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Simultaneous Presence of G1 and G4 Mutations in Growth Differentiation Factor 9 Gene of Iranian Sheep

Research Article

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ABSTRACT

The fecundity in sheep has been much interested for producers due to its economical importance. Growth differentiation factor 9 (GDF9) is an autosomal gene with over dominance effect for two single nucleotide polymorphisms (SNPs), which resulted in sterility in homozygote mutant ewes and additive effect for three SNPs, without showing sterility event. In the present study, DNA was extracted from whole blood of 19 Iranian Afshari and 8 Shal sheep breeds. The extracted DNA was multiplied several folds for G1, G4 and G8 sites in GDF9 gene through polymerase chain reaction (PCR) technique and was sent for sequencing. Results showed that G1-GDF9 mutation (arginine to histidine shift) was detected in Afshari breed, while G4-GDF9 mutation (glutamic acid to lysine shift) was detected in both Afshari and Shal breeds. The observed SNPs were recorded with very low frequency (<8%) in fertile ewes with twining birth, while infertile ewe did not carry the mutant allele. This might indicate an association between these mutations and twin-births in Iranian breeds. Furthermore, these mutations could not be the reason for ewe infertility. In consistent with the rare frequency of infertility in Iranian sheep, the investigated population lacked the G8 or Thoka mutations, which are the most effective mutations in GDF9 gene. This may be due to lack or rare frequency of this SNP in Iranian sheep flocks. In conclusion, G1- and G4-GDF9 SNPs are segregating with very low frequency in Iranian sheep flocks, so that no homozygous ewe for these SNPs was detected in the current study. The same phenomenon could be the case for other important mutations and the sample size should be very large to rule out presence of such mutations in Iranian sheep breeds.

KEY WORDS Afshari, GDF9, G1, G4, Iran, Shal, twin birth.

INTRODUCTION

There are three genes that play key roles in the follicular growth and ovulation rate of sheep (Fabre *et al.* 2006); these include ALK6 (activin receptor-like kinase 6) or BMPR-1B (bone morphogenetic protein receptor 1-B), GDF9 (growth and differentiation factor-9) and the BMP15 (Bone morphogenetic proteins). In sheep, GDF9 gene is located on chromosome 5 (Sadighi *et al.* 2002). This gene (GDF9) is 5.2 kb in length, contains two exons and one intron, 1126 bp ORF encoding an immature polypeptide with 453 amino acids and active polypeptide of 135 amino acids (Sadighi *et al.* 2002). So far, ten mutations in this gene has been observed in Belclair and Cambridge breed of sheep (Hanrahan *et al.* 2004), one mutation has been observed in the Thoka breed (Nicol *et al.* 2009), and one mutation in Norwegian White sheep (Vage *et al.* 2013). Among these, five mutations lead to amino acid changes in the mature polypeptide pattern in which three of these mutations (G7, G8 and Thoka) cause non-conservative alteration of the amino acids and affect on multiple pregnancy or sterility in sheep breeds (Våge *et al.* 2013).

The GDF9 levels in mice (Yan *et al.* 2001) and the simultaneous presence of both BMP15 and GDF9

hormones in sheep (Juengel *et al.* 2002) are important factors influencing the follicular development and ovulation, while in case of their inactivation, folliculogenesis stops at stage 2 of the follicular growth or pre-antral follicular stage (Fabre *et al.* 2006).

Iranian sheep breeds vary greatly in meat and milk production and the prolificacy traits as well (Yalcin, 1979). Shal sheep, a breed of sheep that are kept mainly in Qazvin province situated between 36 ˚16' N and 50 ˚00' E with > 1300 meters elevation above the mean sea level, has an average daily gain of 250 g per day, with the highest percentage of twinning births among Iranian breeds (Ghaffari *et al.* 2009). Studies on G8 mutation in GDF9 and Booroola SNP in BMPR-1B in various Iranian sheep breeds, including Shal, Ghezel, Baluchi and Sangsari, did not indicated the presence of these SNPs (Akbarpour *et al.* 2008; Ghaffari *et al.* 2009; Moradband *et al.* 2011; Kasiriyan *et al.* 2011. Presence of G1 mutation in Ghezel and Moghani breeds (Barzegari *et al.* 2010) as well as G4 mutation in Afshari breed (Eghbalsaied *et al.* 2012) has been confirmed.

However, like Davisdale flock (Juengel *et al.* 2011), the causative SNPs for highly prolific Iranian breeds are still unknown. This study was thus aimed to investigate G1, G4 and G8 mutations in GDF9 gene of Afshari and Shal breeds.

MATERIALS AND METHODS

In this study, a sample of 27 ewes, including 14 ewes with twining births, 12 ewes with single birth, and 1 infertile ewe, was randomly chosen and used for analysis of GDF9 gene. 2-5 mL of blood was collected by vein puncture of the jugular vein; the blood was collected from 8 and 19 Shal and Afshari sheep, respectively. Samples were collected in Qazvin, Zanjan (situated in 36 ˚40 ▪ N and 48 ˚29 ▪ E, and > 1600 meters above the mean sea level as latitude, longitude and altitude, respectively) and Isfahan (situated in 32 ˚39 ▪ N and 51 ˚43 ▪ E, and > 1590 meters above the mean sea level as latitude, longitude and altitude, respectively) provinces by using 15 mL Falcon tubes containing 0.5 mL of 0.5 molar ethylenediaminetetraacetic acid (EDTA) and quickly transported on ice to the laboratory. After centrifugation at 13000 rpm for 5 min and separation of white blood cells, the DNA was extracted using DNA extraction kit (Bioneer Corporation, South Korea) or the phenol-chloroform standard procedure (Sambrook and Russell, 2001). Given that the mutation site do not have a proper cutting action for restriction enzymes, it is necessary to use other techniques to facilitate agarose-gel-based mutation detection pathway for the same PCR-ARMS (tetra-primer amplification refractory mutation system) with 4 simultaneous primers was used (Polley *et al.* 2010). Primers were designed so that the two external primers were able to amplify the desired band combination covering the entire area (the first line of genotype band). Two internal primers were designed to make a mutation in the adjacent 3' region of the primer for higher affinity of either the mutant or the wild type nucleotides (Table1). PCRs were carried out on the extracted DNA from the sheep blood using 12 primer harboring G1, G4 and G8 mutations in GDF9 gene (Polley *et al.* 2010). The following materials were used in each PCR reaction: 1X Buffer, dNTP 250 m*M*, $MgCl_2$ 5 m*M*, primers (forward and reverse 5 pmol each), DNA 100 ng, *Ex-Taq* DNA polymerse 1 IU (Sina Clone, Iran).

PCR conditions were similar for all three mutations (except for annealing temperature) and includes the following steps: initial denaturation at 94 ˚C for 4 min, 35 cycles of denaturation at 94 ˚C for 20 seconds, annealing for 20 seconds and extension at 72 ˚C for 20 seconds, lasted by 4 min final extension (Polley *et al.* 2010).

Ten micro liters of the PCR products was loaded on 1% agarose gel containing ethidium bromide and screened using gel documentation (UNITECH) merchandiser. PCR products for both exons from 27 ewes were sequenced (BioBasic, Canada).

Table 1 Sequence of primers for mutation detection in GDF9-gene in Iranian sheep (Polley *et al.* 2010)

Primer name	(5'->3') Primer sequence
G1IFA	CTGCAGCCAGATGACAGAGCTTTTCA
G1IRG	CGTATGCCTTATAGAGCCTCTTCATGTCGC
G1OF	GCCTGGCTCTGTTTTCCTATTAGCCTTG
G1OR	TCTTCTTCCCTCCACCCATTAACCAATC
G4IFG	TTCACATGTCTGTAAATTTTACATGTGAGG
G4IRA	GCTGAAGGATGCTGCAGCTGGTCGTT
G4OF	CAACAACTCCATTTCTTTTCCCTTTCCTG
G4OR	TAGGCAGATAGCCCTCTC TTCTGGTCAG
G8IFT	AGGGCGGTCGGACATCGGTATGGATT
G8IRC	TGATGTTCTGCACCATGGTGTGAACCGTAG
G8OF	GGATTGTGGCCCCACACAAATACAACCC
G8OR	CATCAGGCTCGATGGCCAAAACACTCAA

Statistical analysis

Allelic and genotypic frequencies among phenotype classes (single-birth, twin-birth and infertile) as well as between breeds (Shal and Afshari) were compared using Chi-Square test in the Genmod procedure of the SAS package (version 9.1), considering P-value < 0.05 as significant level.

RESULTS AND DISCUSSION

Sequence analysis confirmed the PCR-ARMS results for G4 mutation in GDF9 compared to the NM (001142888 accession code in the NCBI website: http://www.ncbi.nlm.nih.gov/nuccore/NM_001142888.2.

The G260A mutation occurred in the mRNA sequence (Figure1) that causes R87H amino acid changes, histidine replaced by arginine. In addition, as depicted in Figure 1, the G4 mutation (G721A) was observed in some sheep. This mutation was observed at the first base of the codon and leads to E241K amino acid change and glutamic acid is replaced by lysine. This change alters an acidic amino acid and is replaced by a basic amino acid, followed by a space charge modification in the polypeptide chain pattern. These two mutations were observed in both Shal and Afshari breeds. The study indicated the presence of G1 and G4 mutants however there were no ewes with G8 mutation. As presented in Table 2, sequencing results from Shal and Afshari ewes indicated very low frequency of G1-GDF9 and G4-GDF9 mutations. None of ewes (of both the breeds) with single-birth carried either G1 or G4 mutations. However, 7.1 and 27.6% of ewes with twin births carried G1-GDF9 and G4-GDF9, respectively. The only infertile sheep (which belonged to the Afshari breed) did not carry any of the above mentioned mutations. In overall, the observed prevalence of G1 SNP in Shal and Afshari breeds were 0 and 2.7%, respectively. Moreover, screening DNAs for G4 SNP showed that 5.3 and 15.8% of Afshari ewes were heterozygous for the mutation. The ARMS-PCR technique with the specific primers reported by Polley *et al.* (2010) was not efficient for discrimination of alleles and all evaluated samples were heterozygous for all three primer pairs. So, we could not find this technique as an accurate tool for determining mutant *vs.* wild type alleles and the sample size was only limited to the sequencing results.

Fine mapping of causative SNPs in BMPR-1B of sheep breeds have been reported from India, China, Australia, New Zealand and some other Pacific Ocean countries (Davis *et al.* 2002). Similarly studies on GDF9 and BMP15 mutations have been reported in some European sheep breeds (Juengel *et al.* 2002; Sadighi *et al.* 2002; Hanrahan *et al.* 2004). Unlike the sheep breeds of west European and Asian origin, major SNPs in GDF9 and BMPR-1B region have not been detected in sheep breeds of middle east countries, including Iran (Eghbalsaied *et al.* 2012). The result of the present genomic analysis indicates that Afshari ewes carried G1 and G4 mutations, both of which occur in pre peptide region of GDF9 protein. Screening of 8 GDF9-DNA samples from Shal breed, however, did not reveal the presence of G1 mutation, though G4 mutation was detected at a frequency of 6.25%.

Results of a study by Barzegari *et al.* (2010) on Moghan and Ghezel Sheep indicated that G1-GDF1 mutation exists in these Iranian breeds. The results of the present study also indicated the presence of mutations in the G1-GDF9 site of Afshari breed, although the B2-BMP15 mutation was not screened. The results of the present study however did not indicate that the homozygosity of G1-GDF9 mutation was associated with sterility and the only sterile ewe was wild type homozygous. G4 mutation was previously reported in Afshari sheep (Eghbalsaied *et al.* 2012) and the present study also indicates the presence of the same mutation in Shal breed. Although G1 and G4 mutations were considered ineffective by Hanrahan *et al.* (2004) because of do not occurring in the mature mRNA, they could make an impact either on the signal peptide or the spherical conformation of the propetide and partially hinder the post-modification of the translated protein.

G1 mutation causes R87H or arginine substituted by histidine without changing amino acid classification (Hanrahan *et al.* 2004). However, different pKa for these two amino acids have suggested to be very effective on side chain spherical form of the polypeptide (Branet, 2010). G4 mutation sound to be more effective than G1 mutation because of shifting in amino acid classification, from acidic to basic group. Mutant allele frequency for G1 was 0 and 2.7% in Shal and Afshari breeds, respectively. The estimated mutant allele frequency in other Iranian breeds, including Sangsari breed, was lower than 19.8% (Kasiriyan *et al.* 2011), 33.3% in Ghezel and Moghani breed (Barzegari *et al.* 2010), and 18% in Baluchi breed (Moradband *et al.* 2011).

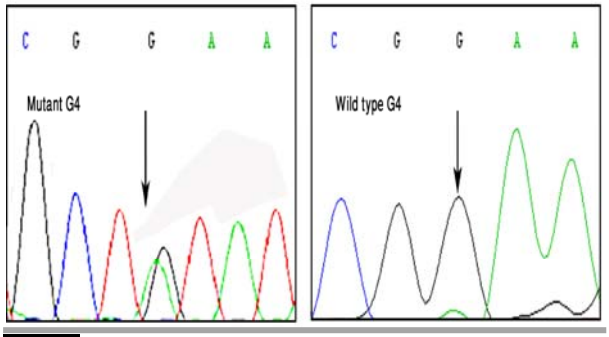

Figure 1 Sequencing graphs of G1 and G4 mutations in Iranian sheep
The G1 (G260A) and G4 (G721A) mutations in the DNA sequences which lead to substitution of the arginine with histidine and glutamic acid with lysine in amino acid polypeptide of the GDF9 prepeptide, respectively

Table 2 Genotypic frequency (with gene frequency in parenthesis) of Iranian Shal and Afshari ewes for G1-GDF9 and G4-GDF9 SNPs

Genotype	Fertility			Breed	
	Twin birth	Single birth	Infertile	Shal	Afshari
G1 / G+	1 out of 14 (3.6%)	0 out of 12 (0%)	0 out of 1 (0%)	0 out of 8 (0%)	1 out of 19 (2.7%)
G4 / G+	4 out of 14 (14.3%)*	0 out of 12 (0%)	0 out of 1 (0%)	1 out of 8 (6.25%)	3 out of 19 (7.9%)

* ($P<0.05$).

However, the observed frequency for G4 mutation was consistent with the only previous report in Afshari breed (Eghbalsaied *et al.* 2012). The observed SNPs were in heterozygote state. This might indicate that crossing with specific rams that are heterozygote or homozygote for these mutations is undergoing. However, identification of the origin of these SNPs could unwrap our understanding about the migration history and possible selection strategies which underwent several years ago in Middle East and Central Asia. Several studies have indicated that the prolific sheep breeds of European / Asian origin usually carry significant mutations (BMPR-1B, G8-GDF9 and B1-BMP15) along with G1 and G4 mutant alleles (Hanrahan *et al.* 2004; Polley *et al.* 2010) and this might subsequent to underestimation of the effect of G1- and G4-GDF9 mutations. Iranian sheep breed have two-breeding season, spring and autumn, while ewes only in one of these season associates with twinning births. This could be due to poor nutrition or other environmental factors like photoperiod. However, the present study indicates the existence of simultaneous/dual mutation (G1 and G4) in Iranian sheep. Similar observations have been reported in Davisdale flock (Juengel *et al.* 2011). The present study further indicates the synergistic effects of minor SNPs in Iranian sheep breeds could partially inactive the GDF9 protein thus enhancing the ovulation rate. The claim can be partially supported by the fact that there are rarely infertile ewes attributed due to complete inactivation of GDF9 or BMP15 proteins, in sheep flocks of Iran. However, complete sequencing of GDF9, BMP15 and BMPR1 genes in the studied breeds could reveal their twinning mechanism.

CONCLUSION

Results of the current study indicated the presence of G1- and G4-GDF9 mutations in two Iranian breeds of sheep. Screening of GDF9 DNA from 27 fertile ewes from Iranian Shal and Afshari breeds did not indicate the presence of G8 mutation. Moreover, the frequency of these two SNPs was estimated to be less than 8% in the evaluated sample size. The observed SNPs were recorded on fertile ewes with twining birth, while infertile and single-birth ewes did not carry the mutant allele. This might indicate an association between these mutations and twin-births in Iranian breeds. Furthermore, these mutations could not be the only reason for infertility of the ewe. Sequencing results did not support the presence of G8 mutations in these breeds. This may be due to lack or rare frequency of this SNP in Iranian sheep flocks. This study was limited to sequencing results of 27 ewes from Iranian Shal and Afshari breeds, since we could not optimized the PCR-ARMS technique for allelic discrimination. However, we need to expand the population size to further elaborate the effect of SNPs and highly scrutinize sheep genome screening for other mutations including G1- and G7-GDF9 and BMP15 mutations.

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REFERENCES

Akbarpour M., Houshmand M., Ghorashi A. and Hayatgheybi H. (2008). Screening for FecGH mutation of growth differentiation factor 9 gene in Iranian Ghezel sheep population. *Int. J. Fertil. Steril.* **2,** 139-144.

Barzegari A., Atashpaz S., Ghabili K., Nemati Z., Rustaei M. and Azarbaijani R. (2010). Polymorphisms in GDF9 and BMP15 associated with fertility and ovulation rate in Moghani and Ghezel sheep in Iran. *Reprod. Domest. Anim.* **45,** 666-669.

Branet M. (2010). A Note on "Amino Acids". Department of Chemistry and Biochemistry, Rose-Hulman Institute of Technology.

Davis G.H., Galloway S.M., Ross I.K., Gregan S.M., Ward J., Nimbkar B.V., Gray G.D. and Inounu I. (2002). DNA tests in prolific sheep from eight countries provide new evidence on origin of the Booroola (FecB) mutation. *Biol. Reprod.* **66,** 1869-1874.

Eghbalsaied S., Ghaedi K., Shahmoradi S., Pirestani A., Amini H., Saiedi T., Nicol L. and McNeilly A. (2012). Presence of SNPs in GDF9 mRNA of Iranian Afshari sheep. *Int. J. Fertil. Steril.* **5,** 225-230.

Fabre S., Pierre A., Mulsant P., Bodin L., Di Pasquale E., Persani L., Monget P. and Monniaux D. (2006). Regulation of ovulation rate in mammals: contribution of sheep genetic models. *Reprod. Biol. Endocrinol.* **4,** 20-31.

Ghaffari M., Nejati-Javaremi A. and Rahimi G. (2009). Detection of polymorphism in BMPR-IB gene associated with twining in Shal sheep using PCR-RFLP method. *Int. J. Agric. Biol.* **11,** 97-99.

Hanrahan J.P., Gregan S.M., Mulsant P., Mullen M., Davis G.H., Powell R. and Galloway S.M. (2004). Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biol. Reprod.* **70,** 900-909.

Juengel J.L., Hudson N.L., Heath D.A., Smith P., Reader K.L., Lawrence S.B., O'Connell A.R., Laitinen M.P., Cranfield M. and Groome N.P. (2002). Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biol. Reprod.* **67,** 1777-1789.

Juengel J.L., O'Connell A.R., French M.C., Proctor L.E., Wheeler R., Farquhar P.A., Dodds K.G., Galloway S.M., Johnstone P.D. and Davis G.H. (2011). Identification of a line of sheep carrying a putative autosomal gene increasing ovulation rate in sheep that does not appear to interact with mutations in the transforming growth factor beta superfamily. *Biol. Reprod.* **85,** 113-120.

Kasiriyan M.M., Hafezian S.H. and Hassani N. (2011). Genetic

polymorphism BMP15 and GDF9 genes in Sangsari sheep of Iran. *Int. J. Gen. Mol. Biol.* **3,** 31-34.

Moradband F., Rahimi G. and Gholizadeh M. (2011). Association of polymorphisms in fecundity genes of GDF9, BMP15 and BMP15-1B with litter size in Iranian Baluchi sheep. *Asian-australas J. Anim. Sci.* **24,** 1179-1183.

Nicol L., Bishop S.C., Pong-Wong R., Bendixen C., Holm L.E., Rhind S.M. and McNeilly A.S. (2009). Homozygosity for a single base-pair mutation in the oocyte-specific GDF9 gene results in sterility in Thoka sheep. *Reproduction.* **138,** 921-933.

Polley S., De S., Brahma B., Mukherjee A., Vinesh P., Batabyal S., Arora J.S., Pan S., Samanta A.K. and Datta T.K. (2010). Polymorphism of BMPR1B, BMP15 and GDF9 fecundity genes in prolific Garole sheep. *Trop. Anim. Health Prod.* **42,** 985-993.

Sadighi M., Bodensteiner K., Beattie A. and Galloway S. (2002). Genetic mapping of ovine growth differentiation factor 9 (GDF9) to sheep chromosome 5. *Anim. Gen.* **33,** 244-245.

Sambrook J. and Russell D.W. (2001). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Våge D.I., Husdal M., Kent M.P., Klemetsdal G. and Boman I.A. (2013). A missense mutation in growth differentiation factor 9 (GDF9) is strongly associated with litter size in sheep. *BMC Genet.* **2,** 14.

Yalcin B. (1979). The Sheep Breeds of Afghanistan, Iran and Turkey. Rome: Food and Agriculture Organization of the United Nations.

Yan C., Wang P., De Mayo J., De Mayo F.J., Elvin J.A., Carino C., Prasad S.V., Skinner S.S., Dunbar B.S. and Dube J.L. (2001). Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol. End.* **15,** 854-866.