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Polymorphism of the exon 3 of leptin gene in Malpura sheep

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ABSTRACT

Leptin (*LEP*) is primarily expressed in the adipose tissues. It regulates the feed intake, energy metabolism and body composition and plays a crucial role in regulating body weight and growth in mammals. The aim of the present study was to find out an allelic variation in leptin gene of Malpura sheep. A total of 112 Malpura sheep were selected and the genomic DNA was isolated by phenol-chloroform extraction method. PCR was carried out in order to amplify the 471 bp fragment of the exon 3 coding sequence of the leptin gene. The genotyping was done by PCR-RFLP technique. PCR products were digested with three (*BcnI, SsiI* and *OliI*) restriction enzymes. For detection of allelic variants, three non-synonymous SNPs were found in Malpura sheep. The A271G and A316C loci were found mono-morphic, while T387G locus was found polymorphic. Two genetic variants (G and T) and three genotypes (GG, GT and TT) were found in Malpura sheep. The allelic frequency of G and T allele at T387G locus was found 0.82 and 0.18, respectively.

Key words: Malpura sheep, Leptin, Polymorphism, PCR-RFLP.

INTRODUCTION

In India, sheep is mainly reared for meat and wool purposes. Malpura sheep is a native mutton type breed found in the semi-arid region of Rajasthan, India. This sheep is reared by the small holders, who graze them on fallow lands, crop residues and community pasture lands. The economic values of sheep production depend upon the growth and reproduction efficiency. The growth performance and energy metabolism of an individual may depend upon the secretion of the metabolic hormones.

Leptin is a 16 kd non-glycosylated protein hormone of cytokine family, which plays an important role in body growth by maintaining the balance between food intake and energy expenditure through signaling to the brain (Friedman and Halaas 1998). Leptin is secreted mainly by white adipocytes and also from the tissues of stomach, skeletal muscle and placenta (Friedman and Halaas 1998). In farm animals, leptin regulates various economically important productive and reproductive traits (Hossner 1998; Lende *et al.,* 2005). Leptin is involved in the synthesis of leptin protein with 167 amino acids, which is secreted into blood after cleavage of 21 amino-acid signal peptide (Zhang *et al*., 1994).

Polymorphism in the bovine leptin gene has been associated with food intake (Lagonigro *et al*., 2003), milk production (Buchanan *et al*., 2003), and carcass and meat quality traits (Schenkel *et al*., 2005) and also with production traits in sheep (Boucher *et al*., 2006). Allelic variants of exon 3 of the leptin gene were also reported in New Zealand sheep breeds (Zhou *et al*., 2009). Tahmoorespur *et al*., (2010) found that the leptin was significantly associated with additive estimated breeding value (EBV) for the growth weight in Baluchi sheep. Similarly, it was significantly associated with the growth traits in Kermani sheep (Shojaei *et al.,* 2010). Polymorphism in the leptin gene was studied in Assaf and improved Awassi sheep breeds and they found the major sequence variation (c.367G>T) (designated as c.387G>T) in the exon 3 of the leptin gene (Reicher *et al.,* 2011). The leptin gene exon 3 polymorphism five PCR-SSCP genotypes associated with heart girth and rump length (Sadeghi *et al.,* 2014) and fat tail dimensions (Hajihosseinlo *et al*., 2015) in fat tail Makooei breed of Iran. Jamuna *et al.,* (2016) also found leptin gene exon 2 polymorphism significantly associated with 305 days milk yield (Lactaion mailk yield) in Murrah buffaloes. In India, there is single study has been carried out on leptin polymorphism in Nilagiri sheep (Cauveri *et al.,* 2014). Keeping in view of the importance of the leptin in the growth and development, the present study was carried out to find out allelic variants in the exon 3 of leptin gene of Malpura sheep through PCR-RFLP technique and to confirm the sequence polymorphism through DNA sequencing.

MATERIALS AND METHODS

Blood samples (1.5 ml) were collected aseptically from jugular vein in ACD (citric acid, sodium citrate, D-glucose) solution in 2 ml eppendorf tubes from 112 animals of Malpura sheep. DNA was extracted from white blood cells

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(WBCs) using standard phenol-chloroform extraction method with minor modifications (Clamp *et al.,* 1993). DNA samples were dissolved in 0.1X TE buffer (pH 8.0).

The forward 5'-AGGAAGCACCTCTACGCTC-3' and reverse 5'- CTTCAAGGCTTCAGCACC -3' primers were used as described by Zhou *et al*., (2009) for amplification of 471 bp fragment of the entire exon 3 coding sequence of the Leptin gene. The PCR reaction was carried out in 20µl of reaction volume, containing 10X PCR buffer (500mM KCl, 100 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pM of each forward and reverse primer, 100 ng sheep genomic DNA and 1U Taq DNA polymerase. The PCR product was checked on 1.5% agarose gel. The PCR reaction conditions were as follows; initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min. Sheep individuals were genotyped for leptin gene polymorphism by PCR-RFLP technique. PCR products were digested by three restriction enzymes (*BcnI, SsiI* and *OliI*) at 37^0C for 12-14 h. PCR products were resolved on a 3% agarose gel along with 100 bp DNA marker. Allelic and genotypic frequencies were calculated for the genetic variants of leptin gene by direct counting. The PCR products showing different band patterns on RFLP gel were selected for DNA sequencing.

RESULTS AND DISCUSSION

In the present study, three SNPs at 271 bp, 316 bp and 387 bp position lies within the PCR product (471bp) of the leptin gene were screened in Malpura sheep. For detection of a particular SNP in coding region of the leptin gene, the restriction sites were mapped by NEB cutter V2.0 software (http:/tools.neb.com/NEBcutter2). The PCR product was digested with *BcnI* restriction enzyme for detection of G271A SNP. The digested products were resolved on 3% agarose gel. All studied samples had only 270 bp and 201 bp bands after digestion with *BcnI* restriction enzyme (Figure 1). The studied samples were homozygous GG genotype, while other two genotypes (GA and AA) were not present in the population. The results are in agreement with the study of Zhou *et al*. (2009) that the exon 3 region of the leptin gene was polymorphic in six New Zealand sheep breeds (Romney, Merino, Coopworth, Corriedale, Poll Dorset and Suffolk). They found four SNPs in all breeds, out of four, 271 bp, 316 bp and 387 bp SNPs were non-synonymous (resulted in amino acid changes).

At position A316C, the PCR products were digested with *SsiI* restriction enzyme. All studied samples produced 263bp, 116bp, 40bp, 29bp, and 23bp bands on 4% agarose gel (Figure 2). The studied samples had only homozygous CC genotype, while other two genotypes (AC and AA) were not detected in the population. It was very difficult to resolve the 40 bp, 29 bp, and 23 bp fragments on 4% agarose gel, since it could not be taken into account. Thus, 263 bp and 116 bp fragments could distinguish the genotypes clearly. It is inferred that the loci G271A and A316C were found monomorphic in Malpura sheep, thus no allelic variants were observed in this breed.

Digestion of the 471bp PCR product with *OliI* restriction endonuclease at the locus T387G resulted two bands (384 bp and 87 bp) as homozygous GG, three bands (471 bp, 384 bp and 87 bp) as heterozygous GT, and one band (471bp) as homozygous TT individuals (Figure 3 and Figure 4). The genotyping of the sample was done on the presence of 384bp and 471bp band which is clearly visible in gel images. The one sample of each genotype was sequenced by both forward and reverse primers. After the alignment of the exon3 leptin gene sequence polymorphic SNP (T387G) clearly visible in sequencing chromatogram in the three genotype of Malpura sheep (Figure 4). The genotypic frequencies of the GG, GT and TT genotype were 0.68, 0.28 and 0.04, respectively (Table 1). The obtained aligned sequence was subjected to NCBI/BLAST, which confirmed the amplification of 471bp fragment of leptin gene in sheep. The BLAST comparison of nucleotide sequence of both allele G and T revealed high similarity (99%) with

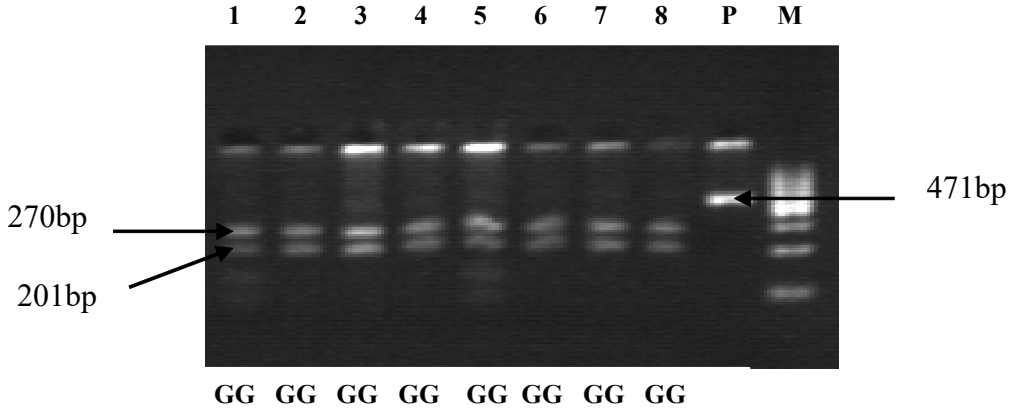

Fig 1: PCR-RFLP profile of the exon 3 of leptin gene digested by *Bcn1* enzyme. Products were resolved on 3% agarose gel. Lane 1 to 8 represents GG genotyping, P: undigested PCR product, M: 100 bp DNA Ladder.

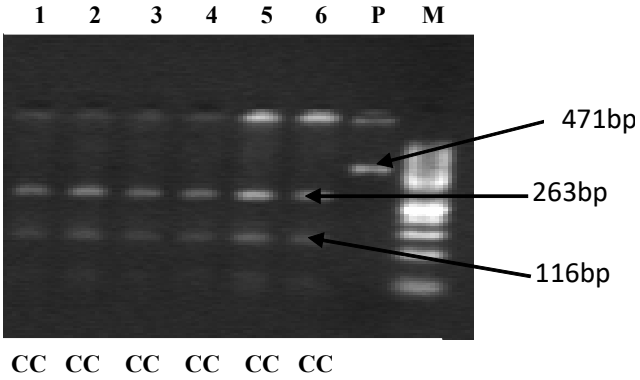

Fig 2: PCR-RFLP profile of the exon 3 of the leptin gene after digestion with *Ssi1* enzyme. Products were resolved on 4% agarose gel. Lane 1 to 6 is represents CC genotype. P - PCR product, M: 100 bp DNA Ladder.

Table 1: Detection of Three SNPs in the exon 3 of the leptin gene in Malpura sheep

SNP position[a]	Number of animals	Genotype frequency			Allelic frequency	
		GG	GT	TT	G	T
G387T (*OliI*)	112	0.68 (76)	0.28 (32)	0.04 (4)	0.82	0.18
G 271 A (*Bcn1*)	100	Found mono-morphic in Malpura sheep, only one genotyping (GG) was detected				
A 316 C (*Ssi1*)	71	Found mono-morphic in Malpura sheep, only one genotyping (CC) was detected				

[a]SNP positions relative to the first nucleotide of exon 3 of leptin gene

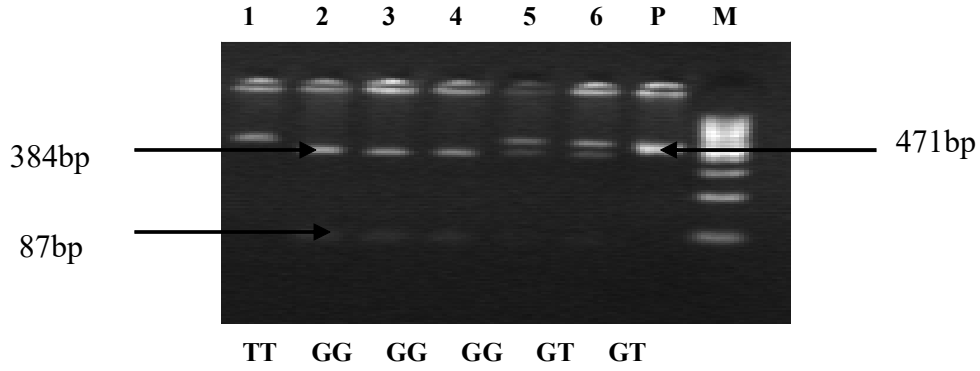

Fig 3: PCR-RFLP profile of leptin gene exon 3 digested by *OliI* enzyme on 3% agarose gel, Lane 1 represents TT, lane 2, 3 and 4 represents GG and Lane 5 and 6 GT genotype, P-undigested PCR product, M : 100 bp DNA Ladder.

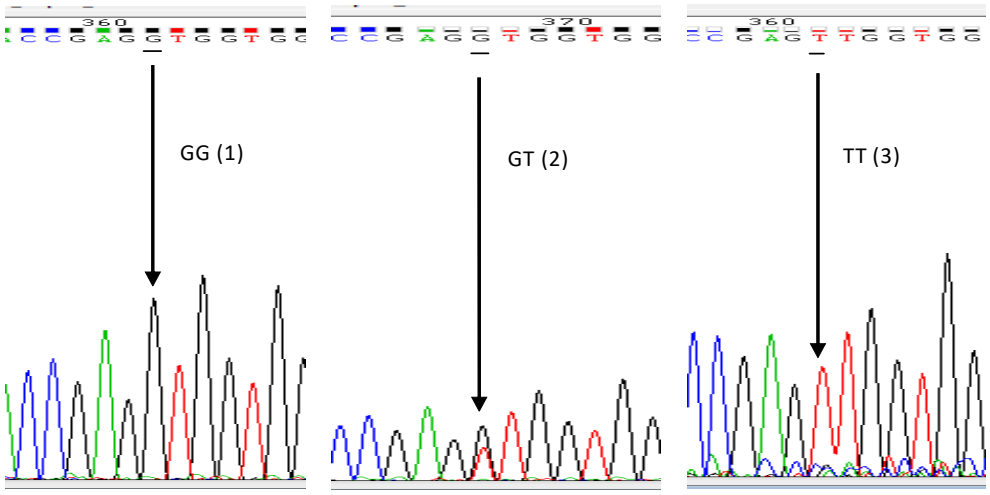

Fig 4: Sequencing of the PCR products showing homozygous GG (1), heterozygous GT (2) and homozygous TT (3) genotypes at G387T locus. Comparison of GG, GT and TT sequences (The arrow sign indicates the endonuclease site for *OliI* or marked SNP).

Ovis aries, 98% with *Capra hircus* followed by 96% with *Bos indicus, Bos taurus, Bos grunniens, Bos frontalis* and *Bubalus bubalis*. The sequences of homozygous samples GG and TT was submitted to GeneBank (Acc. No. KJ918739 and KJ918740, respectively). From the leptin gene amplicon, a SNP was found in T387G which creates a restriction site for endonuclease *OliI* (G/T) (Figure 4). The sequenced product was also verified for the presence of SNP (G271A and C316A) as a mono-morphic locus.

The most common allele in Malpura sheep was 'G' allele with a frequency of 0.82 and the counterpart 'T' allele with a frequency of 0.18. The PCR-RFLP results of the Malpura sheep showed that, there were a dominance of the GG genotype than other genotypes . Li *et al.,* (2008) found three SNPs in the exon 3 of leptin gene, all of which resulted in amino acid changes in Poll Dorsets, Suffolk, Texels and Small Tan sheep. Polymorphism in the entire coding region of an ovine leptin gene was also reported by Zhou *et al.,* (2009) and found four polymorphic SNPs in New Zealand sheep breeds. The Malpura sheep breed one SNP (G387T) was polymorphic, while other two SNPs (G271A and C316A) were mono-morphic in the studied population. Several workers found polymorphism in the exon 3 of the leptin gene in Iranian Baluchi, Kermani sheep with the same primers, which detected the three genotypes by PCR-SSCP technique (Tahmoorespur *et al.,* 2010, Shojaei *et al.,* 2010; Tahmoorespur and Ahmadi, 2012). However in , Makoei sheep of Iran had five genotypes in exon 3 of leptin gene as found in New Zealand sheep breeds (Hashemi *et al*., 2011 and Sadeghi *et al.,* 2014). The exon 3 of leptin gene of Assaf, Awassi and Dorper sheep breeds was found synonymous and non-synonymous mutations (Reicher *et al*., 2010; Reicher *et al*., 2011). Cauveri *et al*., (2014) found two novels SNPs in untranslated regions (UTRs) of an exon 3 of leptin gene in Nilagiri sheep. Nilagiri sheep had only one SNP (SNP-L1) that is mono-morphic (AA genotype), while second SNP (SNP-L2) was polymorphic. The Nilagiri sheep breed was not reported four SNPs as reported by Zhou *et al.* (2009). The five SSCP genotypes of Makooei sheep breed were found significant association with heart girth and rump length while body length, height at back, scrotal circumference did not affected with the genotypes (Sadeghi *et al.,* 2014). The five SSCP patterns (L1 to L5) was also found significant effect on tail length (rump length), fat thickness (thick rump), and tail width (rump width) in Makooei sheep. The L5 genotype of leptin gene have superiority of tail length and fat thickness compared to other (L1, L2 L3 and L4) genotypes. The individuals with L2 genotype have higher tail width (Hajihosseinlo *et al*., 2015). This is the first report on the genetic polymorphism of the leptin (obese) gene in Malpura sheep. Future study may be taken up to associate the exon 3 polymorphism (G387T) with body weight and meat quality traits of sheep breeds of the India with respective genotype.

CONCLUSION

Genetic polymorphism of the leptin gene exon-III in Malpura sheep was investigated by the PCR-RFLP method to identify the possible presence of different single nucleotide polymorphism in the studied population of the sheep breed. The genomic DNA was isolated from 112 blood samples. The PCR-RFLP study on leptin gene in Malpura sheep identified one polymorphic SNP at the positions G387T while absence of SNP at the other two positions. However, further investigation is required to confirm the association of the 'G387T' polymorphic loci with growth data of the animals. It is suggested that polymorphism in the entire leptin gene can be used as a selective marker for the desired genotype for improving the growth performance of Malpura sheep.

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