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Genetic effects of leptin receptor (*LEPR*) polymorphism on litter size in a Black Bengal goat population

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Abstract

The leptin receptor (LEPR) is involved in central signaling for both energy homeostasis and reproduction. The present study investigates the association of the *LEPR* gene with the prolificacy of Black Bengal goat. Two single nucleotide polymorphisms (SNPs) in intron 3 and one SNP in exon 4 in the *LEPR* gene were identified by pooled DNA sequencing. The identified SNPs were genotyped by the direct sequencing method from 84 Black Bengal does. A synonymous mutation (Lysine > Lysine) was found as a polymorphism in exon 4. The effects of the different genotypes on litter size traits were estimated using linear models. Our results show that goats with heterozygous genotype AG at the loci g.104911A>G and g.105151A>G showed the highest prolificacy performance when compared with the other, homozygous genotypes. Dominance and additive effects were observed at the considered loci. No significant allele substitution effects were found for any locus. Our results indicate preliminarily that *LEPR* may have some association with prolificacy and could be a candidate gene to improve the prolificacy in goat.

Keywords

Black Bengal goat; goat; leptin receptor; prolificacy; single nucleotide polymorphism; ungulate

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Introduction

Leptin (LEP), the protein product of the *obese* (*OB*) gene, is involved in central signaling for both energy homeostasis and reproduction (Shi et al., 2010). The leptin receptor is found on proopiomelanocortin (POMC) neurons in the arcuate nucleus and evidence suggests the anorexigenic effect of gonadal estrogen is being mediated through these same POMC neurons (Gao et al., 2007). LEP significantly influences many aspects of the reproductive function, including an association with the onset of puberty as well as with fertility in both males and females (Gale & Van Itallie, 1979; Bivens & Olster, 1997). LEP-deficit male and female obese mice are infertile (Charlton, 1984) and artificially-induced expression of *LEPR* in the brain of mice restores their fertility, implying that *LEPR* has a critical regulatory function in reproduction (Hill et al., 2008).

There are several reports of nucleotide sequence variants in *LEP* and its receptor (*LEPR*) gene in cattle and associations with circulating leptin concentrations (Liefers et al., 2005; Nkrumah et al., 2005; Buchanan et al., 2007), feed intake, carcass merit, body fatness, milk quantity and quality and energy balance (Giblin et al., 2010; Ekerljung et al., 2012; Li et al., 2013). A recent report on goat linking polymorphisms in *LEP* with parameters of physiology and health supports the role of *LEP* in controlling metabolism (Di Gregorio et al., 2014). Regarding polymorphisms in the *LEPR* gene, there seems to be a vital role for them in outcomes associated with reproductive status (lambing rates, age at onset of puberty, ovulation) in sheep in New Zealand (Haldar et al., 2014; Juengel et al., 2016). The present study was designed to detect single nucleotide polymorphisms in the ovine *LEPR* gene in goats, and to investigate whether those polymorphisms are associated with litter size.

This is of relevance because goats are economically important in animal agriculture and a promising animal resource in developing countries, especially in Asia and Africa (Husain, 1999). For example, it is the second-largest ruminant livestock in Bangladesh and occurs across the country. At more than 90% of the local goats (Husain, 1999), the Black Bengal goat (BBG) is the most prolific local breed in Bangladesh. The mean litter size of BBG had been reported to be 2.5 (Chowdhury et al., 2002). Usually, they give birth twice a year or more commonly thrice in two years (Zeshmarani et al., 2007). The litter size varies from singles to quadruplets, with the most common litter size being twins (56.32%) and quadruplets being the least frequent (2.11%) (Hassan et al., 2007).

Materials and methods

Experimental animal and phenotypic data

All procedures involving animals and samples were approved by the ethical review committee of the National Institute of Biotechnology (NIB), Bangladesh, where the experiment was conducted. A total of 84 BBG were chosen from the Natore

and Bandharban districts, including four buck families with 10-25 daughters from each sire under control and contract farming. A total 147 kids from 84 does were selected at farmers from Natore (85) and Bandharban (62) who were rearing at least two goats. Farmers were trained to keep records, vaccinate and deworm on schedule. Most of the time, the goats were allowed to graze freely. Around 100 g/day of rice, maize and wheat mixture were given to each goat as concentrate feed in the morning. Natural services were provided for does showing heat.

Sample and relevant information collection

Blood samples were collected by puncturing the jugular vein. About 5 ml of blood samples were collected from each goat. Besides data on litter size up to sixth parity, body weight, management system and service system during heat period were also obtained using a structured and simple questionnaire. The samples were transferred to the laboratory, maintaining the cold chain. Genomic DNA was extracted from whole-blood samples using a TIANamp Genomic DNA kit (TianGen, Beijing, China) according to the manufacturer's instructions.

Primers

A pair of primers was designed to amplify exon 4 and part of the flanking intronic sequences based on the reference sequence of the ovine *LEPR* gene (GenBank accession no. NC_030810) with the Primer3 web Program (v.0.4.0) (Rozen & Skaletsky, 2000). The expected fragment length was 453 bp. The sequences of the primers were F: 5′-GTG CTT CAC TGT TGC CTC AT-3′, R: 5′-TGA GCT GAC ATT GGA GG TGA-3′. The primer was synthesized by Invitrogen (Invitrogen Life technologies, Beijing, China).

Detection and genotyping of the polymorphisms

A DNA pool (50 ng μl^{-1}/buck) was constructed from the four bucks. PCR amplifications for pooled DNA from four bucks were performed in a final reaction volume of 50 μl consisting of 2 μl containing 100 ng genomic DNA, 25 μl premix, 1.5 μl of each primer (20 pM) and 20 μl ddH_2O using reagents from Invitrogen. The PCR protocol was 5 min at 94°C for initial denaturing followed by 34 cycles at 94°C for 30 s; 56°C for 30 s; 72°C for 30 s; and final extension at 72°C for 7 min. Then, 40 μl of each PCR product from the pooled DNA was sequenced using an ABI3730XL (Applied Biosystems, Foster City, CA, USA). For processing of the chromatographs generated from the sequences, the BioEdit Sequence Alignment Editor (version 7.0.9.0) (Hall, 1999) was used. Both forward and reverse direction sequences were then aligned using the ClustalW (Hall, 1999) multiple sequence alignment program to determine the presence of polymorphisms. The direct sequencing method was used for individual genotyping of the 84 BBG samples.

Statistical analysis

Allele frequencies, genotype frequencies and Hardy-Weinberg equilibria for the three identified SNPs were calculated using POPGENE version 1.32 (http://www.ualberta.ca/~fyeh/) and association analyses were performed in SAS 9.1.0 software (SAS Institute, Cary, SC, USA). Finally, the effects of the different genotypes were estimated using linear models in the procedure PROC GLM in SAS with the random sire effect (residual) using genetic background and/or experiment as fixed effects. The following model was employed to estimate the effects of *LEPR* polymorphic genotypes on the litter size in BBG and among the different genotypes; least squares means were used for multiple comparisons.

$$Y = \mu + S + \mathrm{KS} + P + G + \alpha + e,$$

where Y is the phenotypic value of litter size of does; μ is the general mean; S is the fixed effect of sire; KS is the fixed effect of kidding season; P is the fixed effect of parity, G is the fixed effect of polymorphism genotypes; α accounts for additive genetic effects other than the *LEPR* polymorphism genotype; and e is a random residual.

The effects of the polymorphism in three different genotypes were compared using multiple t-tests with Bonferroni correction in which the significance level of the multiple tests was equal to the significance level of each single test divided by the number of tests. The equation of Falconer and Mackay (Falconer & Mackay, 1996) was employed for the estimation of additive (a), dominance (d) and allele substitution (α) effects, i.e., $a = (\mathrm{AA} - \mathrm{BB})/2$, $d = \mathrm{AB} - (\mathrm{AA} + \mathrm{BB})/2$ and $\alpha = a + d(q - p)$, where AA and BB indicate the two homozygous genotypes, AB indicates the heterozygous genotype, and p and q are the allele frequencies of A and B, respectively.

Results

Screening of single nucleotide polymorphisms and genotypes

Based on the GenBank database (GenBank accession no.: NC_030810), the ovine *LEPR* gene contains 20 exons with a total length of about 156,998 bp. By screening the pooled DNA sample sequences of exon IV and the partial intronic sequences of the ovine *LEPR* gene with a pair of primer, three SNPs were identified in this study, of which two (g.104911A>G and g.104976A>G) were found in intron 3 and the third (g.105151A>G) in exon 4. The identified SNPs were genotyped by direct sequencing (fig. 1) the 84 BBG with an average genotyping success rate of 98.7%. The genotypic and allelic frequencies and the Hardy-Weinberg equilibrium test (χ^2) are summarized in table 1. The chi-square test implied that all genotypic frequencies in the population were in Hardy-Weinberg equilibrium ($P > 0.05$) (table 1) and that selection pressure for litter size did not have a large influence on the genotypic frequencies.

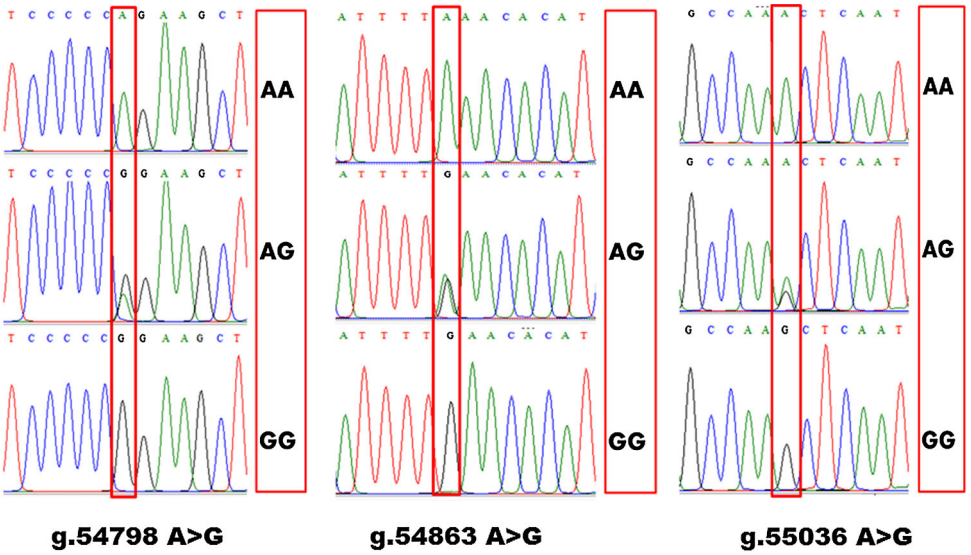

Figure 1. Sequence analysis, polymorphism detection and genotyping of individual polymorphisms of the *LEPR* gene in Black Bengal goat.

Association and effects of SNPs

The effects of the three identified SNPs (g.104911A>G, g.104976A>G and g.105151A>G) on litter size of BBG were estimated and summarized in tables 2 and 3. Out of the three, two were found in an intronic sequence and one was found in exon 4 as a synonymous mutation (Lysine > Lysine). All three SNPs (g.104911A>G, g.104976A>G and g.105151A>G) were found to be highly associated ($P < 0.0001$, $N = 147$) with litter size of BBG with raw P values <0.05; such associations remained significant even after Bonferroni correction for multiple t-testing. Our results revealed that goat with heterozygous genotype AG at the

Table 1.
Genotypic and allelic frequencies *LEPR* locus.

Polymorphisms	Genotypic frequency			Allelic frequency		Hardy-Weinberg equilibrium χ^2 test ($N = 147$)
	AA	AG	GG	A	G	
g.104911A>G	0.37 ($N = 55$)	0.35 ($N = 51$)	0.28 ($N = 41$)	0.55	0.45	$P > 0.05$ ($\chi^2 = 14.38$)
g.104976A>G	0.61 ($N = 90$)	0.25 ($N = 37$)	0.14 ($N = 20$)	0.74	0.26	$P > 0.05$ ($\chi^2 = 10.94$)
g.105151A>G	0.63 ($N = 93$)	0.29 ($N = 42$)	0.08 ($N = 12$)	0.78	0.22	$P > 0.05$ ($\chi^2 = 6.43$)

Table 2.
Least squares means (LSM) and standard errors (SE) for litter size of different *LEPR* genotypes in Black Bengal goat.

Locus	Genotypes	Number of does	Litter size*
g.104911A>G	AA	55	1.73 ± 0.16^{a}
	AG	51	2.00 ± 0.11^{b}
	GG	41	1.83 ± 0.13^{c}
P-value			<0.0001
F-value			6.62
DF			2
g.104976A>G	AA	90	1.80 ± 0.09^{c}
	AG	37	1.85 ± 0.17^{b}
	GG	20	2.27 ± 0.19^{c}
P-value			<0.0001
F-value			6.84
DF			2
g.105151A>G	AA	93	1.78 ± 0.08^{a}
	AG	42	2.17 ± 0.14^{b}
	GG	12	1.33 ± 0.43^{c}
P-value			<0.00015.64
F-value			2
DF			

* Different superscripted letters among the genotypes indicate significant differences ($P < 0.001$).

loci g.104911A>G ($N = 51$) and g.105151A>G ($N = 42$) showed the highest prolificacy performance ($P < 0.0001$) among the genotypes with 0.83 and 0.84 more lambs, respectively, per parturition compared to goat with GG genotypes (table 2). The obtained result further showed that locus g.104976A>G had dominant effects and locus g.105151A>G had additive effects on the prolificacy of BBG (table 3). However, our results did not reach significance for allele substitution effects at any locus.

Discussion

The leptin receptor (*LEPR*) gene, containing 20 exons, is a prominent candidate gene for use in breeding for improved production and reproduction in livestock. A non-synonymous mutation (C>T) in exon 20 at position 115 of bovine *LEPR* causes a change of threonine to methionine in the leptin receptor in Holstein-Friesian cows. Leptin levels in blood were influenced by this *LEPR* SNP in late pregnancy but not in lactation. Cows with homozygous CC genotypes contained higher levels of blood leptin compared to heterozygous CT cows (Liefers, 2004). Schenkel et al. (2006) demonstrated few associations between *LEPR* SNPs and production traits (fat mass, fat yield and fat grade) in Western dairy and beef cattle but

Table 3.
Additive, dominant and allele substitution effects of SNPs on litter size in Black Bengal goat.

Locus	Genetic effects	Litter size
g.104911A>G	Additive (a)	−0.0481
	Dominant (d)	0.2245
	Allele substitution (α)	−0.0695
g.104976A>G	Additive (a)	−0.2318*
	Dominant (d)	−0.1848
	Allele substitution (α)	−0.1437
g.105151A>G	Additive (a)	0.2246
	Dominant (d)	0.6144*
	Allele substitution (α)	−0.1139

* Significant additive effect or allele substitution effect at the $P < 0.05$ level.

recently Guo et al. (2008) genotyped a *LEPR* exon 4 SNP and established links with body measures (height, weight, length and weight gain at 6 and 12 months) in five Chinese cattle that may speed productivity increases in these demographically important breeds. Sun et al. (2009) reported associations of a *LEPR* exon-2 SNP (C155T) with first litter size and percentage of live-born piglets in Large White sows that might help selection for reproductive output in pigs. On the basis of associations of *LEPR* SNPs with age and weight at puberty in gilts, Kuehn et al. (2009) suggest possible quantitative trait loci to select for earlier puberty in pigs. The authors also remarked that worldwide, many breeds are used with different strengths of the association between *LEPR* SNPs and production and reproduction traits.

Normally *LEPR* is expressed in the ventromedial hypothalamus, dorsomedial hypothalamus, lateral hypothalamus, ventral tegmental area, premammillary ventral nucleus and the nucleus of the solitary tract (Mercer et al., 1996; Fei et al., 1997; Elmquist et al., 1998). Leptin receptor-expressing neurons may play key roles in the regulation of reproduction. Several studies indicated that *LEPR*-expressing neurons on the hypothalamic premammilary nucleus regulate the secretion of luteinizing hormone (Donato et al., 2009) and activate the hypothalamus to directly innervate GnRH neurons (Leshan et al., 2009) to regulate reproduction.

The function of the *LEPR* gene in reproduction and literature reports support our study. In the current study we identified two polymorphisms in intron 3 (g.104911A>G and g.104976A>G) and one in exon 4 (g.105151A>G) (synonymous mutation, Lysine > Lysine) of the *LEPR* gene. Although Guo et al. (2008) genotyped several SNPs in exon 4 of the *LEPR* gene including SNPs identified in our study and found a strong association with production in Chinese cattle, the authors did not report on any reproductive traits. Di Gregorio et al. (2014) studied polymorphisms in leptin and their effect on parameters of physiology and health, supporting the role of leptin in controlling metabolism in goat. A recent study by Juengel et al. (2016) showed a significant association of polymorphisms in *LEPR* with reproductive status (age at onset of puberty, ovulation, lambing rates) for ewes

in New Zealand. In our study, we found a significant association between the identified SNPs and litter size in BBG but, to the best of our knowledge, there are no other studies investigating the effect of *LEPR* on reproductive traits in goat for comparison. It is thought that any mutation in the gene will affect gene expression, the rate and the regulation of gene transcription or the amino acid sequence of the gene product (Nott et al., 2003; Zan et al., 2007). The identified SNPs could thus have an effect on litter size in goat.

The present study revealed two SNPs in intron 3 and one synonymous SNP in exon 4 of the ovine *LEPR* gene with a significant association with litter size in our study population. We provide preliminary results for the association between polymorphisms of the *LEPR* gene and litter size in BBG. Thorough scanning and genotyping of the *LEPR* gene had uncovered several potentially meaningful polymorphisms and such novel polymorphisms may provide further evidence that the *LEPR* gene is a key regulator of reproductive function. Due to the lack of functional data, the tentative conclusion about the function of these polymorphisms now needs to be validated with further studies using long-term production, a larger dataset, and *in vitro* biological analysis.

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