Indian Journal of Animal Sciences **89**(7): 801–803, July 2019/Short communication
https://doi.org/10.56093/ijans.v89i7.92057

Polymorphism in inhibin alpha (INHA) gene is not associated with litter size in Markhoz goats

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Received: 1 September 2018; Accepted: 27 November 2018

Key words: INHA gene, Litter size, Markhoz goats, Polymorphism

Investigations in goats on fecundity genes (McNatty *et al.* 2001, Montgomery *et al.* 2001, Davis 2005) are limited. Members of the transforming growth factor beta (TGFβ) super-family and their related cell-surface receptors are important intra-ovarian regulators of ovarian follicular development and ovulation rate (McNatty *et al.* 2001). Inhibin alpha (INHA) is 1 of 5 main groups within the TGFβ super-family (Burt and Law 1994). Biosynthesis of the inhibin alpha-subunit is associated with normal oocyte and follicle maturation, and excessive alpha-inhibin is associated with poor embryo quality in human (Fujiwara *et al.* 2000). But, studies on the effect of INHA on litter size in goat are scarce. Markhoz (Angora) goats, reared in some provinces of Iran (Shokrollahi 2015, Bahmani 2017), provide mohair which is used for making local clothes (Bahmani 2017). Study on reproduction biology is required to increase Markhoz population. Therefore, it is essential to explore the genetics and reproduction in Markhoz goat to identify genes with major effect on prolificacy. The objective of this study was detection of the polymorphism in exon 2 of INHA gene in Markhoz goat breed by RFLP-PCR and association between variations of INHA gene and litter size.

Female Markhoz goats (150) were randomly selected and their litter size data were obtained. Blood samples from selected goats were collected. The whole blood was preserved in ethylenediaminetetraaceticacid (EDTA)-coated tubes and stored at –20°C.

The genomic DNA was extracted from white blood cells using salting-out extraction procedure (Miller *et al.* 1988). Quality and quantity of extracted DNA was measured on 0.8% agarose gel prepared in 0.5× TBE buffer, visualized with ethidium bromide, and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 Pro, USA). The DNA samples were dissolved in TE buffer (pH=8.0) and stored at –20°C for use.

The primers were manufactured by CinnaGen Co. Ltd. The restriction endonucleases (RE) were purchased from Fermentas Co. Ltd. The primers sequences, restriction enzymes, annealing temperature are shown in Table 1. The PCRs were performed in a final volume of 25 µL containing 100 ng of template DNA, 0.5 µL of each primer, 2.5 µL of 10× PCR buffer, 4 µL of 1.25 mM dNTP, 1 µL of 50 mM $MgCl_2$ (CinnaGen, Tehran, Iran), and 0.5 µL of Taq DNA polymerase (CinnaGen) using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany).

Based on the methods reported by Wu *et al.* (2009) for P1 locus, a 332 bp fragment produced from primers made a *Bsp143II* restriction enzyme site G567A. For P2 locus, the wild-type strand of T911C site was cleaved with the same enzyme (*Bsp143II*), restriction digestion of PCR products produces a 335 bp and 143 bp while non-carrier products remained uncut at 478 bp.

A linear mixed model was employed to analyze the data on litter size using SAS 9.4 (SAS Institute Inc, 2012). The following statistical model was used to compare differences in litter size between INHA genotypes by least squares analysis of variance:

$$Y_{ijkl} = \mu + S_i + P_j + G_k + d_{jl} + e_{ijkl}$$

where Y_{ijkl}, phenotypic value of litter size; µ, overall mean; S_i, effect of ith kidding season in 3 class; P_j, effects of jth parity in 2 class; G_k, effects of Kth genotype; d_{jk}, normally distributed random variable with mean zero and variance (σ^2) corresponding to doe l in parity j; and e_{ijkl}, random error. The interactions between fixed effects were not significant and therefore excluded from the model. Mean separation procedures were done using Duncan's multiple range test.

Genetic improvement in reproductive traits in goats and sheep is problematic because reproduction traits have low heritability, expressed in late period of life and only in females. Therefore, studies should be focused on improvements in reproduction efficiency in goats and sheep. Success has been restricted to conventional breeding and improvement programs in goats. Improvement of reproduction efficiency in goats and sheep can be achieved by implementation of marker-assisted selection programs

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Table 1. Primer sequences for RFLP-PCR of INHA gene mutation points.

Locus	Region of the gene	Size of the PCR product	Primer (F: Forward, R: Reverse)	Annealing temperature
P1	Exon 2: 272–604	332 bp	F: GCGGGGATGAGCCAGATG R: GGGCGGAGCAGGAACAGA	64°C
P2	Exon 2: 576–1054	478 bp	F: GCGTTGTCCTCTCTGTTCCT R: GGTTGGGCACCATCTCATAC	63°C

using genetic markers. In this regard, genes are selected to increase ovulation rate and prolificacy. Fecundity genes, viz. BMP15, GDF9, and BMPR1B were identified in sheep and tested in different goat breeds. In our previous studies, we found polymorphism in GDF9 and BMP1RB loci (Shokrollahi and Morammazi 2018) but no polymorphism in BMP15 gene (Shokrollahi 2015) in Markhoz goats. The INHA gene can be used in superovulation and fecundity studies in sheep and goats. In this study, genetic polymorphisms of 2 SNPs in INHA gene in Markhoz goats were tested using the PCR-RFLP method. Two regions in exon 2 within the INHA gene (2 fragments of 332 and 478 bp in length) were successfully amplified. All extracted DNA from Markhoz goat blood samples yielded a specific, single-band PCR product without nonspecific bands for both loci. Therefore, the PCR products were directly used for RFLP analysis. The results showed that the P1 locus was polymorphic and the frequencies of the observed genotypes for this locus were 14, 77.33 and 8.66% for GG, GA, and AA, respectively, and allele frequencies were 52.66 and 47.34% for G and A alleles (Table 2). The results of PCR-RFLP for P2 locus showed that all individuals were wild homozygous hence this locus was monomorphic in Markhoz goats. Wu *et al.* (2009) surveyed polymorphism of these loci and 10 other SNPs in the INHA gene in Boer goats and suggested that all 12 SNPs were polymorphic. Aaolu *et al.* (2015) observed 2 alleles (A and G) and 3 genotypes (AA, AG and GG) for the INHA gene in Honamli and Hair goat breeds. Moreover, Isa *et al.* (2017) identified 4 SNPs including one novel mutation (g.2518G > A) in exon 2 of INHA gene and 3 others namely g. –65C > G in 5'UTR, g. 3041A > G in exon 3 and g. 3234C > T in 3'UTR in Kalahari Red and Nigerian goats. The novel mutation and g. –65C > G were only detected in KR population while other mutations were present in all the populations.

In our study, the association analysis for P1 locus showed that parity and kidding season had no significant effect on litter size (P>0.05) and different genotypes of P1 locus of INHA gene had not any significant effect on litter size in tested Markhoz does (P>0.05). The least squares mean (LSM) and standard error for litter size of different genotypes at the P1 locus INHA gene are given in Table 3. The P1 locus had no association with litter size in Markhoz goats. Isa *et al.* (2017) suggested that polymorphism at g.3234C > T locus in INHA gene may serve as a baseline genetic marker for litter size. Likewise, Hua *et al.* (2007) showed that a new mutation (G284A) in Chinese goats was associated with litter size. Liu *et al.* (2017) detected 2 mutations (G258A and G759A) in INHA in prolific Jining Grey goats and low to medium fecundity breeds, viz. Boer, Liaoning Cashmere, Wendeng Dairy, Taihang and Inner Mongolia Cashmere by PCR-SSCP and sequencing methods, and found that the G759A mutation was associated with high litter size in Jining Grey while C446T, A651G, and A946 were associated with litter size significantly in Nubi, Matou, Boer goats respectively. Wu *et al.* (2009) reported that among 12 studied loci, only A651G with over-dominant effect had association with litter size. In exon 2, a missense mutation (G125A) showed a significant effect on litter size in Dazu Black and Nanjiang Yellow goats (Zhao *et al.* 2012). He *et al.* (2010) showed that there were no polymorphism in GDF9, BMP15 and BMPR1B genes in 3 Chinese goat breeds but they detected 1 mutation in INHA gene in the same animals that had relationship with prolificacy. However, in our study, no association of P1 locus with litter size was found in Markhoz goats.

This study was the first attempt for identification of INHA gene variation in Markhoz goats. Two alleles (G and A) and 3 genotypes (GG, GA, and AA) were observed in the exon 2 region of the INHA gene in Markhoz goat. The most frequent allele and genotype were G (52.66) and GA (77.33%), respectively. One reason could be linked to relatedness, because the blood samples were collected from one farm, therefore, it is likely that the rate of relatedness among animals was very high. In that case, it has an influence on the effective size of the population and in turn, it would have

Table 2. Allele and genotype frequencies of P1 locus of INHA gene in Markhoz goats

Locus	N	Allele frequency (%)		Genotype frequency (%)		
		G	A	GG	GA	AA
P1 (G567A)	150	52.66	47.34	14	77.33	8.67

Table 3. Least squares mean and standard error for litter size of different genotypes at G567A of INHA gene in Markhoz goats

Locus	Genotype	Number of does	Litter size	P value
P1 (G567A)	GG	21	1.2056±0.1203	0.5923
	GA	116	1.3502±0.0764	
	AA	13	1.2781±0.1401	

an effect on the association analysis. Thus, in future studies, it is necessary to extend research on the goats with low relatedness, although the overall population of Markhoz goats is severely diminished. Polymorphism occurring in the exon 2 region of the INHA gene in the Markhoz goat breed had no association with litter size trait. For lack of functional researches in inhibin gene, further studies should be done so as to study the whole gene and its possible relationship with litter size in Makhoz goats. This paper provides preliminarily reference for the investigation of polymorphisms in INHA gene in Markhoz goats.

SUMMARY

The present study was aimed to survey the polymorphisms in 2 loci (exon 2) of inhibin alpha (INHA) gene in Markhoz goats. Blood samples collected from 150 female goats were used for extraction of genomic DNA; and 2 fragments related to exon 2 with 332 bp (G567A, P1 locus) and 478 bp (T911C, P2 locus) in length were amplified using polymerase chain reaction (PCR). PCR products were subjected to digestion using Bsp143II endonuclease. The results showed that 2 alleles (G and A) with the frequency of 0.526 and 0.473, and 3 genotypes (GG, GA and AA) with the frequency of 0.138, 0.085 and 0.773 were identified for P1 locus in Markhoz goats but P2 locus was monomorphic. Investigation on effect of genotypes in P1 locus on litter size trait showed that P1 locus genotypes had no association with litter size in Markhoz goats. The results demonstrated, for the first time, that polymorphism in a locus in exon 2 of the INHA gene had no significant association with litter size in Markhoz goats.

ACKNOWLEDGEMENTS

This work was financially supported by Sanandaj Branch, Islamic Azad University.

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