Effects of genetic variants for the *calpastatin* gene on calpastatin activity and meat tenderness in Hanwoo (Korean cattle)

Hoyoung Chung [a,b,*], Michael Davis [c]

[a] *Animal Genetic Improvement Division, National Institute of Animal Science, Seonghwan, Cheonan 333801, Republic of Korea*
[b] *Bovine Functional Genomics Laboratory ARS/USDA, 10300 Baltimore, Beltsville, MD 20705, USA*
[c] *Department of Animal Sciences, The Ohio State University, Columbus, OH 43210, USA*

ABSTRACT

This study was designed to investigate the effects of calpastatin genotypes determined by PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) on calpastatin activity (CAC) and Warner–Bratzler Shear Force (WBS). Longissimus muscles were prepared from 379 Hanwoo bulls aged approximately 20 months. The selection of PCR primers was based on exons (27 and 28) of the bovine calpastatin cDNA sequences, and genetic variants were detected by SSCP analysis using *Taq I* restriction enzymes. Sequencing analysis confirmed 4 restriction sites (nucleotide positions 52, 67, 796, and 1369), and a genetic variant was verified at a nucleotide position 641 (C/T substitutions) based on sequences (AF281256). The CAST28 genotypes showing allele frequencies of C (0.429) and T (0.571) were significantly associated with CAC and WBS. A significant positive residual correlation (r = 0.121, P = 0.02) between CAC and WBS was obtained.

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1. Introduction

With the development of molecular genetics, new technologies searching genetic variants make it possible to find genetic relationships among economically important traits of domestic animals (Hediger, 1991) and to identify genetic or environmental effects even though most quantitative trait loci (QTLs) are controlled by polygene. In addition, studying candidate genes can be a powerful tool in the area of genetic selection because DNA polymorphisms will be potentially useful markers for quantitative traits (Soller & Beckmann, 1983). If specified genetic variants of marker genes explain phenotypic variations, the improvement of animals will be accelerated, and selection programs will be more efficient. Therefore, a marker typing process will provide broad knowledge about the genome, accuracy for identifying specific genes with important production traits, and diagnosis of genetic diseases of farm animals. However, research is still required for genetic selection using specified genes from the entire genome due to the difficulty of separating marker effects from environmental effects.

Consumer's desire for the prediction of meat tenderness is one of the major issues facing the beef industry because meat tenderization during the postmortem period is highly variable between carcasses. Therefore, studies of biochemical mechanisms for muscle breakdown are essential at the molecular level, issuing genes related to meat tenderness. It has been reported that the calpain family is mainly responsible for improvements of meat tenderness during postmortem storage (Koohmaraie, 1994), suggested by the results of degradation and weakening of the myofibril proteins near the Z-disks (Kendall, Koohmaraie, Arbona, Williams, & Young, 1993; Van den Hemel-Grooten et al., 1997). *Calpastatin*, which is an endogenous inhibitor (EC 3.4.22.17, Ca^{2+} dependent cysteine proteinase), plays a central role in regulation of the calpain activities (Forsberg, Ilian, Ali-Bar, Cheeke, & Wehr, 1989; Murachi, 1983; Murachi, Tanaka, Hatanaka, & Murakami, 1981) and is considered to be one of the major modulators for calpains. In addition, both *calpastatin* and *calpain* have a domain IV, which contains four EF-hand structures consisting of single helix-loop-helix (Raser, Buroker-Kilgore, & Wang, 1996; Sorimachi, Amano, Ishiura, & Suzuki, 1996) structures and is responsible for Ca^{2+} binding in the intracellular environment (Kawasaki, Emori, & Suzuki, 1993; Maki et al., 1989). However, it is not clear whether *calpastatin* inhibits the binding of *calpain* to cell membranes by a regulatory inhibition site or the inhibiting sequences (Kawasaki et al., 1993). Thus, genetic variants in genomic regions corresponding to the calcium binding domain, which essentially regulates calpastatin activity, may account for variation of meat tenderness. Furthermore, *calpastatin* may affect proteolysis of myofibrils due to the regulation of calpains that initiate postmortem degradation of myofibril proteins (Goll, Thompson, Taylor, & Zaleweska, 1992; Huff-Lonergan et al., 1996). Therefore, this study was carried out to investigate effects of the calpastatin genotypes on CAC and WBS, to provide information that will increase the accuracy of selection and improve rates of genetic progress regarding meat tenderness.

* Corresponding author at: Animal Genetic Improvement Division, National Institute of Animal Science, Seonghwan, Cheonan 333801, Republic of Korea. Tel.: +82 41 580 3354; fax: +82 41 580 3369.

E-mail addresses: chung133@korea.kr, hoyoung.chung@ars.usda.gov (H. Chung).

2. Materials and methods

2.1. Sample preparation

A total of 379 purebred Hanwoo (Korean cattle) bulls born at Heongsung in Korea from 2005 to 2007 were randomly selected. This experiment was approved by the ethics and welfare committee of the National Institute of Animal Science (NIAS). Animals were fed a diet formulated to meet nutrient requirements according to NRC (1985), and calves were weaned at an average age of 90 days. Animals were slaughtered at a packing facility in Heongsung, and longissimus muscles were removed from carcasses of bulls aged 20 months. Steaks were cut approximately 5 cm thick from the longissimus muscle between the 12th and 13th rib. Steaks were vacuum packed for aging for 14 days at 3 °C and then stored at −20 °C for 7 days in order to standardize the origin and aging lengths that were not considered in the statistical analysis (Destefanis, Brugiapaglia, Barge, & Dal Molin, 2008).

2.2. Measurements of WBS and CAC

After frozen samples were thawed at 4 °C for 24 h, steaks were cut into 2.54 cm thick pieces and cooked to a final internal temperature of 71 °C for 12 min on a grill. After cooking, samples were equilibrated at room temperature for 4 h, and cores, 1.27 cm in diameter, removed. WBS was measured on six to 10 cores from each steak using the Warner–Bratzler Shear equipment, with a crosshead speed of 200 mm/min, reporting in kilograms.

Five grams of muscle tissue, refrigerated at 4 °C for 24 h, was used to assay calpastatin activities with 25 ml of extraction buffer (150 mM Tris–HCl, 5 mM EDTA, pH 8.3, 0.2% beta - MCE) and homogenized twice at 500 rpm for 30 s (Shackelford et al., 1994). The homogenate was centrifuged for 30 min at $12,000 \times g$, and the volume of the supernatant recorded after filtration. The supernatant was heated for 15 min at 98 °C and cooled on ice for 15 min. After centrifugation for 1 h at $1500 \times g$, the heated samples were assayed for activity with a volume needed for 80% inhibition of known m-calpain activity. The sample volume was made up to 1 ml using the elution buffer (20 mM Tris–HCl, pH 7.35, 0.5 mM EDTA, 0.2% MCE). Twenty microliters of partially purified lung m-calpain were added along with 1 ml of assay media (100 mM Tris, pH 7.5 with 1 N acetic acid, 1 mM NaN3, 0.5% casein and 0.2% MCE) followed by 50 μl of 200 mM CaCl₂. The reaction took place for 1 h at 25 °C and was stopped by addition of 2 ml of 5% trichloroacetic acid (TCA). After centrifugation at $1500 \times g$ for 30 min, A_{278} was determined for the supernatant.

2.3. Design of primers

Primers were selected based on the bovine calpastatin cDNA (Killefer & Koohmaraie, 1993) and focused on exons between 27 and 28 (GenBank accession number L14450) in a calcium binding domain. The forward and reverse primers were GTGCC CAGGA CCCCA TTG (positions at 1941–1958) and AGCAG GCTTC TTGTC TTTGT C (positions at 2024–2044), respectively. Because of the large fragment size (1466 bp) that is not appropriate for direct sequencing analysis, inner primers amplifying approximately 308 bp were designed to complete sequences. The inner forward and reverse primers were GGGGC TTCTC CTCTG GCAT (nucleotide positions at 650–668) and GACAC TCATT TCACT TTTCC TCCT (nucleotide positions at 833–858), respectively, based on AF281256.

2.4. PCR-SSCP procedure

Two microliters of 10 X reaction buffer (10 mM Tris, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂), 25 mM dNTP, 10 pmol of each primer, 50 ng genomic DNA, and 1 unit of Taq DNA polymerase (Gibco BRL, Grand Island, N.Y.) in a final volume of 20 μl were used. PCR conditions were 95 °C for 2 min for the first cycle, 1 min at 94 °C for denaturation, 1 min at 57 °C for annealing, and 1.5 min at 72 °C for polymerization, with a total of 35 cycles (Perkin Elmer Cetus, Norwalk, CT). Because large fragments were not efficiently analyzed in the SSCP (single strand conformational polymorphism) analysis, the amplified products were digested with Taq I restriction enzyme at 65 °C for 2 h in 10 μl of reaction solution consisting of 2.7 μl of distilled H₂O, 1 μl of enzyme buffers, 0.3 μl (3 units) of restriction endonucleases, and 6 μl of PCR products. PCR-SSCP analysis was performed with the digested products (8 μl) with the same volume of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol FF, 90% Formamide), and the separation was conducted on 0.5 X MDE gels at 250 V and 10 °C for 16 h. Genetic variants were visualized using ethidium bromide under the UV transilluminator.

2.5. Confirmation of PCR product and sequence alignment

DNA bands showing different migration patterns in the SSCP analysis were selected and purified using the Nucleotrap gel purification kit (Clontech, Palo Alto, CA). PCR cloning was conducted for the amplified fragments using a pGEM T easy cloning vector (Promega, Madison, WI). Insertion of the PCR fragments into the vector was carried out at 4 °C overnight, and transformation was conducted with JM109 competent cells (Promega, Madison, WI). After purification of plasmid DNA using the Mini-plasmid prep kit (Qiagen, Valencia, CA), sequencing analyses were performed with an ABI 3730XL Genetic Analyzer. The blast search confirmed correctly amplified segments as the bovine calpastatin gene. After confirming genetic variants, inner primers were used for the direct sequencing analysis to make up sequence gaps, and the sequencing analysis was duplicated for both PCR and sequencing reactions to minimize base calling errors. All individual sequences were aligned with the SEQMAN program in DNAstar version 6.0 to verify single nucleotide polymorphisms (SNP).

2.6. Statistical analysis

Allele frequencies were calculated, and least squares means with standard errors determined to investigate genotype effects on WBS and CAC. The analysis of variance estimated residual correlations with the Statistical Analysis System (SAS, 1988). A statistical model included a fixed effect of calpastatin genotype and age of bull as a covariate. Statistical analysis detected significant mean differences based on genotypes with general linear model produces and Fisher's least significant difference tests with a comparison error rate of 0.05.

3. Results and discussion

3.1. Genetic variants

PCR amplified the targeted genomic region approximately 1466 bp that contained exons 27 and 28 including an intron region. After finding clear genetic variants by the SSCP analysis using a restriction enzyme (Taq I) to increase separation abilities of genetic variations (Fig. 1), PCR products of each animal showing different genotypes were cloned with pGEM T easy vectors to verify genomic sequences. After verification of sequences, the nucleotide analysis confirmed the sizes of 5 restriction fragments (15, 52, 97, 573, and 729 bp) by the Taq I enzyme and verified 4 restriction sites (nucleotide positions at 52, 67, 796, and 1369 bp) based on sequences (AF281256), which was submitted into GenBank. The verification of the SNP location at nucleotide position 641 confirmed substitutions