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Polymorphisms of the bovine growth differentiation factor 9 gene associated with superovulation performance in Chinese Holstein cows

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ABSTRACT. Growth differentiation factor 9 (GDF9) belongs to the transforming growth factor β superfamily and plays a critical role in ovarian follicular development and ovulation rate. We examined the bovine *GDF9* gene polymorphism and analyzed its association with superovulation performance. Based on the sequence of the bovine *GDF9* gene, six pairs of primers were designed to detect single nucleotide polymorphisms of two exons and intron 1 of *GDF9* using polymerase chain reaction-single-strand conformation polymorphism. Only the products amplified by primer 3-1 displayed polymorphisms. Sequencing revealed two mutations of A485T and A625T in intron 1 of the *GDF9* gene in 171 Chinese Holstein cows treated for superovulation. Association analysis showed that these two single nucleotide polymorphisms of A485T and A625T had significant effects

on the number of transferable embryos ($P < 0.05$), and the A625T polymorphism was significantly associated with the total number of ova ($P < 0.05$). In addition, a significant additive effect on the number of transferable embryos was detected in polymorphisms of A485T ($P < 0.05$). This study is the first to identify two polymorphisms in bovine *GDF9* and describe their correlation with superovulation traits in Chinese Holstein cows.

Key words: Chinese Holstein cows; Polymorphism; Superovulation; Growth differentiation factor 9

INTRODUCTION

Growth differentiation factor 9 (GDF9) belongs to the transforming growth factor β superfamily and plays a critical role in ovarian follicular development and ovulation rate (Elvin et al., 1999; McNatty et al., 2005). Previous studies have shown that GDF9 is involved in cumulus expansion, hyaluronic acid synthesis signaling, maintenance of an optimal oocyte microenvironment, and synergistic action along with bone morphogenetic protein 15 through the regulation of several key granulosa cell enzymes that are essential for normal ovulation, fertilization, and female reproduction (Eppig et al., 1997; Yan et al., 2001; McNatty et al., 2005). Genetic studies in mice have demonstrated critical roles for GDF9 in ovarian function (Dong et al., 1996). Given the central role of GDF9 in ovulation and reproduction, *GDF9* is a good candidate gene for mutations associated with reproductive performance. This gene has been widely studied in humans, sheep, and goats (McNatty et al., 2003). In sheep, several mutations in *GDF9* have been identified, including the $FecG^H$ (G8) mutation in Cambridge and Belclare sheep (Hanrahan et al., 2004), the FecTT mutation in Thoka sheep (Nicol et al., 2009), the G1 mutation in Iran Moghani and Ghezel sheep (Barzegari et al., 2010; Polley et al., 2010), the $FecG^E$ mutation in Brazilian Santa Ines sheep (Melo et al., 2008; Silva et al., 2011), and the G729T mutation in Small Tail Han sheep (Chu et al., 2011), and they are significantly associated with increased ovulation rate or infertility. Variants of *GDF9* are also associated with litter size in goat breeds - e.g., the G1189A mutation in Guizhou White goats (Du et al., 2008) and the A959C mutation in Jining Grey goats (Feng et al., 2011). In humans, mutations in *GDF9* have been identified in non-symptomatic premature ovarian failure patients, suggesting that altered GDF9 function may also be involved in ovarian dysfunction in women (Dixit et al., 2006; Laissue et al., 2006; Kovanci et al., 2007; Zhao et al., 2007). GDF9 mutations have also been detected in mothers of dizygotic twins, and those variants are significantly related to increased ovulation rate (Palmer et al., 2006), which suggests that some variants may also be linked to a polyovulatory phenotype (Montgomery et al., 2004). Furthermore, Wang et al. (2010) have found that the GDF9 G546A mutation is significantly correlated with poor ovarian stimulation and *in vitro* fertilization outcomes in women with diminished ovarian reserve. However, studies of *GDF9* and bovine reproduction are relatively rare.

Superovulation is a major component of embryo transfer techniques and has been practiced in cattle for more than 60 years. However, it has undergone little improvement during the last 20 years (Hasler, 2003). The major limitation to its development is the reli-

ance on follicle-stimulating hormone (FSH)-induced superovulation and the large variability in response to treatments (Mapletoft et al., 2002). Superovulation is a time-consuming and expensive procedure (Rico et al., 2009). Given the important role of GDF9 in ovulation, fertilization, and ovarian stimulation response, the *GDF9* gene was chosen as a potential candidate gene for superovulation in Chinese Holstein cows. Bovine *GDF9* spans approximately 2.9 kb and contains two exons and one intron in which exons 1 and 2 and the single intron span 431, 1360, and 965 bp, respectively. The objectives of the present study were to 1) detect single nucleotide polymorphisms (SNPs) of *GDF9* in Chinese Holstein cows using polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), and 2) investigate the association between SNPs of *GDF9* and superovulation performance in Chinese Holstein cows.

MATERIAL AND METHODS

Experimental cows and sampling

All procedures involving animals were approved by the Animal Care and Use Committee of Huazhong Agricultural University. The study included 171 Chinese Holstein cows with no relatedness in three generations and which had been successively superovulated one to four times at monthly intervals between 2007 and 2010 using FSH (Beijing Amber Embryo Technology Co. Ltd., China; Table S1). These animals were produced through crossbreeding with Chinese Yellow cattle and imported Holstein cows and were designated Chinese Holsteins by the Chinese Ministry of Agriculture in 1992. Approximately 10 mL blood was collected aseptically from the jugular vein of each cow and kept in a tube containing anticoagulant ethylenediaminetetraacetic acid (EDTA) and held on ice until delivery to the laboratory. Genomic DNA was extracted from white blood cells using a standard phenol-chloroform protocol (Joseph and David, 2002) and stored at -20°C for later use.

Superovulation procedure and embryos harvest

Superovulation was induced via eight intramuscular injections of FSH (Folltropin-V, Bioniche Animal Health Canada Inc.) for 4 days beginning on day 4 after the insertion of an intravaginal progesterone-releasing device (PRID1, Ceva Sante Animale, France). The injections were given at 12-h intervals in a dose step-down manner - i.e., doses of 5.4, 4.4, 3.4, and 2.4 mL on each day - as described in previous studies (Tang et al., 2011). Each cow was twice given 4 mL 0.15 mg/mL prostaglandin F2α (Qilu Animal Health Products Co., Ltd., China) along with the sixth and seventh FSH treatments. The progesterone-releasing device was removed after the second prostaglandin treatment. Estrus detection by visual observation was carried out twice daily for at least 30 min at approximate 12-h intervals. Any cow standing to be mounted by a herdmate was considered to be in heat. All cows were inseminated with frozen-thawed semen from one bull of known good fertility 12 and 24 h after standing estrus. Uteri were non-surgically flushed (Newcomb et al., 1978) on day 7 after artificial insemination by an experienced technician using standard techniques. Each uterine horn was flushed with 500 mL phosphate-buffered saline. Prostaglandin F2α was administered to all cows immediately after the flushing procedure. The recovered lavage was filtered through an

embryo filter (Miniflush Embryo Recovery System, mesh size 44 µm, Minitube, Germany). The fluid was examined for oocytes or embryos under a stereomicroscope, and embryos were isolated and graded as A (excellent), B (good), C (fair), or D (poor) according to the criteria of the International Embryo Transfer Society. After flushing, recovered ova and embryos were separated into transferable embryos (grades A, B, and C), degenerate embryos (grade D), and unfertilized ova. Unfertilized ova were counted on the basis of corpora lutea. Cows with no oocytes or embryos recovered in two successive superovulation treatments were defined as non-responders or cows without superovulation response. Six cows without superovulation response were found in this experimental population (see Table S1). The traits of superovulation included total number of ova (TNO), number of transferable embryos (NTE), number of unfertilized ova, and number of degenerated embryos (NDE).

Primer synthesis and PCR conditions

Six pairs of primers were designed according to the bovine *GDF9* DNA sequence (GenBank accession No. 282574). All of exon 1 and parts of intron 1 and exon 2 of *GDF9* were amplified, in which primers 1-1 and 1-2 were used to amplify exon 1, primers 2-1 and 2-2 were used to amplify part of the exon 2, and primers 3-1 and 3-2 were used to amplify part of intron 1 (Table S2). PCR was performed in 20-µL mixtures containing 10 pmol primers, 200 µM deoxyribonucleotide triphosphate, 2 µL 10X reaction buffer containing 1.5 mM $MgCl_2$, 0.5 U Taq-DNA polymerase (Promega, Madison, WI, USA), and 50 ng genomic DNA as a template. After denaturation at 94°C for 5 min, 34 amplification cycles of 94°C for 45 s, 58° to 61°C (primer specific) for 45 s, an extension at 72°C for 45 s, and a final extension at 72°C for 10 min, PCR products were electrophoretically detected on 2.0% agarose gel (5 V/cm) via staining with ethidium bromide.

SSCP detection and sequencing

Aliquots of 4 µL PCR products mixed with 12 µL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole, and 0.025% bromophenol blue) were heated for 10 min at 99°C and chilled on ice. Denatured DNA was subjected to 10% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 29:1) in 1X Tris-borate EDTA buffer at constant voltage (140 V) for 14 to 16 h. The gel was stained with 0.1% silver nitrate, and the PCR products with different SSCP patterns were sequenced in an ABI PRIZM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) to identify the SNPs.

Genotyping

SNPs in *GDF9* identified using PCR-SSCP and sequencing did not affect common restriction endonuclease cleavage sites. Therefore, primers 3-3 and 3-4 were designed to introduce the *Nsi*I and *Dra*I restriction enzyme sites, respectively, to detect the SNPs according to the sequencing results (Table S3). The digestion mixture contained 4.5 µL PCR products, 1X digestion buffer, and 5.0 U each enzyme and was digested at 37°C overnight. Fragments were detected with 10% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 29:1) and subsequent silver staining.

Statistical analysis

Gene frequencies were determined through direct counting, and Hardy-Weinberg equilibrium was analyzed with the chi-square test using SAS 8.1 (SAS Institute Inc., Cary, NC, USA). Pairwise linkage disequilibrium was measured using the online SHEsis software (Shi and He, 2005). Both additive and dominant effects were estimated using models in the REG procedure of SAS 8.1 (Liu, 1998). The relationship between *GDF9* genotypes and superovulation traits including TNO, NDE, NTE, and number of unfertilized ova were calculated using the general linear model of SAS 8.1 (Yang et al., 2010). Fixed effects of genotypes (three for each mutation locus), age (1.0-2.0, 2.1-3.0, and 3.1-5.0 years) (Malhi et al., 2008), year (2007, 2008, and 2010), season [spring (March to May), summer (June to August), autumn (September to October), and winter (December to February)], repeat time (four times), and their interactions were independent variables. The variables, which were measured in two to four successive treatments, were considered repeated measures (Yang et al., 2010). Their effects on superovulation traits were analyzed using the general linear model procedure and compared using the Duncan multiple range test (SAS 8.1). Only factors that affected the records significantly (genotype, season, and age; $P < 0.05$) were fitted in the final statistical model:

$$y_{ik} = \mu + G_i + A_k + S_j + e_{ikj}$$

where y_{ik} is the phenotypic value of traits; μ is the mean population mean; G_i is the fixed effect of genotype; A_k is the fixed effect of age; S_j is the fixed effect of season, and e_{ikj} is the random residual error.

RESULTS

Genotypic and allelic frequencies

PCR products of six pairs of primers were detected with 1.5% agarose gel electrophoresis. The amplified products were consistent with the target fragments and had good specificity, which could be directly analyzed with SSCP. Only the PCR products amplified by primer 3-1 displayed polymorphisms (Figure S1), and sequencing revealed two nucleotide mutations, A485T and A625T, in intron 1 of *GDF9* (Figure 1). Mutation A485T destroyed the introduced *Nsi*I endonuclease restriction site and resulted in three genotypes: g.485AA (24 and 184 bp), g.485AT (24, 184, and 208 bp), and g.485TT (208 bp) (Figure S2A). Mutation A625T destroyed the introduced *Dra*I endonuclease restriction site and resulted in three genotypes: g.625AA (25 and 351 bp), g.625AT (25, 351, and 376 bp), and g.625TT (376 bp) (Figure S2B). The frequencies of the g.485A, g.485T, g.625A, and g.625T alleles were 0.708, 0.292, 0.465, and 0.535, respectively, in the analyzed population, and the A625T polymorphism was in Hardy-Weinberg disequilibrium ($P < 0.05$; Table 1). The linkage disequilibrium status of two SNPs was weak ($r^2 = 0.270$), and we declined to perform further haplotype analysis.

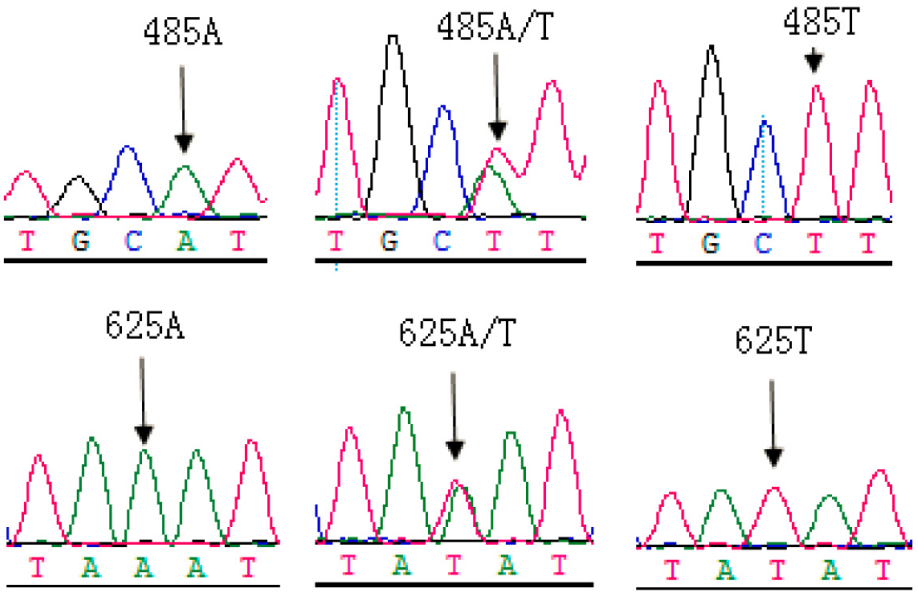

Figure 1. Sequence comparison of different genotypes of the *GDF9* gene in Chinese Holstein cows by primer 3-1.

Table 1. Allelic and genotypic frequencies for sequence polymorphisms in the *GDF9* gene in Chinese Holstein cows and the percentage of non-responders in the first superovulation treatments.

Locus	Genotype	Genotypic frequency	Allele frequency	Genotype distribution of non-responders	χ^2 value (Hardy-Weinberg equilibrium)
A485T	AA (82)	0.480	A 0.708	1.2 (2)	1.751 (P > 0.05)
	AT (78)	0.456	T 0.292	1.8 (3)	
	TT (11)	0.064		0.6 (1)	
A625T	AA (25)	0.146	A 0.465	0.6 (1)	13.43 (P < 0.05)
	AT (109)	0.637	T 0.535	1.8 (3)	
	TT (37)	0.216		1.2 (2)	

Number of cows is shown in parentheses.

Association of genotypes with superovulation traits

The results of association analysis between *GDF9* genotypes and superovulation traits are given in Table 2. In polymorphic locus 485, cows with the g.485TT genotype had an NTE higher than cows with the g.485AA and g.485AT genotypes ($P < 0.05$; see Table 2). In polymorphic locus 625, individuals with the g.625AA genotype collected more ova than individuals with the g.625AT genotype ($P < 0.05$) and had an NTE higher than g.625AT individuals ($P < 0.05$; see Table 2). Conversely, cows with the g.625AA genotype produced more degenerated embryos than cows with the g.625AT and g.625TT genotypes ($P < 0.05$). Both additive and dominance effects of the genotypes are shown in Table 2, and the A485T polymorphisms had a significant additive effect on NTE. Furthermore, TNO and NTE were significantly affected by season ($P < 0.05$); maximal TNO and NTE occurred during the spring (Table S4). The age of the cow at the time of treatment had significant effects on TNO and NTE ($P <$

0.05); least-square means of TNO and NTE increased with the increase in age from 1.0 to 3.0 years (Table S5). No significant differences were found among other genotypes and repeated superovulation (Tables S6 and S7).

Table 2. Association between *GDF9* genotypes and superovulation traits.

Traits	Genotype at A485T			Additive effect	Dominance effect
	g.485AA (82)	g.485AT (78)	g.485TT (11)		
NUO	1.510 ± 0.151	1.618 ± 0.169	1.067 ± 0.279	-0.132 ± 0.240	-0.122 ± 0.144
NDE	1.776 ± 0.136	1.730 ± 0.138	1.933 ± 0.371	0.033 ± 0.209	0.033 ± 0.126
NTE	3.779 ± 0.234[a]	3.701 ± 0.270[a]	5.267 ± 1.030[b]	0.376 ± 0.395*	0.282 ± 0.238
TNO	7.065 ± 0.335	7.050 ± 0.374	8.267 ± 1.139	0.276 ± 0.544	0.193 ± 0.327
Traits	Genotype at A625T			Additive effect	Dominance effect
	g.625AA (25)	g.625AT (109)	g.625TT (37)		
NUO	1.273 ± 0.198	1.654 ± 0.143	1.353 ± 0.223	0.040 ± 0.183	-0.170 ± 0.114
NDE	2.364 ± 0.280[a]	1.630 ± 0.112[b]	1.759 ± 0.204[b]	0.012 ± 0.160	-0.065 ± 0.100
NTE	4.662 ± 0.526[a]	3.446 ± 0.205[b]	4.397 ± 0.420[ab]	-0.05 ± 0.302	0.078 ± 0.187
TNO	8.288 ± 0.690[a]	6.730 ± 0.295[b]	7.509 ± 0.544[ab]	0.189 ± 0.416	0.133 ± 0.258

Data are reported as least square means ± standard error. NUO = number of unfertilized ova; NDE = number of degenerate embryos; NTE = number of transferable embryos; TNO = total number of ova. Values with different superscript letters within the same line indicate significant differences ($P < 0.05$), and values with the same letters, do not differ. *A485T polymorphisms had a significant effect on NTE ($P < 0.05$).

DISCUSSION

GDF9 is thought to be required for ovarian folliculogenesis and, as such, is presumably a candidate gene for ovulation rate (Dong et al., 1996; McNatty et al., 2003, 2005; Orisaka et al., 2006). Currently, 10 mutations have been identified in the exons and intron of sheep *GDF9*, and seven of them result in amino acid substitutions (Hanrahan et al., 2004; Melo et al., 2008; Nicol et al., 2009). *GDF9* also displays abundant polymorphisms in goats. Nine mutations have been identified in the exons and intron of goat *GDF9*, and four of the mutations result in amino acid substitutions (Du et al., 2008; Chu et al., 2011; Feng et al., 2011). However, studies of mutations in bovine *GDF9* have been relatively rare. In the present study, the combined results from sequence data and SSCP analysis of all exon 1 and parts of intron 1 and exon 2 of bovine *GDF9* revealed only two SNPs in intron 1 of *GDF9* in 171 Chinese Holstein cows. The results imply that the *GDF9* gene is very conservative in cows and may play a critical role in bovine reproduction. The absence of detectable SNPs in the *GDF9* exon sequence may also be due to the small number of animals sampled. Therefore, our results should be further validated in larger cow populations.

The variants of *GDF9* are significantly associated with increased ovulation rate, litter size, or sterility in sheep and goats (Du et al., 2008; Chu et al., 2011; Feng et al., 2011; Silva et al., 2011). Similarly, variants of *GDF9* have been significantly correlated with ovulation rate, ovarian stimulation response, and *in vitro* fertilization and are involved in ovarian dysfunction in women (Dixit et al., 2006; Laissue et al., 2006; Kovanci et al., 2007; Zhao et al., 2007). In the present study, the A485T and A625T SNPs had significant effects on NTE, and the A625T polymorphism was significantly associated with TNO. In addition, a significant additive effect on NTE was detected in polymorphisms of A485T. Conversely, the A625T polymorphism had a negative effect

on NDE. The negative effects on NDE and positive effects on TNO and NTE might be explained by a positive genetic correlation between these traits. In this study, although the analyzed polymorphisms, which were found in the intron, did not directly alter any amino acid residue, they are close to the exon-intron junction, which is important for messenger RNA splicing (Krawczak et al., 2007) and this positioning might be directly related to functional variation.

SNPs in introns have been observed to affect phenotype, gene expression, and consequent function. For example, the G3072A mutation has been identified in intron 3 of the pig insulin-like growth factor 2 gene and is significantly associated with muscle growth through the regulation of the expression of insulin-like growth factor 2 messenger RNA in postnatal muscle (Van Laere et al., 2003). An SNP (T392C) in intron 5 of the pig RING finger protein 4 gene has been found to be significantly correlated with litter size by Niu et al. (2009) in two pig populations. Pang et al. (2011) have reported that the T6765C mutation in intron 2 of the vascular endothelial growth factor gene is associated with early development and growth in Chinese domestic cattle. One SNP (A3481C) in intron 1 of the adipocyte fatty acid-binding protein gene is significantly associated with meat quality traits in Junmu No. 1 white swine, and immunoblot analysis has shown that individuals with the g.3481CC genotype had an adipocyte fatty acid-binding protein expression level significantly lower than that of individuals with the g.3481AC genotype (Gao et al., 2011). Therefore, variations in *GDF9* sequences may have important regulatory roles and be directly related to functional variations, but further verification is needed.

In our experiment, TNO and NTE were significantly affected by season. Maximal TNO and NTE occurred during the spring season, which is inconsistent with a previous finding (Lee et al., 2012) that NTE is highest during the fall. However, Lerner et al. (1986) have reported that season has no significant effects on NTE. This difference in results may be due to the variations in environmental and nutritional conditions between studies. The age of cows at the time of treatment in our study was significantly associated with TNO and NTE. TNO and NTE increased with an increase of age from 1.0 to 3.0 years, which is consistent with previous findings (Bastidas and Randel, 1987), but then decreased. This result may be attributable to the small sample sizes, especially the small number of cows aged >3.0 years.

In conclusion, we identified two polymorphisms (A485T and A625T) in bovine *GDF9* and their correlation with superovulation traits in Chinese Holstein cows. Owing to a lack of functional data, the conclusion was not solid, and further studies are necessary for functional validation of these polymorphisms.

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Supplementary material

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