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Polymorphism of the inhibin βA gene and its relationship with superovulation traits in Chinese Holstein cows

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ABSTRACT. Inhibin is a major regulator of secretion of follicle-stimulating hormone, which is involved in follicular development and regulation of steroidogenesis in females. The objectives of this study were to detect polymorphisms of the bovine inhibin beta-A subunit (INHβA) gene and to evaluate its associations with superovulatory responses in 171 Chinese Holstein cows treated for superovulation. Polymerase chain reaction-restricted fragment length polymorphism revealed a C>T transition determining the *Sty*I polymorphism at position 7639 in intron I of the bovine *INHβA* gene, and three genotypes (CC, CT, and TT) were detected. The frequencies of the three genotypes showed a tendency for CT > TT > CC, and this polymorphism was in Hardy-Weinberg equilibrium. Statistical analysis revealed no significant differences of least square means for superovulation traits among the three genotypes (P > 0.05). These results demonstrate, for the first time,

that the detected loci of the *INHβA* gene have no significant effects on superovulation performance in Chinese Holstein cows.

Key words: Chinese Holstein cows; Superovulation; *INHβA*; RFLP

INTRODUCTION

Superovulation is a major component of the embryo transfer technique that has been used in the cattle industry for over 60 years. However, ovarian responsiveness to gonadotropins remains highly variable among individuals, and such unpredictability has become a major limiting factor for the development of embryo transfer (Mapletoft et al., 2002; Mapletoft and Hasler, 2005). Although several sources of this variability have been identified, including follicle stage, age, parity, and nutritional and gonadotropin reparation (Kafi and McGowan, 1997; Thatcher et al., 2001; Bó et al., 2002; Mossa et al., 2007; Malhi et al., 2008), no significant improvement in the superovulatory response has been observed since the early 1990s (Cory et al., 2013). The candidate gene approach has been widely used in genetic association and biomarker studies in animals and humans (Tabor et al., 2002). Therefore, the candidate gene approach appears to be useful to identify potential markers that are correlated with superovulation performance for donor selection.

Inhibin was named for its biological role in inhibiting pituitary synthesis and secretion of the follicle-stimulating hormone (FSH) (Bernard et al., 2001). Neutralization of its biological activity by active (Medan et al., 2004) and passive immunization (Ishigame et al., 2004) against inhibin led to increases in pituitary secretion and the plasma concentration of FSH, which was thought to stimulate development of, or select for, increased follicles in the final maturation stage (Shi et al., 2000; Ozawa et al., 2001; Medan et al., 2004; Ishigame et al., 2004). In addition, because of its inhibitory action on FSH secretion, inhibin has been widely used in the treatment of superovulation. Previous studies have demonstrated that immunization against inhibin could improve the ovarian response to superovulation, resulting in increased ovulation rates and higher yields of transferable embryos in sheep (D'Alessandro et al., 1999), heifers or cows (Li et al., 2009; Mei et al., 2009), and water buffalo (Li et al., 2011). Therefore, inhibin is a potential candidate gene for superovulation performance.

There are two forms of inhibin: inhibin A (α-βA) and inhibin B (α-βB), and the inhibin subunits are encoded by three separate genes: *INHα*, *INHβA*, and *INHβB* (Bernard et al., 2001). *INHβA* was chosen as an intragenic marker for boar and bull fertility, and was found to be significantly associated with sperm quality traits in pigs (Lin et al., 2006) and cattle (Sang et al., 2011). Furthermore, *INHβA* was confirmed to be an important candidate gene for fecundity in the goat. Chu et al. (2007) identified 21 single nucleotide polymorphisms (SNPs) of *INHβA*, which significantly influenced litter size in Small Tail Han sheep. Hou et al. (2012) detected one SNP, C936T, of *INHβA* in Xinong Saanen, Guanzhong, and Boer goats, but did not show statistically significant associations with litter size. To date, no significant association of *INHβA* polymorphisms with female reproductive traits has been revealed in bovines. In this study, the association between *INHβA* polymorphisms and superovulation traits was evaluated in 171 Chinese Holstein cows. To our knowledge, the present study is the first to report the distribution of the *Sty*I polymorphism of the *INHβA* bovine gene in order to investigate its prognostic significance in superovulation responsiveness.

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 unrelated Chinese Holstein cows of three generations, including 6 cows without superovulation response, 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; see Yang et al., 2012). Approximately 10 mL blood was collected aseptically from the jugular vein of each cow, which was kept in a tube containing the anticoagulant ethylenediaminetetraacetic acid on ice until delivery to the laboratory. Genomic DNA was extracted from white blood cells using a standard phenol-chloroform protocol and stored at -20°C for later use.

Superovulation procedure and embryo 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 administered at 12-h intervals in a dose step-down manner; doses of 5.4, 4.4, 3.4, and 2.4 mL each day, as described in previous studies (Yang et al., 2012). Each cow was given 4 mL (0.15 mg/mL) prostaglandin $F_{2\alpha}$ (Qilu Animal Health Products Co., Ltd., China) twice, along with the sixth and seventh FSH treatments. The progesterone-releasing device was removed after the second prostaglandin treatment. Estrus detection was carried out twice daily by visual observation for at least 30 min at approximate 12-h intervals. Any cow appearing 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 starting estrus. Uteri were non-surgically flushed on the 7th day following artificial insemination by an experienced technician using standard techniques. Each uterine horn was flushed with 500 mL phosphate-buffered saline. Prostaglandin $F_{2\alpha}$ 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 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. The superovulation traits analyzed included total number of ova (TNO), number of transferable embryos (NTE), number of unfertilized ova (NUO), and number of degenerated embryos (NDE).

Primer synthesis and polymerase chain reaction (PCR) conditions

One reported SNP of *INHβA*, C7639T (Sang et al., 2011), which destroys the *Sty*I en-

donuclease restriction site, was selected as a marker to evaluate its effects on superovulation traits of Chinese Holstein cows. Primer sequences (F: 5'-GGTGGTTGTTACTGTTTATC-3' and R: 5'-CAGGGTTTCAGAAGTTGG-3') and reaction conditions for the C7639T polymorphism were selected according to a previous study (Sang et al., 2011). PCR was performed in a total volume of 20 µL containing 10 pmol primers, 200 µM dNTP, 2 µL 10X reaction buffer [1.5 mM $MgCl_2$, 0.5 U Taq-DNA polymerase (Promega, Madison, WI, USA)], and 50 ng genomic DNA as the template. After denaturation at 94°C for 5 min, 34 amplification cycles were performed comprising denaturation at 94°C for 45 s, annealing at 64°C for 45 s, and extension at 72°C for 45 s, followed by a further 10-min extension at 72°C.

Genotyping

To detect the SNP *INHβA* C7639T, the *INHβA* PCR products were digested with *Sty*I (Sang et al., 2011) (TaKaRa, Tokyo, Japan). The digestion mixture contained 4 µL PCR products, 1X digestion buffer, and 3.0 U of each enzyme, and was digested overnight at 37°C. Fragments were separated on 2% agarose gels and were visualized with GelRed staining.

Statistical analysis

Gene frequencies were determined through direct counting, and Hardy-Weinberg equilibrium was analyzed using the chi-squared test with the SAS 8.1 software (SAS Institute Inc., Cary, NC, USA). Fixed effects of the independent variables, *INHβA* genotypes (CT, TT, CC), 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, summer, autumn, and winter), repeat time (four times), and their interactions were determined. The variables were considered repeated measures as each variable was measured in two to four successive treatments (Yang et al., 2012). Their effects on superovulation traits including TNO, NDE, NTE, and NUO were analyzed using the general linear model procedure and were compared using the Duncan multiple range test (SAS 8.1) (Tang et al., 2013). Only factors with significant effects (genotype, season, and age; $P < 0.05$) were fitted in the final statistical model:

$$y_{ikj} = u + G_i + A_k + S_j + e_{ikj}$$

where y_{ikj} is the phenotypic value of traits, u 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

In order to best describe different genotypes of the C>T mutation of the bovine *INHβA* gene, we named the alleles INHβA-C and INHβA-T. The mutation C>T is located at position 7639 in intron I and destroys the *Sty*I endonuclease restriction site. Therefore, *Sty*I digestion of amplified products resulted in two fragments (150 and 138 bp) for the INHβA-C allele and

one fragment (288 bp) for the INHβA-T allele (Figure 1). The frequencies of the C and T alleles were 0.456 and 0.544, respectively, and the frequency of the three genotypes showed a tendency of CT > TT > CC (Table 1). This polymorphism was determined to be in Hardy-Weinberg equilibrium (Table 1).

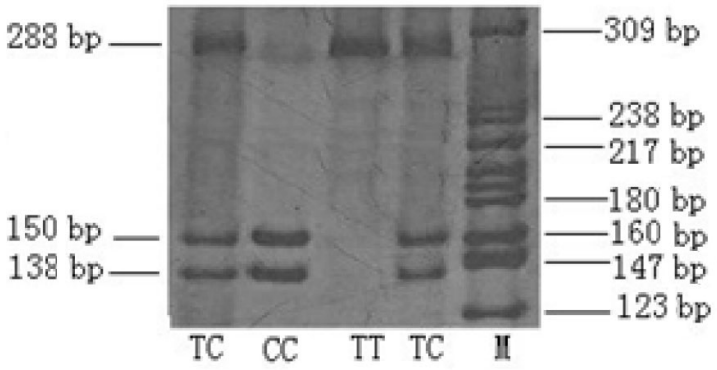

Figure 1. Representative genotyping of the *INHβA* gene at loci C7639T by a polyacrylamide gel electrophoresis. *Lane M* = pBR322 DNA/*Msp*I marker.

Table 1. Allelic and genotypic frequencies for sequence polymorphisms in the *INHβA* gene and the percentage of non-responders for each genotype.

Locus	Genotype	Frequency	Allele frequency	Non-responders	χ^2 value
INHβA (C7639T)	CC (31)	0.181	C 0.456	0.6 (1)	1.987 (P > 0.05)
	CT (94)	0.550	T 0.544	2.3 (4)	
	TT (46)	0.269		0.6 (1)	

Influence of fixed effects on superovulation traits

Superovulation traits were significantly influenced by age and season (all P < 0.05) (see Tang et al., 2013). The *INHβA* genotype had no significant influence on superovulation traits in Chinese Holstein cows (P > 0.05). The least squares means and standard errors for superovulation traits of different *INHβA* genotypes in Chinese Holstein cows are shown in Table 2.

Table 2. Association analysis between *INHβA* genotypes and superovulation traits in Chinese Holstein cows.

Superovulation traits	Genotypes		
	CC (31)	CT (94)	TT (46)
NUO	1.652 ± 0.229	1.507 ± 0.226	1.652 ± 0.259
NDE	1.800 ± 0.283	1.786 ± 0.260	1.730 ± 0.274
NTE	4.038 ± 0.453	3.486 ± 0.415	3.567 ± 0.440
TNO	7.490 ± 0.595	6.779 ± 0.514	7.148 ± 0.971

NUO = number of unfertilized ova; NDE = number of degenerated embryos; NTE = number of transferable embryos; TNO = total number of ova.

DISCUSSION

Inhibin is a major regulator of FSH secretion, which is involved in the regulation of follicular development and steroidogenesis in the female. Passive (Medan et al., 2004) and active (Ishigame et al., 2004) immunization against inhibin results in increased pituitary secretion and plasma concentrations of FSH, which could stimulate follicle development and improve the ovarian response to superovulation, resulting in increased ovulation rates and higher yields of transferable embryos in sheep (D'Alessandro et al., 1999), heifers or cows (Li et al., 2009; Mei et al., 2009), and water buffalo (Li et al., 2011). Due to the important role of inhibin in FSH secretion and as a growth factor in the ovary, *INHβA* was confirmed to be an important candidate gene for improving reproductive performance (Xue et al., 2004; Phillips, 2005; Chu et al., 2007).

Jaeger and Hiendleder (1994) analyzed 1000 lambing records and found that *INHβA* had obvious genetic effects on litter size in sheep. An animal model analysis revealed that the substitution effect of *INHβA* reached 0.04 lambs in Merinolandschaf ewes and 0.09 lambs in East Friesian Milksheep ewes (Hiendleder et al., 1996a). One *Taq*I restriction fragment length polymorphism was detected in sheep breeds, which was significantly associated with litter size (Leyhe et al., 1994; Hiendleder et al., 1996b). Chu et al. (2007) identified 21 new SNPs of *INHβA* that significantly influenced litter size in Small Tail Han sheep. Furthermore, Hou et al. (2012) identified a synonymous mutation, C936T, of *INHβA* in three goat breeds, but a functional analysis revealed that this mutation had no effects on its biological function and was not associated with litter size. To date, no significant association of *INHβA* polymorphisms with female reproductive traits have been identified in bovines. In the present study, one SNP (C7639T) in intron I of the *INHβA* gene was identified in Chinese Holstein cows and association analysis revealed that this mutation (C7639T) had no significant influence on superovulation traits.

The results of the present study are the first to demonstrate that the *INHβA* C7639T mutation has no significant effect on superovulation traits in Chinese Holstein cows, but should be further validated using larger cow populations.

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