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Polymorphisms of the *ATP1A1* gene associated with mastitis in dairy cattle

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ABSTRACT. Mastitis affects the concentrations of potassium and sodium in milk. Since sodium-potassium adenosine triphosphatase (Na^+, K^+-ATPase) is critical for maintaining the homeostasis of these two ions, and is involved in cell apoptosis and pathogenesis, we presumed that polymorphism of the *ATP1A1* gene, which encodes the bovine Na^+, K^+-ATPase α1 subunit could be associated with mastitis. The *ATP1A1* gene was analyzed in 320 Holstein cows using PCR low ionic strength single-strand conformation polymorphism (PCR-LIS-SSCP) and DNA sequencing methods. A C/A SNP was identified at nucleotide position -15,739 in exon 17 of the *ATP1A1* gene, but it did not induce any change in amino acids. We examined a possible association of polymorphism of the *ATP1A1* gene with somatic cell score and 305-day milk yields. Individuals with genotype *CC* in *ATP1A1* had significantly lower somatic cell scores and 305-day milk yields than those with genotype *CA*. We also examined changes in Na^+, K^+-ATPase activity of red cell membranes. The Na^+, K^+-ATPase activity was significantly higher in dairy cows with

genotype *CC* compared to the other two genotypes, and the Na^+, K^+-ATPase activity of the resistant group was significantly higher than that of the susceptible group in dairy cows. We conclude that this polymorphism has potential as a marker for mastitis resistance in dairy cattle.

Key words: Dairy cow; *ATP1A1*; SNP; Mastitis; Somatic cell score; Na^+, K^+-ATPase

INTRODUCTION

Mastitis is a major health problem caused by bacterial infection of the udder tissues of cows (Rupp and Boichard, 2003). This disease is responsible for the reduced milk production and quality and the high milk production cost because of the increased veterinary expenses (Schutz, 1994). Efficient genetic selection for milk productivity is a response associated with unfavorable susceptibility of somatic cells to clinical mastitis (CM) (Strandberg and Shook, 1989; Lund et al., 2003; Carlén et al., 2004). In marker-assisted selection, mastitis resistance is an important breeding objective, but reports on CM are not generally available in dairy farm management. CM has an antagonistic genetic correlation to the production traits (Poso and Mantysaari, 1996). Fortunately, the genetic correlation between somatic cell score (SCS) and CM has been found to be in the range of 0.3 to 0.8, with an average of 0.6 (Hinrichs et al., 2005). In addition, SCS is relatively easily acquired and is widely used in the Dairy Herd Improvement (DHI) program; therefore, most studies have focused on SCS as an indirect measure of mastitis (Heyen et al., 1999; Longeri et al., 2006; Sugimoto et al., 2006).

As a complex genetic trait, mastitis resistance is likely controlled by multiple genes that, when considered individually, may produce only a modest effect on a phenotype (Prochazka et al., 2001). Although pleiotropy has been suggested, recent studies have been unable to identify a common quantitative trait locus for CM and somatic cell count (SCC) (Klungland et al., 2001; Schulman et al., 2004). However, related researches have reported that several genes are associated with mastitis, such as bovine chemokine receptor 2 (Youngerman et al., 2004), bactericidal peptide β-defensin 5 (Yang et al., 2006), lactoferrin gene (Zhou et al., 2006), Toll-like receptor 4 (Wang et al., 2007), and lymphocyte antigen DRB3 (Duangjinda et al., 2008). These genes provide a prospective for identifying valuable candidate genetic markers. According to previous studies, both clinical and subclinical mastitis can affect the composition and manufacturing properties of milk (Auldist and Hubble, 1998; Pyörälä, 2003). The most common effect reported is the dramatic increase of Na^+ concentration and decrease of K^+ concentration (Vandeputte-Van et al., 1993; Auldist et al., 1995; Hoeben et al., 1999). To some extent, variation in the ion concentrations might be correlated with the inhibition of ATPase activity of the iron-transporting system. Na^+, K^+-ATPase is a transmembrane enzyme that utilizes ATP to transport 3 Na^+ out and 2 K^+ into a mammalian cell via the plasma membrane (Rakowski et al., 1989). This helps in establishing and maintaining ionic homeostasis in the cytoplasm, which is required for normal resting membrane potentials, various cellular activities, osmotic balance, and cell volume regulation (Geering, 1997; Pavlov and Sokolov, 2000). In addition, this enzyme plays a role in the mechanism of cell death and apoptosis (Yu, 2003), which is associated with a wide range of disease states. Therefore, the bovine Na^+, K^+-ATPase gene may act as a potential

candidate gene for mastitis. Single nucleotide polymorphisms (SNPs) in this gene may increase or reduce the susceptibility of somatic cells to mastitis.

The bovine Na^+, K^+-ATPase gene consists of 2 major subunits - the catalytic α-subunit and glycosylated β-subunit. The catalytic α subunit is a large polypeptide that catalyzes the ion-dependent ATPase activity and carries the binding sites for ATP and the specific inhibitor ouabain. In mammals, 4 genes (α1-α4) are known to encode the α-subunit (Blanco and Mercer, 1998). Of these 4 isoforms, the α1 isoform of the enzyme, which is predominantly found in red blood cells and nerve tissue, is encoded by the *ATP1A1* gene; this gene is about 20.9 kb long and includes 22 exons. Glorioso et al. (2007) conducted SNP haplotype analysis and reported an association of human *ATP1A1* ($P < 0.000005$) and dear ($P < 0.03$) with hypertension. Polymorphism in intron 1 of the human *ATP1A1* gene could influence the Na^+, K^+-ATPase activity of red cell membranes in the case of C-peptide deficiency (Jannot et al., 2002).

In this study, we analyzed the polymorphisms within the coding region of bovine *ATP1A1* gene by using polymerase chain reaction-low ionic strength single-stranded conformation polymorphism (PCR-LIS-SSCP) and determined their possible associations with SCS and 305-day milk yield in Holstein cows. Furthermore, the Na^+, K^+-ATPase activities of red cell membranes across various genotypic dairy cows were analyzed, and the difference in the activity between the susceptible and resistant groups were compared.

MATERIAL AND METHODS

Animals

In all, 320 unrelated Holstein cows were randomly selected from the Yuanyuan farm of Pingdingshan city, Henan Province, China. Data of 305-day milk yields and blood samples with anticoagulant acid citrate dextrose were collected. The milk samples with the antiseptic were sent to Nanjing Dairy Cattle Centre to determine the SCS.

Twenty cows having the same parity, age, and milk stage were selected from the 320 cows on the basis of SCS. The milk samples were confirmed to be positive for bovine mastitis by the California Mastitis Test. The cows with different SCS levels were then equally divided into 2 groups (SCS for the susceptible group ranged from 4 to 5.32, while that for the resistant group was <4).

DNA extraction and PCR

Genomic DNA was extracted from 1 mL frozen/thawed blood samples by using a standard phenol-chloroform method. Quality and quantity of DNA samples, diluted to a final concentration of 50 ng/μL, were measured using 0.8% agarose gel electrophoresis and spectrophotometry. Primers were designed by the Oligo 6.12 software on the basis of the bovine *ATP1A1* gene DNA sequences (GenBank accession No. NC_007301.3). The E17 primers (forward, 5'-ACA AAC AAA AGG GTC ACA ACA T-3'; reverse, 5'-CTT ACC CTA GAT CCT GGC TCA T-3') were used to amplify a 301-bp fragment of the *ATP1A1* gene, including a partial intron 16, exon 17, and partial intron 17. PCRs were performed in a total volume of 20 μL containing 50 ng genomic DNA, 1X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.25 μM primers, 2.0 mM $MgCl_2$, 0.25 mM dNTPs, and 0.5 U Taq DNA polymerase (TaKaRa)

in a PTC-200 PCR machine (MJ Research). The thermal cycling process included an initial denaturation step at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. The reaction was terminated by a final extension at 72°C for 8 min. PCR products were detected using electrophoresis on a 1.5% agarose gel.

Polymorphism detection

The PCR products of the *ATP1A1* gene were screened for polymorphisms by using the LIS-SSCP analysis. Several conditions were optimized for the analysis, including the amount of PCR product, denaturing solution, acrylamide concentration, cross-linking, voltage, running time, and temperature. To 10 µL LIS loading solution (10% sucrose, 0.01% bromophenol blue, and 0.01% xylenecyanol FF), 2 µL PCR product was added. The mixture was incubated at 98°C for 5 min, cooled on ice, and loaded onto a 12% polyacrylamide gel (acrylamide/bisacrylamide 29:1, v/v). Electrophoresis was carried out using 1X TBE (45 mM Tris-borate/1 mM EDTA) at 160 V for 15 h. LIS-SSCP gel were detected using silver staining. The DNA bands of different LIS-SSCP patterns were purified using the Agarose Gel DNA Purification Kit (TaKaRa), cloned into the PMD19-T Vector (TaKaRa), and then sequenced by Shanghai Invitrogen Biotechnology.

Na^+, K^+-ATPase activity of red cell membranes

Five samples were included in each genotypic group, and 10 samples each were included in the susceptible and resistant groups. For determining the activity, 2 mL fresh blood was centrifuged for 5 min at 3000 *g*. Serum and the white cell layer were carefully removed. Red cells were not washed, and hemolysate was obtained by suspending the cells in 0.1 mM Tris buffer, pH 7.4, and centrifuging for 30 min at 4°C. The hemolysate was then centrifuged at 10,000 *g* for 30 min at 4°C, and the separated red cell ghosts were washed first with the hemolysing solution and then thrice with a solution containing 2 mM Tris, 0.02 mM EDTA, and 15.5 mM NaCl, pH 7.4. The whitish pellet was then suspended in 400 µL distilled ion-free water. The preparation was homogenized using a Potter-Elvehjem homogenizer; the protein concentration was determined by using the Folin phenol method (Lowry et al., 1951). The suspension was used as the source of enzyme. The Na^+, K^+-ATPase activity of red cell membranes was determined using Ultra Micro-Na^+, K^+-ATPase Detection Kit (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer protocol. The activity was expressed as µmol Pi·mg $protein^{-1}$·h^{-1}.

Statistical analyses

The frequency of alleles and genotypes, heterozygosities (*h*), polymorphism information content (PIC), effective number of alleles (N_E), and Hardy-Weinberg equilibrium at the mutation site were calculated using the POPGENE 1.31 software. One-way analysis of variance (ANOVA) as appropriate was used to test the differences between the groups by using SPSS 13.0.

Traits of interest were SCC on test day, which was measured at the Nanjing Dairy Cattle Centre, China, by using the following formula: $SCS = \log_2(SCC / 100) + 3$. The values were rectified to eliminate the effect of lactation days and sampling period on SCS (Wiggans and Shook, 1987).

The general linearity model was applied to analyze the associations of *ATP1A1* gene polymorphisms with SCS and 305-day milk yield in Holstein cows by using SPSS 13.0. The following linear model was used: $Y_{ijkl} = \mu + P_i + M_j + G_k + e_{ijkl}$, where, Y_{ijkl} is the value of analyzed traits of the dairy cows, μ is the overall mean, P_i is the number of parity, M_j is the lactation month of milk sample collection, G_k is the effect of the genotype, and e_{ijkl} is the random error. Data are presented as least squares means with associated standard error. Statistical significance was set at $P < 0.05$.

RESULTS

Polymorphism of the bovine ATP1A1 gene

The 301-bp fragment of the *ATP1A1* gene was amplified by PCR (Figure 1A). DNA sequencing revealed that the fragment included a partial intron 16, complete exon 17, and partial intron 17. Genetic polymorphisms in the E17 locus in the study population were detected using LIS-SSCP; each cow was classified as *AA*, *CC*, or *CA* with respect to the *ATP1A1* genotype (Figure 1B). A C/A SNP at nucleotide -15739 (Figure 1C) in exon 17 of the *ATP1A1* gene was responsible for the polymorphism. The DNA sequence of the *ATP1A1* gene is available in GenBank with accession number NC_007301.3. The genetic polymorphism in the bovine *ATP1A1* gene did not induce any change in the amino acid sequence.

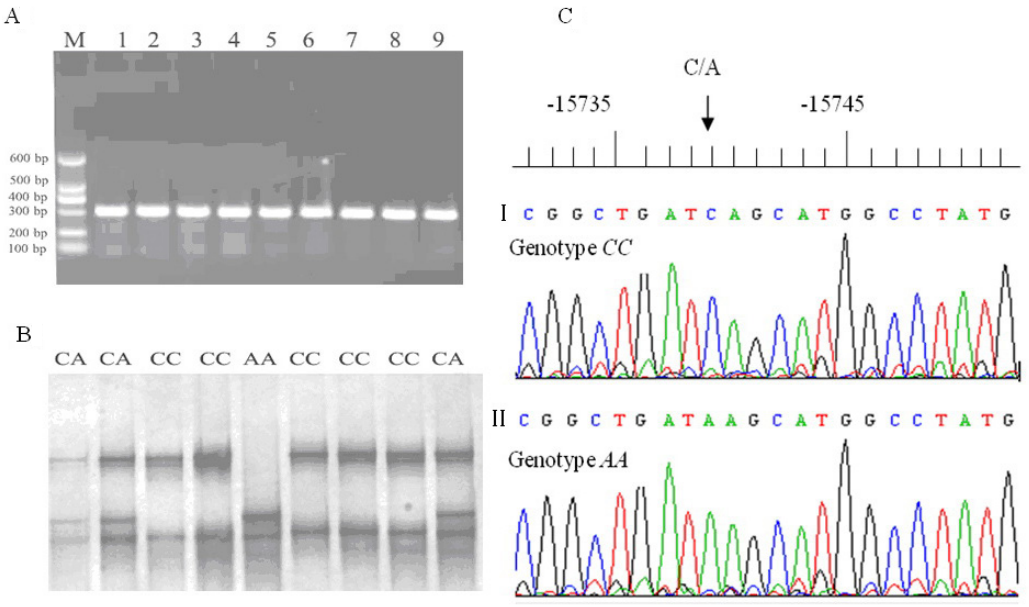

Figure 1. Electrophoresis result of PCR products, LIS-SSCP patterns and sequences of two homozygotes. **A.** PCR results of E17: marker (*lane M*); E17 (*lanes 1-9*). **B.** LIS-SSCP results of the PCR products E17, corresponding to *CC*, *CA*, and *AA* genotypes. **C.** Sequences of two types of homozygotes. Sequence I and II occur in the genotype *CC* and *AA*, respectively.

Genetic characters in the population

The genetic diversity at the E17 locus was calculated. The frequencies of the allele *C*/*A* were 0.86/0.14, and the genotype *CC* showed a higher frequency at 74.55%. These results suggested that the allele C was superior in this population and that it was a superior gene. The *h*, PIC, and N_E were 0.24, 0.21, and 1.32, respectively. The polymorphic site fitted the Hardy-Weinberg equilibrium (P > 0.05).

The relationship between bovine mastitis trait and ATP1A1 gene polymorphisms

The analysis of variance in SCS was calculated using the model with a genetic marker effect (Table 1). Results showed that parity, lactation month, and the *ATP1A1* genotype extremely affected the SCS (P < 0.01); the parity and the *ATP1A1* genotype also had significant effect on the 305-day milk yield (P < 0.05).

Table 1. Effects of different factors on somatic cell score (SCS) and 305-day milk yields.

Factors	Parity	Lactation month	Genotype
SCS	6.84**	6.65**	3.77**
305-day milk yields	2.13*	0.39	2.61*

*P < 0.05 and **P < 0.01 for significant differences from each other.

There was a significant association between the E17 locus genotype and mastitis trait. Given the small number of homozygous *AA* cows, the most frequent genotype classes (*CC* and *CA*) were used in the statistical analysis. The cows with a *CC* genotype showed lower SCS (3.20 ± 0.15) than those with a *CA* genotype (3.96 ± 0.19) (P < 0.01) (Table 2). However, the 305-day milk yield of cows with a *CA* genotype (8377.25 + 218.45) was higher than that of cows with a *CC* genotype (8022.06 + 179.79) (P < 0.05). This result indicated that dairy cows with a *CC* genotype were more resistant to mastitis infection than those with a *CA* genotype. In other words, allele *C* might be beneficial for acquiring mastitis resistance.

Table 2. Least-squares means and standard error for somatic cell score (SCS) and 305-day milk yields of different genotypic dairy cows.

Locus	Genotype	Number	SCS	305-day milk yields
E17	*CC*	237	3.20 ± 0.15**	8022.06 + 179.79*
	CA	77	3.96 ± 0.19	8377.25 + 218.45
	AA	6	4.13 ± 0.26	8465.41 + 198.33

*P < 0.05 and **P < 0.01 for significant differences from each other.

The Na^+, K^+-ATPase activity of red cell membranes in the groups

The Na^+, K^+-ATPase activities of red cell membranes in cows with genotypes *CC*, *CA*, and *AA* were 51.65 ± 2.82, 32.57 ± 2.56, and 40.19 ± 3.17, respectively. There was a significant difference in the activities among the 3 genotypes (P < 0.01; Figure 2). The Na^+, K^+-ATPase activity of the red cell membranes of the resistant group (52.60 ± 4.11) was considerably higher than that of the susceptible group (43.79 ± 3.48; P < 0.01; Figure 3).

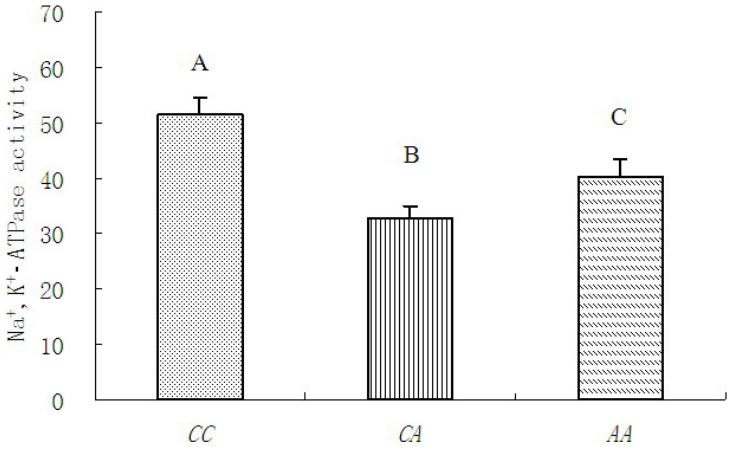

Figure 2. Na^+,K^+-ATPase activity of red cell membranes in different genotypic cows. Different superscript uppercase letters indicate significant differences ($P < 0.01$), and bars represent standard error.

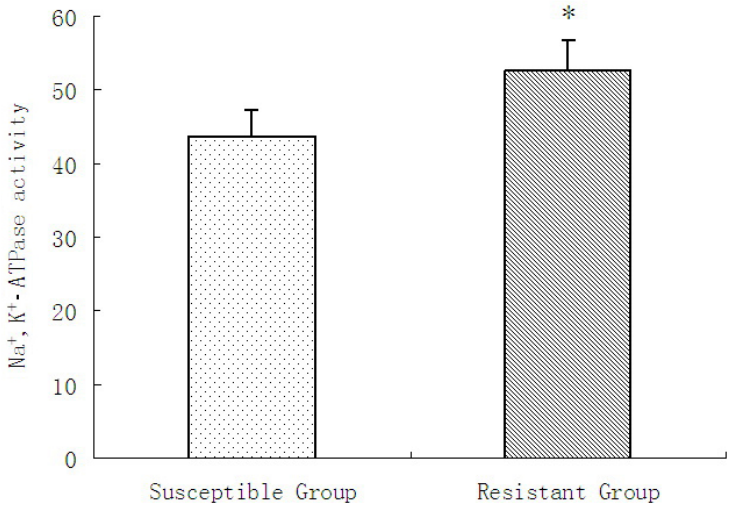

Figure 3. Na^+,K^+-ATPase activity of red cell membranes of different group in dairy cows. *Indicate significant differences ($P < 0.05$), and bars represent standard error.

DISCUSSION

In this study, PCR-LIS-SSCP was used to analyze the polymorphism of the bovine *ATP1A1* gene in Holstein cows. Allele *C* was the predominant allele of the E17 locus in the population, and the genotypic frequency of *CC* was the highest, while that of *AA* was the lowest. The E17 locus in the Holstein population fitted the Hardy-Weinberg equilibrium ($P > 0.05$). This indicated that the *ATP1A1* gene locus was under homeostasis, and artificial selection, migration, and genetic drift had little effect on this polymorphism site.

Hence, artificial selection at this polymorphic site for mastitis resistance needs to be applied in dairy cattle breeding program.

Recent studies showed a positive and moderately high genetic correlation between CM and SCC (Heringstad et al., 2000). SCC of milk might reflect the degree of mastitis (Philipsson, 1995; Barkema et al., 1999). Shook and Schutz (1994) also concluded that genetic correlation is reasonably high between SCS and CM, heritability is relatively higher for SCS than for SCC or CM, and SCS can be obtained relatively easily. Banos and Shook (1990) showed that the heritability of mean SCS for lactation was about 12%. Hence, SCS can be used for indirect selection for genetic improvement of mastitis resistance. Low SCSs are correlated with low probability of CM (Coffey et al., 1986). In this study, SCC was converted to SCS. Our results showed that parity and lactation month significantly affected the SCS ($P < 0.01$), which corroborated the results obtained by previous research (Wang et al., 2007). The association of the *ATP1A1* polymorphism with the mastitis trait in Holstein cows indicated that genotype *CC* of *ATP1A1* was related to mastitis resistance, whereas genotype *CA* was related to mastitis susceptibility. This showed that the bovine *ATP1A1* gene may play an important role in mastitis resistance. However, the 305-day milk yield of cows with genotype *CC* was lower than that of cows with genotype *CA* ($P < 0.05$). Similar results have been reported previously, and simultaneous improvement of milk yield and mastitis resistance appears to be economically undesirable because of the positive correlation between milk yield and mastitis (Strandberg and Shook, 1989; Rogers, 1993). Rupp and Boichard (2003) reported that selection of mastitis resistance by using genetic markers would increase the resistance to mastitis but lower milk production. Therefore, including SCSs with appropriate economic factors in selection indexes will improve the genetic traits and offer an overall advantage (Shook and Schutz, 1994).

Milk contains a high concentration of K^+ relative to Na^+; this is because the latter are actively removed from the secretory cells of the mammary glands by Na^+, K^+-ATPase located on the basolateral membrane (Linzell and Peaker, 1971). However, the concentrations of K^+ and Na^+ in milk are altered during mastitis. In cows affected with subclinical mastitis, the concentration of K^+ decreases and that of Na^+ increases (Auldist et al., 1995; Auldist and Hubble, 1998). This changes the osmolality of milk. Hence, information on Na^+, K^+-ATPase sensitivity to mastitis would be of interest since this system plays an important role in osmoregulation of milk. Changes in the osmolality of milk in mastitis-affected cows might indicate that the Na^+, K^+-ATPase activity is inhibited by mastitis. The Na^+, K^+-ATPase activity has been studied in humans and rats. The Na^+, K^+-ATPase activity is regulated by multiple mechanisms, and its functional roles vary in different conditions, making it vulnerable to pathogenic insults (Hollenberg and Graves, 1996; Therien and Blostein, 2000). Besides ATP dependence, the Na^+, K^+-ATPase activity is regulated by ionic distributions across the membrane (Gloor, 1997). Recent studies have shown that the *ATP1A1* gene polymorphism influences the Na^+, K^+-ATPase activity in the case of complete or partial C-peptide deficiency (Jannot et al., 2002). In the present study, Na^+, K^+-ATPase activity of red cell membranes was higher in the resistant group than in the susceptible group ($P < 0.05$), and dairy cows with genotype *CC* had remarkable mastitis resistance and had the highest Na^+, K^+-ATPase activity of red cell membranes. These findings suggested that the Na^+, K^+-ATPase activity may act as a physical indicator of mastitis resistance.

In conclusion, the polymorphism site described in this study might be a useful molecular marker of assisted selection of the anti-mastitis trait in dairy cattle breeding programs, although further studies on larger population are needed in order to expand its application.

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