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Full Length Research Paper

Allelic polymorphism of Makoei sheep leptin gene identified by polymerase chain reaction and single strand conformation polymorphism

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Leptin, a 16-kDa protein secreted from white adipocytes has been implicated in the regulation of food intake, energy expenditure and whole-body energy balance in animals. In the present study, the polymorphism of the leptin gene (LEP) of Makoei sheep was investigated by polymerase chain reaction and single strand conformation polymorphism technique (PCR–SSCP). Genomic DNA was extracted from whole blood samples collected from 130 sheep. A 471 bp LEP exon 3 segment was amplified by standard PCR, using the locus specific primers. PCR products were subjected to SSCP denaturation and polyacrylamide gel electrophoresis. SSCP bands were visualized with silver staining. Five SSCP patterns, representing five different genotypes, were identified. The frequencies of the observed genotypes were 0.17, 0.09, 0.14, 0.37 and 0.23 for AB, BB, AC, BC and CC, respectively. Allele frequencies were 0.15, 0.37 and 0.48 for A, B and C, respectively. The observed heterozygosity (H_{obs}) value for LEP gene was 0.6769. The chi-square test showed that there was no significant deviation (P>0.01) from Hardy-Weinberg equilibrium for this locus in Makoei sheep population.

Key words: Leptin gene (LEP), polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP) Makoei sheep.

INTRODUCTION

Leptin is a 167-amino acid protein produced by the leptin gene (*LEP*); it plays a key role in regulating energy intake and expenditure, including appetite and metabolism. It is synthesized by adipose tissue and involved in regulation of food intake, energy balance, fertility and immune functions (Fruhbeck et al., 1998). The physiological role and biology of leptin is well reviewed (Hossner., 1998; Houseknecht et al., 1998). Leptin, the product of the *OB* gene, is a protein secreted predominantly by white adipose tissue (WAT) (Frederich et al., 1995; Friedman and Halaa, 1998; Houseknecht et al., 1998).

The leptin gene is located on the fourth chromosome in the bovine genome (Barendse et al., 1994; Stone et al., 1996) and located on chromosome 7 in human. Several SNPs have been previously identified in introns and exons of leptin among different breeds of cattle (GreGreen et al., 1995).

Liefers et al. (2003) observed that mutations in the leptin gene or its promoter are associated with differences in serum leptin concentrations and other economically relevant traits in beef and dairy cattle. Liefers et al. (2003) also, suggested that dry matter intake, live weight, milk yield and energy balance affect the serum leptin concentration.

It functions, through interactions with the leptin receptor in the hypothalamus, as a regulator of body weight, feed intake, energy expenditure and energy balance (Houseknecht et al., 1998; Woods et al., 1998; Chen et

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Abbreviations: LEP, Leptin gene; **PCR-SSCP,** polymerase chain reaction and single strand conformation polymorphism technique.

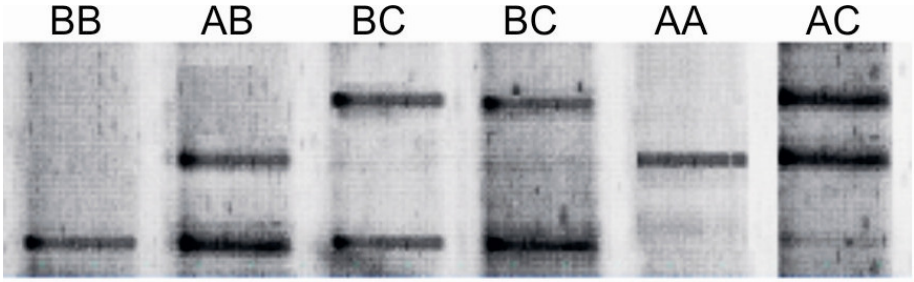

Figure 1. SSCP polymorphism of Makoei sheep *LEP* gene. Five different PCR-SSCP patterns (genotype) were identified.

al., 1996; Asdell, 1947). Endocrine regulation of leptin, gene expression and secretion has been studied (Frederich et al., 1995; MacDougald et al., 1995). The relation of leptin with reproduction and immune system having been scrutinized by many scholars (Garcia et al., 2002; Kirkwood and Aherne, 1985; Liefers et al., 2002; Lord et al., 1998) have shed light on the influence of leptin on both the milk performance and reproduction in beef cattle. Leptin and hematopoiesis have been discussed together (Leitman and Read, 1997; Shivdasani and Orkin, 1996).

Polymorphisms in the bovine *LEP* gene have been described (Haegeman et al., 2003; Leitman and Read, 1997; Pomp et al., 1997; Wilkins and Davey, 1997) and its association with food intake (Lagonigro et al., 2003), milk production (Buchanan et al., 2003; Liefers et al., 2003) carcass and meat quality traits (Schenkel et al., 2005) have been reported.

The objective of this study was to characterize potential variation in the Makoei sheep *LEP* gene using PCR-single-strand conformational polymorphism (PCR–SSCP) analysis and this study aimed to evaluate the genotype and gene frequencies of *LEP* gene of ˝Makoei˝ sheep breed in west Azerbaijan Breeding Station, Iran.

MATERIALS AND METHODS

Sheep, blood sample collection and genomic DNA extraction

Makoei sheep examined in this study were fat-tailed sheep with medium body size, white color with black spots on face and feet. They are kept in east and west Azerbaijan provinces of Iran and their main products are meat and wool (Saadat-Noori and Siah-Mansoor, 1992). Blood samples (approximately 2 to 3 ml) were obtained from 130 unrelated Makoei sheep from different parts of west Azerbaijan province and stored in EDTA-coated tubes. Genomic DNA was extracted from 0.3 ml blood using the genomic DNA purification kit (Cat. No 0512, Fermentas, EU) according to manufacturer's instructions. Quality and quantity of extracted DNA was measured by 0.8% agarose gel electrophoresis.

Amplification of the exon 3 of *LEP* gene

The DNA amplification of the *LEP* gene was achieved by PCR. Two PCR primers, *LEP*-up (5-AGGAAGCACCTCTACGCTC-3) and *LEP*-dn (5´-CTTCAAGGCTTCAGCACC-3´), targeting a fragment of 471 bp was employed as described (Zhou et al., 2009). The PCRs were carried out in 50 µl volumes using PCR mastermix kit (Cinnagen, Iran) containing 2.5 units *Taq* DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer and about 100 ng of extracted DNA as 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 Mastercycler (Eppendorf, Germany).

Single strand confirmation polymorphism (SSCP)

PCR products were mixed with 8 µl of denaturing loading dye [95% (w/v) deionized formamide, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue and 0.02 M EDTA] in a total volume of 15 µl. The mixture was denatured at 95°C for 5 min and was snap chilled on ice (Pipalia et al., 2004). The total volume was applied in a 15% polyacrylamide gel, as described by Herring et al. (1982). The electrophoresis was performed in 0.5 X TBE buffer (Tris 100 mM, boric acid 9 mM, EDTA 1 mM) at room temperature (18°C) and constant 200 V for 3 h. Polyacrylamide gels were stained with silver according to the protocol described (Herring et al., 1982).

Statistical analysis

The allelic and genotypic frequencies, expected means, observed and expected Nei's heterozygosities (HE = 1-Σ P_i^2, where P_i is the frequency of allele i) and Hardy-Weinberg equilibrium were calculated using PopGene32 program (ver 1.31, Canada).

RESULTS

PCR-SSCP analysis of *LEP* gene

All extracted DNAs from sheep blood samples yielded a specific single band PCR product without any nonspecific band. Therefore, the PCR products were directly used for SSCP analysis.

The allelic variation in the *LEP* gene was examined by PCR-SSCP. The non-denaturing gel electrophoresis enabled the visualization of ssDNA and was analyzed for SSCP band patterns. In this study, a total of Five SSCP patterns were observed in the examined sheep (Figure1).

Table 1. Observed Allele and genotypic frequencies for *lep* locus in Makoei sheep.

A	B	C	AB	BB	AC	BC	CC
0.15	0.37	0.48	17%	9%	14%	37%	23%

Table 2. Estimated statistical parameters for *LEP* locus in Makoei sheep.

Exp-Het	Exp-Hom	Het $_{(Nei)}$	Ave-Het	Obs-Hom	Obs-Het
0.6155	0.3845	0.6108	0.6108	0.3231	0.6769

The frequencies of the observed genotypes were 0.0 9, 0.17, 0.37, 0.14 and 0.23 for BB, AB, BC, AC and CC, respectively. Allele frequencies were 0.15, 0.37 and 0.48 for A, B and C, respectively (Table 1).

The observed heterozygosity (H_{obs}) value for calpastatin was 0.6769. The chi-square test showed no significant deviation (P>0.01) from Hardy-Weinberg equilibrium for this locus in the studied population (Table 2).

DISCUSSION

In livestock, variation in the leptin (*LEP*) gene has been characterized in cattle and pig, but it has not been reported in sheep. In this study, variation in the exon 3 coding sequence of the ovine *LEP* gene was investigated using PCR-SSCP analysis. In the present study, three alleles (*A*, *B*, and *C*) and five genotypes (AC, AB, BB, BC, and CC) were identified for exon III of *LEP* gene in "Makoei" sheep in west Azerbaijan, Iran. The most frequent allele and genotype in the "Makoei" sheep were allele C and genotype BC with frequencies of 48 and 37%, respectively. The results obtained from this study revealed the existence of polymorphism in the LEP gene of Makoie sheep.

Positive correlations between leptin serum levels and carcass traits such as marbling, 12th rib back fat thickness, kidney pelvic heart fat and quality grade in beef cattle were reported (Geary et al., 2003). Differences in muscling, fat thickness and marbling were also found (Delavaud et al., 2002).

E2FB (Kpn2I) SNP had allelic frequencies of the C and T alleles of 0.389 and .611, respectively (Schenkel et al., 2005). The C allele was associated with lower fat, more grade fat, and more leanness. It was suggested that the T allele had a high degree of dominance due to the fact that the heterozygote had very similar values with the TT homozygote allelic frequency of the Kpn2I SNP in *Bos indicus* and *Bos taurus* cattle as well as their crossbreeds (Choudhary et al., 2005). The frequencies for the C allele in the crossbreds, Holstein Friesian and Jersey cattle were 0.82, 0.60 and 0.44, respectively. The T allele frequencies were 0.18, 0.40 and 0.56.

The polymorphism in the exon 3 of the *LEP* in sheep was also reported by Zhou et al. (2009) using PCR-SSCP technique. They reported five unique SSCP patterns corresponding to five allelic sequences using the combination of two different electrophoresis conditions in six common New Zealand sheep breeds. One or two of these patterns for each sheep was observed, reflecting the presence of homozygous or heterozygous genotypes at the ovine LEP locus. In comparison with the results obtained in the present study, 3 alleles were identified in one sheep breed.

In another study, leptin gene polymorphism and its association with skeletal muscle growth and meat quality was investigated using single nucleotide polymorphism (SNP) analysis. A total of three SNPs were identified in the ovine LEP gene including two SNP in intron 2 and one in the 3' UTR (Boucher et al., 2006).

There are several studies on the association of *LEP* gene polymorphism and other traits in animals. Almeida et al. (2003) reported that RFLP genetic marker genotypes in the leptin gene were significantly associated with calving date in a Brangus herd in Brazil. A weak correlation between leptin serum levels and cow body condition score (BCS) and a strong relationship between leptin serum levels and the size of adiposities has been described (Delavaud et al., 2002).

Similar results were reported, where TT genotype was not detect in the Hariana, Sahiwal, Gir and Nimari cattle breeds, but reported comparatively high TT genotype frequency (0.30) in Jersey cattle (Choudhary et al., 2005). As TT genotype was not observed by using PCR RFLP, by using PCR-SSCP in our research, AA genotype was not also seen. To find the evolutionary relationships among close populations, leptin is a suitable and informative marker system. The diversity data generated for native Iranian sheep breeds may be utilized for characterizing the genetic relationships with other countries as well.

Very little information is currently available to compare different ovine populations from Iran. Although, we have used only one breed, the present study may be regarded as the beginning of attempts to understand the genetic diversity of local sheep breeds in Iran.

In sheep, analysis of allelic variation of leptin loci could potentially be used to evaluate temporal changes in genetic diversity and our analysis showed that breeds

can be differentiated using leptin variability.

The present study was the first attempt for the identification of *LEP* (Exon III) gene variation in Iranian "Makoei". Further studies are required to investigate the relationship between *LEP* gene polymorphisms and the performance traits, and the association of leptin concentration and genotypes in Makoei sheep.

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