Polymorphisms of Caprine GDF9 Gene and Their Association with Litter Size in Henan Dairy Goat

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Abstract: The Henan Dairy goat is a prolific goat breed in China. The exons 2 of growth differentiation factor 9 (GDF9) gene in three randomly selected does of Henan Dairy goat, Yaoshan goat and Taihang Black goat were amplified and analyzed. The SNPs (T1189C (Val397Ile) and T3615C (Val240Ala) in exon 2) were detected in three goat breeds with different prolificacy, in which T3615C was a new SNP in goats. The product amplified by primers P1 and P2 displayed polymorphisms. For primer P1, three genotypes (GG, GA and AA) were detected in Henan Dairy goats and Yaoshan goats, two genotypes (GG and GA) were found in Taihang Black goats and one mutation (1189T→C) was found by sequencing in GA genotype. The differences of Least Squares Mean (LSM) for litter size between genotypes GG, GA and AA were significant (p<0.05) in Henan Dairy goat and non-significant (p>0.05) in Taihang Black goat. For primer P2, two genotypes (GG and GA) were detected in three goat breeds and one mutation (3615T→C) was found by sequencing. The differences of LSM for litter size between genotypes GG and GA were significant (p<0.05) in Henan Dairy goats. These results preliminarily showed that the detected loci of the GDF9 gene maybe a major gene that influences prolificacy of Henan Dairy goats.

Key words: Goat, growth differentiation factor 9, prolificacy, breeds, genotype

INTRODUCTION

Growth Differentiation Factor 9 (GDF9) is one member of the Transforming Growth Factor Beta (TGF-β) superfamily ligands, it was isolated and characterized for the first time (McPherron and Lee, 1995) and was subsequently proved GDF9 essential for early follicle growth (Dong et al., 1996). Expression of GDF9 was oocyte-specific in ovine and bovine ovaries beginning at the primordial follicle stage (Bodensteiner et al., 1999). GDF9 is secreted by oocytes in growing ovarian follicles (Juengel et al., 2002). The changing concentrations of GDF9 in vivo leads to incremental changes in ovulation rate in sheep (Hanrahan et al., 2004). The mutations in this gene cause increased ovulation rate and twin and triplet births in heterozygotes and complete primary ovarian failure in homozygotes resulting in total infertility in some prolific breeds of sheep (Hanrahan et al., 2004). The GDF9 fecundity alleles show an X-linked and an autosomal over-dominant inheritance pattern with infertility in homozygous females, respectively (Davis et al., 1992; Galloway et al., 2000). It is reported that natural mutations in prolific sheep breeds have shown that the GDF9 is crucial for ovulation and as well as for increasing litter size. Mutations in the gene increased prolificacy in sheep (Vacca et al., 2010). For example, FecGH (G8) mutation was found in Cambridge and Beetalove sheep (Hanrahan et al., 2004), FecGE (also named as FecGST) mutation was found in Santa Ines sheep (Melo et al., 2008; Silva et al., 2011), FecTT mutation was found in Thoka sheep (Nicol et al., 2009) and G1 mutation was found in Mogharian and Ghezel sheep and in Garole sheep (Barzegari et al., 2010; Polley et al., 2009a). Also, for Chinese sheep breed has been studied in this gene (Li et al., 2003; Chu et al., 2005; Chang et al., 2009). Based on results indicating a major gene effecting sheep prolificacy, the possibility of a major gene also affecting the prolificacy capability of some goat breeds was widely investigated using biology information and molecular biotechnology in recent years (Hua et al., 2008; Wu et al., 2006; Zhang et al., 2008; Ran et al., 2009), those researches have provided the basis reference for Chinese goat breeding.

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Livestock play a critical role in supporting families in most parts of rural Henan Province. Henan has a goat population of 19.97 million which accounts for 7.26% of the national total goat flocks in 2009. Henan Dairy goat, Yaoshan and Taihang Black goat are three local Chinese goat breeds. Henan Dairy goat is a prolific and major milk-meat or pearl-meat producing animal. The average litter size of 2.15 does raised under extensive conditions was 2.04 (Bai and Tan, 2010). Studies on Dairy goats have been negligible compared to meat goat in China. It was only in recent years, some researches on Henan Dairy goat were featured in the scientific literature (Wang et al., 2009, 2010, 2011a, b).

Yaoshan goat is also called “Cow leg” goat because their leg are strong. These goats were kept in mountain areas of Lushan County of Pingding Shan City. They have high quality meat and have an average litter size of 1.2 lambs. Taihang Black goat have an average litter size of 1-2 lambs (Wang et al., 2011a). Indigenous goats have many remarkable characteristics when compared with the exotic breeds, these three goat are better adapted to survive and reproduce under the harsh environments and are a natural gene reservoir for improving crossbreed predominance. Protection and use of these indigenous goat breed resources is the most urgent research problem in local goat genetics and breeding. Consequently, it is essential to study the genetics and reproduction of these goat breeds using modern genetic methods. Until now, the association of GDF9 genetic variations with litter size has been reported in some goat breeds but not in the Henan Dairy goat. Since, the GDF9 gene is associated with the folliculogenesis of sheep. Thus, the main objective of this study was to test association between GDF9 polymorphism with litter size in three local goat breeds. Researchers found two mutations which associations litter size of Henan Dairy goat, this is a good reference for us to further establish prolific populations of Henan Dairy goats.

MATERIALS AND METHODS

Animals and DNA isolation: Genomic DNA samples were obtained from 366 female goats belonging to three goat breeds: Henan Dairy goats (166), Yaoshan goats (102) and Taihang Black goats (98). Goats were obtained from different villages of Luyang, Lushan mountain areas and Xiwwu County of Henan Province, China. All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture. Approximately 10 mL of blood was collected from the jugular vein and the collected samples were transported to the laboratory at 4°C. The DNA was isolated according to the procedure described by a routine protocol. Records of kids for different parities of the three goat breeds were collected for statistical analysis. Kidding was partitioned into four 3 months seasons: March through May (season 1, Spring, n = 45), June through August (season 2, Summer, n = 35), September through November (season 3, Autumn, n = 46) and December through February (season 4, Winter, n = 40). For each doe, the number of kids born, the date of kidding, the flock number, the season of kidding and the prolific performance of the first three parities were recorded. Does with incomplete performance records, does lacking birth information and records with other obvious errors were removed.

Primer and PCR amplification: Two pairs of PCR primers for exon 2 of GDF9 was first designed by Oligo 6.0 Software (Biolytic Lab Performance Inc.). The P3 primer was designed based on the sequence of the amplification fragment of P2 for RFLP analysis. Amplification conditions were the same as described above except that extension time was 15 sec. The expected fragments for primers P1, P2 and P3 were 121, 542 and 171 bp, respectively. The sequences of the three pairs of primers were as follows: Primer P1, F: 5'-TOTA CCGCAGTTAG-3', R: 5'-GTTTTACTTGACAGGA G-3'. Primer P2, F: 5'-GATTGTGACCCGCTCC-3', R: 5'-CCATACCAATGTCCCAAACC-3'. Primer P3, F: 5'-AACAG GTCAATTAAACAGGC-3', R: 5'-CACATGTCTGTAA ATTTACATCGC-3'.

Amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 45 sec with a final extension at 72°C for 10 min on Mastercycler 5333 (Eppendorf AG, Hamburg, Germany). The amplification fragments of P1 and P2 from all 45 goats were cloned and sequenced.

The primers were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, People’s Republic of China). Polymerase chain reactions were carried out in 25 μL volume containing approximately 1 μL of 10 μmol L⁻¹ each primer, 2.5 μL of 10×PCR buffer (50 mmol L⁻¹ KCl, 10 mmol L⁻¹ Tris-HCl [pH 8.0], 0.1% Triton X-100, 25 mmol L⁻¹ MgCl₂), 2.0 μL of 2.5 mmol L⁻¹ each dNTP, 2.0 μL of 50 ng μL⁻¹ caprine genomic DNA, 1.0 μL of 2.5 U μL⁻¹ Taq DNA polymerase (Promega, Madison, WI, USA) and the rest is ddH₂O.

PCR-RFLP analysis: The PCR products of P1, P3 primers were detected by 1.5% agarose gels and mixed from 10 samples to sequence to search SNPs. PCR products of P1 and P3 primas were digested by HindII and HhaI.
respectively and detected by 8% PAGE. The genotypes were detected using and Alphalmage TM 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

**Statistical analysis:** In order to study the differences of litter size among genotypes in the condition of fixed effect of goat breed, least square analysis of the variance for litter size of different genotypes was carried out by the follow model:

\[ y_{ilm} = \mu + K_{i} + P_{k} + G_{i} + e_{ilm} \]

Where:
- \( y_{ilm} \) = Phenotypic value of litter size
- \( \mu \) = The population mean
- \( K_{i} \) = Fixed effect of ith buck, \( i = 1, 2, 3 \)
- \( P_{k} \) = Fixed effect of season
- \( G_{i} \) = Fixed effect of the kth genotype
- \( e_{ilm} \) = Random residual error

GLM (General Linear Model) of SAS (V8.12) was used for multiple testing.

**RESULTS**

**Sequence analysis of amplification fragments of primers P1 and P2:** Genomic DNA of goats was successfully amplified using primer pairs P1 and P2. The results showed that the sizes of the amplification fragments were consistent with the target fragments and had sufficient specificity to sequence and analyze. As shown in Fig. 1 and 2.

The sequences of fragments amplified by primer P1 and P2 from Henan Dairy goat, Yaoshan and Taihang Black goats were identical. Two SNPs were identified which were T1189C and T3615C for primer P1 and P2, respectively. Based on the above two mutations, primers from P1 and P3 were designed to detect the SNPs of GDF9 gene by PCR-RFLP.

**Polymorphisms detection in goat GDF9 gene:** As shown in Fig. 3a, G to A transition at the 1189 locus showed three genotypes: GG, GA and AA and the sequences of the genotypes were presented in Fig. 3b. C to T transition at the 3615 locus showed two genotypes: GG and GA, the corresponding sequences of two homozygous genotypes were shown in Fig. 4b.

**Allele and genotype frequencies of GDF9 gene in three goat breeds:** Allele and genotype frequencies of GDF9
Table 1: Allele and genotype frequencies of GDF9 in three goat breeds

<table>
<thead>
<tr>
<th>Primer/Locus</th>
<th>Breed locus</th>
<th>Dairy goat</th>
<th>Yaoshan goat</th>
<th>Taihang black goat</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1/1189</td>
<td>Number</td>
<td>168</td>
<td>98</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.6667 (112)</td>
<td>0.8367 (82)</td>
<td>0.7745 (79)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>0.3214 (54)</td>
<td>0.1633 (16)</td>
<td>0.2255 (23)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.0119 (2)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.827381</td>
<td>0.918367</td>
<td>0.88725</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.172619</td>
<td>0.081633</td>
<td>0.112745</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-W test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>2.65</td>
<td>0.09</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number</td>
<td>166</td>
<td>98</td>
<td>102</td>
</tr>
<tr>
<td>P2/3615</td>
<td>Genotype frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.8494 (141)</td>
<td>0.9286 (91)</td>
<td>0.9510 (97)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>0.1506 (25)</td>
<td>0.07143 (7)</td>
<td>0.04902 (5)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.9247</td>
<td>0.9643</td>
<td>0.9755</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.075301</td>
<td>0.035714</td>
<td>0.02451</td>
<td></td>
</tr>
</tbody>
</table>

The numbers in the brackets are the genotype individuals; *p<0.05, **p<0.01, 0.01

Table 2: Least squares mean and standard error for litter size of different genotypes of the two loci of GDF9 gene in Henan Dairy goats

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Genotype</th>
<th>Number of does</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henan Dairy goat</td>
<td>GG</td>
<td>112</td>
<td>1.91±0.12</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>54</td>
<td>2.32±0.17</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2</td>
<td>1.67±0.12</td>
</tr>
<tr>
<td>3615</td>
<td>GG</td>
<td>141</td>
<td>1.76±0.22</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>25</td>
<td>2.36±0.15</td>
</tr>
</tbody>
</table>

Least squares means with the same superscript for the same locus have no significant difference (p>0.05). Least squares means with the different superscripts for the same locus differ significantly (p<0.05)

Influence of fixed effects on litter size in Henan Dairy goats: Litter size in Henan Dairy goats was significantly influenced by sire, kidding season and parity (all p<0.05). The least squares mean and standard error for litter size of different GDF9 genotypes in Henan Dairy goats were given in Table 2. For the 1189 locus, the Henan Dairy goat does with genotype GA had 0.41 (p<0.05) or 0.65 (p<0.05) kids more than those with genotype GG and GA, respectively. No significant difference (p>0.05) was found in litter size between GG and AA genotypes in Henan Dairy goats. For the 3615 loci, the goat does with genotype GA had 0.60 (p<0.05) kids more than those with genotype GG and had significant difference (p<0.05) was found in litter size between different genotypes in Henan Dairy goats. These results preliminarily revealed a significant correlation between allele G of the 1189 and A of 3615 locus in GDF9 gene with high litter size in Henan Dairy goats.

DISCUSSION

Polymorphisms of GDF9 gene in sheep and goats: Growth Differentiation Factor 9 (GDF9), known as FecG on chromosome 5 (Hanrahan et al., 2004). As a candidate gene with a major effect on litter size in sheep and goats was studied wildly cross the world. Some specific mutations found on this gene have been shown to be associated with different phenotypic effects. It was reported that the Cambridge and Belclare breeds also carry a mutation in GDF9 (FecGH) that causes increased ovulation rates in heterozygous ewes and infertility due to streak ovaries in homozygous carriers (Hanrahan et al., 2004). It was well known that GDF9 increased ovulation in heterozygous mutant ewes because the altered proteins result in increased sensitivity of granulosa cells to FSH which leads to accelerated follicular development and precocious ovulation of small follicles (Moore et al., 2004; Moore and Shimasaki, 2005). Ovulation rates in GDF9 mutants are high in the heterozygotes. The homoygotes, however have small, flattened streak ovaries with follicles that do not develop up to the primary stage (Hanrahan et al., 2004; Galloway et al., 2000; McNatty et al., 2005; Bodin et al., 2007) resulting in

Fig. 4: a) PCR-RFLP analysis of the 3615 locus in goat GDF9 gene using primer P3. Two genotypes: GG, bands 1 and 2 (171 bp); GA, bands 3, 5, 6, 7 and 8 (171/128 bp); AA, M: DNA Marker I (Tiangen, Beijing). b) Sequence of genotypes GG and AA of T3615 C in goat GDF9 gene. The T3615 C mutation resulted in Ala to Val amino acid change at position 240 (A240V)
complete sterility in these animals (Galloway et al., 2000; Hanrahan et al., 2004; Bodin et al., 2007; Montecaglio et al., 2009).

Recently, increased attention has been given to studying candidate genes for fecundity in goats. Five point mutations G1, P2, G6, G7 and G8 in Black Bengal goats were tested. It is found that all Black Bengal goats tested had only the wild type genotype, GDF9 wild type alleles had G, A, G, G and C nucleotides at the G1, P2, G6, G7 and G8 locations corresponding to the arginine, glutamic acid, valine, valine and serine amino acids, respectively. All animals had the wild type homozygote for GDF9 gene (Polley et al., 2009b). The mutation of P223A and G1189A in Jining Grey, Boer, Wendeng dairy, Liaoning Cashmere and Beijing Native goats by PCR-SSCP (Wu et al., 2006). According to reports that mutation G1189A, A959C, C818T and G1188A was detected in Yangtze River Delta White, three mutations G1189A, A959C and G1188A were detected in Huanghai Boer goats and two mutations G1189A, A959C were detected in Huanghai and Boer goats (Zhang et al., 2008). Mutation G1189A in Guizhou White goats was also reported (Du et al., 2008). The G1133T was found in Guizhou Black goats (Huang et al., 2009) and mutation P1288A in intron 1 of GDF9 was firstly reported in Jining Grey goat, Guizhou White goat, Boer, Liaoning Cashmere goat breeds (Feng et al., 2011). Four polymorphic sites from GDF9 mature peptide including V18A, P78Q, V79I and T81A in Guizhou White goats (Ran et al., 2009). In this study, mutation T3615C in intron 2 of GDF9 was firstly reported in three goat breeds. The G1189A had also been detected in several goat breeds (Wu et al., 2006; Du et al., 2008; Feng et al., 2011).

Association between litter size and GDF9 gene in goats: Primarily study showed that the GDF9 was associated with fertility function and GDF9 had been shown to be obligatory for fertility in female mice by loss of function studies and to be essential at multiple steps in the process of female reproduction in vitro studies (Wu and Matzuk, 2002). It has been well known, the variants of GDF9 gene have different effects on ovulation rate and litter size in sheep. Many mutations are associated with increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in many different sheep (Nicol et al., 2009; Barzegari et al., 2010). In these years, more and more researches of GDF9 in Caprine magnified that GDF9 gene was abundant polymorphisms either. It is reported by Du et al. (2008) that g.11893>A mutation heterozygote was identified in eight of 33 high prolificacy Guizhou White goats (exceeding 3 kids per litter) and none was found in 112 low prolificacy Guizhou White goats. This mutation resulted in p.V79I amino acid change which could have an effect on the binding ability with its receptor, ALK5. Huang et al. (2009) tested heterozygous c.1133C>T mutation only in two high prolificacy Guizhou Black goats exceeding 3 kids per litter while nothing was found in low prolificacy Guizhou Black goats (n = 12) and believed that the mutation could affecting on high prolificacy in Guizhou Black goats. Feng et al. (2011) concluded that allele C at the 589 locus of GDF9 gene was associated with high litter size in Jining Grey goats (p<0.01) and also showed that the allele A at G2 locus may have certain correlation with prolificacy in Jining Grey goats. In the study, the results preliminarily revealed a significant correlation between allele C at the 3615 locus and G at the 1189 in GDF9 gene and high litter size in Henan Dairy goats. More and more researches on finding locus of GDF9 or other genes associating with litter size in different goat breeds are being carried out, researchers just hope to find out the main factors affecting on litter size of goat for improving reproductive rate in goat for farmers. Similar to other reports, the study tested relatively a small number of animals, further studies are necessary for function validation of these polymorphisms, a large number of animals is required to confirm the linkage disequilibrium of those loci found in all those researches.

CONCLUSION

Although, there are many reports on mutations in a single gene of fecundity gene family, mutations at the multiple loci of fecundity genes have been reported in several goat breeds. To the best knowledge of the researchers, the Henan Dairy goat is therefore the other goat breed that has existing mutations in fecundity genes GDF9. The findings of this study point to a preliminary supposition that mutations of GDF9 gene might be major determinant to influence prolificacy in the Henan Dairy goat. Further studies on large scale of selective prolific populations are necessary to further verify the gene affections in goat breeding.

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