Original Article

Screening for FecG^{H} Mutation of Growth Differentiation Factor 9 Gene in Iranian Ghezel Sheep Population

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Abstract

Background: Ghezel sheep are highly prolific and one of the local sheep breeds in Iran and Turkey. Growth differentiation factor-9 (GDF9) gene has been found to be essential for growth and differentiation of early ovarian follicles. Novel mutations in GDF9 have been associated with increased ovulation rates and high litter sizes in heterozygous carriers. Therefore, fecundity gene for GDF9 (FecG^{H}) mutation in GDF9 is considered as a possible candidate for the increased litter size observed in Ghezel ewes.

Materials and Methods: The aim was to evaluate the frequency of recently reported SNP (FecG^{H}) using polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) in 110 Ghezel ewes with a history of high litter size reproductive activity and 75 fertile ewes with normal reproductive activity.

Results: The GDF9 gene exon II was investigated by this technique to screen whether they are FecG^{H} (S395F) carriers or not. SSCP analysis identified four fragments that contained conformational differences; however the combined results with sequencing analysis data did not reveal the FecGH mutation (C to T) in GDF9 gene in Iranian Ghezel ewes.

Conclusion: Current results confirmed that FecG^{H} mutation is not present in the selected Iranian Ghezel sheep population and is not associated with Ghezel sheep high prolificacy performance. Therefore, this SNP may not represent a molecular marker for marker assisted selection programs in this population.

Keywords: Differentiation Factor-9, Fecundity, SSCP, Litter Sizes

Introduction

One of the most important questions to be considered in the survival of a species is the basis for determining ovulation quota and litter size (1).

Over the years farmers have carefully established and maintained strains of sheep for their high prolificacy. Typically, such sheep have an increased rate of twin and triplet pregnancies (2).

Recently, this field took a quantum leap forward when an oocyte specific factor, growth differentiation factor 9 (GDF9) (3, 4) was found to play a pivotal role in specifying ovulation rate and litter size (5). Within the ovary, mRNA and protein for GDF9 has been shown to be expressed exclusively in the developing oocyte in humans (6), rodents (7-9), ruminants (10-12), and marsupials (13). In sheep, expression of GDF9 gene can be seen in primordial follicles (10, 11). However, in some primates, granulose cells near the oocyte as well as the oocyte itself have also been shown to express GDF-9 mRNA and protein (14, 15). Members of TGFβ superfamily are known to signal through a receptor complex comprising two types (type I and type II) of membrane-bound serine/threonine kinases. Recently, bone morphogenetic receptor II (BMPRII) was identified as a type II receptor involved in GDF9 and BMP15 signaling (16, 17). GDF9 signals through a separate type I receptor, namely TGFbRI (also known as ALK5) (18). Hanrahan et al has shown that GDF9 is essential for sheep folliculogenesis and reported a presumably inactivating point mutation in GDF9 gene (FecG^{H}), which results in S77F substitution in the mature region of the GDF9 protein (5). This has been associated with both increased ovulation rate in heterozygous carriers with a single copy of mutated GDF9 as well as sterility with primary ovarian failure in homozygous carriers in some Bel-

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clare and Cambridge ewes(5). Compared to sheep, genetic studies using the poly-ovulatory mouse model reveal both basic similarities and important differences in the phenotype of animals with GDF9 mutation (19, 20).

In terms of growth, studies reveal that GDF9 along with BMP15 are potent stimulators of granulose and theca cell mitosis (17, 21-23). GDF9 has also been shown to regulate stem cell factor (SCF) but the effects are inconsistent (21, 24).

Given the crucial role of mitosis in follicle growth the loss of mitotic activity of these molecules provides a possible mechanism to explain infertility in homozygous GDF9- knockout mice. It is likely that mitosis of granulose cells, particularly during the preantral phase, requires bioactive GDF9 in both mice and sheep (1).

GDF9 also stimulate inhibin production (23, 25, 26). Both GDF9 and bone morphogenetic factor 15 (BMP15) may regulate steroidogenesis through suppression of gonadotropin receptors, with GDF9 suppressing binding of hCG (22) and BMP15 suppressing FSH-R mRNA expression (27). GDF9 appears important in maintaining the cumulus cell phenotype in mice and may play a role in luteinization (22, 28-29).

Ghezel sheep, with significant reproductive characteristics of high prolificacy, are one of the native sheep breeds in Iran in which lambing percentages average 261% (30). Based on the essential biological role of GDF9 in folliculogenesis and its association with high litter size in some species, GDF9 gene was considered as a possible potential gene responsible for high prolificacy in Ghezel sheep. The main objective of the study design was screening for $FecG^{H}$ mutation and its polymorphism among this population.

Materials and Methods

Blood Sample Collection and DNA Extraction

Blood samples were collected from 185 Ghezel ewes using EDTA 1mg/ml as an anticoagulant along with data on litter size in W.Azarbijan Breeding Sheep Farm, Iran. Genomic DNA was extracted from blood samples and dissolved in double diluted sterile water.

Primer sequence and PCR Conditions

The primer pair was designed according to the full DNA sequence (GenBank accession number AF078545) of the ovine GDF9 gene reported by Bodensteiner et al. (11) and synthesized by TAG Copenhagen (Denmark). This primer was named GD-SS and was used to amplify a 206bp fragment of exon 2 of the ovine GDF9 gene where the $FecG^{H}$ mutation was reported to be located.

Primer GD-SS sequence

5'-GCGGGACAGCCTGTTTAACA-3'
5'-TGCGGTGACGGGACGATCTTA-3'

To analyze the GDF9 gene, PCR-SSCP technique was performed. PCRs were carried out in 50µl volume containing approximately 10×PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100) 5µl, 2 mM $MgCl_2$, 250µM each dNTP, 1.0µM each primer, 100 ng ovine genomic DNA, and 1 U Taq DNA polymerase (Fermentase, Germany).

PCR conditions were as follows: denaturation at 94ºC for 3minutes, followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 1minute, extension at 72 ºC for 30 seconds, with a final extension at 72 ºC for 10minutes on Techne Gradiant PCR System (Techne, Cambridge; UK). PCR products were detected by electrophoresis on 2% agarose gels.

Single Strand Conformation Polymorphism (SSCP)

The 2µl PCR product was mixed with 5µl loading buffer solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5 mM EDTA (pH 8.0) and 660 µl NaOH, denatured at 98 ºC for 10 minutes, quickly placed on ice for 5minutes, and then 30 µl of each sample applied to a 10% neutral polyacrylamide gel (acrylamide: bisacrylamide = 29:1, TBE buffer 5X, Glycerol 50%, Ammonium per sulfate 10%, TEMED and ddH2O) electrophoresis gel (31). The samples were separated at 220 volts for 12 hours at -4 ºC cold room. Gels were silver-stained according to the method of Sambrook et al (32).

Sequencing data analyzing

Three samples with different electrophoretic patterns on SSCP gel were sequenced (Ceena Gene; Iran) and multiple alignment performed using CLUSTAL W online software.

Results

PCR amplification of ovine GDF9 gene for Ghezel sheep genomic DNA gave a uniform fragment of 206bp after 2% agarose gel electrophoresis. These specific amplified fragments were suitable to be analyzed by SSCP approach (Fig 1).

The SSCP of PCR products was run on neutral polyacrylamide gels. The results identified two (sample G7), four (samples G1-G2 and G4-G6) and five (sample G3) fragments that contained conformational differences (Fig 2).

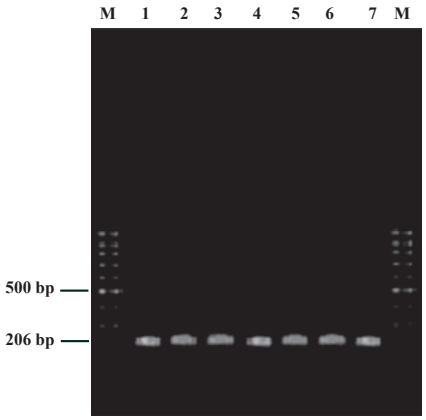

Fig 1: 206bp PCR product of GDF9 gene amplified using GD-SS primers

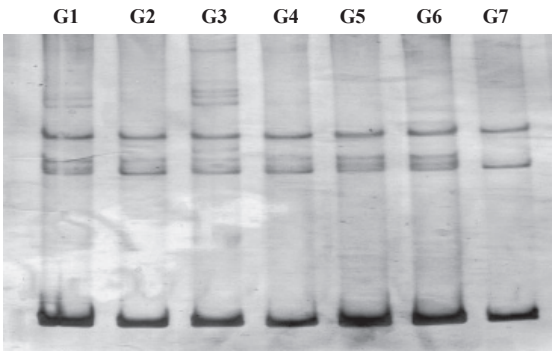

Fig 2: PCR-SSCP analysis of 206bp PCR product of GDF9 gene from Iranian Ghezel sheep which were screened for FecGH mutation (C to T) SNP. Lines G1 to G5, samples with high litter size history of reproductive activity and lines G6-G7, fertile individuals with normal reproductive activity.

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SW: GDF9-G4/1-323          CTGTAAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTANGGCTCTCCGGTTCACACCAT
SW: GDF9-G3/1-323          CTGTAAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTANGGCTCTCCGGTTCACACCAT
SW: GDF9-G7/1-323          CTGTAAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTANGGCTCTCCGGTTCACACCAT
SW: GDF9-NM_001142888/1-1362 ************************************************************
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Fig3: Multiple alignments of sequence data. Highlighted is the SNP position which is the same in all samples compared with NCBI Gene data bank using CLUSTAL W software.

To confirm these differences three samples (G3, G4 and G7) were sequenced but the combined results with sequencing analysis data did not reveal the FecGH mutation (C to T) in exon II of GDF9 gene in Iranian Ghezel sheep ewes with high litter size reproductive activity nor in fertile individuals with normal reproductive activity (Fig 3).

Discussion

Development of the mammalian ovary is characterized by the endowment of a fixed number of primordial follicles throughout fetal life. Follicular growth and differentiation are controlled by pituitary gonadotrophins as well as paracrine factors produced by granulose and theca cells (33).

Oocyte derived growth factors play a crucial role in early folliculogenesis. Bi-directional oocyte-somatic cell communication is an essential requirement for normal folliculogenesis (34). Three members of the TGFβ superfamily, GDF-9, GDF9B (BMP15) and BMP-6 have been shown to be expressed selectively by oocytes from the primary follicle stage in rodents and the primordial stage in ruminants (4, 9, 11, 29, 35). Moreover, targeted deletion of the GDF9 gene in mice leads to arrested follicle development at the primary follicle stage (20) suggesting that oocyte derived GDF9 is an obligatory signal for further growth.

The physiological characteristics of sheep with inactivating mutations in GDF9 have not been well characterized. Ewes with a single copy of the mutated GDF9gene are fertile and have an increased ovulation rate (5). In contrast, ewes homozygous for this mutation are infertile with primary ovarian failure. Ewes heterozygous for mutation in GDF9 are fertile and the effects of this mutation on ovulation rate are additive (5). GDF9 is also involved in follicular luteinization at ovulation (36) and regulates the number of follicles ovulated in sheep, a mammal that normally has a low ovulation rate. However, no such role for GDF9 has been delineated in mice, a mammal that normally has a high ovulation rate (19, 20). These findings also increased strong interest to investigate whether these mutations are responsible for premature ovarian failure and or polycystic ovary syndrome in women (37-40).

In the present study, GDF-9 gene was considered as a possible candidate gene for increased litter size and high prolificacy in Iranian Ghezel sheep. There wasn't any genetic variation within the GDF9 gene by PCR-SSCP among the selected members of the prolific and normal fertile individuals.

Li detected polymorphism of GDF9 gene in Hu, Dorset and Suffolk sheep by PCR-SSCP and reported two genotypes related to population variations, but in that study, FecG^{H} mutation has not been considered as a probable cause of high litter size in those breeds (41). In the current study we

did not find any carrier of this mutation in selected populations either. We described DNA fragment from a part of the GDF9 gene that was reported previously for $FecG^H$ mutation without ruling out the possibility of other variations throughout the GDF9 gene in this population. The entire GDF9 coding region should be sequenced in both high and low fecundity sheep breeds and fully characterized for possible amino acid variation amongst these populations.

At present, the biological function(s) and site(s) of action of GDF9 remain unknown. Further research into the site(s) of action of GDF9 within the mammalian ovary is needed to determine the precise role of GDF9 in the initiation and regulation of folliculogenesis.

The fertility of heterozygous mutant GDF9 ewes increases because of an increase in the number of dominant follicles and eggs ovulated (1). Analysis of the ovaries of heterozygous ewes reveals that the corpora lutea are significantly smaller than those of wild type ewes. The interpretation is that the increase in ovulation quota is caused by precocious development of the pool of dominant follicles that, ultimately, confers an ability to ovulate at a smaller size. Importantly there are no differences in circulating levels of FSH between wild type and heterozygous mutant ewes. A phenomenon has been observed with GDF9 in granulose cells in which GDF9 decreases the FSH-stimulated induction of cAMP (22) and expression of LH-receptor mRNA (29). This increased FSH sensitivity is expected to occur in heterozygous GDF9 mutant ewes and cause an increase in ovulation quota. The major gene involved in Inverdale sheep has been shown to be X-linked and also shown that homozygous carriers are sterile due to ovarian hypoplasia, reflecting a failure of ovarian follicles to progress beyond the primary stage of follicle development. In contrast, the Booroola gene is on chromosome 6 and has an essentially additive effect on ovulation rate. The genes responsible for the Inverdale and Booroola effects have been recently identified. Mutations in bone morphogenetic protein 15 (BMP15, also known as growth differentiation factor 9B) gene are associated with both increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in Inverdale (V31D), Hanna (Q23Ter), Cambridge (Q239Ter) and Belclare sheep (Q239Ter and S367I) (5,10).

A nonconservative substitution (Q249R) in the intracellular kinase domain of the bone morphogenetic protein receptor IB (BMPR-IB) gene has been associated fully with increased ovulation rate in Booroola Merino ewes (42-44). Therefore, both the BMPR-IB and BMP15 genes could be considered as likely candidate genes influencing high prolificacy in both Ghezel and other high prolific sheep populations in Iran.

Conclusion

Although there are conformational differences between normal fertile and high litter size ewes in SSCP derived fragments, the results from sequence data did not reveal $FecG^H$ single nucleotide polymorphism across the coding region of exon II of the GDF9 gene. The results of our study emphasize that among this selected population of Iranian Ghezel sheep, $FecG^H$ mutation is completely absent and not associated with high litter size in prolific Ghezel sheep. Reproductive activity is a multifunctional process and numerous genes, proteins, growth factors, and hormones, etc. are involved in this activity. For further studies to find a suitable marker for increased reproductive activity of Ghezel sheep there are a variety of molecules to be investigated. However, understanding the contribution of ovary-specific genes and pathways will eventually enable to find specific markers to be used in marker assisted selection procedures in animal breeding protocols. Ongoing investigations into the basis of the unexplained sterile phenotypes are likely to reveal further insights into the events controlling follicle and oocyte development. Also, based on recent clinical studies on humans, GDF-9 would be a very compelling target for nonsteroidal contraceptive development as well as a candidate protein for addition in assisted fertility and in vitro fertilization protocols.

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The authors declare that there is no conflict of interest for this article.

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