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Genetic polymorphisms of the *BMAP-28* and *MASP-2* genes and their correlation with the somatic cell score in Chinese Holstein cattle

J. Wu, J.Y. Bai, L. Li, S. Huang, C.M. Li and G.L. Wang

College of Animal Science and Technology,
Nanjing Agricultural University, Nanjing, China

Corresponding author: G.L. Wang
E-mail: glwang@njau.edu.cn

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ABSTRACT. DNA sequencing, nested polymerase chain reaction (PCR), and PCR-restriction fragment length polymorphism were used to investigate mutations in the coding regions of the bovine myeloid antimicrobial peptide-28 (*BMAP-28*) and mannan-binding lectin-associated serine protease-2 (*MASP-2*) genes in 249 Chinese Holstein dairy cows. The associations between the polymorphisms and somatic cell score (SCS) were analyzed to determine the potential of these genes as genetic markers for breeding mastitis-resistant dairy cattle. The results revealed a C-86G synonymous mutation in the *BMAP-28* gene that caused no alteration in the amino acid sequence. G553A mutation was found in the *MASP-2* gene that led to the substitution of glycine with serine. The chi-square test showed that the G553A mutation was in Hardy-Weinberg equilibrium in the Chinese Holstein dairy cows, whereas the C-86G mutation was not. The variance analysis of the influence of group loci and different genotypes on the SCS showed that the SCS of genotype CC was significantly lower than those of genotypes CG and GG ($P < 0.05$) of the C-86G mutation, and that the SCS of genotype GG was significantly lower than those of genotypes

GA and AA ($P < 0.05$) of the G553A mutation. Genotype combination analysis showed that the combination of the *BMAP-28* genotype CC and the *MASP-2* genotype GG was the best one, in which the SCS was significantly lower than those in the other combinations. Thus, this combination might be useful as a molecular and genetic marker of mastitis in Chinese Holstein dairy cows.

Keywords: *BMAP-28*; *MASP-2*; Mastitis resistance; Somatic cell score

INTRODUCTION

Mastitis is one of the most common and important diseases of dairy cows and causes huge economic losses to the dairy industry worldwide. The susceptibility of individual animals to mastitis and their resistance to the disease differ significantly in the same herd (Heringstand et al., 2000). A strong positive genetic correlation is known to be found between the somatic cell score (SCS) and mastitis, ranging from 0.30 to 0.98 (Carlen et al., 2004). Therefore, indirect selection, typically based on indices such as SCSs as indicators of resistance to mastitis, has been practiced widely (Chen et al., 2011). The SCS is an effective method of monitoring bovine mastitis (Ruegg, 2012). More recently, an approach involving the improvement of bovine genetics through molecular marker selective breeding has become widely accepted. Identifying cattle with high resistance to mastitis is very important to facilitate marker-assisted selection strategies and select useful genetic markers for mastitis resistance and breeding in Chinese Holstein dairy cattle.

The bovine myeloid antimicrobial peptide-28 (*BMAP-28*) gene exhibits potent and broad-spectrum antimicrobial activity *in vitro* against gram-positive bacteria (Takagi et al., 2012) and shows anti-inflammatory immune responses (Kindrachuk et al., 2011). It has 3 introns and 4 exons and has a total length of 1953 bp; it is located on bovine chromosome 22. Intraperitoneal administration of 0.2 to 0.8 mg/kg *BMAP-28* was found to confer protection to mice against *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections and *Escherichia coli*-associated peritonitis (Lai and Gallo, 2009). The *BMAP-28* gene has two-way effects on the expression of tumor suppressors in mammary epithelial cells (Tomasinsig et al., 2010). Therefore, analyses of the *BMAP-28* gene polymorphism and investigations of its relationship with mastitis in cattle are important to identify molecular markers associated with mastitis resistance (or susceptibility).

The mannan-binding lectin-associated serine protease (*MASP-2*) gene is located on bovine chromosome 16; it includes 11 exons and encodes a protein of 686 amino acids. The *MASP-2* gene polymorphism exhibits a strong relationship with autoimmune diseases. Varga et al. (2008) found that the lectin pathway might be activated in subjects with chlorine-esterase-inhibitor deficiency, which is associated with low *MASP-2* and complement 4 levels. Thiel et al. (2009) showed that human *MASP-2* gene polymorphisms affect the protein folding of *MASP-2* and binding efficiency of *MASP-2* and mannan-binding lectin (*MBL*), in addition to autologous activation and serine protease activity. Although *MASP-2* gene polymorphisms have been studied extensively in humans and mice, there are few studies of this polymorphism in Chinese Holstein cattle.

The objective of this study was to detect polymorphisms of the *BMAP-28* and *MASP-2* genes in Chinese Holstein cows and to assess the association of these polymorphisms with

the SCSs in order to determine the potential of these genes as genetic markers for breeding mastitis-resistant dairy cattle.

MATERIAL AND METHODS

Animal data

In this study, 249 Chinese Holstein cows aged between 4 and 7 years were selected from the Taizhou Dairy Farm. Data on milk traits (fat content, protein content, adult milk equivalent weight, and somatic cell count) were collected from the Dairy Herd Improvement Center in Jiangsu Province. The hair follicle of the cows was plucked and stored at -20°C. Genomic DNA was extracted using DNA MiniExtract kit (Nanjing Runbang Bio-Tech Company, Nanjing, China). All DNA samples were stored at -80°C for subsequent analysis.

PCR amplification

PCR primers (Table 1) were designed using the Primer 5.0 software, and most of the genomic region of the bovine *BMAP-28* and *MASP-2* genes was sequenced (GenBank accession nos. AC000179 and AC000173, respectively). The PCR analyses were performed in 25 μL volumes containing 100 ng genomic DNA, 2.5 μL 10X PCR buffer, 1.8 mM $MgCl_2$, 0.5 mM dNTPs, 0.8 μM of each primer, and 0.5 U *Taq* DNA polymerase (TaKaRa, Dalian, China). The nested PCR program comprised initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 50-61°C for 30 s, and extension at 72°C for 30 s. The first step involved amplification for 20 cycles and termination. The second step involved amplification for 35 cycles, followed by a final extension at 72°C for 8 min. The PCR products were electrophoresed on 2% agarose gels and sequenced by Shanghai Megiddo Biomedical Technology Co. Ltd.

Table 1. Five couples of primer designed based on *BMAP-28* and *MASP-2* genes in coding region.

Primers	Sequence (5'→3')	Position	Product sizes (bp)	Temperature (°C)
BMAP-28 P1	F: AGAGGGGACAAATGGG R: CAGGGAAGTCACCTCACA	-582-502	1085	60
BMAP-28 P2	F: TCTGCACCTCGGTGTCTC R: CCAGCCGTAATATCTTCCTA	514-1816	1303	61
BMAP-28 P3	F: CATTTCTTCACCTTGCACA R: TCCACAGCACGAAGCAC	-200-139	340	50
MASP-2 P4	F: AGGAGTCATGGGGTCTGTTC R: GCCTGTTAGGGATATTTGCA	-87-837	925	61
MASP-2 P5	F: CTATCGCCTTCGCCTCTA R: GCCTCTAAGCCCGTGAA	290-642	353	56

PCR-restriction fragment length polymorphism

PCR-restriction fragment length polymorphism (RFLP) was performed to determine genotype variations within the amplified regions. The PCR product (5 μL) was mixed with 5 μL *Bcc*I and *Bfa*I, heated in a water bath at 37°C for 8 min, and immediately chilled on ice. Denatured DNA was subjected to 2% agarose gel electrophoresis in 1X TBE buffer at a constant voltage of 100 V for 30 min.

Statistical analysis

The genotype and allele frequencies, χ^2 value, polymorphism information content (PIC), effective number of alleles (N_E), and heterozygosities (H_E) were calculated. The association of single nucleotide polymorphisms (SNPs) or genotypes of the *BMAP-28* and *MASP-2* genes with milk yield and mastitis traits was analyzed using the least squares method and the SPSS software (17.0). The integrity of the animal model was analyzed using the following equation: $Y_{ijkl} = \mu + F_i + T_j + G_k + e_{ijkl}$, where Y_{ijkl} = individual phenotypic value, μ = overall mean, F_i = paternal effect, T_j = parity effect, G_k = genotype marker effect, and $e_{ijk\,l}$ = random error.

RESULTS

The P1 and P2 primer pairs were used to amplify the coding and flanking regions of the bovine *BMAP-28* gene. A novel SNP was detected in the 5'-untranslated region (UTR; Figure 1). PCR products of the g. C-86G locus digested using the *Bcc*I restriction endonuclease produced 115- and 225-bp fragments for genotype GG; 115-, 225-, and 340-bp fragments for genotype CG; and 340-bp fragments for genotype CC.

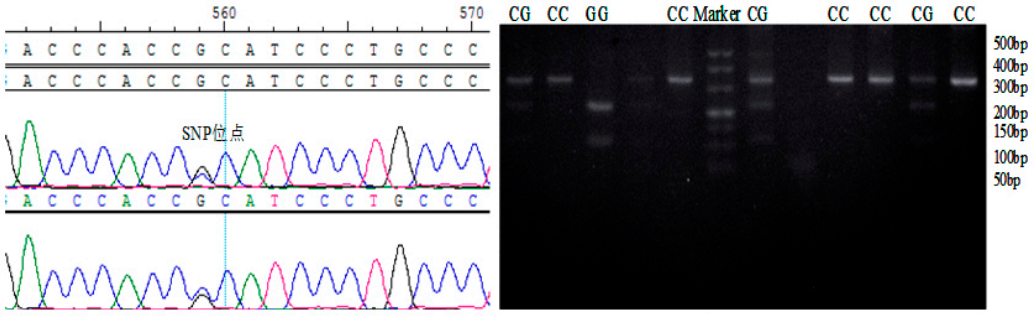

Figure 1. Sequencing result of *BMAP-28* and *Bcc*I restriction enzyme.

The P4 primer pair was used to amplify the region of the bovine *MASP-2* gene. A novel SNP was detected in the third exon (Figure 2). The PCR products of the g. G553A locus digested using the *Bfa*I restriction endonuclease produced 85- and 268-bp fragments for genotype AA; 85-, 268-, and 353-bp fragments for genotype GA; and 353-bp fragments for genotype GG.

Allelic and genotypic frequency

The PCR-RFLP analysis detected 2 alleles and 3 genotypes for both SNPs (Table 2). At the g. C-86G locus, the frequency of the CG genotype was higher than that of the GG and CC genotypes. The G allele was the predominant allele in the Chinese Holstein cattle. At the g. G553A locus, the frequency of the GA genotype was higher than that of the GG and AA genotypes. Allele G was the predominant allele in the population. The PIC, H_E, N_E, and χ^2 values for the 2 SNPs were calculated (Table 3). The results of the χ^2 test indicated that the g. G553A locus in the population was in Hardy-Weinberg equilibrium ($P > 0.05$), whereas the g. C-86G locus was not ($P < 0.05$).

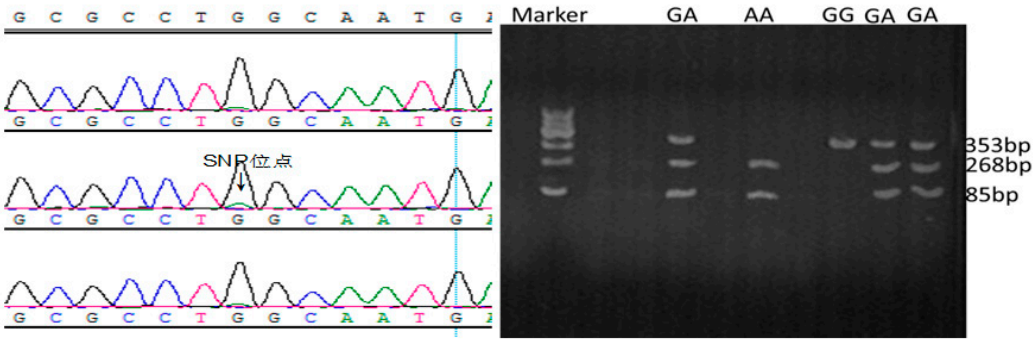

Figure 2. Sequencing result of *MASP-2* and *Bfa*I restriction enzyme.

Table 2. Gene frequency and genotype frequency in -86 bp mutation locus of *BMAP-28* and 553 bp mutation locus of *MASP-2*.

Gene	No.	Genotype frequency			Allelic frequency	
BMAP-28	249	CC	CG	GG	C	G
		0.293 (73)	0.406 (101)	0.301 (75)	0.496	0.504
MASP-2	249	GG	GA	AA	G	A
		0.249 (62)	0.534 (133)	0.217 (54)	0.516	0.484

Table 3. Data of polymorphism information contents (PIC), heterozygosities (H_E), effective number of alleles (N_E).

Loci	Polymorphism information content	H_E	N_E	χ^2
C-86G	0.375	0.499	1.996	8.872*
G553A	0.375	0.499	1.996	1.218

* or ** indicate significant difference at 0.01 or 0.05 levels.

Association of the polymorphisms with the SCS and milk composition traits in Chinese Holstein cows

For the *BMAP-28* C-86G polymorphism, the SCS of the CC individuals was significantly lower than those of the CG and GG genotype individuals ($P < 0.05$). The milk fat and protein content were significantly higher in the CG genotype individuals than in the CC genotype individuals. For the *MASP-2* G553A polymorphism, the SCS score of the GG individuals was significantly lower than those of the GA and AA genotype cows. The milk fat and protein content were significantly higher in the GA genotype cows than in the GG genotype cows. The adult milk equivalent weight was significantly higher in the AA than in the GA genotype cows.

Association of the genotype combinations with milk yield and milk composition traits in Chinese Holstein cows

The association between the genotype combination and milk composition traits was

analyzed in the Chinese Holstein cattle. The individuals with a CGGA haplotype combination had higher fat content than those with GGGA, CCAA, and CGGG genotype combinations ($P < 0.05$) and higher protein content than those with the GGAA, CCAA, and CCGG genotype combinations ($P < 0.05$). The CGAA individuals had the highest adult milk equivalent weight, whereas the individuals with the CGGA genotype had the lowest. Cows with the CCGG genotype had the lowest SCS, implying that they had potentially improved mastitis resistance.

DISCUSSION

In this study, we detected 2 novel SNPs, C-86G and G553A, in the 5′-UTR and in the third exon, respectively, of the *BMAP-28* and *MASP-2* genes. These SNPs might affect the process of exon homing by regulating gene mRNA splicing or transcription (Zan et al., 2007). The PIC, H_E, and N_E indices were used to evaluate the genetic variation in the population. We found that, the higher the values of PIC and H_E, the greater were the levels of genetic variation. The cattle showed moderate polymorphism ($0.25 < PIC < 0.5$) at the g. C-86G and g. G553A loci, revealing that the genetic polymorphisms were abundant. These loci could provide considerable genetic information and might be suitable for linkage analysis of markers and traits. The chi-square test indicated that the g. G553A locus in the population was in Hardy-Weinberg equilibrium ($P > 0.05$). The locus was possibly dynamically balanced due to the action of various factors, such as migration and genetic drift. The pressure of artificial selection on the g. G553A locus was not strong. On the other hand, the g. C-86G locus was not in Hardy-Weinberg equilibrium. The reasons for this may include the following: 1) small number of experimental cows; 2) substantial change in the genetic structure of the genotype of the Chinese Holstein cows because of economic conditions, as well as artificial selection; and 3) foreign genes affecting the primordial allelic and genotypic frequencies of the Chinese Holstein cows because of importing frozen, preserved embryos and semen.

Wang et al., (2012) detected a SNP at the 511 position of the *MASP-2* gene and found that it was significantly associated with the SCS; however, we did not detect this SNP in this study. Its absence might be due to the different herds of experimental cows used in the study. This study indicated that the *MASP-2* SNP and the SCS were significantly correlated (Table 4), which suggests that G553A might be a genetic marker of mastitis traits in Chinese Holstein cows. The G553A mutation in the *MASP-2* gene was identified as a nonsynonymous mutation, which led to the substitution of glycine with serine in the CUB1 domain. Stengaard-Pedersen et al. (2003) found a mutation in the CUB1 domain that caused the substitution of glycine with aspartic acid in the loop connecting beta strands 8 and 9 (i.e., D120G).

Table 4. Least squares mean (LSM) and standard errors (SE) for all production traits among the genotypes of *BMAP-28* and *MASP-2*.

Loci	Genotype	Somatic cell score	Fat content (%)	Protein content (%)	Adult milk equivalent weight (kg)
C-86G	CC	3.17 ± 0.18^{b}	2.97 ± 0.07^{b}	3.02 ± 0.04^{b}	8249.78 ± 274.51
	CG	3.95 ± 0.18^{a}	3.15 ± 0.07^{a}	3.13 ± 0.04^{a}	8123.23 ± 260.76
	GG	3.60 ± 0.16^{c}	3.09 ± 0.06^{ab}	3.11 ± 0.04^{a}	8157.80 ± 231.63
G553A	GG	3.21 ± 0.28^{c}	3.00 ± 0.10^{b}	2.97 ± 0.07^{b}	8300.56 ± 286.87^{ab}
	GA	4.24 ± 0.27^{a}	3.17 ± 0.10^{a}	3.07 ± 0.06^{a}	8135.05 ± 293.43^{b}
	AA	4.05 ± 0.24^{b}	3.06 ± 0.09^{b}	3.02 ± 0.06^{ab}	8621.73 ± 235.68^{a}

Data with different capital letters or small letters in same row indicate significant difference at 0.01 and 0.05 levels.

The G553A mutation caused the loss of MASP-2 binding to MBL and ficolin molecules and resulted in significantly lower serum MASP-2 expression levels. The aspartic acid at this position is conserved in all MASPs and in the similar serine proteases C1r and C1s of the classic complement pathway. Identification of the prevalence of mutations in the CUB1 domain should facilitate the determination of its clinical effect on immune defense and the development of inflammatory diseases. For the *BMAP-28* C-86G polymorphism, the SCS of the CC genotype individuals was significantly lower than that of the CG and GG genotype individuals, which might exhibit the strongest resistance to mastitis (Table 4). The gene polymorphism was located in the 5′ flanking region, which contained several nuclear factors involved in inflammatory reactions during the acute phase, such as interleukin-6, nuclear factor-κB, the interleukin-6 response element, acute phase response factors, and gamma interferon response element sites. Further in-depth study is needed to determine whether the mutation affected the expression and regulation of the *BMAP-28* gene and whether it affected the mastitis resistance of different individuals.

The somatic cell count showed a negative correlation with the adult milk equivalent weight (Tables 4 and 5). This might be due to the increased somatic cell count of the cows with mastitis. As a result, their mammary gland cells might be damaged or destroyed, thereby leading to dysfunctional secretion and subsequent decline in milk yield. The somatic cell count was positively correlated with the fat and protein contents, but the influence of mastitis on the fat content was still uncertain. Santos et al. (2003) asserted that an increase in the somatic cell count leads to a decline in milk production and the resulting effect of concentrated milk compensated for the decrease in milk fat synthesis and secretion; therefore, changes in fat mass fraction can be ignored and even lead to an increase in the total fat content. With respect to the fat content in the cows with mastitis, the permeability of blood-milk can be increased due to the destruction of the mammary gland epithelial cells, resulting in the inclusion of immunoglobulin, lactic acid protein, serum albumin, etc., into the breast milk, thereby increasing the fat content.

Table 5. Least squares mean (LSM) and standard errors (SE) for all production traits of *BMAP-28* and *MASP-2* genotypes combinations.

Genotypes combination (samples)	Somatic cell score	Fat content (%)	Protein content (%)	Adult milk equivalent weight (kg)
CCGG (24)	2.87 ± 0.35^{b}	2.89 ± 0.14	2.89 ± 0.09^{c}	8593.95 ± 471.87
CCGA (21)	3.89 ± 0.36^{a}	3.01 ± 0.15	3.08 ± 0.09	8452.46 ± 454.38
CCAA (38)	3.44 ± 0.28	2.89 ± 0.11^{c}	2.97 ± 0.07^{bc}	8400.72 ± 323.52
GGAA (32)	4.56 ± 0.30^{a}	3.07 ± 0.12	3.10 ± 0.08^{b}	8690.03 ± 355.37^{b}
GGGG (17)	3.50 ± 0.43	2.91 ± 0.17	2.93 ± 0.11	7443.91 ± 546.99
GGGA (28)	4.44 ± 0.32^{a}	3.18 ± 0.13^{b}	3.09 ± 0.08^{a}	8228.27 ± 379.59^{a}
CGGA (16)	4.70 ± 0.43^{a}	3.30 ± 0.17^{a}	3.16 ± 0.11^{a}	6780.61 ± 576.83^{c}
CGGG (26)	3.33 ± 0.34	2.93 ± 0.14^{c}	3.03 ± 0.09	8578.61 ± 400.83^{b}
CGAA (47)	3.97 ± 0.27^{a}	2.99 ± 0.11^{ac}	3.02 ± 0.07	8733.72 ± 277.71

Data with different capital letters or small letters in same row indicate significant difference at 0.01 and 0.05 levels.

This study showed that the CCGG genotype combination was the best combination, with a significantly lower SCS score in cows with this genotype than in those with the other genotype combinations. The CGGA genotype combination, which has a high somatic cell count but a low mild yield, should be gradually phased out in breeding programs (Hughes et al., 2005). Some of the genotypes did not have a beneficial effect on many of the milk pro-

duction traits. These should be eliminated with artificial selection for different genotypes that have positive impacts on these traits. Given the effects of sample size on multiple genes and quantitative traits, molecular biology research of dairy cow breeding should include a larger sample size to obtain more usable data.

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