The Polymorphism of GDF-9 Gene in Hisari Sheep

Yadollah Bahrami, Sajad Bahrami*, Hamid Reza Mohammadi**, Vaheid Chekani-Azar***
and Seyed Azim Mousavizadeh****

Ph.D. student of Biotechnology Animal Science, Young Researchers and Elites Club, Khorasgan Branch,
Islamic Azad University, Khorasgan, Iran,
*Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran,
**Department of Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
***Young Researchers and Elites Club, Ilkhchi Branch, Islamic Azad University, Ilkhchi, Iran,
****Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

ABSTRACT: Application of molecular technologies led to the discovery of mutations with major effects on the reproductive and meat efficiency of sheep and goat. This study aimed to identify mutations in exon 1 region of GDF-9 gene associated with twining was conducted using PCR-RFLP method on Sheep Hisari - Tajikistan race. To identify polymorphisms in these loci Tajikistan blood samples were bled from 110 head of Hisari Tajikestani sheep race. DNA was extracted using the salt in Yashor biotechnology laboratory was located in Dushanbe and to amplification of these fragments in gene loci, quantity and quality of extracted DNA was determined by using electrophoresis gel. Then, piece, 462 bp, is related to GDF-9 positions was used from specific primer and polymerase chain reaction (PCR). GDF-9 gene by the enzyme HhaI showed both wild type allele G (+) and G (-). Also, G (+/+), G (-/+) and G (-/-) genotypes in frequency 93.64, 6.36 and 0 and alleles frequency G (+) and G (-), respectively showed 0.97 and 0.03. The average heterozygosity for GDF-9 is 0.0616. Results of this study indicate that there is genetic polymorphic and mutation in the gene loci GDF-9. Further studies on exon 1 region of GDF-9 gene or other genes are required using larger samples to be done in this race, so that definite the results. Because the Hisari sheep race is an important source of genetic Tajikistan sheep and pay attention to valued production traits, it has ability to become a twining breed.

Key words: Polymorphism, GDF-9 gene, Hisari sheep, twining, PCR-RFLP.

INTRODUCTION

hisari sheep is a light weight fat tailled Tajikestani 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). Improvement of reproductive traits in livestock species has become of increasing interest, especially in sheep, where small increases in litter size can equal large gains in profit. Genetic improvement of reproductive traits has traditionally been restricted to use of quantitative genetic methods but gain has been limited when using these methods. Recently improvement in molecular genetics provided that the major genes associated with reproduction can be utilized in breeding through marker-assisted selection (MAS). Reproductive traits are often suggested as prime targets for MAS for their low heritability and the fact that the trait can be measured only in one sex. Ovulation rate (i.e. the number of mature oocytes released during one reproductive cycle) in mammals is determined by a complex exchange of hormone signals between the pituitary gland and the ovary, and by a localized exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells (Galloway *et al.,* 2000; Eppig, 2001). Many mam sometimes two whereas other mammals such as rats, mice, hamsters, cats, dogs and pigs have ovulation rates that vary between four and 15 (McNatty *et al.,* 2005). Genetic variation in ovulation rate in sheep has been widely documented and the evidence shows substantial differences among breeds and in a number of cases exceptional variations within breeds/strains (Bindon *et al.,* 1996). The latter phenomenon has been explained by segregation of a major gene with a large effect on ovarian function.This hypothesis provided an explanation for high prolificacy of Booroola sheep (Davis *et al.,* 1982; Piper *et al.,* 1982).

Subsequently, putative major genes were invoked to explain the increased litter size and/or ovulation rate in a variety of breeds/strains, including Inverdale (Davis *et al.,* 1991), Cambridge (Hanrahan *et al.,* 2004), Thoka (Jonmundsson *et el.,* 1985), Javanese (Bradford *et al.,* 1986), Olkuska (Radomska *et al.,* 1988), Belclare (Hanrahan, 1991), Lacaune (Bodin *et al.,* 1998) and Woodlands (Davis *et al.,* 2001) sheep breeds. Growth differentiation factor 9 (GDF9) is a growth factor and a member of the transforming growth factor superfamily that is secreted by oocytes in growing ovarian follicles, which is essential for growth and differentiation of early ovarian follicles (McPherron *et al.,* 1993). GDF9 was reported to be expressed exclusively in the ovary, specifically in the oocyte in mice (McGrath et al., 1995; Dube *et al.,* 1998; Lan *et al.,* 2003), rats (Vitt *et al.,* 2000), sheep (Bodensteiner *et al.,* 1999; Juengel *et al.,* 2002), cattle (Bodensteiner *et al.,* 1999) and the human (Vitt *et al.,* 2000). The structure of the GDF9 gene had been reported in rats (Jaatinen *et al.,* 1999) and sheep (Bodensteiner *et al.,* 1999). Female mice lacking GDF9 were infertile due to a block in folliculogenesis at the primary follicle stage (Dong *et al.,* 1996; Carabatsos *et al.,* 1998). Bodensteiner *et al.* (1999) reported the nucleotide sequence of the ovine GDF9 gene (GenBank accession number AF078545). Like the human and mouse genes, ovine GDF9 spans approximately 2.5 kb and contains two exons and one intron. Exon 1 spans 397 bp and encodes for amino acids 1-134, while exon 2 spans 968 bp and encodes for amino acids 135-456. The single intron spans 1126 bp. Bodensteiner *et al.* (2000) reported that this was the first time that the GDF9 mRNA expression was localized exclusively to oocytes of foetal sheep at day 135 of gestation. Sadighi et al. (1998; 2002) mapped the GDF9 gene to ovine chromosome 5. Juengel *et al.* (2004) reported that short-term immunization against GDF9 peptide resulted in an increase in ovulation rate with no apparent detrimental effects on fertilization of released oocytes, the ability of fertilized oocytes to undergo normal foetal development, or the ability of the immunized ewes to carry a pregnancy to term. Cambridge and Belclare are prolific sheep breeds, the origins of which involved selecting ewes with exceptionally high litter size records from commercial flocks. Hanrahan *et al.* (2004) reported a mutation (S395F) in the GDF9 gene that was associated with both increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in Cambridge and Belclare sheep. The aim of this study was to investigate the presence of polymorphism in GDF9 and its possible association with litter size in the hisari sheep breed.

MATERIAL AND METHODS

In this project, 110 Hisami sheep race were randomly sampled. All blood samples was prepared from the jugular vein in the neck and using vacuum tube 10 mM containing EDTA, and in the ice was transported to the Laboratory of Biotechnology Yashour located in Dushanbe. DNA was extracted using DNA extraction method from white blood cells and extracting salt of Miller et al (1988) with a few changes DNA extraction. DNA extraction is electrophoresis on agarose gel 0.7 percent, slightly higher than the first marker bands. To determine the quality and quantity of DNA, done in agarose gels with a concentration of 0.8% and stained with ethidium bromide concentration 10µg/µl. Polymerase chain reaction stages in amplification of the exon 1 region of GDF-9 gene in Hisari sheep was used with the following sequence (Table 1).

The desired gene fragment was amplified by polymerase chain reaction (PCR). Size used in the mixture (PCR), is µl25 so that includes (Table 2)

Temperature program of chain reaction consisted of 35 cycles of amplification with an initial denaturing temperature of 94°C for 3 min, then denaturing temperature of 94°C for 1 min, junction temperature 59°C for 45 seconds, multiply temperature of 72°C for 1 min and final extension temperature was 72°C for 5 min. Polymerase chain reaction products on agarose gel (2% buffer TBE) electrophoresis and in markers size was confirmed accuracy of desired fragment. 462 bp desired fragment after the amplification were under restriction enzyme treatment (GCG C) HhaI. Then products obtained of electrophoresis were isolated on agarose gel (2% buffer TBE). After this time, the gel was taken to Gel documentation system and took it the photo. After completion of the laboratory works and genotyping of all samples, counting the genotypes and determine the allele frequency, check the Hardy - Weinberg equilibrium, observed heterozygote and homozygote has been examined, it was done Pop Gene32 software.

Table 1. Primer sequences used for calpastatin gene (Hanrahan et al., 2004).

GDF9 F (5´-GAAGACTGGTATGGGGAAATG-3´)
GDF9 R (5´-CCAATCTGCTCCTTACACACACT-3´)

Table 2. Concentrations of materials used in the PCR-RFLP* reaction.

Material	Concentration(µl)
Re-distilled water	17/3
Buffer PCR (10X)	2/5
Magnesium chloride (50mM)	0/6
Mixed nucleotide (10mM)	0/6
Forward primer (10Pmol/µl)	0/5
Reverse primer (10Pmol/µl)	0/5
DNA Template (20 ng)	2/5
Polymerase enzyme Taq (5u/µl)	0/5
Total	25

*Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

RESULTS

Extraction of DNA molecules from sheep blood tissue by salt extracting Miller et al (1988) DNA extraction was performed with a few changes. During this procedure, ammonium chloride plays a role cell lysis. Determination of concentration and quality of DNA was done in each sample compared with standard concentration of lambda phage DNA that cut with EcoRI and HindIII shear enzymes. Extracted DNA did not generate any abnormalities samples on the agarose gel that it indicates that DNA molecules are not broken. Also, in this experiment, the contamination effects were found by salt in the extracted solution that signifies on the purity of the extracted DNA.

Electrophoresis of amplified products of exon 1 position of GDF-9 gene confirmed 462 bp fragments from amplification. PCR products obtained by using agarose gel (2%) were electrophoresed that in Fig.1 given an example of the PCR products.

Amplified 462 bp fragment of GDF-9 gene was impressed restriction enzyme HhaI. Restriction enzyme will cut GCG"!C site. About this gene, wild-type allele has 2 cut locals that after digestion created 3 pieces 254, 156 and 52 bp. So, a homozygous individual in

this case is as G (+/+) and with 3 piece. While the mutant type allele has a cut local that after digestion created 2 fragments 410 and 52 bp. Therefore homozygous individual in this state as G (-/-) and has 2 pieces. In animals heterozygous G (-/+) a 4 bands or pieces is visible of 410, 254, 156 and 52 of the digest (Figure 3). After electrophoresis, digested products of all samples in studied Hisari sheep of two genotypes G (+/+) and G (-/+) shown in Fig. 2.
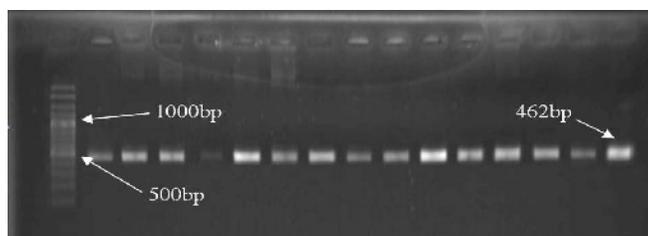
Fig. 1. An example of the PCR product of exon 1 of gene GDF9.
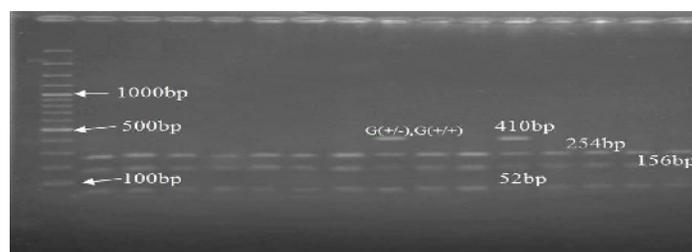
Fig. 2. Wild genotypes G (+/+) and heterozygous G (-/+) base on enzyme digestion pattern 462 bp fragment of exon 1 of GDF9 gene and enzyme HhaI.

In Table 3, the observed number, observed genotype frequencies, the expected frequency, expected number, Chi-square (X2) and observed allele frequencies, position in exon 1 of the GDF-9 gene in Hisari sheep race has shown. In this gene locus was determined two wild type allele G (+) and G (-). Genotype G (+/+) showed maximum frequency of 103 and 93.64 percent in herds. Heterozygous genotype G (-/+) with 7 observations and 6.36 percent showed lower frequency than the genotypes G (+/+). Genotype G (-/-) in testing

herd wasn't found so that this could be due to the low number of samples or low frequency alleles G (-). To evaluate the Hardy - Weinberg equilibrium was used of Chi-square test that in the Table 3 was shown. According to calculation of ^{2} gained from the Table (0.1014) and comparison with the Chi-square Table 3 suggesting that studied population for GDF-9 gene is in the Hardy - Weinberg equilibrium, although the due to the low samples and so on, genotype G (-/-) weren't identified.

Table 3: Observed number, observed genotype frequencies, expected frequency, expected number, Chi-square (^{2}) and observed allele frequencies, of exon 1 of GDF9 gene in Sheep Hisari – Tajikistan.

Genotype	Observed number	Observed genotype frequencies	Expected frequency	Expected number	Chi-square (^{2})	Observed allele frequencies	
						G (+)	G (-)
G (+/+)	103	93.64	94	103.0959	0.0001	0.97	0.03
G (+/-)	7	6.36	5.91	6.8082	0.0054		
G (-/-)	0	0	0.09	0.0959	0.0959		
	110	100	100	110	0.1014	1	

Observed and expected heterozygosity, Nei expected heterozygosity and the average heterozygosity of the GDF-9 gene in the studied herds are given in Table 4. Heterozygous level is considered one of indexes to introduce genetic variation in a population. Average heterozygosity, Nei heterozygous, observed heterozygosity is amount of 0.0616, 0.0616 and 0.0636,

respectively. As it is noticeable, the lower heterozygous level of GDF-9 gene in studied herd that it can be because of closing down of the herd. So that in the system studied herds breeding is used of wild males for mating system itself. Therefore, increasing the breeding and reduce the level of heterozygosity is observed.

Table 4: Observed and expected heterozygosity, Nei expected heterozygosity and the average heterozygosity of exon 1 of GDF9 gene in Sheep Hisari – Tajikistan.

Gene	Observed Heterozygosity	Expected Heterozygosity	Nei Expected Heterozygosity	Average Heterozygosity
Exon 1 region of GDF-9 gene	0.0636	0.0619	0.0616	0.0616

First time Hanrahan *et al.,* (2004), by the molecular analysis of GDF-9 gene in sheep of Cambridge and Belclare breeds were reported mutations in those genes that were associated with multiple birth of these two strains. They discovered new mutations in this gene and expressed these mutations in heterozygous state are associated with increased rates of egg laying and in the homozygous state show the sterility phenotype. The researchers showed that Cambridge and Belclare sheep carrying a mutation in the gene (FecGH) GDF-9 in exons 1 and 2, which increases ovulation rate in heterozygous sheep and the lack of fertility in homozygous sheep. They also stated that a copy of FecGH increase 1.4 ovulation rate in this sheep, that our results confirm this gene as heterozygous in Hisari sheep and according to having this aspect is one of important characteristics in sheep and its importance in meat races that their breeding is done in order to more meat production is most prominent.

GDF-9 gene is autosomal that in homozygous state causes sterility and in heterozygous state provides multiple births. Eight mutations in this gene have been identified that influence of each mutant allele from 0.3 to 1.2 lambs in per lambing has been reported (Naghizadeh *et al.,* 2010). Genetic variation in ovulation rate in sheep mainly has been studied in different races, and it is clear that an important part of the phenomenon of multi birth is by segregation of major genes associated with reproduction and ovulation that also included of the Hisari race. Therefore, it may be argued this point that the nature of Tajiki sheep act in favor of animal competence for survival and according to that Tajikistan in aspect of the geographical location is in mountainous terms. The pasture of this country is relatively poor and sheep rising in Tajikistan done traditionally, presence of such genes in Hisari sheep is a great success for corrective actions, and it should be make the utmost use. Evaluation of Hardy - Weinberg equilibrium using the X2 test showed that the studied population is not in desired locus of the equilibrium.

Statistical analysis of data of genotype effect on the number of lambs produced in any lambing is significant, so that the ewes with the heterozygous genotype compared to other genotypes showed the highest average number of lambs per birth. Because of low heritability, lambing rate in the sheep, the emergence of this trait only in females and also lack of the incidence it in early animal life, obtaining of genetic markers can be used as a convenient and efficient tool in the selection program. The comparison between the observed and expected distribution of genotypes showed that the population is not in position of the Hardy - Weinberg equilibrium study. This imbalance may be due to the chosen strategy which is applicable in this population. By survey recorded information about the type of ewes lambing over five generations, will determine which records all these ewes have lambing record (either single agents or twin-causing). The statistical comparison between genotypes obtained from the enzyme digestion of GDF-9 gene showed that the genotype has the average condition in offspring production. Existing of GDF-9 hormon in rat (Yan *et al.,* 2001)and also exist of hormone dose of the BMP-15 and GDF-9 in sheep (Juengel *et al.,* 2004) have been reported as important factors for the completion folicologensis, and in inactivation case of this factor, the maximum growth of follicular is to phase type 2 (Juengel *et al.,* 2004). Inactivation of a term of GDF-9 factors in the blood by immunization of Romney sheep breeds causes to increase rates of ovulation and multi-birth. Phenotype of sheep that were treated inactivation (Juengel *et al.,* 2004). This mechanism explains how the effect of inactivating mutations in sheep heterozygous for the GDF-9 genes. In the homozygous ewes for mutation in these genes, inactivation of these factors is complete done, and because of their necessary exist to complete folicologensis, oviduct follicles progress to Stage 2 (Juengel *et al.,* 2004). GDF-9 neutralizing was effective on activity of the corpus luteum and therefore in addition to impact on follicle growth, on the health of ovulation and pregnancy induction is also effective (Juengel *et al.,* 2004).

An asexual GDF-9 gene on chromosome 5 has a mutation that can increase the rate of ovulation in heterozygous ewes and sterility in homozygous ewes (Hanrahan and Owen, 1985), GDF-9 is similar to BMP-15 and is stimulants granulosa cell proliferation in rat. Some of its features in relation to its effect on granulosa cells compared with BMP-15 are different that probably due to different signaling pathways. First, it inhibits the expression kit lhgand (Otsuka *et al.,* 2000), secondly, inhibits the GDF-9, FSH-induced progesterone and estradiol, while the basal level of progesterone that is mediated by upregulation of genes StAR (astroidogenic regulatory protein), which in turn by prostaglandins E2 is controlled speeds (Otsuka and Shimasaki, 2002). Furthermore, GDF-9 extends synthesis of LH receptors and the cumulus cells. Even if these two biomolecular haven't similar messaging pathway, in this collaborative mode causes to granulosa cells multiply, increasing the production of inhibin and reduction of progesterone secretion (Stéphane *et al.,* 2006).

The researchers showed that this gene plays a key role in fertility and rising levels of ovulation and follicle maturation and our study also confirms the fact that the GDF-9 gene has effective role on fertility and twining of Tajikistan Hisari sheep. According to phenotypic studies conducted in the examined herd, and its comparison with the famous twin sheep, lambing rate of this sheep than to study twining sheep is low that probably due to unfavorable environmental conditions and raising Hisari sheep region. Because of environment acts against reproductive genes, therefore must provide a suitable environment for the improvement of reproduction and fertility status, because the Tajikistan sheep also have reproductive genes as polymorphic form. For considering mutation in the gene GDF-9, it is necessary to use other techniques such as SNP approach that can be used in the country breeding programs and improve of reproductive state and according to the obtained results, it can be said that this gene has a significant effect on the twining in investigated sheep. Finally it can be stated that in the Tajik ethnics this gene exist as polymorphisms that its different phenotypes cause to reproductive or non-reproductive. Regarding to the effect of increasing the number of born lambs or twining on the amount of meat produced per ewes per year, reducing the number of breeding ewes on pasture

and prevent of destruction of pastures, it seems that finding the genes with a major effect on twining in different races in Tajikistan country is required as GDF-9.

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