



Association between SNPs within Prolactin Gene and Milk Performance Traits in Holstein Dairy Cattle*

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ABSTRACT : Prolactin plays an important role in mammary gland development, milk secretion initiation and maintenance of lactation, so the bovine prolactin gene is considered as a potential quantitative trait locus affecting milk performance traits in dairy cattle. In this study, to determine the association between prolactin and milk performance traits, the genetic polymorphisms of a part of the prolactin gene were detected in a population of 649 cows of Chinese Holstein Dairy Cattle. Three SNPs in the promoter and one SNP in the intron1 of prolactin were identified, which was A/C (-767), G/T (-485), C/A (-247), and C/T (427), respectively. Statistical results indicated that one of SNP within promote, CHBP2, was significantly associated with milk yield ($p < 0.01$), fat yield ($p < 0.05$), protein yield ($p < 0.01$), and protein percentage ($p < 0.05$). The cows with genotype BB of CHBP2 had significantly higher milk yield ($p < 0.01$), fat yield ($p < 0.05$), and protein yield ($p < 0.01$) than those of cows with genotype AA, while cows with genotype AA showed the highest protein percentage ($p < 0.05$). In addition, based on the nine major haplotypes constructed from the four SNPs, the association analysis between diplotypes and milk performance trait was carried out. Results showed that the least square mean for fat yield of diplotype H2H8 was significantly higher than those of other eleven diplotypes ($p < 0.05$). Our findings implied that CHBP2 and H2H8 of prolactin would be useful genetic markers in selection program on milk performance traits in Holstein Dairy Cattle. (**Key Words :** Holstein Dairy Cattle, Prolactin, Haplotype, SNPs, Milk Performance Traits)

INTRODUCTION

Prolactin is a polypeptide hormone and plays crucial roles in mammary gland development, initiation and maintenance of lactation, which is synthesized in lactotrophic cells of the anterior pituitary gland of vertebrate. Previous studies showed that Prolactin could promote JAK2 or STAT5 transduction pathways and activate the promoter activity of casein gene with binding to its receptor (Yu Lee et al., 1998). Gene disruption experiments also proved the mandatory roles of Prolactin and its receptor for mammary gland development, lactogenesis and expression of milk protein genes (Horseman et al., 1997). Therefore, the bovine prolactin gene seems to be an excellent candidate for quantitative

trait loci (QTL) affecting milk production traits. Bovine prolactin spans approximately 9.4 kb and consists of five exons and four introns, which encodes a mature protein with 199 amino acids (Camper et al., 1984; Cao Xin et al., 2002). Hallerman et al. (1988) first mapped it on the bovine chromosome 23.

Single nucleotide polymorphisms (SNPs), one base changes including deletion, insertion and substitution, may play important roles in the regulation of genes transcription and amino acids sequences of mature proteins, which has been used to the association studies between candidate genes and complex traits in domestic animals (Kim et al., 2005; Meng et al., 2005; Yoon et al., 2005). So far, several SNPs within the bovine prolactin gene have been reported, but most studies only described its restriction fragment length polymorphism (RFLP) and single stranded-conformation polymorphism (SSCP) patterns without indicating their nucleotide changes and locations within the gene (Cowan et al., 1989; Hart et al., 1993; Zhang et al., 1994; Chung, 1997). Based on the sequences of 4 cDNA clones, 7 nucleotide substitutions were described by

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Table 1. Means and standard deviations of milk performance traits

Traits	Mean (kg)	SD ¹ (kg)
Milk yield	334.49	432.57
Fat yield	15.12	16.07
Protein yield	10.13	13.22
Fat percentage	0.040	0.185
Protein percentage	-0.012	0.086

¹ Standard deviation.

Sasavage et al. (1982). One of them, recognized by *RsaI*-PCR-RFLP has become a most commonly used genetic marker for genetic characterizations of multiple cattle populations and associations between prolactin variants and milk performance traits (Mitra et al., 1995; Chung et al., 1996; Chrenek et al., 1998; Udina et al., 2001; Dybus 2002). Most recently, six SNPs were identified within the exon4 of prolactin in Black-and-White cows, one of which was shown to be associated with milk yield and fat content (Pawel et al., 2005). In recent years, with the rapid investigation of a large number of SNPs, haplotype-based association analysis offers a powerful approach to identify genes affecting complex traits in humans besides single SNP analysis (Altshuler et al., 2000; Sachidanandam et al., 2001; Knoblach et al., 2002). However, up to now, as for SNPs within the promoter, exon1 and intron1 of bovine prolactin gene, few studies have been reported. Therefore, our aims of this study were to identify SNPs in the promoter, exon1 and intron1 of prolactin in the Chinese Holstein dairy cattles and further analyze their associations with milk performance traits.

MATERIALS AND METHODS

Animals

A total of 649 blood samples of Chinese Holstein dairy cattles were collected from 9 farms in Beijing, including twelve sire families with 43-65 daughters from each sire. Five milk performance traits, namely milk yield, fat yield, protein yield, fat percentage, and protein percentage, were used for association analysis. Table 1 presented the mean

and standard deviations of five traits.

PCR-SSCP analysis

Genomic DNA was isolated from blood samples by the phenol-chloroform method. Six pairs of primers were designed to amplify the promoter, exon1 and intron1 of bovine prolactin according to its genomic sequence (GenBank Accession No. AF426315 and X01452) using the Oligo6.0 software (Table 2). PCR reactions were carried out in a total of 25 µl volume including 50 ng of genomic DNA, 0.20 mM each dNTP, 2.5 mM MgCl₂, 0.20 mM primers and 0.5 U Taq DNA polymerase. Amplification condition was 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 45 sec and a final extension at 72°C for 10 min. The PCR products of prolactin were genotyped by single-stranded conformation polymorphism (SSCP). Two µl PCR products of each individual were mixed with 5 µl denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue), and then denatured at 94°C for 5 min followed by a rapid chill on ice for 10 min. The denatured PCR products were separated for 14 h at 4 V/cm on 12% polyacrylamide gel. The DNA bands were stained by silver staining (Qu et al., 2005). Individual genotypes were defined according to band patterns.

PCR products of each type of homozygotes were purified with DNA Fragment Quick Purification/Recover Kit. The purified PCR products were ligated to the PMD 18-T vector and transformed into DH5- α *Escherichia coli*. Positive recombinant colonies were sequenced on the ABI 377 sequencer.

Statistical models and analysis

Associations between genotypes and diplotypes of 4 SNPs of bovine prolactin gene and five milk performance traits and genetic effects were analyzed using GLM procedure of SAS 8.02 software, respectively. The diplotypes were constructed with the phase 2.0. The following models were used.

Table 2. Primer sequences and information of bovine prolactin gene

Names	Sequences	Length (bp)	Tm (°C)	Amplicons
Primer1	5-TTCCCCAGTATGAACTCCC-3 5-CGTCCCCAGATTGAGAAAC-3	209	55	Promoter: -690 to -898
Primer2	ATCCCACTGTTTCTCAATC-3 5-CTGGCTGAAGTCAAGATTT-3	248	54	Promoter: -457 to -690
Primer3	5-AATCTTGACTTCAGCCAG-3 5-GAGAGATAATGATGGTGAG-3	261	58	Promoter: -211 to -471
Primer4	5-CTCACCATCATTATCTCTC-3 5-AAGAAACACACTTCACCAG-3	280	56	Partial promoter and exon1: -229 to 50
Primer5	5-GTACAGCAGCTTGTTGGAGT-3 5-TATCTCTGTTCTCTACCCC-3	279	60	Intron1: 32 to 310
Primer6	5-GGGGTAGAGAACAGAGATA-3 5-AGACTACTCTGCTGTCACT-3	281	56	Intron1: 292 to 572

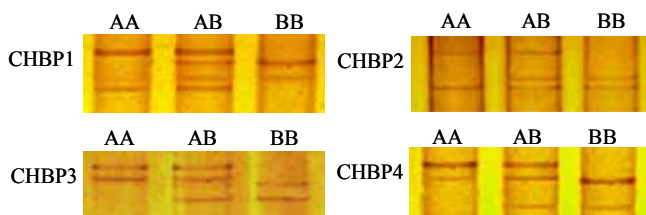


Figure 1. DNA bands patterns for the four SNPs.

Table 3. Frequencies of alleles and genotypes of four SNPs of bovine prolactin gene (%)

SNPs	Genotypic frequencies			Allelic frequencies	
	AA	AB	BB	A	B
CHBP1	2.8	14.8	82.4	10.2	89.8
CHBP2	10.2	38.5	51.3	29.4	70.6
CHBP3	0.6	21.0	78.4	11.1	88.9
CHBP4	1.4	13.3	85.4	8.0	92.0

$$Y_{ijk} = \mu + S_i + G_j + e_{ijk} \quad (1)$$

$$Y_{ijk} = \mu + S_i + H_j + e_{ijk} \quad (2)$$

Where Y_{ijk} = EBVs of five milk performance traits, μ = overall mean, S_i = sire families, G_j or H_j = fixed effects of genotypes of each SNP or diplotype, e_{ijk} = random error.

Considering the phenotypes of five milk performances traits have been converted to EBVs, so other effects including lactation and herd-year-season were not taken in models (1) and (2). Significant differences among least-square means of different genotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

RESULTS

Identification of SNPs within bovine prolactin

Using PCR-SSCP analysis, four SNPs, namely CHBP1, CHBP2, CHBP3 and CHBP4, were identified, which was located at positions of -767 (A/C), -485 (G/T), -247 (C/A), 427 (C/T) of bovine prolactin gene, respectively. Three genotypes were found for each SNP and named as AA, AB, and BB respectively (Figure 1).

Gene and genotypic frequencies were listed in Table 3. From the whole, the frequency of AA for each of four SNPs, 2.8%, 10.2%, 0.6% and 1.4%, was very low, respectively. A

Table 4. Associations between each of SNPs in bovine prolactin gene and milk performance Traits

SNPs	Milk yield	Fat yield	Protein yield	Fat percentage	Protein percentage
CHBP1	NS	NS	NS	NS	NS
CHBP2	**	*	**	NS	*
CHBP3	NS	NS	NS	NS	NS
CHBP4	NS	NS	NS	NS	NS

* $p \leq 0.05$, ** $p \leq 0.01$.

Table 6. Frequencies of Haplotypes based on the 4 SNPs in bovine prolactin gene (%)

Type	Haplotype	Observation	Frequency
H1	AGAT	698	53.78
H2	AGAC	51	3.93
H3	AGCT	63	4.85
H4	ATAT	264	20.34
H5	ATAC	18	1.39
H6	ATCT	46	3.54
H7	ATCC	13	1.01
H8	CGAT	76	5.86
H9	CTAC	40	1.38

relatively high frequency of the genotype BB was 82.4%, 51.3%, 78.4% and 85.4%, respectively.

Associations between SNPs with milk performance traits

The association analysis of the 4 SNPs within bovine prolactin gene with the milk performance traits was carried out using least square estimation. Statistical results indicated that, among the 4 SNPs, only CHBP2 in the promoter was significantly associated with milk yield ($p < 0.01$), fat yield ($p < 0.05$), protein yield ($p < 0.01$), and protein percentage ($p < 0.05$) (Table 4). Further, multiple comparisons analysis showed that cows with genotype BB had significantly higher milk yield ($p < 0.01$), fat yield ($p < 0.05$), and protein yield ($p < 0.01$) than those of genotype AA, while cows with genotype AA had significant higher protein percentage than cows with genotypes BB and AB ($p < 0.05$). Other three SNPs showed no association with milk performance traits ($p > 0.05$). Table 5 presented the result of multiple comparisons.

Association between diplotypes with milk performance traits

Haplotypes were constructed based on 4 SNPs in the

Table 5. Association of CHBP2 genotypes (least squares means \pm SD) with milk performance traits

Traits	AA	AB	BB
Milk yield	-105.53 \pm 214.46 ^B	255.07 \pm 54.5 ^{A, B}	358.73 \pm 21.40 ^A
Fat yield	-3.60 \pm 8.06 ^b	12.38 \pm 2.05 ^{a, b}	15.87 \pm 0.81 ^a
Protein yield	-1.98 \pm 6.44 ^B	5.93 \pm 1.63 ^{A, B}	11.33 \pm 0.64 ^A
Fat percentage	0.011 \pm 0.093	0.033 \pm 0.023	0.040 \pm 0.009
Protein percentage	0.034 \pm 0.04 ^a	-0.043 \pm 0.011 ^b	-0.003 \pm 0.004 ^b

A and B mean significant at $p < 0.01$ level, a and b mean significant at $p < 0.05$ level.

Table 7. Associations between diplotypes of bovine prolactin gene and milk performance traits¹

Diploypes	Percent (%)	Milk yield	Fat yield	Protein yield	Fat percent	Protein percent
H1H1	30.66	272.3±30.37 ^b	13.650±1.14 ^b	8.354±0.926 ^b	0.05343±0.013	-0.00504±0.006 ^{a,b}
H1H4	20.34	410.4±37.15 ^{a,b}	18.035±1.39 ^b	12.214±1.133 ^{a,b}	0.04047±0.015	-0.01626±0.007 ^{a,b}
H1H6	5.86	420.4±68.61 ^{a,b}	16.59±2.57 ^b	11.632±2.093 ^{a,b}	0.01472±0.029	-0.04085±0.013 ^{a,b}
H1H3	6.78	364.4±64.59 ^{a,b}	15.739±2.43 ^b	10.751±1.971 ^{a,b}	0.03131±0.027	-0.02488±0.013 ^{a,b}
H1H7	1.54	190.1±135.49 ^b	11.230±5.08 ^b	5.650±4.134 ^b	0.09824±0.058	0.00844±0.027 ^{a,b}
H2H3	1.23	417.2±151.47 ^{a,b}	18.304±5.68 ^b	12.152±4.623 ^{a,b}	0.05378±0.065	-0.01613±0.030 ^{a,b}
H1H8	4.62	309.6±78.22 ^{a,b}	12.038±2.93 ^b	9.313±2.387 ^b	0.00357±0.033	-0.01270±0.016 ^{a,b}
H4H8	3.98	328.1±93.49 ^{a,b}	14.430±3.51 ^b	12.060±2.853 ^{a,b}	0.02902±0.040	0.02271±0.019 ^a
H1H5	2.16	350.0±114.51 ^{a,b}	13.180±4.30 ^b	9.399±3.494 ^b	0.00960±0.049	-0.04757±0.023 ^b
H1H2	3.39	324.4±91.34 ^{a,b}	16.763±3.43 ^b	9.359±2.787 ^b	0.07373±0.039	-0.01801±0.018 ^{a,b}
H4H4	6.93	344.7±62.49 ^{a,b}	13.986±2.34 ^b	11.010±1.907 ^{a,b}	0.00014±0.026	-0.00081±0.012 ^{a,b}
H2H8	1.08	609.3±161.93 ^a	29.657±6.08 ^a	18.675±4.942 ^a	0.10706±0.069	-0.01910±0.032 ^{a,b}

¹ Least squares means±standard deviation.

^{a,b} Different superscript letters of least squares mean within a row mean significant difference at $p < 0.05$.

experiment population by use of the Phase 2.0 program. Eleven haplotypes with the minor allelic frequencies of above 1% were identified, of which four main haplotypes, AGAT, AGCT, ATAT, and ATCT, accounted for 82.51% of the observations (Table 6). Twelve diplotypes with the minor frequency of higher than 1% were further obtained from these haplotypes, which accounted for 88.57% of the observations (Table 7). Association analysis indicated that there was significant association between diplotype H2H8 and fat yield ($p < 0.05$). By multiple comparisons, we found that the least square mean of fat yield for diplotype H2H8 was significantly higher than those of other diplotypes ($p < 0.05$), respectively (Table 7).

DISCUSSION

Prolactin, one of pituitary hormone, regulates important physiological functions, ranging from mammary gland development to initiation and maintenance of lactation. Previous study reported the associations between polymorphisms in the coding regions of the bovine prolactin gene and economically important traits (Pawel et al., 2005). In this study, we first identified three SNPs within the promoter and one SNP in the intron1 of the bovine prolactin gene. The frequencies of genotype AA for the four SNPs were lower than genotypes AB and BB, and the frequencies of genotype BB were up to above 50% (Table 3). The differences between genotypes are possibly due to long-term artificial fertilization and selection towards high fat and protein contents of milk.

Further, our findings that there were significant associations between CHBP and milk yield, fat yield, protein yield, and protein percentage traits implies that CHBP2 could be a potential QTN (quantitative trait nucleotide) affecting milk yield, fat yield, protein yield, and protein percentage in dairy cattle. Further studies in larger population are needed to confirm this result. Furthermore,

putative Transcription Factors binding sites were predicted for the four SNPs by the TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>). Results showed CHBP2 leads to a CdxA site which is a common promoter element like AP-2, Pax-2, SRY, STAT1 and STAT5A within the core promoter region of human, chicken and rat genes. (Bajic VB, 2004). It is reported that, as a homeobox gene, CdxA may be part of a regulatory network coupled to axial determination during gastrulation in the early chicken embryo (Frumkin, 1993, 1994; Hiramatsu, 2004). However, if the exact molecular mechanisms underlying the association of the SNP of CHBP2 with milk traits reported in the present study is related to the regulation of CdxA site is unknown, the possible functionality of the promoter variants of the bovine prolactin, CdxA, can only be appreciated from *in vivo* and *in vitro* experiments in the future.

Besides single SNP, haplotype or haplotype block also provides a practical solution to identify the QTL for complex traits (Daly et al., 2001). In this study, based on the construction of haplotypes with the 4 SNPs, the diplotype H2H8 was found to be significantly associated with milk fat yield, which is consistent with the result reported by Pawel et al. (2005). Therefore, H8 and H2 may be the most advantageous haplotype for milk fat yield because of nucleotide substitution interaction between different positions (Putt et al., 2004).

In conclusion, 4 SNPs were first identified in the promoter and intron1 of bovine prolactin gene in this study. The SNP located in the promoter, CHBP2, was significantly associated with the most milk performance traits. Further, there was significant association between diplotypes H2H8 based on 4 SNPs with milk fat yield. This implied that prolactin gene could be a potential QTL which affects milk performance traits in dairy cattle, and hope it would be useful genetic marker in the selection of some milk performance traits.

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