Characterization of Gene Expression of Fatty Acid Binding Proteins (FABP) in Largescale Shoveljaw Fish (*Onychostoma macrolepis*)

Xiaofei Yang, Shaogang Xu, Wentong Li, Ding Yuan, and Shiquan Ma

***Abstract*—The Fatty acid-binding proteins (FABP) is a 14-16 kDa conserved multifunctional protein that plays key roles in binding fatty acid ligands, metabolism of fatty acids and metabolic homeostasis. In this study, the complete cDNA of FABP was isolated and characterized from *Onychostoma macrolepis* to study the functions of FABP in fish metabolism, we report the FABP (GenBank number:JN254629), it contains 405 bp open reading frame(ORF) encoding predicted 134 amino acids. Phylogenetic analysis was showed that *Oma-FABP* shared 94% sequenced identity with *Cyprinus carpio* and *Rutilus rutilus*, suggest that FABP have conserved amino acid sequence and domains in fishes. Expression diversifications of *Oma-FABP* are analyzed in tissues, feeding stimulation, and starvation challenge using a qRT-PCR approach. In *Onychostoma macrolepis*, FABP are expressed ubiquitously although transcript levels varied between organs, the highest mRNAs levels are detected in muscle. During feeding stimulation, *Oma-FABP* was induced and downregulated by dietary protein of 52% in liver ($P<0.05$), 42% in intestine ($P<0.05$). In starvation challenge, *Oma-FABP* modifies its expression, increasing its transcripts at 2 days, 6 days and 9 days after treatment($P<0.05$), the expression of *Oma-FABP* was highly expressed from 3d to 6d. Overall, these results demonstrate the existence of FABP, and differentially regulated by changes in conditions.**

Index Terms*—Onychostoma macrolepis, *FABP, cloning, qRT-PCR, expression.

I. Introduction

Fatty acid-binding proteins (FABPs) are members of a conserved multigene family, approximately 14-16 kDa intracellular protein and consist of 126-134 amino acids [1], [2]. The cellular functions of FABPs are versatile, FABPs are involved in pathways of metabolism by binding fatty acid ligands, FABPs are involved in shuttling fatty acids and regulating intracellular lipid metabolism and gene expression, metabolism of fatty acids and metabolic homeostasis [3]-[5]. FABPs are generating a large number of tissue-specific homologs and consists of several types, liver-type (L-), adipocyte-type(A-), epidermal-type(E-) and heart-type(H-) FABP [6], [7]. A-FABP could play a critical role in efficient fat storage and utilization and the balance between lipolysis and lipogenesis in adipocytes, may be a candidate for meat quality traits [5], [8]. H-FABP could transport fatty acids from cellular membranes to the site of their oxidation in the mitochondria, it should be considered as a candidate gene responsible for intramuscular fat (IMF) content in animals [9], [10].

Now, the FABP has been identified in many fishes, such as *Danio rerio* [11], *Cyprinus carpio* [12], *Cryodraco antarcticus*, *Chaenocephalus aceratus* and *Gobionotothen gibberifrons* [13]. Sequence analysis comparisons suggest that FABP gene is highly conserved across some fish species in protein sequence. The largescale shoveljaw fish (*Onychostoma macrolepis*) is a omnivorous species belonging to the family *Cyprinidae*, it is a ancient wild species distributed in China [14], however, no data is available on the FABP and its expression in *Onychostoma macrolepis*. Consequently, this study investigated FABP through homology cloning and quantitative real-time PCR (qRT-PCR), our focus was to clarify the FABP sequence and the association of the gene expression with feed challenge and starvation challenge in largescale shoveljaw fish.

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II. Materials and Methods

A. Materials Collection

This study was based on two dietary experiments. Exp I was performed at National Engineering Research Center for Freshwater Fisheries in Fangshan District(Beijing, China) from March 2011 to May 2011(total of twelve weeks). Appro-ximately 240 Largescale shoveljaw fish, 21±4 g in body mass, 12.3±3.2 cm in body lengh, were distributed to 0.2m^3 glass tanks. Two experimental diets was devised by 42% and 52% content of protein, the diet containing Tubifex(*Limnodrilus hoffmeisteri*) as the control group. Exp II was starvation challenge, liver was collected from 1 to 14 day post starvation from the mature Largescale shoveljaw fish, 0 day as the control group. For the cloning and examination of FABP in the organizations: the gonad, ovary, liver, heart, brain, cheek, intestine, muscle, eye, spleen were collected from the mature fish. All the samples were washed with RNase-Free water, frozen directly in liquid nitrogen and stored at -80 ℃ until analysis.

B. RNA Isolation

The mRNA was isolated using the Trizol RNAprep Kit (Tiangen, Beijing, China), the isolation was performed following the manufacturer's protocol. The purity of RNA

was verified by measuring the absorbance at 230, 260 and 280 nm by Smart spec™ plus spectrophotometer (Bio-Rad, Hercules, US) and its quality and integrity was confirmed by 1.2% agarose electrophoresis.

C. Clone of Oma-FABP Sequence

First-strand cDNA synthesis was performed by Reverse Transcriptase kit (TaKaRa, Dalian, China). A fragment encoding FABP was amplified by PCR on basis of primers derived from fishes (GenBank datebase), primers showed in Fig. 1. Amplification PCR cycles used: An initial cycle of 95 ℃ for 60s, followed by 35 cycles at 95 ℃ for 30s, 55 ℃ for 30s, 72 ℃ for 60s and 72 ℃ 300s, using a Eppendorf PCR cycler(Eppendorf, Hamburger, Germany). Then, the BD SMART RACE Kit(Clontech, Carlsbad, US) was used to obtain the 5′ and 3′ cDNA ends of *Oma-FABP*, two pair of primers were used for 3′ and 5′ ends RACE(Fig. 1).

D. Sequence and Phylogenetic Analysis

Sequences Similarity analysis were performed by Primer Premier 5.0(Premier Biosoft International, Palo Alto, CA) and protein alignment was performed by ClustalW2(http://www. ebi.ac.uk/Tools/msa/clustalw2/). Gene translation and predi-ction of the deduced protein were performed with the tool of Open Reading Frame Finder (ORF, http://www.ncbi.nlm.nih. gov/). Sequence assembly was performed using a DNA man software (Lynnon Biosoft). The Molecular Evolution Genetics Analysis (MEGA) version 4.0 and ClustalX were used to construct a phylogenetic tree using the neighbor-joining(NJ) method.

E. Quantitative Real-Time PCR Analysis of Oma-FABP

Before cDNA synthesis, the total RNA was purified using PrimeScript®RT Enzyme(Takara, Dalian, China), reaction included increasing the reaction volume to 10 μL, using 500 ng total RNA, a mix of random 6 mers and oligo-dT, 5×Prime Script Buffer and PrimeScript RT Enzyme Mix Ⅰ, The reactions were incubated at 37 ℃ for 15 min and 85 ℃ for 5 s. Quantitative real-time PCR was performed using FAM Fluorophore on an Funglyn FTC-3000 cycler(Funglyn Biotech, Toronto, Canada). The reaction mixture(20 μL) contained primers(10 μM), 10 μl 2×SYBR Premix Ex Taq(Takara Dalian China), 0.8 μl each of 10 μM forward and reverse primer, 0.4 μl ROX and 6 μl DNase-RNase free water(Takara, Dalian, China) and 2 ul cDNA. The amplification program was set to: 95°C for 5s, followed by 45 cycles of 95 ℃ for 5s, 60 ℃ for 34s. The gene of *β*-actin were were used as internal controls. The relative quantification between ACBP and *β*-actin transcript were calculated using the comparative CT method. These experiments were repeated two times in order to confirm the results obtained.

III. Results

A. Characterization of Oma-FABP

The *Oma-FABP* was cloned and characterized, the *Oma-FABP* sequence was identified on the basis of similarity to FABP of *Cyprinus carpio*. The full nucleotide sequence of FABP mRNA was 405 bp encoding a predicted 134 amino acid protein, and including a conserved FABP domain (Fig. 2), the sequence was submitted to GenBank database under accession number JN254629. The sequence identity index of *Oma-FABP* ranged from 81% to 94% to FABP compared to other fish species. ClustalW2 was used to align FABP protein, the longest identity conserved of 15 amino acid without mismatches was found from the 25-39 amino acid position, and clearly suggested that *Oma-FABP* was one isoform of FABP family.

B. Phylogenetic Analysis of Oma-FABP with other Species

A phylogenetic tree was constructed from all published sequences of fish members of FABP, using *Ictalurus furcatus* FABP as outgroup (Fig. 3). We performed phylogenetic analyses of *Oma-FABP* and other fish FABP closely related to the *Oma-FABP* sequence identified in this study (Fig. 4). The *Oma-FABP* shared 94% sequenced identity with *Cyprinus carpio* and *Rutilus rutilus*, 89% with *Danio rerio*.

Primers	Sequence(5′→3′)	Position	Product(bp)
For the cloning of conserved sequence			
P1	ggaaaccggaccaagccc	100-305	206
P2	ttgccatcccaggtctgt		
For the cloning of 3’ end and 5’ end RACE			
P3RACE-1	agtcgcagagcaccttc	161-405	245
P3RACE-2	gcagaaacagacctgggatgg	256-405	150
P5RACE-1	gttttgaaggtgctctgcgact	1-182	182
P5RACE-2	gtttgccgttctcaagagtcac	1-277	277
Primers used for quantitative real-time PCR			
P-QF	gcggatgatagaaagactacgac	229-375	147
P-QR	gccaccacatcacccattg		
actinF	tcccctcaatcccaaagcc		121
actinR	ggagtccatcacgataccagt		

Fig. 1. *Oma-FABP* and *β*-actin genes, PCR primers used for gene fragment amplified and quantitative real-time PCR.

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1     atggttgacaaattcgtgggaacatggaagatgaccaccagtgataactttgacgagtac
        M  V  D  K  F  V  G  T  W  K  M  T  T  S  D  N  F  D  E  Y
61    atgaaggctataggtgtgggtttcgctactcgtcaggtgggaaaccggaccaagcccaac
        M  K  A  I  G  V  G  F  A  T  R  Q  V  G  N  R  T  K  P  N
121   ttggtcgtgtgcgtggatgatcaggggctcatatgcatgaagtcgcagagcaccttcaaa
          L  V  V  C  V  D  D  Q  G  L  I  C  M  K  S  Q  S  T  F  K
181   actactgagatcaaatttaaactcaatgagtcattcgaggagatcaccgcggatgataga
          T  T  E  I  K  F  K  L  N  E  S  F  E  E  I  T  A  D  D  R
241   aagactacgactgttgtgactcttgagaacggcaaacttatgcagaaacagacctgggat
          K  T  T  T  V  V  T  L  E  N  G  K  L  M  Q  K  Q  T  W  D
301   ggcaaagagtcgacgatagagagggaggtgacggatgggaaattagtagctaaatgcaca
          G  K  E  S  T  I  E  R  E  V  T  D  G  K  L  V  A  K  C  T
361   atgggtgatgtggtggctgtgaggacatatgtgaaggaggcatga
          M  G  D  V  V  A  V  R  T  Y  V  K  E  A  *
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Fig. 2. The full length cDNAs and deduced amino acid sequences of *Oma-FABP*.

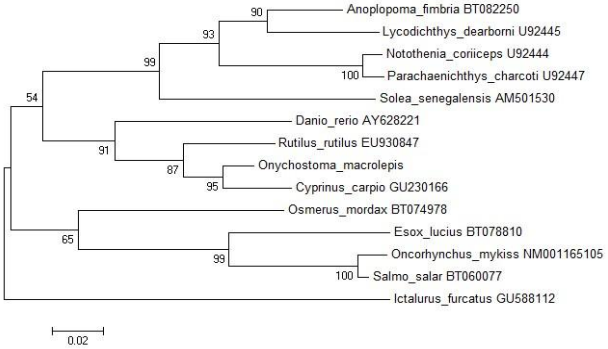

Fig. 3. Phylogenetic analysis of *Oma-FABP* with other known sequences.

Species	Score	Expected value	Max ident(%)	GenBank No.
Cyprinus carpio	636	5e-179	94%	GU230166
Rutilus rutilus	563	2e-157	94%	EU930847
Danio rerio	533	4e-148	89%	AY628221
Cryodraco antarcticus	401	2e-108	81%	U92443
Chaenocephalus aceratus	401	2e-108	81%	U92442
Parachaenichthys charcoti	398	2e-107	81%	U92447
Gobionotothen gibberifrons	398	2e-107	81%	U92446
Notothenia coriiceps	398	2e-107	81%	U92444
Anoplopoma fimbria	392	9e-106	81%	BT082250
Perca flavescens	389	1e-104	81%	FJ826533

Fig. 4. Sequence similarities of *Oma-FABP* with other species.

C. qRT-PCR Analysis of Oma-FABP Expression

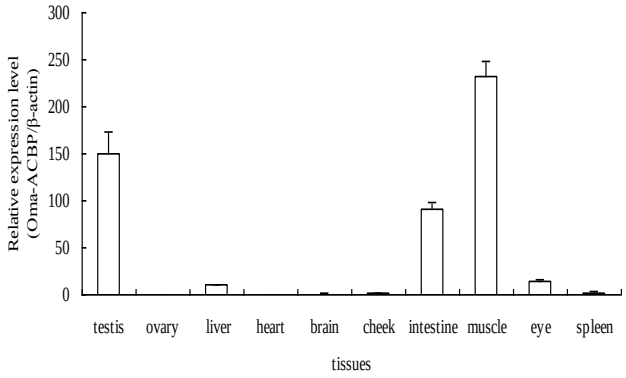

Fig. 5. Quantitative real-time PCR analysis of *Oma-FABP* expression in tissues.

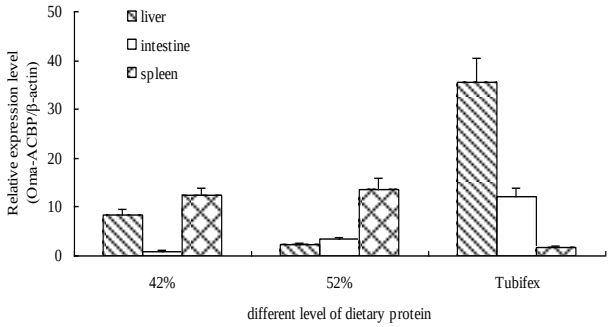

Fig. 6. Quantitative real-time PCR analysis of *Oma-FABP* expression in dietary protein of 42% and 52% in liver, intestine and spleen, Tubifex as the control group.

The *Oma-FABP* gene expression analysis was performed using specific primers designed on full nucleotide sequence to obtain amplification(Fig. 1). The expression analysis in tissues show that *Oma-FABP* was detected in intestine, spleen, liver, testis, ovary, heart, brain, cheek, muscle and eye, the expression show significant changes among muscle and other tissues (P<0.05, Fig. 5). In order to investigate the interaction between regulation and feeding stimulation on *Oma-FABP* gene expression, different fishes were obtained post twelve weeks feeding stimulation (dietary protein of 42% and 52%, Tubifex, *Limnodrilus hoffmeisteri*) showed in Fig. 6. The *Oma-FABP* gene expression analysis showed that FABP was induced and downregulated by 52% in liver (P<0.05), 42% in intestine (P<0.05) and Tubifex group in spleen (P<0.05). In starvation challenge, there are three relatively peaks, respectively, 2d, 6d, 9d (P<0.05), and then showed a gradual downward trend (Fig. 7).

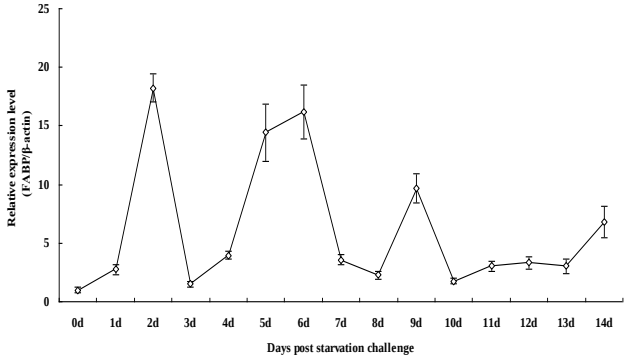

Fig. 7. Quantitative real-time PCR analysis of *Oma-FABP* expression in liver post starvation challenge from 0 to 14day

IV. Discussion

The current work was performed to isolate the full length mRNA encoding FABP. Its role was studied in pathways of metabolism by binding fatty acid ligands, FABPs are involved in shuttling fatty acids and regulating intracellular lipid metabolism. The full length of *Oma-FABP* mRNA sequence was isolated, 405 bp encoding a predicted 134 amino acid protein. Earlier studies in heart of rainbow trout and Atlantic salmon(*Salmo salar*) consist of 126-134 amino acids [1], [2], the result of sequence alignment was obtained and including a conserved FABP domain, therefore, the *Oma-FABP* gene have been cloned and characterized. Phylogenetic analyses showed that *Oma-FABP* is more similar to FABP of *Cyprinus carpio* than to the other members of the FABP family. These data suggest that that *Oma-FABP*, FABP-H6, FABPH have diverged from a common ancestor.

FABP is widely distributed in brain, eye, gonad, heart, intestine, liver, muscle, skin [15]. In bovine, the FABP mRNA and protein levels are potentially good indicators of the muscle metabolic type at a given age [16]. In the present study, the expression of analyses in tissues showed that *Oma-FABP* was highly expressed in muscle, weakly expressed in testis, intestine, spleen, liver, ovary, heart, brain, cheek and eye, these results confirm that *Oma-FABP* have a

tissue-specificity, and may plays an important regulatory function during adipogenesis in muscle cells of *Onychostoma macrolepis*.

In bird, the results found that feeding stimulation was a critical factor inducing FABP gene expression irrespective of light condition, however, feeding stimulation only slightly stimulated expression of the FABP gene, and was not always its primary determinant [17]. The studies in RNAi and transgenic animals have showed that the expression of the FABPs are in many cases interdependent, FABP can be considered that grave functional consequences of the loss of a particular FABP are often prevented by the compensatory overexpression of another member of FABP family [18]. FABP of *Salmo salar* is considered as a gene marker during the differentiation process, the cells change their morphology from an unspecialised fibroblast-like cell type to a mature adipocyte filled with lipid droplets and with a significant increase in the expression of FABP [19]. Low and high IMF(intramuscular fat) levels in muscle tissue showed scientifically correlation between intramuscular fat content and transcript level of the FABP gene [20]. On the other hand, the study of the relationship between nutrients and FABP gene expression, found that ingestion of a docosahexaenoic acid enriched diet had no effect on FABP gene expression in ducks [21]. FABP expression may link FABP to the process of fatty acid oxidation in red muscle related to coordinate changes in energy demanding parts of the *Salmo salar* production life cycle, the muscle changes in total fatty acid oxidation capacity and muscle FABP protein expression patterns respond similarly to dietary inclusion of RO [22]. In this initial study, there was obvious difference of *Oma-FABP* transcript post feeding stimulation, *Oma-FABP* transcription may be associated with the nutrition need for organ and fatty acid metabolism, it was clear that the content of dietary protein induced *Oma-FABP* expression. In the study of the starvation challenge, the expression of *Oma-FABP* was highly expressed from 1d to 2d, and 3d to 6d, the trend line from can be speculated that the cycle of consume the nutrients is about 2 days until 7d. The relationship of *Oma-FABP* expression with different diet and starvation challenge in metabolism implies critical importance for cellular function, *Oma-FABP* might be expected to play essential roles in regulating postnatal nutrients and energy metabolism, further studies are needed to elucidate mechanisms for fatty acid transport in *Onychostoma macrolepis* and to determine the specific in vivo function of *Oma-FABP* tissues, especially in muscle.

In conclusion, this study reports the first cloning the FABP(JN254629) gene from *Onychostoma macrolepis*. *Oma-FABP* expression has been characterized in intestine, spleen, liver, testis, ovary, heart, brain, cheek, muscle and eye, showed that it may be a muscle-specificity type FABP. For liver, instine and spleen, changes in fatty acid metabolism and *Oma-FABP* expression patterns respond similarly to the content of dietary protein. FABP may link to the process of fatty acid metabolism in organ related to coordinate changes in energy demanding of the metabolism requirement. However further studies are clearly needed to clearly define mechanisms for fatty acid transport and metabolism in *Onychostoma macrolepis*.

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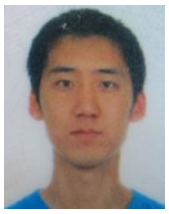

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