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A Novel SNP Polymorphism in the Ovine Leptin Gene Related to Back Fat Depth

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ABSTRACT: The objective was to identify potential polymorphisms in exon 3 coding sequence of the *oLEP* gene in two- and three-breed crossbred lambs, involving Blackbelly, Pelibuey, Dorper and Charollais sheep, and to evaluate potential transcriptional and phenotypic associations. Polymerase chain reaction–single-strand conformational polymorphism (PCR–SSCP) analyses were performed. Five unique SSCP patterns defined by six different bands were observed, from which, amplicons of animals with two of the identified patterns were re-amplified and sequenced. A non previously reported, non synonymous C→G transversion SNP in exon 3 of the *oLEP* gene was identified at position 355, which resulted in a predicted amino acid change from aspartic acid to glutamic acid at the 118 codon position. Lambs with the CG genotype had 1.02 ± 0.4 mm more ($P < 0.05$) back fat depth at slaughter than those with the CC genotype.
Keywords: Sheep; *LEP* gene; Fat deposition

Introduction

Leptin is a protein hormone involved in the regulation of feed intake, energy metabolism and body weight. It is expressed by the *LEP* gene and synthesized mainly in the adipose tissue (Friedman and Halaas (1998)). In lambs, Altmann et al. (2006) found that leptin blood concentrations increased with growth until a 40 kg body weight was reached. At this point, they observed a positive correlation with visceral ($r = 0.58$) and carcass fat ($r = 0.41$). Thus, the *oLEP* gene is a good candidate to look for SNP markers related to sheep productivity, in addition to the use of leptin as an indicator of blood metabolism chemistry. To date, different studies have shown some relationships of SNPs in the *LEP* gene with carcass traits (Schenkel et al. (2005)) and feed intake (Lagonigro et al. (2003)) in bovine, and with intramuscular fat (Villalba et al. (2009)), body weight and back fat (Pérez-Montarelo et al. (2012)) in swine. However, information on polymorphisms in the *oLEP* gene and their relationship to growth and fat deposition is limited (Zhou et al. (2009); Barzehkar et al. (2009)). The objective was to identify SNPs in the *oLEP* gene in crossbred lambs in México and their potential association with leptin relative gene expression, feed intake, feed efficiency, growth and fat deposition.

Materials and Methods

Animals. Forty two crossbred non castrated lambs were used. Twenty two (10 males and 12 females) came from a two-way cross between Blackbelly and Pelibuey ewes with Dorper rams and 20 (10 males and 10 females) came from a three-way cross between two-way cross (as mentioned before) ewes and Charollais rams. Each lamb was randomly allocated in a 1.2 x 2.4 m pen and fed an ad libitum mixed ration including corn (46.1 %), alfalfa hay (20 %), dry distillery grain (20 %), cotton seed meal (1.9 %), bypass fat (3.0 %), cane molasses (3.9 %), soybean meal (2.7 %), minerals premix (1.1 %), $CaCO_2$ (0.7 %) and salt (0.7 %). Feed was served daily with an estimated 10 % surplus and lambs, previously fasted for 12 h, were weighed every 14 d until slaughter (Group 1: 70 d and 42.6 ± 4.8 kg, n=21; Group 2: 91 d and 42.8 ± 5.5 kg, n=21). Previous to slaughter, animals were fasted for 24 h.

Feeding and slaughter data. The quantified variables were: average daily weight gain (ADG), feed intake (FI), feed to gain ratio (F:G), residual feed intake (RFI), kidney fat (KF), back fat (BF) and rib eye area (REA) measured between the 12 and 13th rib of the chilled carcass.

Hormone assay data. Preprandial jugular blood samples were collected into tubes at day 28 and previous to slaughter. Serum was collected and stored at −20°C until leptin concentrations (SLC) were determined in duplicate by ELISA (Creative Biomart®) on days 28 and 70 samples for both groups of lambs and on day 91 for Group 2. The intra-assay CV was 7 %.

Gene expression data. Subcutaneous adipose tissue samples were collected from the base of the tail at day 28 and at slaughter. Samples were submerged into 1 ml of TRIzol® (Invitrogen™), placed in liquid nitrogen and stored at −80°C. Total RNA was extracted from the adipose tissue using TRIzol® reagent procedures. Complementary DNA was synthesized from 100 ng of total RNA, using the SuperScript® VILO ™ (Invitrogen ™) kit. The RNA abundance of *oLEP* was measured using TaqMan® Gene Expression Assay with MGB probes labeled with FAM dye (Applied Biosystems). Ribosomal protein L19 (RPL19) gene was used as an internal control for relative gene expression analyses (Peletto et al. (2011)). Oligonucleotide sequences of primers and probes for *oLEP* gene were: *oLEP*-up (5'-GACCAGACATTGGCAATCTA CCA-3'), *oLEP*-dn (5'-CGGAGGTTCTCCAGGTCATTA GATA-3') and Probe (5'-CTGCCTTCCAGAAATG-3'); and for RPL19 gene were: RPL19-up (5'-GCTCAGACGA TACCGTGAATCTAAG-3'); RPL19-dn (5'-ACACGTTA CCCTTCACCTTCAG-'3) and Probe (5'-CCGCCACATG TATCAC-3'). Each reaction was carried out with duplicates in a real time PCR StepOne® system (Applied Biosystems ™). Thermal cycling condition was: 95ºC for 10

min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative gene expression was calculated by the delta-delta CT method, based on the sample with the least expression as a calibrator in each day (Livak and Schnittgen (2001)).

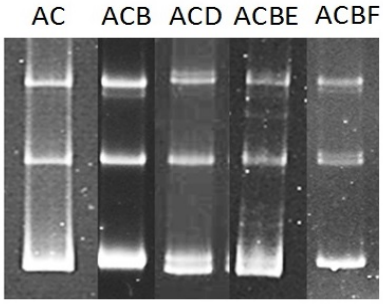
Genotyping. Polymerase chain reaction–single-strand conformational polymorphism (PCR–SSCP) analysis and DNA sequencing were used to identify potential polymorphisms in the entire exon 3 coding sequence of the *oLEP* gene. Primers used for amplification were o*LEP*-up (5'-GCAAGGTCCAGGATGAC-3') and o*LEP*-dn (5'-GGACTGAGGTCCAGCTG-3'), based on the published mRNA sequence of *oLEP* (GenBank access number U84247.1), which flank a fragment of 387 pb containing polymorphisms reported by Zhou et al. (2009). Amplification was performed in a 50 µL reaction with 200 ng of cDNA, Platinum® Taq DNA Polymerase (Invitrogen ^{TM}), reaction buffer, primers and dNTPs (as recommended by the enzyme manufacturer), and BSA 0.2 µg µl^{-1} (New England BioLabs®). The thermal cycler profile was 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56.3°C, 30 s at 72°C, with a final extension of 7 min at 72°C. Amplicons were visualized by electrophoresis in a 1% agarose gel stained with ethidium bromide at 10 mg ml^{-1}. They were concentrated down to 10 µL by incubation at 35°C and mixed with 1 µL of denaturant alkaline buffer (NaOH, EDTA). After denaturation at 95°C for 15 min, samples were cooled on wet ice and then mixed with 6 µL of loading dye (formamide, EDTA, bromophenol blue and xylene cyanol). The sample was loaded on 18% acrylamide:bis-acrylamide (37.5:1) gel. Electrophoresis was carried out at 450 V and 4° C for 7 h. Gels were stained with SYBR ® Green I 1x (Invitrogen^{TM}) for 15 min. The bands of the different patterns of SSCP were cut and subjected to reamplification for sequencing with an automatic DNA sequencer Perkin Elmer (Applied Biosystems). The sequences were analyzed with Clustal Omega (http://www.ebi.ac.uk) for the identification of SNPs.

Statistical analyses. PROC MIXED of SAS (SAS Inst. Inc., Cary, NC) was used for statistical analyses. The fold change on leptin mRNA levels and the serum leptin concentrations were log transformed. For all variables, the adjusted model included fixed effects of genotype, type of cross (two and three breeds), sex, slaughter group and their interactions. For those variables with repeated measurements such as (BW, FI and ADG), the model also included random effects of animal within subclasses defined by genotype, cross, slaughter group and sex.

Results and Discussion

Detected polymorphisms. Figure 1 shows the five unique SSCP patterns defined by six different bands that were observed. There were reasonable amounts of animals with the AC (n = 10) and ACB (n = 27) PCR-SSCP patterns to continue with sequencing and phenotypic association analyses. The rest of the patterns were discarded from those analyses due to limited numbers to insure genuine polymorphisms and not PCR or sequencing artifacts. Sequencing analyses of band a as well as band c resulted in the same sequence, while the sequence of band b revealed the presence of an SNP in exon 3 of the *oLEP* gene at position 355, being a C→G transversion. This was confirmed by amplifying and sequencing the same band from five different animals.

Figure 1: PCR-SSCP of exon 3 of the *oLEP* gene in lambs tested.



	Pattern				
Band	AC	ACB	ACD	ACBE	ACBF
d					
a					
b					
e					
f					
c					
n=	10	27	2	1	1

The lambs with the AC pattern were identified as homozygous CC and those with the ACB pattern as heterozygous CG. Genotype frequencies were 0.27 for CC, 0.73 for CG, with no presence of the GG genotype, and were similar for both crosses (Table 1), but were higher for the CG genotype in the slow growth lambs (Slaughter Group 2; 0.10) than in the fast growth lambs (Slaughter Group 1; 0.47).

Table 1: Genotypes distribution according to cross type, sex and slaughter group.

	Genotype	
Factor	**CC**	**CG**
Cross type		
Two-breed	5	14
Three-breeds	5	13
Sex		
F	7	14
M	3	13
Slaughter group		
1	8	9
2	2	18
Total	10	27

When comparing the C and G *oLEP* alleles to the ones reported by Zhou et al. (2009), the C allele has the same sequence as the 01 allele of that study, and the G allele has not been reported in the GeneBank. This SNP is

not synonymous, which results in a prediction of an amino acid change from aspartic acid to glutamic acid at the 118 codon position. In part, leptin activity has been located between amino acids 106 to 140 (Grasso et al., 1997). Therefore, it can be hypothesized that an amino acid substitution in this region can affect the activity of the hormone and cause changes in the phenotype of lambs.

Phenotypic associations to polymorphism. There were not clear differences between the two genotypes (CG vs CC) for traits measured during the feeding trial (Table 2), except for body weight at day 91 (Group 2). Nonetheless, there were only two animals in this group for the CC genotype.

Table 2: Least squares means (± SE) of traits measured at the feeding trial

Time	Genotype	
	CC	**CG**
	Body weight, kg	
28 d	27.49 ± 0.63	28.23 ± 0.37
70 d	37.46 ± 1.21	39.73 ± 0.62
91 d	36.85 ± 2.67 [a]	43.00 ± 0.87 [b]
	Average daily gain, kg d^{-1}	
0-28 d	0.24 ± 0.02	0.27 ± 0.01
29-70 d	0.26 ± 0.02	0.27 ± 0.01
0-70 d	0.25 ± 0.02	0.27 ± 0.01
71-91 d *	0.26 ± 0.05	0.30 ± 0.01
	Average daily feed intake, kg d^{-1}	
0-28 d	1.01 ± 0.06	1.11 ± 0.03
29-70 d	1.36 ± 0.07	1.38 ± 0.04
0-70 d	1.21 ± 0.05	1.26 ± 0.03
71-91 d *	1.40 ± 0.18	1.68 ± 0.06
	Feed to gain ratio	
0-28 d	4.54 ± 0.35	4.34 ± 0.20
29-70 d	5.16 ± 0.57	5.40 ± 0.33
0-70 d	4.88 ± 0.21	4.76 ± 0.12
71-91 *	5.88 ± 1.50	6.19 ± 0.49
	Residual feed intake kg d^{-1}	
Across trial	0.005 ± 0.029	0.009 ± 0.017

[a, b] Within a row, means without a common superscript differ (P<0.05)
*Only animals in Group 2 were included in this period

The most clear phenotypic association to the novel SNP was observed for BF. Lambs with the CG genotype had 1.02 ± 0.4 mm (P < 0.05) more back fat than lambs with the CC genotype (Table 3); though, higher levels of gene expression and serum leptin concentrations for the CG genotype were not statistically detected (Table 4).

Conclusions

A non previously reported, non synonymous C→G transversion SNP in exon 3 of the *oLEP* gene was identified at position 355, which resulted in a predicted amino acid change from aspartic acid to glutamic acid at the 118 codon position. A phenotypic effect of the novel SNP was observed on back fat depth of lambs, but no evident effects on levels of gene expression, serum leptin concentrations, feed intake, feed efficiency or growth were detected. Breed specificity of the G transversion SNP deserves further investigation to evaluate its potential in marker assisted selection schemes.

Table 3: Least squares means (± SE) for traits measured on the carcass

Slaughter Group	Genotype	
	CC	**CG**
	Back fat depth, mm	
1	2.99 ± 0.39[c]	4.10 ± 0.35[d]
2	2.06 ± 0.72	3.11 ± 0.23
Average	2.55 ± 0.32[a]	3.58 ± 0.19[b]
	Kidney fat weight, kg	
1	0.57 ± 0.06	0.46 ± 0.05
2	0.45 ± 0.15	0.69 ± 0.05
Average	Genotype*Group interaction (P=0.04)	
	Rib eye area, cm^2	
1	14.61 ± 0.73	14.63 ± 0.65
2	13.73 ± 1.48	15.46 ± 0.48
Average	14.90 ± 0.68	15.00 ± 0.39

Within a row, means without a common superscript differ:
[a, b](P<0.05); [c, d](P<0.1)

Table 4: Least squares means (± SE) for the relative[1] expression of *oLEP* normalized by *RPL19* and serum leptin concentration

	Days on trial		
		Slaughter	
Genotype	28	70	91
	Log *oLEP* expression, Fold-change		
CC	1.81 ± 0.28	1.79 ± 0.31	2.43 ± 1.15
CG	2.08 ± 0.20	1.67 ± 0.24	3.26 ± 0.37
	Log serum leptin concentration, pg ml^{-1}		
CC	5.00 ± 0.68	4.75 ± 0.45	---
CG	5.33 ± 0.28	5.22 ± 0.26	5.11 ± 0.41

[1] Relative to the lamb with the least expression level

Cited Literature

Altmann, M., Sauerwein, H., and von Borell, E. (2006). Meat Science, 74:600-604.

Barzehkar, R.A., Salehi, A.A.R., and Mahjoubi, F. (2009). Iran. J. Biotechnol. 7:241-246.

Friedman, J. M., and Halaas, J. L. (1998). Nature 395:763-770.

Grasso, P., Leinung, M.C., Ingher, S.P. et al. (1997). Endocrinology 138:1413–1418.

Lagonigro, R., Wiener, P., Pilla, F. et al. (2003). Animal Genetics 34:371–374.

Livak, K.J., and Schmittgen, T.D. (2001). Methods 25:402–408.

Peletto, S., Bertuzzi, S., Campanella, C. et al. (2011). Int. J. Mol. Sci. 12:7732-7747.

Pérez-Montarelo, D., Fernández A., Folch, J.M. et al. (2012). Animal Genetics 43:805-809.

Schenkel, F.S., Miller, S.P., Ye, X. et al. (2005). J Anim. Sci. 83:2009-20.

Villalba, D., Tor, M., Vidal, O. et al. (2009). Livestock Science 121:335-338.

Zhou, H., Hickford, J.G.H., and Gong, H. (2009). Mol. Biotechnol. 41:22-25.