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Association of single nucleotide polymorphism in leptin gene with growth traits of Jamunapari goat

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ABSTRACT

The current study sought to identify single nucleotide polymorphisms (SNPs) in the leptin gene and investigate their relationship with growth traits in goats. Phenotypic data for birth weight (BW), weaning weight (WW), body weight at six month (6MW), body weight at nine month (9MW) and body weight at twelve month (12MW) from Jamunapari goats were recorded. Seven pairs of primers were designed to amplify the leptin gene spanning promoter, exonic, intronic and 3' UTR regions by polymerase chain reaction. High Resolution Melting (HRM) was employed for genotyping, and DNA sequencing was utilized to mining SNPs. Four novel SNPs at g.584C>T, g.4226T>C, g.4158T>C and g.4133T>A were identified in promoter region and 3' UTR region of leptin gene. Statistical analysis revealed that SNPs at g.584C>T was associated with weaning and post weaning stages of growth. Moreover, goats with the CT genotype had higher body weight at WW, 6MW, 9MW and 12MW than those with CC genotype. The results of this study demonstrated that SNPs in leptin gene was significantly associated with growth traits in goat. These findings suggest that an SNP in the caprine leptin gene could be one of the candidates for improving body weight in the Jamunapari goat.

Keywords: Association, Goat, Growth, Jamunapari, Leptin, Single nucleotide polymorphism

Leptin is a 16 KDa hormone encoded by LEP gene. It is synthesized predominantly by adipocyte (Houseknecht *et al.* 1998). It plays a significant role in regulation of body weight, feed intake, energy expenditure, fat deposition, immune function, milk yield and fertility (Macajova *et al*. 2004, Banos *et al*. 2008, Batista *et al*. 2013). It acts on cell receptor in the nucleus of the hypothalamus (Brennan *et al*. 2006). The caprine leptin gene has been mapped on fourth autosome (Perucatti *et al*. 2006) and it consists of three exons. In goat rearing, growth is a very important trait that determines the economic value of the animals. Jamunapari goat is well known for its growth and milk production. With an average body weight of 24.49±0.08 kg at 12 months, Jamunapari is a large, tall with large pendulous ears and a pronounced Roman nose (Dige *et al.* 2021). Identification of novel molecular genetic marker and their validation for growth traits is first step to establish MAP programme (Allan *et al*. 2007). Several polymorphism studies have been carried out in different species, viz. cattle, sheep, goat, buffalo and pig to find out its association with economically important production traits (Batista *et al*. 2013, Mankowska *et al.* 2015, Wang *et al.* 2015, Jonas *et al*. 2016, Mishra *et al*. 2018). Polymorphism in leptin gene has been shown to influence the production and reproduction traits in livestock. Leptin polymorphism has been linked to growth (Clempson *et al*. 2011), feed intake, dry matter intake (Batista *et al*. 2013), carcass traits (Silva *et al*. 2014), daily milk yield (Batista *et al*. 2013) and fertility traits (Trakovicka *et al*. 2013) in cattle. Similarly, the leptin gene polymorphism has been associated with production and carcass traits in sheep (Jonas *et al*. 2016, Quirino *et al*. 2016) and pig (Kennes *et al*. 2001, Chen *et al*. 2004, De *et al*. 2006). In rabbit, polymorphism in leptin gene influence carcass and meat traits (Midgal *et al*. 2018). There has been little progress to date that has linked polymorphism in leptin gene to the growth traits in Indian goat. The primary aim of the present study was to identify polymorphism in relation to growth traits in goat.

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MATERIALS AND METHODS

Animal and herd management: The institute farm (ICAR-Central Institute for Research on Goat) is located on the banks of river Yamuna, at 78°02′E Latitude and 27°10′N Longitude at an altitude of 169 meters above mean sea level. The farm climate is semi-arid in nature while the average temperature varies from minimum 2°C (winter) to maximum 48.5°C (summer). The average rainfall is around 379 mm, received mostly during the monsoon season from July to September. Under a semi-intensive management

system, the goats were maintained with 6–7 h of grazing and stall feeding with seasonally available green fodder like lucern, barseem, maize etc. *ad lib.* supplemented with concentrate mixtures based upon age of the animals. Animals were housed separately depending on their ages, health status and sex. Breeding was practiced in two breeding seasons with the does being bred during months of May to June and October to November followed by kidding in the months of October to November and March to April, respectively. Does were mated with bucks by natural mating. After kidding, each kid was assigned with an identification number by ear tattooing followed by records of date of birth, sex, birth type and live body weights were taken. Kids were stall-fed up to weaning (3 months age), and then allowed to graze nearby areas. Flocks were vaccinated against Peste-des-petits ruminants (PPR), enterotoxaemia (ET) and Foot and mouth disease (FMD). Targeted deworming was carried out during the pre-monsoon season (June–July) and in the post-monsoon season (September to October) for the control of gastrointestinal nematodes. The soil was sandy with natural pasture and bush as the main vegetation type. The pastures were mainly *Cenchrus setigerus* and *Cenchrus ciliaris*, along with native annual and perennial flora.

Data collection and DNA extraction: The body weight was recorded at birth (BW), weaning weight (WW), body weight at six month (6 MW), body weight at nine month (9 MW) and body weight at twelve month (12 MW). About 2 ml of blood samples were collected from 191 Jamunapari goats under aseptic condition in EDTA coated vacutainer tube (BD Biosciences, Franklin Lakes, NJ, USA) and stored at –20°C. Two hundred thirty blood samples were also collected from Barbari, Jakhrana, Jamunapari and Sirohi for direct sequencing. During sample collection, animals were handled strictly in accordance to the ethical guidelines. The genomic DNA was isolated by DNA extraction kit (Macher-Nage, Gmbhand Co, Germany). The quantity and purity of genomic DNA was evaluated by biophotometer (Eppendorf, Germany). Genomic DNA samples showing OD ratio (260/280) in the range of 1.7 to 1.9 were retained for further analysis. The extracted DNA was stored at –20°C for subsequent experiments.

Primer designing and polymerase chain reaction (*PCR*): The primers were designed using IDT online software based on published leptin sequences in NCBI. The seven different pair of primers were used to amplify different fragment covering maximum possible target regions of the of leptin gene (Table 1). Seven regions of leptin gene spanning over part of promoter, intron, exonic and 3′ UTR regions were amplified. For amplification, 25 µl of PCR reaction mixture was prepared by adding ready to use master mix (Emerald Amp® Max PCR Master Mix, Clonetech Takara). The amplification was carried out in pre-programmed thermal cycler (Bio-Rad) with following conditions: Initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing temperature as shown in Table 1 for 30 sec and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. The amplified PCR products were separated by electrophoresis on 3% agarose gel in parallel with 100 bp DNA marker.

SNP genotyping by HRM (*High resolution melting*): Genotyping was carried out by HRM as per the protocol standardized in our laboratory. PCR products of each fragment amplified from first six primers (Leptin1 to Leptin 6) were prepared for HRM analysis by heating at 95°C for 1 min, then rapid cooling at 40°C and incubation for 1 min. HRM plate was heated from 65°C to 95°C performing 25 acquisitions per 1°C. Finally, HRM was performed using initial denaturation at 95°C for 10 min, continued with 45 cycles for final denaturation at 95°C for 10 sec, annealing at 61°C for 20 sec and extension at 72°C for 25 sec. Then HRM products were checked on 3% agarose gel electrophoresis for further confirmation of primers set generating single HRM products of the expected size. Data were visualized using fluorescent-based normalized and temp-shifted plot then analyzed using the automated grouping functionalities provided by the Light Cycler 480 Gene Scanning Software Module (www.roche-applied-

Table 1. Primers used for the amplification of the Leptin gene

Name of primer		Primer sequence (5′→3′)	Fragment size (bp)	Region covered	Annealing temperature (°C)
Leptin 1	F1	TACCGATTCCTGTGGCTTTG	129	Exon 1 and intron 1	60.0
	R1	CTACCGTGTGTGAGATGTCA			
Leptin 2	F2	CAGAGCTCTTTCCTCCTGTATTG	133	5′ UTR	60.5
	R2	GATAATGTCAGACGCAGTGCT			
Leptin 3	F3	GACAGCAGATCTCGTTGTTATC	156	5′ UTR and Exon 1	61.0
	R3	TCCACAGCGCATTTTCCTTC			
Leptin 4	F4	GATATGCCTGAAGTCGTGCA	143	5′ UTR	61.0
	R4	AGGTGCGGTGGAATCAAGAA			
Leptin 5	F5	AACAGAGGGTCACTGGTTTG	152	Exon 2	62.0
	R5	GAGGTTCTCCAGGTCATTAGA			
Leptin 6	F6	GACCTTCTCCACCTGCTG	165	Exon 2	61.5
	F6	CTGCCGCAACATGTCCT			
Leptin 7	F7	GCTCTGAATGGATCTCGAAGG	670	3′ UTR	62.0
	R7	GCCTCCTCCTTTGTTCTACTG			

science.com). The HRM curve (sequence variation in the PCR product) was analyzed using the Light-Cycler 480 Gene Scanning software version 1.5 (www.roche-applied-science.com). Melting curves were normalized by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals to obtain uniform value.

DNA sequencing and sequence analysis: The variants obtained from HRM analysis as well as 670 bp directly amplified segment of leptin gene were subjected to DNA sequencing using ABI 3130 (48 capillary) DNA analyzer to identify the SNPs. Nucleotide sequence data were analyzed using chromas software (Ver 1. 45, http://www.technelysium.com.autochromas.html). The multiple sequence alignment was performed with Clustal W programme (http://align.genome) and MegAlign program of LASERGENE software (DNASTAR, Inc., Madison, WI, USA) to identify the nucleotide variations. The alignments of reverse and forward sequences were applied to produce consensus sequences. The sequences of each individual DNA fragments were aligned with original sequences to identify the presence of SNPs. PopGen32 software was used to estimate the allele and genotypic frequencies, Exp. Homozygosity (Hm), Exp. Heterozygosity (He), Effective number of allele (Ne), Nei's heterozygosity, Shanon's information index (I) and Chi Square test (χ^2). Sequence data were edited by Chromas Application Ver.1.0.0.1. Multiple sequences alignments were performed with SeqMan program of DNASTAR software to identify SNPs.

Statistical analysis: Data on 191 animals were used for the analysis. The data were analyzed using general linear model (GLM) by SPSS 22 version to assess the effect of non-genetic factors and genotype on the growth data. The following statistical model was used:

$$Y_{ijklmno} = \mu + S_i + C_j + N_k + T_l + P_m + G_n + \varepsilon_{ijklmno}$$

where, S_i , effect of sex (2 levels); C_j , effect of j^{th} year/ cohort of birth (6 levels); N_k, season of birth (2 levels: spring and autumn); T_l, effect of type of birth (2 levels: single and multiple); P_m, effect of m^{th} parity of dam (5 levels); G_n, effect of possessing n^{th} genotype for leptin gene, and $\varepsilon_{ijklmno}$, random error term for corresponding observation $Y_{ijklmno}$ for the o^{th} individual.

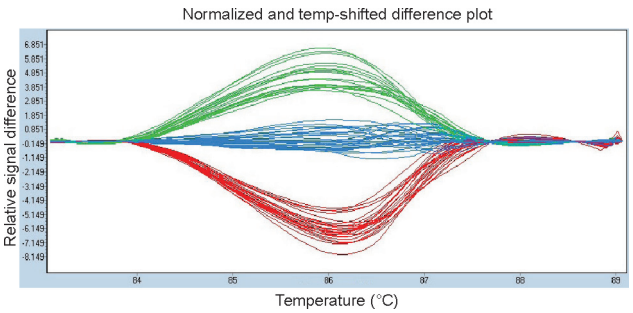

Fig. 1. Difference plot of amplified fragment 143 bp (promoter region) of leptin gene.

RESULTS AND DISCUSSION

The seven different fragments of leptin gene covering promoter, intronic, exonic and 3′ UTR region were amplified. The lengths of amplified fragments of leptin gene were 129, 133, 156, 143, 152, 165 and 670 base pair. Among first six fragments, the 143 bp fragment revealed distinct HRM groups based on HRM analysis, which were selected and sequenced. HRM difference plots were used to assess genotypes, which were adjusted by pre and after melting temperatures (Fig. 1). HRM indicated no variation for the other five segments of leptin gene (129, 133, 156, 152, and 165 bp). HRM is a sensitive tool for analysing genetic variation that is mostly applied to the study of mutation (Liew *et al*. 2004). We found that in HRM analysis, fragments with less than 200 bp length produced good results.

Direct sequencing was used to identify SNPs in a 670 bp fragment of leptin gene. A comparison of the amplified nucleotide sequences of 143 and 670 bp fragments with the published sequence revealed four SNPs at g.584C>T positions (143 bp fragment of promoter region), g.4226T>C, g.4158T>C, and g.4133T>A (670 bp fragment of 3' UTR region). In the population investigated, other segments of leptin gene (129, 133, 156, 152, and 165 bp) were found to be monomorphic. The homology analysis revealed that the nucleotide sequence of leptin gene (g.584C>T locus) of goat with other species, viz. sheep, buffalo, and yak showed high similarity and it was 97.2, 95.8, and 93.7%, respectively. Table 2 shows the estimated allele and genotype frequencies of the SNP g.584C>T locus of the leptin gene in Jamunapari goat. Allele C was preponderant allele at g.584C>T position of leptin gene. The result of Chi-square test indicated that a SNP locus fitted in Hardy-Weinberg equilibrium.

The results of a single nucleotide polymorphism association study demonstrated that a detected SNP g.584C>T in the promoter region of the leptin gene was substantially ($P<0.05$) linked with body weight in Jamunapari goats at weaning and post weaning stages of growth (Table 3). CT genotype goats had significantly higher body weight at WW, 6 MW, 9 MW, and 12 MW than CC genotype goats.

Table 2. Genotypic and allele frequencies at SNP g. 584C>T locus in Jamunapari goat

Gene	SNPs	CC	CT	C	T	χ^2	P	Hm	He	Ne	Nei	I
Leptin gene	*g.584C>T*	0.79	0.21	0.89	0.11	124.03	0.00	0.81	0.19	1.24	0.19	0.34

SNPs, Single nucleotide polymorphisms; χ^2, Chi Square; P, Probability; Hm, Exp. homozygosity; He, Exp. heterozygosity; Ne, Effective number of allele; Nei, heterozygosity; I, Shanon's information index.

Table 3. Effect of genotype on body weights in Jamunapari goats

Genotype	BW (kg)	WW (kg)	6 MW (kg)	9 MW (kg)	12 MW (kg)
CC (150)	3.230±0.045	11.590±0.209[a]	15.842±0.291[a]	19.668±0.420[a]	23.616±0.600[a]
CT (41)	3.536±0.871	13.619±0.400[b]	18.275±0.558[b]	24.726±0.804[b]	31.531±1.148[b]

Effect of genotype was showed significant (different superscript 'a' and 'b' column-wise) (P<0.05) at different age group. BW, Birth weight; WW, weaning weight; 6 MW, body weight at six month; 9 MW, body weight at nine month; 12 MW, body weight at 12 months.

Similar to our finding, polymorphism in leptin gene were also reported in various goat and sheep populations (Boucher *et al.* 2006, Singh *et al*. 2009, Shojaei *et al*. 2010, Hajihosseinlo *et al*. 2012, Maitra *et al*. 2014, Wang *et al*. 2015, Jonas *et al*. 2016, Hartalik *et al*. 2020). SNPs were identified in exon 2 of the Dorset and Suffolk sheep breeds by Boucher *et al*. (2006). Polymorphism in the leptin gene was also reported in the exon 2 and intron 2 regions in Barbari and Jamunapari goats (Singh *et al*. 2009). Maitra *et al.* (2014) reported variation in leptin gene at 22 places in Indian goats by comparing with Garganica goat of Italy. In Nanjiang yellow goat, six SNPs were reported in Exon 1, introns 1 and 3′ flaking region by Wang *et al.* (2015). In Awassi-Merino sheep, Jonas *et al.* (2016) reported a variant (C11T) in exon 2 with a non-significant (P>0.05) relationship with body weight. Four SNPs were identified in leptin intronic region (introns 1 and introns 2) gene of Bligon goat (Hartalik *et al*. 2020). In accordance with our findings, Shojaei *et al*. (2010), Hajihosseinlo *et al*. (2012) and Wang *et al*. (2015) reported a significant association of leptin polymorphism with growth traits in Kermani sheep, Makooei sheep and Nanjiang yellow goat, respectively. In addition to sheep and goats, leptin gene polymorphism has been linked to various traits in different species. Significant associations were found between the leptin gene polymorphism and marbling, red muscle colour intensity, post-slaughter fat thickness, and carcass fat distribution traits in Nellore cattle (De *et al*. 2006). Four SNPs in the promoter region of the porcine leptin gene were found to have no effect on growth traits in the Polish Landrace, Large White, and Pietrain breeds (Stachowiak *et al*. 2007). Ten SNPs and two non-synonymous mutations in the exonic regions of the leptin gene were found to influence variable fatty acid composition in Simmental bull muscle fat (Orru *et al*. 2011). Likewise four SNPs in leptin gene in Holstein cow were associated with early skeleton growth, fertility and milk production (Clempson *et al*. 2011). The leptin haplotype positively influenced daily milk yield, feed intake, and dry matter intake in UK dairy cows (Batista *et al*. 2013). The SNP in the leptin gene (g.321C>T) of Nellore cattle was linked to post-slaughter rump fat thickness and muscle colour (Silva *et al*. 2014). In Polish Landrace pigs, polymorphism in the 3′ UTR of porcine leptin was found to have a weak relationship with abdominal fat weight (Mankowska *et al*. 2015). However, no polymorphisms in the leptin gene were detected in the New Zealand White rabbit population (Dige *et al*. 2015).

In the current investigation, four SNPs at g.584C>T, g.4226T>C, g.4158T>C and g.4133T>A were detected in leptin gene. SNP at the promoter region (g.584 C>T) was demonstrated to have significant (P<0.05) association with body weight in Jamunapari goat. These findings suggested that SNP in caprine leptin gene could serve as potential genetic marker for improving body weight in Jamunapari goat. However, these results are preliminary and further research into the validation of this association in a different and larger populations are needed before it can be incorporated for goat breeding programme.

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