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Polymorphism of the ovine BMP15, INHBA and INHA candidate genes for litter size in four Iranian Indigenous sheep using PCR-sequencing

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Abstract The inhibin α (INHA), inhibin βA (INHBA) and bone morphogenetic protein 15 (BMP-15) were investigated as candidate genes for reproductive traits in four Iranian sheep breeds (Bahmaei, Lak Ghashghaei, Lori-Bakhtiari and Karakul). Based on the ovine sequences of BMP-15, INHA and INHBA genes, three pairs of primers were designed to identify single nucleotide polymorphisms (SNPs) in exon 2 of BMP-15, INHA and INHBA in multiparous ewes by DNA sequencing. Two SNPs were detected in exon 2 of the ovine BMP15 gene at positions 367 and 430, which lead to amino acid substitutions at position 231 and 252 in the BMP15 protein sequence, respectively. Substitution of Leucine to Proline at position 252 is predicted to affect the protein function. A synonymous mutation was found in the amplified fragment of exon 2 at position 752 in ovine INHBA gene. In addition, the c752C>T mutation was only found in heterozygous condition in only one Lori-Bakhtiari ewe, while other breeds were in wild type genotype for c752C>T mutation. The INHA gene was shown to be highly polymorphic. A total of 7 SNPs including 6 nucleotide substitutions and one insertion were found in the amplified fragment of the INHA locus. The insertion mutation was found in two animals of Bahmaei and Karakul breeds. Interestingly, homozygous condition for the mutant alleles in all identified SNPs in BMP15, INHA and INHBA loci was absent in these breeds. Generally, these breeds showed different genetic structures with regard to the identified SNPs in BMP15, INHBA and INHA genes. However, further research with larger sample size and phenotype data on reproductive performance is required to investigate the definitive effect of the identified mutations in this study.

Keywords: BMP15, INHA, INHBA, litter size, sheep breeds

Introduction

Currently, there are more than 1400 sheep breeds in the world that differ in many performance features, including the reproductive traits (National Sheep Association, 2021). The ewes usually gives birth to 1 or 2 lambs at each lambing, but there are some highly prolific sheep breeds with litter size ranging from 3 to 6 lambs (Kaczor, 2017). Currently, the genetic mechanisms of some reproductive features, including the number of mature follicles and ovulating rate in many sheep breeds, have been investigated. It has been confirmed that sheep fertility can be determined by polygenic or determind by the segregation of a major gene named the fecondity *(Fec)* gene (Kaczor, 2017). Many studies have reported that genetic polymorphisms in *Fec* gene family co-

uld be associated with different reproductive traits like fertility, ovulation rate and litter size. For example, Booroola gene was the first identified gene that affected prolificacy traits in sheep (Davis et al., 1982; Piper and Bindon, 1982). So far, many fecundity genes such as bone morphogenetic protein 15 (BMP15) and inhibins have been identified that have major effects on reproductive traits.

Inhibin, a glycoprotein hormone, belongs to superfamily of transforming growth factor-β (TGF-β) that suppresses the synthesis and secretion of follicle-stimulating hormone (FSH) (Bernard et al., 2001; Chu et al., 2018; Robertson et al., 1985; Woodruff et al., 1996). It consists of two subunits (α and β) that are linked by disulfide bonds. The α-subunit form the common part of two inhibins, while for the β-subunit two different components (βA or βB) have been identified (Mason et al., 1985; Williams et al., 2021). Although, follicles are the major source of inhibin expression in sheep (Bao et al., 2021; Rodgers et al., 1989), there is some evidence for its other sources of inhibin production as well (Kondi-Pafiti et al., 2013; McNatty et al., 1992). Inhibin immunization improve the development of ovarian follicular, ovulation rate and transferrable embryos by increasing FSH secretion (Ishigame et al., 2004; Li et al., 2011; Medan et al., 2004).

Bone morphogenetic protein 15 (BMP-15), a growth factor and expressed specifically in oocytes, is also a member of the TGF-β superfamily (Galloway et al., 2000). BMP-15 regulates proliferation and differentiation of cells by mitosis promotion, expression suppression of FSH receptor, and expression stimulation of kit ligand; all of these mechanisms play a pivotal role in mammalian female fertility (Juengel et al., 2002; Moore and Shimasaki, 2005; Otsuka and Shimasaki, 2002; Otsuka et al., 2000). Thus, it has been confirmed that inhibins and BMP-15 are essential for normal folliculogenesis and fertility in sheep. On the other hand, mutations reported in the inhibins and BMP-15 genes were associated with reproductive traits in some sheep breeds (Hanrahan et al., 2004; Niu et al., 2021; Saleh et al., 2020).

In Iran, the average litter size has been reported to be less than 10% in most native sheep breeds under nomadic breeding conditions. It means that non-additive genetic variance is a major component of the phenotypic variance. For example, an over dominant autosomal major gene was identified that influences the litter size in Baluchi sheep (Saneei and Nejati-Javaremi., 2000). Therefore, identification of major genes affecting reproductive traits could play an important role in enhancing fecundity in native sheep breeds (Ghiasi et al., 2006; Muhaghegh Dolatabady and Habibizad, 2019). In Iran, extensive research has been conducted to identify genes that affect fecundity traits in different breeds of sheep. Because inhibins and BMP15 are associated with the folliculogenesis, their genes were considered as possible candidates for fecundity traits in sheep. Therefore, the aim of this study was to investigate the presence of polymorphism in exon 2 of the inhibin α (INHA), inhibin βA (INHBA) and BMP-15 genes in four native sheep breeds; namely, Bahmaei, Lak Ghashghaei, Lori-Bakhtiari and Karakul.

Materials and methods

A total of 24 randomly multiparous ewes were selected, Lori-Bakhtiari sheep from Chaharmahal and Bakhtiari province (n=6), Karakul sheep from Fars province (n=6), Bahmaei (n=6) and Lak Ghashghaei sheep (n=6) from Kohgiloueh and Boyer-Ahmad province. Jugular vein blood was collected into 2 mL sterilized tubes containing EDTA. Genomic DNA was isolated by AccuPrep® genomic DNA extraction kit (BiONEER, South Korea), according to the manufacturer's instructions.

The primers were designed based on the ovine INHA (NC_040253.1), INHBA (EF192431.1) and BMP15 (AH009593) genes using Primer3plus (http://www.bioin forma tics.nl/cgi-bin/prime r3plu s/prime r3plus.cgi) (Table 1). Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume. Each reaction contained 2.5 µL10 × buffer, 200 µM of each dNTP, 1.5 mM MgCl2, 0.5 U Taq DNA polymerase, 15 pm of each primer and 50 ng genomic DNA template. The polymerase chain reaction was carried out in initial denaturation at 94 °C for 5 min and then, 35 cycles at 94 °C for 45 s, annealing temperature, and 72 °C for 45 s followed by 72 °C for 5 min (Table 1).

The PCR products were checked by electrophoresis using 1.0% agarose gel in 1 × TBE buffer along with DNA ladder and visualized with ethidium bromide solution. Then, the purified PCR products were sequenced using the forward primers (two repeats) by TOPAZGENE Ltd and the data were analyzed using BLAST in GenBank, CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and FinchTV software (version 1.4.0, Geospiza Inc ., Seattle, WA, USA).

The SIFT (Sorting Intolerant from Tolerant) tool was also applied to predict that amino acid substitution can affect protein function. For this purpose, SIFT considers the type of amino acid change and the position at which the amino acid to be altered, and computes a SIFT score. The SIFT score, ranges from 0 to 1, indicate probability of observing the new amino acid at that position, and a value of between 0 and 0.05 is predicted to influence the protein function.

Results

In this study, three pairs of primers were used to amplify approximately full length of exon 2 ovine BMP15, INHA and INHBA genes, and then the PCR products were separated on 1.0% agarose gels. The amplified products were consistent with the expected size of target fragments and showed good specificity, which could be directly used for sequencing.

Table1. Primer sequence, expected product size, amplified region and annealing temperatures for amplification of the exon 2 in BMP15, INHA and INHBA genes

Gene	Primer sequence	Product size (bp)	Amplified region	Annealing tem.	Access Number
BMP15	5'- CTCTGAGACCAAACCGGGTA-3' 5'- TCTGATCCACCAGCTCACTG-3'	699	5659-6357	62	AH009593
INHA	5'-TTTCGTGTGGGCACCTAGCAG-3' 5'-GCCTAGACCTCACCCTGACA-3'	946	2002-2948	63	NC_040253.1
INHBA	5'-AAAGAGCACCTGGCACATCT-3' 5'-CACACAGCACGACTTGAGGT-3'	532	750-1281	62	EF192431.1

The sequence alignment revealed two single nucleotide polymorphisms (SNPs) at positions 367 (T→A) and 430 (T→C) exon 2 in ovine BMP15 gene (Figure 1a and 1b). The c367T>A mutation with alteration of the TTC codon to the TAC codon, leads to amino acid substitution of Phenylalanine (F) to Tyrosine (Y) at position 231 in the BMP15 protein sequence. The c430T>C SNP also results in a shift of the amino acid Leucine (L) to Proline (P) at position 252 in BMP15 protein by changing the CTG codon to the CCG codon. In addition, the c430T>C SNP was observed only in one Bahmaei ewe in heterozygous condition (CT) while other breeds were in wild type genotype (CC) for the identified mutation. Then, SIFT tool was applied to predict that identified nonsynonymous mutations can affect the function of BMP15 protein. Based on obtained the SIFT score (0.28), substitution of Phenylalanine to Tyrosine at position 231 is predicted to be tolerated for BMP15 protein but substitution at position 252 from Leucine to Proline is predicted to affect the protein function with a score of 0.05.

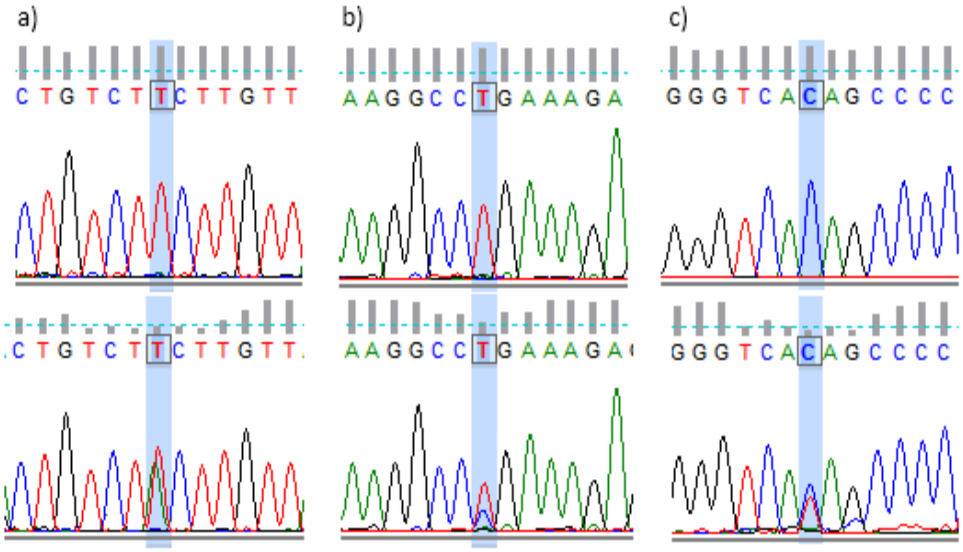

Figure1. Nucleotide changes of identified SNPs in BMP15 and INHBA genes a) T to A at position 367 in exon 2 BMP15; b) T to C at position 430 in exon 2 BMP15; c) C to A at position 752 in exon 2 INHBA.

Sequencing of amplified fragment of INHBA gene revealed one SNP (C→T) at base 752 of exon 2 (Figure 1c). This mutation was synonymous in nature and, thus could not change an amino acid in the INHBA protein. In addition, the identified mutation was only found in one Lori-Bakhtiari ewe in heterozygous condition (CT) but other breeds were monomorphic (CC) for c752C>T mutation.

A 946 bp amplified fragment of the ovine INHA gene revealed 7 novel mutations including 6 nucleotide substitutions at positions 276G>A, 317C>A, 387T>A, 683C>T, 9500C>T and 1100G>A (Figure 2) and an insertion of A at position 271 (Figure 3) coding sequence

(CDS) of ovine INHA sequence according to GenBank accession number NC_040253.1. Subsequent analysis revealed that the two identified SNPs 317C>A and 683C>T resulted in the change of Threonine amino acid to Lysine and Methionine at positions 106 and 228 of the INHA protein, respectively. The SIFT score for amino acid substitutions at positions 106 and 228 were 1.00 and 0.37, respectively, indicating that these amino acid alterations could not influence the protein function of INHA.

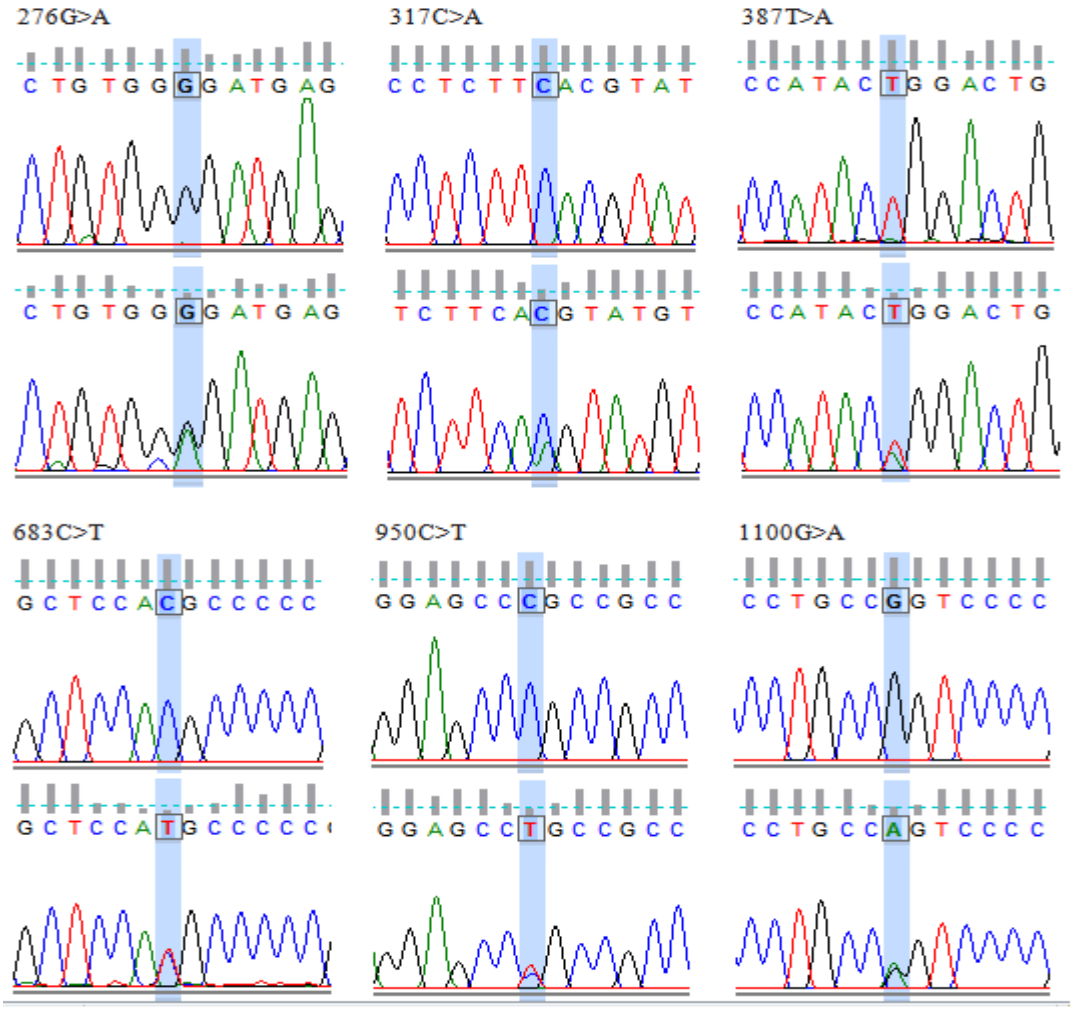

Figure 2. Nucleotide changes of the six identified SNPs in exon 2 INHA gene.

The observed frequencies of 7 identified SNPs in INHA fragment were different in these breeds (Table 2). The 387T>A, 9500C>T and 1100G>A mutations were observed only in one Bahmaei ewe in heterozygote condition, while 276G>A, 317C>A and 683C>T mutation were only found in Lak Ghashghaei, Karakul and Lori-Bakhtiari sheep, respectively. In addition, no mutant homozygous genotypes were observed.

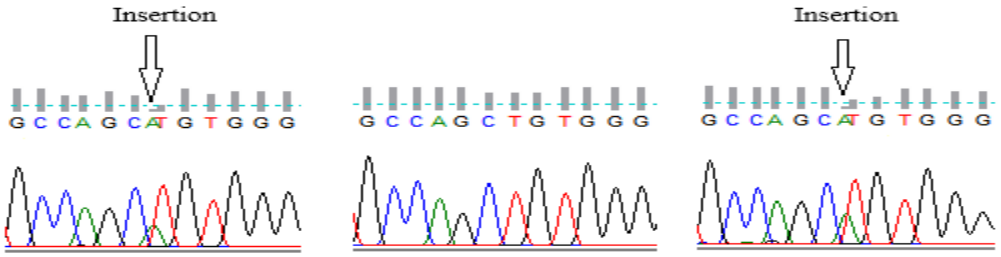

Figure 3. Nucleotide insertion at position 271 in INHA gene.

An interesting finding of the sequencing was an insertion of A nucleotide at position 271 in coding CDS of INHA gene (Figure 3). This insertion was observed in only one Bahmaei and one Karakul ewe (Table 2).

Table 2. Genetic structures and number of ewes containing identified mutations in INHA gene in four sheep breeds

	276G>A	317C>A	387T>A	683C>T	950C>T	1100G>A	A insertion
Bahmaei	-	-	1	-	1	1	1
Lak Ghashghaei	-	1	-	-	-	-	-
Karakul	-	-	-	-	-	-	1
Lori-Bakhtiari	1	-	-	2	-	-	-

Discussion

The growth factor TGF-β affects the ovarian functions, including follicular growth (Fortune et al., 2010), proliferation of granulosa cell (Chen et al., 2015), and ovulation (Fang et al., 2014). The BMP15, INHBA and INHA proteins, produced by oocytes, belong to superfamily of TGF-β which in turn affect not only the normal development of follicles but also play key roles in ovulation rate, at least in some species. Therefore, based on the important functions of BMP15, INHBA and INHA genes in female fertility, their genes were considered as possible candidate genes for the improvement of reproductive traits in sheep. Many polymorphisms at the ovine BMP15, INHBA and INHA genes were identified that show obvious genetic effect on reproduction traits like litter size in sheep. For example, a total of eight identified mutations in the ovine BMP15 gene have been associated with prolificacy in various sheep breeds (Galloway et al., 2000; Hanrahan et al., 2004). These reported mutations include nonsynonymous amino acid substitution ($FecX^{I}$, $FecX^{B}$, $FecX^{L}$, $FecX^{O}$ and $FecX^{Gr}$), premature stop codons ($FecX^{G}$ and $FecX^{H}$), 17 bp deletion in open reading frame of gene (Martinez-Royo et al., 2008; Monteagudo et al., 2009) and missed CTT bases in exon1 leading to deletion of leucine amino acid in the BMP15 protein (Abdoli et al., 2018; Niu et al., 2021). Among identified mutations in the ovine BMP15 locus; $FecX^{I}$, $FecX^{H}$, $FecX^{L}$, $FecX^{G}$, $FecX^{B}$ and $FecX^{R}$, in spite of their different molecular mechanism, result in the same phenotype in different sheep breeds. The ewes with heterozygous condition give birth to more lambs, while homozygous ewes for these mutations are infertile (Bodin et al., 2007; McNatty et al., 2005; Mingxing et al., 2005). However, the $FecX^{O}$ and $FecX^{Gr}$ mutations function differently, and the ewes harboring one and two copies of the mutant allele are more prolific (Demars et al., 2013). In the present study, two newly identified mutations, c367T>A and c430T>C, led to amino acid substitution at positions 231 and 252 in the BMP15 protein, respectively, but only c430T>C could affect the function of the target protein according to the SIFT score (0.05). Therefore, the amino acid substitution at position 252 in BMP15 protein may play an important role in the fertility difference among ewes. For this mutation, heterozygous (E+) and wild-type (++) genotypes were identified in Cele black sheep where the heterozygous ewes (E+) for p.L252P mutation had significantly higher litter sizes as compared with the wild-type genotype (Niu et al., 2021). It means that p.L252P could be a mutation that affects fecundity in sheep. The low frequency and lack of homozygous genotype for the identified mutations in four studied breeds could indicate the detrimental effects of these mutations in the homozygous condition on sheep fertility. Therefore, further research is needed to determine the association of these newly identified mutations with reproductive traits in sheep.

In this study, a 532 bp fragment was investigated from exon 2 (750-1281) of the ovine INHBA gene in four sheep breeds using DNA sequencing methodology. One novel nucleotide mutation (752C→T) was identified in exon 2 of the *INHBA* in a single animal of Lori-Bakhtiari breed in heterozygous condition. It did not alter any amino acid in the sequence of ovine *INHBA* gene. In addition, seven mutations were identified in amplified fragment of *INHA* gene. Among them, the c58C>A and c488C>T SNPs led to changes in amino acids at positions 106 and 228 in INHA protein sequence, respectively, but they did not affect the protein performance based on the SIFT scores. In addition, the genetic structures of the breeds were different for identified mutations. Generally, many SNPs have been reported for the coding and flanking regions of the inhibin genes, some of which have been associated to reproductive performance in domestic animals. For example, in a comprehensive study, the entire coding region and partial 3' flanking region of the INHBA gene were investigated in eight sheep breeds (Chu et al., 2007). A total of 21 SNPs were identified for exon 2 of the INHBA gene in these breeds, of which 17 SNPs were found only in a single breed (Hu sheep), which is a highly efficient breed for reproductive performance (Chu et al., 2007). Of the 21 SNPs identified for exon 2 in INHBA gene, 8 mutations were in the region covered by the present study. Furthermore, in another study, the coding sequences of exons 1 and 2 of the *INHBB* gene in five different sheep breeds (Chu et al., 2011) were examined for genetic variation. Only one SNP (A267G) was identified in exon 2 of a single breed. Although this polymorphism did not alter the amino acid, but there was a significant relationship (P=0.01) between the genotypes of this mutation and the lambs number in this breed (Chu et al., 2011). Furthermore, in a genetic diversity investigation of the *INHBA* gene in Chinese Bamei sheep, an SNP was detected in exon at position 857 (G857A). Although it did not result in amino acid sub-

stitution, it caused a significant increase (P= 0.01) in litter size in heterozygotes (Suo et al., 2012). In the same study, the A288T mutation in exon 1 of the *INHBA* gene also showed a significant association with the litter size (Zhou et al., 2007). Three SNPs were found in the 5'-UTR and exon 1 of the *INHBA* gene in Kazakh sheep, and their combined genotypes were significantly correlated with the number of lambs in this breed (Zhou et al., 2007). Three mutations were identified in the promoter region and exon 2 of *INHA* gene in Tan, Mongolian and Small Tailed Han sheep breeds and their genotypes were significantly associated with average litter size in three sheep breeds (Tian et al., 2010). A synonymous mutation (G617A) was identified in exon 2 INHA in Olkuska sheep (Kaczor, 2017). This mutation did not alter any amino acid and although it did not have a significant effect on the number of lambs at birth, the GG and GA genotypes had more lambs than the AA genotype for G617A mutation (Kaczor, 2017).

Conclusion

In the present study, the amplified region of exon 2 in the INHA gene was highly polymorphic. The homozygous ewes for the mutant alleles were absent for all identified mutations in *BMP15*, *INHA* and *INHBA* loci in four Iranian sheep breeds. This could be attributed to the low sample size and the detrimental effect of mutated alleles in homozygous condition on animal adaptation and survival. However, to identify the definitive reason for these finding, large sampling, mating animals with different genotypes in identified mutations and DNA analysis would be required.

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