Gene 537 (2014) 132–139

Contents lists available at ScienceDirect

Gene

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

Short communication

Detecting novel SNPs and breed-specific haplotypes at calpastatin gene in Iranian fat- and thin-tailed sheep breeds and their effects on protein structure

Mohsen Aali [a,*], Mohammad Moradi-Shahrbabak [a,b], Hosein Moradi-Shahrbabak [a], Mostafa Sadeghi [a]

[a] Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[b] Excellent Center for Improving Sheep Carcass Quality and Quantity, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

ARTICLE INFO

Article history:
Accepted 11 December 2013
Available online 5 January 2014

Keywords:
Ovine *CAST* gene
Single-strand conformational polymorphism (SSCP)
Single nucleotide polymorphism (SNP)
Breed-specific haplotype

ABSTRACT

Calpastatin has been introduced as a potential candidate gene for growth and meat quality traits. In this study, genetic variability was investigated in the exon 6 and its intron boundaries of ovine *CAST* gene by PCR-SSCP analysis and DNA sequencing. Also a protein sequence and structural analysis were performed to predict the possible impact of amino acid substitutions on physicochemical properties and structure of the *CAST* protein. A total of 487 animals belonging to four ancient Iranian sheep breeds with different fat metabolisms, Lori-Bakhtiari and Chall (fat-tailed), Zel-Atabay cross-bred (medium fat-tailed) and Zel (thin-tailed), were analyzed. Eight unique SSCP patterns, representing eight different sequences or haplotypes, *CAST*-1, *CAST*-2 and *CAST*-6 to *CAST*-11, were identified. Haplotypes *CAST*-1 and *CAST*-2 were most common with frequency of 0.365 and 0.295. The novel haplotype *CAST*-8 had considerable frequency in Iranian sheep breeds (0.129). All the consensus sequences showed 98–99%, 94–98%, 92–93% and 82–83% similarity to the published ovine, caprine, bovine and porcine *CAST* locus sequences, respectively. Sequence analysis revealed four SNPs in intron 5 (C24T, G62A, G65T and T69-) and three SNPs in exon 6 (c.197A > T, c.282G > T and c.296C > G). All three SNPs in exon 6 were missense mutations which would result in p.Gln 66 Leu, p.Glu 94 Asp and p.Pro 99 Arg substitutions, respectively, in *CAST* protein. All three amino acid substitutions affected the physicochemical properties of ovine *CAST* protein including hydrophobicity, amphiphilicity and net charge and subsequently might influence its structure and effect on the activity of Ca2 + channels; hence, they might regulate calpain activity and afterwards meat tenderness and growth rate. The Lori-Bakhtiari population showed the highest heterozygosity in the ovine *CAST* locus (0.802). Frequency difference of haplotypes *CAST*-10 and *CAST*-8 between Lori-Bakhtiari (fat-tailed) and Zel (thin-tailed) breeds was highly significant ($P < 0.001$), indicating that these two haplotypes might be breed-specific haplotypes that distinguish between fat-tailed and thin-tailed sheep breeds.

1. Introduction

In living muscles, calpain system is one of the four proteolytic systems (Li et al., 2009) which have a considerable role in myofibrillar protein degradation in the skeletal muscle (Goll et al., 2003), which suggests their active roles in muscle growth. This system is also believed to initiate post-mortem degradation of myofibril proteins that is the biochemical basis of meat tenderization (Koohmaraie, 1996). *CAST* is the calpain-specific endogenous inhibitor (Koohmaraie, 1992) and thus regulates growth rate and meat tenderness. So, *CAST* is a functional candidate gene for growth and meat quality traits.

CAST gene consists of an N-terminal leader (L) domain containing two subdomains of XL and L and four calpain-inhibitory domains also known as inhibitory repeats (Killefer and Koohmaraie, 1994), each of which constitute three subdomains of A, B and C (Takano et al., 1999; Fig. 1). Each of the four inhibitory repeats independently neutralizes an activated calpain with exquisite specificity and potency (Cong et al., 1998; Wendt et al., 2004) while the L domain does not appear to have any calpain inhibitory activity but might be involved in targeting or intracellular localization (Takano et al., 1999). *CAST* gene contains 36 exons and 35 introns in sheep (Kemp et al., 2010). Exon 6 is the largest exon of ovine *CAST* gene (Zhou et al., 2007; Fig. 1).

Several studies have reported genetic variation in the intron and exon 1 region (Palmer et al., 1998; Roberts et al., 1996), exon 6 (Zhou et al., 2007) and exons 24, 25, 27 and 28 (Chung and Davis, 2012) of

Abbreviations: *CAST* gene, calpastatin gene; SNP, single nucleotide polymorphism; PCR-SSCP, polymerase chain reaction single-stranded conformation polymorphism; QTL, quantitative trait loci; Gln, glutamine acid; Glu, glutamic acid; Asp, aspartic acid; Pro, proline acid; Arg, arginine acid; Leu, leucine acid; HapMap, haplotype map; LD, linkage disequilibrium; NCBI, National Center for Biotechnology Information; BLAST, Basic Local Alignment Search Tool; *RCAN1* gene, regulator of calcineurin 1 gene; BMS, beef marbling score; IMF, intramuscular fat.

* Corresponding author at: Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj 31587-11167, Iran. Tel.: +98 9155072452; fax: +98 26 32248082.
E-mail address: Mohsen.Ali@ut.ac.ir (M. Aali).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.12.023

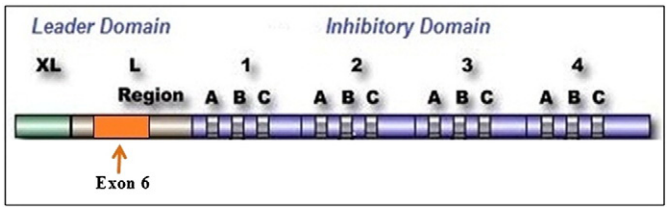

Fig. 1. The structure of the calpastatin protein. Calpastatin consists of an N-terminal leader (L) domain containing two subdomains of XL and L and four calpain-inhibitory domains also known as inhibitory repeats, each of which constitutes three subdomains of A, B and C. Exon 6, the largest exon of the ovine *CAST* gene, encodes L domain of *CAST* protein.

the ovine *CAST* gene. In some studies, the significant associations were observed between the genetic variability within the ovine *CAST* gene and growth traits (Byun et al., 2008; Chung and Davis, 2012; Greguła-Kania, 2012). Zhou et al. (2007) identified five unique SSCP patterns, representing five different haplotypes, and also six new SNPs in the exon 6 and its intron boundary region of the ovine *CAST* gene. However, all five sheep breeds investigated in the study of Zhou et al. (2007) were nonfat-tailed (thin-tailed) and no investigations have concentrated in this locus on the fat-tailed sheep breeds. Also no researches have focused on the possible impact of the SNPs identified in the ovine *CAST* gene on the physicochemical properties and structure of *CAST* protein.

Fat-tail and its modification are very important in the countries which graze fat-tailed sheep breeds. Recently, Moradi et al. (2012) performed a genome-wide scan using ~50,000 single nucleotide polymorphisms (SNPs) in an attempt to identify genomic regions associated with fat deposition in fat-tailed and thin-tailed breeds. They contrasted thin- and fat-tailed breeds in two independent experiments using either Iranian or ovine HapMap genotyping data and were able to identify three novel regions associated with fat deposition located on chromosomes 5, 7 and X in thin- and fat-tailed sheep breeds. The regions located on chromosomes 5 and X were associated with increased homozygosity in the fat-tailed breeds.

Interestingly, *CAST* gene is located on chromosome 5, a chromosome including one of the three genomic regions associated with fat deposition, of sheep (Moradi et al., 2012).

So, the genetic difference of *CAST* gene between fat-tailed and thin-tailed sheep breeds might be due to the effect of *CAST* gene on fat deposition, displaying the linkage disequilibrium (LD) of this gene with quantitative trait loci (QTL) associated with fat deposition in chromosome 5 of sheep (Moradi et al., 2012). Hence, a study on genetic variability of the *CAST* gene and its protein structure in the sheep with different fat metabolisms could be highly desirable.

With this purpose in mind, genetic variation in the entire exon 6, the largest exon of the ovine *CAST* gene, and partial introns 5 and 6 of the *CAST* gene was investigated in the four sheep breeds with different fat metabolisms (fat-tailed, medium fat-tailed and thin-tailed) (Fig. 2) using PCR-SSCP analysis and DNA sequencing. Frequency difference of haplotypes between fat-tailed and thin-tailed breeds was analyzed using "t" test to identify the possible breed-specific haplotypes. Also a protein sequence and structural analysis were performed to predict the possible impact of amino acid substitutions on physicochemical properties and structure of the *CAST* protein.

2. Materials and methods

2.1. Animals

In order to have broad representation of sheep breeds, 487 animals (from both sexes with different ages) belonging to four ancient Iranian sheep breeds from four different provinces of Iran were analyzed.

These breeds involved Lori-Bakhtiari (n = 243: 128 male and 115 female) from Shooli Breeding Station in Chaharmahal and Bakhtiari province, located in the south western part of Iran close to Zagros Mountain Range (situated at longitude between 49° 30′ and 51° 26′ E, latitude between 31° 09′ and 32° 48′ N and altitude of 2040 m above sea level), Chall (n = 29: 14 male and 15 female) from Qazvin Industrial Abattoir in Qazvin province, located in north western part of Iran close to Elburz Mountain Range (situated at longitude of 48° 45′ to 50° 50′ E, latitude of 35° 37′ to 36° 45′ N and altitude of 1278 m above sea level), Zel breed (n = 175: 55 male and 120 female) from Shirang Breeding Station and Zel-Atabay cross-bred (n = 40: 26 male and 14 female) from Gorgan Industrial Abattoir in Golestan, one of the northern provinces of Iran, located in south east of Caspian Sea and north of Elburz Mountain Range (situated at longitude of 53° 57′ to 56° 22′ E, latitude of 36° 30′ to 38° 08′ N and altitude of 174 m above sea level) (Fig. 2). It should be mentioned that Zel breed was also grazed in another northern province of Iran, Mazandaran, located in south of Caspian Sea and north of Elburz Mountain Range (situated at longitude of 50° 34′ to 54° 10′ E, latitude of 35° 47′ to 36° 35′ N and altitude of 54 m above sea level); (Fig. 2) but, in this study, animal sampling of Zel breed was only performed in Golestan province, Iran.

These breeds had different fat metabolisms: Lori-Bakhtiari and Chall are fat-tailed breeds, Zel-Atabay cross-bred is medium fat-tailed and Zel is thin-tailed breed (Fig. 2).

2.2. DNA isolation

Biological samples of these animals were used to extract Genomic DNA from blood (n = 428) using the salting out procedure described by Miller et al. (1988) and meat (n = 59) using DNA Extraction Kit according to the manufacturer's protocol (BioTech, Daejeon, China).

2.3. PCR primers and amplification

A region of the ovine *CAST* gene spanning over a part of intron 5, complete exon 6 and a part of intron 6 was amplified using a set of forward (5′-GTTATGAATTGCTTTCTACTC-3′) and reverse (5′-ATACGATTGA GAGACTTCAC-3′) primers designed by Zhou et al. (2007). PCR was carried out from 50 ng of genomic DNA in a final volume of 20 μl containing 1× reaction buffer, 200 μM dNTP, 1U of Taq DNA polymerase, 2.5 mM $MgCl_2$, and 0.5 μM of each primer and ddH_2O. PCR reactions were performed by a Genecycler (Bio-Rad, Hercules, CA, USA) with the following thermo-cycling profile: initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. The final extension was 5 min at 72 °C. PCR products were visualized by electrophoresis on 2% agarose gel using 1× TAE buffer and staining with 200 ng/ml of ethidium bromide.

2.4. Single-strand conformational polymorphism analysis

For genotyping the *CAST* locus, 6 μl of each PCR product was diluted with 12 μl of loading dye that included 98% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturation at 95 °C for 10 min, the samples were immediately placed on ice and then loaded on 18–20 cm, 12% acrylamide:bisacrylamide (37.5:1) gels. The mixture was electrophoresed using Protean II xi cells (Bio-Rad, Hercules, CA, USA) in 0.5× TBE buffer for 20 h at 300 V in a 10 °C room with 4 °C water circulating through the cell core. The gels were silver-stained according to the method described by Bassam et al. (1991).

2.5. DNA sequencing

For each of the ovine *CAST* variants identified by PCR-SSCP, PCR products of two animals were re-amplified in total volume of 25 μl followed by purification from the gel using Accuprep® PCR purification kit (Bioneer, Daejeon, Korea). Sequencing was performed in both directions with forward and reverse primers using ABI 3730 XL DNA Analyzer (BioNeer, Daejeon, South Korea).