Original Article

The relationship of *GH* and *LEP* gene polymorphisms with fat-tail measurements (fat-tail dimensions) in fat-tailed Makooei breed of Iranian sheep

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Abstract

Background: The present study was designed to investigate the association of *GH* and *LEP* genes' single-nucleotide polymorphisms with fat-tail measurements (fat-tail dimensions) in Makooei sheep.

Materials and Methods: DNA was extracted from whole blood samples collected from 100 sheep. Polymerase chain reaction (PCR) products were subjected to single-strand conformation polymorphism (SSCP) denaturation and polyacrylamide gel electrophoresis. Data were collected at the Makooei Sheep Breeding Station in Makoo (36°, 35′S and 48°, 22′E) of West Azerbaijan province. Climatically, this location has temperate summers and cold winters and receives a mean annual rainfall of about 400 mm. Ewes are raised in an annual breeding cycle starting in September. In general, the flock is managed under a semi-migratory system.

Results: In the tested Makooei sheep population, significant statistical results were found in all traits of fat-tail measurements for *GH* and *LEP* genes. Individuals with the G4, L4 genotype of *GH* and *LEP* genes had lower tail length (rump length), fat thickness (the thick rump), and tail width (rump width) when compared to those of individuals with other genotypes ($P < 0.05$). In addition, the results demonstrated that individuals with the G5, L5 genotype of *GH* and *LEP* genes had superiority of tail length (rump length) and fat thickness (the thick rump) compared to those individuals with other genotypes ($P < 0.05$). Individuals with the G2, L2 genotype of *GH* and *LEP* genes had superiority of tail width (rump width) compared individuals with other genotypes ($P < 0.05$).

Conclusion: These results confirmed potential usefulness of *GH* and *LEP* genes in marker-assisted selection programs of sheep breeding.

Key Words: Fat-tail dimensions, growth hormone, leptin, Makooei sheep breed, polymorphism

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INTRODUCTION

Sheep are an important source of meat in Iran. Indigenous sheep form a valuable genetic resource for sustainable utilization of arid and semi-arid lands which form approximately more than 90% of total land mass of the country. There is evidence that they are resistant to many endemic diseases in Iran. Artificial

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selection that has resulted in tremendous change and wide diversity in phenotypic (morphological, physiological, and behavioral) characters of domesticated animals results in a variety of genotypes with specific production properties and adaptation capability.[1] Almost all Iranian sheep breeds have large fat tails and other adipose depots that negatively affect the sale of sheep by sheep industries in some countries like Iran. Fat tail is not desirable to customers even though it appears to be affordable (the price of 1 kg of fat tail is less than one-sixth of that of 1 kg red meat). Fat tail plays an important role as a source of energy for adult ewe during periods of food shortage (autumn and especially winter). In young lambs, carcass adiposity, particularly the fat tail, reduces the meat value. Lean lamb can be produced by manipulation of nutritional regimen, slaughter at an early age, docking the fat tail in early days after birth, or by a genetic selection program.[2] In the early 1990s, a nucleus flock of Makooei sheep was established in Makoo, West Azerbaijan province, Iran. The aim was to establish a nucleus source for improving other flocks in the region. From the time the first lambs were born, information on growth performance has been recorded and stored in the station. The Makooei sheep is one of the fat-tailed, medium-sized breeds of Iran. They are distributed in the mountainous areas of the country, especially in West Azerbaijan province. Also, they are found in Turkey and are called White Karaman. They are valuable primarily for meat and also for their wool and milk. The wool produced is coarse and usually used for carpet weaving.[3] Today, there are about 2,700,000 heads of Makooei sheep in West Azerbaijan, and due to the large population of this breed, there is an increasing interest in the genetic improvement of this breed. The Makooei sheep examined in this study were fat-tailed sheep with medium body size and were white in color with black spots on the face and feet. They are kept as livestock for meat and wool production in East and West Azerbaijan provinces of Iran.[4] Data were collected at the Makooei Sheep Breeding Station (MSBS) in Makoo (36°, 35′S and 48°, 22′E) in West Azerbaijan province. Animals are kept on natural pasture during spring, summer, and autumn. Range conditions are poor during the winter months and, therefore, the animals are kept indoors during winter. Young ewes are mated so as to lamb for the first time at approximately 1.5 years of age. There is one breeding season in August–October. Lambs are weaned at approximately 100 days of age. In general, the flock is managed under a semi-migratory system.[4-5]

Growth in animals is controlled by a complex system, in which the somatotropic axis plays a key role. For growth, many genes work together to achieve bone formation, birth weight, weaning weight, body condition, and muscle growth.[6] Growth hormone (GH) is a peptide of 22 kda with two disulfide bonds. It has two variants; the first variant is composed of 191 amino acids with terminal alanine and the second variant has 190 amino acids with phenylalanine at the end.[7] This hormone is a major regulator of postnatal growth and metabolism in mammals and has a role in body composition, health, and milk production.[8] The leptin (*LEP*) gene was discovered in 1994 by Zhang.[9] The expression product of this gene is a protein with the same name which is composed of 167 amino acids that act as a satiety signal regulating body weight, feed intake, and has a role in reproductive, immunity, growth and metabolism, and expansion of energy reserves in the body. Leptin is an effective candidate gene for important economic traits in livestock.[10] Therefore, the aim of this study was to investigate the relationship between GH and leptin conformational patterns with fat-tail measurements (fat-tail dimensions) using single-strand conformation polymorphism (SSCP) method in Makooei sheep.

MATERIALS AND METHODS

Animal genomic screening and data collection

Data collection

Data were collected at the MSBS in Makoo (36°, 35′S and 48°, 22′E) in West Azerbaijan province. Climatically, this location has temperate summers and cold winters and receives a mean annual rainfall of about 400 mm. Ewes are raised in an annual breeding cycle starting in September. In general, the flock is managed under a semi-migratory system.[11] Ewes are raised in an annual breeding cycle starting in August. There is one breeding season in August–October. Ewes in heat are exposed to pre-defined rams in the morning. Lambing begins in mid-January and continues until April. Ewes are supplemented, depending upon their requirements, for a few days after lambing. All lambs are identified at birth, and birth weights, as well as sex, birth type, and pedigree information are recorded. During the suckling period, lambs are fed with their mothers' milk and are also allowed dry alfalfa after 3 weeks of age. Lambs are weaned at approximately 100 days of age. Animals are kept on natural pasture during spring, summer, and autumn. Range conditions are poor during the winter months and, therefore, animals are kept indoors during winter. Animals are kept on natural pasture during spring, summer, and autumn.[12]

Animal genomic screening

Blood samples (approximately 2–3 ml) were obtained from 100 unrelated Makooei sheep from different

parts of West Azerbaijan province and stored in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Genomic DNA was extracted from 0.3 ml blood using the genomic DNA purification kit (Fermentas, CinnaGen Inc., Tehran, Iran) according to manufacturer's instructions. The quality and quantity of extracted DNA was measured by 0.8% agarose gel electrophoresis.

Amplification of exon 3 of LEP gene

DNA amplification of the *LEP* gene was achieved by polymerase chain reaction (PCR). Two PCR primers, LEP-up (5-AGGAAGCACCTCTACGCTC-3) and LEP-dn (5′-CTTCAAGGCTTCAGCACC-3′), targeting a fragment of 471 bp were employed as described.[13] The PCRs were carried out in 50 μl volumes using PCR Master Mix kit (CinnaGen Inc., Tehran, Iran) containing 2.5 U of Taq DNA polymerase in reaction buffer, 4 mM MgCl2, 50 μM of each of dATP, dCTP, dGTP, and dTTP, 0.5 μM of each primer, and about 100 ng of extracted DNA as a template. The thermal profile consisted of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, with a final extension of 5 min at 72°C. Amplification was carried out in a Mastercycler (Eppendorf, Homburg, Germany).

Amplification of exon 4 of GH gene

PCR was carried out on about 25 ng genomic DNA in a reaction volume of 25 μl. PCR reactions were performed in a final volume of 25 μl according to the following conditions: 25 ng of genomic DNA, 10 pmol of each primer, 1.5 unit of Taq DNA polymerase, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP. The amplification included an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s followed by a final extension at 72°C for 5 min. Each amplification product was analyzed by electrophoresis on a 2% agarose gel (5 V/cm) using ethidium bromide staining. Two pairs of oligonucleotide primers were designed and a standard PCR protocol was used to amplify three fragments. Sequences of the primers that were used in PCR had previously been reported by Marques *et al*. in 2006.[14] The sequences of each of the primers were as follows:

Forward: 5'- CTGCCAGCAGGACTTGGAGC-3', respectively

Reverse: 5'- GGAAGGGACCCAACAATGCCA-3'.

Single-strand confirmation polymorphism

For SSCP analysis, several factors were tested to optimize the methodology. The amount of PCR product (4–15 μl), dilution in denaturing solution (20–85%), denaturing solution (A: 95% of formamide, 10 mM NaOH, 0.05% xylene cyanol, and 0.05% bromophenol blue; B: Same as A, plus 20 mM of EDTA), acrylamide concentration (6–14%), percentage of cross linking (1.5–5%), presence (10%) or absence of glycerol, voltage (100–350 V), running time (2–12 h), and running temperatures (4°C, 6°C, 10°C, and 15°C). Each PCR was diluted in denaturing solution, denatured at 95°C for 5 min, chilled on ice, and resolved on non-denaturing polyacrylamide gel.

Studied traits

The traits measured were tail length (rump length), fat thickness (the thick rump), and tail width (rump width).

Statistical analysis

For the association studies, the traits of interest were analyzed using the general linear model (GLM) procedure of the SAS program, according to the following statistical model:

$$Y_{ijklm} = \mu + G_i + Ld + S_j + e_{ijklm},$$

Where

Y_{ijklm} is growth traits or fat-tailed measus, μ the overall mean, G_i the fixed effect of the ith genotype for GH, Ld the fixed effect of the dth genotype for leptin, S_j the fixed effect of sex (j = 1, 2), and e_{ijklm} is the random residual error.

RESULTS

The PCR-SSCP analysis of exon 3 *LEP* gene revealed five distinct patterns. The frequencies of the observed genotypes were 0.09, 0.17, 0.37, 0.14, and 0.23 for BB (L1), AB (L2), BC (L3), AC (L4), and CC (L5), respectively. Allele frequencies were 0.15, 0.37, and 0.48 for A, B, and C, respectively. The observed heterozygosity (H_{obs}) value for leptin was 0.6769. PCR-SSCP analysis of the of the ovine *GH* gene (a part of intron 3, exon 4, and a part of intron 4) revealed the following five banding patterns: AA (g1), AB (g2), BB (g3), CC (g4), and CD (g5), respectively. Four alleles A, B, C, and D with frequencies of 0.63, 0.327, 0.028, and 0.015 were detected. Genotype frequencies were 31.3, 64.6, 1, 1, and 2.1 for AA, AB, BB, CC, and CD, respectively.

The relationship between *GH* and *LEP* genes single nucleotide polymorphisms with fat-tail measurements

Evaluation of the relationships between genotypes and fat-tail measurements was done with 100 samples. Levels of significance, least squares means, and standard errors are reported in Tables 1 and 2.

Table 1: Least square means and standard errors for fat-tail dimensions of Makooei sheep according to the different LEP patterns

	Fat-tail dimensions		
Leptin	Tail length	Tail width	Fat thickness
L1	26.44^{ab}±0.25	32.06^{a}±0.93	1.19^{ab}±0.11
L2	28.20^{ab}±0.86	34.48^{a}±1.43	1.297^{ab}±0.17
L3	27.41^{ab}±0.67	33.55^{a}±0.97	1.33^{ab}±0.12
L4	23.95^{b}±1.34	25.25^{b}±2.04	0.88^{b}±0.11
L5	28.23^{a}±1.15	33.82^{a}±1.50	1.73^{a}±0.18
F value	3.32*	3.57*	3.26*

*Dissimilar letters in the same column show significant difference (P<0.05).
LEP: Leptin

Table 2: Least square means and standard errors for fat-tail dimensions of Makooei sheep according to the different GH patterns

	Fat-tail dimensions		
GH	Tail length	Tail width	Fat thickness
G1	27.54^{a}±0.25	32.74^{a}±0.77	1.39^{ab}±0.09
G2	29.10^{a}±0.86	35.30^{a}±1.26	1.33^{ab}±0.16
G3	28.51^{a}±0.67	34.13^{a}±0.84	1.51^{ab}±0.10
G4	22.97^{b}±1.34	25.85^{b}±1.86	1.09^{b}±0.24
G5	29.33^{a}±1.15	34.75^{a}±1.31	2.00^{a}±0.17
F value	3.42*	3.94*	3.34*

*Dissimilar letters in the same column show significant difference (P<0.05).
GH: Growth hormone

In the tested Makooei sheep population, significant statistical results were found in all traits of fat-tail measurements for *GH* and *LEP* genes.

Effects of GH and leptin SSCP variants on the fat-tail measurement traits in Makooei sheep are presented in Tables 1 and 2. The table demonstrates a significant effect of this polymorphism on tail length (rump length), fat thickness (the thick rump), tail width (rump width) (all traits) in GH and leptin conformational patterns.

Individuals with the G4, L4 genotype of *GH* and *LEP* genes had lower tail length (rump length), fat thickness (the thick rump), and tail width (rump width) (all traits) compared to individuals with other genotypes ($P < 0.05$). In addition, the results demonstrated that individuals with the G5, L5 genotype of *GH* and *LEP* genes had superiority of tail length (rump length) and fat thickness (the thick rump) compared to individuals with other genotypes ($P < 0.05$). Also, individuals with the G2, L2 genotype of *GH* and *LEP* genes had superiority of tail width (rump width) compared to individuals with other genotypes ($P < 0.05$).

DISCUSSION

Fat tail is considered as an adaptive response of animals to a hazardous environment and it is a valuable energy reserve for the animal during migration and winter. It had additional value to the herder because it was used to preserve cooked meat for longer periods of time and also as an energy reserve during times of drought and famine. Therefore, the climatic variation as well as the associated requirements of humans led to artificial selection for higher fat tail weight across generations.[15] Nowadays, in intensive and semi-intensive systems, most of the advantages of large fat tail have reduced their importance, and therefore, a decrease in fat tail size is often desirable for producers and consumers.[16] In contrast to natural populations, domesticated species provide an exciting opportunity to understand how artificial selection promotes rapid phenotypic evolution.[17] With a hypothesis that different selection pressures operated in thin and fat tail breeds over time and somehow the selection acts on a variant that is advantageous only in one breed, it is expected that the frequency of that variant may differ across populations to a greater extent than predicted for variants evolving neutrally in all populations.[18] Identifying these genome regions, which have been subject to such selective sweeps, could reveal the mutations that are responsible for fat deposition in these breeds.

To date, a relatively small number of studies have successfully identified genomic regions subject to positive selection in different domestic animals.[18-21] The constraint to identify selection signatures in sheep has been the limited density of markers. Fat-tail average was shown to be 1.03 kg. Furthermore, tail fat as a proportion of carcass also varied widely. This could be a biological diversity criterion in this breed and could be used for selecting lambs with smaller fat tail. Fat-tail plays an important role as a source of energy for adult ewe during periods of food shortage (Fall and specially winter).[22-23] There is limited information regarding the relationship between gene polymorphisms and fat-tail measurements in sheep. There is very little information about polymorphism of the *GH* and *LEP* genes and its association with fat-tail measurement traits. Many studies have been conducted on the association of polymorphism of the genes with carcass traits. It has been reported that the relationship between band patterns with tail length and tail down circumference was close to the significance level in the fourth exon of *GH* gene in Kermani sheep.[24] Also, the statistical analysis showed significant relationship between the band patterns (exon 3 ovine *LEP* gene) and tail, chest, abdomen, and neck circumference ($P < 0.05$) and body length ($P < 0.01$) in the Zel breed. Also, in the Bakhtiari breed, the patterns were associated with stature, gap tail length, and middle and down tail width ($P < 0.05$).[25]

Also, statistical analysis showed significant association between band patterns (part of exon and intron 2 *LEP* gene in fat-tailed Lori-Bakhtiari and tailed Zel breed) and hip circumference, tail length, and blood triglyceride in the Zel breed ($P < 0.05$). But there was no significant association between the observed bands and the measured traits in the Bakhtiari breed.[26] Also, statistical analysis showed significant association between band patterns 16-17 exon of *DGAT1* gene in Lori-Bakhtiari sheep (LB) and Zel sheep (Z) breeds. At the DGAT1 locus, CC sheep showed significantly greater fat-tail weight ($P < 0.05$) and backfat thickness ($P < 0.01$). The results of this study demonstrate novel associations in which the C allele of *DGAT1* gene had a positive effect on fat-tail weight and backfat thickness in Lori-Bakhtiari sheep (LB) and Zel sheep (Z) breeds.[27]

In the tested Makooei sheep population, significant statistical results were found in all traits of fat-tail measurements for *GH* and *LEP* genes. Effects of GH and leptin SSCP variants on the fat-tail measurements in Makooei sheep are presented in Table 1. The table demonstrates a significant effect of this polymorphism on tail length (rump length), fat thickness (the thick rump), and tail width (rump width) (all traits) in GH and leptin conformational patterns. These results confirmed potential usefulness of *GH* and *LEP* genes in marker-assisted selection programs of sheep breeding.

To date, this is the first study that attempted to detect association between allele variation in the ovine *GH* and *LEP* genes and fat-tail dimensions in Iranian sheep breeds. The previous breeding programs in most research centers of Iran were based on only phenotypic characters. Thus, adding sheep varieties, expanding samples, and doing further correlation studies are still needed to accumulate quantitative molecular genetic data in studying the relationship between the *GH* and *LEP* genes and fat-tail dimensions in sheep.

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