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CHARACTERIZATION OF EXON 14 AND INTRON 5 OF THE SREBP-1 GENE IN SARDA BREED SHEEP

Giovanni COSSO [1], Cinzia DAGA [1], Sebastiano LURIDIANA [1], Maria Consuelo MURA [1], Sara BODANO [1], Marta PALUDO [1], Maria Luisa DIAZ [1], Giuseppe Massimo VACCA [2], Vincenzo CARCANGIU [2]

ABSTRACT

SREBP-1 (Sterol Regulatory Element Binding Protein) is a transcription factor that regulates the expression levels of some genes and plays a central role in energy homeostasis. The aim of the present study was to screen for single nucleotide polymorphisms (SNPs) or insertions/deletions in the exon 14 and intron 5 of the SREBP-1 in Sarda sheep breed. Two-hundred multiparous ewes were randomly chosen from four farms located in Sardinia. From each ewe a blood sample was taken to perform DNA extraction and PCR-SSCP analysis of the fragments above. 20 PCR products for each fragment were sequenced in both directions, then compared and aligned with sequence of *Bos taurus* (GenBank AAFC03008483). Five nucleotide substitution (G19C, G22A, C52T, G53A and A68G) in the exon 14 and eighteen (C20T, G23A, T25G, G26A, C35G, C39G, A64G, T109C, T179C, G185A, G220A, G229A, G241A, G245A, G250A, C287T, A298T and G300A) in the intron 5 were detected in the sheep sequence compared to cattle. In the intron 5 of sheep SREBP-1 gene no deletion was found. Our data evidenced no variation in exon 14 and intron 5 of the SREBP-1 gene in Sarda sheep contrarily to that found in cattle. However, to extend the knowledge about SREBP-1 gene in this breed of sheep it would be necessary to enlarge the number of samples and to study other parts of this gene.

Key words: sheep / breeds / Sarda sheep breed / molecular genetics / SREBP-1 gene / milk traits

1 INTRODUCTION

Sterol regulatory element binding proteins (SREBPs) are transcription factors that play a key role in the energy homeostasis by promoting glycolysis, lipogenesis, and adipogenesis. SREBPs belong to the original basic helix-loop-helix-leucine zipper family of transcription factors (Eberle *et al.*, 2004). In human, the family consists of two genes: SREBP-1, mapped to chromosome 17 (Hua *et al.* 1995) and involved in the lipid synthesis (Shimano *et al.* 1996, 1997), and SREBP-2, mapped to chromosome 22 (Miserez *et al.* 1997) and involved in regulation of cholesterol biosynthesis (Horton *et al.* 1998). In human and mouse, the SREBP-1 gene produces two proteins by alternative splicing, SREBP-1a and 1c (Eberle *et al.* 2004). It has been known that SREBPs regulate gene transcription activation by binding to Sterol Regulatory Element (SRE) sequences (5'-TCACNCCAC-3') contained in the promoter of their downstream genes, including the Stearoyl-CoA Desaturase (SCD) gene (Shimano 2001). Therefore, differences in expression level and/or mutation(s) in the SREBP gene may affect the expression level of SCD gene, leading to differences in fatty acid composition in the adipose tissue of cattle (Ohsaki *et al.*, 2009). Furthermore, in cattle, mutations in this gene within intron 5 and exon 14, were found to be associated with fat content of milk (Cecchinato *et al.*, 2012; Rincon *et al.*, 2012; Shogo *et al.*, 2007).In sheep, however, the SREBP-1 gene is still little studied and its relationship with production traits is not yet well known. Thus, the aim of this paper was to study the exon 14 and the intron 5 of SREBP-1 gene in order to highlight possible SNPs and their correlation with the milk traits.

1 Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Via Vienna 2, 07100, Sassari, Italia
2 Same address, e-mail: endvet@uniss.it

2 MATERIAL AND METHODS

2.1 ANIMALS AND SAMPLING

The study has been conducted on 200 ewes of Sarda breed from four farms located in Sardinia. During the day the animals grazed on leguminous and gramineous grasses, moreover they received, as supplement, 300 g/head/day of concentrate commercial food (crude protein 20.4% and 12.5 MJ ME/kg DM) in two doses, during the morning and the evening milking. The sheep were penned at night when they had free access to hay (crude protein 11.1% and 7.2 MJ ME/kg DM), and water. Animals used were 4 to 5 years old, multiparous, lactating and in their third to fifth lactation. A jugular blood sample was collected from each ewe using vacuum tubes with EDTA as an anticoagulant (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK). On day 30 and 60 from lambing individual milk yield has been recorded and for each sample content of fat, protein and lactose were analysed (CombiFoss 6000, Foss Electric, Denmark).

2.2 DNA EXTRACTION AND AMPLIFICATION

Genomic DNA was extracted from whole blood using a commercial DNA extraction kit (Genomic DNA from blood, Macherey-Nagel Germany) and then kept at −20 °C until use. Primers for exon 14 were designed using Primer3-plus software (Ex14 forward : 5'-AGCCATGTTGACCGCCTGT-3' and reverse: 5'-GCAGAAACTCAGCCACACTG-3'); Primers for intron 5 were forward: 5'-CCACAACGCCATCGAGAAACGCTAC-3' and reverse: 5'-GGCCTTCCCTGACCACCCAACTTAG-3' (Barton *et al.*, 2010). An aliquot of 100 ng of genomic DNA was used for the amplification of the exon 14 and the intron 5 of the SREBP-1 gene. The PCR reaction for both fragments were carried out in 25 µl volume containing 2.5 µl of 10x PCR Buffer (20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM Sodium Phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% (v/v) glycerol), 0.5 µl of each primer 10 µM for exon 14 and 1.25 µl for intron 5,4 µl of each dNTP 1.25 mM, 0.75 µl of $MgCl_2$ 50 mM; 1.25U of Taq DNA polymerase (Platinum® Taq DNA Polymerase, Invitrogen, Carlsbad, CA, USA). PCR conditions were as follows: denaturation at 95 °C for 2.5 min followed by 35 cycles of denaturation at 95 °C for 20s, annealing at 61 °C for 30s, extension at 72 °C for 16s, and a final extension at 72 °C for 10 min (Mastercycler epgradient S, Eppendorf AG, Hamburg, Germany) for the exon 14; denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30s, extention at 72 °C for 1 min, and a final extension at 72 °C for 7 min (Mastercycler gradient S, Eppendorf AG, Hamburg, Germany) for the intron 5. Electrophoresis of PCR products were performed in 1.5% (w/v) agarose gel (GellyPhor, Euroclone, UK), in parallel with 100 bp DNA marker (Invitrogen, Carlsbad, CA, USA), in 1X TBE buffer at a constant voltage of 100V for 30 min. After ethidium bromide coloration, products were visualized by using of ultraviolet transilluminator (UVItec, Cambridge, UK).

2.3 SSCP ANALYSIS

Aliquots of 4.0 µl of PCR products, of both fragment of each ewe, were later denatured at 95 °C for 10 min in a 6.0 µl denaturing solution containing 1 mg/ml xylenecyanole (SIGMA-ALDRICH Corporation, St. Louis, Missouri, USA), 1 mg/ml bromophenol blue (Pharmacia Biothec, Uppsala, Sweden) and 25 mM EDTA (pH 8) 95% formammide (SIGMA-ALDRIC Corporation, St. Louis, Missouri, USA) and chilled on ice. Single Strand Conformation Polymorphism (SSCP) analysis, has been conducted using the vertical electrophoresis DCodeTM Universal Mutation Detection System for SSCP (Bio-Rad Laboratories, Segrate, MI, Italy). Denatured DNAs were subject to electrophoresis at constant voltage (25W, 1000V, 150 mA), in a 15% acrylamide gel of 40 ml: 40% acrylammide solution (acrylamide/bis-acrylamide 37,5:1) 15 ml; 1X TBE (Tris-borate-EDTA) buffer 8 ml; glycerol 2 ml; Temed (Tetramethylethylenediamine) 40 µl and 10% APS (Ammonium Persulfate) 200 µl and pure water 14.76 ml. Running time was 20h; running temperature was 15 °C. The gels were stained with syber gold 1X for 30 min. 20 PCR products of each fragment were purified and sequenced using a commercial service in forward and reverse directions.

2.4 STATISTICAL ANALYSIS.

These data, normally distributed ($P > 0.05$, Anderson-Darling'test), were expressed as mean ± standard deviation (SD). Statistical analysis was performed using R-Project software (The R Foundation for Statistical Computing, R-2.13.1). ANOVA (one-way) was used to analyse the time variation of milk yield, protein, fat and lactose contents.

3 RESULTS

The resulting fragments were of 222bp for exon 14 and 432bp for intron 5 (Fig. 1).

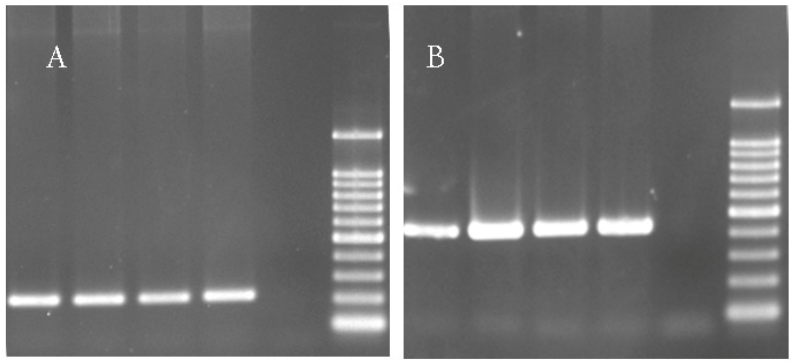

Figure 1: *Electrophoresis on a 2% agarose gel of exon 14 (222bp) (A) and intron 5 (432 bp) (B) (marker 100bp)*

No differences were found in the SSCP analysis for both fragments, as they always showed the same migration patterns. The sequencing confirmed the amplification of the desired gene sections and no differences emerged from the comparison among the obtained sequence. Five nucleotide substitution (G19C, G22A, C52T, G53A and A68G) in the exon 14 and eighteen (C20T, G23A, T25G, G26A, C35G, C39G, A64G, T109C, T179C, G185A, G220A, G229A, G241A, G245A, G250A, C287T, A298T and G300A) in the intron 5 were detected in the sheep sequence compared to cattle (GenBank AAFC03008483) (Fig 2). After sequence comparison between cattle and Sarda sheep a 89% genetic similarity was revealed. The milk yield, the content of fat, protein and lactose showed no differences in the time of collection.

4 DISCUSSION

Identifying the genes responsible for phenotypic variation is important not only to increase our understanding about milk fatty acids synthesis, but also to in-

Exon 14

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                10        20        30        40        50        60        70        80        90
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bos taurus      GGAGTTCTCAGATGCCCTGGGGTACCTGCAGCTGCTGAACAGCTGTTCGGACGTGGCCGGAGCTCCTACCTGCAGCTTCTCCATCAGCTC
Ovis aries      ..................C..A.............................TA.............G.....................

                100       110
                |....|....|....|....|
Bos taurus      CAGCATGGCTGCCACCCCCG
Ovis aries      ....................
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Intron 5

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                10        20        30        40        50        60        70        80        90
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bos taurus      GTGTGGGCTGAGGCCCTAACAGGGTGGCTCTGGGCAAGCAGGCACCTGGGAGGAGGAGGAGGAAGATGGGGCTGGGCAGACAGTCCTGGG
Ovis aries      ..................T..A.CA........G...G.........................G.........................

                100       110       120       130       140       150       160       170       180
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bos taurus      GCCCCAGCCTCCTCGGGCTTGGCAGCTCTGTTCAGCTGAGCTTCAGGGAAGCCCCGGGTGGCACAGGCTCTTCCAGGTGCTGGGGATTTA
Ovis aries      ..................C.....................................................................C.

                190       200       210       220       230       240       250       260       270
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bos taurus      GCAGGGGACAGACAGAGATCCTGCTGTTGAGTAGCTGACGTTCTAGCAGGGTGCGGGGGCGCCAGCAGAGCTGGGTTGGGGTACGCCAGG
Ovis aries      ....A.................................A........T..........A...A....C.....................

                280       290       300       310       320       330       340
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....
Bos taurus      TGGCATAAGTGCTGTGCTGGAGAGAAGAGGGATGGGGGAAGTTGCAAACCTAAGTTGGGTGGTCAGGGAAGGCC
Ovis aries      ................T..........T.A.............................................
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Figure 2: *Comparative sequence analysis of exon 14 and intron 5 of the SREBP-1 gene between cattle and Sarda sheep*

crease milk-fat contents in ruminants (Bionaz and Loor, 2008). Among the genes indirectly involved in the milk fat synthesis, the SREBP-1 is of great importance because it regulates the transcription of several genes in the mammary gland including the SCD gene (Hoashi *et al.*, 2007). Therefore mutation(s) of the SREBP gene may affect the expression level of SCD, leading to differences in fatty acids composition both in tissue and milk in cattle (Conte *et al.*, 2010). Indeed the 84-bp deletion in intron 5 of the bovine SREBP-1 gene could indirectly contribute to fat quality characteristics in cattle, affecting the level of translation efficiency of SREBP-1 itself (Hoashi *et al.* 2007; Cecchinato *et al.*, 2012). Moreover mutations in exon 14 of the SREBP-1 gene in cattle determine an influence on milk fat content (Rincon *et al.*, 2012). Our data about Sarda sheep are not in agreement with that found in cattle, because the exon 14 and the intron 5 lack differences in their nucleotide sequences. This could be due intense genetic selection of Sarda breed sheep aimed to improve its milk traits, both for quantity and quality (Carta *et al.*, 2009). Indeed this dairy breed produces large amounts of milk, with high fat content (more than 6%). The lack of 84bp deletion within the intron 5 confirmed this, but is not applicable for that found in the exon 14. In fact in our sample of the Sarda breed sheep only CC genotype in position 66 was found, which causes a lower fat concentration in cow's milk (Rincon *et al.*, 2012). Probably the C allele in sheep does not determine an influence such as to diminish fat contents unlike in cattle.

In conclusion our data evidenced that in the analyzed fragments of the SREBP-1 gene in Sarda sheep breed there are no variation, contrarily to that found in cattle. However, in order to deepen the knowledge about SREBP-1 in this sheep, the expansion of sampling and the study of other parts of this gene are necessary.

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