003198001)	1E-35
Integral membrane protein 2Bb	JX477190	*Danio rerio* (NP_998141)	5E-64
Antimicrobial			
Liver-expressed antimicrobial peptide 2B	JX477191	*Oncorhynchus mykiss* (NP_001117937)	6E-18
Apoptosis and cell cycle			
Programmed cell death 10	JX477192	*Ictalurus punctatus* (NP_001187364)	4E-37
Calmodulin	JX477193	*Homo sapiens* (AAH07965)	1E-66
Complement c1q, tumor necrosis factor-related protein	JX477194	*Danio rerio* (XP_001919564)	2E-41
Myeloid cell leukemia sequence 1B	JX477195	*Danio rerio* (AAL32470)	5E-41
Cellular defense effecter			
α-1-microglobulin	JX477196	*Danio rerio* (NP_957412)	4E-119
α-2-macroglobulin	JX477198	*Cyprinus carpio* (ABC47740)	1E-99
β-2-microglobulin	JX477197	*Labeobarbus intermedius* (CAD44964)	6E-44
MHC class I antigen	JX477199	*Cyprinus carpio* (AFO38426)	4E-23
Eukaryotic translation initiation factor 3 subunit M, dendritic cell protein	JX477200	*Danio rerio* (NP_001019906)	7E-47
Leukocyte cell-derived chemotaxin 2	JX477201	*Danio rerio* (NP_001041520)	3E-78
Chemokine CCL-c25s	JX477202	*Danio rerio* (BAF98242)	4E-23
Immune regulator			
Kininogen 1	JX477203	*Danio rerio* (NP_001005981)	1E-91
Secreted immunoglobulin domain 4	JX477204	*Danio rerio* (NP_001029354)	4E-107
Carboxypeptidase B2	JX477206	*Danio rerio* (NP_001018539)	4E-48
Signal sequence receptor, γ Translocon-associated protein subunit γ	JX477207	*Danio rerio* (NP_956347)	2E-67
Fetuin-A, α-2-HS-glycoprotein	JX477208	*Danio rerio* (NP_997787)	3E-35
Basic transcription factor 3	JX477209	*Danio rerio* (NP_001070026)	1E-39
Proteases and protease inhibitor			
Inter-α (globulin) inhibitor H3	JX477210	*Danio rerio* (NP_001018424)	1E-96
Inter-α-trypsin inhibitor heavy chain H4 precursor	JX477211	*Danio rerio* (NP_001020335)	3E-48
Serine (or cysteine) proteinase inhibitor, clade C (antithrombin)	JX477212	*Danio rerio* (NP_878283)	2E-103
Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor)	JX477214	*Danio rerio* (CAQ14779)	7E-40
Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase), member 1	JX477215	*Danio rerio* (AAH95615)	1E-60
Serpin peptidase inhibitor, clade A (α-1 antiproteinase), member 7	JX477213	*Danio rerio* (AAI53542)	4E-100
Serpin peptidase inhibitor, clade F, member 2B	JX477217	*Danio rerio* (NP_001073479)	3E-57
C1 inhibitor precursor	JX477216	*Danio rerio* (NP_001116757)	8E-60
Trypsin	JX477218	*Cyprinus carpio* (BAL04385)	2E-82
Complement C1r/s-A	JX477219	*Cyprinus carpio* (BAB17845)	4E-36
Cathepsin E	JX477220	*Danio rerio* (AAI65335)	2E-34
Cathepsin L, 1 a	JX477221	*Cyprinus carpio* (BAD08618)	7E-95
Cathepsin D	JX477222	*Ctenopharyngodon idella* (AAN62917)	1E-38
Cathepsin B	JX477223	*Cyprinus carpio* (BAE44111)	2E-40
Prothrombin-like	JX477224	*Oreochromis niloticus* (XP_003457819)	2E-25
60S ribosomal protein L40, Ubiquitin A-52 residue ribosomal protein fusion product 1	JX477228	*Danio rerio* (NP_001032190)	8E-45
FBR-musv ubiquitously expressed; ribosomal protein S30	JX477230	*Danio rerio* (NP_957031)	2E-45
Redox			
Selenoprotein P, plasma, 1a	JX477233	*Danio rerio* (NP_840082)	3E-54
Glutathione peroxidase 1a	JX477232	*Cyprinus carpio* (ADK26519)	2E-59
Glutathione peroxidase 3 (plasma)	JX477233	*Danio rerio* (NP_001131027)	6E-36
Glutathione peroxidase 4a	JX477234	*Carassius auratus* (ABO36294)	9E-67
Glutathione *S*-transferase theta 1a	JX477235	*Danio rerio* (XP_692427)	2E-151
Pi-class glutathione *S*-transferase	JX477237	*Carassius auratus* (ABF57553)	2E-41
Rho-class glutathione *S*-transferase	JX477236	*Cyprinus carpio* (ABD67511)	3E-79
Catalase	JX477239	*Ctenopharyngodon idella* (ACL99859)	7E-40
Ferritin, middle subunit-like	JX477240	*Danio rerio* (XP_687175)	5E-108
Natural killer cell enhancing factor B (NKEF-B)	JX477241	*Cyprinus carpio* (ABC59223)	4E-140
SOD (Cu/Zn)	JX477242	*Danio rerio* (NP_571369)	6E-57

Table 2 (*continued*)

Putative function	GenBank	Species (Accession no.)	*E*-value
Signal transduction			
Integrin, β1b	JX477244	*Danio rerio* (AAI62706)	2E-46
Stress protein			
ATP synthase F0 subunit 6	JX477246	*Carassius auratus* (ABQ01626)	2E-46
Hsp5	JX477247	*Salmo salar* (NP_001135114)	7E-34
Hsp10	JX477248	*Danio rerio* (NP_571601)	5E-43
Tumor rejection antigen (gp96)	JX477250	*Danio rerio* (NP_937853)	7E-98
Acute phase response/inflammation			
Complement component C3	JX477251	*Cyprinus carpio* (BAA36619)	4E-66
Complement component C4-1	JX477252	*Cyprinus carpio* (BAB03284)	4E-35
Complement component C4-2	JX477253	*Cyprinus carpio* (BAB03285)	2E-63
Complement component C7	JX477254	*Hypophthalmichthys molitrix* (AFH01333)	1E-52
Complement component C9	JX477255	*Ctenopharyngodon idella* (ABN49522)	1E-47
Complement B/C2-A2	JX477256	*Cyprinus carpio* (BAA78416)	7E-65
Complement control protein factor I-B,	JX477257	*Cyprinus carpio* (BAB88921)	1E-75
Complement factor H like 4	JX477258	*Danio rerio* (CAP09610)	7E-31
Heme oxygenase	JX477259	*Ictalurus furcatus* (ADO27993)	4E-77
Macrophage stimulating 1	JX477260	*Danio rerio* (AAH84690)	1E-45
Transferrin variant B	JX477264	*Carassius gibelio* (AAL57602)	3E-30
Transferrin variant C	JX477262	*Carassius gibelio* (AAL57603)	2E-120
Transferrin variant D	JX477263	*Carassius gibelio* (AAM90970)	4E-64
Transferrin variant E	JX477261	*Carassius gibelio* (AAM90971)	4E-131

Abbreviations: FBR-musv, Finkel-Biskis-Reilly murine sarcoma virus; Hsp, heat shock protein; SOD, superoxide dismutase.

innate immune response and the adaptive immune system of animals with functionally divided two gene categories, inducible chemokines (inflammatory) and constitutive chemokines (homeostatic) [1,34]. Based on the number and location of conserved cysteine residues in the N-terminus, chemokines are classified into CCL, CXCL, CX3CL, and XCL subfamilies. However, an appropriate phylogenetic backbone or gene annotation criteria are still not available in the teleost chemokine subfamily as yet. Therefore, the identification of chemokines and a relevant study in fish may provide fundamental views concerning the potential role of each chemokine gene in the complexity of the immune system. Of the chemokine family, we identified one CC chemokine cDNA designated as *Ca-CCL25* gene in the crucian carp. The BLAST search and amino acid analysis showed that *Ca-CCL25* gene was close to zebrafish *CCL25s* gene (BAF98242) with 72% sequence similarity. As shown in Fig. 4, the *Ca-CCL25* transcript was detected strongly in kidney and spleen tissues. In head kidney, gill, and intestine tissues, moderate transcript levels of the *Ca-CCL25* gene were observed but only slight expressions were observed in other tissues. This result was similar from that reported previously in several fish such as *Pseudosciaena crocea* [59,61], *Cynoglossus semilaevis* [26], *Rachycentron canadum* [48], and *Oplegnathus fasciatus* [20] but different transcriptional profiles were also observed in *Oncorhynchus mykiss* [24] and *Miichthys miiuy* [10,56]. Thus, these results suggested that different types of CC chemokine genes would have evolved in different fish tissues. Also several reports supported this hypothesis. For example, Laing and Secombes [23] reported that both inducible and constitutive types of CC chemokines were presented in teleosts as different inducible patterns of 18 CC chemokines were observed upon recombinant TNF-α stimulation in the rainbow trout *O. mykiss*. Also, Borza et al. [4] suggested that CC chemokines would be regarded as either inducible or dual-function rather than a constitutive one based on tissue-specific different inducibilities of 12 CC chemokines of the Atlantic cod *Gadus morhua*. Particularly, high levels of different CC chemokine transcripts in kidney and spleen tissues were strongly correlated with the immune response against immune challenges in teleosts [29,59,61,8,26,48]. In teleosts, Trede et al. [50] suggested that kidney and spleen tissues were important immune-regulating organs for activation and interaction of chemokines as diverse lymphocytes, T and B cells, and myeloid cells were also found in these tissues. Although CCL25 is essential for colonization of T cells in thymus during early development of zebrafish [28], less is known in other teleosts. Therefore, the tissue-specific expression profile of *Ca-CCL25* gene will be helpful to understand its potential immune-relevant roles such as inflammatory and/or homeostatic functions in kidney and spleen tissues of the crucian carp.

3.3.3. Cathepsin

Cathepsins are lysosomal cysteine proteases for maintaining homeostasis and are among the most thoroughly studied hydrolases [3]. To date, over a dozen cathepsin proteases have been cloned and characterized in a number of mammalian and fish species (cathepsins B, C, F, H, K, L, O, S, T, U, V, W, and X). Cathepsins are classified into different subfamilies based on conserved domains/motifs and phylogenetic relationship with their substrates [22]. In the crucian carp, four cathepsin homologues (*Ca-CatB*, *Ca-CatD*, *Ca-CatE*, and *Ca-CatL*) were identified by pyrosequencing and *in silico* analysis. Based on overall transcriptional expressions of these genes in different tissues (Fig. 5), the constitutive expression of these cathepsins in immune and non-immune tissues indicates the pleiotropic nature of their functions and their crucial roles in homeostasis. Particularly, transcriptional abundance of *Ca-CatB* was measured in crucian carp, and revealed that the *Ca-CatB* transcript was mainly expressed in liver, intestine, and both kidney tissues. The *Ca-CatD* transcripts were distributed ubiquitously in most tissues except in eye, muscle, and skin. However, mRNA levels of *Ca-CatD* in kidney, liver, and spleen were greater than in other tissues. Study of expression of *Ca-CatE* at different tissues of the crucian carp indicated that the highest levels of transcripts were observed in kidney and liver. Heart, head kidney and intestine showed almost the same levels of mRNA expression. Tissue-specific differences in the transcriptional abundance of *Ca-CatL* were observed. In kidney, liver, and spleen, mRNA levels of *Ca-CatL* were highly detected, while in other tissues, the *CatL* transcripts were distributed ubiquitously in all the tissues but at a low level. Similarities and discrepancies of these four genes on tissue-specific transcriptional expressions have been reported in several fish such as *Paralichthys olivaceus* [59,61], *Cynoglossus semilaevis* [7], and *Oplegnathus fasciatus* [54] for *CatB* gene; *Oncorhynchus mykiss*

Table 3
Oligonucleotides used for real-time RT-PCR.

#	Gene	Oligonucleotide sequence	Tm (°C)	Length (bp)
1	Coagulation factor II	F: TTCCACCTCGGTCATCATC	56	148
		R: AATGCCAATCTGATACCAGC	55	
2	Coagulation factor VII	F: TTGTAGAGGACATGATGGCAG	56	118
		R: GGTGTAAATCCCATAGTTCCC	55	
3	Coagulation factor X	F: CAGAAGTTAACGGTGCCATAC	55	119
		R: CCCTGACATGCATCTTTCTC	55	
4	Coagulation factor V	F: TGGCCAGACTACATAATACAGG	55	124
		R: TGCTTGGCTCCTTGTGTG	55	
5	C-type lectin	F: AGTGTGGAAATGGGTGGAC	54	158
		R: TGAAATCACATGGCACGTC	55	
6	Fibrinogen, β polypeptide	F: ATTCTATCAGCTGGTCGTTACTC	55	151
		R: CTCCAGGTATCCATTTGTCG	55	
7	Fibrinogen, γ polypeptide	F: GACTACGCCACATTCAAACTC	55	106
		R: CGAAGTCGAAACCATCAAAG	56	
8	Fibrinogen α chain	F: TGATAGGGACAGCGATAAGTG	55	148
		R: AATCTCATAAGGGACTTTGGTG	56	
9	F11 receptor	F: TCTTCAATGGTAGCCTGGAG	55	148
		R: GCTGGTGTTTGGCTGTTG	55	
10	Interlectin 3	F: GAGATGGTACATGGGCAAAC	55	132
		R: GCTTATTATTGGGAACGTGC	55	
11	CD59 glycoprotein	F: CCCTGAGATGTCAAAACTGTG	55	139
		R: ATGCACCTCCGGAAGTAAG	55	
12	Integral membrane protein 2Bb	F: CATCATCACCCTGAACACATC	55	139
		R: GCTGTCCATCCTTTCTGTAATC	56	
13	Liver-expressed antimicrobial peptide 2B	F: ATACTTCATCGCAAGATCCG	55	135
		R: AGGCTCATTAAACGAACAGTG	55	
14	Programmed cell death 10	F: CTGCAGATGACGTGGAAGAG	56	183
		R: CCGTGTCCAGTAGTTCTTTTATG	57	
15	Calmodulin	F: AAGATGAAGGACACGGACAG	54	137
		R: TCCTCATCTGTTAACTTCTCCC	55	
16	Complement c1q, tumor necrosis factor-related protein	F: CAAGTACAGAGGATACAAACCG	54	152
		R: GCCTGAGTACCTGAAGTAATACAC	55	
17	Myeloid cell leukemia sequence 1B	F: CGTTGTGAAGCACGAACTG	55	120
		R: GTGATGCCATCGCTGAAG	55	
18	α-1-microglobulin	F: AGTGTGTTCCAGGTGAGCAG	55	138
		R: GGGACTTACACGCTTCAGG	55	
19	α-2-macroglobulin	F: TCAACAACAGAATGAAGGGTG	56	131
		R: GGACTTACAACATGACAAACCTC	56	
20	β-2-microglobulin	F: GTGCTGTACATCACTGTCCAAG	55	130
		R: TGATATCTGGAGGGTGGAAG	54	
21	MHC class I antigen	F: CAGTGCCACAAGGAATCATC	56	135
		R: TCAGGCTGCTCTTTCCATAC	55	
22	Eukaryotic translation initiation factor 3 subunit M, dendritic cell protein	F: TCTTGCAATAGACCAGACACAG	55	117
		R: AGATTCTGTTTCCAGGAGCAC	56	
23	Leukocyte cell-derived chemotaxin 2	F: TACGAAGTATGGCTGTGGAAG	56	108
		R: AATGGAGCATAAACTGTGGC	55	
24	Chemokine CCL-c25s	F: CCTGAGAAATGTTGCTGGTC	55	111
		R: TGTCATAACCACAATGCCAG	55	
25	Kininogen 1	F: ACCTGCAACAAACGGATAAG	55	129
		R: GACACTGGGCGACTTTAGAG	55	
26	Secreted immunoglobulin domain 4	F: CAGTCATCTCCTGGTCGAAG	55	109
		R: ATGTACTGGCCTGAGTCCTG	54	
27	Carboxypeptidase B2	F: AGGAGGCCTCAACAAAGATAC	55	143
		R: AAGCTCAAAGGTGAAGGAATAC	55	
28	Signal sequence receptor, γ	F: CAAGCACAAAGTCGCTCAG	55	111
		R: ATCCTCTCGTCCTTCTCTTTG	55	
29	Fetuin-A, α-2-HS-glycoprotein	F: AGACTAGAACAGAAACACAGACG	56	116
		R: TGTGAAGCTGGTTCGGTG	55	
30	Basic transcription factor 3	F: GAAATTAGCCAAGTTGCAGG	55	148
		R: GCCAGAGATGTTATTTACTCCC	55	
31	Inter-α (globulin) inhibitor H3	F: CAGAGTCAAACCCAAACAGC	55	101
		R: TGATGAATGTACCGTATGCG	55	
32	Inter-α-trypsin inhibitor heavy chain H4 precursor	F: AGTTGCTCGCAGGATTTATG	56	133
		R: GCTGGTCTTGGTAAGGTTTG	55	
33	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin)	F: GACGATAAATGAATGGATAGCC	56	120
		R: TGACCTTTGAAGTAGATTGCG	56	
34	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor)	F: AGTGAATGAAGAAGGTACCGAG	56	141
		R: CCCATGAACACAACGCAG	55	
35	Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase), member 1	F: TAGAGGGAAGTGGGATAAACC	55	138
		R: TGGTTGACAGGGTCTTGATAG	55	
36	Serpin peptidase inhibitor, clade A (α-1 antiproteinase), member 7	F: TCGAACTCACTTCTAAAGTCCTC	55	141
		R: TCCACTTTTCAATGTGCTGTC	56	
37	Serpin peptidase inhibitor, clade F, member 2B	F: AACTTGACTTCAACCAGGACC	56	130
		R: CTGGACATATGCTGTACACTGG	56	
38	C1 inhibitor precursor	F: TTCTCTGCCTCTGTCTACGC	55	114

Table 3 (*continued*)

#	Gene	Oligonucleotide sequence	Tm (°C)	Length (bp)
		R: ACTTCGAGCACCCAATAGC	55	
39	Trypsin	F: CCGCTACCCTCAATCAGTAC	55	107
		R: AGTGGAGCTCATGGTGTTTC	54	
40	Complement C1r/s-	F: CTGTAATGGAGGGCAAGATG	56	139
		R: TCAGTGACCAAATAATCCTCG	55	
41	Cathepsin E	F: CATCAACTGGATTCCAGTCAC	55	138
		R: ATTAGAGAAGTTCCCGTGTCC	55	
42	Cathepsin L, 1a	F: GATGAATGGCTACAAACACAAG	55	127
		R: ATCTTTCACGGGAGTCACG	55	
43	Cathepsin D	F: TGGGTTGACTCTGTGTAAAGG	55	147
		R: TTACAGTCTACCAGGAACTCTCC	55	
44	Cathepsin B	F: TGAGCATCATGTGAATGGC	55	122
		R: TTCCAAAGTGTTTGTCCTCC	55	
45	Prothrombin-like	F: ATAAAGAGGAAAGAGTTGCTGC	56	116
		R: GTAATGTTTCCGCCTGCTC	56	
46	60S ribosomal protein L40,	F: CTACAACATTCAGAAAGAGTCCAC	56	123
		R: CACTTGCGGCAGATCATC	55	
47	FBR-musv ubiquitously expressed; ribosomal protein S30	F: TGTCTCTTCTGGACTGTGGTG	55	139
		R: TTCTCCTGTTTGTCCACTTTG	55	
48	Selenoprotein P, plasma, 1a	F: TGAATATCACCCTCTACGCC	55	125
		R: GGGAAGAGACAGATGGTAAGTG	56	
49	Glutathione peroxidase 1a	F: GGATTACACCCAGATGAACG	55	128
		R: TCAGAGACAGCAGAATTTCATC	54	
50	Glutathione peroxidase 3 (plasma)	F: CACTGCAACAGGAGTTACGAG	56	128
		R: ATTGCCTGGACGGACATAC	55	
51	Glutathione peroxidase 4a	F: TTGGAAATCAGGAACCAGG	55	98
		R: GCCATTCACATCAATCTTACTG	55	
52	Glutathione *S*-transferase theta 1a	F: TGGAACTGTATCTCGATTTGC	55	126
		R: ATTCCTCTCCATATTGTTCACC	55	
53	Pi-class glutathione *S*-transferase	F: ACCTTCTGCTGAATCATAAAGTC	55	124
		R: AAGTTTTCACACTCCAGGAGG	56	
54	Rho-class glutathione *S*-transferase	F: AAGGAGAGAGACATGAATCAGC	55	123
		R: TGAAGTTCTTACCAGCGAGG	55	
55	Catalase	F: CTTTACAATGCCATCTCCAAC	55	140
		R: ATCAGAGGAAAGTCTTTATGGG	55	
56	Ferritin, middle subunit-like	F: AGACCCTCATCTGTGTGACTTC	55	114
		R: TTGTTGCCAGCATCCATC	55	
57	Natural killer cell enhancing factor B (NKEF-B)	F: TCTGTAGACGAGACCCTGAGAC	56	112
		R: GCACATCTGGAACAATAGTGTC	55	
58	SOD (Cu/Zn)	F: CGCACTACAACCCTCATAATC	55	110
		R: GTCAATTTCTGCAACACCATC	55	
59	Integrin, β1b	F: ATCTGCCCAACTTGTCCTG	55	143
		R: AGTTCCTCCTTCTTCTTCACC	54	
60	ATP synthase F0 subunit 6	F: ATCGGAATGCGAAATCAAC	56	124
		R: GGGCTAATGGTCGAATAAGTAG	56	
61	Hsp 5	F: GACCTGAAGAAGACCGACATC	56	119
		R: TCCGGGTTGATTCCTCTG	56	
62	Hsp 10	F: AAAGTCTCAAGCCAAAGTGC	55	119
		R: CTGGCAGCAGAACTTTATCC	55	
63	Tumor rejection antigen (gp96)	F: AGTTCGGCACCAACATTAAG	55	140
		R: CTTCTCCTTCATCCTCTCCAC	55	
64	Complement component C3	F: TGAAACAGGACTCGGACATC	55	147
		R: GGTACGACTTGCCTTCCAC	55	
65	Complement component C4-1	F: AACGACGGACAACATTTCAG	56	111
		R: AAGAAGATGGCAGGCAGC	56	
66	Complement component C4-2	F: GTGTCAATGTGCAGAAAGAGG	55	130
		R: ATCCACAATGACTTCAAATGC	55	
67	Complement component C7	F: CTCGTTTGTGTAATAATCCTGC	55	133
		R: GAAGGATCAAAGCAGTGGG	55	
68	Complement component C9	F: GAGTGTAAATGCATGTGCCTAC	55	103
		R: CCCAGTTTCCCAGTTGTTC	55	
69	Complement B/C2-A2	F: TTGGAGTTGGAGATGAGGTG	55	143
		R: TACACTGGTGCTCTCATCAATC	55	
70	Complement control protein factor I-B,	F: GACATCGCTTTGGTGCAG	55	127
		R: TGGTGCAGGTGTCATTGG	55	
71	Complement factor H like 4	F: CGAAGTGGACACAGGTTGAC	56	123
		R: TGCCTCAACACATTTCGG	55	
72	Heme oxygenase	F: GAGTATCTGGTGGCTCATGC	55	116
		R: AACAACAAGCCCTCTCCG	55	
73	Macrophage stimulating 1	F: TACACCACTGACCCTGAACC	55	130
		R: GACACTCTTTCCCGCTCAC	55	
74	Transferrin variant B	F: GAGAAGATGCACGGAAAGAC	54	134
		R: CTCCTGAAGGCATTTAGTGG	55	
75	Transferrin variant C	F: GTTATGATGAAATCCCACCG	55	136
		R: TCCTTGCGGCTGATGAC	55	

(*continued on next page*)

Table 3 (*continued*)

#	Gene	Oligonucleotide sequence	Tm (°C)	Length (bp)
76	Transferrin variant D	F: TATTATGGCTATGATGGGGC	55	126
		R: CTTCAGATCCTTAGCCCACTC	56	
77	Transferrin variant E	F: AGGGCATCATCTGTGGAAG	55	126
		R: TTGCTCAACCATAACTGGAAC	55	

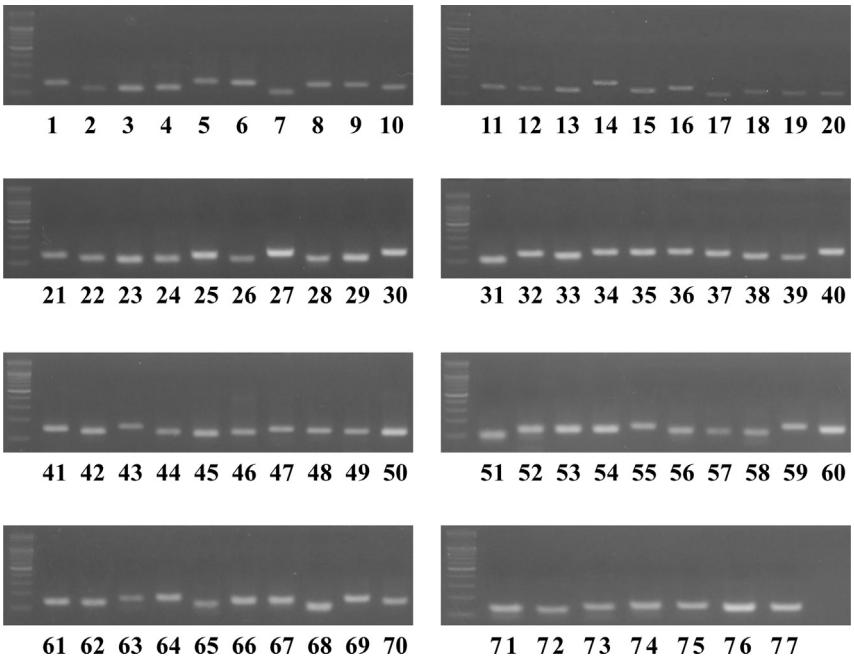

Fig. 2. Amplicon identity of entire immune-relevant genes annotated in the EST library from *C. auratus*. Gene name for each number was incorporated in Table 3. A 100 bp ladder marker was used for each load.

[6], *Danio rerio* [39], *Scophthalmus maximus* [19], *Paralichthys olivaceus* [33], *Ictalurus punctatus* [16], and *Ctenopharyngodon idella* [13] for *CatD* gene; *Ictalurus punctatus* [57] and *Oplegnathus fasciatus* [54] for *CatL* gene, respectively, indicating that the gene regulation of cathepsins is conserved with some divergence in fish.

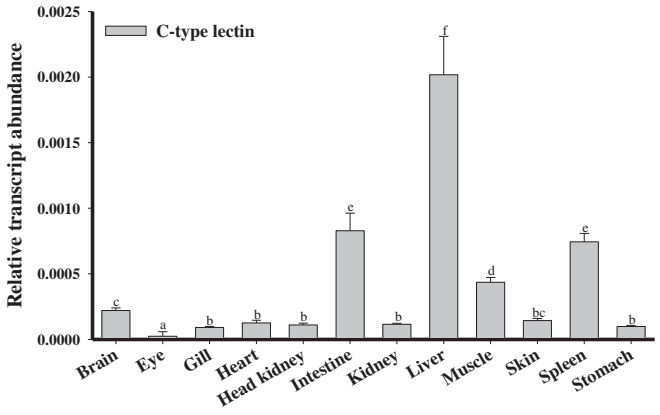

Fig. 3. Tissue-specific mRNA expressions of *Ca-CTL* gene in different tissues of the crucian carp. *C. auratus β-actin* gene was used as a reference housekeeping gene to normalize the expression. Each value represents an average of three replicate samples (±S.D). Significant difference over the lowest value are indicated by different small letters on the data bars ($P < 0.05$) analyzed by multiple-comparison ANOVA.

In vertebrate tissues, cathepsins are known to be ubiquitously expressed with different and specific roles in almost all cell types [51], suggesting that specialized function of cathepsins would be related to their transcriptional levels in the different tissues of the crucian carp as well. Also, our results suggest that the cathepsin family of the crucian carp is involved in a number of physiological processes that have a potent role in host defense through innate immunity. Due to the versatility of expression of the cathepsin family, further studies are needed to unveil their functions in each tissue of crucian carp.

3.3.4. Natural killer cell enhancing factor

Natural killer (NK) cell enhancing factor (NKEF) belongs to the newly defined peroxiredoxin (Prx) family. Originally, NKEF as a cytosolic protein of the human red blood cells has been known to enhance NK cytotoxic activity against the K562 tumor cell line [44]. In mammals, NKEF protein is consisted of two highly homologous genes as Prx1 (NKEF-A) and Prx2 (NKEF-B). Recently, NKEFs have been cloned from diverse teleosts, and their susceptibility against immune challenges suggested a role for NKEFs in inflammation as an important regulator in antitumoral and antiviral defense (summarized in a recent publication; [15]). In the crucian carp, *NKEF-B* gene (*Ca-NKEF-B*) was annotated from EST database, and its tissue-preferential transcript expression was analyzed. As shown in Fig. 6, transcriptional abundance of *Ca-NKEF-B* was ubiquitously detected in almost tissues, while high levels were measured in kidney, heart, and head kidney tissues. This kind of expression

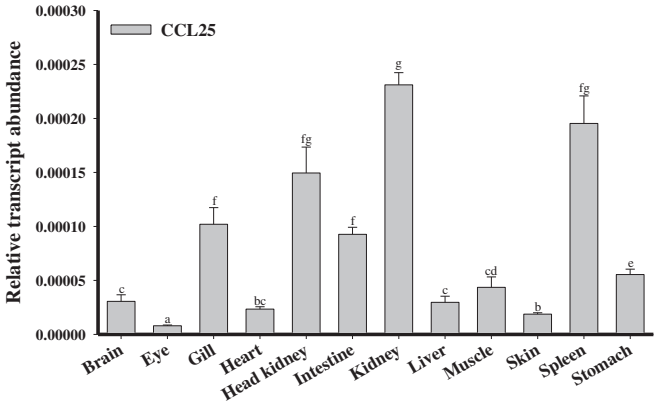

Fig. 4. Tissue-specific mRNA expressions of *Ca-CCL25* gene in different tissues of the crucian carp. *C. auratus β-actin* gene was used as a reference housekeeping gene to normalize the expression. Each value represents an average of three replicate samples (±S.D). Significant difference over the lowest value are indicated by different small letters on the data bars ($P < 0.05$) analyzed by multiple-comparison ANOVA.

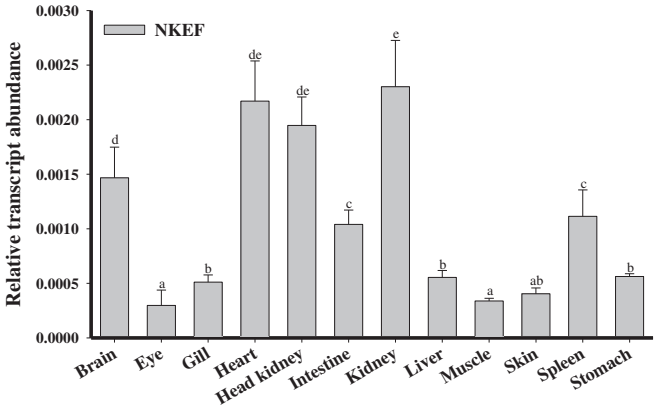

Fig. 6. Tissue-specific mRNA expression of *NKEF* gene in different tissues of the crucian carp. *C. auratus β-actin* gene was used as a reference housekeeping gene to normalize the expression. Each value represents an average of three replicate samples (±S.D). Significant difference over the lowest value are indicated by different small letters on the data bars ($P < 0.05$) analyzed by multiple-comparison ANOVA.

patterns were observed in different tissues of diverse teleosts but its tissue-specific function is not clear as yet. Possibly, ubiquitous profiles can be explained as NKEFs are probably associated with forming tissue [43]. In fact, peroxiredoxins are important proteins in the antioxidative defense system and have relevant functions in immunity [38]. Marionnet et al. [30] reported that several immunostimulants (lipopolysaccharide, dextran sulfate, and tilorone) could induce intracellular ROS production by macrophages in the head kidney and the spleen tissues of common carp. Thus, we suggest that *Ca-NKEF-B* might have an antioxidant role in diverse tissues to prevent immune challenges-induced oxidative stress and further damage, resulting in ubiquitous transcript expression in the crucian carp.

3.3.5. Complement component

Complement system is a part of the immune defense system and is regulated by complement control proteins. Activation of the complement system as a supportive defense line of innate immunity triggers the assembly of the terminal complement complexes against immune challenges, resulting in the induction of the characteristic complement-mediated cytolysis [41]. In the crucian carp, five complement components, *Ca-C3*, *Ca-C4-1*, *Ca-C4-2*, *Ca-C7*, and *Ca-C9*, were annotated from the EST database. The complement system is composed of three major pathways such as the classical complement pathway (CCP), the alternative complement pathway (ACP), and the lectin complement pathway (LCP) [5]. Of complements, the complement component 3 (C3) is a central protein of the entire complement system. Upon immune challenges, C3 induces an amphipathic pore structure as a membrane attack complex (MAC) that is composed by C5b, C6, C7, C8, and C9 molecules [40]. C4 molecule is known to be involved in the prevention of the autoimmune disease with maintenance of the B-cell tolerance in mammal [9]. Also, C4 is a key molecule for the activation of both the CCP and the LCP in teleosts [18]. These complement components were highly detected in liver tissues of the crucian carp (Fig. 7). Our results strongly supported the common

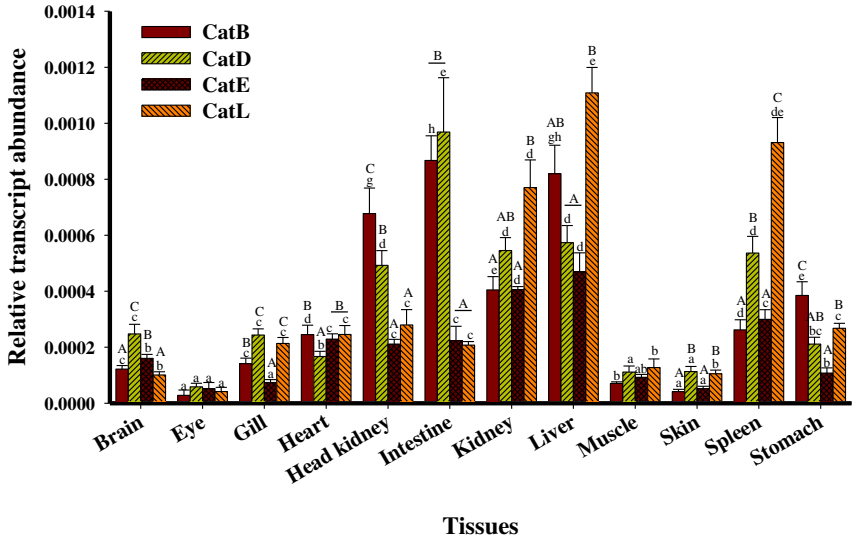

Fig. 5. Tissue-specific mRNA expressions of four cathepsin genes in different tissues of the crucian carp. *C. auratus β-actin* gene was used as a reference housekeeping gene to normalize the expression. Each value represents an average of three replicate samples (±S.D). In each gene, significant differences in different tissues over the lowest value are indicated by different small letters on the data bars ($P < 0.05$) analyzed by multiple-comparison ANOVA. In each tissue, significant difference between different transcripts is indicated by capital letters on the data bars ($P < 0.05$) analyzed by one-way ANOVA followed by Tukey's test. When none of the characters between data bars match, values are considered statistically insignificant.

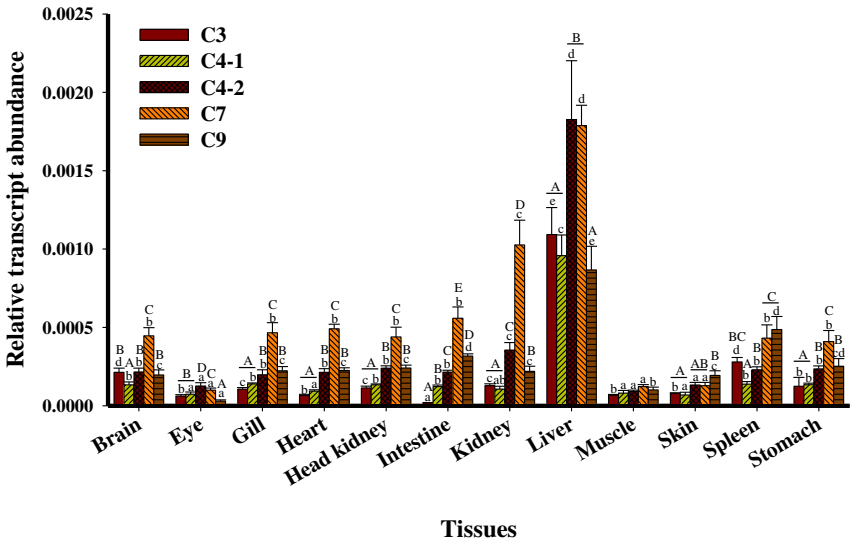

Fig. 7. Tissue-specific mRNA expressions of five complement components in different tissues of the crucian carp. *C. auratus β-actin* gene was used as a reference housekeeping gene to normalize the expression. Each value represents an average of three replicate samples (±S.D). In each gene, significant differences in different tissues over the lowest value are indicated by different small letters on the data bars ($P < 0.05$) analyzed by multiple-comparison ANOVA. In each tissue, significant difference between different transcripts is indicated by capital letters on the data bars ($P < 0.05$) analyzed by one-way ANOVA followed by Tukey's test. When none of the characters between data bars match, values are considered statistically insignificant.

feature that complement components are primarily synthesized as inactive precursors in hepatocytes. Also, transcriptional expression of the complement components in other tissues of the crucian carp can be explained by extrahepatic synthesis of several complement proteins, as different tissues are known to be associated with complement production for further inflammatory responses [25,32]. In most complement components identified in teleosts, a vast majority of transcriptional abundance was observed in liver tissues but only slightly expressed in other tissues. Thus, we can conclude that liver of the crucian carp synthesizes complement components with several extrahepatic sources.

4. Conclusion

Using the pyrosequencing method, we confirmed putative immune-related gene information in the crucian carp, *C. auratus* as studied in numerous previous reports, and demonstrated the effectiveness of the gene cloning method with pyrosequencing. Immune-related gene information of the crucian carp provides clues to develop immune response analysis in this species, as the crucian carp has been known to be one of most important commercial species in Asian aquaculture. Ongoing studies are targeting the specific role of the entire set of immune-relevant genes upon immune challenge in this species.

Acknowledgments

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