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Association between SREBP-1 gene expression in mammary gland and milk fat yield in Sarda breed sheep ☆

Vincenzo Carcangiu *, Maria Consuelo Mura, Cinzia Daga, Sebastiano Luridiana, Sara Bodano, Giovanni Antonio Sanna, Maria Luisa Diaz, Giovanni Cosso

Dipartimento di Medicina Veterinaria, Università di Sassari, 07100 Sassari, Italy

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SUMMARY

The aim of this study was to examine the expression patterns of SREBP-1 gene in milk somatic cells and its association with milk fat yield during early lactation in Sarda breed sheep. A sample of 20 Sarda ewes, aged between 4 and 5 years, in their third to fourth lactation were chosen. From each ewe 28 days after lambing milk yield was measured, and a 160 ml milk sample for the RNA extraction and to test somatic cells count and lactose, fat and protein contents were collected. From the obtained RNA, total cDNA was synthesized and the quantitative PCR was performed. The fat, proteins and lactose content showed many differences among the animals, but these variations were no correlated with the milk yield. The SREBP-1 gene expression resulted higher in the high milk fat producing ewes. The correlation analysis showed that the SREBP-1 expression level is directly related to the amount of milk fat (g/die) ($P < 0.001$), while the total RNA obtained from each sample was found to be related to the somatic cells number ($P < 0.001$). Instead the expression of this gene showed no relations with the concentration of fat in milk. Our data highlight that in sheep SREBP-1 gene is expressed in the mammary gland during early lactation. Moreover, the positive relationship between SREBP-1 gene expression and the milk fat yield suggests that SREBP-1 gene is required for the lipid synthesis in the sheep mammary gland.

* Corresponding author at: Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Via Vienna 2, 07100, Sassari, Italy. Tel.: +39 079229436; fax +39 079229592.
E-mail address: endvet@uniss.it (V. Carcangiu).

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1. Introduction

The synthesis of fatty acids and cholesterol, which in turn gives rise to multiple lipid compounds, can occur in any cell, but this biochemical process is particularly important in tissues such as liver, adipose tissue and mammary gland, organs specialized in lipogenesis or/and in lipolysis (Harvatine and Bauman, 2006; Hoashi et al., 2007). Both processes of the biosynthesis of cholesterol and fatty acids are controlled by a family of transcription factors (SREBPs) (Harvatine et al., 2009). These transcription factors play a central role in energy homeostasis by promoting glycolysis, lipogenesis, and adipogenesis. SREBPs belong to the original basic helixloop–helix–leucine zipper family of transcription factors (Eberle et al., 2004). SREBP are synthesized as ~125 kDa precursors that are bond to the membrane of the endoplasmic reticulum until proteolytically cleaved to release the amino terminal fragment of ~68 kDa that migrates to the nucleus to activate gene transcription (Brown and Goldstein, 1997; Horton et al., 2002). Two genes are accountable for the production of the 3 isoforms of SREBP: SREBP-1a and 1c are transcribed from a single gene through the use of alternative start sites and are associated with fatty acid metabolism, whereas SREBP-2 is transcribed from a distinct gene and controls primarily cholesterol metabolism (Horton et al., 2002). The two isoforms of SREBP-1 (a and c) can be expressed at different levels in tissues and differ by only 84 nucleotides at the first exon (Eberle et al., 2004). Then SREBP-1 gene is considered a candidate gene as it plays a role in the regulation of the synthesis of milk fat (Cecchinato et al., 2012; Hoashi et al., 2007), and controls the expression of more than 30 genes (McPherson and Gauthier, 2004). The expression of this gene in bovine mammary tissue plays a central role in milk fat synthesis regulation and highlights a pivotal function for a concerted action among PPARG, PPARGC1A, and INSIG1 genes (Bionaz and Loor, 2008). To date, there have been no published studies demonstrating the expression of SREBP-1 in ovine mammary gland, and an influence of this gene on the milk fat yield. The objective of the current study was to examine the expression patterns of SREBP-1 gene in milk somatic cells and its association with milk fat yield during early lactation in Sarda breed sheep.

2. Material and methods

2.1. Animals

The study was conducted on 20 Sarda breed sheep from a farm located in north Sardinia (40° 48′N). During the day the animals grazed on leguminous and gramineous grasses, and then they received each a supplement of 300 g/day of concentrate commercial food (crude protein 20.4% and 12.5 MJ ME/kg DM). The sheep were penned at night, and received hay (crude protein 11.1% and 7.2 MJ ME/kg DM) and water *ad libitum*. The chosen ewes (aged between 4 and 5 years and in their third to fourth lactation) lambed between 2010 November 01 and 03 and suckled their lambs until Day 21 after parturition.

2.2. Sampling

From each ewe, at Day 28 after lambing, milk yield was measured, and a 150 ml milk sample for the RNA extraction was collected. In the same date another 10 ml milk sample from each ewe was collected to test somatic cells count and some milk quality parameters (lactose, fat and protein contents). Milk samples were analyzed by using an infrared spectrophotometer (Milko-Scan 133B; Foss Electric, DK-3400 Hillerød, Denmark) to assess fat, lactose and protein percentage according to the International Dairy Federation standard (IDF 141C:2000). SCC was measured using an automatic cell counter (Fossomatic 90, Foss Electric) according to IDF 148A:1995.

2.3. Total RNA extraction and analysis

RNA extraction from whole milk was performed in accordance to Mura et al. (2012). Briefly the samples were centrifuged at 2000 rpm for 15 min at 6 °C. The supernatant was carefully removed by overturning the tubes and the remaining cell pellet was washed twice with 10 ml of PBS (Phosphate Buffered Saline), pH 7.2, and supplemented with 0.5 mM of EDTA (Ethylene-D-amine-Tetra-Acetic-Acid) and DEPC 0.1% (D-Ethyl-Pyro-Carbonate) to limit formation of casein micelles and fat globules and

to inhibit RNase; 404 μl of 5 mM TCEP (tris(2-carboxyethyl) phosphine) + lysis solution was added directly to the cell pellet and homogenized by vortex for 2 min. Then total RNA was extracted by using of a commercial kit (PerfectPure RNA Tissue Kit, 5 PRIME GmbH, Hamburg, Germany). Genomic DNA was removed from RNA with DNase. The total RNA was eluted in 50 μl of elution solution and then stored at −80 °C. Purity and concentration of the extracted RNA were measured with a spectrophotometer (Eppendorf BioPhotometer). RNA integrity was assessed by electrophoretic analysis in denaturing agarose gel of 28S and 18S rRNA subunits.

2.4. RNA retro-transcription and qPCR

From the obtained RNA, total cDNA was synthesized using 500–600 ng of extracted RNA in a total volume of 20 μl by using of iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol: 5X iScript reaction mix, 4 μl; iScript reverse transcriptase, 1 μl; RNA template, 7 μl (corresponding to 500–600 ng, approximately); Nuclease-free water, 8 μl. The reaction was performed in an Eppendorf Mastercycler® gradient according to the kit's protocol: 25 °C for 5 min, at 42 °C for 30 min, 85 °C for 5 min, held at 4 °C. The cDNA was sequenced in both direction, and the nucleotide sequence was compared with sequences deposed in the public database NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast). The cDNA was then diluted 1:3 with DNase/RNase free water. Before starting to perform the quantitative PCR by using of RealMasterMix SYBR ROX (5 PRIME GmbH, Hamburg, Germany) in one individual tube (1.0 ml) of the 2.5X RealMasterMix SYBR ROX, 125 μl of SYBR ROX solution was added and thoroughly mixed. Finally the quantitative PCR reaction was performed in triplicate in a 12 μl final volume containing cDNA diluited 1:3, 4 μl of cDNA and 8 μl of qPCR mastermix, distributed as follows: 2.5X RealMasterMix SYBR ROX (0.05U/μl HotMaster Taq DNA Polymerase, 10 mM Magnesium Acetate (4.0 mM final), 1.0 mM dNTPs with dUTP (0.4 mM final) with the addition of 20X SYBR solution as above specified) 5 μl; 10 pmol/μl of each primer, 0.4 μl for forward primer and 0.4 for reverse primer (333 nM final); DNA/RNA-free water, 2.2 μl. Samples were placed in 96-well semiskirted Eppendorf plates in Eppendorf RealPlex ep gradient S (Eppendorf AG, Hamburg, Germany). Primers for

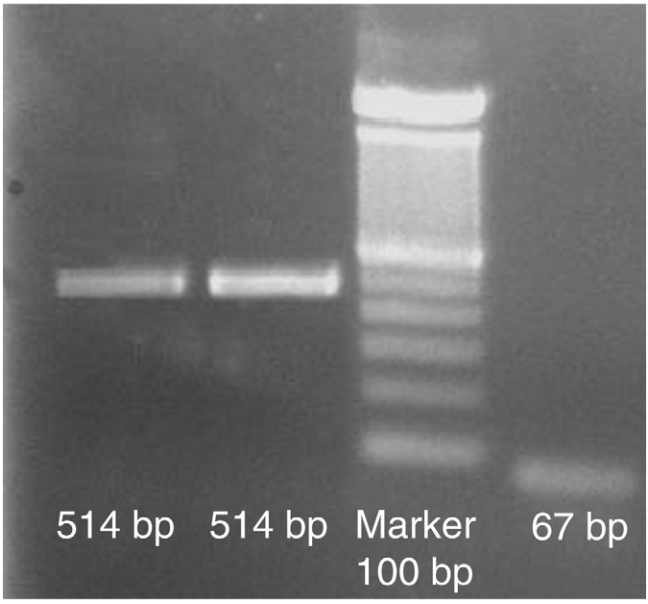

Fig. 1. Gel electrophoresis (1.5% agarose) of SREBP-1 gene PCR product using genomic DNA and the single-stranded cDNA from the extracted RNA. Line 1 and 2: genomic DNA (514 bp); line 3: 100 bp DNA Marker; line 4: cDNA (67 bp).

SREBP and UXT were the same used by Bionaz and Loor (2008). The qPCR conditions were as follows: 95 °C for 2 min; followed by 40 cycles shared in denaturation at 95 °C for 15 s and annealing + extension at 60 °C for 1 min. The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures up to 95 °C for 15 s plus 65 °C for 15 s (repeated for 20 min), and then up to 95 °C for 15 s.

The relative quantities of SREBP mRNA were determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), the ΔC_T value was determined by subtracting the average reference gene UXT C_T value from the average target SREBP C_T value, by GenEx software. A single reference gene was used as its invariant expression was confirmed by others studies (Bionaz and Loor, 2007, 2008), where UXT was defined internal gene that can be used for normalization in future studies. PCR-normalized data are presented as n-fold change relative to a reference sample used as a calibrator with a SSC <100,000 and fat percentage of 5.37.

2.5. Statistical analysis

Pearson's correlation analysis was used to determine the relationship among milk yield and quality (fat, proteins and lactose content), SCC and RNA concentration. Statistical analysis was performed using the R software package.

3. Results

Polymerase chain reaction products of cDNA using primers for SREBP-1 gene gave a single 67-bp band after agarose-gel electrophoresis, while the genomic DNA shows a 514 bp band (Fig. 1). The cDNA sequence comprised the final part of the exon 16 and the initial part of the exon 17 of the SREBP1 gene, as expected. No differences emerged from the alignment of the obtained sequence with goat (GenBank accession number DQ483057.1) or cattle sequences (GenBank accession number GQ477182). Table 1 shows the data on milk production, and content of fat, proteins and lactose in the 20 analysed ewes, at Day 28 of lactation. The animals had very different levels of productions: there were high productive ewes, with 2.0 kg of milk per day, and low-productive ewes, with 0.7 kg of milk per day. Also the content of fat, proteins and lactose showed many differences among the animals, but these variations were no correlated

Table 1
Milk yield, and fat, protein and lactose percentage, in the 20 analysed ewes.

Subject	Milk yield (g)	Fat (%)	Protein (%)	Lactose (%)
1	1342	5.33	6.84	4.97
2	1339	4.93	5.80	5.19
3	1354	5.84	6.90	4.47
4	1553	6.13	6.56	5.17
5	1262	5.85	5.94	5.02
6	854	5.93	5.97	4.85
7	977	6.81	6.51	5.09
8	1315	6.06	6.00	4.82
9	1051	6.04	6.29	4.90
10	780	7.13	8.33	4.97
11	943	6.10	6.27	5.05
12	1217	7.58	6.59	5.07
13	974	7.52	7.05	4.98
14	1034	5.11	7.01	5.24
15	811	5.37	6.69	4.88
16	1303	4.78	5.96	5.20
17	2282	5.50	5.22	5.21
18	991	7.06	6.10	5.15
19	1315	5.80	6.60	5.11
20	725	6.03	5.91	5.27
mean	1171	6.05	6.43	5.03
sd	±349.2	±0.8	±0.6	±0.2

Table 2
RNA concentration, Somatic Cells Count (SCC), fat yield and fold change in the 20 analysed ewes.

Subject	RNA (μg/ml)	SCC ($\times 10^3$ cells/ml)	Fat yield (g)	Fold change[a]
1	48.5	92.0	71.5	5.15
2	55.0	64.0	66.0	3.54
3	48.8	97.0	79.1	3.77
4	21.3	47.0	95.2	5.95
5	60.0	69.0	73.9	7.45
6	19.0	35.0	50.7	6.99
7	74.3	86.0	66.5	3.06
8	39.5	73.0	79.7	4.18
9	121.0	224.0	63.5	5.88
10	21.0	66.0	55.6	4.32
11	47.6	86.0	57.5	5.46
12	20.0	53.0	92.2	7.92
13	58.0	68.0	73.3	7.55
14	77.0	88.0	52.9	6.97
15	125.0	207.0	43.6	0
16	140.0	74.0	62.3	−1.84
17	49.0	54.0	125.5	10.38
18	27.3	41.0	70.0	4.97
19	34.0	44.0	76.3	7.66
20	52.0	64.0	43.7	2.91
mean	56.9	81.6	69.9	
sd	±35.3	±49.1	±19.2	

[a] Fold change is the measure of the change in the level of expression of the SREBP-1 gene.

with the milk yield. Table 2 shows results on RNA concentration, Somatic Cells Count (SCC), fat yield and fold change for each ewe. SCC were quite low, with an average value around 81,600 (Table 2). The amount of total RNA extracted from each sample resulted variable and it was depending on the number of somatic cells in the sample. The milk fat amount is mainly influenced by the individual milk yield and when the animals show the same milk production level, the milk fat concentration influences the total amount of milk fat. The SREBP-1 gene expression resulted higher in the high milk fat producing ewes. The correlation analysis (Table 3) showed that the SREBP-1 expression level is directly related to the amount of milk fat ($P < 0.01$), while the total RNA obtained from each sample was found to be related to the somatic cells number ($P < 0.001$).

4. Discussion

Our main objective was to detect the expression of SREBP-1 gene in the mammary gland of sheep during early lactation using the somatic cells as RNA source. The obtained results show that in sheep the gene above is highly expressed, and this suggests that also in dairy sheep the SREPB-1 gene plays an important role in the secretion of milk fat. Indeed the synthesis and secretion of milk fat by the mammary gland involve an integration of different biochemical processes coordinated by several lipogenic genes (Horton et al., 2003; Liang et al., 2002). SREBP-1 gene is highly expressed in the lactating bovine mammary gland and many of the key enzymes involved in the lipogenesis are transcriptionally regulated by SREBP-1

Table 3
Correlation values among fat yield, fat, protein and lactose percentage, Somatic Cells Count (SCC), fold change and RNA concentration.

	Fat yield	Fat %t	Protein %	Lactose %	SCC
Fold change[a]	0.56**	0.31	−0.08	0.08	–
Milk yield	0.89***	−0.34	−0.45*	0.12	–
RNA conc.	–	–	–	–	0.73***

Significance level: *=$P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$.
[a] Fold change is the measure of the change in the level of expression of the SREBP-1 gene.

(Bionaz and Loor, 2008). In mice, SREBP-1 gene is upregulated at the beginning of lactation, and disruption of this gene results in a decrease of 41% in milk fat concentration (Rudolph et al., 2007). The expression levels of SREBP-1 gene we observed may be compared only with those observed in cattle (Bionaz and Loor, 2008), since there are no research in sheep mammary expression of this gene during lactation. We observed at 28 days a greater SREBP-1 expression level than that found at 30 days of lactation in cows (Bionaz and Loor, 2008). Considering that cows produce a daily amount of milk fat much greater than the sheep, our expectation was that the expression of this gene was greater in cattle than in sheep. However, the concentration of fat in the Sarda sheep milk is approximately double compared to the cattle's milk and this may have led to increased expression of SREBP-1 in sheep. Our results also suggest that SREBP-1 gene increase lipid synthesis in the mammary gland of the sheep. Indeed, the highest expression of the gene SREBP-1 is found in ewes producing the greater amount of fat (g/die). This gene controls the synthesis of novel fatty acids in mammary gland and, as found in bovine, the peak of new synthesis of fatty acids in mammary gland, starting from acetate and butyrate, is observed around 30 days of lactation (Bionaz and Loor, 2008). Therefore, we could speculate that also in sheep the higher de novo synthesis of fatty acids in milk occurs in the same period and consequently it justifies the high values of SREBP-1 gene expression found in greater milk fat producing sheep at 30 days of lactation.

Our data showed no relationship between the expression of the gene SREBP-1 and the concentration of fat in milk despite the animals had a great different fat percentage in milk. In fact, we found sheep with a concentration of 4.73% of milk fat and others with 7.13%. This fact suggests that high expression of SREBP-1 gene is necessary for the milk fat yield, but the regulation of the fat concentration is due to the intervention of other genes which are involved in the uptake of fat as INSIG. However, in order to confirm the data obtained it could be necessary to extend sampling to a larger number of animals and to assess the expression of the SREBP-1 gene during the course of lactation.

5. Conclusion

Results of this study evidenced the importance of this gene in the milk fat yield, and highlight, for the first time, the positive relationship between SREBP-1 gene expression and the milk fat yield in sheep. Moreover the expression of this gene is not associated with milk fat concentration. On the basis of these data further studies seem to be required to clarify the real role of the genes involved in the control of synthesis of milk fat.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed equally to the design and conception of this study, conducted computational experiments, performed and interpreted data and drafted the manuscript. All authors contributed to and approved the final manuscript.

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