Molecular and Cellular Probes 30 (2016) 266–272

Contents lists available at ScienceDirect

Molecular and Cellular Probes

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

Polymorphisms of *FLII* implicate gene expressions and growth traits in Chinese cattle

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ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form
30 June 2016
Accepted 20 July 2016
Available online 21 July 2016

Keywords:
FLII
Polymorphism
Association analysis
Growth trait
Gene expression
Cattle

ABSTRACT

Flightless-1 (*FLII*) is essential for early embryogenesis, structural organization of indirect flight muscle and can inhibit adipocyte differentiation. We therefore aimed to identify common variations in *FLII* gene and to investigate their effects on cattle growth traits. By DNA sequencing and forced PCR-RFLP methods, we evaluated two synonymous mutations (rs41910826 and rs444484913) and one intron mutation (rs522737248) in four Chinese domestic breeds (n = 628). Association analysis indicated that these SNPs were associated with growth traits and gene expressions ($P < 0.05$). At rs41910826, individuals with TT and/or CT genotypes had some better body sizes in Jiaxian, Nanyang, and Qinchuan breeds. Consistently, among adult Qinchuan cattle muscles, quantitative real-time PCR study witnessed considerable increases of mRNA level of *FLII* in cattle with CT genotype. For rs444484913, TT and/or TC genotypes were significantly associated with increased body traits of Qinchuan cattle while the qPCR data showed that the TT genotype was more conducive to *FLII* expression in fetal muscle. At rs522737248, performances of cattle with AA genotype showed compelling superior merits in all four breeds, and allele A had an increasing tendency for the mRNA expression of *PPARγ* in adult adipose and *FLII* in fetal muscle. These findings strongly demonstrate that the three SNPs of *FLII* gene could be utilized as molecular markers for future assisted selection in cattle breeding program.

1. Introduction

There are more than 70 indigenous cattle breeds in China [1], whereas most of them have such drawbacks as low beef productivity, small body size, and low intramuscular fat content, especially compared with foreign cattle breeds that have been specifically bred for meat production for a long time [2]. Over the last decades, much attention has been paid on identifying candidate genes or alleles underlying favorable phenotypic traits in the early stage to improve the accuracy and efficiency of cattle breeding in the long run [3,4].

Flightless-1 (*FLII*) is a member of the gelsolin superfamily and consists of 15 tandem LRR domains (N-terminal) and 5 tandem gelsolin-like domains (GLD, C-terminal) [5]. There are substantial evidences indicating that *FLII* play essential roles in the development of adipose and muscle profoundly in animals. With the two domains, *FLII* functions on actin remodeling, anti-inflammation, and anti-immune responses [6,7]. Choi et al. [8] discovered that *FLII* could repress transcriptional activity of *PPARγ*, as a co-regulator competing with retinoid X receptor α (RXRα) and interrupting formation of the PPARγ/RXRα complex, to inhibit adipocyte differentiation. By interacting with estrogen receptor (ER), *FLII* can promote the expression of hormone-stimulated gene [9]. Accordingly, our lab observed a significant up-regulation of *FLII* in adult adipose compared to fetal adipose based on high-throughput sequencing [10]. Also, FLII protein is necessary for the formation of actin/myosin fibers and M-lines and Z-lines of the *Drosophila* muscle [11], while mutants will show actin disorganization in the fast muscle [12].

In current study, we hypothesized that there might be an association between genetic variations of *FLII* and phenotypic

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http://dx.doi.org/10.1016/j.mcp.2016.07.005
0890-8508/

differences of livestock growth. However, similar research has not been reported up to now. The object of this research was to get some pregnant information for the influence of mutations on gene functions and cattle breeding pioneering. Polymorphisms of *FLII* gene were detected in four Chinese native cattle breeds, and their effects on body sizes and mRNA expression were valuated.

2. Materials and methods

2.1. Sample preparation and data collection

To investigate allelic variation of the bovine *FLII* gene, a total of 628 blood samples of female cattle, 2 years old, were sampled from four Chinese indigenous cattle breeds: Qinchuan (QC, n = 166), Nanyang (NY, n = 94), Jiaxian (JX, n = 223), Jinnan (JN, n = 145). They were fed in the reserved farms located in Shaanxi (QC), Henan (NY, JX), Shanxi (JN) according to the INRA (National Institute for Agricultural Research, France) standards. Genomic DNA were isolated from the blood samples and stored at −80 °C based on standard procedures [13]. To evaluate the effects of polymorphisms on meat production related performance traits, the phenotypic traits of cattle were measured as described in a previous association study [14]. Objective body size traits included abdominal girth (AG), body mass (BM), body height (BH), body length (BL), chest breadth (CB), circumference of cannon bone (CCB), chest depth (CD), chest girth (CG), high commend (HC), height at hip cross (HHC), hip width (HIW), hucklebone width (HUW), and rump length (RL). All animal experiments were implemented in compliance with relevant laws and institutional guidelines and were approved by Northwest A&F University Institutional Animal Care and Use Committee.

2.2. DNA pool sequencing

To explore the allele variation of the bovine *FLII* gene, a total of 9 primer pairs were designed to cover the exons and the adjacent introns (Table S1, GenBank accession number: AC_000176.1). Fifty DNA samples were randomly selected from each breed to construct four DNA pools. PCR reactions were carried out using a Touchdown PCR System Thermal Cycler Dice (TaKaRa, Dalian, China). About 50 μL PCR products were sequenced in both directions (Shanghai Sangon Biotech Co., Ltd., P. R. China; Applied Biosystems 3730xl DNA sequencer, Foster City, CA, USA). The sequences were imported into the BioXM software 2.6 to identify single nucleotide polymorphisms (SNPs).

2.3. Genotyping by forced PCR-RFLP

To facilitate genotyping of three SNPs identified above, forced PCR-RFLP technique was developed for creating expected restriction sites for endonucleases. Aliquots of 10 μL of PCR products were digested with 3U of *Hinc*II, *Ban*II, and *Sac*II for 8 h at 37 °C. The digested products were detected by electrophoresis in 3.0% agarose gel stained with 200 ng/mL ethidium bromide at a constant voltage (120 V) for 0.5 h. The information for enzyme primers, restriction enzymes, and fragment sizes were shown in Table 1.

2.4. Total RNA isolation

To investigate the expression pattern of *FLII*, tissue samples of female QC cattle in three growth periods (embryo, newborn, and adult, n = 3) were obtained from slaughter farm Tumen Abattoir (Xi'an, China). Bovine preadipocyte (0 day) and differentiated adipocytes (10 day) were provided by Li et al. [15] in our lab. In an effort to analyze the influence of allele variations on mRNA expression levels, skeletal muscles and adipose were separately sampled from female QC cattle at fetal (n = 30) and adult (n = 27) stages (adipose in fetal stage were not obtained). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All samples were subjected to reverse transcription using a cDNA high capacity kit (Invitrogen, Carlsbad, CA, USA).

2.5. Selection of reference genes and quantitative real-time PCR (qPCR)

For evaluation of relative gene expression of *FLII* and *PPARγ*, seven candidate reference genes were tested for stability (Table S2). *HMBS, PPIB, EMD,* and *LRP10* were chosen because they had been previously reported to be the most stable reference genes in fat and muscle tissues [16,17]. *GAPDH, ACTB,* and *RPL19* were included because these genes have been utilized as reference genes in numerous studies [18–20]. Gene stability values were estimated using the geNorm software which can identify the optimal normalization gene among a set of candidates. All candidate reference genes were ranked by stepwise elimination of the genes with the highest M value, and normalization would be carried out with the geometric mean of the most stable reference genes [21,22].

Quantitative real-time PCR (qPCR) was carried out using SYBR Green (TaKaRa, Dalian, China). The amplification efficiency and specificity of primers were verified by melting curve analysis. Each experiment was performed at least three times. According to the geNorm manual [22], relative mRNA expression levels from qPCR were calculated.

2.6. Statistical analysis

Genotypic and allele frequencies were estimated by direct counting. Population genetic indexes including gene homozygosity (*Ho*), effective allele numbers (*Ne*) and polymorphism information

Table 1
Primers designed for genotyping of the three SNPs in the *FLII* gene.

Loci	Position (AC_000176.1)	Primers	Tm (°C)	Production size (bp)	Restriction endonuclease	Genotype pattern (bp)
rs41910826	g. 2870-2982 (g. C2890T)	F1- *Hinc*II 5′- CCTGG*A*TTG*T*G*A*CGC<u>*G*</u>TCAA-3′ R1 5′- GAGG*T*AAGCTGGTTCCGGGA-3′	58	113	*Hinc*II	CC:95,18; CT:113,95,18 TT:113
rs444484913	g. 6852-6962 (g. C6877T)	F2-*Ban*II 5′- CTGATGACTTTTAGCCA*T*CC<u>*G*</u>AG-3′ R2 5′- AGTCCAGAATGAGCCTACAGCCA-3′	53	111	*Ban*II	TT:111; TC:111,86,25; CC:86,25
rs522737248	g. 8180-8350 (g. C8245A)	F3 5′- TCACGG*A*GCTCTT*A*CTG*A*CGC-3′ R3 5′- CCGTCCAGGTCCTCGTT-3′	53	171	*Sac*II	CC:102,69; CA:171,102.69; AA:171

Note: The italics and underlined nucleotide showed mismatch to create restriction sites for variation detection. The italics nucleotides showed mismatches to enhance the amplification efficiency and primers specificity.