Short Communication: An association analysis between one missense polymorphism at the *SREBF1* gene and milk yield and composition traits in goats

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Key words: Goat *SREBF1* gene, milk fat content and composition

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Mots clés: Gène *SREBF1*de la chèvre, composition de la matière grasse du lait

Sterol regulatory element binding transcription factor 1 (SREBF1) plays a key role in energy homeostasis by regulating the expression of lipogenic genes (Brown and Goldstein 1997). At a cellular level, inactive SREBF1 remains anchored to either the nuclear envelope or the endoplasmic reticulum, while the N- and C-terminal ends localize to the cytoplasm (Párraga et al. 1998). Low levels of cholesterol activate SREBF1 through the proteolytic cleavage of the N-terminal segment by site-1 and site-2 proteases (Shimano 2001). Subsequently, SREBF1 is translocated to the nucleus where it forms homodimers and upregulates the expression of as many as 30 genes involved in cholesterol and FA biosynthesis (Párraga et al. 1998; Shimano 2001). In lactating cows, measurement of *SREBF1* mRNA levels has shown that they increase after parturition, a finding consistent with the key role of this molecule in the coordination of the mammary gland lipogenic pathways (Harvatine and Bauman 2006; Rudolph et al. 2007; Viturro et al. 2009). The main goal of the current work was to characterize the genetic variability of the caprine *SREBF1* coding region and find out if it is associated with phenotypic variation of milk fat content and composition traits in dairy goats.

Total RNA was extracted from liver samples obtained from three Murciano-Granadina and two Malagueña

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goats following protocols reported by Zidi et al. (2008), and cDNA was synthesized with the ThermoScript RT-PCR kit (Invitrogen S.A., Barcelona, Spain). The amplification of the coding region and part of the 3′UTR of the goat *SREBF1* gene was performed with six primer pairs, following the protocols detailed at Supplementary Table 1. After enzymatic purification (ExoSAP-IT, Amersham Biosciences), PCR products were sequenced by using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reaction products were purified with the Montage SEQ_{96} cleanup kit (Millipore Corporation, Billerica, MA) and electrophoresed in an ABI PRISM 3730 DNA Analyser (Applied Biosystems, Foster City, CA).

Molecular analyses yielded the near-complete sequence of the coding region and part of the 3′UTR of the goat *SREBF1* gene, consisting of 3561 bp and encoding a 1139 amino acid protein (GenBank accession number: HM443643, Supplementary Fig. 1). Partial sequences had previously been submitted to GenBank, but assembled together they just covered half of the coding region of this gene (GenBank accession numbers: DQ487874, DQ455606, DQ480338 and DQ483057). Blast analysis of the goat *SREBF1* cDNA sequence showed that it was very similar to its bovine (96% identity), porcine (88%) and human (86%) orthologs. Functional domains were inferred according to the UniProtKB entry for human SREBF1 (http://www.uniprot.org/uniprot/P36956). The caprine SREBF1 consisted of an N-terminal transcription factor portion, including a basic region/helix–loop–helix/leucine zipper (bHLHZ) DNA-binding motif, a hydrophobic region with two membrane spanning regions, and a C-terminal regulatory segment. The helix-loop-helix (HLH) domain of SREBF1 displayed a 60–100 amino acid residue motif, encoding two amphipathic α helices separated by a variable loop that is essential for forming homodimers (Párraga et al. 1998). This motif is flanked at its N-terminal and C-terminal ends by a highly conserved basic region that binds to DNA, and a leucine zipper motif, respectively (Supplementary Fig. 1). Finally, it is worth highlighting the existence of an acidic transactivation domain, which it is fundamental to activate transcription, adjacent to a proline-rich region.

Alignment of goat *SREBF1* cDNA sequences allowed us to detect a c.353C > T missense polymorphism that involved a proline to leucine replacement at position 118 (Supplementary Fig. 2). A primer-extension based protocol was implemented to genotype it. Genomic DNA isolation and amplification reactions (PCR composition and thermal cycling) were performed as reported in Zidi et al. (2010), with the only exception of the annealing temperature ($T_{ann} = 64°C$). Primer Express software (Applied Biosystems, Foster city, CA) was employed to design primers to amplify the polymorphic region as well as to make the extension reaction (Supplementary Table 1). The primer-extension reaction was carried out by following manufacturer instructions and using the following thermal cycling profile: 25 cycles of 10 s at 96°C, 5 s at 50°C and 30 s at 60°C. Extension products were run in an ABI PRISM 3730 capillary electrophoresis device (Applied Biosystems, Foster City, CA) and analyzed with the ABI PRISM GeneMapper software v.4.0 (Applied Biosystems, Foster city, CA). In silico prediction of amino acid substitutions was carried out with the Polyphen software (Ramensky et al. 2002). This tool allows one to infer the functional consequences of an amino acid substitution by building a multiple alignment of the query protein sequence and related sequences and calculating a profile-matrix. Elements of this matrix (PSIC scores) are logarithmic ratios of the likelihood of given amino acid occurring at a particular position to the likelihood of this amino acid occurring at any position. Subsequently, PolyPhen calculates the absolute value of the difference between PSIC scores of both alleles in the variable site. If this difference is high (i.e., above 1.5), it means that the analyzed substitution is rarely or never observed in the protein family. According to this criterion, substitutions are classified as benign (do not involve a functional change) or possibly/probably damaging (they may involve a functional change).

Genotypic frequencies of c.353C > T polymorphism were calculated in a sample of 427 Murciano-Granadina goats. Frequencies were 0.39, 0.46 and 0.15, for CC, CT and TT genotypes, respectively (C and T allele frequencies: 0.62 and 0.38, respectively). Hardy-Weinberg equilibrium was assessed with an online Microsoft Excel tool available at http://www.tufts.edu/∼mcourt01/Documents (Court Lab-HW calculator file). This approach allowed us to demonstrate that genotype frequencies matched the Hardy Weinberg equilibrium expectation ($\chi^2 = 0.155$, P value: 0.693). The effects of this mutation were predicted in silico with the Polyphen software (Ramensky et al. 2002), obtaining a PSIC score difference of 2.55. This result suggested that this polymorphism might be likely damaging. With the aim of gaining additional insights into the biological effects of this substitution, we decided to investigate if the goat *SREBF1* genotype is associated with milk yield and quality traits.

In order to achieve this goal, we used as animal material two subsamples obtained from the 427 Murciano-Granadina population mentioned above (Badaoui et al. 2007; Zidi et al. 2010). Subsample 1 consisted of 116 goats distributed in three herds. These goats had records for dairy traits i.e., milk yield, milk protein, fat, lactose and dry extract contents and logarithm of the total somatic cell count (logSCC). The management of this population and phenotype recording are fully described in Badaoui et al. (2007). Subsample 2 was composed of 174 goats belonging to a single herd. For each goat, whole milk in the first of the two daily milkings was collected beginning 3 mo after parturition (average 97.7 d) and with a periodicity of

Table 1. Association between c.353C > T *SREBF1* genotype and dairy traits in Murciano-Granadina goats

Data available	CC	CT	TT	Total
Number of goats	114	135	41	290
Number of records	361	437	142	940
Traits	Least square means ± SE			*P* value
Milk yield (kg d^{-1})	1.70 ± 0.11	1.72 ± 0.11	1.80 ± 0.13	0.58
Protein (%)	3.61 ± 0.05	3.59 ± 0.05	3.65 ± 0.06	0.51
Fat (%)	5.74 ± 0.18	5.67 ± 0.18	5.67 ± 0.20	0.78
Lactose (%)	4.92 ± 0.04	4.92 ± 0.04	4.97 ± 0.04	0.36
LogSCC	2.71 ± 0.07	2.74 ± 0.07	2.73 ± 0.08	0.75

every 2 mo (65.5 d average sampling interval). Protein, fat, lactose, dry extract contents and somatic cells were measured with a CombiFoss 600 FC instrument, as reported by Zidi et al. (2010). A small sample of milk from each goat was freeze-dried and stored at −20°C until it was used for the gas chromatography analyses of FA. Samples were weighted before performing all milk FA composition analyses. Separation and quantification of the FA methyl esters were carried out using a gas chromatograph Agilent 6890N Network GS System (Agilent, Santa Clara, CA), as reported by Zidi et al. (2010).

As a whole, the number of goats with registers for dairy traits (milk yield, milk protein, fat, lactose and dry extract contents and logSCC) was 290 (116 goats from subsample 1 and 174 from subsample 2), while for milk FA composition, we had data from only 174 goats (subsample 2). A mixed model for repeated measurements (Littell et al. 1998) implemented with the SAS software (SAS 9.2 Institute, Inc., Cary, NC) was used to detect associations between *SREBF1* genotypes and traits under study. This model included *SREBF1* genotype, ordinal number of lactation, number of offspring born, month of lactation, season of sampling and herd as fixed effects; the random animal effect and the residual error term. Milk FA composition phenotypes were obtained in goats belonging to a single herd, so this fixed effect was not considered in the statistical model. In contrast, season of sampling was included as a fixed effect because there is a seasonal effect on lipid composition of plant pastures (Mel'uchová et al. 2008). Besides, logSCC was used as a covariate in the statistical model. In this sense Jensen (2002) suggested that udder health might affect milk FA composition.

As shown in Tables 1 and 2, the association analysis did not yield significant results. We just found a statistical tendency for omega-3 FA content. This latter result is consistent with the key role of SREBF1 in the synthesis of unsaturated FA. In this way, increased expression of SREBF1 in the mammary gland of rats has been shown to induce an augmentation in the expression of stearoyl-CoA desaturases (Rodríguez-Cruz et al. 2006). Interestingly, when comparing previous studies aimed to associate *SREBF1* genotype with fat content and composition (Hoashi et al. 2007; Ohsaki et al. 2009; Bartoň et al. 2010; Matsuhashi et al. 2011), contrary results were noticed. For instance, Hoashi et al. (2007) identified an association between a 84 bp-indel at intron 5 of the *SREBF1* gene and muscle monounsaturated FA content in Japanese Black cattle. Similarly, Bartoň et al. (2010) found an association between this indel and muscle C14:1 cis-9 FA content in Fleckvieh cattle. In contrast, Ohsaki et al. (2009) analyzed the segregation of the 84 bp-indel in two groups of Japanese Black cattle and, overall, they did not find any significant association with an array of seven carcass and fourteen FA composition traits (although significant associations for myristic, palmitic and elongation index were observed in one group of cows). Similarly, Matsuhashi et al. (2011) performed an association study between the aforementioned 84-bp indel and FA composition of the longissimus thoracis muscle and carcass and meat quality traits in 480 commercial Japanese Black cattle and did not find any significant relationship. Our study is closely aligned with the work carried out by Ohsaki et al. (2009) and Matsuhashi et al. (2011)

Table 2. Association between c.353C > T *SREBF1* genotype and milk fatty acid composition traits in Murciano-Granadina goats

Data available	CC	CT	TT	Total
Number of goats	71	80	23	174
Number of records	194	224	65	483
Trait[z]		Least square means ± SE		*P* value
SFA	76.15 ± 0.44	76.34 ± 0.42	76.56 ± 0.56	0.79
MUFA	19.86 ± 0.40	19.68 ± 0.38	19.79 ± 0.50	0.82
PUFA	4.15 ± 0.07	4.41 ± 0.06	3.97 ± 0.10	0.26
Ratio PUFA/MUFA	0.20 ± 0.008	0.21 ± 0.007	0.19 ± 0.01	0.40
Total CLA	0.72 ± 0.02	0.73 ± 0.02	0.68 ± 0.04	0.51
cis-9, *trans*-11 CLA	0.39 ± 0.03	0.40 ± 0.03	0.36 ± 0.04	0.61
C18:1 n9c	13.84 ± 0.25	14.03 ± 0.23	14.0 ± 0.38	0.79
Omega 3 FA	0.31 ± 0.007	0.31 ± 0.007	0.29 ± 0.01	0.07
Omega 6 FA	3.03 ± 0.05	3.0 ± 0.05	2.90 ± 0.08	0.42

[z]Fatty acid content was expressed as the percentage of total methyl esters. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid.

in the sense that we did not find a significant association between goat *SREBF1* genotype and milk yield and quality traits. Notably, the results of the association analyses outlined in Table 1 and Table 2 challenge the in silico prediction obtained with PolyPhen that indicated that the P118L might have functional consequences. Recently, Rincon et al. (2011) identified a non-synonymous SNP at exon 14 of the bovine *SREBF1* gene, which, according to in silico analyses with SIFT (Ng and Henikoff 2003) and PolyPhen, involved a functional change. Interestingly, this SNP displayed significant associations with milk fat and polyunsaturated FA content in cows suffering milk fat depression, but not in their healthy counterparts. As a whole, these results illustrate the complexity of assessing the functional effects of SNP on production traits through the performance of association studies, since results can be strongly affected by a plethora of biological (linkage phase between markers and causal mutations, genotype by environment interactions, dominant and epistatic effects, etc.) and experimental (sample size, number of records, phenotype recording etc.) factors. This circumstance stresses the need of validating associations between genetic markers and phenotypes in multiple populations and breeding environments in order to reach meaningful conclusions.

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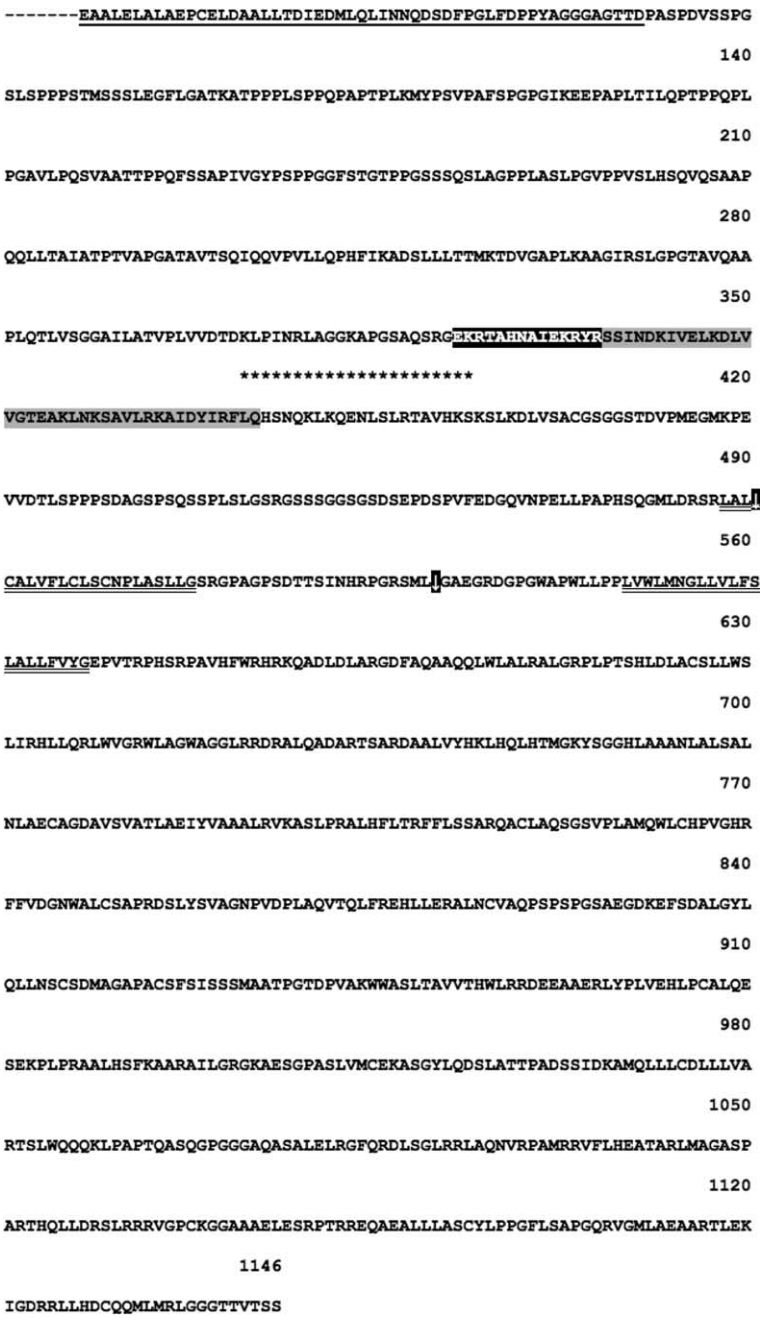
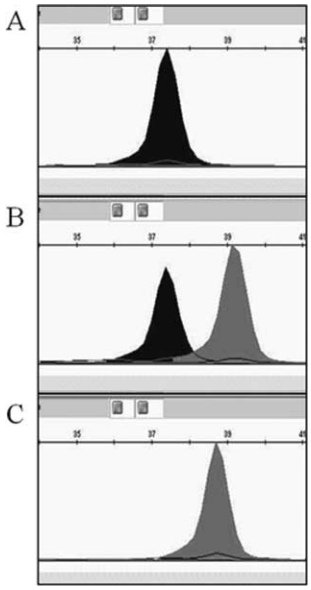
Supplementary Table 1. Sequences of primers employed to amplify the goat *SREBF1* gene cDNA and to genotype the c.353C > T polymorphism.

Primer sequence[z]	T^{y}_{ann} (°C)
Init-Fw, 5′-ATGGACGAGCCACCCTTCA-3′	64
Init-Rw, 5′-GCTGGCTCCTCCTTGATCC-3′	
F1- Fw, 5′-TGCTTCAGCTCATCAACAACCAAG-3′	64
F1-Rw, 5′-GTTGATGGGCAGCTTGTCAGTGT-3′	
F2-Fw, 5′-CTGCAGCCCCACTTCATCAAGGC-3′	64
F2-Rw, 5′-GACAGAGGAAGACGAGCGCGCA-3′	
F3-Fw, 5′-CCCTCCCAGAGCAGCCCCTTGT-3′	61
F3-Rw, 5′-CTGGGCTGGGCCACGCAATT-3′	
F4-Fw, 5′-TTGCCATGCAGTGGCTCTGC-3′	64
F4-Rw, 5′-AAGGAGCAGGTCACACAGGAGCA-3′	
Fin-Fw, 5′-GCCTCTCTGACAGCTGTGGT-3′	66
Fin-Rw, 5′-CAGGGGCCAGCACTATCCT-3′	
F5, 5′-ACCCACCCCCCTGAAGATGTA-3′	64
R2, 5′-AATGGGGGCAGAGCTGAACT-3′	
S2, 5′-TTGATCCCAGGCCCC-3′	

[z]Primers Init, F1, F2, F3, F4 and Fin were used to amplify and sequence the goat *SREBF1* coding region. F5 and R2 primers were employed to amplify the region containing the c.353C > T polymorphism, whilst S2 was used to carry out the extension reaction.
[y]Annealing temperature. The amplification conditions for all fragments were as follows: an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing temperature for 1 min and extension at 72°C for 2 min, plus a final extension step at 72°C for 10 min. Amplification reactions were performed by using a reaction mixture containing 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each primer, 2 μL of reverse transcription reaction, and 1.25 U of Taq DNA polymerase (Ecogen S.R.L., Barcelona, Spain) in a final volume of 25 μL.



Supplementary Fig. 1. Functional domains of the goat SREBF1 transcription factor according to the UniProtKB database (entry P36956) for human SREBF1: Acidic domain (underlined), putative transmembrane domains (double underlined), basic DNA-binding domain (black box), helix-loop-helix (HLH) motif (grey box), leucine zipper region (*) and protease cleavage sites (boxed arrows).



Supplementary Fig. 2. Electropherograms showing CC (lane A), CT (lane B) and TT (lane C) *SREBF1* genotypes detected by primer-extension analysis.