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Identification of *Ovis aries* Gelsolin isoform b, a candidate gene for milk quality

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ABSTRACT

In this report, we describe the identification of the complete ovine gelsolin (*GSN*) isoform b cDNA. We sequenced exons 12 and 13 and parts of introns 11, 12 and 13 in 263 sheep of three breeds. We found 11 novel SNPs, of which 2 were missense mutations. *GSN* is a Ca^{2+}-dependent actin-regulatory protein that modulates actin assembly and disassembly. Because actin is involved in the regulation of intracellular lipid metabolism, particularly in the formation of cytoplasmic fat droplets and in the transport of lipid constituents, we tested the hypothesis that different *GSN* genotypes might influence actin activity and, consequently, milk yield and quality. An association analysis was performed between each SNP and the following traits, which were evaluated in 464 recorded lactations of the same sheep: milk yield, fat and protein content, as well as somatic cell score. Significant associations ($P = 0.02$) were found between three SNPs in intron 12 and fat content in the Altamurana breed. For two of the SNPs, the effect was also noted in the Gentile breed ($P = 0.09$). Suggestive associations were also found between the SNPs and other traits, albeit not in all of the breeds. The three SNPs that affected fat content were located in the consensus sequences of binding sites for the following transcription factors: Tal-1 alpha, Evi-1 and Egr-3. Therefore, we hypothesised that the incorrect modulation of actin assembly occurs in one of the *GSN* genotypes, with a particularly evident effect on fat content.

1. Introduction

Gelsolin is representative of a class of actin-modulating proteins found from lower eukaryotes through mammals. Gelsolin is a Ca^{2+}-dependent actin-regulatory protein that modulates actin assembly and disassembly and regulates cell motility through the modulation of the actin network (Kusano et al., 2000). Actin is a globular, multifunctional protein found in all eukaryotic cells and is the monomeric subunit of two types of cell filaments: microfilaments, which are among the three major components of the cytoskeleton; and thin filaments, which are part of the contractile apparatus in muscle cells. The cytoplasmic and secreted forms of gelsolin are the most potent actin filament-severing proteins identified to date (Spinardi and Witke, 2007). After severing a filament, gelsolin remains attached to the rapidly growing end of the filament, forming a cap, so that a large number of very short actin filaments that cannot reanneal or elongate are generated. In this way, the actin network is disassembled. The two isoforms of gelsolin are isoform a, which is a 782-amino acid secretory (plasma) protein, and isoform b, which is a

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731-amino acid cytosolic protein. The former isoform contains a 27-amino-acid signal sequence at the N-terminus of the protein. Both isoforms are encoded by a single gene and differ as a result of alternative initiation sites/splicing (Pottiez et al., 2010). Gelsolin is an 82-kDa protein composed of six homologous subdomains referred to S1–S6; all of these domains are important for the folding and enzymatic activity of the protein (Dos Remedios et al., 2003).

Signorelli et al. (2010) produced evidence of the differential protein expression of gelsolin isoform b in the mammary glands of sheep that differed highly in terms of milk production traits. Addis et al. (2011) showed a significant overrepresentation of gelsolin isoform b protein in samples of milk fat globules extracted from the milk of sheep with infectious mastitis.

Because evidence of an ovine *gelsolin* transcript was absent from GenBank and because previous works had focused on the gelsolin protein, the aims of our work were to identify the following: (1) the complete sequence of the ovine transcript of *gelsolin* (*GSN*) isoform b; (2) causative variants in the complete sequence of the mRNA; and (3) additional genomic variants, particularly in the flanking intronic regions of the exons in which missense mutations were detected, with the aim of performing association analyses with milk traits in dairy sheep.

2. Materials and methods

2.1. Animals and phenotyping

Within the GENZOOT project funded by the Italian Ministry of Agriculture, this study was conducted at the Segezia Experimental Station of the Italian Agricultural Research Council (CRA) for two years, from December 2010 to July 2012. A total of 263 ewes of three breeds (155 Altamurana, 62 Gentile and 46 Sarda) were maintained on the same experimental farm and were traditionally managed. The ewes were housed on straw litter in boxes with outdoor pens. The ewes were offered 0.5 kg of a pellet concentrate and 1.8 kg of vetch/oat hay daily, provided as 2 meals per day (at 07:30 and 15:30 h). Water was available ad libitum. The ewes were milked twice daily (at 07:00 and 15:00 h) from weaning (mid December) until drying off (the end of July), using pipeline milking machines (Alfa Laval Agri, Tumba, Sweden). Monthly, at both daily milking of each ewe, the individual milk production was measured by means of graduated measuring cylinders attached to individual milking units. The milk samples were collected and transported to the laboratory at 4 °C in transport tankers. Using a mid-infrared spectrophotometer (MilkoScan FT120, Foss Electric, Hillerød, Denmark), the following analyses were performed: fat, total protein, and lactose contents. The somatic cell count was evaluated using a disk cytometry cell counter (Fossomatic 90, Foss Electric), following the regulations of the International Committee for Animal Recording (ICAR). The milk yield, fat and protein content, as well as the somatic cell score and length of lactation, were recorded for 464 lactations.

2.2. RNA isolation and Quantification

RNA was extracted from the milk somatic cells of 6 sheep (Comisana breed). Milk somatic cells were isolated from 50 mL freshly collected milk by centrifugation at 2000 g for 5 min at 4 °C, and EDTA was added to a final concentration of 0.5 mM at pH 8.0.

The fat layer on the top of the supernatant was removed with a sterile pipette tip, and the skim milk was discarded. The cell pellet was washed with 8 mL buffer (0.5 mM EDTA pH 8.0 in Dulbecco's PBS). After centrifugation, the somatic cell pellet was resuspended in 1 mL QIAzol® lysis reagent (Qiagen, CA, USA) and stored at −80 °C.

Total RNA was extracted from the stored somatic cells using RNeasy® Lipid Tissue (Qiagen), following the manufacturer's protocol, including on-column DNase digestion.

The RNA quality and quantity were assessed with RNA Nano Chips® on an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA).

2.3. DNA isolation and quantification

DNA was extracted from 5 mL blood with the NucleoSpin® Blood kit (Macherey-Nagel, Germany), following the manufacturer's protocol. The quantity and purity of the DNA were evaluated using the NanoPhotometer® Pearl (Implen GmbH, Germany), and the integrity was determined by electrophoresing 250 ng on an 0.8% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., CA, USA).

2.4. Primer design

The *Bos taurus GSN* genomic sequence (accession NC_007306–Chromosome 8 Btau_4.6.1) showed that the gene produced two transcripts that differed both in the 5′ UTR sequence and in the number of exons: variant 1, corresponding to isoform a, with 17 exons (accession NM_001113284) and variant 2, corresponding to isoform b, with 16 exons (accession BC104560). Isoform b of the *Bos taurus GSN* gene was submitted to a BLAST search against GenBank expressed sequence tags (ESTs) from sheep, and a putative ovine cDNA 2373 base pairs in length was inferred. Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to design eight primers for this sequence to amplify and sequence the full-length cDNA (id. 1–8, Table 1), and six primers were designed to amplify different regions of the DNA (id. 9–14, Table 1).

2.5. RT-PCR

cDNA was synthesised from 1.0 μg of total RNA using RevertAid™ Premium Reverse Transcriptase (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions, on the GeneAmp® PCR System 9700 (Life Technologies, CA, USA). To amplify the complete *gelsolin* isoform b transcript, 50 ng of cDNA was used in a total reaction volume of 50 μL using Herculase II Fusion DNA Polymerase (Agilent Technologies), according to the manufacturer's instructions for cDNA samples. The forward and reverse primers are shown in Table 1 (id. 1 and 8, respectively).

PCR was performed on the iCycler iQ™ (BioRad Laboratories, CA, USA) with the following optimised cycling parameters: an initial denaturation at 95 °C for 2 min; 35 cycles of denaturation (20 s at 95 °C), annealing (20 s at 58 °C) and extension (72 °C for 3 min and 30 s); and a final extension at 72 °C for 20 min.

2.6. cDNA and DNA sequencing

To sequence the complete cDNA primers, id. 1–8 in Table 1 were used. The amplicons and sequencing products were purified with the Agentcourt® AMPure® XP and Agentcourt® CleanSEQ® kits (Beckman Coulter, IN, USA), respectively, according to the manufacturer's instructions.

The purified products were sequenced on an Applied Biosystems 3500 Genetic Analyzer (Life Technologies, CA, USA), and the sequences were processed using the Sequencing Analysis v 5.3.1 software (Life Technologies).

2.7. DNA Genotyping

To genotype the nonsynonymous mutations identified in the cDNA, we amplified three DNA fragments spanning exons 11–12, 12–13 and 13–14 (primers id. 9–14, Table 1) to obtain the intronic sequences. We used 50 ng of genomic DNA, 0.5 U of AmpliTaq® Gold DNA Polymerase (Life Technologies), a final concentration of 2 mM of $MgCl_2$, 1× Gold buffer, 200 μM of each dNTP and 0.5 μM of each primer in a 20-μL total reaction volume. The following optimised cycling parameters were applied: an initial denaturation at 95 °C for 5 min; 35 cycles of denaturation (95 °C for 30 s), annealing (20 s) and extension (72 °C for 1 min and 30 s); and a final extension at 72 °C for 7 min.

The same primers were used for the DNA genotyping and the sequencing protocol (see above).