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POLYMORPHISM AND BIOINFORMATICS ANALYSIS OF GROWTH DIFFERENTIATION FACTOR *9* GENE IN LORI SHEEP

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Abstract

Growth differentiation factor *9* (*GDF9*) is a fecundity major gene affecting prolificacy in sheep. In the present study, genetic variation of a 380-bp fragment in *GDF9* gene exon 1 was investigated in 100 Lori ewes. Single-strand conformational polymorphism (SSCP) and DNA sequencing methods were used to detect single nucleotide polymorphism (SNP) of the studied fragment. A SNP (g.306G>A), known as G1 mutation, with two genotypes (GG and AG) was found in two different SSCP patterns of *GDF9* gene exon 1, deducing an amino acid (AA) exchange (p.Arg87His). Frequencies of the AG and GG genotypes were 37.65% and 62.35%, respectively. Also, the estimated allelic frequencies for the A and G alleles were 18.825% and 81.175%, respectively. The observed nucleotide sequences were subjected to alignment analysis and it was found that the studied fragment had more than 99.7% similarity with some sequences reported for other breeds of sheep. Two different secondary and 3D protein structures were predicted for A and G alleles. Moreover, the A and G alleles had different isoelectric pH values (8.7 and 9, respectively). The observed genotypes tended to have a significant association with litter size (P<0.10) where average litter size of GG ewes was slightly (20%) higher than for AG animals. With respect to the results of the present study, it seems that more studies are needed to evaluate the mutations in other fragments of this gene or other genes in Lori sheep.

Key words: *GDF9*, mutation, PCR-SSCP, bioinformatics analysis, sheep

The interest in evaluation and identification of major genes and their relationships with prolificacy in sheep has increased since 1980 (Davis, 2004). The sheep is considered as an ideal species for studying genes affecting reproduction and mechanisms controlling ovulation rate (Polley et al., 2010). Three fecundity genes have been identified in sheep. These three genes belong to a large family of TGFβ and are called *BMPR-1B* (bone morphogenetic protein receptor 1B), *BMP15* (bone morphogenetic protein 15) and *GDF9* (growth differentiation factor *9*) which are located

on chromosomes 6, X and 5 in sheep, respectively (Galloway et al., 2000; Souza et al., 2001; Hanrahan et al., 2004). The *GDF9* is a major gene affecting prolificacy and is essential for normal folliculogenesis in sheep (Hanrahan et al., 2004). A mutated allele of *GDF9* gene (*FecG*^{H}) is associated with increase of ovulation rate in heterozygous animals (Hanrahan et al., 2004). Bodensteiner et al. (1999) reported that the nucleotide sequence of ovine *GDF9* gene (GenBank accession number AF078545), mapped to chromosome 5, is similar to human and mouse genes and spans approximately 2.5 kb, containing 2 exons separated by a single 1126 bp intron. Eight different point mutations (G1–G8) have been identified in exons 1 and 2 of the *GDF9* gene, while only five mutations led to an exchange of deduced amino acid (AA) sequence (Polley et al., 2010). The first mutation (G1) with an AA exchange (p.Arg87His) was found in exon 1 of *GDF9* gene (Polley et al., 2010; Hanrahan et al., 2004). Moreover, a mutation in *GDF9* gene, called Thoka gene has been found in prolific Icelandic sheep (Nicol et al., 2009). Another mutation, called Embrapa mutation has also been found in the Brazilian Santa Inês sheep, which differs from previous mutations in the *GDF9* gene, because homozygous ewes are not sterile but show an increase in ovulation rate (Silva et al., 2011).

The aims of this study were to evaluate the presence of single nucleotide polymorphisms in *GDF9* gene exon 1 using PCR-SSCP and DNA sequencing methods and to use bioinformatics tools to study this fragment in Lori sheep.

Material and methods

Experimental animals and DNA isolation

Blood samples were taken from 100 Lori ewes belonging to three different flocks in Lorestan province in west Iran. A total of 85 ewes were selected randomly and 15 ewes were selected based on high litter size. Approximately 10-ml blood per sheep was collected from the jugular vein and the genomic DNA was extracted from whole blood using CinnaGen kit (CinnaGen Co, Iran) and stored at –20ºC.

PCR amplification

A pair of primers was designed to amplify a 380-bp fragment in exon 1 of the *GDF9* gene (GenBank No. AF078545). The primers were designed based on forward primer of Hanrahan et al. (2004) using Primer 3 software online (Rozen and Skaletsky, 1998). The primer sequences were as follows:

Right: 5′-GAAGACTGGTATGGGGAAATG-3′

Left: 5′-TGTAGAGGTGGCGTCTGTTG-3′

PCR was carried out in a 50 µL final volume, consisting of PCR buffer 10X [50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100], $MgCl_2$ (2.5 mM), 200 µM of each dNTPs, 10 pM of each primer, 2.5 units of Taq DNA polymerase (CinnaGen, Tehran, Iran), 50–100 ng of DNA template and distilled water. PCR reactions were run on a Mastercycler (ASTEC, Japan) using the following program: an initial denaturation at 94ºC for 5 min, followed by 32 cycles of denaturation at 94ºC for

30 s, annealing at 60ºC for 45 s and extension at 72ºC for 45 s, and finally extension at 72ºC for 5 min. The PCR products were separated by electrophoresis on 2% agarose gels to ensure that the reactions worked. The gels were visualized by ethidium bromide and photographed under UV light using a BTS-20.M gel documentation system (UVItec Ltd, UK).

SSCP and sequence analysis

For SSCP analysis, 4 μL of PCR sample was aliquoted into separate tubes. Then 7 μL of SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA) was added and mixed. After heat denaturation at 98ºC for 10 min, the samples were immediately chilled on ice to prevent heteroduplex formation and then run (22 h, 300 V, 5ºC) on 8% acrylamide:bis-acrylamide gel (29:1 acrylamide to bisacrylamide), in 1x TBE buffer on a 21×22 cm gel casting vertical electrophoresis (Payapajoohesh Pars, Iran). DNA visualization was done by silver staining (Sanguinetti et al., 1994). Two samples of each SSCP pattern were randomly selected for DNA sequencing. The primers used for sequencing were the same as those for the PCR reaction. The PCR products were sequenced by Bioneer Co., Korea.

Bioinformatics analysis

The observed sequences of the studied fragment of *GDF9* exon 1 were compared with a sequence reported for sheep (GenBank No. AF078545.2) and some other sequences reported for Belclare and Cambridge sheep (Hanrahan et al., 2004), Norwegian white sheep (GenBank No. HE866499.1) and *Capra hircus* (GenBank No. AY682555.1). The nucleotide sequences were translated to the codified AA sequences using ExPASy translate tools website (http://us.expasy.org/translatetool). Nucleotide and AA Sequence alignments were carried out using the MegAlign module of DNASTAR software (DNASTAR Inc., Madison,WI. USA) and compared by ClustalW method. The identified SNPs were compared with the sheep and goat NCBI dbSNP databases using BLAST (http://www.ncbi.nlm.nih.gov/SNP/index.html). The DNASTAR software (DNASTAR Inc., Madison, WI. USA) was used for calculation of percent similarities of nucleotide sequences of partial exon 1 of *GDF9* gene in the studied population of Lori ewes and some reported sequences in sheep and goat.

Secondary structures of proteins were predicted using Protean section of DNASTAR software (DNASTAR Inc., Madison, WI. USA). Moreover, 3D structures of proteins were predicted using PS2-V2 section of Protein Structure Prediction Server, available at Molecular Bioinformatics Center (MBC) website (http://ps2v2.life.nctu.edu.tw). Titration curves were drawn using Protean, titration curve section of the DNASTAR software.

Statistical analysis

The allelic and genotypic frequencies were calculated by direct counting in 85 randomly selected ewes. Association of different genotypes with litter size was investigated in all animals (100 ewes), using Kruskal-Wallis test. The NPAR1WAY procedure of SAS (2004) was employed for this test.

Results

SSCP and sequence analysis

The SSCP analysis of the studied fragment revealed two distinct patterns (GG and AG genotypes) on the polyacrylamide gel. The AG and GG genotypes exhibited different band patterns on SSCP gel (Figure 1). Two different allelic forms were confirmed by nucleotide sequencing. The sequencing results showed a transition of g.306G>A in the studied fragment (Figures 2 and 3). This mutation deduces an AA exchange, (p.Arg87His) in the codified AA chain (Figure 4).

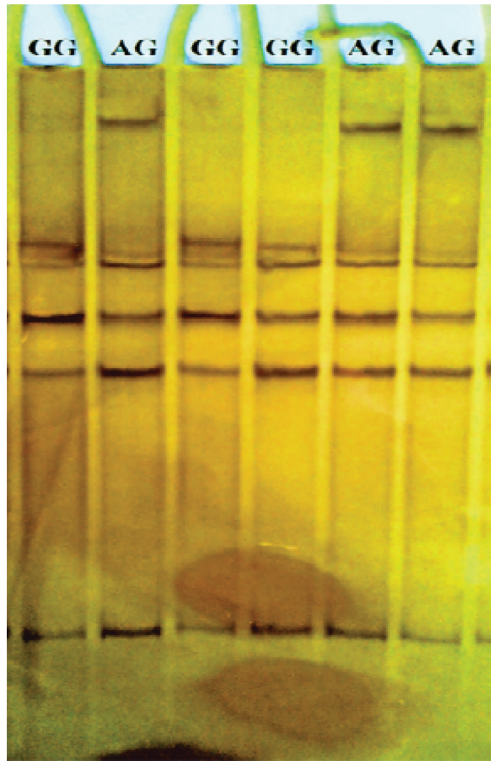

Figure 1. Different SSCP genotypes (GG and AG) of 380-bp fragment of *GDF9* gene exon 1

The overall frequencies of two genotypes of the studied fragment (AG and GG), in 85 randomly selected ewes, were 37.65% and 62.35%, respectively (Table 1). Also, the overall allelic frequencies of A and G alleles were 18.825% and 81.175%, respectively (Table 1). In 15 ewes, selected based on high litter size records, the frequencies of AG and GG genotypes were 20% and 80%, respectively. In other words, the frequency of GG genotype in high prolific ewes was slightly higher than in the randomly selected animals.

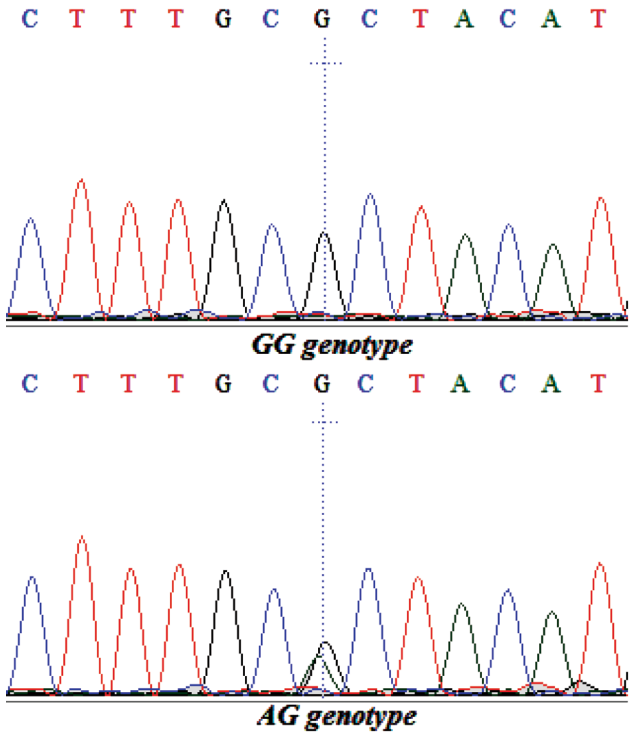

Figure 2. Sequencing results and two genotypes (GG and AG) of *GDF9* gene exon 1

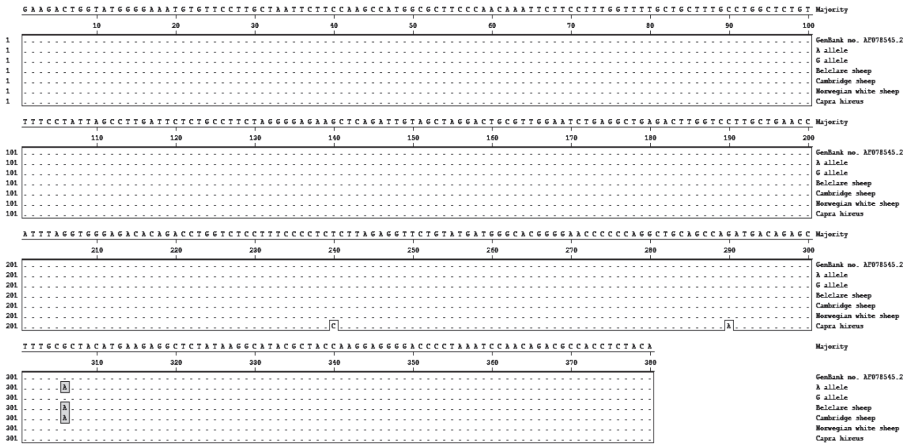

Figure 3. The comparison between sequences of G and A alleles of *GDF9* exon 1 and GenBank accession number AF078545.2, with other sequence in NCBI for Norwegian white sheep (GenBank No. HE866499.1), Belclare and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1)

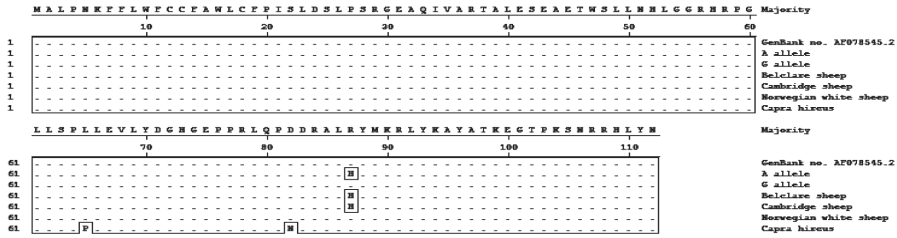

Figure 4. The codified protein sequence and deduced amino acid exchange of different alleles (G and A) of *GDF9* exon 1 in comparison to GenBank accession number AF078545.2 and other sequences in NCBI for Norwegian white sheep (GenBank No. HE866499.1), Belclare and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1)

Table 1. Frequencies of the observed variants in 85 randomly selected Lori ewes

Frequency	Genotype			Allele		
	AG	GG	total	A	G	total
N	32	53	85	32	138	170
%	37.65	62.35	100	18.82	81.18	100

Nucleotide sequence comparison

The nucleotide G, presented in position 306 of the designated allele G has been reported in other breeds of sheep (GenBank numbers AF078545.2 and HE866499.1). This position was replaced by nucleotide A in the allele A, which was previously reported in Cambridge and Belclare sheep (Hanrahan et al., 2004) (Figure 3). The results of alignment analysis as sequence similarity between the studied fragment and some reported sequences are presented in Table 2. A sequence similarity, higher than 99.2%, was found between the observed genotypes and other reported sequences, including GenBank No. AF078545.2, Belclare and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1). The designated allele A had a 100% homology with sequences of Belclare and Cambridge sheep (Hanrahan et al., 2004) and 99.2% homology with *Capra hircus* (GenBank No. AY682555.1). Similarity of the allele A with allele G and Norwegian white sheep (GenBank No. HE866499.1) was 99.7%. The allele G had a 100% homology with sequences of Norwegian white sheep (GenBank No. HE866499.1) and a high similarity of 99.7% with Belclare and Cambridge sheep (Hanrahan et al., 2004). The G allele had also a high similarity (99.5%) with *Capra hircus* (GenBank No. AY682555.1).

Table 2. Similarity and divergence percentages of the observed alleles (A and G) and other reported sequences for sheep and *Capra hircus*

Percent Identity

Divergence	1	2	3	4	5	6	7		
1		99.7	100.0	99.7	99.7	100.0	99.5	1	GenBank no. AF078545.2
2	0.3		99.7	100.0	100.0	99.7	99.2	2	A allele
3	0.0	0.3		99.7	99.7	100.0	99.5	3	G allele
4	0.3	0.0	0.3		100.0	99.7	99.2	4	Belclare sheep
5	0.3	0.0	0.3	0.0		99.7	99.2	5	Cambridge sheep
6	0.0	0.3	0.0	0.3	0.3		99.5	6	Norwegian white sheep
7	0.5	0.8	0.5	0.8	0.8	0.5		7	Capra hircus
	1	2	3	4	5	6	7		

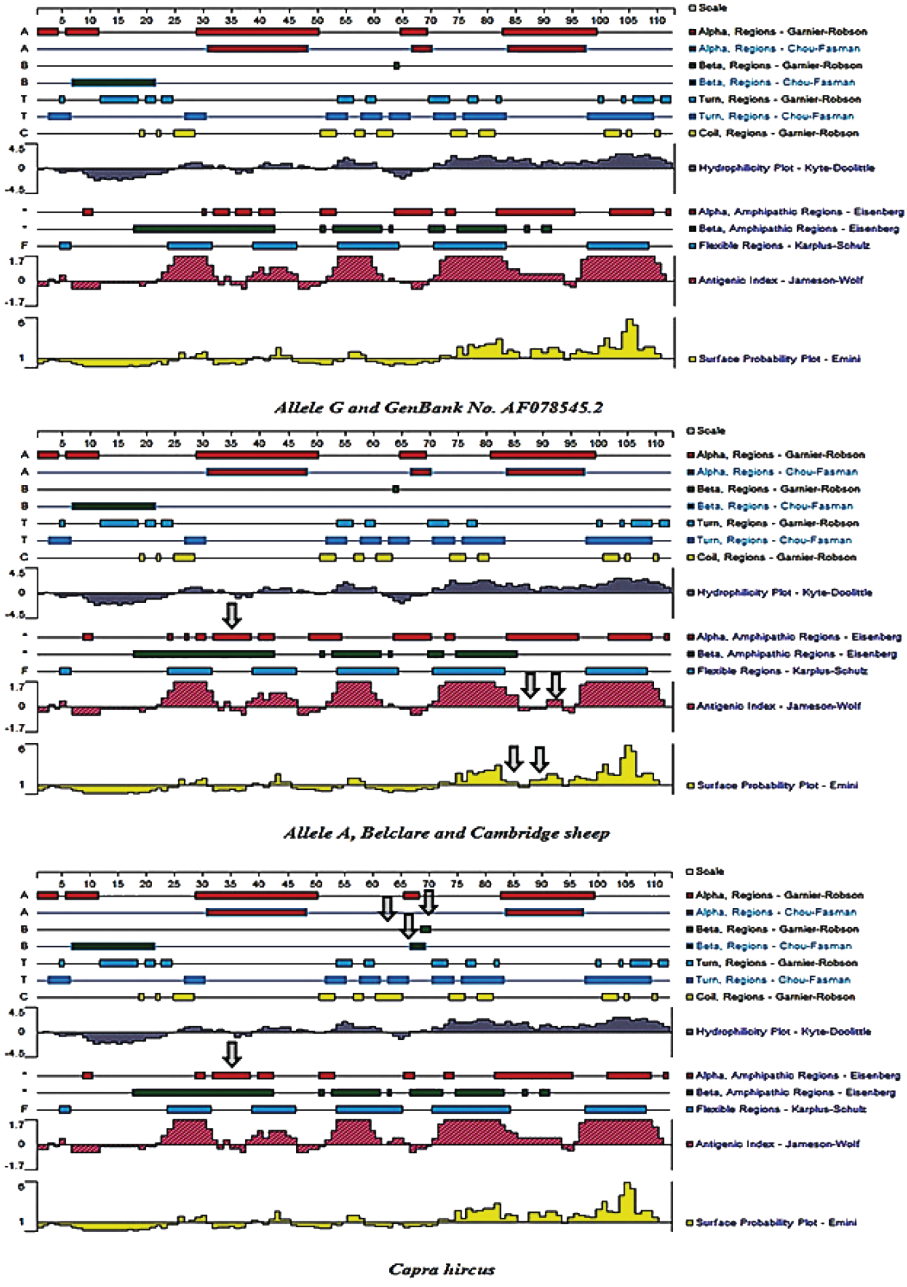

Figure 5. The changes in the secondary structure of protein and the comparison between the referring sequence (GenBank accession number AF078545.2), different alleles (G and A) of *GDF9* exon 1 and other sequences in NCBI for Belclare and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1). Differentiations are presented by ↓ symbol

Protein structure changes

The allele A showed a different predicted secondary protein structure in comparison to the allele G and the referred GenBank sequence (GenBank accession number AF078545.2). The most obvious changes in the allele A in comparison to the allele G and GenBank sequence were found in antigenic index and surface probability plot. While most of differences between the allele G and the *Capra hircus* sequence (GenBank No. AY682555.1) were found in alpha and beta regions (Figure 5).

Moreover, the results of the predicted 3D protein structures for the alleles A and G, a referring sequence for sheep (GenBank accession number AF078545.2), the sequence reported for Belclare and Cambridge sheep (Hanrahan et al., 2004) and a sequence reported for *Capra hircus* (GenBank No. AY682555.1) are illustrated in Figure 6. The alleles G and A had different predicted 3D protein structures. However, the 3D protein structures predicted for the alleles G and A were similar to the referring sequence of sheep (GenBank accession number AF078545.2) and Belclare and Cambridge sheep (Hanrahan et al., 2004), respectively. The observed alleles (G and A) and all referred sheep sequences had different 3D protein structures, in comparison to *Capra hircus* (Figure 6).

Figure 6. The changes of 3D structures of protein predicted for the alleles G and A, the referring sequences for sheep (GenBank No. AF078545.2) and Belclar and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1)

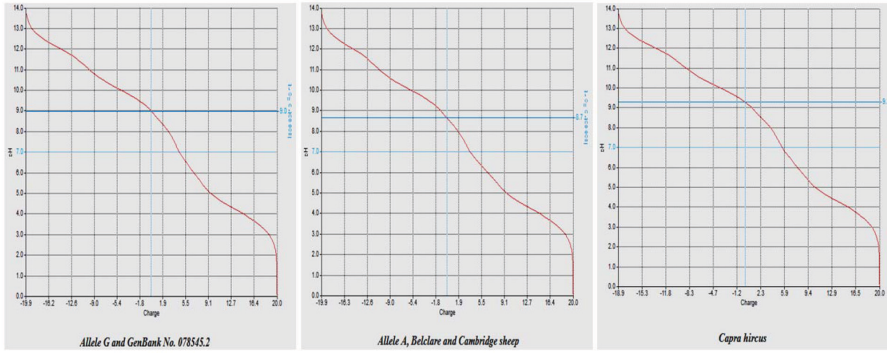

Figure 7. Titration curves with isoelectric pH values for the observed alleles (A and G), the referring sequence (GenBank No. AF078545.2) and other sequences in NCBI for Belclare and Cambridge sheep (Hanrahan et al., 2004) and *Capra hircus* (GenBank No. AY682555.1)

Table 3. Association of the observed genotypes with litter size

Genotype	Litter size
AG	1.310±0.52
GG	1.568±0.66
P-value*	0.0956

* Obtained by Kruskal-Wallis test.

The results of the drawn titration curves, detected the isoelectric pH values of 8.7, 9 and 9.3 for the allele A, allele G and *Capra hircus*, respectively (Figure 7).

Association of the observed genotypes with the studied traits

Evaluation of the associations between different genotypes and litter size did not show any significant association (Table 3). However, the association of the observed genotypes with litter size tended to be significant ($P<0.10$) where average litter size of GG ewes was slightly (20%) higher than in AG animals.

Discussion

A common assumption in animal breeding, called infinitesimal model assumes an infinite number of loci with an equal and small effect on a quantitative trait. However, the major gene model suggests that few genes may be responsible for a relatively high proportion of genetic variation. The genes involved in the biology of a trait of interest are candidates for association studies and can be considered as "candidate genes". The candidate gene approach is justified when the effects of the genes on the traits of interest have been previously identified in the species of interest or in other species (Yu et al., 1995).

The *GDF9* is produced as a precursor in the oocyte and encodes a prepropeptide of 453 AA residues. The active mature peptide is 135 AA long (Bodensteiner et al., 1999; Juengel et al., 2004). *GDF9* is essential for ovarian follicular development and normal ovulation in sheep (Knight et al., 2006). *GDF9* mRNA and protein are present in germ cells during follicular stage, in oocytes of primordial follicles and all subsequent stages of follicular growth (Juengel et al., 2004). Hanrahan et al. (2004) reported eight point mutations (G1, G2, G3, G4, G5, G6, G7 and G8) occurring in exons 1 and 2 of *GDF9* gene in Cambridge and Belclare sheep. The first mutation (G1) with one AA exchange of p.Arg87His was found in allele A of the *GDF9* gene exon 1 in the present study (Figure 4).

The mutation G→A (allele A), called G1 by Hanrahan et al. (2004) is also reported in other Iranian breeds such as Ghezel and Moghani (Barzegari et al., 2010), Zel (Farajzadeh et al., 2005), Baluchi (Moradband et al., 2011), Sangsari (Kasiriyan et al., 2010), Kordi and Arabi (Ghaderi et al., 2010), and Mehraban sheep (Abdoli et al., 2013). In *Capra hircus* (GenBank No. AY682555.1), the nucleotide G is presented in this position. However, the nucleotides at positions 240 and 290 in the studied fragment of *GDF9* gene in the present study and other mentioned reports for sheep

are different from those of *Capra hircus* (GenBank No. AY682555.1). In the present study and other reported sequences for sheep (GenBank No. AF078545.2), Belclare and Cambridge sheep (Hanrahan et al., 2004) and Norwegian white sheep (GenBank No. HE866499.1), in positions 240 and 290, the nucleotides T and G are replaced by C and A nucleotides, respectively in *Capra hircus* (GenBank No. AY682555.1) (Figure 3).

In the present study, no homozygous (AA) individual was observed. This is possibly due to the low frequency of the allele A (0.19) and consequently a low expected frequency (0.036) for its homozygous genotype (Table 1). The missing of the genotype AA may not be due to lethal or sterility effects of this genotype, because the genotype AA has been found in other breeds, including Mehraban (Abdoli et al., 2013) and Baluchi sheep (Moradband et al., 2011).

Protein secondary structure can be described by hydrogen-bonding pattern of the peptide backbone of the protein. Amino acids vary in their ability to form the various secondary structure elements. On the other hand, any change of secondary structure could change the 3D structure of protein. Thus, any amino acid change could change features of the protein structures and properties. The best modern methods of secondary structure prediction in proteins reach about 80% accuracy which allows the use of the predictions as feature improving fold recognition and protein structure prediction (Pirovano and Heringa, 2010). Therefore, the substitution may affect the activity of mature *GDF9* protein. Although both replacing amino acids (arginine and histidine) are basic polar, they exhibit different ionization rates in different physiological situations. Arginine has a pKa of 12.5 which essentially always has protonated side-chains at physiological pH but histidine has various values of pKa depending on the environment (Moradband et al., 2011). The isoelectic pH in the *Capra hircus* was the highest (9.3) and in allele A was the lowest (8.7). Moreover it was medium for allele G (9). This suggests that the G and A alleles sequences are acid and alkaline properties. Thus, different ionization rates can induce various protein structures and isoelectric pH in different physiological situations (Figure 5, 6 and 7).

The G1 mutation induces an arginine to histidine shift, having minimal or no effect on sheep fecundity (Hanrahan et al., 2004). On the other hand, the G8 mutation which caused an over dominance phenotype in Belclare and Cambridge breeds (Hanrahan et al., 2004) has not been found in Iranian breeds of sheep. For example, Eghbalsaied et al. (2012) detected three mutations (G2, G3 and G4) in Afshari sheep, an Iranian breed of sheep. In that study, G2 (C471T) and G3 (G477A) mutations caused no substitution in the translated amino acid chain. But the G4 mutation replaced glutamic acid with lysine at position 241 of amino acid residue of the unprocessed protein.

The association of the genotypes observed in the present study and some reproduction traits has been evaluated in previous studies. Hanrahan et al. (2004) found eight mutations in exons 1 and 2 of *GDF9* in Cambridge and Belclare sheep and only G8 mutation in exon 2, with deduced S395F AA exchange had a significant effect on litter size and ovulation rate. Non significant effect of the allele A on litter size was also reported by Barzegari et al. (2010) and Abdoli et al. (2013). However, in another study, Moradband et al. (2011) showed that the G1 mutation in exon 1 of

GDF9 gene (allele A in the present study) has a major effect on litter size in Iranian Baluchi sheep.

Non significant associations of the observed genotypes (AG and GG) with the studied reproductive traits in the present study and other studies could be attributed to similar secondary and 3D structures of the codified proteins for the alleles A and G (Figures 5 and 6). Therefore, it could be expected that the codified proteins might have similar structural properties. However, more studies are still needed in the future.

Reproductive ability has an important role in profitability of sheep production. Therefore, it is necessary to look for new mutations with positive effects on prolificacy in different breeds and populations. A mutation with a significant positive effect on reproductive performance could enhance the efficiency of animal breeding to improve the profitability of animal production enterprise. Incorporation of a major gene for prolificacy into a flock can be achieved using marker assisted selection (MAS), artificial insemination and embryo transfer programs (Davis, 2004). Regarding the results obtained in the present study and previous reports, it seems that more studies are needed to evaluate the mutations in other fragments of this gene or other genes such as *BMP15*, and *ESRα* in other breeds and populations. Combined effects of different genes on fertility could also be considered as an interesting subject for similar studies in the future.

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