

Polymorphisms of the ovine *leptin* gene and its association with growth and carcass traits in three Iranian sheep breeds

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Abstract

Leptin (*LEP*), the expression product of the obese gene produced primarily in the adipose tissue, is related to feed intake, growth and lipid metabolism. The aim of this study was to study the possible association between polymorphism of the *LEP* gene with growth and carcass traits among three Iranian sheep breeds of the Shal, Zandi and Zel varieties. A total of 180 pure-bred animals of the Shal, Zandi and Zel, breeds were chosen for this study. Three flocks, each comprising of 60 ewes of three breeds, were derived from Aboureyhan sheep populations. The Shal (n=18), Zandi (n=24) and Zel (n=17) lambs were screened for polymorphism of the *LEP* gene. Following genomic DNA extraction from whole blood samples, polymerase chain reaction (PCR) was carried out in order to amplify a 260 bp fragment of the target gene. Polymorphisms were detected using the single strand conformational polymorphism (SSCP) technique. Two genotypes of AA and AG with frequencies of 0.53 and 0.47 in the Shal 0.70 and 0.30 in the Zandi and 0.65, 0.35 in the Zel breed were observed, respectively. The frequencies of alleles A and G were 0.74, 0.26 in the Shal breed, 0.85, 0.15 in the Zandi breed and 0.82, 0.18 in the Zel breed, respectively. Chi-Square test (χ^2) confirmed the Hardy-Weinberg equilibrium for the *LEP* loci. Average heterozygosity (30%) of the *LEP* locus for the three breeds was slightly low. Comparison of the sequence of the target gene available in the GeneBank with the results of the present study showed two single nucleotide polymorphisms (SNP) A→G and T→C transitions at 113 and 165 bp positions, respectively. In the Shal breed, the A113G SNP associated with an increase in cold carcass weight ($p < 0.01$), fat-tail percent ($p < 0.05$) and total body fat weight ($p < 0.05$). In the Zel breed, the A113G

SNP was associated with an increase in fat-tail percent ($p < 0.05$) and reduction in slaughter weight ($p < 0.05$), cold carcass weight ($p < 0.01$) and lean meat weight ($p < 0.01$). Therefore, a significant association between SNP within the *LEP* gene and certain carcass traits in the Shal and Zel breeds is proposed. In the Zandi breed the A113G SNP was not associated with carcass traits but showed a reduction in weaning weight ($P < 0.05$).

Keywords: Leptin; Polymorphism; Carcass traits; Shal; Zandi; Zel

INTRODUCTION

The recently discovered hormone *LEP* is the main factor in regulation of appetite (Thomas *et al.*, 2001). The *LEP* gene was discovered in 1994 by positional cloning techniques. The gene consists of two introns and three exons that are involved in the synthesis of a protein with 167 amino acids, which is secreted into blood after cleavage of the 21-amino-acid signal peptide (Dela *et al.*, 1996; Zhang *et al.*, 1994). The adipose tissue is the main source of *LEP*. In fact, *LEP*'s receptors are found in many organs including the brain, adipose tissue, stomach, gonads, muscles, hypothalamus and hypophysis (Houseknecht *et al.*, 1998; Dyer *et al.*, 1997). *LEP* acts as an adipostat or circulating signal that carries information from fat depots or energy reserves to regulatory appetite centers and is thought to play an important role in many physiological functions; lipogenesis, angiogenesis, thermogenesis, feed intake regulation, fertility, fetal growth, immunological processes, body weight, hypothalamus-hypophysis-adrenal and hypophysis-gonads axis (Barb *et al.*, 2001; Houseknecht *et al.*, 1998; Schneider *et al.*, 2000). Genetic differences in the *LEP* gene were

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first reported in mice and humans (Ohshiro., 2000; Halaas *et al.*, 1995). In recent years studies have been performed on the association between *LEP* gene polymorphisms and production traits in dairy cattle (Fiona *et al.*, 2002), sheep (Boucher *et al.*, 2006) and poultry (Taouis *et al.*, 1998). The Shal and Zandi breeds are known as fat-tailed breeds. These two breeds differ in body size and breeding conditions. The Shal is large in size while the Zandi is medium in size. The only tailed sheep breed of Iran is the Zel. The aim of this study was to identify polymorphisms of the ovine *LEP* gene and its possible association with growth and carcass traits in three Iranian sheep breeds.

MATERIALS AND METHODS

Animals: A total of 180 animals belonging to the Shal, Zandi and Zel breeds were selected for this study. Three flocks, each consisting of 60 ewes of the three breeds, were derived randomly. In each breed, ewes were mated with rams of the same breed. Population sets of randomly selected purebred Shal (n=18), Zandi (n=24) and Zel (n=17) lambs were selected. The choice of these three breeds was based on their opposite phenotypes for fat deposition and muscle accretion. The Shal and Zandi are known as fat-tailed native breeds of the Tehran province. These two breeds differed in size and living conditions. The Shal is large in size while the Zandi is medium in size. The only tailed sheep breed of Iran is the Zel, living as a native of two Northern provinces of the country. This breed is small in size and early-maturing (Kashan *et al.*, 2005). Lambs were reared with their dams until an average age of 90 days. At the time of weaning (approximately 90 days old), only the male lambs were selected in each breed. All lambs were then raised under the same controlled-environmental conditions. Animals were fed a ration without limitation to finishing weight. All lambs were slaughtered according to their targeted fattening period (90 days) after a 12 h fasting period.

Growth and Carcass data: From birth to weaning and during the 90-days fattening period, the lambs were weighed once a week. At the end of the fattening period all lambs were weighed. The weights of carcasses were also recorded. The carcasses were then chilled at 4°C for 24 h and weighed again. Subsequently, the carcasses were split longitudinally into two parts. The left side of the carcass was dissected into lean meat, bone, subcutaneous fat (SCF), intramuscular fat (IMF), trimmings and weighed separately according to Kashan *et al.* (2005). Internal fat was

obtained from the fat surrounding the intestine and kidney. The whole soft tissue (lean meat and fat) of the left side carcasses was grounded and passed twice through a plate with a 4mm orifice. Dry matter (DM), crude protein (CP), ash and chemical fat (ether extract) contents were detected according to the Association of Official Analytical Chemist (AOAC) standardized methods (1995).

Blood samples and PCR amplification: Blood samples were obtained from three pure breeds of Shal, Zandi and Zel lambs. Each sample contains 3-5 ml blood taken from jugular vein. Genomic DNA was purified from 100 µl of blood samples using the Diatom DNA prep100 (DNA extraction kit) (gene Fanavaran Co., Tehran) The Primers were designed based on available ovine genomic sequences (GeneBank accession numbers: U84247 and AY831682). The primer sequences are as follows: forward 5'-CGCAAGGTCCAGGATGACACC-3'; and reverse 5'-GTCTGGGAGGGAGGAGAGTGA-3'. The amplicons were 260 bp in length that covered exon 2 and part of intron 2 of the ovine *LEP* gene. The amplification reactions were performed in a total volume of 25 µl containing 200 ng of genomic DNA, 1 µl of each primer (10 pM), 0.5 µl of dNTPs (10 mM), 0.8 µl of MgCl₂ (1.6 mM), 1.2 U of *Taq* DNA polymerase and 2.5 µl of 10X PCR buffer in 35 Cycles (initial denaturation: 94°C, 2 min; denaturation: 94°C, 30s; annealing: 62.5°C, 25s; extension: 72°C, 45s) followed by a final extension step at 72°C for 7 min.

SSCP and sequencing: PCR products were purified using DNA purification kit (Gene Fanavaran Co., Tehran) before being sequenced. For genotyping of the *LEP* locus, SSCP was carried out on the PCR product according to the standard protocol (Sambrook *et al.*, 1989). One microliter aliquots of the PCR products were mixed with 4 µl of dye 95% formamide, 25 mM EDTA, 0.025% xylene-cyanol and 0.025% bromophenol blue), incubated at 95°C for 10 min and then chilled on ice. Denatured DNA was loaded on a 6% acrylamid gel in 1×TBE buffer and electrophoresed at a constant voltage of 150 V for 15 h. The gel was stained with 0.1% silver nitrate solution. (Xianyong *et al.*, 2007). The PCR fragments from different SSCP patterns in different breeds were cleaned up and sequenced by the ABI PRIZM 377 DNA sequencer (Perkin-Elmer Corporation).

Statistical analysis: The genotypic and allelic frequencies, expected mean, observed and Nei's heterozygosities were calculated using the Pop Gene 32

(version 1.31) software. The Hardy-Weinberg equilibrium in the populations was also tested. The sequences were aligned using the Sequencer Software (version 4.2). The General Linear Model (GLM) procedure of Statistical Analysis System SAS (SAS Institute Inc, version 9.2, USA) was used to test the association of SNP marker genotypes of the *LEP* gene with growth and carcass traits. The initial and final live weights of the lambs were used as co-variate for body and carcass weight, while empty body weight was used as a co-variate for the carcass components.

The linear model used was as follows:

$$Y_{ijk} = \mu + G_i + b(x_i - \bar{x}) + \varepsilon_{ijk} \quad (1)$$

Where Y_{ijk} is the considered dependent variable (growth and carcass traits); μ , the overall mean, G_i , the fixed effect of the i th genotype at the *LEP* locus ($i = 1, 2, 3$ for AA, AG and GG, respectively); $b(x_i - \bar{x})$, the covariate and ε_{ijk} is the residual effect. Analyses were performed for each breed, separately.

RESULTS

PCR-SSCP and sequencing: The 260 bp PCR product consisting of exon 2 and part of intron 2 of the *LEP* gene was amplified. Following PCR-SSCP and sequencing, two alleles (A and G) were found and two genotypes (AA and AG) were observed. (Fig. 1). Two SNPs in intron 2 were noticed by A→G transition at the 113 bp position and T→C transition at the 165 bp position of the ovine *LEP* gene (Fig. 2). According to the results of sequencing, C→T SNP for all samples was similar.

Genetic variability: The *LEP* allelic and genotypic frequencies and chi-square (χ^2) values are summarized in Table 1. The genotypic frequencies of AA and AG were 0.53, 0.47 in the Shal lambs and 0.70, 0.30 in the Zandi lambs and 0.65, 0.35 in the Zel lambs, respec-

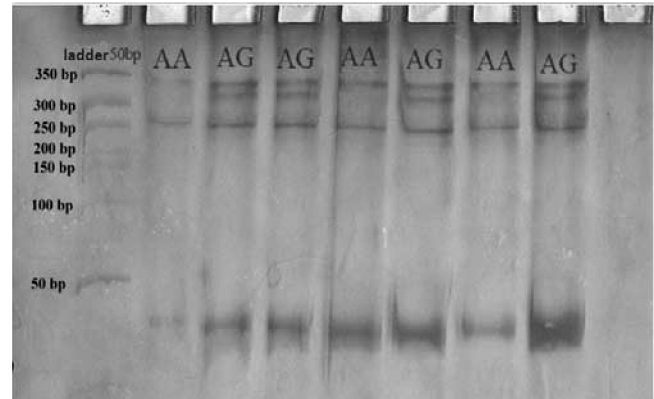


Figure 1. Acryl amide electrophoresis of different Leptin genotypes. Molecular marker is M50 (gene Fanavaran Co., Tehran).

tively. The GG genotype was not observed in these populations. Allelic frequencies for A and G were 0.76, 0.24 in the Shal lambs and 0.85, 0.15 in the Zandi lambs and 0.82, 0.18 in the Zel lambs, respectively. The χ^2 test confirmed the Hardy-Weinberg equilibrium in the studied populations. Observed and expected heterozygosities, Nei's and average heterozygosities of the *LEP* gene for the three Iranian sheep populations were slightly low (Table 2).

Association analysis: The GLM procedure was performed to determine possible association between the *LEP* gene and growth and carcass traits. Significant associations were found for the A→G SNP (Table 3). In the Shal breed, the presence of the A→G SNP is associated with an increase in cold carcass weight (2473 g; $p < 0.01$), fat-tail (3.3%; $p < 0.05$) and total body fat (1074.1 g; $p < 0.05$) of the carcass traits. In the Zel breed, the presence of the A→G SNP is associated with an increase in fat-tail (1.6%; $p < 0.05$) and a reduction in slaughter weight (-577.9 g; $p < 0.05$), cold carcass weight (577.4 g; $p < 0.01$) and lean meat weight (303.3 g; $p < 0.05$). This study did not indicate any obvious association between the intron 2 SNP and growth traits in the Shal breed.

Table1. Allelic and genotypic frequencies and χ^2 values of the exon 4 region of the *LEP* gene in three Iranian sheep breeds.

	Allele		Genotypes		χ^2
	A	G	AA	AG	
Shal	0.74	0.26	0.47	0.53	1.38
Zandi	0.85	0.15	0.70	0.30	0.62
Zel	0.82	0.18	0.65	0.35	0.63
Total	0.82	0.18	0.63	0.37	2.75

A, G and AA, AG are alleles and genotypic frequencies; χ^2 is Chi-Square value.

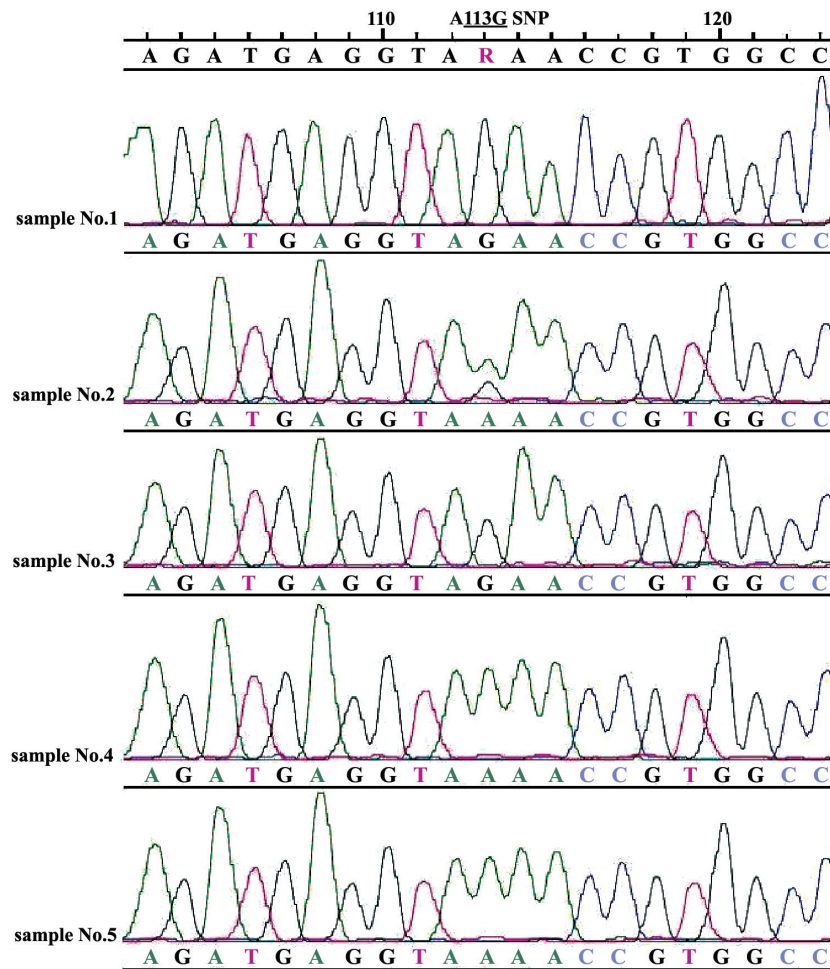


Figure 2. The sequencing results and comparisons of the *LEP* gene in Shal breed representing SNP A to G transition in base 113 (A113G SNP).

Table 2. Observed, expected and average heterozygosities of the *LEP* gene.

	Locus	Ob-Het	Exp-Het*	Nei**	Ave-Het
Shal	LEP	0.53	0.40	0.36	0.30
Zandi	LEP	0.30	0.26	0.26	0.30
Zel	LEP	0.35	0.30	0.29	0.30
Total	LEP	0.37	0.30	0.30	0.30

Ob-Het, Exp-Het and Ave-Het are Observed, Expected and Average heterozygosities. *Expected heterozygosity was computed using the Levene (1949) formula. **Nei (1973) is the expected heterozygosity.

DISCUSSION

Genetic variability: We observed two SNP in intron 2 in these three Iranian sheep populations whereas, three SNPs, A→G, C→T and C→G in intron 2 of the *LEP* gene in the Suffolk and Dorset breeds have been detected (Boucher *et al.*, 2006).

Two genotypes have been identified (AA and AG) with frequencies ranging from 0.53 to 0.70 for AA and 0.30 to 0.47 for AG genotype in Shal, Zandi, and Zel lambs. Results of this study are to some extent almost similar to published data on other sheep breeds. The genotypic frequencies of AA and AG in the ovine *LEP* gene of Dorset and Suffolk breeds have been found to

Table 3. Effect of the A113G SNP on growth and carcass traits measured in Shal, Zandi and Zel lambs.

Traits	Breed					
	Shal		Zandi		Zel	
	AG	AA	AG	AA	AG	AA
Growth performance (g)						
Birth weight	4657.5 ± 241.1	4491.7 ± 201.3	3912.5 ± 241.7	4093.4 ± 199.8	ND	ND
Average daily gain	259.9 ± 14.3	241.4 ± 10.6	180.9 ± 23.5	192.8 ± 13.4	ND	ND
Weaning weight	28054 ± 1345.4	26220.5 ± 978.3	20189.7±2294 ^a	21445 ± 1249 ^b	ND	ND
Start weight	37750 ± 1636.6	34555 ± 1821	25813 ± 2592	28306 ± 1592.5	18816 ± 995.1	20781 ± 898.6
Average daily gain	169 ± 11.6	167.9 ± 13.1	158.2 ± 11.6	137.2 ± 9.0	152 ± 8.64	138.8 ± 9.4
Slaughter weight	53000 ± 1510.6	49661 ± 2365	40056 ± 3100.2	40662 ± 1914.3	32525 ± 607.1 ^a	33268.2 ± 1330 ^b
Cold carcass weight	25525 ± 936 ^A	33052.8 ± 1228 ^B	18466 ± 1555.6	18866 ± 1104.2	14006.7 ± 387 ^A	14584.6 ± 671 ^B
Carcass components (g)						
Lean meat	5923.9 ± 331.8	5961.9 ± 266.7	4513.1 ± 299	4636.6 ± 205.2	3629 ± 53 ^A	3932.3 ± 148.3 ^B
Bone	1820 ± 81.3	1781.7 ± 90.7	1410.6 ± 117.3	1513 ± 57.9	1202.5 ± 37.9	1194.5 ± 33.5
Fat-tail	1815 ± 108.1	1425 ± 198.4	1014.4 ± 175.1	1304.4 ± 178.6	226.5 ± 60.5	123 ± 19.3
Subcutaneous fat (SCF)	1844.4 ± 202.5	1458.9 ± 199.9	1236.3 ± 232	1128.4 ± 119.5	890 ± 137.1	1010.5 ± 140.1
Intramuscular fat (IMF)	710 ± 89.2	695.5 ± 108.3	535.6 ± 61.8	528.8 ± 36.0	648.3 ± 91.9	562.3 ± 59.5
Internal fat	1093.8 ± 121.3	809.4 ± 134.1	602.5 ± 128.68	521.6 ± 52.5	768.3 ± 97.8	848.2 ± 103.5
Total body fat	5463 ± 281.5 ^a	4388.9 ± 394.6 ^b	3388.8 ± 489.1	3483 ± 345.6	2684.2 ± 206.8	2625.9 ± 282.1
Carcass components (%)						
Lean meat	49.6 ± 1.7	51.2 ± 1.3	50.3 ± 2.0	50.9 ± 1.1	52.7 ± 1.0	55.9 ± 1.6
Bone	15.2 ± 0.6	15.4 ± 0.4	15.8 ± 0.7	16.5 ± 0.5	17.5 ± 0.8	17 ± 0.53
Fat-tail	15.2 ± 0.9 ^a	11.9 ± 1.1 ^b	10.9 ± 1.6	13.1 ± 1.2	3.3 ± 0.8 ^a	1.7 ± 0.2 ^b
Subcutaneous fat (SCF)	15.4 ± 1.8	12.9 ± 1.9	12.9 ± 1.7	11.7 ± 0.8	12.8 ± 1.8	13.8 ± 1.6
Intramuscular fat (IMF)	6 ± 0.8	5.8 ± 0.7	5.9 ± 0.39	5.7 ± 0.3	9.4 ± 1.3	7.8 ± 0.7
Internal fat	4.2 ± 0.5	3.5 ± 0.6	3.1 ± 0.5	2.7 ± 0.2	5.4 ± 0.67	5.6 ± 0.5
Total body fat	21.2 ± 1.1	18.1 ± 1.1	17.2 ± 1.4	17.7 ± 1.1	15.5 ± 0.9	14 ± 1.2
Chemical composition (%)						
Protein	14.4 ± 0.99	15.7 ± 0.46	13.9 ± 0.45	14.6 ± 0.69	18.0 ± 0.5	17.7 ± 1.03
Lipid	32.5 ± 2.12	28.4 ± 1.51	28.6 ± 1.75	27.9 ± 1.78	28.7 ± 2.11	26.2 ± 2.11
Ash	3.1 ± 0.2	3.0 ± 0.2	2.8 ± 0.2	2.9 ± 0.2	2.4 ± 0.1	2.6 ± 0.1
Water	50.8 ± 1.1	48.2 ± 1.5	46.4 ± 1.4	46.9 ± 1.2	50.6 ± 2.5	47.3 ± 0.6

Values are least square means. Within each breed, means with capital and small superscript letters differ, $P < 0.01$ and $P < 0.05$, respectively. ND: Not Determined.

be 0.75, 0.25 and 0.87, 0.13, respectively (Boucher *et al.*, 2006).

The frequencies of alleles were (0.76 and 0.24), (0.85 and 0.15) and (0.82 and 0.18) for A and G, in the Shal, Zandi and Zel lambs, respectively. Boucher *et al.* (2006) found allelic frequencies of 0.87 and 0.13 for A and G in Dorset sheep, respectively, which is in agreement with our results. Allelic frequencies of A and T for E2JW SNP in the bovine *LEP* gene of the Charolay cattle have been found to be 0.91 and 0.09, respectively (Schenkel *et al.*, 2005). However, our results of sequencing suggested that C→T mutation is fixed in all studied population.

The studied populations show a low degree of genetic variability for the *LEP* gene. Our explanation is that a few number of ram which have been used for breeding may lower the effective number of population with conservation so that heterozygosity decreases as a result of inbreeding.

Association analysis: We observed significant association in the presence of A→G SNP with some carcass traits in Shal and Zel breeds. Published data indicated a similar association between lean meat and the *LEP* locus (Boucher *et al.*, 2006). The UASMS1 SNP is associated with fat yield, grade fat and lean meat yield

(Schenkel *et al.*, 2005). It has previously been reported that the A→G SNP in the ovine *LEP* gene has no significant association with total dissected fat (Boucher *et al.*, 2006). Although no significant association was observed between A→G SNP and carcass traits in the Zandi breed, but a significant reduction (1255.3g) in weaning weight was detected ($p < 0.01$). It has been reported that intronic mutations, such as the A→G variant, may affect gene regulation and transcription levels (Tokuhiko *et al.*, 2003). Intronic mutations can also result in splicing abnormalities, which often change the structure of mature protein (Faustino *et al.*, 2003).

CONCLUSION

This study demonstrated the polymorphism of the *LEP* gene and its association with growth and carcass traits in three Iranian sheep breeds. The results obtained from this study suggest a significant association between the A113G variant of the *LEP* gene and carcass traits in the Shal and Zel breeds only. Generally, the lambs with the G allele had a higher carcass fat. However, the A→G SNP in intron 2 of the *LEP* gene did not have such an effect on growth traits and only in the Zandi breed a significant reduction in weaning weight was observed. To date, this was the first study that attempted to detect allele variation in the ovine *LEP* gene in Iranian sheep breeds. Further studies will be needed before application of this SNP to the breeding industry.

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