Research Article

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Genome-wide identification, phylogeny and expression analysis of *G6PC* gene family in common carp, *Cyprinus carpio*

G6PC gen ailesinin sazan, *Cyprinus carpio*, balığındaki filogenisinin ve ekspresyonunun analizi

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Abstract

Objective: The Glucose 6-phosphatase (G6Pase) catalytic subunit (G6PC) catalyzes glucose 6-phosphate (G6P) to inorganic phosphate and glucose, playing a critical role in endogenous energy supply. Here, the *G6PC* gene family was investigated and characterized in common carp (*Cyprinus carpio*).

Methods: Sequence alignment and phylogenetic analysis were performed using MEGA5. The HMM profiles, motif structure were analyzed using Pfam and MEME, respectively. Quantitative real-time PCR was used to test the expression profiles.

Results: Four assumptive members of *G6PC* family in common carp whole-genome sequence were identified

as *cg6pca.1*, *cg6pca.2a*, *cg6pca.2b* and *cg6pcb* which were classified into *g6pca* and *g6pcb* subtypes, respectively. Evolutionary analysis revealed that *cg6pca.2a* and *cg6pca.2b* have a closer evolutionary relationship, and the same subtype members have higher homology among different species. A classical PAP2-glucose phosphates domain is found in four genes and were highly conserved. The expression patterns revealed that only *cg6pca.2a* elevated significantly after 12 and 24 h of both starvation and cold treatment ($p < 0.05$).

Conclusions: This study performed a comprehensive analysis of *G6PC* gene family in common carp. Moreover, *cg6pca.2* may be the major functional gene in cold and fasting stress. And the transactors, PLAG1 and Sox8, may be concerned with expression regulation of *cg6pca.2*.

Keywords: Abiotic stress; Common carp; *G6PC* gene family; Phylogenetic analysis; Transcriptional levels.

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Öz

Amaç: Glikoz 6-fosfatın (G6Pase) katalitik alt birimi (G6PC), glikoz 6-fosfat (G6P)'ı inorganik fosfat ve glukoz katalize ederek endojen enerji kaynağında kritik bir rol oynar. Buradaki G6PC gen ailesi, sazanlarda (*Cyprinus carpio*) araştırılmış ve karakterize edilmiştir.

Yöntemler: Dizileme ve filogenetik analiz MEGA5 kullanılarak yapılmıştır. HMM profilleri, motif yapısı sırasıyla Pfam ve MEME kullanılarak analiz edilmiştir. Ekspresyon profillerini test etmek için kantitatif gerçek zamanlı PCR kullanılmıştır.

Bulgular: Sazanların tüm genom dizisinde G6PC ailesinin dört varsayım üyesi, sırasıyla *g6pca* ve *g6pcb* alt tipleri olarak sınıflandırılan *cg6pca.1*, *cg6pca.2a*, *cg6pca.2b* ve *cg6pcb* olarak tanımlanmaktadır. Evrimsel analizler, *cg6pca.2a* ve *cg6pca.2b*'nin daha evrimsel bir ilişkisi

sahip olduğunu ve aynı alt tipteki üyelerin farklı türler arasında daha yüksek homolojiye sahip olduğunu ortaya çıkarmıştır. Dört gende klasik bir PAP2-glukoz fosfat bölgesi bulunur ve bu yüksek oranda korunmuştur. Ekspresyon paternleri, hem *cg6pca.2a*'nın hem açlık hem de soğuk tedavisinden 12 ve 24 saat sonra anlamlı derecede yükseldiğini ortaya koymuştur ($p < 0.05$).

Sonuçlar: Bu çalışma, sazanlarda *G6PC* gen ailesinin kapsamlı bir analizini gerçekleştirmiştir. Ayrıca, *cg6pca.2*, soğuk ve açlık stresinde temel fonksiyonel gen olabilir. *PLAG1* ve *Sox8* transfaktörleri, *cg6pca.2* ekspresyon regülasyonu ile ilgili olabilir.

Anahtar Kelimeler: Abiyotik stres; Ortak sazan; *G6PC* gen ailesi; Filogenetik analiz; Transkripsiyon seviyeleri.

Introduction

Glucose 6-phosphate (*G6Pase*, EC 3.1.3.9) is an important enzyme in the hepatic glucose production, catalysing glucose-6-phosphate (glu-6P) to glucose, which is then released into blood [1]. *G6Pase* catalysis (*G6PC*) carry out the last step in glycogenolysis and gluconeogenesis, and thus plays a crucial role in blood glucose homeostasis [2, 3]. At present three *G6PC* members were identified in *Homo sapiens* and *Danio rerio* [4], while only one member was identified in *Branchiostoma japonicum* [5].

In teleost, the regulation of glucose homeostasis is a complicated and species-specific process, opposite to mammals, in which blood glucose maintains at a stable level. The strictly carnivorous rainbow trout has a “glucose-intolerant” character testified by sustaining hyperglycemia when fed with a diet high in carbohydrate since the lack of molecular inhibition of gluconeogenic enzyme. Oppositely, common carp, an omnivorous fish, does not show postprandial hyperglycemia [6]. Marandel et al. [7] found that *g6pcb2* may play a part in glucose intolerance of trout and may partly interpret its inefficiency of carbohydrate utilization. However, there are few studies about the phylogenetic relationships and functions of the *G6PC* genes in common carp.

Common carp (*Cyprinus carpio*) is not only the oldest, most domesticated commercial fishes in the world, but also is highly suitable for fundamental research for its taxonomic status and appropriate body size for obtaining sufficient tissue material for advanced studies [8]. While majority studies about *G6Pase* in common carp have been well documented when it comes to enzyme kinetics, this enzyme system has never been lucubrated from

the respective of whole genome. Fortunately, the genome assembly of common carp published in 2014 [9] provides a prerequisite to studies of *G6PC* gene family in whole genome.

The purposes of this study, taking common carp hepatopancreas gland (the main gluconeogenic tissue) as object, were firstly to characterize the phylogenetic relationships, physicochemical properties, HMM profiles, motif structures in whole genome. Moreover, given the fact that abiotic stress, such as low temperature, can influence the synthesis of glucose in fish [10], expression profiles of *cg6pca.1*, *cg6pca.2a*, *cg6pca.2b*, and *cg6pcb* in common carp under cold or fasting conditions were analyzed using real-time quantitative PCR.

Materials and methods

Sequence information

The nucleotide and protein sequences of common carp genome assembly V1.0 and annotation file were extracted from the European Nucleotide Archive (ENA, PRJE7241) (<https://www.ebi.ac.uk/ena/data/view/PRJE7241>) and the common carp database (<http://www.carpbase.org/>), respectively. The upstream sequences of the target genes were downloaded from the common carp genome via an in-house perl program. The amino acid sequences of zebrafish (*Danio rerio*) genome assembly (GRCz10, GCA_000002035.3) were extracted from Ensembl (ftp://ftp.ensembl.org/pub/release-90/fasta/danio_rerio/dna/). Other species' protein sequences were obtained from the NCBI protein databases (<http://www.ncbi.nlm.nih.gov/protein>).

Sequence alignment and phylogenetic analysis

The multiple alignment of DNA or protein sequences was conducted with ClustalW (Belfield, Dublin, Ireland) [11], and gene phylogeny were analyzed by MEGA5 software (Hachioji, Tokyo, Japan) [12]. The phylogenetic trees, based on protein sequences, were constructed by the Neighbor-Joining method. The bootstrap method was used to test the reliability of the inferred trees with 1000 replications. Percent identity matrix of paralogous teleost sequences were calculated using the MUSCLE program.

Physicochemical properties and motifs analysis

The biophysical and biochemical parameters of the proteins belonging to common carp *G6PC* family were conducted using GPMW program (Cambridge, MA, USA) [13]. Hidden Markov Model (HMM) profiles were analyzed using the Pfam program v27.0 (St. Louis, MO, USA) (<http://pfam.xfam.org>). The novel protein motif analysis was determined using MEME Suite v4.10 (Seattle, WA, USA) [14], and visual procedures were conducted by TBtools (Guangzhou, Guangdong, China) (<https://github.com/CJ-Chen/TBtools>).

Starvation or cold stress

For the starvation or cold stress, 270 juvenile common carps weighing 66.73 ± 6.16 g that acclimated to 24°C (the temperature of control) were sampled and randomized to three groups equally (30 individuals per group): 24°C control group, 24°C starvation stress group and 4°C stress group. In the starvation test, none food was supplied during the test period while the temperature was kept to 24°C. While, in the cold stress, the reduction of water temperature from 24°C to 4°C for the experimental groups was finished in 1 h by a refrigerated cooler and ice block in an air-conditioning room (Gree, Hefei, Anhui, China) and the same food (30% protein, 8.0% total fat, 8.0% fiber, supplied by a commercial mixed feed, Yangzhou, Jiangsu, China) was supplied similar to control group. The control temperature was maintained at 24°C. During the course of the experiments, oxygen supplies was adequately given to the cold stress, starvation and control group. The sampling started when the water temperature of the cold-stress group reached at 4°C. The hepatic tissues of three fishes were collected in starvation or cold stress and the

control groups were stored at liquid nitrogen until analyses of the transcript levels (Yangzhou, Jiangsu, China), with the time intervals of 0, 12, 24, 36 and 48 h. Every treatment was repeated three times.

Real-time quantitative PCR (qRT-PCR)

Total RNA was isolated from hepatopancreas gland using the RNAeasy Fibrous Tissue Mini-kit (Qiagen, Duesseldorf, Germany) following the instruction manual. Quality and integrity of mRNA were verified spectrophotometrically and by agarose gel electrophoresis. The cDNA was synthesized by PrimeScript®II 1st Strand cDNA Synthesis Kit (Takara, Dalian, Shandong, China) in a 20 µL reaction system with 5 µg total RNA using an iCycler (Bio-Rad, Hercules, CA, USA) programmed at 42°C for 60 min, and 95°C for 5 min. The real-time PCR reaction contained 1× Power SYBR Green Premix Ex Taq (Takara, Dalian, Shandong, China), 0.3 µM of each forward and reverse primer and 5 ng of cDNA template. Real-time qPCR was conducted in a Light Cycler 480® real-time qPCR system (Roche, Basel, Basel-Stadt, Switzerland) with the conditions: 3 min at 94°C, followed by 45 cycles of 10 s at 94°C, 20 s at 58°C. Melting curve assay was done to verify the primers specificity. Every sample was run in triplicate, and each assay was set three repetitions. When the procedure was completed, the threshold cycle (Ct) values were obtained from each reaction. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantity [15]. According to previous researches [16, 17] and the preliminary experiment, *actin-β* was determined as the housekeeping control genes for the normalization of target genes. The specific primers showed in Table 1 were designed using Oligo v7.0 software (Vondelpark Colorado Springs, CO, USA) (Applied Biosystems) and Primer Premier 6.0 (PREMIER Biosoft, Palo Alto, CA, USA).

Table 1: The sequences of primers used in this experiment.

Target gene	Primer sequence(5'-3')	Product length (bp)	Gene ID
<i>actin-β</i>	F:GAAGAGTTACGAGCTGCCTGA R:CATGGATACCGCAAGATTCC	106	M24113(GeneBank ID)
<i>cg6pca.1</i>	F:AGCCTGGCAGGCTGCCTTCA R:AGTCCAGGTACTCCGTCTGCA	171	cycg043353
<i>cg6pca.2a</i>	F:CATCCAACATTGACTGCTTAG R:GTAACCTGCTGCCACAAT	125	cycg044960
<i>cg6pca.2b</i>	F:ATCACTCTTGACCACT R:TCAGATCTGCCGCAA	170	cycg043354
<i>cg6pcb</i>	F:GACCTGCGCACAACA R:ACCAATAAGACGCTCGC	166	cycg044064

Statistical analyses

R statistical software (Version 3.1.3) (Auckland, Auckland Region, New Zealand) was used for statistical analysis. Before statistics and analyzing the information, the data were log transformed as to maintain assumptions of normality as determined by a Shapiro-Wilk's test. Duncan's multiple test was conducted to test the significance of differences in the time-course measurements at each sampling times ($p < 0.05$). The data are presented as mean $\pm$ SEM. The diagrams were generated by R and OriginPro 2015 (Origin Lab, Northampton, MA, USA).

Results and discussion

Phylogenetic relationships of common carp G6PC gene family

G6PC gene family has been identified in many species including both invertebrates and vertebrates [18–20].

By analyzing common carp genome, we identified four paralogs sharing high sequence homology with the zebrafish (*Danio rerio*) orthologs, namely *cg6pca.1*, *cg6pca.2a*, *cg6pca.2b*, and *cg6pcb* classed into *g6pca* and *g6pcb* subtypes, respectively (Figure 1A), encoding 354, 376, 252 and 353 amino acids, respectively (Table 2). In mammals, the G6PC gene family includes *g6pc*, *g6pc2*, and *g6pc3* three members in all [19]. While, Marandel et al. [5] found rainbow trout (*Oncorhynchus mykiss*) has five members, which were grouped into *g6pca.1*, *g6pca.2* and *g6pcb* orthologs, respectively. In this study, we identified in total four members in common carp. The identity of nucleotide and amino acid sequences showed that the four isoforms revealed a moderate sequence homology, except that *cg6pca.2a* and *cg6pca.2b* shared high sequence identity (Tables 3 and 4).

Phylogenetic tree analysis based on the full-length protein sequences showed that Teleost *g6pc3* formed a clade itself, while the *g6pca* and *g6pcb* were clustered together, and the same subtype members have higher homology among different species. Common carp *cg6pca.1*, *cg6pca.2a* and *cg6pca.2b* have a closer

Figure 1: Phylogenetic relationship of common carp G6PC proteins (A), domain (B) and partial amino acid sequence of PAP2 (C).

(A) Shows the phylogenetic tree of G6PC proteins. (B) Indicates the protein motifs corresponding to (A). We used the *Danio rerio* *g6pc3* amino acid sequence as outgroup. (C) Shows the partially conserved regions.

Table 2: The G6PC gene family members in common carp V1.0 genome.

Sub-family	Gene ID	Symbol	Locus tag	Localization	Gene length (CDS bp)	Protein length (aa)
<i>g6pca</i>	cycg043353	<i>cg6pca.1</i>	LN598274.1	44,873–48,794	1062	354
<i>g6pca</i>	cycg044960	<i>cg6pca.2a</i>	LN592114.1	13,565–15,903	1128	376
<i>g6pca</i>	cycg043354	<i>cg6pca.2b</i>	LN598274.1	52,465–54,957	756	252
<i>g6pcb</i>	cycg044064	<i>cg6pcb</i>	LN590703.1	8,925,238–8,927,076	1059	353

Table 3: Matrix mRNA(CDS) identity of *g6pc* gene family among common carp.

	<i>cycg044064(cg6pcb)</i>	<i>cycg043353(cg6pca.1)</i>	<i>cycg043354(cg6pca.2b)</i>	<i>cycg044960(cg6pca.2a)</i>
<i>cycg044064</i>	–	57.0	62.0	62.5
<i>cycg043353</i>		–	60.5	61.3
<i>cycg043354</i>			–	92.5
<i>cycg044960</i>				–

Table 4: Matrix protein identity of *g6pc* family among common carp.

	<i>cycg044064(cg6pcb)</i>	<i>cycg043353(cg6pca.1)</i>	<i>cycg043354(cg6pca.2b)</i>	<i>cycg044960(cg6pca.2a)</i>
<i>cycg044064</i>	–	56.3	52.7	59.8
<i>cycg043353</i>		–	53.1	55.2
<i>cycg043354</i>			–	96.4
<i>cycg044960</i>				–

evolutionary relationship than *cg6pcb* (Figures 1A and 2). This topology was similar with *Oncorhynchus mykiss* [6] and *Amphioxus* [5]. Yu et al. [5] constructed a genetic evolution model of *G6PC* family and considered that an original *g6pc* gene generated two paralogs in an early genome duplication event, and then one of the derived genes evolved into *g6pc3*, while the other one undergone a specific genetic multiplication on the vertebrate lineage when it had been split from the protochordate stem. The *g6pc3* class is absent in common carp genome, contrary to relative species, *Danio rerio*. It

could be that the gene was lost during the evolution. But for all this, we cannot rule out alignment errors caused by assembly of genome.

Structural properties of common carp *G6PC* gene members

The physicochemical characters of common carp *G6PC* family proteins indicated that the estimated molecular weight (MW) of *cg6pca.1*, *cg6pca.2a*, *cg6pca.2b*, and

Figure 2: The phylogenetic relationships of common carp *G6PC* proteins among the fishes and other vertebrates. The tree was constructed using the neighbor-joining method. The bootstrap values for 1000 replicates are represented at the nodes of clades.

cg6pcb monomers are approximately 39.7, 42.2, 27.3, and 39.4 kDa, while isoelectric points (pI) are 9.75, 10.13, 10.27 and 9.91, respectively. The structural properties analysis revealed that common carp G6PC family genes all contain the classical PAP2-glucose-6-phosphatase (Figure 1) which is characterized of all G6Pase isoforms [5], and no signal peptide was identified by Signal P, which is also a typical property among all known G6PC members. Additionally, the conserved motifs were identified using the full-length protein sequences of common carp G6PC family members, and three motifs were screened out (Figure 3). Although the four isoforms showed a moderate amino acid sequence homology, their motif sequences were very similar (Figure 3C).

Gene expression variations under starvation or cold stress

In mammals, all the gluconeogenic enzymes are primarily determined by altering the content of the protein [20]. Moreover, G6Pase are also affected by short-term regulation via covalent or allosteric modification [21]. Previous

studies mostly focused on the biological significances of G6PC in different nutritional status of fish, however few documents have presented the gene expression differential display under abiotic stress. Given the critical role of G6Pase in maintaining blood glucose homeostasis, in this study, the transcriptions of G6PC family were detected in hepatic tissues of common carp under two common abiotic stresses, starvation or cold condition. The results showed that *cg6pca.1*, *cg6pca.2a* and *cg6pca.2b* were transcribed under normal condition, except for that *cg6pcb* was not detected at control or any treatment condition. And contrary to expectations, only *cg6pca.2a* was significantly elevated after 12-h 4°C or after 24-h fasted treatment, respectively. Long et al. [22], Long et al. [23] and Hu et al. [24] also found *Danio rerio* *g6pca2* were up-regulated by cold stress in the transcriptome analyses during cold stress. When the ambient temperature lower than the optimal temperature range, there will be a significant reduction in food intake and conversion rates [25]. Meanwhile, the response to cold stress itself is an energy-demanding process. The organisms have to mobilize more energy substance to cope with it. For the limited capacity to store abundant glycogen during cold

Figure 3: The motifs in common carp G6PC family (A), the motif logos (B) and partial amino acid sequence of the three motifs (C). (A) The annotated amino acid sequence motifs and p-values. (B) The consensus motif sequences. (C) The specific motif sequences.

Figure 4: The relative expressions of common carp *G6PC* members in cold or starvation stress. (A) The relative expression of *cg6pca.1*. (B) The relative expression of *cg6pca.2a*. (C) The relative expression of *cg6pca.2b*. Time-course changes in expression difference of *cg6pca.1*, *cg6pca.2a*, *cg6pca.2a* and *cg6pcb* under starvation (gray bars) or cold stress (black bars) were depicted (white bars for control group). Data were normalized to *actin-β*. Values represent mean $\pm$ SEM (n=3). The letters indicate the differences are significant difference between any two means (p<0.05).

stress, carps' gluconeogenic pathway had been activated, in order to steadily support glucose. As the data represented in Figure 4, the levels of *cg6pca.2a* in cold stress was greater than starvation. This could be a sign that common carp need more glucose at low temperature than fast challenges.

In consideration of the regulation of upstream control elements (UCE) to target gene, we identified the proximal common carp *G6PC* promoter binds multiple transcription factors as well. We scanned the 1 kb upstream regions of the 5'UTRs of common carp *G6PC* genes, which were the UCE-rich regions using the motif-finding algorithm MEME. Annotations of the detected motifs were conducted by TOMTOM program with JASPAR database. The workflow is shown in Figure 5A, copied from Hu et al. [26]. The proximal *G6PC* family promoter-binding transcription factors, including Alx1, CDX1, Foxa2, HNF4, PLAG1, RORA and SOX8 (Figure 5B). Among them, Foxa2 and HNF4 are the common factors in common carp and human [16, 19]. Except for *cg6pcb*, *cg6pca.1*, *cg6pca.2a* and *cg6pca.2b* have the similar transfactor, PLAG1 and Sox8 are two unique transfactors in *cg6pca.2a* among the three genes. Consequently, the different progress of cis-regulatory elements and trans-factors among common carp *G6PC* members may result in partially the diverse expression patterns, and PLAG1 and Sox8 may be concerned with abiotic stress in common carp.

In conclusion, this study firstly identified all *G6PC* members in common carp genome, revealed the phylogenetic relationships, physicochemical properties, HMM profiles, motif structures of common carp *G6PC* members. In addition, the expression profiles of common carp *G6PC* genes under different abiotic stresses figured out

Figure 5: The identification of the cis-regulatory elements and transcription factors. (A) The screening process for enriched motifs in the proximal promoter regions of target genes. (B) Comparison of transcription factors binding the common carp *G6PC* members' promoters.

cg6pca.2a played an important role in resisting adverse situation.

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