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Full Length Research Paper

Effects of *Dra*I, *Sty*I, and *Msp*I polymorphisms and haplotypic combinations of the transferrin (*Tf*) gene on the sperm quality of Chinese Holstein bulls

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Transferrins (*Tfs*) are a class of related metal-binding transport glycoproteins with high specificities for iron. Testicular *Tf* was found to be synthesized by sertoli cells and involved in the net transport of ferric ions to germ cells. In the present study, the polymerase chain reaction-restriction fragment length polymorphism method was applied to detect the g.−1748G>A (5′ flanking region), g.13942T>C (exon8), and g.14037A>G (intron 8) polymorphisms of the *Tf* gene in 327 Chinese Holstein bulls. The associations of these polymorphisms with sperm quality traits were analyzed. Statistical analyses revealed a significant association between g.13942T>C and sperm abnormality traits. Six haplotypes were constructed from 14 genotype combinations. Association analyses of these haplotypes showed that bulls with the combined haplotypes GAT/GAT had the highest ejaculate volumes, and that bulls with the combined haplotypes AAT/AAT had the highest fresh sperm motility. The quantitative data indicated that the *Tf* mRNA level was significantly higher in liver tissue than in the heart, spleen, lung, kidney, and testis tissues. The *Tf* mRNA level was higher in testis tissue than in spleen, lung, and kidney tissues, but there was no significant difference among them. Therefore, GAT/GAT and AAT/AAT can be possible candidates for marker-assisted selections in bull breeding programs.

Key words: Chinese Holstein bull, genotypic combination, semen quality traits, SNPs, transferrin.

INTRODUCTION

Artificial insemination from superior sires is one of the main tools for genetic improvement of the economically important traits of dairy cattle herds (Parmentier et al., 1999). The conception rate with artificial insemination depends on the quantity and quality of semen. These characteristics are affected by the environment, management, physiological status, and genetic factors (Mathevon et al., 1998). Semen density, motility, and rate have usually been used as criteria for semen quality evaluation (Colenbrander et al., 1993). However, the early direct selection of semen quality traits including semen volume per ejaculate, motility, density, and so on is difficult because of their low heritability (Mathevon et al., 1998). In contrast, molecular markers that reveal polymorphisms at the DNA level are key players in animal genetics. There are many recent reports regarding the use of candidate genes as markers for semen quality and fertility in swine (Huang et al., 2002; Wimmers et al., 2005; Lin et al., 2006), goats (Wang et al., 2011), and Holstein bulls (Dai et al., 2009; Liu et al., 2011;Yang et al., 2011; Gorbani et al., 2009a, b).

Transferrin (*Tf*), a major protein for iron metabolism in higher vertebrates, ensures the transfer of iron (III) ions

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through biological fluids from uptake to utilization sites (Lambert et al., 2005). *Tf* is an 80 kDa single glycoprotein composed of two structurally similar lobes that each contains an iron-binding site. *Tf* is synthesized by hepatocytes, Sertoli, ependymal, and oligodendrodial cells (Gomme and McCann, 2005). Plasma *Tf* supplies most body tissues with iron, whereas testicular *Tfs* are synthesized by sertoli cells. The primary functions of these cells are to provide essential growth factors, create the proper environment for the development of germinal cells, and transport iron to germ cells (Sylvester and Griswold, 1993). Several studies have demonstrated the association of *Tf* polymorphisms with milk production traits (Sanz et al., 2010; Ju et al., 2011) and mastitis resistance by limiting microbial access to iron (Chaneton et al., 2008). However, few studies have examined their effects on the semen quality traits of bulls.

A linear relationship between testicular *Tf* mRNA transcripts and testicular weight in rats has been demonstrated (Hugly and Griswold, 1987). Mutant mice that lack the ability to synthesize normal amounts of *Tf* also have reduced abilities in producing functional spermatozoa (Bernstein, 1987). Furthermore, there appears to be a relationship between *Tf* and sperm concentrations in humans and bulls (Barthelemy et al., 1988; Gilmont et al., 1990). These findings suggest a possible relationship between *Tf* and semen quality traits. The bovine *Tf* gene is located on chromosome 1q41-q46, which is composed of 17 exons and 16 introns spanning about 39 kb. The present study aims to elucidate the effects of *Dra*I, *Sty*I, and *Msp*I polymorphisms as well as genotypic combinations in the *Tf* gene on semen quality. Another aim is to assess *Tf* gene expression levels in the different tissues (heart, liver, spleen, lung, kidney, and testis) of Chinese Holstein bulls by fluorescence quantitative real-time polymerase chain reaction (qPCR).

MATERIALS AND METHODS

Experimental animals and phenotypes

A total of 327 normal mature Chinese Holstein bulls used in the present study were from the Beijing, Shanghai, and Shandong bull stations. Semen and blood samples of 134 bulls from the Beijing Dairy Center, 137 bulls from the Shanghai Bright Dairy and Food Co. Ltd., as well as 56 bulls from the Shandong OX Bio-Technology Co. Ltd. were examined. For each bull, repeated determinations of semen quality traits were made from 2005 to 2009. Semen from each bull was collected at intervals of 3 to 6 day using an artificial vagina. Immediately after collection, the ejaculates were stored at 37°C in a water bath prior to evaluating fresh semen quality traits. The traits include semen volume per ejaculate (ml), semen motility (%), semen density ($\times 10^8$/ml), and abnormal sperm rate. Fresh semen was diluted with glycerol-egg yolk-citrate in the Beijing and Shanghai bull stations or with Bioxcell (IMV Biotechnology, France) in the Shandong bull station.The semen samples were packaged in 0.25 ml straws and cryopreserved. After storage in liquid nitrogen for 5 to 7 day, two straws were randomly obtained from each ejaculate and thawed at 38°C for 20 s. The straws were immediately evaluated for post-thaw frozen semen quality traits. These traits include post-thaw frozen semen motility and percentage of abnormal spermatozoa. The evaluations were performed using light microscopy and in accordance to the guidelines of the World Health Organization.

The ejaculate volume was directly measured in a semen-collecting vial. The motilities of fresh and post-thaw frozen semen were viewed on a TV monitor connected to a camera mounted on a phase-contrast microscope (Olympus-BX40, Minitüb, Tiefenbach, Germany) at 400 × magnification. For the viewing, a drop of semen was placed onto a pre-warmed (37°C) slide, which was then overlain with a cover slip. Semen density was determined using a semen densitometer (ACCUCELL, IMV, L'Aigle, France) as calibrated by the hemocytometer method of determining sperm concentration. The percentage of viable semen was calculated by examining over 100 semen per sample at a magnification of 400 ×. Eosin Y–Aniline blue was used for the staining. Percentage semen deformities were similarly calculated at magnifications of 400 × and 1000 × by staining with Giemsa. To minimize variation, the quality trait assessment of all semen samples was performed by one well-trained technician in each semen collection station.

Both semen and blood were collected from each bull. An anticoagulant (ethylenediaminetetraacetic acid, EDTA) was added to the blood samples, which were then stored at -20°C. Genomic DNA was extracted from whole blood using the standard phenol-chloroform extraction protocol (Ju et al., 2011). The sperm, which had been cryopreserved, was thawed, and their DNAs were extracted (TakaRa, Dalian, China) for examination. The DNA samples were dissolved in Tris-EDTA buffer containing 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA (pH 8.0) before storing at -20°C.

Polymerase chain reaction (PCR) condition and sequencing

Three primer pairs (Table 1) were designed to amplify the bovine *Tf* gene (NW_001493777) according to Ju et al. (2011). The PCR mixture with a final volume of 25 µl consisted of 2.5 µl of 10× buffer, 1.2 µl of 50 mM Mg^{2+}, 0.6 µl of 10 mM dNTPs, 0.8 µl of 10 µM primer, 1.0 µl of 50 ng/µl genomic DNA, 0.5 µl of 5 U/µl Taq DNA polymerase (TaKaRa, China), and 17.6 µl of distilled deionized H_2O (ddH_2O). The PCR was performed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing (see Table 1 for temperatures) for 30 s, and 72°C for 30 s, as well as a final extension at 72°C for 8 min. The PCR products were evaluated by electrophoresis in 1% agarose gels after staining with ethidium bromide. The products were directly sequenced using an ABI PRISMTM 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator v3.1 sequencing kit (Shanghai Sangon, China). The sequence data were analyzed with the DNAMAN software (Version 4.0, Lynnon Corporation, Quebec, Canada) to identify SNPs. The SNPs were then confirmed using PCR-restriction fragment length polymorphism (RFLP) or created restriction site (CRS) PCR.

Genotyping tests

The enzymes *Sty*I, *Dra*I, and *Msp*I were used to digest the PCR products. The PCR product using primer pair P1 included a natural *Dra*I endonuclease restriction site (TTT^AAA). On the other hand, the DNA fragment obtained from the PCR using primer pairs P2 and P3 had no suitable endonuclease restriction sites. Hence, this fragment was genotyped by CRS-PCR. The primers for CRS-PCR that contained nucleotide mismatches enabled the use of restriction enzymes to discriminate sequence variations. Specifically, the second base A from the 3' end of primer P2R was replaced by C, which created a *Sty*I restriction site (C^CWWGG). The second base T from the 3' end of primer P3F was replaced by C, which created

Table 1. Primer information on the PCR amplification of the *Tf* gene.

Primer	Loci position	Primer sequence (5'–3')	T_m (°C)	Size of amplification (bp)
Tf P1	13728~14609 Exon 8	F: GGTCTGACTGCCCTCTCTC R: GTTCAAACACACCTCTAATG	57	882
Tf P2	-1903~-1727 5'flanking region	F: CACTCCCTAATGCCTGATAC R: CAGGGACTTTCTGTTTAC**C***A	54	177
Tf P3	14014~14177 Intron 8	F:AGAGAAAGTAAACGTAAGTAT**C***C R: ATTTATCATCCGTCTAACACTG	55	164

The GenBank number of the reference sequence for the PCR is NW_001493777. Three primer sequences were synthesized according to Ju et al. (2011). In primer P2R, the asterisk indicates a mutation (A to C) that creates a *Sty*I restriction site (C^CWWGG). In primer P3F, the asterisk indicates a mutation (T to C) that creates an *Msp*I restriction site (C^CGG).

the *Msp*I restriction site (C^CGG) (Table 1). Aliquots (5 µl) of the PCR products were digested with 10 units of restriction enzyme for 8 h at 37°C following the supplier's instructions. The digested PCR products were subjected to 10% PAGE (80 × 73 × 0.75 mm) electrophoresis in 1 × 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 qPCR

Thirty different tissues from five culled Chinese Holstein bulls were divided into six groups: heart (n = 5), liver (n = 5), spleen (n = 5), lung (n = 5), kidney (n = 5), and testis (n = 5). Total RNA was isolated from the thirty tissues using the TRIzol reagent (Bioteke, Beijing, China) according to the manufacturer's instructions. cDNA was synthesized using the transcriptor first-strand cDNA synthesis kit (TaKaRa, Dalian, China). Real-time PCR was performed in a 20 µl mixture containing 50 ng of cDNA, 0.4 µM each of sense and antisense primers, 6.8 µl of ddH_2O, 10.0 µl of SYBR® Premix Ex TaqTM (2×), and 0.4 µl of ROX Reference Dye (50×; TaKaRa, Dalian, China). 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'). The PCR was monitored by the ABI PRISM 7000HT Fast Real-Time PCR system (Applied Biosystems, USA). Relative quantification of the *Tf* gene expression was performed using the standard curve-based method for relative real-time PCR (Larionov et al., 2005).

Statistical analyses

The genotypic frequencies, allelic frequencies, polymorphism information content (*PIC*), heterozygosity (H_e), and effective number of alleles (N_e) were calculated using POPGENE version 1.31 (Molecular Biology and Biotechnology Centre, University of Alberta, Canada). The linkage disequilibrium and haplotype frequencies were estimated using the SHEsis software (Shi and He, 2005).
The associations of SNP markers and combined genotypes of the *Tf* gene with semen quality traits were analyzed by the least-squares method of the general linear model procedure of the SAS software V8.1 (SAS Institute Inc, Cary, NC, USA) according to the following linear model:

$$Y_{ijkl} = \mu + G_i + A_k + P_j + H_l + e_{ijkl}.$$

Y_{ijkl} is the observed value of each semen quality trait. μ is the overall mean. G_i is the fixed effect of the genotype or combined genotype. A_k is the fixed effect of age [k = 2 to10; (1) 2 to3 years, (2) 4 to 5 years, (3) 6 to 10 years)]. P_j is the fixed effect of the bull origin. H_l is the effect of the source farm (l = 1 to 3). e_{ijkl} is the random residual error.

$P < 0.05$ and $P < 0.01$ values were regarded as significant.

RESULTS AND DISCUSSION

Three reported SNPs, namely, g.1748G>A (5' flanking regions), g.13942T>C (exon 8), and g.14037A>G (intron8), of the bovine *Tf* gene were evaluated for their effects on the sperm quality traits of 327 Chinese Holstein bulls (Figure 1). These SNPs formed *Sty*I (g.1748G>A), *Dra*I(g. 13942T>C), and *Msp*I (g.14037A>G) endonuclease restriction sites (Figure 2). Polymorphisms of bovine *Tf* gene were detected by PCR-RFLP and CRS-PCR (Figure 2). Digestion of the PCR product by *Sty*I (containing the *Tf* g.1748G>A locus) generated fragments with lengths of 111 and 66 bp for genotype GG; 111, 93, 66, and 18 bp for genotype GA; as well as 93, 66, and 18 bp for genotype AA. Digestion

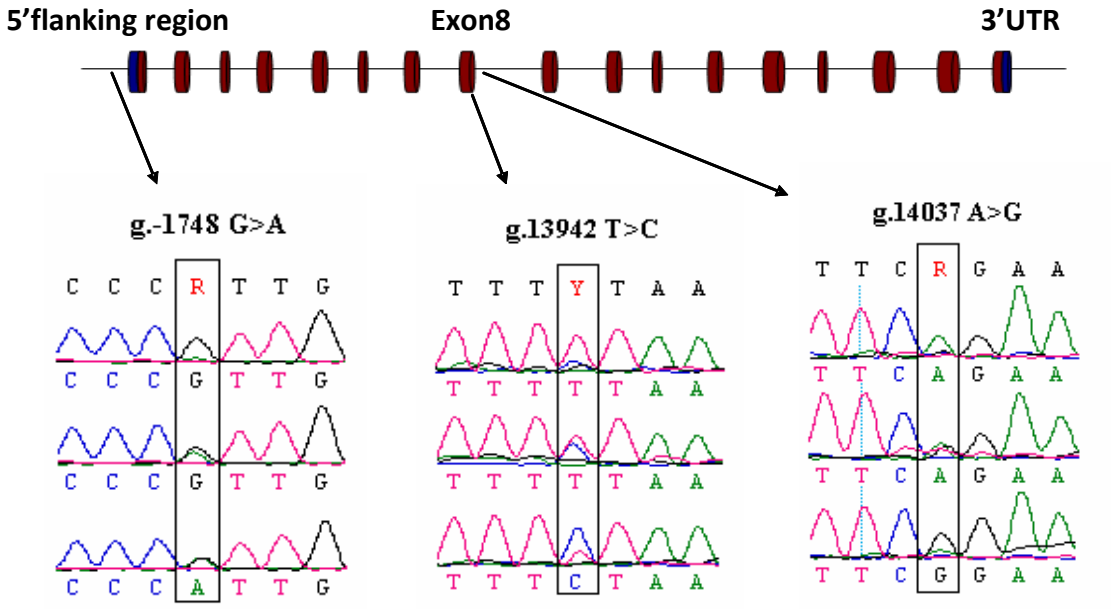

Figure 1. *Tf* structure, location of SNPs, and sequencing results of the three genotypes g.−1748G>A, g.13942T>C, and g.14037A>G.

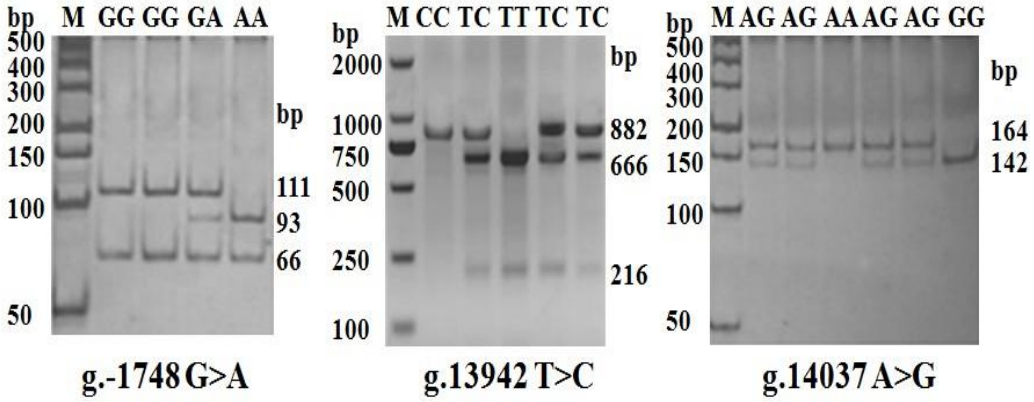

Figure 2. Representative genotyping of *Tf* gene at g.−1748G>A, g.13942T>C, and g.14037A>G locus. Silver-stained gels showing the band patterns of the SNPs g.−1748G>A, g.13942T>C, and g.14037A>G digested with *Sty*I, *Dra*I and *Msp*I, respectively. Bands smaller than 50 bp are not shown.

of the PCR product by *Dra*I (containing the *Tf* g.13942T>C locus) generated fragments with lengths of 666 and 216 bp for genotype TT; 882, 666, and 216 bp for genotype TC; as well as 882 bp for genotype CC owing to a nucleotide substitution. Digestion with *Msp*I (containing the *Tf* g.14037A>G locus) generated fragments with lengths of 164 bp for genotype AA; 164, 142, and 22 bp for genotype AG; as well as 142 and 22 bp for genotype GG. The 22 bp fragment was not noticeable on the gel due to its small size (Figure 2).

The allelic and genotypic frequencies of the three SNPs in bovine *Tf* gene are shown in Table 2. The alleles G, T, and A were predominant at positions g. 1748G>A, g. 13942T>C, and g.14037A>G, respectively. The frequencies of allele G at g.1748G>A, allele T at g.13942T>C, and allele A at g.14037A>G were 0.85, 0.61, and 0.56, respectively. The distribution patterns of all three SNP loci in the bull *Tf* gene were similar to those of the same SNPs identified in the other three breeds/populations. In a previous study, Ju et al. (2011) demonstrated that Chinese Holstein cattle, Luxi Yellow, and Bohai Black breeds all had three genotypes at the same three SNP loci.

The χ^2 test (Table 2) indicated that SNP g.−1748G>A met the Hardy–Weinberg equilibrium(P > 0.05). In contrast, positions g.14037A>G and g.13942T>C did not

Table 2. The allelic and genotypic frequencies, Hardy–Weinberg equilibrium χ^2 test, as well as the *PIC*, H_e, and N_e of the *Tf* gene in 327 Chinese Holstein bulls.

Loci	Genotype/ No	Genotypic frequency (%)	Allelic frequency (%)	*PIC*	H_e	N_e	HWE (*P* value)
	GG/230	70.34	G/84.40				
g.1748G>A	GA/92	28.13		0.22	0.25	1.34	0.16 Equilibrium
	AA/5	1.53	A/15.60				
	TT/131	40.06	T/61.16				
g.13942T>C	TC/138	42.20		0.36	0.48	1.90	0.043 Disequilibrium
	CC/58	17.74	C/38.84				
	AA/82	25.08	A/55.66				
g.14037A>G	AG/200	61.16		0.37	0.49	1.97	1.53×10^{-5} Disequilibrium
	GG/45	13.76	G/44.34				

Abbreviations: H_e, heterozygosities; N_e, effect of alleles; *PIC*, polymorphism information content; HWE, Hardy–Weinberg equilibrium.

meet the equilibrium ($P < 0.05$) in Chinese Holstein bulls. These results implied that the selection pressure on the g.−1748G>A locus in the population was not powerful. The differences in genotype frequencies among Holstein bulls may have resulted from long-term artificial selection. The tested Holstein bulls were mostly bred by implanting imported embryos or by artificial insemination in the nuclear herd with imported semen from USA or Canada. The genetic indices (H_e, N_e, and *PIC*) showed moderate genetic polymorphisms of g.13942T>C and g.14037A>G loci, as well as a low polymorphism for the g.1748G>A locus.

SNPs (FSHβ-U2 and FSHβ-3; g.16904G>T and g.22696T>C of SPAG11; GnRHR286, GnRHR340; bGH-*Msp* Ⅰ) may be useful markers for identifying genes that contribute to semen quality (Dai et al., 2009; Liu et al., 2011; Yang et al., 2011; Gorbani et al., 2009a, b). An SNP analysis may be an efficient tool for characterizing genes that predispose to better semen quality. Hence, the effects of the three loci of the *Tf* gene on the semen quality traits (ejaculate volume, semen density, fresh semen motility, post-thaw frozen semen motility, and percentage of abnormal spermatozoa) of 327 Chinese Holstein bulls were determined (Table 3). Statistical analyses revealed that the polymorphism at g.13942T>C correlated with the percentage of abnormal spermatozoa ($P < 0.05$). Animals with the homozygous genotype CC at position 13942 had a significantly lower percentage of abnormal spermatozoa than those with genotype TT ($P < 0.05$). This result indicated that the allele g.13942T>C-C may be associated with a lower percentage of abnormal spermatozoa in the population. Therefore, bulls with genotype CC can be selected for breeding. The possible explanation for this is that 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. The primary function of testicular *Tf*s was to transport Fe^{3+} ions to areas unreachable by plasma (Skinner et al., 1984). This finding is consistent with previous reports. Gilmont et al. (1990) reported significant correlations between seminal Tf concentration and spermatogenic capability in bulls. There also appears to be a relationship between human *Tf* levels in seminal plasma and spermatogenesis (Ber et al., 1990; Zalata et al., 1996). Moreover, Mariola et al. (2007) suggested the relation of semen motility parameters to *Tf* polymorphism in carp seminal plasma. Therefore, polymorphism may be related to semen competitive ability. All these results suggest a possible relationship between *Tf* and semen quality traits in bulls. However, no significant correlation between g.1748G>A/g.14037A>G polymorphisms and semen quality traits was found ($P > 0.05$). The consistency of this finding in other cattle breeds and populations is unknown and warrants further studies.

Pair-wise linkage disequilibrium (LD) in the 327 Chinese Holstein bulls showed three mutations

Table 3. Least squares means and standard errors for the semen quality traits of different *Tf* genotypes in 327 Chinese Holstein bulls

Loci	Genotype/No	Ejaculate volume (ml)	Semen density (×10^8/ml)	Fresh semen motility (%)	Post-thaw frozen semen motility (%)	Abnormal spermatozoa (%)
g.−1748G>A	GG/230	5.89±0.11	10.23±0.17	72.35±0.49	40.13±0.49	15.31±0.26
	GA/92	5.60±0.17	9.87±0.27	71.79±0.76	40.42±0.76	15.31±0.42
	AA/5	5.48±0.78	10.06±1.22	75.06±3.47	42.19±3.33	15.01±2.60
g.13942T>C	TT/131	5.84±0.14	10.07±0.23	71.90±0.64	40.36±0.64	15.85±0.36^{a}
	TC/138	5.74±0.14	10.14±0.22	72.19±0.64	40.04±0.64	15.27±0.30^{ab}
	CC/58	5.88±0.21	10.24±0.33	73.04±0.94	40.37±0.90	14.23±0.54^{b}
	AA/82	5.94±0.18	10.12±0.28	73.22±0.79	40.49±0.78	15.63±0.39
g.14037A>G	AG/200	5.71±0.12	10.09±0.18	71.75±0.52	40.13±0.53	15.26±0.28
	GG/45	6.02±0.24	10.36±0.37	72.64±1.07	40.17±1.03	14.64±0.64

Lowercase (a and b) superscripts within the same row in the same locus denote significant difference at $P < 0.05$.

(g.−1748G>A, g.13942T>C, and g.14037A>G) with a weak LD (D' = 0.884, 0.999, and 0.827; R^2 = 0.086, 0.139, and 0.545, respectively). These *Tf* SNPs were used for haplotype construction, including H1: AAT, H2: GAC, H3: GAT, H4: GGC, H5: GGT, and H6: AAC. The estimated frequencies were 0.143, 0.032, 0.376, 0.351, 0.092, and 0.005 for H1–H6, respectively. Among these six haplotypes, H3 had the highest frequency and H6 had the lowest. Only 14 combined genotypes of three SNPs were found, although 27 combinations were possible in the tested Chinese Holstein population. There were two explanations for this finding. One was that there were insufficient samples to test the other genotype combinations; the other was that some individuals with the other genotype combinations could not survive because of difficulties adapting to the environment (Liu et al., 2010). Five combined genotypes H2H3(3), H4H5(1), H5H5(2), H1H2(3), and H3H6(2) were not included in the association analyses due to small sample sizes (<4) (Table 4).

Statistical analyses showed that ejaculate volume, semen density, and fresh semen motility were significantly different ($P < 0.05$) in different combinations of haplotypes (three SNPs). No significant difference in post-thaw frozen semen motility and deformity rate was observed in various haplotype combinations (Table 4). Individuals with the haplotype combination H3H3 showed significantly higher ejaculate volumes than the haplotype combinations of H1H5 ($P < 0.05$). Individuals with the haplotype combination H3H3 had significantly higher semen densities than the haplotype combinations H1H3 ($P < 0.01$) and H1H5 ($P < 0.05$). Individuals with the haplotype combination H1H1 had significantly higher fresh semen motilities than the haplotype combination H1H5 ($P < 0.05$). The single SNP correlation results showed that bulls with g.13942T>C-CC genotypes had a significantly lower percentage of abnormal spermatozoa than TT individuals ($P < 0.05$). No significant correlation was observed between each of the three SNPs and other semen quality traits (ejaculate volume, semen density, fresh semen motility, and post-thaw frozen semen motility) in the analyzed Chinese Holstein bulls ($P > 0.05$). Interestingly, significant differences were found between combined genotypes of the three SNPs as well as the ejaculate volume, semen density, and fresh semen motility. This result suggested that the genotype of one SNP may be influenced by other SNPs. Therefore, the analysis of haplotype combinations for marker-assisted selection is more valuable than the study of a single SNP. This deduction is consistent with the conclusion drawn in the human SNP and inheritance studies of Fallin et al. (2001). Based on the data, there were significant correlations between the ejaculate volume and haplotype combination H3H3, semen density and haplotype combination H3H3, as well as fresh semen motility and haplotype combination H1H1. Hence, the haplotype combinations H3H3 and H1H1 are

Table 4. Effects of different combinations of three SNPs on sperm quality traits in 327 Chinese Holstein bulls.

Genotype/No	Genotypic frequency (%)	Ejaculate volume (ml)	Semen density (×10^8/ml)	Fresh semen motility (%)	Post-thaw frozen semen motility (%)	Abnormal spermatozoa (%)
H1H1/4	1.2	5.48±0.78	10.07±1.22	75.11±3.46^{a}	42.33±3.36	15.02±2.57
H1H3/26	8	5.73±0.30	9.17±0.48^{B}	72.63±1.37	42.19±1.37	15.99±0.79
H1H4/40	12.3	5.77±0.26	10.75±0.40	73.70±1.12	40.50±1.10	14.92±0.52
H1H5/18	5.6	5.24±0.37^{b}	9.31±0.58^{b}	66.31±1.64^{b}	37.55±1.71	16.59±1.27
H2H4/14	4.3	5.52±0.42	9.99±0.65	74.21±1.85	41.15±1.80	13.62±1.05
H3H3/46	14.2	6.20±0.24^{a}	10.77±0.37^{Aa}	73.61±1.05	39.17±1.02	15.67±0.46
H3H4/91	28.1	5.81±0.17	9.96±0.27	71.72±0.76	39.62±0.77	15.49±0.36
H3H5/35	10.8	5.77±0.29	10.11±0.45	71.47±1.27	42.07±1.28	16.12±1.48
H4H4/42	13	6.03±0.25	10.30±0.39	72.59±1.10	40.24±1.08	14.48±0.66

Values with different superscripts (a>b; A>B) within the same row denote significant difference $P < 0.05$ and $P < 0.01$, respectively. Means marked with the same superscript or without any superscript do not differ statistically. Abbreviations: H1, AAT; H2, GAC; H3, GAT; H4, GGC; H5, GGT; and H6, AAC.

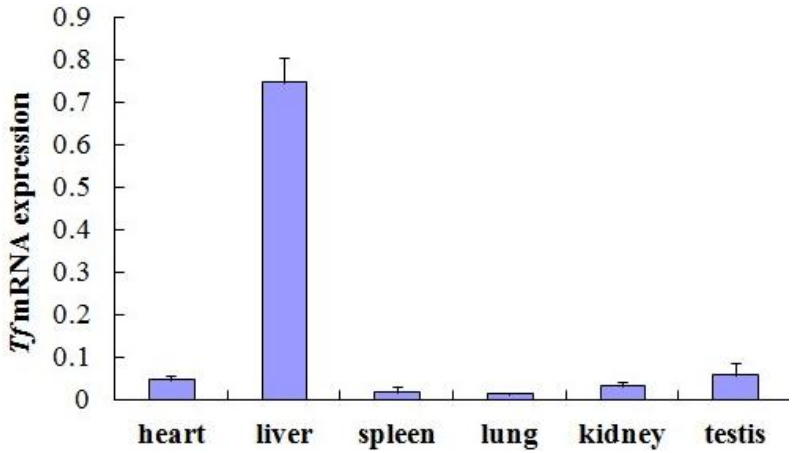

Figure 3. qPCR analysis of *Tf* mRNA expression in heart, liver, spleen, lung, kidney, and testis tissues.

convincing molecular markers for high ejaculate volume, semen density, and fresh semen motility. However, the manner by which these mutations alter the structure and conformation of *Tf* remains unknown. Therefore, further studies are required to clarify the biochemical reproduction trait effects of the various isoforms of Tf resulting from these polymorphisms.

Plasma Tf is known as a polymorphic blood protein synthesized by the liver (Fletcher and Huehns, 1968). Plasma Tf is also detected in the semen (Gilmont et al., 1990) and in milk (Kmiec, 1998), to which it is transferred from blood serum. Hence, we investigated the differences in *Tf* gene expression among heart, liver, spleen, lung, kidney, and testis tissues in 5 bulls (Figure 3). The quantitative data indicated that the *Tf* mRNA level was highest in liver tissue, and that a significant

difference was found between liver tissue and the other five tissues.

These experimental results agreed with the values of *Tf*, which were 70 mg/l in human semen (Tauber et al., 1975) and 2 to 3 g/L in human serum (Goya et al., 1972). The *Tf* mRNA level was highest in testis tissue, but there was no significant difference among them. These findings were similar to those previously reported in rats (Skinner et al., 1984). Testicular *Tf*s were synthesized by Sertoli cells and were found to transport iron to areas that could not be reached by plasma (Skinner et al., 1984).

In conclusion, the associations of *Dra*I, *Sty*I, and *Msp*I polymorphisms as well as haplotypic combinations of the *Tf* gene with semen quality traits in Chinese Holstein bulls were reported. Additionally, *Tf* gene expression levels were investigated in heart, liver, spleen, lung, kidney, and testis tissues. The haplotype combinations H3H3 and H1H1 should be used as the molecular markers for selecting bulls with high ejaculate volume, sperm density, and fresh sperm motility, respectively. The results suggested that *Tf* influences semen quality traits and should be used to breed Chinese Holstein bulls with excellent semen quality traits.

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