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Full Length Research Paper

cDNA structure, genomic organization and expression patterns of visfatin in silver Prussian carp (*Carassius auratus gibelio*)

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Visfatin was a newly identified adipocytokine, which was involved in various physiologic and pathologic processes of organisms. The cDNA structure, genomic organization and expression patterns of silver Prussian carp visfatin were described in this report. The silver Prussian carp visfatin cDNA cloned from the liver was ~2.0 Kb long and contained a 1482 bp open reading frame (ORF), which encoded a protein of 493 amino acids. Furthermore, the complete gene was isolated from fin ray total genomic DNA. The whole gene was ~10 Kb and consisted of 11 exons and 9 introns. The coding sequence of silver Prussian carp visfatin had a close relationship with some other species, such as common carp, green spotted puffer fish, house mouse, porcine, human and so on. However, the non-coding sequences showed great variation from others. Particularly, the length and complexity of silver Prussian carp visfatin gene were less complicated. The results of tissue distribution analysis revealed that silver Prussian carp visfatin mRNA was broadly expressed and had higher levels in gill, heart, ovary and mesenteric adipose, indicating that visfatin played an important role in silver Prussian carp.

Key words: Silver Prussian carp, visfatin, cDNA cloning, exon-intron organization, expression profiles.

INTRODUCTION

Accumulating evidence has demonstrated that adipose tissue is a significant endocrine organ but not just the inert energy storage, since a number of bioactive substances, the so-called adipocytokines, were discovered to be secreted from it; such as adipsin (Cook et al., 1987), adiponectin (Scherer et al., 1995; Maeda et al., 1996), leptin (Friedman and Halaas, 1998), resistin (Steppan et al., 2001) and so on. Visfatin, a new member of this family, was found to be preferentially expressed in visceral fat for the first time by Fukuhara et al. (2005).

Recently, visfatin was characterized originally as a cytokine named pre-B cell colony-enhancing factor (PBEF), and as an enzyme named nicotinamide phosphoribosyltransferase (Nampt). PBEF could synergize with stem cell factor and interleukin 7 (IL-7) to promote the formation of B-cells (Samal et al., 1994), and Nampt was a rate-limiting enzyme involved in NAD biosynthesis, which converted nicotinamide to nicotinamide mononucleotide (NMN), a key nicotinamide adenine dinucleotide (NAD) intermediate (Rongvaux et al., 2002). Thereupon, visfatin played an essential part in various physiologic and pathologic processes and drew much attention.

In addition, visfatin was suggested to possess inflammatory and immuno-modulating properties (Moschen et al., 2007). It was up-regulated in normal fetal membranes after labor and severely infected amnion membranes, thus protecting amniotic epithelial cells from apoptosis (Ognjanovic and Bryant-Greenwood, 2002; Kendal-

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Wright et al., 2008). Likewise, the increased visfatin could inhibit neutrophils from apoptosis in response to multiple inflammatory stimuli (Jia et al., 2004). It has also been showed that visfatin was elevated in a mass of acute and chronic inflammatory diseases like sepsis, acute lung injury, rheumatoid arthritis, inflammatory bowel disease and myocardial infarction (Luk et al., 2008). Fujiki et al. (2000) concluded a similar viewpoint in common carp as a result of increasing visfatin mRNA expression by injection of non-specific immunostimulants. Moreover, visfatin could modulate metabolic response to nutritional availability in various tissues and cellular response to a crowd of stresses and damages through sirtuins which enhanced the deacetylation needed to consume NAD (Imai and Guarente, 2008; Schwer and Verdin, 2008; Yang et al., 2007)

Thus far, reliable and complete visfatin mRNA sequences of certain species have been reported, including *Suberites domuncula*, common carp, zebra fish, spotted green puffer fish, western clawed frog, chicken, house mouse, Norway rat, pig and human. However, the complete visfatin gene sequences were only determined in house mouse, Norway rat and human. It was not difficult to point out that the structural information of visfatin gene was far from enough, particularly in the fish. Silver Prussian carp (*Carassius auratus gibelio*) is classified in the order Cypriniformes, family Cyprinidae, and genus *Carassius*. In recent years, silver Prussian carp has become one of the most important aquaculture species in freshwater regions, and has been a new model organism in aquatic animals (Gui et al., 2007). Therefore, we aimed to clone the silver Prussian carp visfatin cDNA sequence, analyze its genomic organization and detect its tissue distribution in this study, with the hope of enriching the study of visfatin and contributing to a better understanding of the molecular evolution of visfatin family in both lower vertebrate and mammals as a whole.

MATERIALS AND METHODS

Animals and sample collection

For cDNA cloning and analysis of mRNA expression, female silver Prussian carps with body weight 350 ± 50 g, were purchased from local aquatic products market and then reared in indoor tanks under the natural photoperiod and water temperature. Fishes were anesthetized with phenobarbital (10 mg/L) before sampling. Ten tissues (gill, brain, heart, spleen, liver, head kidney, intestine, ovary, mesenteric fat and muscle) were rapidly collected on ice and frozen in liquid nitrogen immediately, and then stored at -80°C until assayed.

RNA extraction and cDNA synthesis

Total RNAs were isolated from silver Prussian carp tissues by TRIzol reagent (Invitrogen, USA), and then digested with RNase-free DNase I (Takara, Japan) to remove contaminating genomic DNA. The first strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Japan) in a 50 µl reaction mixture according to the manufacture's instruction. In brief, a mixture of 2 µg total RNA, [5 µl oligo(dT)] was incubated at 72°C for 5 min to break the RNA secondary structure. The mixture was then chilled on ice for at least 2 min and then 10 µl 5X RT buffer, 5 µl dNTPs (10 mM each), 50 U RNase inhibitor and 250 U ReverTra Ace reverse transcriptase were added for a total volume of 50 µl. The RT mix was incubated at 42°C for 60 min. Finally, the reverse transcriptase was inactivated by 5 min incubation at 99°C.

Isolation of full-length cDNA of silver Prussian carp visfatin

In an attempt to isolate the sequence that encoded silver Prussian carp visfatin, common carp mRNA sequence of visfatin (GenBank Accession no. AB027712) was compared to all complete visfatin mRNA sequences available in the GenBank database using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Based on the highly conserved segments, gene- specific primer pairs (V1 and V2, Table 1) were designed to amplify the partial cDNA fragment of silver Prussian carp visfatin. The cycling conditions consisted of 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 7 min.

To obtain the full-length cDNA sequence, 3′-RACE was employed to amplify silver Prussian carp visfatin 3'-end. Total RNA isolated from the silver Prussian carp liver served as template to generate first strand cDNA using 3′-Full RACE Core Set Ver.2.0 (Takara, Japan) following the manufacturer's instructions. Briefly, 3′ RACE Outer Primer (V3 Table 1) and an antisense specific primer (V4 Table 1) were used to amplify the first-strand cDNA. The nested PCR was performed using 3' RACE Inner Primer (V5 Table 1) and a nested specific antisense primer (V6 Table 1). The procedures of the two rounds of PCR were identical: 95°C for 3 min, then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and the extension time was increased to 7 min in the last cycle.

The PCR products were separated by electrophoresis on 1.0% agarose gel and purified using a Gel Extraction Kit (Sangon, Shanghai, China). The purified products were subcloned into the pMD18-T vector (Takara, Japan) and sequenced by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd.

Sequence analysis

The homology of the visfatin nucleotide sequence in silver Prussian carp was confirmed with that in common carp using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), where all parameters were suggested as default. Multiple alignments of the visfatin coding sequences and deduced amino acid sequences were achieved by using the program of ClustalW (http:// www.ebi.ac.uk/Tools/msa/ clustalw2/) with the default settings, and the results were displayed by using the program of BoxShade Server (http://www.ch.embnet.org/software/BOX_form.html). A phylogenetic tree based on the deduced amino acid sequences of silver Prussian carp visfatin and other species was constructed by the MEGA4 software using the Neighbor-Joining method with 500 bootstraps.

Expression pattern analysis of the visfatin gene in silver Prussian carp

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was applied to determine the tissue distribution of silver Prussian carp visfatin mRNA. A visfatin-specific primer pairs (V7 and V8 Table 1) were designed to amplify a 382 bp fragment and

Table 1. The information of used primers in the study.

Primer	Sequence (5'-3')	Use
V1	AGTCAAGTCAAGGGGAAGATGGAGCAG	Partial amplification of visfatin
V2	GCGGAGTCAGTAGGCAGAAACATAGAACAG	
V3	TACCGTCGTTCCACTAGTGATTT	For 3'-RACE of visfatin
V4	TTAGCGTGTTTCCCATCAGC	
V5	CGCGGATCCTCCACTAGTGATTTCACTATAGG	
V6	CCGCTGTTCTATGTTTCTGCCTAC	
V7	ACATTACAAACAGTATCCACCCAACG	For semi-quantitative PCR of visfatin
V8	TGCCCAGTTAGTGAGCCAGTAGC	
V9	TCCACCCTACATCCGAGTCAT	For real-time PCR of visfatin
V10	CAGTCCGTTCGTCACCACAT	
S1	TTGGTTCTCGGGAGTGGG	For semi-quantitative PCR of 18S rRNA
S2	AGCGGGTCGGCATAGTTT	
S3	AGCGAAAGCATTTGCCAAG	For real-time PCR of 18S rRNA
S4	AGGTTTACGGTCGGAACTACG	
I01	TCGGGATCCCTCGCCGTGGACTTCAATTTCTTGTTAG	To amplify intron i
I02	CCCAAGCTTCCGTTTTGTCGTATTTGACTTTTCTGAGCT	
I03	CACATTACCAACAGTATCCACCCAACGC	To amplify intron ii
I04	GTTCCCTGTCCACCTCTTTTGCTTCCT	
I05	AGGGGAAGATTCAGGAAGCAAAAGAGGTGT	To amplify intron iii
I06	TTATCTGGATGGGCAGGTGACCGTTGT	
I07	TGAAGGCTGTGCCGGAGGGAAGCGTTAT	To amplify intron iv
I08	GCGTCGGTGATGGGGTACCAGGTCTGAA	
I09	TGGACGACTTCGGCTACA	To amplify intron v
I10	ACAGGAACTGGGTCTTTGG	
I11	TTCAAAGGAACAGACACCGTGGCCGGGATC	To amplify intron vi
I12	TCGGGGGCGTTGTAGATGTCGTAGCTGTCG	
I13	AGCACCACCACTGCCTGGGGAAAAGAC	To amplify intron vii
I14	CACCGCCACCCTGAATGACTCGGATGT	
I15	GTAACGGGCTTCCACCCTACATCCGAGTCATTC	To amplify intron viii
I16	GCAGCAAAGAACCTCCTGAGCCAAACGAGATGT	
I17	TTCTAAGTGTAGTTATGTGGTGACGAACGGAC	To amplify intron ix
I18	CCTTGCCCTCAACCAGAGTAACGAAATT	
I19	GTTACTAAGGAAGAGGGCAAGGGTGATC	To amplify intron x
I20	GCGGAGACAGTAGGCAGAAACATAGAAC	

the PCR parameters were a denaturation cycle of 95°C for 3 min, followed by 32 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 40 s, and by a final extension at 72°C for 7 min. An internal control PCR was performed in a separate tube, using two specific 18S rRNA (GenBank accession no. EF189737) primers (S1 and S2 Table 1). The PCR condition for 18S rRNA was similar to the visfatin PCR, except for annealing at 50°C instead of 59°C and using 28 cycles instead of 32 cycles. Negative control was included in which reverse transcriptase was omitted from the cDNA synthesis reactions.

The expression level of silver Prussian carp visfatin in ten different tissues was detected by SYBR Green real-time quantitative PCR on an ABI 7300 real-time PCR thermal cycle instrument (ABI, USA). Each real-time PCR reaction (in 20 µl) contained 10 µl SYBR® Green Real-time PCR Master (Toyobo, Japan), 0.2 µM of gene-specific primers (V9 and V10 Table 1), and 2 µl 1/20 diluted cDNA solution. The PCR amplification was 95°C initial denaturation for 60 s, then 40 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 45 s. The specificity of PCR products were confirmed by melting curve analysis. 18S rRNA was used in each sample in order to standardize the results by eliminating variations in mRNA and cDNA quantity and quality. The quantitative results were expressed as the ratio of target gene/18S rRNA using Gene Expression Macro software (ABI, USA) by employing an optimized comparative Ct (ΔΔCt) value method (Livak and Schmittgen, 2001). Expression levels were considered not detectable when the Ct value of the target gene exceeded 35 in the sample tissue.

Splicing of the complete visfatin gene in silver Prussian carp

With the purpose of achieving the complete sequence, the identified encoding sequence of silver Prussian carp visfatin was compared to *Homo sapiens* chromosome 7 genomic contig (GenBank accession no. NW_923640) and *Mus musculus* chromosome 12 genomic contig (GenBank accession no. NW_001030500), which

contained the complete sequence of human and mouse visfatin. Then, it was divided into 11 segments (putative exon 1 to 11). On the basis of these 11 fragments, ten new primer pairs (I01 to I20 Table 1) were designed to amplify the sequences between every two neighboring exons (so-called intron I to X) in turn.

The genomic DNA served as template was isolated from the silver Prussian carp fin rays in accord with Aljannbi and Martinez (1997) description, and then was checked in advance by electrophoresis to escape from degradation. The thermal profiles were carried out as follow: 95°C initial denaturation for 5 min, 35 cycles of 94°C denaturation for 30 s, different annealing temperature consistent with different primer pairs for 40 s, and 72°C extension for different time according to different intron length. The final extension was performed at 72°C for 10 min. The products were analyzed by electrophoresis on 1.5% agarose gel. The appearing bands were excised, purified, subcloned into the pMD18-T vector (Takara, Japan), and sequenced by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. All the doubtless non-coding segments were spliced with encoding fragments to compose the whole genomic sequence of silver Prussian carp visfatin eventually.

Statistical analysis

Quantitative results were presented as means and standard error of the mean (SEM). Statistical differences were estimated by one-way ANOVA followed by Tukey's post hoc test, a p value <0.01 was considered significant. All statistical analyses were performed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Molecular cloning and characterization of the silver Prussian carp visfatin

A cDNA fragment, approximately 1600 bp in length, was amplified by the initial RT-PCR from total RNA isolated from the liver of silver Prussian carp using the specific primers V1 and V2. Through 3′-RACE experiment, products of approximately 400 bp were yielded. Consequently, a 1990 bp length silver Prussian carp visfatin cDNA was obtained by assembling the overlapping PCR fragments. The silver Prussian carp visfatin cDNA consisted of a 12 bp 5'-untranslated region (UTR), a 1482 bp open reading frame (ORF) that encoded a protein of 493 amino acid residues, and a 496 bp 3'-UTR which contained a consensus polyadenylation signal sequence (AATAAA) (Figure 1).

The amino acid sequence deduced from silver Prussian carp visfatin cDNA had a molecular mass of approximately 55.95 kDa, and its isoelectric point (pI) was about 6.69 as calculated by Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Corresponding with other species, silver Prussian carp visfatin also lacked a signal peptide which was confirmed by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). In silver Prussian carp visfatin, there were six cysteine residues which could help to form disulfide bonds, one more than in common carp (Fujiki et al., 2000); three N-glycosylation sites were identified by using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/ services/NetNGlyc/), two of which had high similarity with human and common carp visfatin (Fujiki et al., 2000) and just one tyrosine sulfation site was determined by SulfoSite (http://sulfosite.mbc. nctu.edu.tw/); one less than porcine visfatin (Chen et al., 2007).

Alignment and phylogenetic tree analysis of silver Prussian carp visfatin with other sources

Comparison of the coding sequence of visfatin between silver Prussian carp and common carp showed high similarity (up to 94%), which strongly indicated that this sequence cloned from silver Prussian carp was a homologue of common carp visfatin. Henceforth, the encoding sequence and deduced amino acid sequence of silver Prussian carp visfatin was aligned to other sources. The encoding sequence comparison exhibited that the silver Prussian carp visfatin shared 79% identities with green spotted puffer fish, 74% with chicken, 73% with rat, 72% with house mouse, 71% with western clawed frog, 71% with porcine, 71% with human, and 69% with zebra fish. The amino acid sequences comparison displayed that silver Prussian carp visfatin shared 95% identities with common carp, 91% with green spotted puffer fish, 86% with western clawed frog, 84% with chicken, 84% with house mouse, 84% with rat, 84% with porcine, 84% with human and only 58% with zebra fish (Figure 2).

The phylogenetic tree analysis revealed that visfatin was remarkably conserved from the point of molecular evolution in vertebrates, and silver Prussian carp visfatin had a closer relationship with common carp and green spotted puffer fish than other species (Figure 3).

Tissue distribution of visfatin gene in silver Prussian carp

Semi-quantitative RT-PCR analysis was performed to examine the relative mRNA levels of silver Prussian carp visfatin in ten different tissues including liver, head kidney, spleen, mesenteric adipose, muscle, heart, intestine, ovary, gill and brain. The results suggest that visfatin gene was expressed widely in silver Prussian carp (Figure 4). SYBR Green real-time RT-PCR analysis further confirmed that the mRNA levels of visfatin were abundant in these ten tissues, and the levels were observed higher in mesenteric adipose, heart, ovary and gill than any other tissues detected in this study (Figure 5).

Genomic sequence of visfatin in silver Prussian carp

Through amplification of non-coding sequences using specific primers I01 to I20, 9 intron sequences were

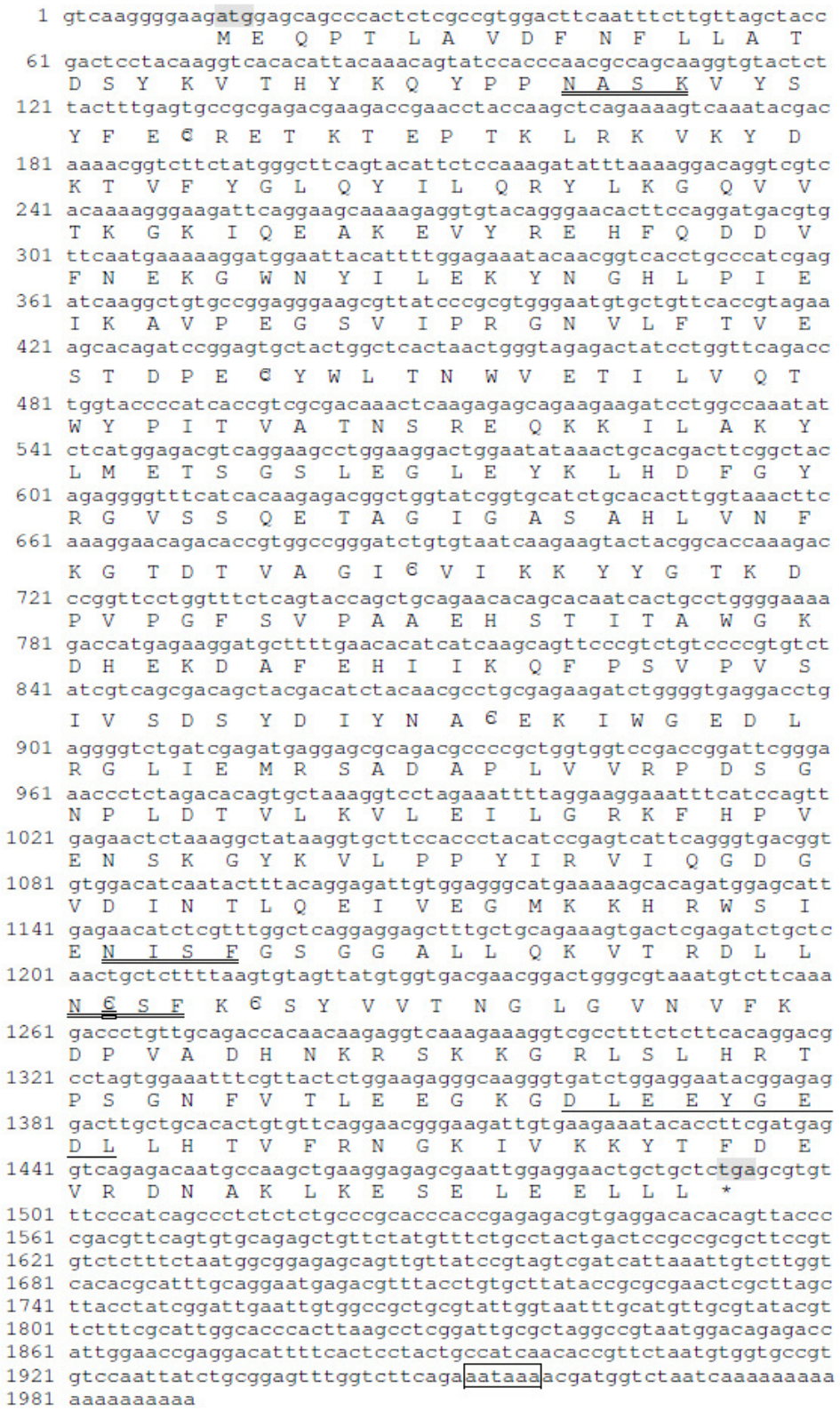

Figure 1. The nucleotide sequence and the deduced amino acid sequence of silver Prussian carp visfatin. The nucleotide sequence is in lowercase, and the deduced amino sequence is in uppercase. The initiation condon and termination codon are shaded in grey. The polyadenylation signal is boxed. The cysteine residues are circled. Double underlining indicates N-glycosylation sites. The tyrosine sulfation site is underlined. The coding sequence has been submitted to GenBank under Accession No.EU253480.

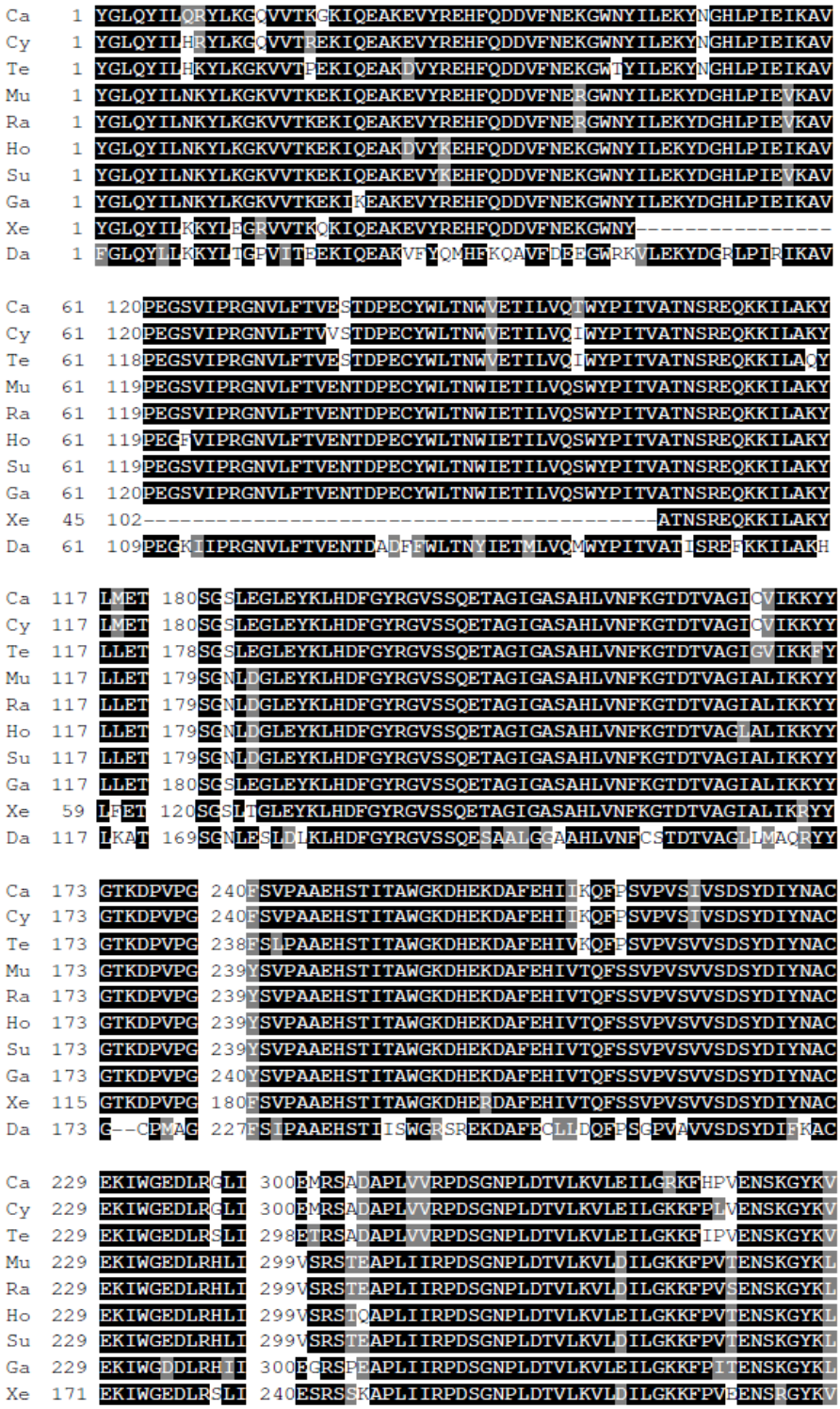

Figure 2. Multiple alignments of amino acid sequences of silver Prussian carp visfatin and several vertebrate species. The amino acid sequences used for comparison were extracted from Genbank Database and their accession numbers are: Cy, common carp, *Cyprinus carpio* (AB027712); Te, green spotted puffer fish, *Tetraodon nigroviridis* (DQ002887); Mu, mouse, *Mus musculus* (NM_021524); Ra, rat, *Rattus norvegicus* (NM_177928); Ho, human, *Homo sapiens* (NM_005746); Su, porcine, *Sus scrofa* (DQ001974); Ga, chicken, *Gallus gallus* (AY946242); Xe, western clawed frog, *Xenopus (Silurana) tropicalis* (NM_001011388); Da, zebra fish, *Danio rerio* (NM_212668). Ca means silver Prussian carp, *Carassius auratus gibelio*.

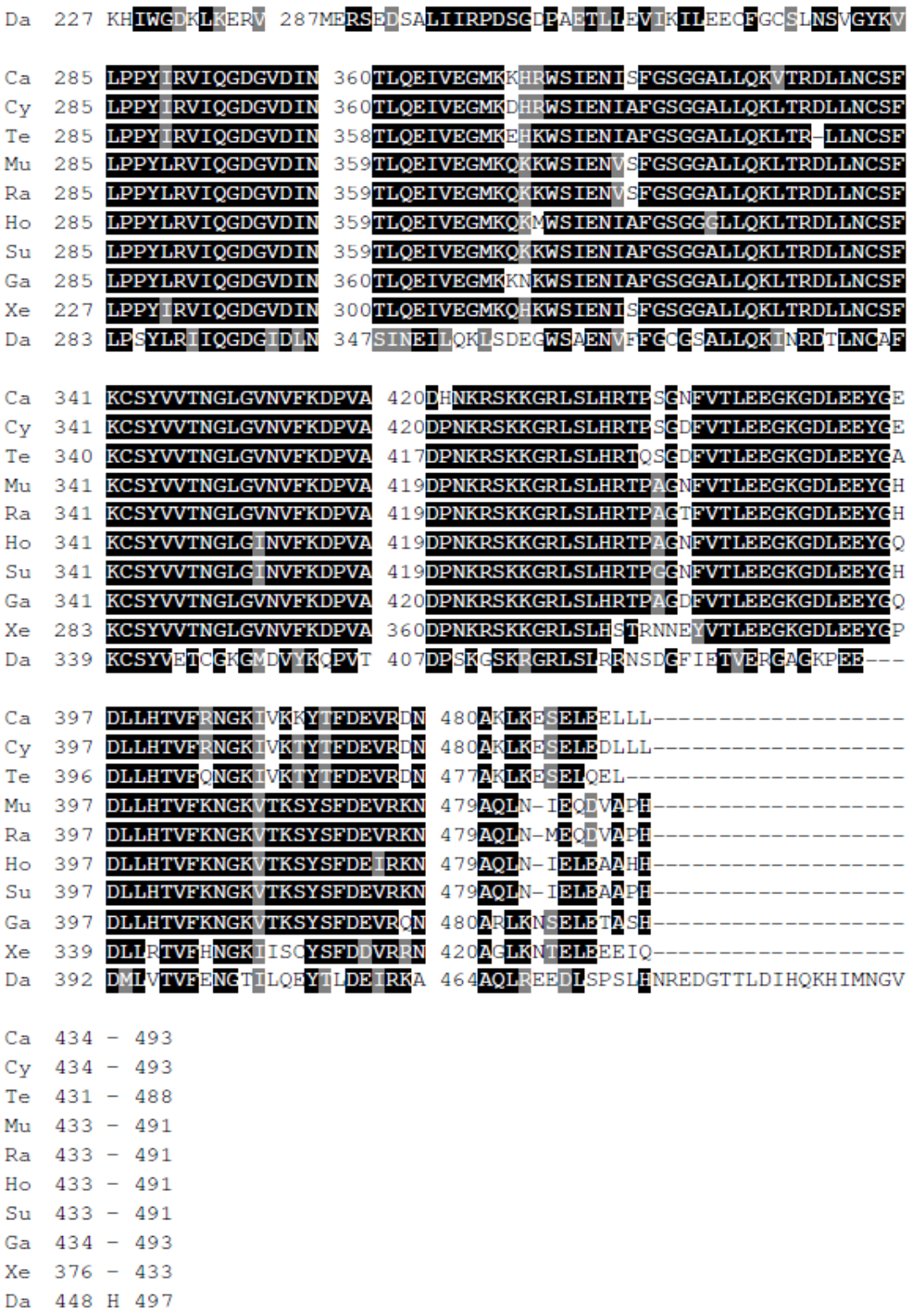

Figure 2. Contd.

obtained. By splicing exons and introns fragments, the entire genomic sequences of silver Prussian carp visfatin were acquired. These sequences had been deposited in GenBank under the accession numbers GQ351348 and

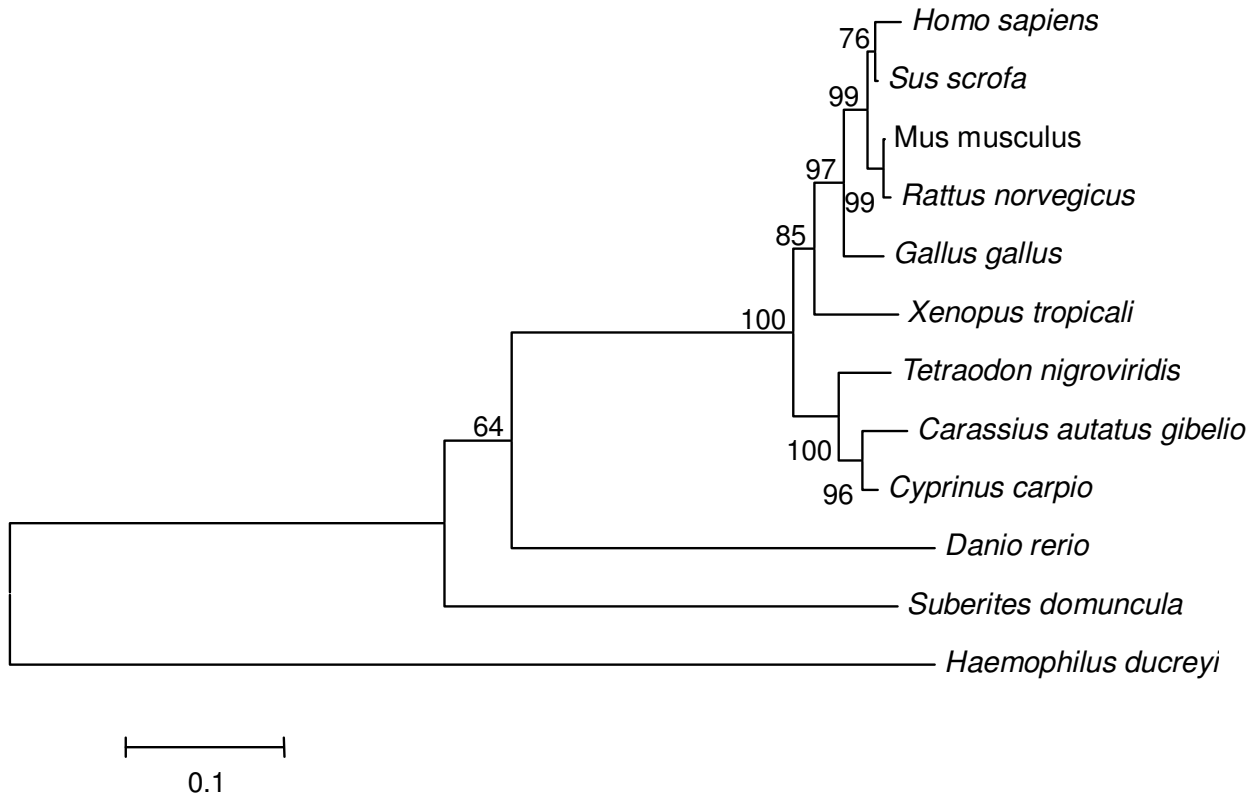

Figure 3. Phylogenetic analysis of visfatin amino acid sequences. The tree was generated by the Neighbor-Joining method with 500 bootstraps. The scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages. The Genbank accession numbers of the sequences used for analysis are as follows: *Homo sapiens* (NM_005746); *Sus scrofa* (DQ001974); *Mus musculus* (NM_021524); *Rattus norvegicus* (NM_177928); *Gallus gallus* (AY946242); *Xenopus tropicalis* (NM_001011388); *Tetraodon nigroviridis* (DQ002887); *Cyprinus carpio* (AB027712); *Danio rerio* (NM_212668); *Suberites domuncula* (Y18901); *Haemophilus ducreyi* (AF273842).

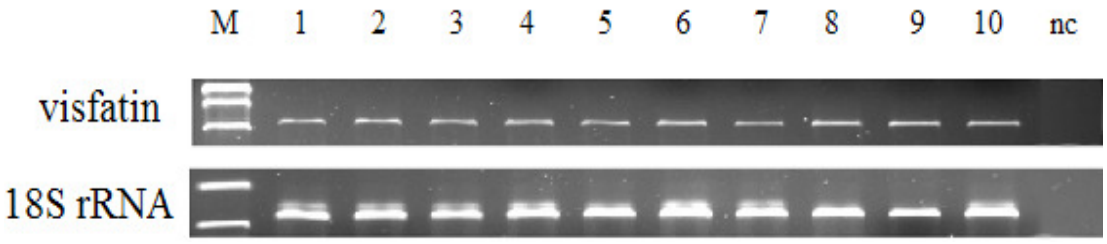

Figure 4. Silver Prussian carp visfatin expression profiles in different tissues obtained by semi-quantitative PCR. M, Marker; 1, liver; 2, head kidney; 3, spleen; 4, mesenteric fat; 5, muscle; 6, heart; 7, intestine; 8, ovary; 9, gill; 10, brain; nc, negative control.

GQ351349. The complete sequences of silver Prussian carp visfatin were approximately 10 Kb and composed of 11 exons and 9 introns (Figure 6). There was no great difference in the length of exons. The nucleotide sequences varied from 60 to 224 bp and the average length was about 135 bp. However, the distinction was absolutely more obvious among different introns. The longest intron was 4472 bp (Intron I), whereas the shortest intron was 81 bp (Intron VI variant 2). All the splice junction sequences of the introns conformed to the GT-AG rule (Mount, 1982) (Table 2). Although no sequence was located between Exon 4 and 5, the GT-AG sequence was still found at the boundary of Exon 4 and 5. Therefore, it was still considered that there were 11 exons in silver Prussian carp visfatin gene. Moreover, another phenomena needed to point out was that Intron VI possessed two variants, variant 1 (190 bp) had 109 nucleotides more than variant 2 (81 bp), and they were

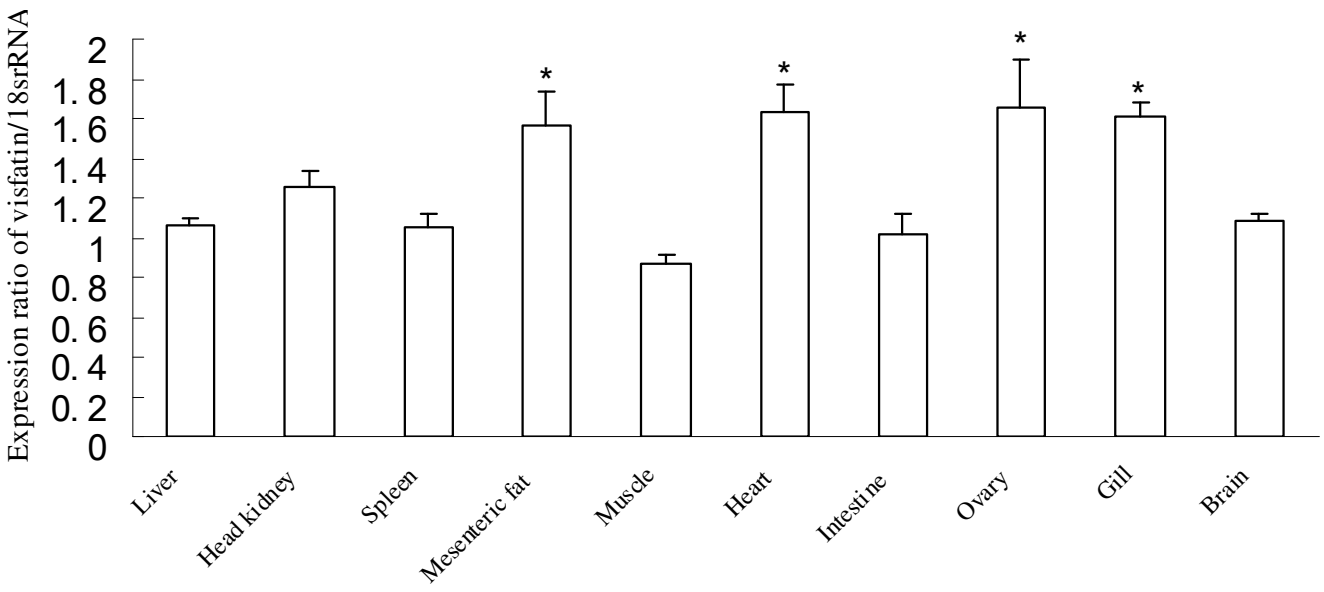

Figure 5. Expression patterns of silver Prussian carp visfatin in different tissues by real-time RT-PCR. The mRNA levels ratio of visfatin/18S rRNA in the intestine is regarded as 1. Data is represented as means ± SEM (n=3). *Significantly differs from the intestine tissue at $p < 0.01$.

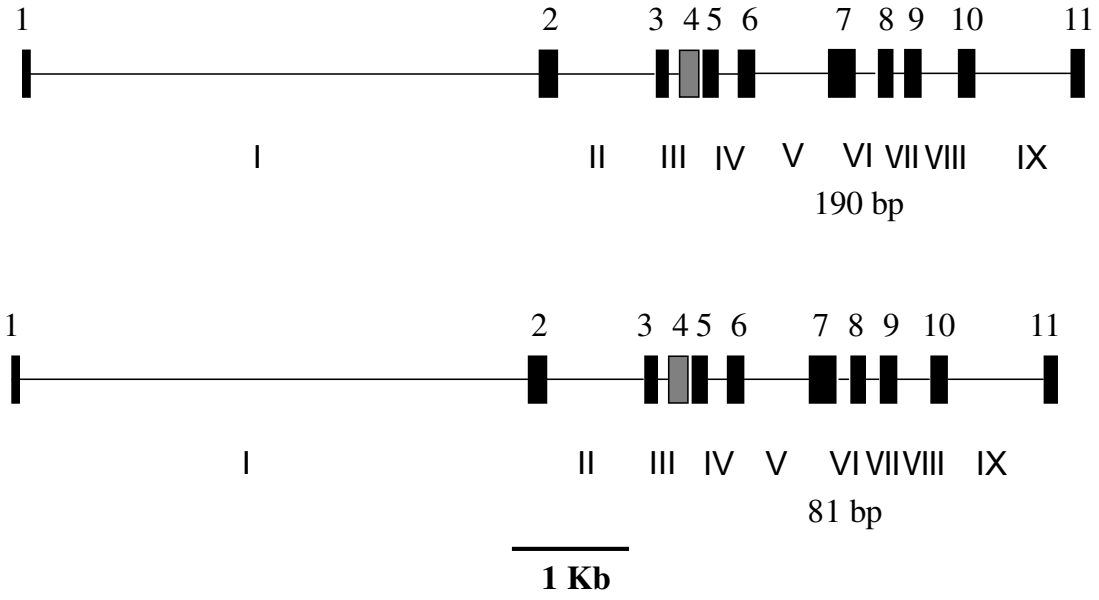

Figure 6. The complete genomic structure of visfatin in silver Prussian carp. Boxes indicate exons, and Arabic numerals 1 to 11 represent Exon 1 to 11. Lines indicate introns, and Roman numerals I to IX represent Intron I to IX. The different length of Intron VI is noted under "VI".

just analogical at the last 40 nucleotides (Figure 7).

DISCUSSION

Previous reports mainly focused on the characterization of mammalian visfatin. Only a few literatures have recorded the nucleotide fragments or amino acid fragments of teleost visfatin.

In this study, the cDNA sequence and its deduced amino acid sequence of silver Prussian carp visfatin were identified. Analysis of amino acid sequence showed that

Table 2. Exon-intron junctions of the silver Prussian carp visfatin gene.

Exon			Intron				Exon	
Number	**Size (bp)**	**3' junction**	**5' splice donor**	**Number**	**Size (bp)**	**3' splice acceptor**	**5' junction**	**Number**
1	60	…TCCTACAAG	gtgagcagag...	1	4472	…ttcttgcag	GTCACACAT…	2
2	157	…ATTTAAAAG	gtatgtagg...	2	880	…cgttcacag	GACAGGTCG…	3
3	104	…ATTTTGGAG	gtatggaga…	3	86	…tcactgcag	AAATACAAC…	4
4	125	…TAACTGGGT					AGAGACTAT…	5
5	163	…TCACAAGAG	gtgacattt…	4	248	..ccgtttcag	ACGGCTGGT…	6
6	137	..AGAACACAG	gtgcaacaa…	5	627	…gtgttgcag	CACAATCAC…	7
7	224	…CAGTGCTAA	gtaagcact/gtagtcact…	6	190/81	…attttccag	AGGTCCTAG…	8
8	121	…TTTACAGCA	gtgtgtatg…	7	100	…ggtatacag	GATTGTGGA…	9
9	141	…CGGACTGGG	gtaagtgtc…	8	317	…taatttcag	CGTAAATGT…	10
10	136	…TACGGAGAG	gtacatgac…	9	812	…ccctcgcag	GACTTGCTG…	11
11	114							

silver Prussian carp visfatin contained several essential amino acid residues and regions. Residues Tyr98, Tyr188 and Ser398 were necessary for phosphorylation modification in human visfatin, and mutations at Asp219, His247 and Arg311 could reduce activity towards nicotinamide (Khan et al., 2006). Furthermore, two nicotinamide ribonucleotide binding domains in human visfatin had been mapped to residues 311 to 313 and 353 to 354. According to Kim et al. (2006), rat visfatin appeared as a homodimer to be activated, and substrates bound at or close to the interface between the two subunits. Some highly conserved amino acid residues were located within the active site. They were Asp16, Tyr18, Phe193, Asp219, Arg311, Aap313, Gly353, Asp354 and Gly384. All of the residues and regions were well-conserved in silver Prussian carp visfatin (amino acid residues Tyr99, Tyr189, Ser399, Asp220, His248, Arg312, Asp17, Tyr19, Phe194, Aap314, Gly354, Asp355, Gly385 and regions 312-314, 354-355). However, the positions of these residues were shifted one locus backwards. By comparing the 3D structure of silver Prussian carp visfatin with human and rat using the SWISS-MODLE programme (http:// swissmodel.expasy.org/), it was found that this translocation did not affect the spatial structure a lot, since they had a similar constitution of helixes, strands and turns. This approved that these functional sites were likely to be evolutionarily conserved and the silver Prussian carp visfatin may have a same or similar way of activation.

The amino acid sequence of silver Prussian carp visfatin was also aligned with other published sequences. Through phylogenetic analysis, it could be referred that silver Prussian carp visfatin had a most proximal relationship with common carp, as they were located in the same group. Even though silver Prussian carp visfatin was classified into a different group, it still gained high scores by comparing with other teleosts, amphibians, birds and mammalians, indicating that visfatin protein was probably conservative during the molecular evolution. Furthermore, to assist in comprehending the structure formation, the complete sequence of silver Prussian carp visfatin was cloned and characterized. The composition of exons was almost in conformity with human and house mouse visfatin gene, whereas introns varied a lot. Every intron in silver Prussian carp visfatin gene was less than half the length of homologous intron in human or house mouse, resulting in the overall length of silver Prussian carp visfatin much shorter than human or mouse visfatin. That no sequence existed between Exon 4 and 5 and two variants of Intron VI also made silver Prussian carp visfatin distinguishable from human or mouse visfatin. In view of this, it was simple to conclude that from silver Prussian carp to mouse and human, visfatin gene became more and more complex. Introns had no longer been considered as junk DNA, and they had many functions including for regulation and structural purposes (Bergman, 2001). The complication of visfatin gene (or introns in visfatin gene) may give us some implications that introns may do a favor in structure and composition.

In addition, on the basis of broad expression in porcine and human, ten tissues involving liver, spleen, head kidney, mesenteric adipose, muscle, heart, intestine, ovary, gill and brain, which played

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Variant 1    GTAAGCACTGTTGCATCAAAAAGCATATCAGCTGTGGCTGGCAGTTGATTAATCATGCTT 60
Variant 2    ------------------------------------------------------------


Variant 1    TAATATTATATTTTTTTCATGATATGGTTGTGTTTTTTTTTTTTTTACGCAGCTCGTCTCA 120
Variant 2    -----------------------------------------------GTAGTCACT 9

* *** *

Variant 1    AATTCTTCCT--TCGTTTCAAAGCATAGAGTGATATGCAT-GTCCATTAAGTCTATTAAG 177
Variant 2    ATTGCTGATTGATTATTCTAGAATTTTATAT-ATATGCATAGTCCATTAAGTCTATTAAA 68
                * * **  *   *  **   * *   *     * ******** ******************

Variant 1    TCTTATTTTCCAG 190
Variant 2    TCTTATTTTCCAG 81
               ++++++++++++
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Figure 7. Alignment of two variants nucleotide sequences of Intron VI in silver Prussian carp visfatin gene by using the ClustalW program. The symbols (*) represented completely identical nucleic acid bases.

significant parts in physiological activities of fish, were chosen to test the mRNA levels of silver Prussian carp visfatin and it was revealed that the transcripts could be detected in all these tissues. This ubiquitous distribution showed that silver Prussian carp visfatin may have a fundamental role in a great many of basic physiologic and pathological activities as well as human or porcine visfatin did. Nevertheless, transcriptional levels were a bit higher in mesenteric fat, heart, ovary and gill tissue. High level in mesenteric fat was in accord with human or mouse visfatin that was predominantly expressed in visceral fat (Fukuhara et al., 2005). At the same time, high levels in heart, ovary and gill may intimate silver Prussian carp visfatin functioned differently from other species. For human and porcine visfatin, Samal et al. (1994) and Palin et al. (2008) discovered three and six different variants generated by alternative splicing, respectively. And one of porcine visfatin variants had a little difference in expression profiles from others (Chen et al., 2007). However, no variant was detected in the transcriptional process of silver Prussian carp visfatin, and only one kind of transcripts could be found in all the ten tissues. Moreover, Ognjanovic et al. (2001) identified that differential transcripts may also partially be attributed to the alternative use of two promoters in human visfatin. Therefore, whether one visfatin transcript is ascribed to the change of promoter or a different splicing mechanism in silver Prussian carp needs to be further researched.

In summary, we isolated and characterized the silver Prussian carp visfatin gene. The cDNA formation and genomic organization presented in this study make a necessary complement to visfatin genetic information in teleosts even in vertebrates and will potentially lead to a better understanding of visfatin evolutional development. Conservative constitution and broad distribution provide a structural and expression basis for future studies on silver Prussian carp visfatin function, and the similarities and differences of silver Prussian carp visfatin with other sources visfatin may shed more light on investigating silver Prussian carp visfatin comprehensively.

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