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Three novel SNPs of the bovine *Tf* gene in Chinese native cattle and their associations with milk production traits

Z.H. Ju, Q.L. Li, J.M. Huang, M.H. Hou, R.L. Li, J.B. Li, J.F. Zhong and C.F. Wang

Dairy Cattle Research Center, Shandong Academy of Agricultural Sciences, Jinan, Shandong Province, China

Corresponding author: C.F. Wang
E-mail: wcf1967@yahoo.com.cn

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ABSTRACT. Transferrin (Tf) is a β-globulin protein that transports iron ions in mammalian cells. It contributes to innate immunity to microbial pathogens, primarily by limiting microbial access to iron. Thus, polymorphisms present in bovine Tf could potentially underlie inherited differences in mastitis resistance and milk production traits. We detected three novel single-nucleotide polymorphisms of the *Tf* gene in Chinese native cattle by screening for genetic variation of *Tf* in 751 individuals of three Chinese cattle breeds, namely China Holstein, Luxi Yellow and Bohai Black, using PCR-RFLP and DNA sequencing techniques. The three new SNPs, g.-1748G>A ss250608649, g.13942T>C ss250608650, and g.14037A>G ss250608651, had allele frequencies of 85.9, 86.3 and 92.5%, 64.5, 73.3 and 65.0%, and 67.6, 73.7 and 60.0%, respectively. SNP g.-1748G>A was located in the 5' flanking region of *Tf*. SNP g.14037A>G was located in intron 8 of *Tf*. SNP g.13942T>C, located in exon 8 of *Tf*, was a synonymous mutation (TTA > CTA), encoding a leucine (326 aa) in the Tf protein. Associations of the *Tf* SNPs with milk traits were also analyzed. Significant ($P < 0.05$) relationships among the *Tf* polymorphisms, somatic cell scores (SCS), and milk productive traits were observed. Cows with genotypes *TT* (g.13942T>C),

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GG (g.-1748G>A) and *AG* (g.14037A>G) had a lower SCS and higher protein levels and 305-day milk yield. Nineteen combinations of different haplotypes from the three SNPs were identified in Chinese Holstein cattle. The haplotype combination ATA/GCA, GCA/GCA and GCG/GTA was dominant in cows with a lower SCS, a higher protein level and a higher 305-day milk yield, respectively. Moreover, the gene expression level of Tf was higher in mastitis-affected mammary tissues than in normal mammary tissues. These results suggest that the *Tf* gene affects milk production, as well as mastitis-resistance traits, in Chinese Holsteins.

Key words: Chinese native cattle; Transferrin; SNPs; Milk production traits; Somatic cell score

INTRODUCTION

The health status of the mammary gland greatly affects the biological value of collected milk (Sevi et al., 2001). Mastitis in dairy cattle is a common and costly inflammatory disease of the mammary gland caused by intramammary infections, and leads to reduced yield, degraded quality, reduced lactation persistency, and early culling of cow (Seegers et al., 2003). Environmental and contagious pathogens including *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, and *Streptococcus agalactiae* are by far the main causes of mastitis (Mason, 2006). Breed improvement programs have been proven useful for the selection of positive milk production traits, but are expensive and time-consuming. Therefore, marker-assisted selection that supports fast and low-cost genetic progress and improves the accuracy of selection is desirable. In this regard, it is useful to study the genetic variations of candidate genes and their associations with milk production and somatic cell count (SCC) (Khatib et al., 2007; Huang et al., 2010), which have a high genetic positive correlation with mastitis (with an estimated average coefficient of 0.7) (Pösö and Mäntysaari, 1996; Heringstad et al., 2000).

Transferrin (Tf) is an iron-binding β-globulin plasma protein synthesized by the liver with a molecular weight of about 80 kDa (Fletcher and Huehns, 1968). It has two separate iron-binding sites, and each of them is capable of binding one atom of ferric iron (Fletcher and Huehns, 1968). In human plasma, transferrin is normally at 2.0-3.2 mg/mL and is typically one-third saturated with iron (Macgillivray et al., 1982). Transferrin may contribute to innate host defense against bacterial and fungal pathogens by limiting microbial access to iron (Chaneton et al., 2008). Transferrin also inhibits bacterial adhesion (Ardehali et al., 2003) and has an iron-independent antifungal effect (Bond et al., 2005). In addition, in animals with diagnosed mastitis, the transferrin concentration in milk is higher than that in healthy animals (Kmiec, 1998). These results suggest a possible relationship between the *Tf* gene and mastitis in dairy cattle.

The *Tf* gene is located on bovine chromosome 1q41-q46 (Chowdhary et al., 1998), where it consists of 17 exons and spans about 39 kb of genomic DNA. Many polymorphisms have been found in the bovine *Tf* gene (Ashton et al., 1964; Zhang et al., 2008; Sanz et al., 2010). Twelve co-dominant alleles of *Tf* have been identified from a single locus using starch gel electrophoresis. A, D1, D2, and E are the major variants that have been described (Gahne et al., 1977). However, little is known about the *Tf* single nucleotide polymorphisms (SNPs) and their associations with infectious diseases such as bovine mastitis.

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Chinese Holstein cattle are derived from crossbreeding and selection between the Chinese native cow and pure-bred Holstein bull. The frequency of mastitis in the breed is about 38-50% (Tao et al., 2007). Luxi Yellow cattle and Bohai Black cattle are two representative indigenous bovine (*Bos taurus*) breeds in China, which have been bred as beef and draft dual-purpose cattle for thousands of years because of their low disease frequencies and high endurance with unfavorable feeding conditions. Here, we detected polymorphisms of the *Tf* gene in three Chinese cattle breeds, and assessed the associations of these polymorphisms with milk production and mastitis-related traits. *Tf* gene expression in normal and mastitis mammary tissues in Chinese Holstein was also assessed by fluorescence quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIAL AND METHODS

Animals

A total of 751 individuals of three cattle breeds in China, including Chinese Holstein (N = 576), Luxi Yellow cattle (N = 135), and Bohai Black cattle (N = 40), were used in the study. Milk production (305-day milk yield), somatic cell score (SCS), and fat and protein contents in Chinese Holstein from 23 sires were obtained by the Dairy Herd Improvement Laboratory, Dairy Cattle Research Center, Academy of Agricultural Sciences of Shandong Province, using a milk composition analyzer (Foss MilkScan FT 6000, Denmark).

Genomic DNA extraction

Genomic DNA was isolated from bovine blood samples by the phenol-chloroform method. DNA concentration was estimated spectrophotometrically and then adjusted to 50 ng/μL. All DNA samples were stored at -20°C.

PCR amplification and sequencing

Seven primer pairs (Table 1) were designed using the PRIMER PREMIER 5.0 software (Premier, Canada) to amplify the bovine *Tf* gene (NW_001493777) including exon 6-8, boundaries of each exon and intron, and the 5' flanking region. Primers P1-P5 were used to sequence the 5' flanking region and exon 6-8. The PCR mixture with a final volume of 25 μL consisted of 2.5 μL 10 X buffer, 1.2 μL 50 mM Mg^{2+}, 0.6 μL 10 mM dNTPs, 0.8 μL 10 μM of each primer, 1.0 μL 50 ng/μL genomic DNA, 0.5 μL 5 U/μL Taq DNA polymerase (TaKaRa, China), and 17.6 μL ddH_2O. PCR was performed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing (temperatures see Table 1) for 30 s, and 72°C for 30 s, and a final extension at 72°C for 8 min. PCR products were evaluated by electrophoresis on 1% agarose gels after staining with ethidium bromide, and directly sequenced using an ABI PRISM™ 3730 DNA Sequencer (Applied Biosystems, USA) and a BigDye terminator v3.1 Sequencing Kit (Shanghai Sangon, China). Sequence data were analyzed with the DNAMAN software (Version 4.0, Lynnon Corporation, Quebec, Canada) to identify SNPs, and SNPs were then confirmed using restriction fragment length polymorphism (RFLP) or created restriction site PCR (CRS-PCR).

Table 1. Primer sequence and PCR conditions.

Loci	Position	Primer sequence (5'-3')	Tm (°C)	Size of amplicon (bp)
Tf P1	12398~13256	F: CCTGCAAGAACCTCCCTAATG R: GAACACAGAATGACTTCCACA	60	859
Tf P2	13728~14609	F: GGTCTGACTGCCCTCTCTC R: GTTCAAACACACCTCTAATG	57	882
Tf P3	-2055~-1158	F: TGGGCAGATTTGCAAGCTC R: ATCCAACTCGATCAGATGGTC	60	898
Tf P4	-1230~-366	F: GACATATCTTCTAAGCCTGG R: CTGGTCCTGAATCAACCTC	60	865
Tf P5	-459~41	F: GGTCTCTGCCTGTCTTCCTCCTA R: AGAACCGCGCAGGCTAACAG	60	501
Tf P6	-1903~-1727	F: CACTCCCTAATGCCTGATAC R: CAGGGACTTTCTGTTTAC**C***A	54	177
Tf P7	14014~14177	F: AGAGAAAGTAAACGTAAGTAT**C***C R: ATTTATCATCCGTCTAACACTG	55	164

In primer P6, the asterisk indicates a mutation (A to C) that creates an *Sty*I restriction site (C^CWWGG). In primer P7, the asterisk indicates a mutation (T to C) that creates an *Msp*I restriction site (C^CGG).

Genotyping tests

According to sequencing results, primer pairs P6, P2 and P7 were used to test for genetic variation. Enzymes *Sty*I, *Dra*I and *Msp*I were used to digest PCR products. The PCR product using primer pair P2 included a natural *Dra*I endonuclease restriction site (TTT^AAA), whereas the DNA fragment obtained from PCR using primer pairs P6 and P7 had no suitable endonuclease restriction site and was therefore genotyped by CRS-PCR. Primers for CRS-PCR contained nucleotide mismatches that enabled the use of restriction enzymes to discriminate sequence variations. Specifically, the second base A from the 3' end of primer P6R was replaced by C, which created an *Sty*I restriction site (C^CWWGG); the second base T from the 3' end of primer P7F was replaced by C, which created an *Msp*I restriction site (C^CGG) (Table 1). Aliquots (5 µL) of PCR products were digested with 10 units of restriction enzyme for 8 h at 37°C following supplier instructions. Digested PCR products were subjected to 10% PAGE (80 x 73 x 0.75 mm) electrophoresis in 1X TBE buffer at a constant voltage of 110 V for 3.5 h at room temperature. Gels were stained with 0.1% silver nitrate, and the genotype was determined based on different electrophoresis patterns.

Fluorescence quantitative real-time PCR

Twelve mammary tissues from 10 culled Chinese Holstein cattle with clinical mastitis were divided into two groups: normal (N = 6) and mastitis (N = 6) mammary. Total RNA was isolated from the 12 mammary tissues using TRIzol reagent (Bioteke, Beijing, China) according to the manufacturer instruction. cDNA was synthesized using the transcriptor first-strand cDNA synthesis kit (TaKaRa). Real-time PCR was performed in a 20-µL mixture containing 50 ng cDNA, 0.4 µM sense and antisense primers each, 6.8 µL ddH_2O, 10.0 µL SYBR® Premix Ex Taq™ (2X), and 0.4 µL ROX Reference Dye (50X) (TaKaRa). To normalize the differences in the amount of total cDNA added to each reaction, the *β-actin* gene was used as an endogenous control. The reaction mixture was denatured for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. The primers used were *Tf* (NM_177484) sense

(5'-ATGCTCAACCTCAAAACTCC-3'), *Tf* antisense (5'-ATCACTCAGACCAGCGAAAC-3'), *β-actin* (NM_173979) sense (5'-GCACAATGAAGATCAAGATCATC-3'), and *β-actin* antisense (5'-CTAACAGTCCGCCTAGAAGCA-3'). PCR was monitored by the ABI PRISM 7000HT Fast Real-Time PCR system (Applied Biosystems). Relative quantification of the *Tf* gene expression was calculated using the standard curve-based method for relative real-time PCR (Larionov et al., 2005).

Statistical analysis

The genotypic frequencies, allelic frequencies, polymorphism information content (*PIC*), heterozygosity (H_e) and effective number of alleles (N_e) were calculated using the POPGENE, version 1.31, software (Molecular Biology and Biotechnology Centre, University of Alberta, Canada). The genotype distributions in different breeds were tested for Hardy-Weinberg equilibrium using the appropriate chi-square (χ^2) test. The linkage disequilibrium and haplotype frequencies were estimated using the SHEsis software (Shi and He, 2005).

The distribution frequency of SCC is usually inhomogeneous and SCS is a commonly used parameter based on SCC. Cow SCS was calculated with $SCS = \log_2 (SCC / 100) + 3$, where SCC is cell number/μL (Rupp and Boichard, 1999).

The associations among genotypes, haplotype combinations of *Tf* gene, and milk production and healthy traits were analyzed using the least-squares method of the general linear model procedure of the SAS software V8.1 (SAS Institute Inc., Cary, NC, USA). Fixed effects of genotypes, haplotype combinations, and season of birth and parity were included as independent variables. In the formula $Y_{ijkl} = \mu + G_i + S_j + H_k + P_l + e_{ijkl}$, Y_{ijkl} is observed value, μ is mean value, G_i is fixed effect of *ith* genotype or *ith* haplotype, S_j is fixed effect of *jth* season (j = 1 to 2), H_k is fixed effect of *kth* farm (k = 1 to 20), P_l is fixed effect of *lth* parity (l = 1 to 4), and e_{ijkl} is random residual effect. Additive and dominance effects of every locus were evaluated separately. Values of $P < 0.05$ were regarded as significant.

RESULTS AND DISCUSSION

Genetic polymorphisms of *Tf* gene in Chinese cattle

Three novel SNPs g.-1748G>A (5' flanking regions), g.13942T>C (exon 8) and g.14037A>G (intron 8) were revealed from 50 samples (15 from Luxi Yellow, 15 from Bohai Black, and 20 from Chinese Holstein randomly selected from different farms) by direct sequencing and comparisons with the reference sequence (NW_001493777) (Figure 1). No SNPs were detected in the P1, P4, and P5 loci. The sequences of SNPs g.-1748G>A, g.13942T>C and g.14037A>G were submitted to the National Centre for Biotechnology Information under accession Nos. ss250608649, ss250608650, and ss250608651, respectively.

Digestions of the PCR products of *Tf* g.-1748G>A locus with *Sty*I produced 111- and 66-bp fragments for genotype *GG*, 111-, 93- and 66-bp fragments for genotype *GA*, and 93- and 66-bp fragments for genotype *AA*. Digestions of the PCR products of *Tf* g.13942T>C locus with *Dra*I produced 666- and 216-bp fragments for genotype *TT*, 882-, 666- and 216-bp fragments for genotype *TC*, and an 882-bp fragment for genotype *CC*. Digestions of the PCR products of *Tf* g.14037A>G locus with *Msp*I produced an 164-bp fragment for genotype *AA*, 164-, 142- and 22-bp fragments for genotype *AG*, and 142- and 22-bp fragments for genotype *GG* (Figure 2).

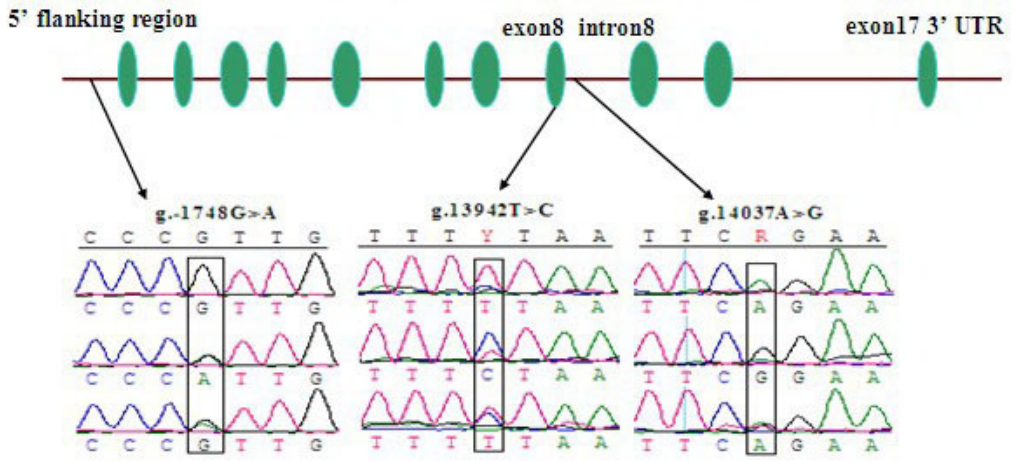

Figure 1. *Tf* structure, location of SNPs and sequencing results of the three genotypes g.-1748G>A ss250608649, g.13942T>C ss250608650, and g.14037A>G ss250608651.

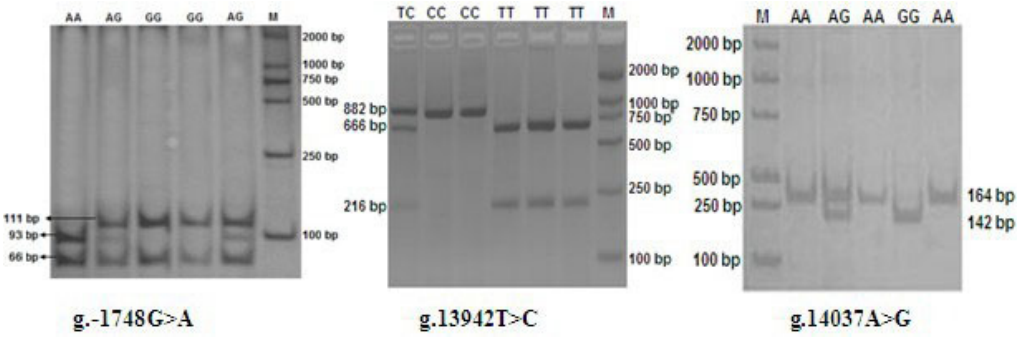

Figure 2. Silver-stained gels showing band patterns of SNPs g.-1748G>A, g.13942T>C and g.14037A>C digested with *Sty*I, *Dra*I and *Msp*I, respectively. Digestions of PCR products of *Tf* g.-1748G>A locus with *Sty*I produced 111- and 66-bp bands for homozygous genotype *GG*, 111-, 93- and 66-bp bands for heterozygous genotype *AG*, 93- and 66-bp bands for homozygous genotype *AA*. Digestions of PCR products of *Tf* g. 13492T>C locus with *Dra*I resulted in 666- and 216-bp bands for genotype *TT*, 882-, 666- and 216-bp bands for genotype *TC*, and a 882-bp band for genotype *CC*. Digestions of the PCR products of *Tf* g.14037A>G locus with *Msp*I generated 164-, 164- and 142-bp bands, and an 142-bp band for genotypes *AA*, *AG* and *GG*, respectively. Bands smaller than 50 bp are not shown.

The genotypic frequencies, allelic frequencies and genetic indices (*PIC*, H_e, N_e, and χ^2 test) in the three Chinese cattle populations are summarized in Table 2. Alleles *G*, *T* and *A* were the dominant alleles at positions g.-1748G>A, g.13942T>C and g.14037A>G in the three cattle breeds, respectively. At locus g.-1748G>A, the genotypic frequencies of the three cattle populations with genotype *GG* were higher than the ones with genotype *AA*, and the locus possessed low genetic diversity ($P < 0.25$) (Table 2A). The three Chinese cattle breeds possessed intermediate genetic diversity ($0.25 < P < 0.50$) at g.13942T>C and g.14037A>G loci, which suggested that there was an intermediate genetic diversity for the Chinese bovine

Tf gene in the populations analyzed (Table 2B and C). The *PIC*, H_e, and N_e of loci g.13942T>C and g.14037A>G in Chinese Holstein and Bohai Black cattle were higher than those in the Luxi Yellow breed, indicating that the polymorphism and genetic variation of Chinese Holstein and Bohai Black cattle were higher than those of the Luxi Yellow breed at these two loci. The results of the χ^2 test showed that all SNPs in the three populations agreed with the Hardy-Weinberg equilibrium (Table 2) ($P > 0.05$), indicating that the selection pressure on the three SNPs in the populations was not too powerful. In most cases, the theoretical numbers of the genotypic and allelic frequencies in beef cattle were different from the actual numbers in dairy cattle (Zhang et al., 2010; Liu et al., 2010), but they were almost the same in this study, which implied that the *Tf* gene could be very conserved. This coincides with a previous finding that transferrins had a substantial degree of conservation in amino acid sequence (>70%) and a very similar overall tertiary structure (Retzer et al., 1996). In addition, phylogenetic studies showed that the exon/intron pattern was very similar among human transferrin, mouse lactoferrin and chicken ovotransferrin genes (Retzer et al., 1996). In our study, the linkage disequilibrium among the three SNPs in the population was estimated (data not shown), and the SNPs were unlinked ($r^2 = 0.032$-0.374).

Table 2. Genotypic and allelic frequencies and Hardy-Weinberg equilibrium χ^2 test of *Tf* gene at positions: **A.** g.-1748G>A, **B.** g.13942T>C and **C.** g.14037A>G.

A.

Breed	Observed genotype			Allelic frequency		*PIC*	H_e	N_e	χ^2 test (P)
	GG	GA	AA	G	A				
CH	424	141	11						
576	0.736	0.245	0.019	0.859	0.141	0.213	0.243	1.321	0.033 (0.855)
LY	100	33	2						
135	0.741	0.244	0.015	0.863	0.137	0.209	0.236	1.310	0.152 (0.697)
BB	35	4	1						
40	0.875	0.100	0.025	0.925	0.075	0.129	0.139	1.161	3.120 (0.077)

B.

Breed	Observed genotype			Allelic frequency		*PIC*	H_e	N_e	χ^2 test (P)
	TT	TC	CC	T	C				
CH	239	265	72						
576	0.415	0.460	0.125	0.645	0.355	0.353	0.458	1.845	0.012 (0.912)
LY	72	54	9						
135	0.533	0.400	0.067	0.733	0.267	0.315	0.391	1.642	0.069 (0.792)
BB	15	22	3						
40	0.375	0.550	0.075	0.650	0.350	0.351	0.455	1.835	1.743 (0.187)

C.

Breed	Observed genotype			Allelic frequency		*PIC*	H_e	N_e	χ^2 test (P)
	AA	AG	GG	A	G				
CH	255	269	52						
576	0.443	0.467	0.090	0.676	0.324	0.342	0.438	1.779	2.547 (0.111)
LY	77	45	13						
135	0.570	0.333	0.096	0.737	0.263	0.313	0.388	1.633	2.648 (0.104)
BB	14	20	6						
40	0.350	0.500	0.150	0.600	0.400	0.365	0.480	1.923	0.069 (0.792)

CH = Chinese Holstein breed; LY = Luxi Yellow breed; BB = Bohai Black breed; H_e = heterozygosities; N_e = effective of alleles; *PIC* = polymorphism information content.

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Association of *Tf* gene polymorphism with milk and health traits in Chinese Holsteins

SNPs may be useful markers for identifying genes that contribute to susceptibility to common diseases (Kruglyak, 1999), and SNP analysis may be an efficient tool for characterizing genes that predispose to iron overload or deficiency. We analyzed the associations of these three SNPs with milk production traits including fat content, protein content, 305-day milk yield, and SCS in Chinese Holsteins (Table 3). At locus g.13942T>C, cows with genotype *CC* had higher SCS than the ones with genotypes *TC* ($P < 0.05$) and *TT* ($P < 0.01$), and the additive effect of SCS was significant ($P < 0.05$). This suggests a possible role of this SNP in the host response against mastitis, which is in agreement with the report that there is a significant association between transferrin genotype and somatic cell count in ewe milk (Steppa et al., 2009). Also, some transferrins are associated with resistance and susceptibility to disease (Enns and Sussman, 1981; Brandon et al., 1999). The possible explanations for this are 4-fold. First, the SNP g.13942T>C in exon 8 is a synonymous mutation [TTA (Leu) > CTA (Leu)] at the 326th amino acid near the C-terminal Fe^{3+} binding sites. Second, bacterial pathogens are the most common causes of mastitis in cows. As a major glycoprotein in iron metabolism in many species including cattle, transferrin ensures the transfer of Fe^{3+} ions from sites of absorption and heme degradation, through biological fluids, to sites of storage and utilization (Lambert et al., 2005). The antibacterial function of Tf is related to the sequestration of free iron necessary for bacterial growth. Third, the antimicrobial action of Tf is likely independent of iron, and is related to the ability of apo-Tf to reduce the adhesion of Gram-positive and Gram-negative bacteria to surfaces (Ardehali et al., 2003). Lastly, some transferrin variants are linked to diseases. For example, Beckman and Beckman (1986) propose that in humans, Tf C2 is associated with diseases of free radical etiology; while Wedekind (1994) suggests that selection after mating may favor heterozygosity or even certain allele combinations at loci that are involved in parasite-host co-evolution.

Table 3. Least squares mean and standard errors for milk production traits of different *Tf* genotypes in 576 Chinese Holstein.

Locus	Genotype	Fat percentage	Protein percentage	305-day milk yield (kg)	SCS
g.-1748 G>A	GG	3.40 ± 0.08	3.03 ± 0.06	6638.6 ± 243.2^{ab}	4.31 ± 0.27
	GA	3.34 ± 0.08	3.03 ± 0.07	6922.6 ± 250.8^{a}	4.19 ± 0.28
	AA	3.26 ± 0.19	2.84 ± 0.16	6179.4 ± 528.3^{b}	5.04 ± 0.53
Additive effect		-0.07 ± 0.09	-0.09 ± 0.07	-229.6 ± 239.7	0.37 ± 0.24
Dominance effect		0.002 ± 0.10	0.10 ± 0.08	517.6 ± 268.5*	-0.48 ± 0.27
g.13942T>C	TT	3.30 ± 0.09	3.02 ± 0.06	6820.9 ± 243.7	4.10 ± 0.21^{Bb}
	TC	3.29 ± 0.09	3.03 ± 0.06	6676.8 ± 248.4	4.15 ± 0.21^{b}
	CC	3.28 ± 0.10	3.06 ± 0.08	6555.1 ± 292.3	4.51 ± 0.24^{Aa}
Additive effect		-0.006 ± 0.03	0.02 ± 0.03	-132.9 ± 96.7	0.20 ± 0.08*
Dominance effect		0.004 ± 0.04	-0.01 ± 0.04	-11.2 ± 132.1	-0.15 ± 0.11
g.14037A>G	AA	3.39 ± 0.08	3.02 ± 0.06^{ab}	6888.6 ± 247.6^{a}	4.19 ± 0.27
	AG	3.40 ± 0.08	3.07 ± 0.06^{a}	6616.9 ± 243.8^{b}	4.34 ± 0.27
	GG	3.26 ± 0.11	2.89 ± 0.09^{b}	6854.4 ± 303.9^{ab}	4.17 ± 0.34
Additive effect		0.06 ± 0.05	0.07 ± 0.04	17.1 ± 110.2	0.009 ± 0.12
Dominance effect		0.08 ± 0.06	0.12 ± 0.05*	-254.7 ± 141.6	0.18 ± 0.16

Values with small (a and b) and capital (A and B) superscript letters within the same row in the same locus denote significant difference at $P < 0.05$ and $P < 0.01$, respectively. Asterisks indicate differences of the locus with additive effect or dominance effect at $P < 0.05$. SCS = somatic cell score.

Our results showed that cows with genotype *GA* at locus g.-1748G>A had higher 305-day milk yield ($P < 0.05$) than the ones with genotype *AA*, and the dominance effect of 305-day milk yield was significant ($P < 0.05$). When using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) to predict the 5' flanking sequence of *Tf* gene, we found that the SNP g.-1748G>A allele G was located in a putative selective T lymphocyte-specific transcription factor binding site. When G was converted to A, the binding sites were eliminated. Douabin-Gicquel et al. (2001) reported that g.-733A>G and g.+47G>A SNPs were located in the 5'-UTR of the human transferrin gene. 5'-UTRs are known to play crucial roles in post-transcriptional regulation, including modulations of the transport of mRNAs out of the nucleus, translation efficiency, and subcellular localization (Jansen, 2001) and stability (Bashirullah et al., 2001). Under this assumption, changes in gene transcription caused by mutations at g.-1748G>A in the essential region (5'-UTR) of the *Tf* gene could result in the variations of milk traits of cattle.

Cows with genotype *AG* at locus g.14037A>G had higher protein content than the ones with genotype *GG* ($P < 0.05$), cows with genotype *AA* had higher 305-day milk yield than the ones with genotype *AG* ($P < 0.05$), and the dominance effect of the protein content was significant ($P < 0.05$). Majewski and Ott (2002) reported that the regions within 20 bp from the intron recognition sites GT and AG had major impacts on intron splicing. Since the SNP g.14037A>G is 9 bp downstream of the 5' intron splicing site (GT), it may directly affect the splicing of intron 8. In addition, although introns do not encode proteins, they are important in regulating gene expression and splicing (Nott et al., 2003). Whether the detected mutations in intron 8 regulate bovine *Tf* gene expression or not needs to be clarified.

The above results suggest that cattle with genotypes *GA*, *TT* and *AA* had higher 305-day milk yield, higher protein content and lower SCS. Accordingly, genetic variations of *Tf* gene in dairy cattle are significant for breeding.

Bovine transferrin is encoded by the *Tf* gene, which is located at 125 cM on bovine chromosome 1 (Kappes et al., 1997). Suggestive quantitative trait loci have been detected for fat yield at 50 cM (Casas et al., 2004). Sanz et al. (2010) reported that SNP c.1455A>G in exon 12 of *Tf* had a significant association ($P < 0.0006$) with high fat production in Holstein-Friesian animals. However, in the present study, no significant associations of these SNPs with fat content were detected at the three loci in the populations analyzed ($P > 0.05$).

Three SNPs (g.-1748G>A, g.13942T>C and g.14037A>G) were used for haplotype reconstruction. The haplotypes were H1 (GTA), H2 (GCG), H3 (ATA), H4 (GCA), H5 (GTG), H6 (ATG), H7 (ACA), and H8 (ACG), and the estimated haplotype frequencies were 45.4, 25.0, 11.9, 9.8, 5.7, 1.5, 0.5, and 0.2%, respectively. H1 had the highest haplotype frequencies and H8 had the lowest haplotype frequencies. Combinations of two of the haplotypes were used to analyze the correlation among haplotype combinations, milk production traits and health traits (Table 4). Nineteen haplotype combinations of *Tf* were detected in the cows tested. The number of individuals with haplotype combinations H2H8 (GACCGG), H5H6 (GATTGG), H6H6 (AATTGG), H3H6 (AATTAG), H5H5 (GGTTGG), and H4H7 (GACCAA) was less than 5, without statistical significance. Accordingly, the associations were not analyzed among these haplotype combinations, milk traits and health traits of Chinese Holsteins.

Table 4. Effect of different combinations of three SNPs (g.-1748G>A, g.13942T>C and g.14037A>G) on milk production traits in Chinese Holstein (N = 576).

Haplotype combination	Number of combinations	Fat content (%)	Protein content (%)	305-day milk yield (kg)	SCS
H1H2 (GGTCAG)	159	3.47 ± 0.09	3.08 ± 0.07	6697.0 ± 269.7^{ab}	4.64 ± 0.29
H1H1 (GGTTAA)	113	3.48 ± 0.09	3.05 ± 0.08	6866.1 ± 278.9	4.41 ± 0.31
H1H3 (GATTAA)	67	3.39 ± 0.11	3.06 ± 0.09	7159.1 ± 299.9^{acd}	4.46 ± 0.33
H1H8 (GATCAG)	41	3.38 ± 0.12	3.11 ± 0.10	6636.7 ± 329.9^{e}	4.49 ± 0.37
H1H4 (GGTCAA)	40	3.39 ± 0.13	3.06 ± 0.11	6652.8 ± 346.7^{f}	4.45 ± 0.38
H2H2 (GGCCGG)	30	3.29 ± 0.14	3.00 ± 0.11	6602.8 ± 361.7^{cgH}	4.41 ± 0.40^{a}
H1H5 (GGTTAG)	27	3.33 ± 0.15	3.15 ± 0.12	6368.2 ± 368.2^{i}	4.01 ± 0.42
H2H4 (GGCCAG)	23	3.58 ± 0.15	3.19 ± 0.12^{a}	6416.4 ± 387.4^{dj}	4.95 ± 0.43^{ab}
H1H6 (GATTAG)	17	3.47 ± 0.17	3.16 ± 0.14	7251.6 ± 391.8^{g}	4.33 ± 0.44
H2H5 (GGTCGG)	15	3.31 ± 0.18	2.85 ± 0.15^{ab}	7584.8 ± 450.1^{befijH}	4.56 ± 0.51
H4H4 (GGCCAA)	14	3.60 ± 0.18	3.24 ± 0.15^{b}	6849.2 ± 453.8	4.52 ± 0.52
H3H4 (GATCAA)	10	3.17 ± 0.19	2.95 ± 0.16	6907.2 ± 494.4	3.64 ± 0.57^{b}
H3H3 (AATTAA)	7	3.48 ± 0.24	2.92 ± 0.19	6493.5 ± 676.4	4.83 ± 0.64

Means with the same small letters within the same row differ at $P < 0.05$. Means with the same capital letters within the same row differ at $P < 0.01$. Means marked with different superscript or without any superscript do not differ statistically. H1 = GTA; H2 = GCG; H3 = ATA; H4 = GCA; H5 = GTG; H6 = ATG; H7 = ACA; H8 = ACG.

The association analysis results showed that individuals with haplotype combination H4H4 had the highest protein and fat contents, which suggested that this haplotype combination (N = 14) was a dominant combination of alleles for protein and fat contents. Individuals with haplotype combination H2H5 had the highest 305-day milk yield, and it was higher than in the ones with haplotype combinations H1H2, H1H8, H1H4, H1H5, H2H2, and H2H4 ($P < 0.05$). As a result, high 305-day milk yield can be obtained by breeding cows with haplotype combination H2H5. Individuals with haplotype combination H3H4 had the lowest SCS. Accordingly, H4H4, H2H5 and H3H4 should be used as molecular markers in the future for the selection of cattle with high protein content, high 305-day milk yield, and low SCS, respectively. Fallin et al. (2001) considered that the inheritance of haplotype combinations was more effective than that of a single SNP. The genotype effect of one SNP may be influenced by other SNPs; while a haplotype combination provides a comprehensive way of assessing the relationship between multiple-site variation and traits. Consequently, the analysis of haplotype combination is superior to the analysis of a single SNP. Furthermore, these results may be instructional for early breeding selection of the Chinese Holstein breed.

Although the effect of the detected genetic variation on *Tf* gene expression remains to be explored, the SNPs of the *Tf* gene possibly contribute to association analysis, and *Tf* can be used as a genetic marker in milk production and mastitis-related traits for animal breeding and genetics.

Expression of the bovine *Tf* mRNA

We also investigated the differences in *Tf* gene expression between normal and mastitis mammary tissues in 10 cows (Figure 3). Our quantitative data indicated that the *Tf* mRNA level was higher in mastitis mammary tissue than in normal mammary tissues, and that no significant difference was found between the two types of mammary tissues. Interestingly, Kmiec (1998) reported that the transferrin content in milk was higher in animals with diagnosed mastitis than that in healthy animals. Also, the lactotransferrin gene expression

in *S. uberis*-infected mammary tissues was higher than that in healthy mammary tissues [3.4-fold (22 EST) in a microarray experiment and 6.5-fold in qRT-PCR analysis] (Swanson et al., 2009). The possible explanation is that Tf inhibits bacterial growth by chelating iron, an important nutrient element for many bacteria and viruses. When mammary tissue is invaded by bacteria and develops mastitis, the *Tf* gene expression in the mammary tissue is increased, so g.13942T>C of *Tf* may play an important role in genetic susceptibility to bovine mastitis.

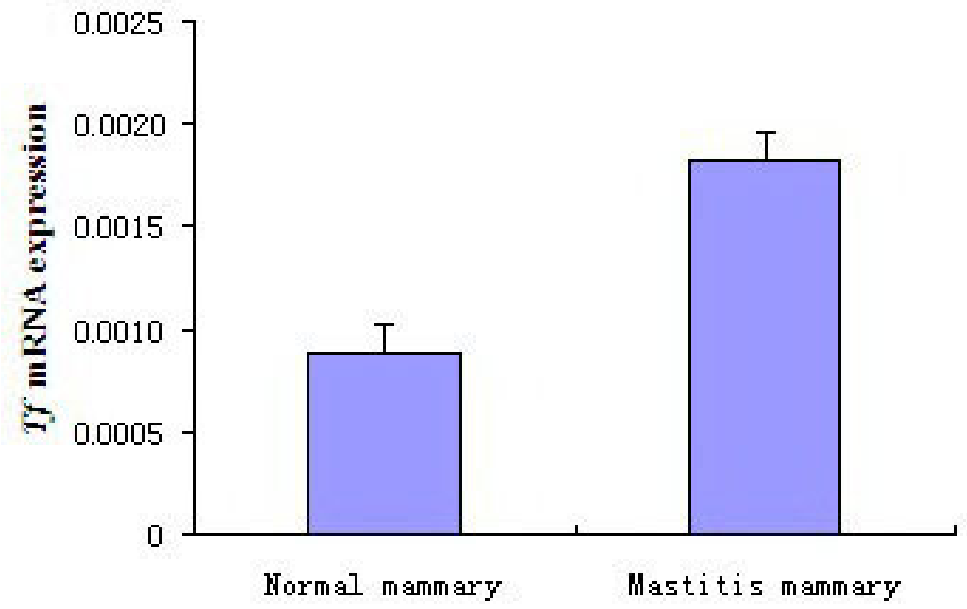

Figure 3. Quantitative polymerase chain reaction analysis of *Tf* mRNA expression in normal and mastitis tissues.

In summary, three novel SNPs and the association between *Tf* gene and milk production and health traits in dairy cattle are reported. In addition, the *Tf* gene expression levels were investigated in normal and mastitis mammary tissues. Cows with haplotype combinations H3H4, H4H4 and H2H5 should be used as the molecular markers for the selection of cattle with low SCS, high protein content, and high 305-day milk yield, respectively. These results suggest that *Tf* gene is a candidate gene that influences milk production traits and that can be implemented in breeding programs to obtain healthy Chinese Holstein with excellent milk quality.

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