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Novel genetic polymorphisms in caprine *GPR54* gene associated with reproductive functions

A MAITRA[1], REKHA SHARMA[2], SONIKA AHLAWAT[3] and M S TANTIA[4]

National Bureau of Animal Genetic Resources, Karnal, Haryana 132001 India

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ABSTRACT

The objective of this study was to characterize caprine *GPR54* gene, a key mediator of molecular mechanism of reproduction (puberty and prolificacy) in mammals. A panel of 9 Indian goat breeds differing in sexual precocity and prolificacy (Black-Bengal, Malabari, Beetal, Barbari, Osmanabadi, Sangamneri, Jakhrana, Ganjam and Sirohi) were utilized for polymorphism scanning of *GPR54* gene by direct DNA sequencing. Exon 1, 2 and 3 along with adjacent intronic regions of the *GPR54* gene were amplified with 5 primer pairs and 2 novel SNPs, 1 each in exon 1 (C1122T) and intron 1 (T1830C) were recorded. All the variations and SNPs were synonymous. The results showed that these mutations existed in both, sexually precocious and late-maturing goat breeds as well as low and high prolificacy goat breeds. The results extend the limited information on genetic variation of the caprine *GPR54* gene, which might contribute towards molecular breeding to enhance productivity of goat.

Key words: Goats, *GPR54*, *KiSS1*, Precocity, Prolificacy

G-protein coupled receptor 54 (*GPR54*), which is also referred as *KiSS1R, AXOR12* and *OT7T175* was initially cloned from rat brain and subsequently identified in the human genome (Lee *et al.* 1999, Muir *et al.* 2001). It is an integral part of the Kisspeptin/*GPR54* pathway which is considered as a key gatekeeper of pubertal development and reproductive function. G - protein-coupled receptor (*GPR54*) acts as cognate receptor for a family of neuropeptides, kisspeptins which are the peptide products of *KiSS1* gene. These peptides have emerged as essential upstream regulators of neurons that produce gonadotropin releasing hormone (*GnRH*) (Popa *et al.* 2005). These neurons reside in the basal forebrain, which drives reproductive functions in mammals in various species and physiological settings (Knoll *et al.* 2013). Kisspeptins directly stimulate GnRH hormone release via *GPR54* and then stimulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion to result in the initiation of puberty (Tena-Sempere 2006).

Human *GPR54* gene maps to chromosome 19p13.3 and contains 5 exons corresponding to an open reading frame of 1197 bp encoding a 398 amino acid protein (Muir *et al.* 2001). Both *KiSS1* and *GPR54* genes are found to be predominantly expressed in the hypothalamus (Clarke and Pompolo 2005). *GPR54* transcripts are co-localized with *GnRH* neurons in hypothalamus of fish, rat, mouse and rhesus monkey demonstrating that *GnRH* neurons are direct targets for regulation of kisspeptins (Cao *et al.* 2010).

Activated mutations of *GPR54* (Luan *et al.* 2007, Teles *et al.* 2008) gene caused central precocious puberty (CPP) in humans with maturation of the hypothalamic-pituitary-gonadal (ovarian) axis (HPGA) by a gonadotropin dependent manner. Contrarily, inactivating mutations in the *GPR54* gene are reported to be associated with idiopathic hypogonadotropic hypogonadism (IHH) and absence of puberty in humans, which is characterized by complete or partial failure of pubertal development due to impaired secretion of LH and FSH (Lanfranco *et al.* 2005). This indicated that kisspeptin-GPR54 system regulates the year-round estrus of mammals.

Due to the importance of *KiSS1/GPR54* as a regulator of puberty onset, it is probable that the polymorphisms of these genes have some relationship with reproductive traits such as high prolificacy, sexual precocity and year-round estrus phenotypes of goats. Polymorphism detection of *GPR54* gene in sexual precocious and sexual late maturing goat breeds of China was attempted (Feng *et al.* 2009, Cao *et al.* 2010) and preliminary association between different alleles of *GPR54* and high litter size as well as sexual precocity was reported. However, up to now, there is no literature on characterization of *GPR54* gene in Indian goats. Hence, the objectives of the present study were to sequence characterize Indian goat *GPR54* gene through polymerase chain reaction (PCR) and sequence assembly and secondly, to identify single nucleotide polymorphisms (SNPs) of this gene by sequence alignment between goat

Present address: [1]Senior Research Fellow (maitra.avishek @gmail.com), [2]Senior Scientist (rekvik@gmail.com), [3]Scientist (sonika.ahlawat@gmail.com), [4]Principal Scientist (tantiams @gmail.com).

breeds (Black-Bengal, Malabari, Beetal, Barbari, Osmanabadi, Sangamneri, Jakhrana, Ganjam and Sirohi) differing in fecundity and age at puberty.

MATERIALS AND METHODS

Animal selection, sample collection and DNA isolation: Nine well recognized breeds of Indian goats from different geographic and agro-climatic parts across India were selected which differ in prolificacy (number of kids per kidding) and age of sexual maturity (Table 1). Each breed was represented by five animals. Unrelated animals were selected at random from their breeding tracks by picking up only 2 samples per herd and only 2 herds per village. Five milliliter blood per goat was collected aseptically from the jugular vein in a vacutainer tube containing EDTA as anticoagulant. All samples were delivered back to the laboratory in an ice box. The genomic DNA was extracted from white blood cells using standard phenol-chloroform extraction protocol (Sambrook *et al.* 1989).

Primers and PCR amplification

For amplification of 5′ UTR, exon 1, 2 and 3 along with adjacent introns of *GPR54* gene, five pairs of primers were utilized (Table 2). These included three pair of primers designed from *GPR54* gene sequence of Chinese goat (GU142846) and two pairs were taken from literature. The primers were synthesized by IDT.

Polymerase chain reaction was carried out in 25 µl reaction volume with about 50–100 ng genomic DNA using i-cycler. The reaction mixture consisted of 200 µM each of dATP, dCTP, dGTP, dTTP, 1.5 mM $MgCl_2$, 50 pmol primer, 0.5U *Taq* polymerase and corresponding *Taq* buffer. Amplification conditions were as follows: initial denaturation for 1 min at 94°C; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at precise temperature (Table 2) for 1 min, extension at 72°C for 1 min; and finally extension at 72°C for 5 min. The PCR products were separated by electrophoresis on 1.8% agarose gels in parallel with a 100 bp DNA ladder, enzymatically purified and sequenced from both directions using DNA sequencer.

Polymorphism detection: Sequence data were edited manually using Chromas Ver.2.33, (http//www.technelysium.com.au/chromas.html). Multiple sequence alignments were performed with MegAlign program of LASERGENE software to identify SNPs. The sequence analysis was carried out by various modules of DNASTAR Version 4.0, Inc., USA. The coding DNA sequences of different exonic regions were conceptually translated to amino acid sequences using EDITSEQ software. Nucleotide

Table 1. Geographical distribution and physiological characteristics of selected Indian goat breeds

Goat breed	Geographical distribution	Prolificacy	Twinning percentage (%)	Sexual maturity (age in months at puberty)
Beetal	Punjab	High	>50	Medium (10–12)
Barbari	Uttar Pradesh	High	>50	Medium (10–12)
Black-Bengal	West Bengal,Bihar, Jharkhand	High	>50	Early (6–10)
Malabari	Kerala	High	>50	Early (6–10)
Osmanabadi	Maharashtra	Medium	25–50	Medium (10–12)
Sangamneri	Maharashtra	Medium	25–50	Medium (10–12)
Jakhrana	Rajasthan	Medium	25–50	Medium (10–12)
Ganjam	Orissa	Low	<25	Late (13–18)
Sirohi	Rajasthan	Low	<25	Late (13–18)

Table 2. Primer sequences for *GPR54* gene for screening polymorphisms in Indian goats

Primer name and Gene region	Primer sequence (5′ to 3′)	Amplicon size	Annealing temperature (p°C)	References
G1 (Partial 5′ UTR)	F: CCCCTGGTATATGGAAACTT R: TGCCCTGAGTATCACTGCT	519 bp	56.0	Cao *et al.* 2010
G2 (Partial 5′ UTR and exon 1)	F:GCCTACCGCTCCCCCTGCTCTG R:GGTGCCACTGCGCCCTCTCATAG	643 bp	64.0	Self designed
G3 (Exon1 and partial intron 1)	F: ACTGGTCATCTTCGTCATCT R: GCACAGCAGGAACATTAAGT	1163 bp	56.0	Cao *et al.* 2010
G4 (Exon 2 and partial intron 2)	F: GCCGCCCTGCTGGTCACTCG343 bp R: CCCCGCCCGCCTCCACAATC	343bp	63.6	Self designed
G5 (Exon 3 and partial intron 3)	F: AGTCTCAGTCTTGGCGGTTTTAGG R: GGTGAGCCCAGGGACGAAGACT	433 bp	63.6	Self designed

Gene region and amplicon position refers to the sequence of goat *GPR54* gene (GenBank accession no. GU142846).

BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used for sequence homology search in public databases. Cluster analysis of Indian goat sequence with that of other species was performed following Neighbour-Joining procedure using CLC Free Workbench software. The transcriptional factor binding sites were identified using MATCH software (http://www.cbil.upenn.edu/cgi-bin/pub/programs/match/bin/match.cgi) (Kel *et al.* 2003).

RESULTS AND DISCUSSION

PCR amplification of Indian goat GPR54 *gene:* Genomic DNA of 9 indigenous goat breeds was successfully amplified using 5 pairs of primers for *GPR54* gene. The results showed that amplification fragment sizes were consistent with the target ones and had a good specificity, which could be directly analyzed by sequencing (Fig. 1).

The sequenced region corresponding to *GPR54* gene in Indian goat is 2819 bp which includes complete 5′UTR

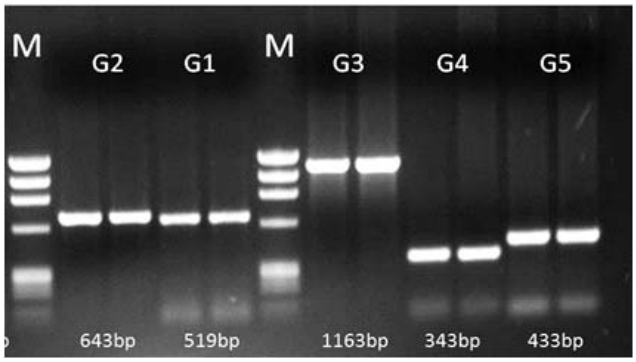

Fig. 1. Amplification results (amplified product of each primer in duplicate on 1.8% agarosege gel stained with ethidium bromide) of goat *GPR54* gene (M: 100 bp ladder).

(1–1026 bp), exon 1 (1027–1270 bp), intron 1 (1271-2333 bp), exon 2 (2334–2458 bp), exon 3 (2952–3087 bp) and partial intron 2 (2459–2564 and 2768–2951 bp) and intron 3 (3088–3149 bp) according to goat sequence (GU142846) and GenBank accessions were received in Indian goat: KJ425415 (intron1), KJ425417 (5′UTR to exon1), KJ425420 (exon 2 and partial intron 2) and KJ425419 (exon 3 and partial intron 2 and 3).

Goat GPR54 *sequence analysis:* Six variations were observed in the sequenced region of Indian goat as compared to Chinese goat (GU142746) which included variation in intron 1 (insertion of 2 bases (AG) between 1558–59, deletion of A corresponding to 2281 bp and T to C transition at 1830 bp) and exon 1 (T to C transition at 1199 bp and C to T transition at 1122 bp) although no change was observed in the 5′UTR. As expected, many variations (97) were recorded in Indian goat *GPR54* as compared to cattle (AC000164) with majority (73.2%) being transitions. Moreover, InDels were recorded at 7 positions which included deletion of 6 consecutive base pairs (1020–1025) in 5′UTR and addition of 283 bp fragment in intron 1 of Indian goat *GPR54* as compared to cattle sequence (in between 1369–1370 bp) (AC000164). Chinese goat sequence (GU142746) has insertion of 281 bp instead of 283 bp in Indian goat as latter has 2 additional nucleotides (AG in between 1558–1559 bp) (GU142846). 5′UTR region has 40 variations in caprine *GPR54* as compared to exotic cattle. Three putative transcription factor binding sites (HNF-4, COMP1, Oct–1) were identified in promoter of *GPR54* of cattle whereas 2 were identified in goats. This was due to a variation (T to C) resulting in the loss of putative transcription factor binding site for HNF-4 in goats (Fig. 2).

BLAST analysis was carried out to find the percentage homology in the DNA sequence of investigated genes of

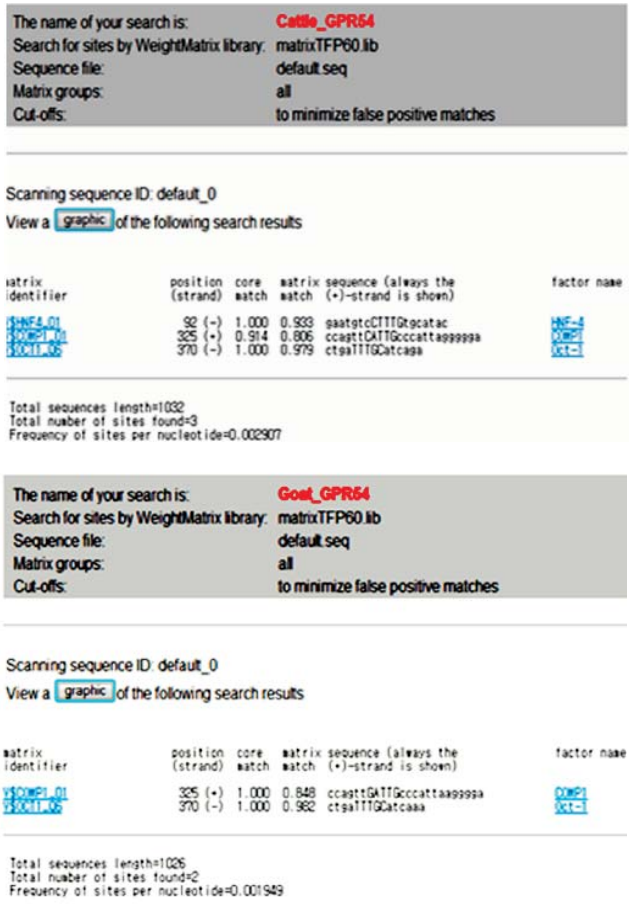

Fig. 2. Comparison of transcription factor binding sites (TFBS) in promoter of *GPR54* gene of cattle and Indian goats.

Indian goat with other species. Coding DNA sequence of *GPR54* region in Indian goat showed maximum similarity (99%) with exotic goat (GU142746) followed by cattle (97%) (AC000164), pig (87%) (NC_010444) and mouse (83%) (NC_000076).

SNPs identification and genotypes: In the current study, comparison of 45 amplified sequences of indigenous goats resulted in identification of 2 novel SNPs (Table 3), one each in exon 1 (C1122T) and intron 1 (T1830C) in Indian goat *GPR54*. The sites of mutation in the present study were numbered according to goat *GPR54* sequence (GU142746). All the variations and SNPs were synonymous. Allele and

Table 3. Novel polymorphisms identified in *GPR54* gene in nine Indian goat breeds

SNP	Position on reference sequence	Primer	Region	Genotype frequency	Allele frequency	Reported/ novel
C1122T	1122	G2	5′UTR	CC=0.902 CT=0.098	C=0.95 T=0.05	Novel
T1830C	1830	G3	Intron 1	TT=0.16 TC=0.41 CC=0.43	T=0.365 C=0.635	Novel

The number corresponds to the sequence of goat *GPR54* gene (GenBank accession no. GU142846).

genotype frequencies of *GPR54* gene in Indian goat are presented in Table 3.

SNP T1830C of *GPR54* presented all the possible genotypes whereas C1122T expressed 2 of the 3 possible genotypes in explored goats (Fig. 3). Wild allele was predominant for C1122T locus of *GPR54*. The genotype distribution of the two mutations did not show obvious difference between sexual precocious breeds and the sexual late-maturing goat breeds and no consistency with the high or low prolificacy goat breeds.

Restriction enzyme site which recognize the SNPs in *GPR54* were identified using NEB cutter V2.0 (Vincze *et al*. 2003). The C1122T mutation of *GPR54* can be detected by restriction fragment length polymorphism (RFLP) as nucleotide variation changes the recognition site of a restriction endonuclease (*BstUI*). This polymorphism can be detected by PCR–RFLP even by laboratories with a limited level of technology since PCR-RFLP method is efficient, low cost, reproducible and convenient. Primer product of G2 can be used to type C1122T mutation of *GPR54*.

GPR54 gene displays abundant polymorphisms in many mammalian species (Tang *et al*. 2012). Several SNPs were identified in idiopathic hypogonadotropic hypogonadism (IHH) (de Roux *et al*. 2003, Luan *et al*. 2007, Lanfranco *et al*. 2005, Cerrato *et al*. 2006) and CPP patients (Teles *et al*. 2008). de Roux *et al*. (2003) identified a homozygous deletion of 155 nucleotides of *GPR54* in all five IHH siblings of a large consanguineous family. Seminara *et al*. (2003) detected three mutations of *GPR54* in one IHH patient which included one homozygous T443C (L148S) mutation and compound heterozygous mutations (C991T [R331X] and T1195A [X399R]). Lanfranco *et al*. (2005) reported a homozygous insertion (1001–1002insC) in human *GPR54* in 1 male IHH patient and 2 common mutations G24A and A1091T (H364L) in human *GPR54*. Besides L148S, R331X and X399R mutations mentioned above, Semple *et al*. (2005) found 2 mutations (T667C [C223R] and G891T [R297L]) of *GPR54* in a boy with IHH. Four rare variants in humans: C9T (T3T), G565A (A189T), 509–55 GNT and 1197+112 CNG were found by Cerrato *et al*. (2006), the latter 2 were variants in non-coding region.

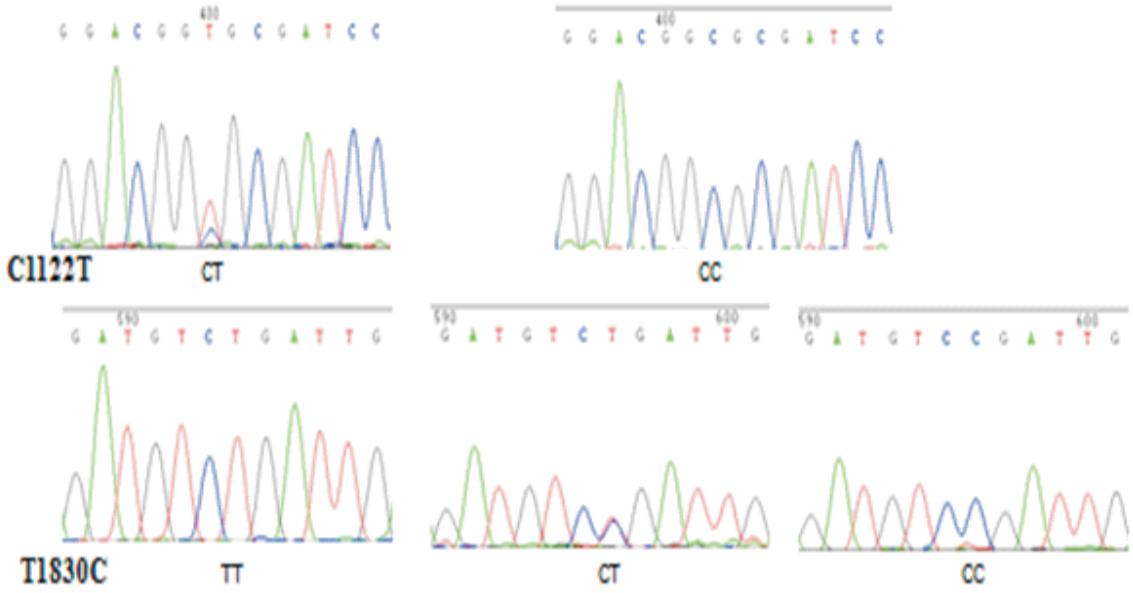

Fig. 3. Sequencing chromatogram of different genotypes at SNP loci identified in *GPR54* gene in Indian goat breeds.

Luan *et al.* (2007) found 6 polymorphisms in human *GPR54* which were G855061A, A855765G, G856737A, C859955A, G860460A and C860868G. Interestingly, Teles *et al.* (2008) identified G1157C (R386P) of *GPR54* in a central precocious puberty (CPP) girl. Seven mutations of *GPR54* gene were identified in White Duroc × Erhualian intercross pigs, which were T245C, C384T, T1411C, A1635G, G1766T, C2448A and T3295C (Li *et al.* 2008). By aligning the sequences between Small Tail Han and Corriedale sheep, two mutations (A125G and a 5 bp deletion/ insertion [TTCTT] at 163–167 locus) in the 5′-regulatory region of *GPR54* gene were identified (Tang *et al.* 2012). Feng *et al.* (2009) found 3 nucleotide mutations (C96T, T173C [I58T] and G176A [G59E]) in goat exon 1 by PCR-SSCP which are 1122, 1198 and 1202 loci in GU142846 respectively. Mutations have been reported in exon 5, viz. G825A and C981T by PCR-SSCP which correspond to 3821 and 3976 loci in GU142846 respectively by Huijbregts *et al.* (2012) and G4014A, G4136A and C4152T mutations in five goat breeds by Cao *et al.* (2010). There were no base pair changes corresponding to these loci in present study and 2 mutations present in Indian goats are different from any mutation reported till date in *GPR54* gene. No mutation has been observed in caprine exon 2 till date (Cao *et al.* 2010, Feng *et al.* 2009). Hence the absence of polymorphism in exon 2 of Indian goats is consistent with earlier reports.

To date, there are a few studies about polymorphisms of *GPR54* related with litter size in livestock. Alleles B and D in exons 1 and 5′ (Feng *et al.* 2009) and allele T at the 4152 locus (Cao *et al.* 2010) of exon 5 in *GPR54* gene were reported to have association with high litter size in Jining Grey goats. Similarly in sheep the C allele at 163–167 locus in the 52 regulatory region of *GPR54* gene was a potentially effective DNA marker which would improve litter size in Small Tail Han sheep (Tang *et al.* 2012). Polymorphisms of *KiSS1* and *GPR54* genes were potentially associated with litter size in Small Tail Han and Hu sheep (Chu *et al.* 2012). However all these are preliminary studies, and as such, additional data is needed to confirm the significant effect before using these results for marker-assisted selection (MAS). The SNPs identified in the present study could be useful for researchers willing to work with genetic markers in goats, genotyping in case-control association or population genetic studies. Hence our results in goats add up to the existing knowledge and extend the spectrum of genetic variation of caprine *GPR54* gene, which might contribute to improvement of goat genetic resources and breeding.

The present study explored the genetic polymorphism of *GPR54* gene resulting in identification of two novel SNPs in indigenous goats. These polymorphisms may play an important role in reproductive functions such as age of sexual maturity and litter size. If association can be established among the identified SNPs and precocity and/or prolificacy trait, these SNPs could be used for propagation of goats with these desirable traits.

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