Polish Journal of Veterinary Sciences Vol. 19, No. 2 (2016), 281–289

DOI 10.1515/pjvs-2016-0035

Original article

Identification of point mutations in exon 2 of *GDF9* gene in Kermani sheep

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Abstract

Screening the fertile ewes from national herds to detect the major genes for prolificacy is an effective way to create the fertile flocks. Growth differentiation factor (*GDF*) 9 is a member of the transforming growth factor β superfamily that is essential for folliculogenesis and female fertility. The aim of this study was to detect single nucleotide polymorphisms (SNPs) in exon 2 of *GDF*9 gene in Kermani sheep breed using PCR-SSCP. Genomic DNA was extracted from whole blood of collected samples using salting-out method. Whole exon 2 of *GDF*9 gene was amplified (634 bp and 647 bp fragments) using designed specific primers. The single stranded conformation polymorphism (SSCP) patterns of PCR products were studied using electrophoresis on acrylamide gel and silver-nitrate staining method. Finally, 4 banding patterns for the first primer pair and 4 banding patterns for the second primer pair were obtained. Also, indices of population genetic per SNP were calculated using Gen Alex 6.41 software. The sequencing results showed the presence of 3 mutations (SNP) (443, 477 and 721 positions) in the studied population.

Key words: fertility, *GDF9* gene, Kermani sheep, PCR-SSCP, SNP.

Introduction

For decreasing the number of low-efficient ewes on pastures and preventing pastures demolition, animal breeding programs are necessary to identify effective candidate genes for the economical traits, especially genes with major effects affecting litter size in Iranian sheep breeds. Genetic variation in ovulation rate in sheep has been widely documented and the evidences show substantial differences among breeds and in a number of cases exceptional variations within breeds/strains (Bindon et al. 1996).

Growth differentiation factor (GDF) 9 is a member of the transforming growth factor β superfamily that is secreted from oocytes during folliculogenesis (Aaltonen et al. 1999) and is essential for folliculogenesis and female fertility (Juengel et al. 2004). GDF9 deletion results in decreased granulosa cell proliferation, abnormal oocyte growth and failure of the follicles to develop past the primary stage (Dong et al. 1996). GDF9 also inhibits granulosa cell apoptosis and follicular atresia (Orisaka et al. 2006). Homozygous mutations of *GDF9* detected in infertile sheep emphasize their essential role in this species (Aaltonen et al.

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Table 1. Polymorphic sequence variations reported by Hanrahan et al. (2004) in *GDF9* (growth differentiation factor 9 gene) within the Cambridge and F700-Belclare flocks.

Base change	Variant	Coding base (bp)	Coding residue (aa)	Mature peptide residue (aa)	Amino acid change
G – A	G1	260	87		Arg (R)-His (H)
C – T	G2	471	157		Unchanged Val (V)
G – A	G3	477	159		Unchanged Leu (L)
G – A	G4	721	241		Glu (E)-Lys (K)
A – G	G5	978	326	8	Unchanged Glu (E)
G – A	G6	994	332	14	Val (V)-Ile (I)
G – A	G7	1111	371	53	Val (V)-Met (M)
C – T	G8	1184	395	77	Ser (S)-Phe (F)

1999). The gene spans about 2.5 kilo bases (kb) and contains 2 exons separated by a single 1126-base pair (bp) 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 (Bodensteiner 1999). Bodensteiner et al. (2000) for the first time reported that the *GDF9* mRNA expression was localized exclusively to oocytes of fetal sheep at day 135 of gestation. Sadighi et al. (1998; 2002) mapped the *GDF9* gene to ovine chromosome 5.

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 (Vittet al. 2000), sheep (Bodensteiner et al. 1999, Juengel et al. 2002), cattle (Bodensteiner et al. 1999) and the human (Vitt et al. 2000). The combined results from sequence data and SSCP analysis of *GDF*9 by Hanrahan et al. (2004) revealed eight single nucleotide polymorphisms across the entire coding region (G1-G8; Table 1) and these differences correspond to one SNP in exon 1, one SNP in the intron, and five SNPs in exon 2. It is proven that exon 2 is more important than exon 1 and intron. Hence, we decided to study exon 2 in Kermani sheep.

Furthermore, a species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. In addition, the ability of a population to respond adaptively to environmental changes depends on its level of genetic variability or diversity (Askari et al. 2011).

Thus, genetic diversity in indigenous breeds is a major concern considering the necessity of preserving what may be a precious and irreplaceable richness, regarding new productive demands. Conservation should be based on a deep knowledge of the genetic resources of the specific breed. Therefore, it is important to try to genetically characterize indigenous breeds (Shojaei et al. 2010) and the applications of molecular genetics have many important advantages (Mousavizadeh et al. 2009). There are more than 50 million heads of sheep in Iran, of 27 breeds and ecotypes (Zamani et al. 2011). One of the most important breeds of Iranian sheep is Kermani sheep. This local breed lives in the south-eastern of Iran and is a fat-tail breed and well adapted to a wide range of harsh environmental conditions in Kerman province. The ability to adapt to different environmental circumstances is a desirable characteristic of this breed. This breed is one of the best Iranian wool sheep breeds which fleece is white and the wool is coarse and curly. The average weight for the rams is 48-50 kg and for the ewes, 45-48 kg. Using molecular genetics methods similar to DNA markers is one of the best choices for faster and better accomplishment of animal breeding programs. Therefore, the aim of this study was to investigate the presence of polymorphism in *GDF9* gene in the Kermani sheep breed.

Materials and Methods

Experimental animals and DNA extraction

The blood samples were randomly collected from Kermani sheep (102 animals) from both sexes and with different ages (Kerman, Iran), using vacuum tubes with 0.25% ethylene diamine tetra acetic acid (EDTA). The blood samples were transferred in dry ice to the laboratory and stored at -20°C pending assays.

DNA isolation

Blood samples of the animals were used to extract genomic DNA using the salting out procedure described by Mohammad Abadi et al. (2009). The quality of DNA was checked by spectrophotometry taking ratio of optical density (OD) value at 260 and 280 nm. Good quality DNA having OD ratio between 1.8 and 2 was used for further work. The poor quality DNA

was re-extracted with the aforementioned method. Also, the quantity of extracted DNA was measured on 0.8% agarose gel.

PCR primers and amplification

The sheep *GDF9* gene was amplified using the polymerase chain reaction (PCR) with designed specific primers (nucleotide sequence GenBank with accession number of AF078545).

The used PCR primers were as follows:

The first primer pair (Product length = 634 bp):

F:5'-GATTCCTTGATTTGACTTCCTGTT-3'; R: 5'-TGGCACTCTCCTGGTCTCTG-3'

The second primer pair (Product length = 647 bp):

F: 5'-TCACTGCTTTTGTATCTGAACGA-3'; R: 5'-CCAAAGGCATAGACAGGGGC-3'

These primers were used to amplify two fragments 634 bp and 647 bp of the first and second halves of the exon 2 for the sheep *GDF9* gene, respectively.

The PCR reaction was performed in a 25 µL reaction volume containing 2 µL of genomic DNA (50 ng/µL), 1 µL of MgCl2 (3 mM), 1 µL of each forward and reverse primers (10 pmol each), 0.5 µL of dNTPs (500 µM each), 0.3 unit of Taq DNA polymerase (Cinna Gene, Iran) and 10X PCR buffer. DNA amplifications were performed using thermo cycler (CLEMENS, Germany) programmed for a preliminary step of 5 min at 94°C, followed by 33 cycles of 30 s at 94°C, 50 s at 62.5°C for the first primer pair and 63.6°C for the second primer pair and 50 s at 72°C, with a final extension of 8 min at 72°C. Amplification was verified by electrophoresis on 1% (w/v) agarose gel in 1 x TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 100bp ladder as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 µg/mL).

Single stranded conformation polymorphism analysis

The SSCP technique was used to allow the sequence variants to be detected from the migration shift in PCR amplified fragments of the gene (Orita et al. 1989a,b). For SSCP analysis, 6 µL of each PCR product was mixed with 12 µL of denaturing loading buffer (19 mL formamide, 0.98 gr NaOH (3% NaOH solution), 0.01 gr xylene cyanol and 0.01gr bromophenol blue). The samples were denatured by heating at 95°C for 10 min, then immediately chilled on ice and loaded onto 8% polyacrylamide gel (37.5:1). Gels were run at 170-180 V for 7-8 hours at 4°C. The electrophoresis was carried out in a vertical unit in 1x TBE buffer (Tris 100 mM, boric acid 9 mM, EDTA 1mM). The gels were stained with silver nitrate to observe the conformational patterns according to the method of Bassam et al (1991).

Sequencing PCR products

After revealing the single stranded conformation polymorphism (SSCP) patterns for this locus, from each of the ovine *GDF9* variants identified by PCR-SSCP, one sample was sequenced (Mahan Gene, Iran).

Statistical analysis

The raw sequence data were edited using Bioedit 7.0 software. Multiple sequence alignments were performed with Bioedit 7.0 and DNAMAN software to identify single nucleotide polymorphisms (SNPs) in the exon 2 of the *GDF9* gene in Kermani sheep. The nucleotide sequence of exon 2 was translated to amino acid sequence for each particular allelic variant. The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) databases for comparison of the ovine *GDF9* sequences with homologous sequences of other animals to determine similarity percentage and detect the novel SNPs in the studied locus.

Population genetic parameters

Population genetic parameters including the allelic and genotypic frequencies, Hardy-Weinberg equilibrium, Na (number of different alleles), Ne (number of effective alleles = 1 / (Sum pi^2)), I (Shannon's information index = -1* Sum (pi * Ln (pi))), Ho (observed heterozygosity = number of Hets / N), He (expected heterozygosity = 1 – Sum pi^2) and UHe (unbiased expected heterozygosity = (2N / (2N-1)) * He) in the ovine *GDF9* locus in the studied population were obtained using GenAlex6.41 software. We reported allelic frequency of the ovine *GDF9* locus for two alleles of each single nucleotide polymorphism (SNP) and allelic sequences as well.

Also, haplotypes sequences forming each genotype for two loci were predicted using the R software.

The study protocol was approved by the Iranian ethics commission and analgesic and anesthetic procedures were not used.

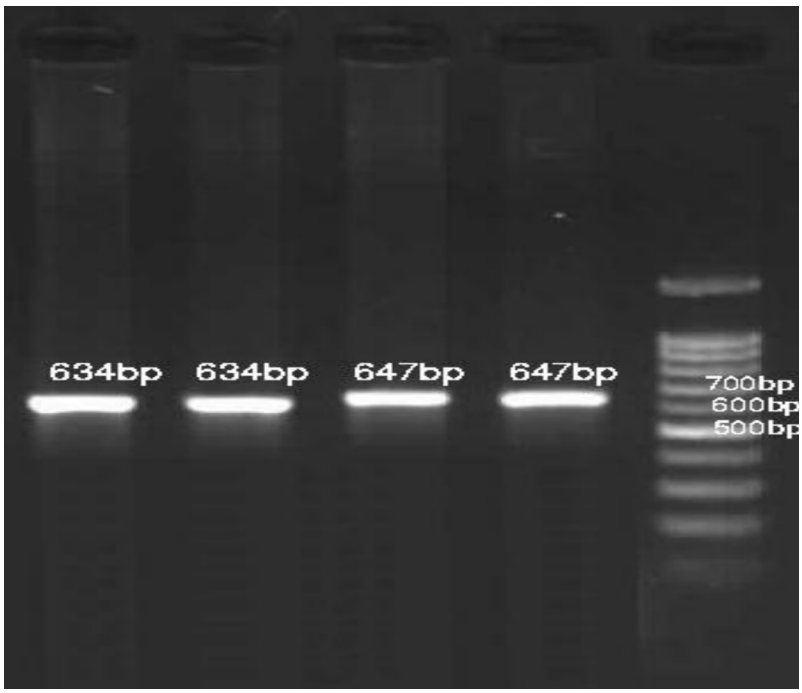

Fig. 1. Electrophoresis of PCR products on 1% agarose gel for exon 2 of *GDF9* gene in Kermani sheep.

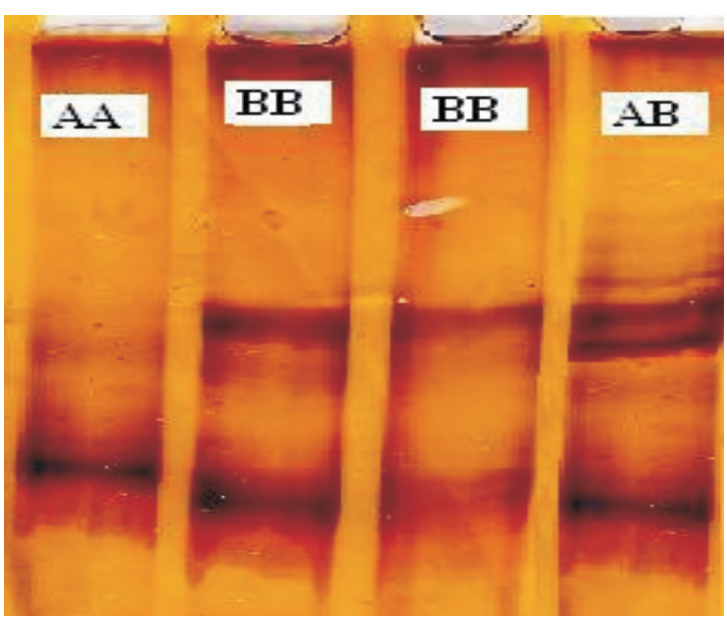

Fig. 2. PCR-SSCP patterns of 634 bp fragment of the first half of the exon 2 in the Kermani sheep *GDF9* gene.

Results

As expected, PCR amplification of the ovine *GDF9* gene for Kermani sheep gave uniform fragments of 634 bp and 647 bp by running on 1% agarose gel (Fig. 1) and the amplified fragments sizes were consistent with the expected size and subsequently sequencing of the ovine *GDF9* amplicons confirmed them to be either 634 bp or 647 bp in size. PCR products were also assessed with M100 size marker (Cinna Gene, Iran).

The SSCP analysis revealed three unique banding patterns for the first half of the exon and four unique banding patterns for the second half of the exon representing different allelic variants (Fig. 2, 3). These seven banding patterns constituted different genotypes in the Kermani sheep breed (Fig. 2, 3). The individual animals with one or two different SSCP

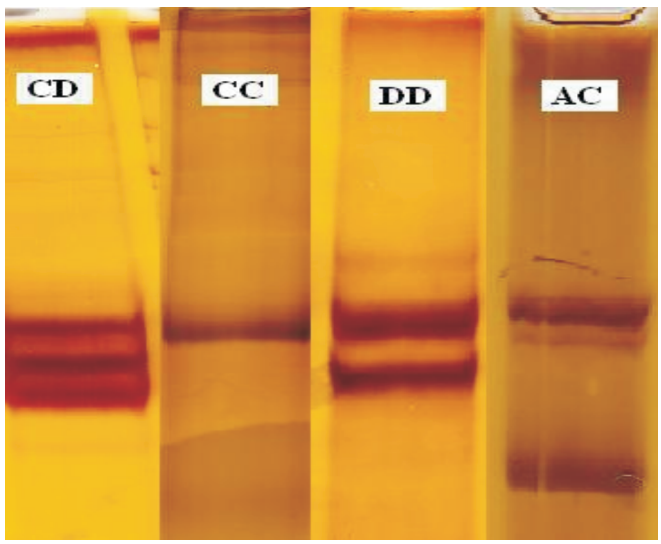

Fig. 3. PCR-SSCP patterns of 647 bp fragment of the second half of the exon 2 in the Kermani sheep *GDF9* gene.

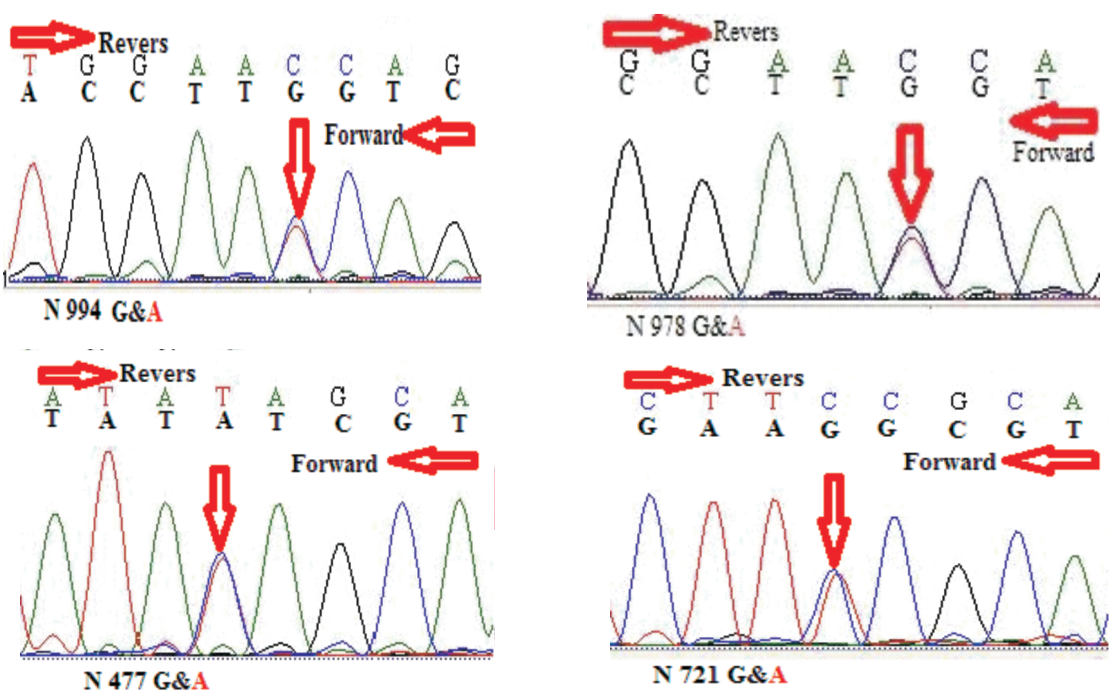

Fig. 4. Partial sequence comparison of the altered bases of *GDF9* in Kermani sheep.

patterns were observed, which are the representative of the homozygous and heterozygous genotypes at the *GDF9* locus, respectively.

Based on our sequence analysis, the SNPs identified for the first and second halves of the exon 2 constituted three and four different sequences, respectively, which confirmed the results of the SSCP analysis. This insured that these sequences represented genuine ovine sequences rather than being the result of PCR and/or sequencing errors. All consensus sequences showed 98-99%, 94-98%, 92-93% and 82-83% similarity to the published ovine, caprine, bovine and porcine *GDF9* locus sequences, respectively. Two SNPs detected in exon 2 were non-synonymous substitutions which would result in 721Gln/Lys and 994 Val/Ilu. Also, two SNPs detected were synonymous substitutions which would result in 477 for Leu and 978 for Glu substitutions in domain L of the mature GDF9 protein.

In the studied population, three different geno-

Table 2. Detected genotypes in the exon 2 of *GDF9* gene in Kermani sheep population.

SNP Position in sequence of *GDF9* gene			
frequency	721	477	number of genotypes for the first half of the exon 2
0.187	GG	GG	1
0.333	GG	AA	2
0.480	GA	GA	3
frequency	994	978	number of genotypes for the second half of the exon 2
0.137	GG	AA	1
0.411	AA	GG	2
0.265	GA	GA	3
0.187	GA	AA	4

Table 3. Detected haplotypes in the exon 2 of *GDF9* gene in Kermani sheep population.

SNP Position in sequence *GDF*9 gene			
frequency	721	477	number of haplotypes for the first half of the exon 2
0.186	G	G	1
0.574	G	A	2
0.240	A	G	3
frequency	994	978	number of haplotypes for the second half of the exon 2
0.362	G	A	1
0.338	A	G	2
0.300	A	A	3

Table 4. SNPs detected in the ovine *GDF9* gene, and their allelic and genotypic frequencies for the studied population.

P-value	genotypic frequency			allelic Frequency		SNP position
0.856	**AA**	GA	GG	A	G	477^{ns}
	0.332	0.482	0.186	0.574	0.436	
0.001	AA	GA	GG	A	G	721^{**}
	0.000	0.481	0.519	0.24	0.76	
0.000	GA	AG	AA	G	A	978^{***}
	0.412	0.265	0.323	0.544	0.456	
0.804	**A**A	GA	GG	A	G	994^{ns}
	0.411	0.450	0.137	0.637	0.363	

ns: not significant (population is in Hardy-Weinberg Equilibrium); ** and *** significant in 0.001 and 0.0001 respectively (population is not in Hardy-Weinberg equilibrium); wild allele (correspond to haplotype 1) is underlined and bolded.

Table 5. Population genetic parameters in each of the four SNPs identified in the ovine *GDF9* locus in the Kermani sheep population.

UHe	He	Ho	I	Ne	Na	SNP Position
0.492	0.489	0.480	0.682	1.958	2.000	477
0.367	0.365	0.480	0.551	1.575	2.000	721
0.499	0.496	0.265	0.689	1.985	2.000	978
0.465	0.462	0.451	0.655	1.860	2.000	994

types and three haplotypes were observed for the first half of the exon 2 and four different genotypes and three haplotypes were observed for the second half of the exon 2. Frequencies of the detected genotypes and haplotypes in the studied population are provided in Table 2 and 3. In total, in this population, genotype 3 in the first half of the exon 2 and genotype 2 in the second half of the exon 2 were most common with a frequency of 0.480 and 0.411, respectively.

For the four identified SNPs, the number of genotyped individuals and the allele frequencies in the studied sheep breed are presented in Table 4. The

SNP g.721G>A located in the first half of the exon 2 displayed the least variability, with a value of the least frequent allele below 0.24 in studied population. Also, homozygosity of mutant allele for this SNP was not detected in this population.

In total, frequency of the least frequent alleles in the first and second halves of the exon 2 ranged from 0.24 (SNP g.721G>A) to 0.36 (SNP g.994 G>A) in this population.

Values for the observed number of alleles (Na), the effective number of alleles (Ne), the Shannon's index (I), observed heterozygosity (Ho), expected heterozygosity (He) and unbiased expected heterozygosity (UHe) of each of the four SNPs identified in the ovine *GDF*9 locus for the studied sheep population are provided in Table 5.

Discussion

In this research, we analyzed the polymorphic variations of the gene coding for the growth differentiation factor (GDF) 9, a member of the transforming growth factor β superfamily that is essential for folliculogenesis and female fertility, in Kermani sheep breed. Thus, the complete exon 2 of this gene was analyzed for 102 animals using designed specific primers, PCR-SSCP method and DNA sequencing.

It should be noted that Hanrahan et al. (2004) studied some parts of exon 2 of *GDF*9 gene using four specific primers pairs while we amplified total exon 2 of *GDF9* gene using two pairs of designed specific primers covering total exon 2 of *GDF9* gene leading to decreased expenses and experiment time. Observation of only one band on agarose gel indicated that the designed primers were specific and there was not DNA sequence similarity at other situations, which could be directly analyzed by SSCP.ą

Using the SSCP technique and sequence analysis of the exon 2 together four SNPs were observed. Two SNPs were located in the first half of the exon 2 (A477→G and A721→G) and two SNPs were located in the second half of the exon 2 (G978→A and A994→G) for the *GDF*9 gene. After the BLAST search of amplified sequences of exon 2 of *GDF*9 gene in NCBI to get open reading frame for start properly translation to protein sequence, we got from +1 (+1 means that translation to protein sequence started from the first nucleotide of the first codon, +2 means that translation to protein sequence started from the second nucleotide of the first codon and +3 means that translation to protein sequence started from the third nucleotide of the first codon) for both amplified sequences. Two of the four polymorphisms were nucleotide changes that do not result in an altered amino acid (G3 at nucleotide 477, and G5 at nucleotide position 978). The two remaining nucleotide changes, i.e. G4 and G6, gave rise to amino acid changes (conservative changes) (Table 1). Both G6, valine to isoleucine change at amino acid residue 332 of the unprocessed protein (residue 14 of the mature coding region), and G7, valine to methionine at residue 371 of the unprocessed protein (residue 53 of the mature coding region), substitute nonpolar groups with nonpolar groups. The remaining two changes result in non-conservative substitutions. G4, glutamic acid to lysine change at amino acid residue 241 of the unprocessed protein, replaces an acidic group with a basic group, but this occurs at a position before the furin processing site and it is unlikely to affect the mature active coding region.

Hanrahan et al. (2004) discovered eight variants (G1 to G8) of *GDF9* gene in Cambridge and Belclare sheep breeds using PCR-SSCP and sequencing. However, G8 variant caused serine to phenylalanine substitution at residue 395 which replaced an uncharged polar amino acid with a nonpolar one at residue 77 of the mature coding region and may change the function of *GDF9* in sheep (Hanrahan et al. 2004). Nikol et al. (2009) discovered 4 variants (G3, G4, G5 and G6) of *GDF9* gene in Icelandic Thoka sheep that is in agreement with the result of the present study. Guan et al. (2005) detected the G8 mutation of *GDF9* gene in Hu sheep using PCR-RFLP and the mutation rate was rare (0.645%). They found two SNPs (T558C and T692C (Leu231Thr)) in the exon 2 of *GDF*9 in small tail Han, Tong, Tan and Oula sheep using PCR-SSCP (Guan et al. 2005). Recently, two variants of sheep *GDF*9 have been reported, FecGSI (T1034G mutation of CDS region resulting in Phe27Cys change of mature protein) in Brazilian Santa Ines sheep (Melo et al. 2008) and FecTT (A1279C mutation of CDS region resulting in Ser109Arg of mature protein) in Icelandic Thoka sheep (Nikol et al. 2009). Chang et al. (2009) identified G2 mutation of *GDF9* gene in small tail Han, white Suffolk, Texel and Tibetan sheep using PCR-SSCP. They detected G4 mutation of *GDF*9 in small tail Han, Poll Dorset, Suffolk, German Mutton Merino and Chinese Merino (Xinjiang type) (including prolific strain, meat strain and large frame strain) by PCR-SSCP. In the present study, we did not find any genetic variations for G2 (G471→C), G7 (A1111→G) and G8 (T1184→C) within the *GDF9* gene using PCR-SSCP among the studied animals of the Kermani breed. Until now among Iranian sheep breeds, G8 mutation of *GDF9* was not detected. Our samples were randomly taken from this flock and one would expect to find all possible genotypes of *GDF9* if it really segregates in this breed. Moreover, G8 mutation of *GDF9* was not detected in small tail Han, Hu,

Dorset, Texel and German Mutton Merino sheep (Chu et al. 2005), Suffolk, Dorset, Charollais, Romney Hills and Chinese Merino sheep (Guan et al. 2007), Cele and Duolang sheep in China (Bai et al. 2005), Shal sheep in Iran (Ghaffari et al. 2009), Tan sheep in China (Sun et al. 2009), Barbarine, Queue Fine de L'Ouest, Noire dehibar, Sicilo-Sarde, D'man sheep in the North Africa (Vacca et al. 2010) that is in agreement with the present study. Eghbal Saied et al. (2012) discovered that the arisen G4 mutation in Iranian Afshari sheep has led to the maximum ovulation rate compared with other breeds that they studied. Therefore, this mutation may be responsible for the increased ovulation rate in some sheep breeds.

Observed heterozygosity (H_O) in each of the three SNPs 477, 721 and 994 was 0.480, 0.480 and 0.451, respectively. SNP G978→A displayed the lowest average heterozygosity across the studied sheep breed (0.265). These results showed that genetic variation for *GDF9* gene in the studied sheep breed is moderate to high.

Findings demonstrated that the G8 mutation changes an uncharged polar serine residue (residue 77 of mature GDF9) to a nonpolar phenylalanine in a region of the molecule that is likely to be involved in binding to the type I receptor. Therefore, this mutation could affect the ability of the ligand to bind to a receptor. However, this change also occurs only three residues away from a conserved histidine of the mature GDF9 peptide. GDF9 lacks the interchain disulphide bond that forms a covalent link between monomers of the biologically active dimer in most other members of the TGFb superfamily. Thus, it is possible that in GDF9, the hydrogen bonds between monomers are even more critical for maintaining dimer stability and the G8 mutation could affect biological activity by disrupting dimerization.

The high level of genetic variability observed in the coding region of the ovine *GDF*9 gene in this study suggests that this region of the *GDF9* gene probably affects folliculogenesis and female fertility in sheep; hence further association studies using appropriate populations are needed to identify genetic variants that can be used as markers related to fertility.

Conclusion

The results of our study emphasized that in this selected population of Kermani sheep, there are G3 (A477→G), G4 (A721→G), G5 (G978→A) and G6 (A994→G) mutations in the ovine *GDF9* gene. G2 (G471→C), G7 (A1111→G) and G8 (T1184→C) mutations are completely absent and they are not associated with litter size in Kermani sheep. Reproductive activity is a multifunctional process and numerous genes, proteins, growth factors and hormones are involved in this activity. The SNPs discovered in this study can be used for the ovine SNP chip design project in future. The discovered alleles and genotypes can also be used as markers in marker-assisted selection of sheep for economic traits in future.

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