



## Search for polymorphism in growth and differentiation factor 9 (GDF9) gene in prolific beetal and tali goats (*Capra hircus*)

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### Abstract

Growth and differentiation factor 9 gene is one of the main genes controlling prolificacy in sheep and goat. Mutations in this gene affect litter size in a dosage sensitive manner so that heterozygous animals have litter size records higher than wild types while the mutant homozygous animals are sterile. In this study, we analyzed exon 2 of the growth and differentiation factor 9 as a candidate gene for high prolificacy in Tali and Beetal goats, the common prolific breeds of goats reared in southern and southeast Iran. The results showed a cytosine to thymine nucleotide change at the position of 881 in Beetal goats causing the replacement of alanine with valine at the position of 273 in the unprocessed protein. The results provided further evidence that this amino acid change can affect functions of the protein through changing the conformation of its third structure in Beetal goats.

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## Introduction

Growth and differentiation factor 9 (GDF9), located on chromosome 5 in sheep (Sadighi *et al.*, 2002) and goat (Nicol *et al.*, 2009), is one of the fecundity genes which have been shown to affect prolificacy in sheep (Hanrahan *et al.*, 2004) and goat (Ran *et al.*, 2009). Protein coding region length for this gene is 1362 base pairs that produce a preproprotein with 453 amino acids. Mature peptides include 135 amino acids (Bodensteiner *et al.*, 1999). GDF9 belongs to transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily. This oocyte-secreted paracrine factor plays a critical role during folliculogenesis (Juengel *et al.*, 2002; Juengel *et al.*, 2004). Specific mutations in this gene affect litter size in a dosage-sensitive manner in sheep (Hanrahan *et al.*, 2004) and goat (Feng *et al.*, 2001). Heterozygous Belclare and Cambridge sheep for S77F mutation of GDF9 gene show increased ovulation rate/litter size whereas mutant homozygous ones show complete primary ovarian failure resulting in total infertility (Hanrahan *et al.*, 2004). Homo- and heterozygous Jining Grey goats carrying Q320P mutation show more litter size than non-carriers (Feng *et al.*, 2001). GDF9 is essential for both normal follicular development and ovulation rate (Juengel *et al.*, 2002). The primary cause of infertility in ewes homozygous for the GDF9 mutations is the lack of bioactive GDF9 (McNatty *et al.*, 2005). Physiologically, development of ovarian follicles is restricted to the antral stages and most of them are abnormal with respect to oocyte morphology and the arrangement and appearance of the granulosa and cumulus cells (McNatty *et al.*, 2005). Since GDF9 has been shown in rats to inhibit FSH-induced cAMP synthesis (Vitt *et al.*, 2004), An explanation for the higher ovulation rate observed in heterozygous animals with a lower than normal concentration of GDF9 may be to enhance the sensitivity of the ovarian follicle to FSH and thereby increase the ovulation rate (McNatty *et al.*, 2005).

Beetal and Tali goats are high prolific breeds reared in Iran. The Beetal goat breed is, originally, from the Punjab region of India and Pakistan. Tali or Saheli is

a typical breed of Iran. Both Beetal and Tali goats are kept for milk and meat production. The aim of the present study was to detect the polymorphism of exon 2 of the caprine GDF9 gene and, if detected, to investigate its association with litter size in Beetal and Tali goats.

## Materials and methods

### *Blood sampling and DNA extraction*

A total of 110 blood samples were collected from Tali (n= 60) and Beetal (n=50) goats. The Tali goats were selected from Tali breeding station in Bandar Abbas, Hormozgan province, southern Iran. The Beetal goats were from a personal breeding station of Beetal goats with high kidding records in Zarand, Kerman province, southeast Iran. Animals were selected based on their individual kidding rate. Litter size of experimental goats was obtained. No sterile animal was found in the stations. Blood samples were collected from animals of three groups of different kidding records; animals which commonly produce 1, 2 or 3 and more kids in subsequent kidding. The ear tag number and kidding records of experimental individuals were obtained. Blood samples of 10 mL were taken from jugular vein into tubes containing EDTA as an *anticoagulant* and were carried to laboratory on ice. Genomic DNA was extracted using salting out method (Miller *et al.*, 1998), dissolved in sterile deionized water, and kept at -20°C until further processing.

### *Polymerase Chain Reaction (PCR) amplifications prior to sequencing*

Amplification of the exon 2 in the GDF9 gene was performed using a template PCR reaction containing 2  $\mu$ l of 10X PCR buffer, 1.75 mM of MgCl<sub>2</sub>, 10 pmol of each primer, 2 units of Taq DNA polymerase, 500  $\mu$ M of each dNTP and 15 ng of template DNA adjusted to a final reaction volume of 20  $\mu$ L with deionized water. Sequences of the primers G-5 and G-7 used in PCR reaction were as follow:

G-5: 5'-ATCCCACCCTGACGTTTAAGGC

G-7: 5'-TCCTCCCAAAGGCATAGACAGG

Since the result from a study by Wu and colleagues (2006) showed 99% homology of the nucleotide sequence of GDF9 exon 2 between goat and sheep (Wu *et al.*, 2006), we recruited primers previously used for screening of this gene in sheep by Hanrahan and colleagues (Hanrahan *et al.*, 2004). In addition, matching of G-5 and G-7 primer sequences to target sequence of the caprine DNA (Accession no. FJ665810) was conformed by Vector NTI software.

An initial denaturation step of 5 min at 94°C was followed by 30 cycles of 30 s at 94°C, 45 s at 62°C, and 70 s at 72°C. The reaction was stopped after a final extension time of 10 min at 72°C. The size (1069 bp) and purity of the PCR products were observed by subjecting the samples to 1% agarose gel electrophoresis in parallel with a 100 bp DNA marker.

*DNA sequencing and analysis*

Five PCR products related to the individuals having different kidding records from Beetal (n=3, common kidding records=1, 2 and 4) and Tali (n=2, common kidding records=1 and 3) goats were sequenced. DNA sequencing was performed at Bioneer Corporation, South Korea. The obtained sequences were analyzed by Vector NTI software.

*PCR-Restriction fragment length polymorphism*

Analyzing the DNA sequencing results by vector NTI software revealed *DdeI* as digestion enzyme for PCR RFLP assay. The PCR products (20µl) of all 110 samples from two breeds were prepared. Digestion of 8 mL of PCR products using 3 U of *DdeI* in a final volume of 20 µl was carried out at 37°C over night. The obtained products were separated on 2.0% agarose gel, visualized with ethidium bromide, and detected by UV transilluminator.

*Statistical analysis*

The following statistical model was fitted to compare differences in litter size between GDF9 genotypes by least squares analysis of variance:

$$Y_{ijklm} = \mu + S_i + Y_j + P_k + G_l + e_{ijklm}$$

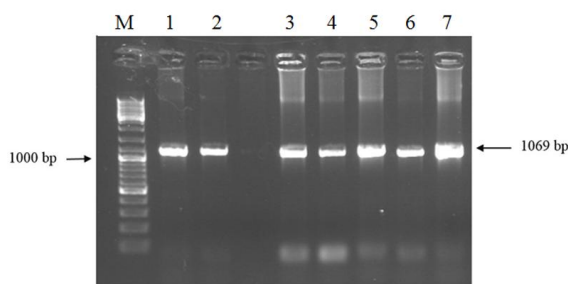
where  $Y_{ijklm}$  is the observed value of litter size;  $\mu$  is the overall mean;  $S_i$  is the fixed sire effect ( $i=1, \dots, 5$ );  $Y_j$  is

the fixed effect of kidding year ( $j=1, 2$ );  $P_k$  is the fixed parity effect ( $k=1, 2, 3$ );  $G_l$  is the fixed effect of GDF9 genotype ( $l=1, 2$ ); and  $e_{ijklm}$  is the residual random term. The model was analysed using the general linear model (GLM) procedure of SAS (Ver 8.1) (SAS Institute Inc., 1998). Comparison of the least squares means was performed using the least significant difference procedure.

**Results**

*PCR Amplification of the exon 2 in the GDF9 Gene*

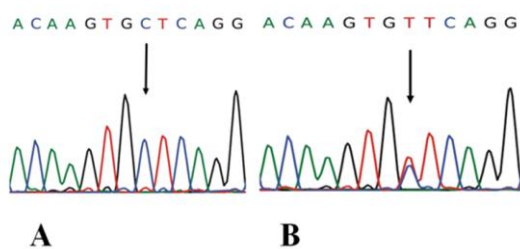
Genomic DNA was amplified using a pair of primers that covered the exon 2 sequence of GDF9 gene. The results showed that amplification fragment sizes were consistent with the target ones and had good specificity so that they could be sequenced directly. The PCR amplification of GDF9 gene by G5 and G7 primers is shown in fig. 1.



**Figure 1.** Amplification of the exon 2 in GDF9 gene using specific primers.

*Sequencing results*

Sequencing of exon 2 in the GDF9 gene revealed a single nucleotide polymorphism (SNP) at the coding base position of 818 (C to T) (Fig. 2) in one of the Beetal goats with the highest records (1, 5, 4, 4 and 4 kids in the five consecutive kidding) which caused a conservative amino acid change. This nucleotide change caused replacement of alanine with valine on amino acid residue of 273 in the coding residue (Fig. 3). This SNP was confirmed by repeated sequencing.



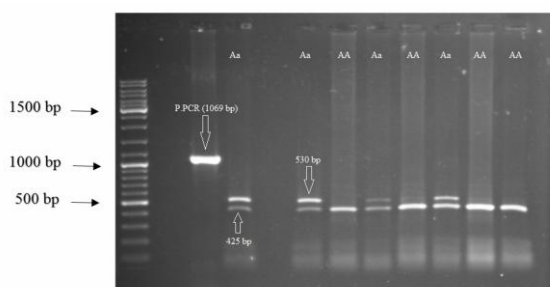
**Figure 2.** Sequence comparison of AA and Aa genotypes of GDF9 gene in Beetal.

	241	240	250	260	270	280	290	300	310				
Beetal-mut	108	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Beetal-non	108	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Tali	108	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Lezhi	241	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Black	241	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Tibetan	241	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Markhoz	241	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS
Rayini	108	KDQLQHP	SARDSL	FNMTLL	VAPSL	LLYLND	TSAQAF	FRHWSL	HPKRFPS	QSPDQ	RRELSAY	PVGEAAE	GVRS

**Figure 3.** Amino acid alignment for C881T mutation leading to conservative V273A shift in unprocessed amino acid peptide.

*Forced PCR restriction fragment length polymorphism assay*

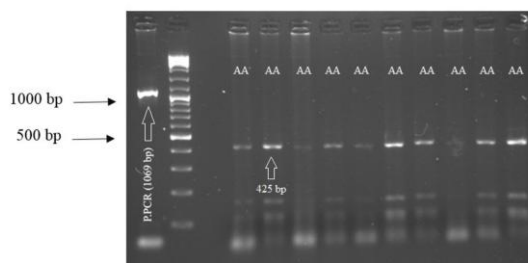
All PCR samples from both Tali (n=60) and Beetal (n=50) goats were digested in this study. Restriction digestion of the PCR products from the wild-type animals resulted in cleavage of the 1069-bp product into nine fragments of 59, 63, 66, 89, 95, 105, 110, 158 and 425bp. Animals heterozygous for the mutation have fragments of all ten sizes (59, 63, 66, 89, 95, 105, 110, 158, 425 and 530bp). All samples from Tali goats were wild-type (Fig. 4). Furthermore, No Beetal goat homozygous for the mutation was found in this study (Fig. 5). In Beetal goats, genotype frequencies for AA and Aa were 0.74 and 0.26, respectively. Allele frequencies for A and a were 0.87 and 0.13, respectively.



**Figure 4.** An AGE electrophoretogram for the products digested by DdeI showing different Beetal's genotypes. The 425 bp fragment is related to the wild type samples, while the 425 and 530 bp fragments together come from heterozygous mutant animals.

*Statistical analyzing*

Mean litter size in experimental goats was 2.23 (SD=0.93) and 2.20 (SD=0.94) for Beetal and Tali, respectively. As shown in Table 1, the Beetal goats with genotype Aa had 1.43 (P < 0.05) kids more than those with genotype AA. The results indicated that allele a was significantly correlated with high prolificacy in Beetal goat.



**Figure 5.** An AGE electrophoretogram for the products digested by DdeI showing Tali's genotype. The 425 bp fragment come from the wild type samples.

**Table 1.** Least squares means and standard error for litter size of different genotypes of GDF9 gene in Beetal goat. Least squares means with the different superscripts differ significantly (P< 0.05).

Genotype	Number	Litter Size
AA	37	1.90 <sup>a</sup> ± 0.22
Aa	13	3.33 <sup>b</sup> ± 0.41

**Discussion**

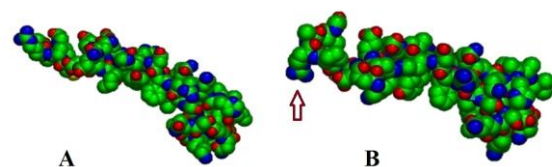
GDF9 gene is specifically expressed in oocytes and is essential for female fertility in sheep. Partial defects in function of GDF9 are associated with the increased ovulation rate in sheep (Galloway *et al.*, 2000). Recently, the genetics of goat litter size has been investigated (Ran *et al.*, 2009; Ren *et al.*, 2010). Some important prolificacy genes of sheep such as GDF9 have also been screened in goats. FecG<sup>H</sup>, FecG<sup>TT</sup> and FecG<sup>E</sup> on GDF9 gene are mutations that affected fecundity in sheep (Hanrahan *et al.*, 2004;

Nicol *et al.*, 2009; Silva *et al.*, 2001). Ovulation rate in GDF9 mutants is high in the heterozygous individuals while the homozygous mutants show a primary ovarian failure resulting in complete sterility (Hanrahan *et al.*, 2004). In recent years, increased attention has been given to investigation of candidate genes for fecundity in goats. Ran *et al.* (2009) confirmed that heterozygous White goats contained one copy of mutation at position of 791 (G791A) in GDF9 exon 2 which resulted in the substitution of valine to isoleucine at residue 79 of GDF9 mature peptide (V79I). Ren and colleagues (Galloway *et al.*, 2000) find a single nucleotide mutation (A562C) in exon 2 of GDF9 gene in Qianbei-pockmarked goats which resulted in an amino acid change of glutamine to proline. The results indicated that there were two genotypes (AA and Aa) detected in Qianbei-pockmarked goats. Genotypic frequencies were 0.7273 and 0.2727 for AA and Aa genotypes, respectively. Frequencies of A and a allele were 0.8637 and 0.1363, respectively. Similarly, our results of PCR-RFLP revealed no homozygous carrier in the present research. Since males produced in the experimental flock were not used for mating in the same flock to prevent inbreeding in the population, and instead external Beetal males were used to produce next generation, we conclude that these external males were probably all wild type. Genetically, when the sire is wild type, none of the offspring has the chance to be homozygous mutant. Therefore, we could not investigate and do not know the effect of aa genotype on phenotype in Beetal. Although homozygous carriers in some sheep breeds showed infertility (Hanrahan *et al.*, 2004), but we concluded from results of some studies in goats (Feng *et al.*, 2011, Wang *et al.*, 2011) that mutations in the main genes for prolificacy may affect litter size but not essentially cause anovulation/infertility, and the effect of these mutations on prolificacy and fertility may be in part different between sheep and goat.

We have investigated the presence of naturally occurring mutation in GDF9 gene of Beetal and Tali goats and confirmed that a different mutation from

sheep was found in exon 2 of GDF9 gene in Beetal goats, which resulted in the substitution of valine to alanine at amino acid 273 of GDF9 coding residue (V273A). Statistical analysis conformed that this amino acid change increases litter size ( $P < 0.05$ ) in heterozygous Beetal goats. Although enzyme digestion of PCR samples resulted in fragments of all ten sizes in Beetal but it excluded 530 bp fragment in Tali goats. Therefore, all experimental Tali goats were wild type. Sequencing results showed that similar to the other TGF $\beta$  superfamily members GDF9 in Beetal and Tali goats was translated as prepropeptides composed of a signal peptide, a large proregion and a smaller mature peptide of 135 residues in GDF9.

Considering the role of different amino acids in formation of third structure of protein, some amino acids such as alanine, glutamic acid and leucine tend to participate in  $\alpha$ -helix and some others tend to take part in  $\beta$ -sheet. Therefore, based on our finding alanine with high tendency to form  $\alpha$ -helix is replaced with valine with high tendency to form  $\beta$ -sheet which changes the third structure of the obtained protein leading to the change in conformation of third structure of the protein (Fig. 6) and its function.



**Figure 6.** Modeling of the conformation of third structure of the wild type (A) and mutant (B) proteins. An arrow shows the domain in which replacement of alanine with valine caused a change in the third structure conformation (third structure was assessed through specific web site of ps2).

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