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Investigation of biochemical changes of the ovine calpain 3 exon-10 polymorphism

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ABSTRACT

Calpain 3 (CAPN3) is a tissue specific calpain, and its mRNA is the most expressed calpain isoform in skeletal muscles. Many mutations and polymorphisms within the human CAPN3 gene have been reported and related to limb-girdle muscular dystrophy. Several reports link CAPN3 polymorphisms and meat quality. An association between three allele variants in exon-10 of ovine CAPN3 and the yield of fat trimmed meat cuts has been reported. This research investigated the biochemical significance of polymorphic variation in CAPN3. CAPN3 mRNA sequences were obtained from muscle samples collected from lambs which were homozygous for each of the three alleles. Four single base substitutions were found besides those in exon-10, but none of them, including the variations within exon-10, caused a change in amino acid sequence. The expression of CAPN3 mRNA and the amounts of CAPN3 protein were also compared among genotypes, and no significant differences were found. These results suggest that the reported association of specific allele variants within CAPN3 exon-10 to phenotype variations were not direct effects of CAPN3 polymorphisms. Interspecies analyses of the CAPN3 sequences indicated that the sequence reported here is more likely to be the correct common ovine CAPN3 sequence than the reference sequence.

1. Introduction

Calpains are calcium-dependent, cysteine proteases that have been implicated in a variety of physiological and pathological processes. They form a superfamily with numerous structurally related isoforms in organisms ranging from microorganisms to mammals [1]. Two categories occur in mammals, ubiquitous and tissue-specific isoforms. Ubiquitous calpains are expressed in all cell types. The major ones are calpain 1 (μ-calpain) and calpain 2 (m-calpain) which differ in their calcium sensitivity *in vitro* [1,2].

This study focusses on a tissue specific calpain, calpain 3 (CAPN3), first reported in 1989 [3] and also called p94 in reference to its molecular mass, or nCL-1 for "novel calpain large subunit". Northern blot analysis detected CAPN3 mRNA only in skeletal muscle samples [3]. Moreover, it was approximately 10-fold more abundant in skeletal muscles than mRNA for calpains 1 and 2 [4]. Therefore, CAPN3 was designated as a skeletal muscle specific calpain [3], the first tissue specific calpain. The structure of CAPN3 is similar to the large subunits of calpains 1 and 2 with four domains; I, II, III and IV, and three CAPN3 specific sequences called NS, IS1, and IS2 located at the N-terminus, in the DII protease domain, and between the DIII and DIV Ca^{2+} binding domain, respectively [5].

An important characteristic of CAPN3 is its rapid autolysis at physiological calcium concentrations which makes purification and characterisation extremely difficult. The human CAPN3 gene has been linked to limb-girdle muscular dystrophy and many mutations and polymorphisms have been reported [6]. Polymorphisms of CAPN3 in livestock animals have also been identified, and possible associations with muscle growth suggested [7–11].

The roles of CAPN3 in muscle yield were analysed on the basis of a CAPN3 gene marker study in livestock. Several reports have proposed the association between CAPN3 polymorphism and phenotype variations within muscle yields based on statistical data of muscle traits from a range of mixed genotypes of animals. In chicken, single nucleotide substitutions were identified in CAPN3 intron-8 and exon-10, and associations between these polymorphisms and carcass traits such as body weight, carcass weight, and meat yields of breast and leg were identified [9]. Another group reported an association between allelic variants within CAPN3 exon-11/12 and birth weight in sheep [8]. Associations between

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specific allele variants (A, B and C-alleles) within CAPN3 exon-10 [7] and increased proportions of shoulder yield [11] and leg yield in sheep [10] have been suggested. Bickerstaffe, Gately and Morton [10] reported that the genotypes containing A-allele had 2.5% greater leg weight and 3.2% greater foreshank weight compared with the other genotypes. The genotypes containing C-alleles had an increase of 1.8% of leg weight. The presence of a B-allele did not show any significant link to the product yield. Although these associations between specific allele variants within CAPN3 exon-10 and the yield of fat trimmed meat cuts were reported, no information was given as to how genetic variation could influence muscle size.

In this study, nucleotide variation within the CAPN3 gene from lambs homozygous for allelic variants (allele-A, B and C designated by variation in exon-10) was analysed to investigate possible mechanisms that could link specific allele variants to increased meat yield. Although the allelic variants within exon-10 do not change the amino acid sequence, they may be linked to other polymorphisms which influence meat yield. Therefore, homozygote samples of each allele (AA, BB and CC) were analysed, and the CAPN3 mRNA sequences were obtained. During this study it became apparent that the published ovine CAPN3 consensus sequence contains sequences that did not match with the obtained sequence. So an interspecies analysis was performed. It has been suggested that synonymous mutations (mutations that alter the coding DNA/RNA sequence without affecting the amino acid sequence of the protein produced) within exon-10 may affect CAPN3 mRNA stability [12]. Therefore CAPN3 mRNA expression and CAPN3 protein levels were analysed to investigate the possible mechanisms underlying the associations that have been observed between CAPN3 allelic variants and meat yield.

2. Methods

2.1. Muscle sample collection

Genotypes of one year old Coopworth lambs were identified by SSCP gel electrophoresis. The frequency of C-allele is very low [7] and it was difficult to find CC-genotype animals randomly. Therefore CC-genotype lambs were traced from the farm with a history of CC-genotypes and two CC-genotype lambs were found. Two of CC-genotypes and some AA, BB, and AC-genotypes were slaughtered at the Johnstone Memorial Lab, Lincoln University in accordance with the New Zealand Animal Welfare Act (1999). Muscle samples were collected from *Longissimus thoracis et lumborum* (LTL), *Gracilis* (G), *Semimembranosus* (SM), and *Semitendinosus* (ST) and cut into 2.5 × 2.5 × 2.5 cm cubes. These were wrapped in aluminium foil, snap-frozen by immersion in liquid nitrogen and kept at −80 °C prior to grinding in a mortar and pestle under liquid nitrogen. The ground frozen muscle samples were stored at −80 °C until required.

2.2. RNA extraction and cDNA synthesis from skeletal muscle samples

Total RNA was extracted from ground frozen muscle samples using RNeasy mini kits (Qiagen, Hilden Germany) following the manufacturer's protocol with some modifications. Sample disruption was enhanced by incubation with 33% (w/v) proteinase-K (New England Biolabs, MA, USA) for 20 min at 55 °C [13]. Traces of DNA were removed from the extracted RNA by the addition of 2 μL of DNase-1(Boehringer Ingelheim, Ingelheim, Germany) and 1 h incubation at room temperature followed by storage at −80 °C. Copy DNA (cDNA) was synthesised by reverse transcription from the extracted RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA, USA) with oligo-dT primers. Synthesised cDNA was stored at −20 °C until required for PCR analysis.

2.3. Sequencing of CAPN3 of each exon-10 genotype

The CAPN3 sequence was obtained from cDNA of four overlapping fragments of CAPN3 using four different primers sets (Table 1) designed from the published ovine CAPN3 mRNA sequence (AF087570, CDS 46-2514bp), or primers F01 and F02 to amplify CAPN3 exon-10 [7]. Primer sets and products sizes are summarised in Fig. 1.

Amplifications were performed in 20 μL reactions containing 1 μL of 2.5 mM dNTP, 0.5 μL of 5 μM forward primer, 0.5 μL of 5 μM reverse primer, 2 μL of 10x PCR buffer, 4 μL of 5x Q-solution, 0.8 μL of 25 mM $MgCl_2$, 0.2 μL of 5units/μL *Taq* DNA polymerase (*Taq* DNA polymerase kit, Qiagen, Hilden, Germany), 10 μL dH_2O and 1 μL of cDNA. After initial denaturing at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 30sec, annealing at 55 °C for 30sec, and extension at 72 °C for 1 min were performed followed by final extension steps at 72 °C for 10 min and at 20 °C for 1 min.

Amplicons were loaded onto 1.2% agarose gels for electrophoresis at 90 V for 1 h. Target PCR amplicons were cut out and collected into tubes for DNA extraction using Mini elute gel extraction kits (Qiagen). Purified DNA samples were sequenced by the Allan Wilson Centre Genome Service (AWCGS), Massey University, Palmerston North, New Zealand. Amplified DNA samples were prepared from four different muscles collected from two animals of each genotype (AA, BB and CC). The quality of results varied depending on the purity of sample DNA. Therefore, PCR, DNA purification, and DNA sample preparation were repeated until confirmed continuous results and overlaps were obtained. Results were interpreted using GeneDoc (http://genedoc.software.informer.com) and Chromas Lite (Technelysium) to locate positions based on the published ovine CAPN3 sequence (AF087570).

Because of the low frequency of the C-allele, AC heterozygote samples were also sequenced as an alternative way to identify the C haplotype gene sequence. The AC-genotype should show a mixture of A and C sequences. Once the AA-genotype sequence had been determined, sites in AC that were heterozygous were used to identify the C sequence as the alternative to the A sequence at these positions. To confirm this approach, exon-10 PCR products of AC-genotypes were also sequenced.

2.4. RT-PCR for CAPN3

The relative quantification of RNA for each genotype was determined by the standard curve method using SYBR-green. Total RNA was extracted from four of AA-genotypes and two of BB, CC, and AC-genotype ground frozen muscle samples, and cDNA was synthesised from the extracted RNA as explained previously, except the amount of RNA was measured and 1 μg of RNA was used for this experiment. Because the association of A and C-alleles toward meat yield has been reported [10], the standard curve was created from AC-genotype samples.

The cDNA of AC-genotype was diluted with diethylpyrocarbonate (DEPC) treated water from 10^{-1} to 10^{-5} to create a standard curve. The cDNA of the other test samples (AA, BB and CC-genotypes) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were diluted to 10^{-3} to fit within the standard curve. PCR cycling started with an initial heating at 94 °C for 10min, then cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, were repeated 40 times. The fluorescence emission caused by SYBR-green binding to PCR products was monitored and Ct values determined with the i-Cycle data analysis program.

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