Comparative Biochemistry and Physiology, Part B 212 (2017) 24–31

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular characterization and expression analysis of the myostatin gene and its association with growth traits in Noble scallop (*Chlamys nobilis*)

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ARTICLE INFO

Keywords:
Chlamys nobilis
MSTN
SNP
Growth traits
Noble scallop

ABSTRACT

Myostatin (MSTN), also called growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily and an inhibitor of muscle differentiation and growth. In this report, we identified and characterized a MSTN gene (CnMSTN) from the scallop *Chlamys nobilis*. The open reading frame of CnMSTN was 1374 bp in length, encoding 457 amino acids. The structure of CnMSTN included a putative signal peptide, a TGF-β propeptide domain, and a conserved TGF-β domain. Phylogenetic analysis showed that the CnMSTN gene was clustered in the same subgroup with the MSTN gene found in Mollusca. Quantitative real-time PCR showed that the CnMSTN gene was widely expressed in all tissues tested, with the highest expression level observed in the adductor muscle. Six single nucleotide polymorphisms (SNPs) were identified in the promoter region, but no SNP was detected in the exon regions. Association analysis showed that SNP g.-579A/C had significant effects on body mass, soft-tissue mass, and adductor muscle mass. The CC and AC genotypes of g.-579A/C had significantly higher growth trait values than that of genotype AA ($P < 0.05$). These results suggest that CnMSTN could be used as a candidate gene for the selective breeding of *C. nobilis*.

1. Introduction

Noble scallop *Chlamys nobilis*, *C. farreri* and *Argopecten irradians* are three of important edible marine bivalve shellfish commonly cultured in China. *C. nobilis* is cultured in south of China and the others are cultured in northern China. All of them are fast growth and short culturing period, which are grown up to 5 cm of shell height in one year. The adductor muscle is the major edible part that confers its commercial importance. Studies on scallop adductor muscle have reported color to be a qualitative trait, with orange adductor muscle having significantly higher carotenoids content than that of brown adductor muscle (Zheng et al., 2010) and white adductor muscle found to be dominant over yellow (Liu et al., 2012a, 2012b). In addition, adductor muscle weight appears to be related to the edible portion as well as shell width (Liu et al., 2009).

Myostatin (MSTN) is a member of the transforming growth factor-β (TGF-β) superfamily. It was first identified and characterized in mice (Mcpherron et al., 1997). Subsequently, MSTN has been cloned and identified in many animals, including mammals, birds, fish, shrimp, and sea cucumber (Muroya et al., 2009; Dall'Olio et al., 2010; Dushyanth et al., 2016; De et al., 2008; Qian et al., 2013; Núñez-Acuña and Gallardo-Escárate, 2014). Recently, the MSTN gene was obtained in several Mollusca species, including *A. irradians*, *C. farreri*, *Pinctada fucata*, *Mytilus chilensis*, *Nodipecten subnodosus*, and *Sinonovacula constricta* (Lin et al., 2012; Hu et al., 2010; Núñez-Acuña and Gallardo-Escárate, 2014; Morelos et al., 2015; Niu et al., 2015; Huang et al., 2016). These studies indicate that the MSTN protein sequence has been conserved throughout evolution.

As a negative growth factor, MSTN can inhibit muscle differentiation and growth (Mcpherron and Lee, 1997). Mutations of the MSTN gene can cause muscle hypertrophy, which can, in turn, generate the "double muscle" phenotype (Mosher et al., 2007; Mcpherron and Lee, 1997). It has been demonstrated that knockout of the MSTN gene induces muscle fiber hypertrophy and changes muscle fiber composition, whereas overexpression of MSTN decreases muscle mass and fiber size (Wang and Mcpherron, 2012; Hennebry et al., 2009; Reisz-Porszasz et al., 2003). In transgenic medaka, giant muscle and increased myofiber quantity have been observed when MSTN is suppressed (Sawatari et al., 2010). The relationship between MSTN expression and the size and number of adductor muscle fibers in *N. subnodosus* also suggests

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http://dx.doi.org/10.1016/j.cbpb.2017.07.004
Received 4 May 2017; Received in revised form 26 June 2017; Accepted 13 July 2017
Available online 16 July 2017

that MSTN is involved in adductor muscle growth regulation, as is observed in vertebrate skeletal muscle (Morelos et al., 2015). In addition, several SNPs in MSTN are related to growth traits in mammals, fish, and sea cucumber (Wang et al., 2016; Tu et al., 2012; Liu et al., 2012a, 2012b; Li et al., 2016). Furthermore, SNPs have been detected in the mollusk MSTN gene exon, with some found to be significantly associated with growth (Guo et al., 2011; Wang et al., 2010; Niu et al., 2015). Thus, MSTN is considered as a possible candidate gene for growth improvement in animals.

In this study, the MSTN gene transcript of *C. nobilis* was isolated and characterized, with its expression in different tissues then determined. Six SNPs were detected in the promoter region of MSTN and their associations with *C. nobilis* growth traits were analyzed.

2. Materials and methods

2.1. Animals and phenotypic data

Ten healthy *C. nobilis* adults, averaging 51 mm in shell height, were collected from Shenzhen, Guangdong Province, China, and kept in seawater for 3–4 h until arrival at the laboratory. For tissue-specific expression determination, the mantle, adductor muscle, gill, testis, ovary, and foot were collected and rapidly frozen in liquid nitrogen, then stored at − 80 °C until RNA isolation.

A total of 152 *C. nobilis* (average shell length 65.69 ± 5.81 mm) at the one years old were chosen randomly from a scallop farm in Sanya, Hainan Province, China. Their body mass, soft-tissue mass, adductor muscle mass, shell width, shell length, and shell height were recorded. The adductor muscles of each individual were sampled and preserved in 95% ethanol for DNA extraction. Total genomic DNA was extracted from 25 mg of the adductor muscle using a Marine Animals DNA kit (Tiangen, China) and dissolved in sterile water with a concentration of 50 ng/ml and stored at − 20 °C.

2.2. Cloning and sequencing of full-length cDNA

Total RNA was extracted from the tissues of *C. nobilis* with RNAiso Plus (Takara, Japan) following the manufacturer's instructions. Total RNA (1 μg) from each tissue was used as the template for reverse transcription reaction with a Prime Script II 1st strand cDNA Synthesis kit (Takara, Japan).

Three cDNA sequences of the MSTN gene were obtained from the transcriptome sequences through BLASTX searching the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST). The unknown sequences among them were amplified successfully by two pairs of primers, M1S/M1A and M4S/M4-1A (Table 1). The PCR program was 1 cycle at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finally 1 cycle at 72 °C for 5 min. The PCR products were isolated using a DNA gel extraction kit (Omega, USA) and sequenced directly by Shanghai Shenggong (China).

2.3. Bioinformatic analysis

The entire nucleotide sequence was analyzed by BLAST searching the NCBI. ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to deduce the amino acid sequences of MSTN. The presence and location of the signal peptide was predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The identification and annotation of the MSTN protein structures were conducted using conserved domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignments were generated and a phylogenic tree was constructed using MEGA 7.0 software. Neighbor joining was used and branching reliability was tested by bootstrap resampling with 1000 pseudo-replicates (Kumar et al., 2016). The N-glycosylation sites in the MSTN gene were predicted using the Net NGly 1.0 Server (Blom et al., 2004).

Table 1
Primers employed in the study.

Primers	Sequences(5` → 3`)	Purpose
M1S	GCCGTAGCGATCAACCAAACC	cDNA cloning
M1A	CCAAACGCAACGAAATCAACCT	
M4S	TGTAGATACCCGTTGGAGGTTGATT	cDNA cloning
M4-1A	AATCTGCCCTATTCAATCGTCCC	
SP1	GGTTGCCCATACTCCTCTTGC	Genomic walking
SP2	GTATCGTAGCCTTTCCTCCTTCC	
SP3	TTGGTAATCGCTTTGCTGTCG	
MyW1s	TGTTCTTTGCGCTGCTGTCTG	SNP identification in exon 1
MyW1a	GTTCTTTCCGGTTGCCCATAC	
MyW2s	GCTGAAATGACATCGGAATTTGAGA	SNP identification in exon 2
MyW2a	CGCCTCCACAACAAGTCCAAG	
MyW3s	GAACCAATGCTTGACATGCGTACTT	SNP identification in exon 3
MyW3a	TATGATCGAAGAAAAGCATGGCTAG	
Wpros	ACACGGCGAAAAAATGGAGC	SNP identification and genotyping in promoter region
Wproa	TTCCAAAGTCAACTCCGGTAAA	
18sF-shan	GACCTACCTCCCGGTTTTAC	qPCR of 18S
18sR-shan	CCCGTCTGTCCCTCTTAATC	
MsQ-S	CGACAGCAAAGCGATTACCA	qPCR of CnMSTN
MsQ-A	AATTCCGATGTCATTTCAGC	

2.4. Real time quantitative PCR (RT-PCR)

The mRNA expression levels of CnMSTN in various tissues were determined by RT-PCR using a pair of gene-specific primers, MsQ-S and MsQ-A. The 18S rRNA of *C. nobilis* was used as an internal reference (Shi et al., 2014). The RT-PCR was performed in triplicate for each sample using the CFX96 real-time PCR Detection System (Eppendorf, Germany) in a 20 μl reaction system, with the following components: 10 μl of 2 × SYBR Green Real-time PCR Master Mix (Takara, Japan), 0.4 μl of each primer (10 μM), 1.0 μl of cDNA, and 8.2 μl of RNase-free water. The PCR program was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. To assess the specificity of the PCR amplification, a melting curve was obtained at the end of the reaction, and a single peak was observed. Data were quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The amplification efficiencies of the target and reference genes were verified and found to be approximately equal. All RT-PCR data were analyzed using SPSS 19.0. Differences between means were considered significant at the 95% confidence level ($P < 0.05$).

2.5. Cloning and sequencing of the promoter region

Genome walking was carried out using a Genome Walking kit (Takara, Japan) according to the user manual. The specific primers SP1, SP2, and SP3 were designed and synthesized based on the MSTN gene sequence (Table 1). Subsequently, three nested PCRs were carried out using primer pairs containing the specific primer and compound annex primers (AP1, AP2, AP3, and AP4) provided with the kit, with genomic DNA used as the template. The final PCR product was separated by 1.2% agarose gel electrophoresis, with the desired band then excised and purified using a DNA gel extraction kit (Omega, Germany) and finally cloned into the pMD18-T vector (Takara, Japan) and sequenced.

2.6. SNP detection and association analysis with growth traits

Twenty *C. nobilis* individuals were collected for SNP detection. Four pair of primers, including MyW1s/a, MyW2s/a, MyW3s/a, and Wpros/a, were used to amplify the partial gDNA sequence of MSTN (Table 1). The PCR products were purified with a Cycle Pure kit (Omega, Germany) and sequenced directly by Shanghai Shenggong (China). The