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Full Length Research Paper

Genetic polymorphism BMP15 and GDF9 genes in Sangsari sheep of Iran

Mohammad Mehdi Kasiriyan[1]*, Seyed Hassan Hafezian[2] and Nosratollah Hassani[1]

[1]Institute of Scientific Applied Eligner Education of Jihad-e-Agriculture, Semnan, Iran.
[2]Animal Improvement and Genetics Department, Sari Agricultural Sciences and Natural Sources University, P. O. Box 737, Sari, Mazandaran, Iran.

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Different mutations in the bone morphogenetic protein 15 (BMP15) and the Growth Differentiation Factor 9 (GDF9) genes cause increased ovulation rate and infertility in a dosage-sensitive manner in sheep. In this study, blood samples (140 ewes and 10 rams) were initially taken from 150 Sangsari sheep breed in Damghan animal breeding Centre using venojects treated with the anti-clot substance (EDTA) and subsequently their DNA content were salted out and extracted. Using two pairs of specific primers, two DNA fragments were amplified from exon 1 of GDF-9 (462 bp) and exon 2 of BMP15 (141 bp) genes. The resulted PCR products were digested using *Hha*I and *Hinf*I restriction enzymes for GDF9 and BMP15 genes, respectively. Digested PCR products with *Hha*I enzyme showed a G to A substiuation in GDF9 locus. The wild type allele of this gene (G/+) with two restriction site resulted DNA fragments of 156.52 and 254 bp while the mutant allele (G/-) with one restriction site resulted two DNA fragments with the size of 52 and 410 bp. Genotype frequencies for G (+/+),G (+/-) and G (-/-) were 70.72, 36.88 and 1.40%, respectively. Restriction digested of PCR products for BMP15 locus with *Hinf* I enzyme showed C to T transition. BMP15 luci was not polymorphic. From studied luci, only GDF9 was polymorphic in Iranian Sanghsari sheep.

Key words: PCR, polymorphism, GDF9, BMP15, Sangsari sheep.

INTRODUCTION

Sangsari sheep is a light weight fat tailled Iranian breed considered to be of major economic importance because of its meat. In sheep, genetic variation in ovulation rate has been widely documented. Evidence shows substantial difference among breeds and in a number of cases exceptional variations within breeds/strains (Galloway et al., 2000). Ovulation rate was determined by a complex exchange of endocrine signals between the pituitary gland and the ovary. Three related oocyte-derived members of the transforming growth factor-β (TGF-β) superfamily, namely growth differentiation factor 9 (GDF-9), bone morphogenic protein 15 (BMP15) and bone morphogenic protein-IB have been shown to be essential for ovulation rate and follicular growth. From examination of inherited patterns of ovulation rate in sheep, several breeds have been identified with point mutations in two growth factor genes (BMP15 and GDF9) and a related receptor (ALK6) that are expressed in oocytes. Five different single nucleotide polymorphisms (SNP) have been identified in the BMP15 gene (Galloway et al., 2000; Hanrahan et al., 2004) eight SNP_s in GDF9 (Hanrahan et al., 2004) and one SNP in ALK6 namely FecB (Wilson et al., 2001; Souza et al., 2001; Mulsant et al., 2001; Davis et al., 2006). In sheep animals, heterozygous for these mutations or heterozygous for two of these mutations or homozygous for the ALK6 mutation had higher ovulation rate than their wild-type contemporaries; of course from BMP15 mutations, only B2 ($FecX^G$) and B4 ($FecX^B$) and from GDF9 mutations and only G8 ($FecG^H$) had high ovulation rate and fertility (Davis, 2005; Davis et al., 2001). Animals homozygous for BMP15 or GDF9 mutations are sterile due to arrested follicular development from the primary stage of growth. The BMP15 and GDF9 mutations are thought to result in reduced levels of mature protein or altered binding to cell-surface receptors (McNatty et al., 1997). From examination

*Corresponding author. E-mail: banaeyan_123@yahoo.com.

of phenotypes of these mutations and subsequent physiological studies, it is clear that GDF9 and BMP15 are essential for ovarian follicular development and normal ovulation and/ or corpus luteum formation in sheep. Moreover, it is evident that GDF9 (Hanrahan et al., 2004; McPherone and Lee, 1993; Dong et al., 1996; Laitinen et al., 1998; Hayashi et al., 1999; Hsueh et al., 2000; Vitt et al., 2000; Juengel et al., 2004) and BMP15 (Galloway et al., 2000; Hanrahan et al., 2004) an X-linked gene that increased ovulation rate by about 1.0 but caused sterility in homozygous carrier females was first described in Romney sheep and named the *Inverdale* gene (FecX) (Davis et al., 1991, 1992). The infertile ewes have small undeveloped 'streak' ovaries which never ovulate. It was discovered that *Inverdale* sheep carried a mutation in an oocyte-derived growth factor gene, bone morphogenetic protein 15 (BMP15; also known as GDF9B). Four different alleles of BMP15 (FecXI , $FecX^{H}$, $FecX^{G}$, $FecX^{B}$) all causing the same phenotype have been identified in Romney, Belclare and Cambridge sheep (Galloway et al., 2000; Hanrahan et al., 2004). The gene is well suited to sheep farming systems in which specialist flocks of prolific ewes are mated to meat breed sires and all offspring of both sexes are slaughtered. The specialist ewe flock which all carry the BMP15 mutation and have a litter size of about 0.6 higher than non-carrier ewes is maintained by mating other non-Inverdale ewes with carrier Inverdale rams and retaining the daughters (Galloway et al., 1996). The Inverdale gene was mapped to a 10 centiMorgans (cM) region at the centre of the sheep X-chromosome (Galloway et al., 1996). DGF9 and BMP15 are growth factors and members of transforming growth factor superfamily that are secreted by oocytes in growth ovarian follicles Unlike BMP15, GDF9 is an autosomal gene located on chromosome 5 (Sadighi et al., 1998). Measurements from a small sample of ewes suggest that the effect of the GDF9 mutation on ovulation rate is greater than the BMP15 mutations, with one copy of $FecG^{H}$ increasing ovulation rate by about 1.4 in the Cambridge and Belclare breeds (Davis et al., 2006). The gene spans about 2.5 kb and contains 2 exons separated by a single 1126 bp intron and encodes a pre-propeptide of 453 amino acid residues. The active mature peptide is 135 amino acids long.

MATERIALS AND METHODS

DNA extraction

In this study, blood samples (140 ewes and 10 rams) were initially taken from 150 Sangsari sheep breed in Damghan animal breeding centre using venojects treated with the anti-clot substance (EDTA) and subsequently their DNA content were salted out and extracted between 2004 and 2007. Genomic DNA was extracted using salting out method. Genomic DNA was dissolved in TE buffer and kept at -20°C. In a total volume of 25 µl which template PCR reaction contained: 2.5 µl PCR buffer 10-X, 2.5 µl $Mgcl_2$,10 pm of each primers, 0.2 µl *Taq* DNA polymerase, 0.2 µl dNTPs and 0.8 µl template DNA. The amplification BMP15 for primers B2-F: CAC TGT CTT CTT GTT ACT GTA TTT CAA AC (forward) and B2-R: GAT GCA ATA CTG CCT GCT TG (reverse) was carried out using 35 cycles at 94°C for 5 min, 94°C for 45 s, 62°C for 40 s and 72°C for 45 s, followed by 72°C for 5 min. The amplification for GDF9 for primers G9-1734: GAA GAC TGG TAT GGG GAA ATG (forward) and G9-2175: CCA ATC TGC TCC TAC ACA CCT (reverse) was carried out using 35 cycles at 94°C for 5 min, 94°C for 45 s, 58°C for 40 s and 72°C for 1 min, followed by 72°C for 10 min. Digestion with restriction enzyme used for GDF9 is *Hha* Iand BMP15 is *Hinf* I. Digestion reaction contain 5 µl of PCR product, 5 U appropriate enzyme, 2 µl buffer 10× in 20 µl final volume incubated for 3 to 6 h at 37°C.

RESULTS

A DNA fragment with the size of 462 bp was amplified from exon 1 of GDF-9 and 141 bp from exon 2 of BMP15 genes successfully. The resulted PCR products were digested with *Hha* 1 restriction enzyme for GDF9 and *Hinf* I for BMP15 luci and genotypes of each individual were detected by electrophoresis. Restriction digested of PCR products with *Hha1* restriction enzyme showed a mutation where the G nucleotide changed to A at this locus (G-A). The wild type allele of this gene (G/+) had two restriction sites and resulted 3 DNA fragments of 156, 52 and 254 bp while the mutant allele (G/-) with one restriction site, resulted in two DNA fragments with the size of 52 and 410 bp. In heterozygous animals, four DNA fragments with the size 52, 156, 254 and 410 bp were detected. Restriction digested of PCR products with for BMP15 with *Hinf* I restriction enzyme showed a mutation where the c nucleotide has changed to T at this locus (C-T). The wild type allele of this gene (B+) with one restriction site resulted DNA fragments with 30 and 111 were not detected for BMP15 hemozygote and hetrozygote shapes. BMP15 luci was monomorphin studied individuals.

Genetic variability

Genotype frequencies for G (+/+), G (+/-) and G (-/-) were 70.72, 36.88 and 1.40%, respectively. Allele frequencies for G (+) and G (-) were 0.80 and 0.19%, respectively. Average heterozygosity (0.36) of GDF9 locus for Sangeh sari sheep was slightly low. χ^2 test (12.48) confirmed the Hardy-Weinberg's equilibrium in this population (Table 1).

DISCUSSION

Resulted polymorphism in GDF9 confirmed previous observations that were reported by (Hanrahan et al., 2004). The present study in Sangsari sheep showed same results obtained in Hanrahan et al. (2004). The present study showed similar results reported by Hanrahan et al. (2004), Juengel et al. (2004), Chu et al. (2004), Chu et al. (2007) and Liao et al. (2004). Ninety sheep were hemozygous and had medium fertility (out of

Table 1. Frequency distribution of Gene BMP15 and GDF9 genotypes.

Genes	Alelles		Genotypes		
BMP15	B+	B-	B+ B+	B+ B-	B- B-
Freq	-	100	-	-	100
GDF9	G+	G-	G+ G+	G+ G-	G- G-
Freq	80.16	19.84	70.72	36.88	1.40

90, 8 samples were rams). Thirty samples of studied sheep had mutant type allele with (-/-) genotype and minimum fertility which was not in agreement with other reports (Galloway et al., 2000; Davis et al., 2006; Juengel et al., 2004; Liao et al., 2004). They reported that homozygous genotype were sterile in Belcarair and Cambrige sheeps. But our result showed that hemozygous genotype had reduced fertility rate, and so our study indicated the similar result as reported by Galloway et al. (2000). 30 sheep were heterozygous genotype and maximum fertility (out of 30 samples, 2 were rams). This indicates that the presence of one copy of mutant GDF9 gene increase fecundity rate in Sangsari sheep; the present study showed the same result reported by Hanrahan et al. (2004), Davis et al. (2006), Juengel et al. (2004) and Liao et al. (2004). Ewes heterozygous for GDF9 mutations have increased ovulation rates, whereas homozygous ewes are sterile due to a failure of normal ovarian follicular development (Galloway et al., 2000; Hanrahan et al., 2004; Davis et al., 1991). Generally, many different loci effect reproduction and ovulation rate between different breeds of sheep, more than genetic background, is under control of age, season and nutrition. According to these and the high prolificacy in these breeds, it is concluded that high prolificacy may be under control of other factors such as age, season and nutrition or maybe there is another major gene in Sangsari sheep. Result digest in BMP15 gene of Sangsari sheep showed only fargments 111, 30 bp. BMP15 locus was monomerphic in our studied which disagrees with the result obtain by Hanrahan et al. (2004), Davis et al. (2006), Chu et al. (2004, 2007) and Guan et al. (2006).

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