Comparative Biochemistry and Physiology, Part B 218 (2018) 44–53

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular cloning, expression pattern of follistatin gene and association analysis with growth traits in bighead carp (*Hypophthalmichthys nobilis*)

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ARTICLE INFO

Keywords:
Bighead carp (*Hypophthalmichthys nobilis*)
Follistatin
Myostatin
Expression
SNP
Association analysis
Growth-related traits

ABSTRACT

Follistatin (FST) is a single-chain gonadal protein involving in various biological effects. *FST* plays important roles in not only ovary development but also body growth, whereas myostatin (MSTN) negatively regulates muscle growth. In this study, *FST* gene in bighead carp (*HynFST*) was cloned and characterized. A 5797 bp genomic sequence of *HynFST*, consisting six exons and five introns were cloned. The full-length cDNA of *HynFST* (2134 bp) has an open reading fragment encoding a polypeptide of 349 amino acids. Sequence comparison and phylogenetic analysis confirmed that FSTs are conserved throughout the vertebrates and *HynFST* belongs to *FST-1* isoform. Nine single nucleotide polymorphisms (SNPs) of the *HynFST* were identified and three of them (g.2443 T > C, g.2852 T > C and g.5483A > G) were significantly associated with four growth-related traits. The average body weight of those fish with the combined genotype (CC CC GG) was 12.15–22.63% higher than that of triplotype (TT TT AA) in two bighead carp populations. *HynFST* was expressed in most of the development stages and various tissues with highest level in ovary. The co-expression results for *FST* and *MSTN* in brain and muscle of divergent weight groups showed that FST may inhibit MSTN expression, thus enhancing growth in bighead carp. Our results suggest that *FST* has significant genetic effects on the regulation of early growth in bighead carp. This study would facilitate the elucidation of multiple functions of FST gene in fish and exploration of the potentials as a gene marker in selective breeding programs for growth of bighead carp.

1. Introduction

Follistatin (FST) is a monomeric glycoprotein rich in cysteine, originally isolated from bovine and porcine follicular fluid in 1987 (Robertson et al., 1987; Ueno et al., 1987). The primary function of follistatin is the binding and neutralization with members of the transforming growth factor factor-b (TGF-β) superfamily, which particularly focus on activin. Follistatin is part of the inhibin-activin-follistatin axis, and plays important roles in modulating activin-action by its binding property (Murakami et al., 2012). It can regulate the differentiation of ovarian granulosa cell by repressing the anterior pituitary's secretion of follicle-stimulating hormone (FSH), thus involving in regulation of follicular development, atresia and lutealization processes of an animal. The suppressive effect of FST on FSH secretion is exerted by its binding to activin with high affinity, resulting in activin unavailable to combine with its own receptor (Act-RII) (Nakamura et al., 1990; Michel et al., 1993). Researchers found that *FST* expresses and effectively modifies the paracrine/autocrine of activin in nearly all tissues of higher animals (Tortoriello et al., 2001; Ni et al., 2012), whose highest concentration is found in the ovary.

Myostatin (MSTN), another member belongs to TGF-β proteins, is a potent negative regulator of muscle growth in mammals (Mcpherron et al., 1997; Amthor et al., 2004; Nakatani et al., 2008; Benabdallah et al., 2009). It regulates skeletal muscle development and growth by inhibiting proliferation and terminal differentiation of myogeneic cells. As a competitive binding protein of MSTN, FST can inhibit its function and accelerate muscle growth in vivo (Amthor et al., 2004). Mutant mice lacking *FST* showed muscle deficiency, such as decreased mass of the diaphragm and intercostal muscles (Matzuk et al., 1994). Whereas mice overexpressing *FST* resulted in a gross increase in muscle fiber hyperplasia and hypertrophy compared to wild-type animals (Lee and Mcpherron, 2001), and even greater effects were detected in *MSTN* knokout mice (Mcpherron et al., 1997). Several other members of TGF-β proteins, such as bone morphogenetic proteins (Fainsod et al., 1997; Iemura et al., 1998; Amthor et al., 2002; Canalis et al., 2003) and growth differentiation factor-9 (Lin et al., 2003; Funkenstein et al., 2009) could also be antagonized by FST at lower affinities compared to activin, demonstrating that FST involves in a wide range of functions

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https://doi.org/10.1016/j.cbpb.2018.02.003
Received 23 November 2017; Received in revised form 12 February 2018; Accepted 16 February 2018
Available online 26 February 2018
1096-4959/

besides ovary development.

Growth is one of the most important factors in the commercial success of particular domestic animals including fish. Nowadays, association study using single nucleotide polymorphisms (SNPs) is a common strategy to elucidate major genes and quantitative trait nucleotides which affect quantitative polygenic traits (Liu et al., 2012), and several genes have been reported to be associated with fish growth-related traits (Liu et al., 2012; Xiu et al., 2015; Liu et al., 2016; Wang et al., 2016). Three SNPs in *MSTN* gene showed significant effects on dry body weight in sea cucumber (*Apostichopus japonicus*) (Li et al., 2016). A SNP mutation located in a CpG-rich region of exon one in the pituitary adenylate cyclase activating polypeptide gene of male half smooth tongue sole (*Cynoglossus semilaevis*) is significantly associated with growth traits, serum physiological and biochemical parameters (Si et al., 2016). Two *FST* transcripts (*FST-1* and *FST-2*) have been found in some fishes, such as zebrafish (Bauer et al., 1998; Dal et al., 2006), fathead minnow (Macqueen and Johnston, 2008), catfish (Macqueen and Johnston, 2008) and grass carp (Zhong et al., 2013). Recently, Li et al. (2011) reported that *FST-1* over-expression could promote muscle growth by enhancing myofiber hyperplasia in zebrafish. Nevertheless, association analysis between *FST* polymorphisms and growth-related traits has been scarcely reported in fish.

The bighead carp (*Hypophthalmichthys nobilis*) is one of the four Chinese Major Carps, which is endemic and has been cultivated in China for more than a thousand years (Yan et al., 2011). Together with silver carp (*Hypophthalmichthys molitrix*), bighead carp has also been introduced to many countries for aquaculture production as food fish and biological control of plankton in a variety of water systems (Xie and Liu, 2014), with an annual world production of bighead carp about 3.4 million tons (FAO, 2015). However, the natural populations of bighead carp have been declined dramatically for the past decades due to habitat fragmentation and overfishing in China (Chen et al., 2002; Liu, 2008; Zhao and Zhao, 2011), hence selective breeding programs are necessary to be initiated for bighead carp. The present study aims are to (1) clone and characterize *FST* gene of bighead carp (*HynFST*); (2) detect possible genetic associations between *HynFST* SNP and growth-related traits in bighead carp populations; (3) illustrate its spatial and temporal expression patterns, and co-expression with *MSTN* in extreme-growth individuals. Results of this study would give insights into the elucidation of function of *FST* gene on growth and provide genetic information for further gene (marker)-assisted selective breeding in bighead carp.

2. Materials and methods

2.1. Sample collection and preparation

In the current study, animal experiments were approved by the Animal Care and Use Committee at the Institute of Hydrobiology, Chinese Academy of Sciences. All efforts were made to minimize the number of animals used and the animals were treated in a humane manner.

24 brooders and two populations with multiple families of bighead carp were collected from the Xinzhou Fish Farm, Wuhan, for single nucleotide polymorphism (SNP) genotyping and association study. These two populations were generated by artificial propagation in 2011 (population 1) and 2012 (population 2), and the progenies of each population were hatched at the same time and raised in the same pond following standard culture condition (24–30 °C, pH = 6–9) with pellet feed, respectively. Five parameters for growth-related traits including body length (BL), body height (BH), head length (HL) and body weight (BW) were recorded. Fulton's Condition factor (K) was calculated as follows: K = 100BW/BL (Nash et al., 2006). Fish were randomly measured from population 1 (n = 181 with average BW of 0.50 ± 0.12 kg) and population 2 (n = 183 with average BW of 0.33 ± 0.06 kg) in the winter at six months age, and fin clips were sampled from each brooder and progeny and soaked in 95% alcohol. Total genomic DNA was isolated from fin clips using a traditional proteinase-K digestion and phenol-chloroform extraction protocol (Sambrook et al., 1989).

A full-sib family of bighead carp was produced by artificial propagation in reproductive season at Xinzhou Fish Farm, Wuhan and cultured in a muddy pond with standard condition. Whole embryos of different development stages (multi-cell stage, blastula, gastrula, optic vesicle, muscular effect, heart beating), newly hatched larvae and larvae of 1 day post-hatch (1 dph), 2 dph, 3 dph and 6 dph were collected from this family. 30 embryos prior hatch, 20 newly hatched larvae, and 10 larvae of various day post-hatch stages were sampled for each stage. Embryos and larvae of each stage were pooled during sampling. After six months of culture, a total of six progenies (mixed sexes) with extreme-light and extreme-heavy body weights were sacrificed and designated as the Light group (n = 3, mean 0.071 ± 0.03 kg) and Heavy group (n = 3, mean 0.362 ± 0.02 kg). Four tissues, including brain, intestine, skin and muscle were collected from these sacrificed bighead carps. Various tissues of six adult bighead carps (three females and three males) with sexual maturity after four years culturing were sampled for gene expression analysis. The fish were dissected after they were over-anesthetized, and fourteen tissues including pituitary, brain, liver, spleen, kidney, intestine, heart, skin, red-muscle, white-muscle, gill, eye, ovary and testis were collected. All embryo, larval and tissue samples were immediately preserved in RNA safer (Omega, US) and subsequently stored at −80 °C until further processing.

2.2. Molecular cloning of HynFST

Total RNA was isolated from liver of an adult bighead carp using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of isolated RNA was monitored on 1% agarose gels and RNA concentration was measured by NanoDrop 2000 UV–VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). After removing genomic DNA using DNase I (Fermentas, Vilnius, Lithuania), first-strand cDNA was reverse-transcribed from 500 ng total RNA using Reverse Transcriptase M-MLV (TaKaRa, Tokyo, Japan) with oligo-dT primer following the manufacturer's instructions. Based on the highly conserved cDNA sequences of other fish species, a pair of primers (follistatin, Table S1) was designed for amplifying partial cDNA fragments of bighead carp *FST* using reverse transcription (RT)-PCR method. A 749-bp partial intermediate fragment of the *HynFST* was cloned, sequenced and used to design nested gene-specific primers (Table S1) for 5′ and 3′ RACE analysis. The 5′ and 3′ ends of the *FST* cDNA were amplified using the Smart™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. PCR products were cloned into pMD 18-T vector (TaKaRa, Japan) and sequenced using an automated DNA sequencer (ABI3730, Foster City, CA, USA).

To gain the genomic sequence of *HynFST*, five primer pairs were designed to amplify the DNA fragments containing five introns and parts of six exons (Table 1).

2.3. Sequence and phylogenetic analysis

The amino acid sequence was deduced from the coding region via DNAStar (version 6.13). Sequence homology comparisons were carried out by the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple alignments of protein sequences were accomplished with Clustal X. A phylogenetic tree of FST putative amino acid sequences was built using the neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates by MEGA 4.0 software.

2.4. Genetic sex identification in the two test populations

A pair of male-specific marker named ArS-9-1 (Liu et al., 2018) was