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*GDF*9 as a candidate gene for prolificacy of Small Tail Han sheep

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Abstract Growth differentiation factor 9 (*GDF*9) which controls the fecundity of Belclare, Cambridge, Santa Ines, Moghani, Ghezel and Thoka ewes was studied as a candidate gene for the prolificacy of Small Tail Han sheep. According to the sequence of ovine *GDF*9 gene, six pairs of primers were designed to detect single nucleotide polymorphisms of two exons of *GDF*9 gene in both high fecundity breed (Small Tail Han sheep) and low fecundity breed (Dorset sheep) by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). Only the products amplified by primers 2-1 and 2-2 displayed polymorphisms. For primer 2-1, three genotypes (*AA*, *AB* and *BB*) were detected in both sheep breeds. Sequencing revealed one silent mutation (G477A) in exon 2 of *GDF*9 gene in the *BB* genotype in comparison with the *AA*, which was known as G3 mutation of *GDF*9 gene in Belclare and Cambridge ewes. The relationship of least squares means for litter size was $AA > AB > BB$ in Small Tail Han sheep ($P > 0.05$). For primer 2-2, two genotypes (*CC* and *CD*) were detected in both sheep breeds. Sequencing revealed one novel single nucleotide mutation (G729T) in exon 2 of *GDF*9 gene in the *CD* genotype in comparison with the *CC*, which resulted in an amino acid change (Gln243His). The ewes with mutation heterozygous genotype *CD* had 0.77 ($P < 0.01$) lambs more than those with wild type *CC* in Small Tail Han sheep. These results preliminarily indicated that allele *D* of *GDF*9 gene was a potential genetic marker for improving litter size in Small Tail Han sheep.

Keywords Sheep · Prolificacy · Growth differentiation factor 9 gene · PCR-SSCP

Introduction

Growth differentiation factor 9 (GDF9) is a member of the transforming growth factor-β superfamily. Sadighi et al mapped *GDF*9 to ovine chromosome 5 between markers BM7247 and BMS2258 [1]. Sheep *GDF*9 spanned approximately 2.5 kb and contained two exons and one intron, in which exon 1, exon 2 and the single intron spanned 397, 965 and 1126 bp, respectively. GDF9 was an oocyte-secreted paracrine factor and played a critical function as a growth and differentiation factor during early folliculogenesis, as well as granulosa cells and theca cells in ovine ovaries [2–4]. Dong et al. intercrossed the heterozygous ($gdf9^{ml/+}$, deletion exon 2 of *GDF*9 gene) mice to obtain $gdf9^{ml}/gdf9^{ml}$ mice (*GDF*9-deficient) and found that $gdf9^{ml}/gdf9^{ml}$ male mice were fertile and female mice with $gdf9^{ml}/gdf9^{ml}$ were infertility for failing to demonstrate any normal follicles beyond the primary one-layer follicle stage [5]. Several mutations in sheep *GDF*9 gene were associated with increased ovulation rate or infertility including $FecG^{H}$ (G8) mutation in Cambridge and Belclare sheep [6], $FecG^{SI}$ mutation, also named as $FecG^{E}$ in

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Brazilian Santa Ines sheep [7, 8], and FecTT mutation in Thoka sheep [9], respectively. On the whole, *GDF*9 was an obvious candidate gene with a major effect on litter size in sheep [10].

The mean litter size alive of Small Tail Han sheep was 2.61 [11], and that of Dorset sheep was 1.41 [12] and 1.77 [13]. Based on the important role of *GDF*9 gene in reproduction, *GDF*9 gene was considered as a potential candidate gene for the prolificacy of Small Tail Han sheep. The objectives of the present study were firstly to detect single nucleotide polymorphisms (SNPs) of *GDF*9 gene in both high prolificacy breed (Small Tail Han sheep) and low prolificacy breed (Dorset sheep) by PCR-SSCP, and secondly to investigate the association between SNPs of *GDF*9 gene and prolificacy in Small Tail Han sheep.

Materials and methods

Animals

All procedures involving animals were approved by the animal care and use committee at the respective institutions where the experiment was conducted. All procedures involving animals were approved and authorized by the Chinese Ministry of Agriculture.

Venous jugular blood samples (10 ml per ewe) were collected from 126 Small Tail Han ewes lambed in 2007, along with data on litter size in the first, second, or third parity (Jiaxiang Sheep Breeding Farm, Shandong Province, P.R. China) and 30 Dorset ewes (HITEK Ranch [Beijing] Ltd. Co., Beijing, P.R. China) with acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by phenol–chloroform method, and then dissolved in TE buffer (10 mmol/l Tris–HCl [pH 8.0], 1 mmol/l EDTA [pH 8.0]) and kept at −20°C.

The 126 Small Tail Han ewes were selected at random and they were the progeny of four rams ($n = 30$, 31, 31, 34). Because the four rams were sold, their blood was not collected and they were not genotyped. No selection on litter size or other fertility traits was performed in the flock over previous years. Lambing seasons consisted of 3-month groups starting with March through to May as season 1 (spring, $n = 33$), June through to August as 2 (summer, $n = 29$), September through to November as 3 (autumn, $n = 37$) and December through to February as 4 (winter, $n = 27$).

Primers and PCR amplification

Six pairs of primers were designed according to coding sequence of ovine *GDF*9 gene (GenBank No. AF078545). All two exons of *GDF*9 gene were amplified, in which primers 1-1 and 1-2 were amplified exon 1 and other four pairs of primers (primers 2-1 to 2-4) for exon 2, respectively. These primers were synthesized by Shanghai Invitrogen Biotechnology Limited Corporation (Shanghai, P.R. China). Primer sequence, location corresponding to ovine *GDF*9 (AF078545) and product size were listed in Table S1 (see electronic supplemental data).

Polymerase chain reactions were carried out in 25 μL volume. The optimal amplification systems and conditions of PCR for six pairs of primers were listed in Table S2 (see electronic supplemental data) and Table S3 (see electronic supplemental data), respectively.

SSCP detection

A volume of 1.5 μL PCR product was transferred in an Eppendorf tube, mixed with 6 μL gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0) and 10% glycerol. The mixture was centrifugalized and denatured at 98°C for 10 min, then chilled on ice for 5 min and loaded on neutral polyacrylamide gels. The optimal concentrations of neutral polyacrylamide gels and conditions of electrophoresis were shown in Table S4 (see electronic supplemental data). After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed and analyzed by an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Sequencing of PCR products

After SSCP analysis, PCR products of different genotypes were sequenced from both directions using an ABI3730 automatic sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) by Shanghai Invitrogen Biotechnology Limited Corporation (Shanghai, P.R. China).

Statistical analysis

The following fixed effects model was employed for analysis of litter size in Small Tail Han ewes and least squares mean was used for multiple comparison in litter size among different genotypes.

$$y_{ijklm} = \mu + S_i + LS_j + P_k + G_l + e_{ijklm},$$

where y_{ijklm} is the phenotypic value of litter size; μ is the population mean; S_i is the fixed effect of the ith sire ($i = 1$, 2, 3, 4); LS_j is the fixed effect of the jth lambing season ($j = 1, 2, 3, 4$); P_k is the fixed effect of the kth parity ($k = 1$, 2, 3); G_l is the fixed effect of the lth genotype ($l = 1, 2, 3$, or $l = 1, 2$) and e_{ijklm} is the random residual effect of each observation. Analysis was performed using the general linear model procedure of SAS (Ver 8.1) (SAS Institute

Inc., Cary, NC, USA). Mean separation procedures were performed using a least significant difference test [14, 15].

Results

PCR amplification

PCR products of six pairs of primers were detected by running a 2% agarose gel electrophoresis. The amplified products were consistent with the target fragments and had a good specificity, which could be directly analyzed by SSCP.

SSCP analysis

Only the PCR products amplified by primers 2-1 and 2-2 displayed polymorphisms. Three genotypes (*AA*, *AB* and *BB*) were detected by primer 2-1(Fig. 1) and two (*CC* and *CD*) were detected by primer 2-2 (Fig. 2).

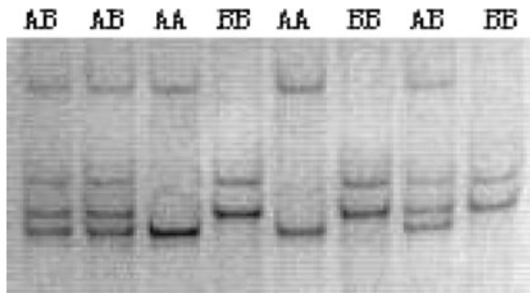

Fig. 1 SSCP analysis of PCR amplification using primer 2-1

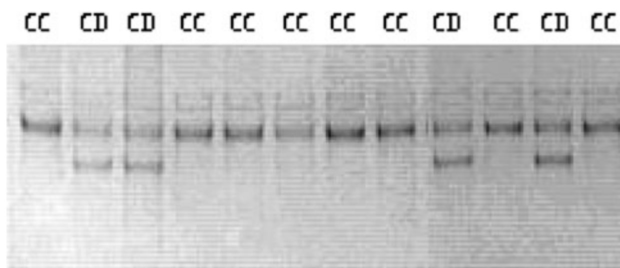

Fig. 2 SSCP analysis of PCR amplification using primer 2-2

Sequencing of different genotypes and nucleotide mutations

For primer 2-1, sequencing revealed one nucleotide mutation (477G→A) (Fig. 3) of *GDF*9 gene between genotype *BB* and *AA*, and which did not cause amino acid changes.

For primer 2-2, sequencing revealed one nucleotide mutation (729G→T) (Fig. 4) of *GDF*9 gene between genotype *CD* and genotype *CC*, and which caused an amino acid change glutamine→histidine at 243 position (Q243H) in GDF9 protein.

Allele and genotype frequencies of GDF9 gene in two sheep breeds

The frequencies of different genotypes and alleles in Small Tail Han and Dorset sheep were presented in Table 1.

Influence of fixed effects on litter size in Small Tail Han sheep

Litter size was significantly influenced by sire, lambing season and parity (all $P < 0.05$). The least squares mean (LSM) and standard error for litter size of different genotypes of *GDF*9 gene in Small Tail Han sheep were given in Table 2.

As shown in Table 2, for primer 2-1, the differences of the LSM for litter size between *AA*, *AB* and *BB* were not significant ($P > 0.05$) in Small Tail Han sheep. For primer 2-2, the Small Tail Han ewes with genotype *CD* had 0.77 ($P < 0.01$) lambs more than those with genotype *CC*. The results indicated that allele D was significantly correlated with high prolificacy in Small Han Tail sheep.

Discussion

Polymorphisms of GDF9 gene in sheep

Li et al. identified one single nucleotide mutation (A152G) of *GDF*9 gene in Hu, Dorset and Suffolk sheep by

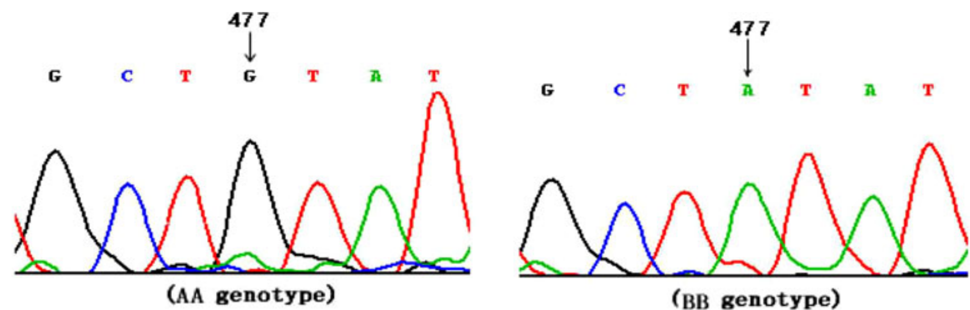

Fig. 3 Sequence comparison of *AA* and *BB* genotypes of *GDF*9 gene in sheep

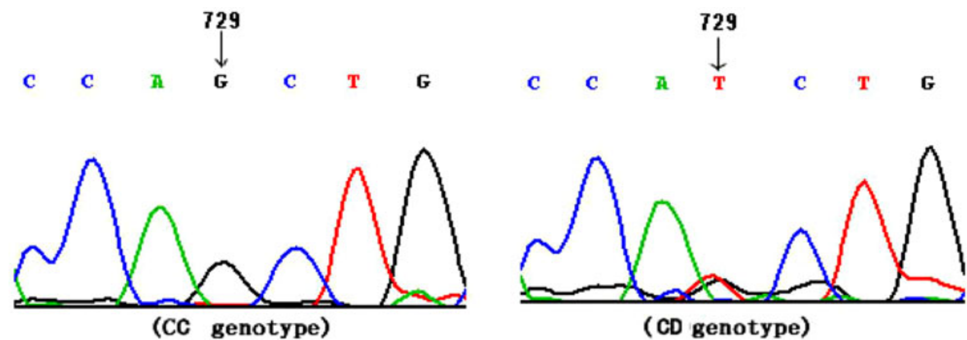

Fig. 4 Sequence comparison of *CC* and *CD* genotypes of *GDF*9 gene in sheep

Table 1 Allele and genotype frequencies of *GDF*9 gene in two sheep breeds

Breed	Number	Primer 2-1					Primer 2-2			
		Allele frequency		Genotype frequency			Allele frequency		Genotype frequency	
		A	*B*	*AA*	*AB*	*BB*	*C*	*D*	*CC*	*CD*
Small Tail Han sheep	126	0.603	0.397	0.302 (38)	0.603 (76)	0.095 (12)	0.909	0.091	0.817 (103)	0.183 (23)
Dorset sheep	30	0.333	0.667	0.100 (3)	0.467 (14)	0.433 (13)	0.967	0.033	0.933 (28)	0.067 (2)

The numbers in the brackets are the individuals that belong to the respective genotypes

Table 2 Least squares mean and standard error for litter size of different genotypes of *GDF*9 gene in Small Tail Han sheep

Primer	Genotype	Number	Litter size
Primer 2-1	*AA*	38	$2.30^{a} \pm 0.17$
	AB	76	$2.25^{a} \pm 0.11$
	BB	12	$2.03^{a} \pm 0.21$
Primer 2-2	*CC*	103	$2.11^{a} \pm 0.10$
	CD	23	$2.88^{b} \pm 0.19$

Least squares means with the different superscripts for the same pair of primer differ significantly ($P < 0.01$)

PCR-SSCP, which resulted in an amino acid change Asn51Asp. And this mutation was not found in Small Tail Han sheep [16]. Hanrahan et al. discovered eight variants (G1 to G8) of *GDF*9 gene in Cambridge and Belclare sheep by PCR-SSCP and sequencing. Three nucleotide changes of the eight polymorphisms did not alter amino acids (G2, G3 and G5). Four G→A mutations of eight SNPs resulted in amino acid changes (G1, G4, G6 and G7) which occurred at a position before the furin processing site or unprocessed protein and were unlikely to affect the mature active coding region. However, G8 variant caused serine to phenylalanine 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 *GDF*9 in sheep [6]. Guan et al. detected the G8 mutation of *GDF*9 gene in Hu sheep by PCR–RFLP and the mutation rate was rare (2/310, 0.645%) [17]. Gao found two SNPs (T558C and T692C [Leu231Thr]) in exon 2 of *GDF*9 in Small Tail Han, Tong, Tan and Oula sheep by PCR-SSCP [18]. Recently, two variants of sheep *GDF*9 were reported, which were $FecG^{SI}$ (T1034G mutation of CDS region resulting in Phe27Cys change of mature protein) in Brazilian Santa Ines sheep [7] and FecTT (A1279C mutation of CDS region resulting in Ser109Arg of mature protein) in Icelandic Thoka sheep [9]. Chang et al. identified G2 mutation of *GDF*9 gene in Small Tail Han, White Suffolk, Texel and Tibetan sheep by PCR-SSCP [19]. Chen et al. 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 [20]. Besides, G1 mutation of *GDF*9 was identified in Moghani and Ghezel sheep in Iran [21] and Garole sheep in India [22]. Moreover, G8 mutation of *GDF*9 was not detected in Small Tail Han, Hu, Dorset, Texel and German Mutton Merino sheep [23], Suffolk, Dorset, Charollais, Romney Hills and Chinese Merino sheep [17], Cele and Duolang sheep in P.R. China [24], Shal sheep in Iran [25], Tan sheep in P.R. China [26], Barbarine, Queue Fine de L'Ouest, Noire de Thibar, Sicilo-Sarde, D'man sheep in Nouth Africa [27]. The major mutations of *GDF*9 gene identified in sheep were summarized in Table 3.

In the present study, two mutations were detected in exon 2 of *GDF*9 in Small Tail Han and Dorset sheep, in which 477G→A mutation known as G3. A new mutation, 729G→T was identified in Small Tail Han and Dorset sheep, which resulted in amino acid change Gln→His at 243rd position. Moreover, the reasons for *DD* genotype at

Table 3 Major mutations of *GDF*9 gene identified in sheep

Mutation	Base change	Coding base (bp)	Coding residue (amino acid)	Mature peptide residue (amino acid)	Amino acid change	References
G1	G→A	260	87	–	Arg→His	Hanrahan et al. [6]
G2	C→T	471	157	–	Unchanged Val	
G3	G→A	477	159	–	Unchanged Leu	
G4	G→A	721	241	–	Glu→Lys	
G5	A→G	978	326	8	Unchanged Glu	
G6	G→A	994	332	14	Val→Ile	
G7	G→A	1111	371	53	Val→Met	
G8	C→T	1184	395	77	Ser→Phe	
$FecG^{SI}$	T→G	1034	345	27	Phe→Cys	Melo et al. [7]
FecTT	A→C	1279	427	109	Ser→Arg	Nicol et al. [9]
A152G	A→G	152	51	–	Asn→Asp	Li et al. [16]
T692C	T→C	692	231	–	Leu→Thr	Gao [18]
G729T	G→T	729	243	–	Gln→His	Present study

site 729G→T was not detected in two sheep breeds joined were (1) genotype *DD* was really not exist in Small Tail Han and Dorset sheep, (2) the sample numbers detected were relatively less.

Association between reproduction and GDF9 gene in sheep

Chu et al. reported that least squares means of litter size in the first and the second parity for genotype *AA* were 0.30 ($P < 0.05$) and 0.77 ($P < 0.0001$) lambs more than those for genotype *AB* detected in exon 1 of the *GDF*9 gene in Small Tail Han sheep, respectively [28].

Hanrahan et al. found that the least squares means for ovulation rate of Belclare ewes with wild type and G8 mutation heterozygote were 1.92 ± 0.28 ($n = 11$) and 2.67 ± 0.89 ($n = 1$), respectively, and which of Cambridge ewes were 2.27 ± 0.49 ($n = 10$) and 4.28 ± 0.31 ($n = 28$), respectively. Ewes with G8 mutation homozygote were total infertility in Belclare and Cambridge sheep [6]. Gao discovered that ewes with genotype *CC* had 0.63 ($P < 0.05$) lambs more than those with genotype *CD* at the P2 locus of *GDF*9 exon 2 in Small Tail Han sheep [18]. Regarding the $FecG^{SI}$ mutation, the average number of corpus luteum in the homozygous ewes was more than heterozygote or wild type animals after estrus synchronization, which were 2.4 ± 0.2, 1.3 ± 0.1 and 1.3 ± 0.2, respectively ($P < 0.001$) [7]. Concerning G1 variant of *GDF*9 gene in Moghani and Ghezel sheep ($n = 97$), ewes with G1 heterozygous mutation could increase fecundity (out of thirteen ewes with G1 heterozygote, seven gave twin birth and the others gave single birth), but ewes with G1 homozygote ($n = 4$) found to be fertile and all gave single birth. The only one infertile ewe was homozygous for both G1 variant of *GDF*9 gene and B2 ($FecX^G$) variant of *BMP*15 gene [21]. As for FecTT mutation in Thoka sheep, ewes in heterozygote were 0.68 more lambs per ewe lambing than wild type animals and infertility in homozygote [9].

In the present study, ewes with genotype *CD* had 0.77 ($P < 0.01$) lambs more than those with genotype *CC*. These results preliminarily showed that the *GDF*9 gene is either a major gene that influences the prolificacy in Small Tail Han sheep or a molecular genetic marker in close linkage with such a gene. Further extensive sampling and DNA analysis would be required to verify these results.

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