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Genetic Variations of the Bovine *MX1* and Their Association with Mastitis

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ABSTRACT

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The primary agent of mastitis is a wide spectrum of bacterial strains; however, viral-related mastitis has also been reported. The *MX dynamin-like GTPase 1* (*MX1*) gene has been demonstrated to confer positive antiviral responses to many viruses, and may be a suitable candidate gene for the study of disease resistance in dairy cattle. The present study was conducted to investigate the genetic diversity of the *MX1* gene in Chinese cattle breeds and its effects on mastitis in Holstein cows. First, polymorphisms were identified in the complete coding region of the bovine *MX1* gene in 14 Chinese cattle breeds. An association study was then carried out, utilizing polymorphisms detected in Holstein cows to determine the associations of these single nucleotide polymorphisms (SNPs) with mastitis. We identified 13 previously reported SNPs in Chinese domestic cattle and four of them in Holstein cattle. A novel 12 bp indel was also discovered in Holstein cattle. In addition, haplotype frequencies and linkage disequilibrium of four SNPs detected in Holstein cows were investigated. Analysis of these four SNPs in Chinese Holstein cows revealed two SNPs (g.143181370 T>C and g.143182088 C>T) significantly ($P < 0.05$) associated with somatic cell score (SCS). The results indicated that SNPs in the *MX1* gene might contribute to the variations observed in the SCS of dairy cattle. Therefore, implementation of these two mutations in selection indexes of the dairy industry might be beneficial by favouring the selection individuals with lower SCS.

Keywords: *MX dynamin-like GTPase 1*; polymorphism; marker assisted selection; disease resistance; viral infection; somatic cell score

The *Mx dynamin-like GTPase (MX) 1* gene plays an important role in the immune response, induction of apoptosis, and signal transduction (Haller et al. 2015). *MX1* possesses a N-terminal GTPase domain, a bundle signalling element, and a C-terminal stalk domain. The N-terminal region of MX1 is essential for the regulation of GTPase activity and the C-terminal portion contains a leucine zipper motif that is involved in forming a homooligomer (Melen et al. 1992). The MX1 protein has been demonstrated to have potent activity against several RNA viruses, such as the influenza virus, vesicular

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stomatitis virus (VSV), and Thogoto virus (THOV) (Verhelst et al. 2013; Haller et al. 2015). Loss of MX1 function in human or mice enhances infection by multiple viruses, including orthomyxoviruses, paramyxoviruses, and hepadnaviruses (Verhelst et al. 2015). The *MX1* gene increases fitness through antiviral activity in mice, human, cows (Gerardin et al. 2004; Nakatsu et al. 2004), chickens (Ko et al. 2002), and pigs (Asano et al. 2002). In cattle, overexpression of the MX1 protein inhibits replication of foot-and-mouth disease virus (FMD) (Diaz-San Segundo et al. 2013). The porcine *MX1* gene has been proposed as a candidate gene for immunity traits (Wang et al. 2012). Furthermore, a polymorphism in the promoter region of the *MX1* gene is associated with porcine reproductive traits and respiratory syndrome virus infection (Li et al. 2015). These findings suggest that *MX1* is an interesting candidate gene for disease resistance in farm animals.

Bovine *MX1* cDNA (GenBank Acc. No. AF047692) was originally cloned from an Angus-Gelbvieh cross-bred cow by Ellinwood et al. (1998). The bovine *MX1* gene is mapped to chromosome 1 and is made up of 13 exons (Gerardin et al. 2004) that encode a 654 amino acid protein (Ellinwood et al. 1999). A total of 23 polymorphisms have been identified in the bovine *MX1* gene so far, including 10 synonymous mutations, one missense substitution, and a 13 bp indel mutation in the 3' untranslated regions (UTR) (Nakatsu et al. 2004). In addition, the *MX1* gene of other domestic animals has been identified, however, its impact on disease susceptibility remains unclear (Ellinwood et al. 1999; Asano et al. 2002; Ko et al. 2002). In light of this potential application, the present study was designed to detect polymorphisms of the *MX1* gene in native Chinese cattle.

Mastitis is the most significant health problem affecting dairy cattle. The primary agents of mastitis include a wide spectrum of bacterial strains; however, the incidence of viral-related mastitis has also been reported. Viruses can either be directly or indirectly involved in the etiology of bovine mastitis. In natural cases of mastitis, bovine herpesvirus 1 (BHV1), BHV4, FMD, and PI3 viruses have all been isolated from milk. It is likely that some viruses (BHV2, cowpox, pseudocowpox, FMD, VSV, and papillomaviruses) cause teat lesions, thereby damaging the integrity of the bovine udder, and indirectly contribute to mastitis. In addition, most studies that identify quantitative trait loci (QTL) for mastitis resistance use the somatic cell score (SCS) as an indicator trait for both subclinical and clinical mastitis (CM). Breeding schemes often use the SCS because it shows a moderate to high genetic correlation to CM and it is easier to record (Lund et al. 2007). Taking the functions of the MX1 protein into consideration, we hypothesized that *MX1* might also have an indirect impact on mastitis in dairy cattle. Therefore the aims of the present study were to detect polymorphisms of the bovine *MX1* gene in 13 Chinese cattle breeds (including Yellow cattle and Holstein cows), and to determine the association of these SNPs with milk SCS (considered a mastitis indicator) in 297 Chinese Holstein cows. We expect to discover candidate markers related to CM in Chinese dairy cattle.

MATERIAL AND METHODS

Animals. We investigated genetic variation in the bovine *MX1* gene using 466 individuals from 13 cattle breeds in China. All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture. A total of 120 animals from 12 yellow cattle breeds (ten animals per each breed) were selected from different Chinese farms: Mongolian cattle, Anxi cattle, Qinchuan cattle, Jinnan cattle, Luxi cattle, Jiaxian cattle, Zaosheng cattle, Nanyang cattle, Xuanhan cattle, Wannan cattle, Leizhou cattle, and Hainan cattle. In addition, a total of 346 Chinese Holstein cattle were selected for the study. For the association analysis, 297 Chinese Holstein cows were randomly selected from the Agricultural Machinery Corporation of Xi'an city in the province of Shaanxi. Blood samples were collected and genomic DNA was extracted from leukocytes using the standard phenol-chloroform method (Green and Sambrook 2012). Genomic DNA concentrations were measured and working solutions were prepared and adjusted to concentrations of 50 ng/μl. Ten DNA samples from each breed were selected to construct genomic DNA pools for yellow cattle. DNA samples from 50 Holstein cows were randomly selected for a DNA pool. The genomic DNA pool provided the templates for PCR amplification to explore genetic variation in the *MX1* gene.

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Phenotypic data. All of the dairy cows were 4.0–7.0 years old with the same lactation period. Milk samples were taken from each cow once each month for the entire lactation period. The test-day milk yield was recorded, SCS, fat, and protein percentages were collected from the laboratory of a dairy herd improvement (DHI) center (Shaanxi, China) using a milk composition analyzer (Foss Milk Scan FT 6000, Denmark) (Supplementary Table S1). In the cow, SCS is calculated according to Shook (1993):

$$SCS = \log_2(SCC/100) + 3$$

where:
SCC = cells/μl

The mean and standard error of test day record of average milk yield, fat percentage (%), protein percentage (%), and SCS were 20.779 ± 0.208 kg, 4.309 ± 0.019%, 3.161 ± 0.009%, and 4.962 ± 0.075, respectively, in the tested animals (Supplementary Table S1).

PCR amplification, SNP detection, and genotyping. The coding region of the bovine *MX1* gene was amplified with 15 primers designed according to the sequence obtained from GenBank (Accession No. NC_007299.5) using Primer 5.0 software (Supplementary Table S2). PCR amplification was conducted in a 50 μl volume containing 5 μl of 10× buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 μM of each primer, 1.5 U *Taq* DNA polymerase (TaKaRa, China), and 10 ng of genomic DNA. The PCR conditions were as follows: an initial step at 95°C for 5 min, 35 cycles at 94°C for 35 s, another 35 s at a specific annealing temperature for each primer pair (Supplementary Table S2), and 35 s at 72°C, followed by a final extension for 10 min at 72°C. SNPs were scanned via construction of genomic DNA pools and the sequencing method. The PCR product from the pooled DNA samples was confirmed by agarose gel electrophoresis and purified using an Agarose Gel DNA Purification kit (Qiagen Science, USA). Both strands were then sequenced using the ABI PRISM 377 DNA Sequencer (PerkinElmer, USA) (Chen et al. 2015). The sequences were imported into the BioXM software (Version 2.6) and were analyzed and searched for SNPs. Four SNPs (g.143181370 T>C, g.143181451 A>G, g.143182088 C>T, and g.143189365 T>C) within the *MX1* gene identified in Chinese Holstein cows were genotyped by PCR-restricted fragment length polymorphisms (PCR-RFLP) (Table 1). Three different genotypes of each SNP were confirmed by DNA sequencing again. Aliquots (6 μl) of the PCR products were digested with 10 U *Ava*I for g.143181451 A>G, *ScrF*I for g. 143182088C>T, and *Hae*III for g.143189365 T>C at 37°C for 10 h. The locus g.143181370 T>C has no natural restriction endonuclease cleavage site, so an artificially created restriction site-PCR (ACRS-PCR) primers were designed, forward primer MX1-g.143181370 T>C with G instead of A at the 3' end was designed to introduce a *Hinc*II recognition site. In addition, a 12 bp indel could be genotyped by different length of PCR products. The products were separated by 12% polyacrylamide gel (PAGE) in 1× Tris-borate-EDTA (TBE) buffer under constant voltage (200 V) for 2 h. The gels were stained with ethidium bromide and the genotypes were determined based on difference in electrophoretic patterns.

Statistical analysis. Gene frequencies were determined by direct counting. Population genetic indexes, such as heterozygosity (H_e), homozygosity (H_o), effective allele numbers (N_e), and polymorphism information content (PIC) were calculated according to Nei's and Botstein's methods. The formulas used were as follows:

$$H_o = \sum_{i=1}^{n} P_i^2,\ H_e = 1 - \sum_{i=1}^{n} P_i^2,\ N_e = 1 \Big/ \sum_{i=1}^{n} P_i^2$$

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where:
P_i and P_j = frequency of the i and j alleles, respectively
n = number of alleles

Hardy–Weinberg equilibrium (HWE) analysis was also conducted. The linkage disequilibrium (LD) structure and haplotypes were determined using the SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He 2005). The correlation coefficient r^2 is the most commonly used measure of LD.

SPSS software (Version 18.0) was used to analyze the relationship between the *MX1* genotypes and SCS in the Chinese Holstein group (Chen et al. 2015). The test day linear model was as follows:

$$Y_{ijklmno} = \mu + A_i + S_j + E_k + C_l + L_m + G_n + P_o + e_{ijklmno}$$

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Table 1. Primers for identifying mutations in the *MX1* gene by PCR-RFLP and ACRS-PCR

Primer name[1]	Primers (5'-3')	Size (bp)	T_m (°C)	Enzyme	Cleavage sites	Genotype (bp)
MX1-g.143181370 T>C	F: -ATATGAAGAGAAGGTGCGGCCCTGCGTTGA-	224	55.2	*Hinc*II	GTY↓RAC	*TT*: 224 *CT*: 224, 196, 31 *CC*: 196, 31
	R: -TCTGAGGTCCTCTGTTTCTG-					
MX1-g.143181451 A>G	F: -TCTGGCTGGCATTCTGTC-	550	55.2	*Ava*I	C↓YCGRG	*AA*: 550 *AG*: 550, 438, 112 *GG*: 438, 112
	R: -TCTGAGGTCCTCTGTTTCTG-					
MX1-g.143182088 C>T	F: -CTTGGGAATGAAGACGAG-	186	59.0	*Scr*FI	CC↓NGG	*CC*: 150, 36 *CT*: 186, 150, 36 *TT*: 186
	R: -AGTGGGAACAGTGACAGG-					
MX1-g.143189365 T>C	F: -CACAGGGAAGTTTGAGACC-	585	59.9	*Hae*III	GG↓CC	*TT*: 348, 170, 67 *CT*: 348, 170, 132, 67, 38 *CC*: 348, 132, 67, 38
	R: -CAGCCAACCTTGGACAG-					
MX1-intron3-indel	F: -AAGGGTGAGAACAGGGAG-	361	59.0			*Del 12/Del 12*: 349 *Del 12/Wt*: 361, 349 *Wt/Wt*: 361
	R: -AGTGGGAACAGTGACAGG-					

PCR-RFLP = PCR-restricted fragment length polymorphisms, ACRS-PCR = artificially created restriction site-PCR, T_m = annealing temperature, G = mismatch base
[1]four SNP were g.143181370 T>C (rs209535571), g.143181451A>G (rs210498846), g.143182088 C>T (rs110669418), g.143189365 T>C (rs208244814);

where:

$Y_{ijklmno}$ = test day record of the SCS
μ = herd overall SCS mean
A_i = effect of age
S_j = fixed effect of sire
E_k = fixed effect of season
C_l = calving number
L_m = fixed effect of lactation number
G_n = fixed effect of genotype/combined genotype for each SNP
P_o = random permanent environmental effect
$e_{ijklmno}$ = random residual effect

The Least Squares Means estimates with standard errors for different genotypes and SCS were used. Differences between the means of each group were considered significant at $P < 0.05$.

RESULTS

Polymorphisms and genotypes. The bovine *MX1* gene is made up of 13 exons. Comparison of the sequences of the *MX1* gene with reference sequence (GenBank Acc. No. NC_007299.5) revealed 13 SNPs in 13 Chinese breeds (Table 2). In Chinese yellow cattle, 13 SNPs were detected. Comparison of *MX1* mRNA and mature protein sequences with the reference sequences (GenBank Acc. No. NM_173940.2 and NP_776365.1) revealed four SNPs in Chinese Holstein cows (rs209535571: g.143181370 T>C, rs210498846: g.143181451 A>G, rs110669418: g.143182088 C>T, and rs208244814: g.143189365 T>C), all in the coding region (Table 2). In addition, a novel 12-bp indel was found in intron 3 (Table 2).

Among the four SNPs detected within the *MX1* gene of Chinese Holstein cows, three SNPs (g.143181451A>G, g.143182088C>T, and g.143189365T>C) could be identified by their natural endonuclease restriction sites, whereas g.143181370T>C was genotyped through the introduction of artificial restriction sites (Table 1 and Figure 1). For the g.143181451 A>G locus, the 550 bp PCR fragment (digested with *Ava*I restriction endonuclease) was amplified by the pair of primers MX1-g.143181451 A>G, and this resulted in fragments of different lengths (550 bp for the *AA* genotype; 550, 430, and 112 bp for the *AG* genotype; 430 and 112 bp for the *TT* genotype). For the g.143181370 A>G locus, digestion

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Table 2. SNPs identified in the *MX1* gene in Chinese cattle (GenBank Acc. No. NC_007299.5)

No.	SNPs and indel	Position (cDNA)[1]	rsID[2]	Allele	Amino acid[3]	Type of SNP	Detected in breeds
1	g.143181370 T>C	**exon 3 (129)**	rs209535571	*T*/*C*	43 Asn (gaT)/Asn (gaC)	synonymous	YC, HC
2	g.143181451 A>G	**exon 3 (210)**	rs210498846	*A*/*G*	70 Ser (taA)/Ser (taG)	synonymous	YC, HC
3	g.143182013 T>C	exon 3 (270)	rs470636442	*T*/*C*	90 lle (atT)/lle (atC)	synonymous	YC
4	g.143182088 C>T	**exon 4 (345)**	rs110669418	*C*/*T*	115 Phe (ttC)/Phe (ttT)	synonymous	YC, HC
5	g.143189365 T>C	**exon 7 (852)**	rs208244814	*T*/*C*	284 Ala (gcT)/Ala (gcC)	synonymous	YC, HC
6	g.143192302 T>C	exon 9 (1074)	rs109905056	*T*/*C*	358 Ser (agT)/Ser (agC)	synonymous	YC
7	g.143196228 G>A	exon 11 (1256)	rs719403072	*G*/*A*	419 Arg (cGt)/His (cAt)	missense	YC
8	g.143200788 T>C	exon 13 (1518)	rs520852688	*T*/*C*	506 Ala (gcT)/Ala (gcC)	synonymous	YC
9	g.143204549 C>T	3' UTR (2020)	rs455195411	*C*/*T*			YC
10	g.143204625 G>A	3' UTR (2086)	rs517650155	*G*/*A*			YC
11	g.143204631 C>T	3' UTR (2092)	rs459729171	*C*/*T*			YC
12	g.143204831 A>G	3' UTR (2292)	rs460590827	*A*/*G*			YC
13	g.143204845 T>C	3' UTR (2306)	rs722367644	*T*/*C*			YC
14	indel	**intron3**		12 bp deletion			HC

SNP = Single Nucleotide Polymorphism, YC = Yellow cattle, HC = Holstein cows

[1]comparison of the sequences of the *MX1* mRNA (GenBank Acc. No. NM_173940.2) and four SNPs and the indel detected in Holstein cows written in bold and used for association analysis

[2]rsID in database of dbSNP in NCBI

[3]comparison of the sequences of the MX1 mature protein sequence with reference sequence (NP_776365.1) (http://asia.ensembl.org/Bos_taurus/Gene/TranscriptComparison?db=core;g=ENSBTAG00000030913;r=1%3A143176083-143204865;t1=ENSBTAT00000012035;t2=ENSBTAT00000043742)

of the 224 bp PCR fragment with *Hinc*II restriction endonuclease resulted in different fragment lengths (224 bp for the *TT* genotype; 224, 196, and 28 bp for the *TC* genotype; 196 and 28 bp for the *CC* genotype). For the g. 143182088 C>T locus, digestion of the 186 bp PCR fragment with *ScrF*I restriction endonuclease resulted in different fragment lengths (150 and 36 bp for the *CC* genotype; 186, 150, and 36 bp for the *CT* genotype; 186 bp for the *TT* genotype). Similarly, for the g.143189365

Table 3. Genotypic and allelic frequencies, value of χ^2 test, and diversity parameters of the *MX1* gene analyzed in Chinese Holstein cows

SNPs	Genotypes[1] number/frequencies			Allele frequencies		H_e	N_e	PIC	HWE *P*-value[2]
g.143181370 T>C	*TT* 99/0.286	*TC* 200/0.578	*CC* 47/0.136	*T* 0.575	*C* 0.425	0.4887	1.9558	0.3693	**11.5599**
g.143181451 A>G	*AA* 102/0.295	*AG* 200/0.578	*GG* 44/0.127	*A* 0.584	*G* 0.416	0.4860	1.9453	0.3679	**12.4242**
g.143182088 C>T	*CC* 105/0.303	*CT* 199/0.575	*TT* 42/0.122	*C* 0.591	*T* 0.409	0.4834	1.9358	0.3666	**48.7607**
g.143189365 T>C	*TT* 99/0.286	*TC* 191/0.552	*CC* 56/0.162	*T* 0.562	*C* 0.438	0.4923	1.9696	0.3711	5.0964

SNP = Single Nucleotide Polymorphism, HWE = Hardy–Weinberg equilibrium, H_e = heterozygosity, N_e = effective allele numbers, PIC = polymorphism information content

[1]number of animals investigated n = 346

[2]values in bold indicate that the genotype distribution was not in agreement with the HWE ($P < 0.05$)

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Table 4. Linkage disequilibrium (LD) analysis of the *MX1* gene SNPs in Holstein cows

SNPs[1]	D'			r^2		
	g.143181451 A>G	g.143182088 C>T	g.143189365 T>C	g.143181451 A>G	g.143182088 C>T	g.143189365 T>C
g.143181370 T>C	0.886	0.730	0.929	0.758	0.263	0.790
g.143181451 A>G	–	0.812	0.956	–	0.337	0.868
g.143182088 C>T	–	–	0.813	–	–	0.356

SNP = Single Nucleotide Polymorphism, D' = linkage disequilibrium coefficient, r^2 = correlation coefficient
[1]four SNPs were g.143181370 T>C (rs209535571), g.143181451 A>G (rs210498846), g.143182088 C>T (rs110669418), g.143189365 T>C (rs208244814)

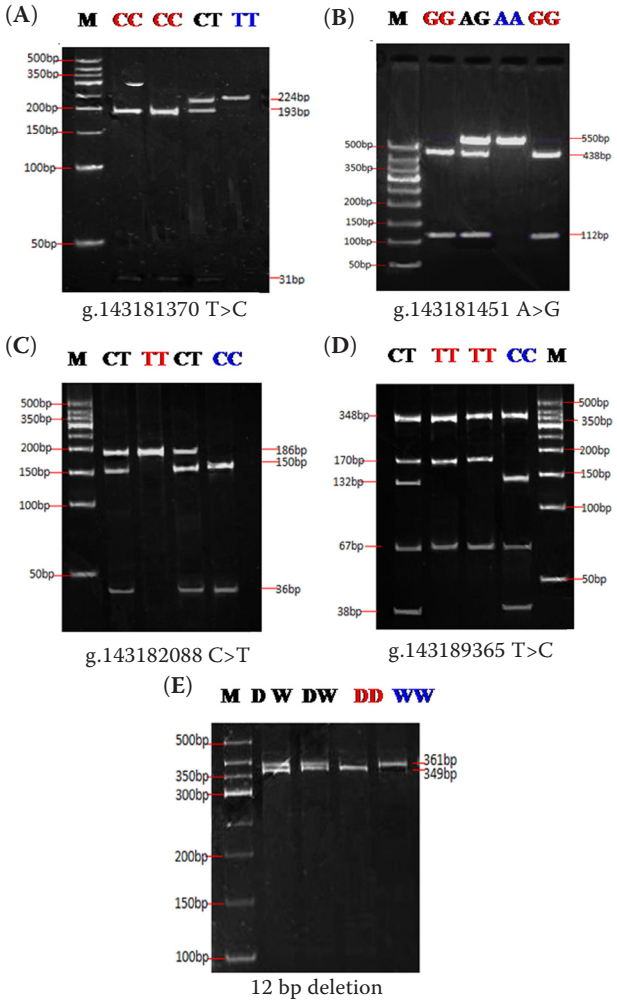

Figure 1. PCR-RFLP detection results of *MX1* gene PCR product

Gel = 12% polyacrylamide gel (PAGE) electrophoretic patterns, M = DNA molecular weight marker is Marker I (500, 400, 350, 300, 250, 200, 150, 100, and 50 bp), D = Deletion, W = Wild type

(**A**) g.143181370 T>C genotypes: *TT* (224 bp), *CT* (224, 196, 31 bp), *CC* (196, 31 bp); (**B**) g.143181451 A>G genotypes: *AA* (550 bp), *AG* (550, 438, 112 bp), *GG* (438, 112 bp); (**C**) g.143182088 C>T genotypes: *CC* (150, 36 bp), *CT* (186, 150, 36 bp), *TT* (186 bp); (**D**) g.143189365 T>C genotypes: *TT* (348, 170, 67 bp), *CT* (348, 170, 132, 67, 38 bp), *CC* (348, 132, 67, 38 bp); (**E**) 12 bp deletion in intron 3: DW (361, 349 bp), DD (349 bp), WW (361 bp)

T>C locus, digestion of the 585 bp PCR fragment with *Hae*III restriction endonuclease resulted in different fragment lengths (348, 170, and 67 bp for the *TT* genotype; 348, 170, 132, 67, and 38 bp for the *CT* genotype; 348, 132, 67, and 38 bp for the *TT* genotype) (Figure 1).

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Table 5. Haplotype frequencies of the *MX1* gene in Holstein cows

Haplotypes	SNPs				Frequency[1]
	g.143181370 T>C	g.143181451 A>G	g.143182088 C>T	g.143189365 T>C	
H1	*C*	*A*	*C*	*C*	0.028
H2	*C*	*A*	*C*	*T*	0.008
H3	*T*	*A*	*C*	*C*	0.008
H4	*T*	*A*	*C*	*T*	0.176
H5	*T*	*A*	*T*	*C*	0.002
H6	*T*	*A*	*T*	*T*	0.362
H7	*C*	*G*	*C*	*C*	0.354
H8	*C*	*G*	*C*	*T*	0.003
H9	*C*	*G*	*T*	*C*	0.032
H10	*T*	*G*	*C*	*C*	0.014
H11	*T*	*G*	*T*	*T*	0.013
Total					1.000

SNP = Single Nucleotide Polymorphism
[1]number of animals investigated n = 346

Genetic characteristics of the four SNPs in the bovine MX1 gene of Chinese Holstein cows. To evaluate the genetic diversity of the Chinese Holstein cows under investigation, H_e, N_e, and PIC, genotypic frequencies, and allelic frequencies of the four detected loci were calculated (Table 3). The results showed that the frequencies of the alleles *T*, *A*, *C*, and *T* were dominant, and for each of the four loci, the heterozygote was the most frequent among the three genotypes. Genetic indexes (H_e, N_e, and PIC) of the Chinese Holstein populations are presented in Table 3. These data demonstrated that the ranges of H_e, N_e, and PIC of the four SNPs were from 0.483 to 0.492, 1.9358 to 1.9696, and 0.3666 to 0.3711, respectively. We also estimated LD among four SNPs in the Holstein group. According to the definition of strong pairwise linkage ($r^2 > 0.33$), there was little linkage between the g.143181370 T>C and g.143182088 C>T loci, whereas there were strong linkages between other loci in the Holstein population (Table 4). Haplotype analysis of the four SNPs identified 11 haplotypes in these animals (Table 5). Haplotype 6 (H6: -TATT-) had the highest frequency (36.2%), followed by Haplotype 7 (H7: -CGCC-) (35.4%).

Relationships between MX1 polymorphisms and SCS. We analyzed the association between four SNPs, a 12 bp indel, and SCS in 297 Chinese Holstein cattle. The results are presented in Table 6. For g.143181370 T>C, animals with the *TT* genotype had a significantly higher value of average of SCS compared to those with *CT* and *CC* genotypes ($P < 0.05$) (Table 6), suggesting that

Table 6. Associations of polymorphisms of the *MX1* gene with SCS in Holstein cows

SNPs	Genotypes (n)[1]	Average of SCS[2]
g.143181370 T>C	*TT* (83)	5.208[a] ± 0.141
	CT (173)	4.851[b] ± 0.097
	CC (41)	4.966[b] ± 0.200
	pooled SE	0.264
	P	0.045
g.143181451 A>G	*AA* (86)	5.150 ± 0.138
	AG (172)	4.875 ± 0.098
	GG (39)	4.967 ± 0.206
	pooled SE	0.273
	P	0.223
g.143182088 C>T	*CC* (95)	5.231[aa] ± 0.131
	CT (168)	4.795[ba] ± 0.980
	TT (34)	5.083[ab] ± 0.218
	pooled SE	0.275
	P	0.007
g.143189365 T>C	*TT* (49)	5.164 ± 0.183
	CT (164)	4.893 ± 0.100
	CC (84)	4.850 ± 0.140
	pooled SE	0.252
	P	0.162
12 bp Deletion	*Del 12*/*Del 12* (83)	5.165 ± 0.141
	Del 12/*Wt* (162)	4.917 ± 0.101
	Wt/*Wt* (52)	4.824 ± 0.178
	pooled SE	0.249
	P	0.248

SCS = somatic cells score, SNP = Single Nucleotide Polymorphism, pooled SE = pooled standard error
[1]number of animals investigated n = 297
[2]values with different superscripts within the same column differ significantly at $P < 0.05$ (a and b); there is a significant difference between the mean values without common letters

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Table 7. Effect of the *MX1* combined genotypes on somatic cell score (SCS)

Combined genotype of four SNPs[1]	Number (281)	Average of SCS[2]
CTAACTCT	6	$4.681^{ab} \pm 0.534$
CTAACTTT	5	$5.358^{ab} \pm 0.585$
TTAACCTT	7	$5.472^{a} \pm 0.495$
TTAACTTT	35	$5.208^{ab} \pm 0.221$
TTAATTTT	30	$5.044^{ab} \pm 0.239$
CCAGCCCC	10	$4.724^{b} \pm 0.414$
CTAGCCCT	50	$5.436^{a} \pm 0.185$
CTAGCTCT	101	$4.602^{b} \pm 0.130$
CCGGCCCC	18	$5.299^{ab} \pm 0.309$
CCGGCTCC	13	$4.665^{b} \pm 0.363$
CTGGCCCC	6	$4.473^{ab} \pm 0.634$
Pooled SE		1.646
P-value		0.029

SNP = Single Nucleotide Polymorphism, pooled SE = pooled standard error

[1]four SNP were g.143181370 T>C (rs209535571), g.143181451 A>G (rs210498846), g.844048 C>T (rs110669418), g.143189365 T>C (rs208244814)

[2]values with different superscripts within the same column differ significantly at $P < 0.05$ (a and b); there is a significant difference between the mean values without common letters number of samples < 3 was not included in the significance test

the *T* allele might be associated with SCS. For g.143182088 C>T, individuals with the *CC* genotype had a significantly higher value of average of SCS compared to those with the *TT* genotype ($P < 0.05$).

To verify the effects of single SNPs on mastitis, we also correlated the combined genotypes of four SNPs with SCS. The combined genotype analysis of the four loci (Table 7) showed that animals carrying the TTAACCTT and CTAGCCCT genotypes had higher value of average of SCS than those with CCAGCCCC, CTAGCTCT, and CCGGCTCC genotypes. This finding is consistent with the higher average of SCS observed in the g.143181370 T>C *TT* and g.143182088 C>T *CC* genotypes ($P < 0.05$).

DISCUSSION

Many viral diseases are known to affect cattle. Previous research has demonstrated that the bovine MX1 protein can be used as a specific marker of acute viral infections in the health monitoring of dairy cattle, because its expression is increased following RNA viral infection (Muller-Doblies et al. 2004). The MX1 protein is known to confer specific resistance against a panel of single-stranded RNA viruses, raising the possibility that the identification of new SNPs of the bovine *MX1* gene could facilitate the implementation of improved genetic selection programs (Gerardin et al. 2004). We hypothesized that MX1 might also be relevant to mastitis resistance in dairy cattle.

Previous studies have suggested that the *MX1* gene is extremely polymorphic in domestic animals (Gordien et al. 2001; Ko et al. 2002; Nakatsu et al. 2004). In the chicken, 25 nucleotide substitutions have been detected, of which 14 were deduced to have caused amino acid exchanges. This suggests that the chicken *MX1* gene is polymorphic (Ko et al. 2002). In addition, 10 nucleotide substitutions that caused four amino acid exchanges were identified in the porcine *MX1* gene (Asano et al. 2002). In cattle, 23 *MX1* gene SNPs have been found in Holstein, Japanese Black, Hereford, and Brahman breeds (Nakatsu et al. 2004). In the present study, the complete coding region of the bovine *MX1* gene was examined in 13 Chinese breeds, and 13 SNPs were detected. Five SNPs were 3' UTR variants, and seven were synonymous mutations, among which one was a missense mutation (Table 2). In addition, a novel 12 bp indel was found in intron 3 of Holstein cattle. Our result demonstrated that the bovine *MX1* gene is highly polymorphic in Chinese cattle. Holstein cows have been intensively selected over the past decades for improved milk production. This could be the reason that only four out of 13 SNPs were found in Chinese Holstein cows (Table 2). According to the population genetic indexes and PIC value (0.369, 0.368, 0.367, and 0.371), these four loci exhibited abundant polymorphisms. The Holstein cows were not in HWE at three loci (g.143181370 T>C; g.143181451A>G; and g.143182088C>T). This could be attributed to artificial selection, which is consistent with the genetic background of Holstein cattle. Furthermore, genetic drift and migration might have also contributed to the disequilibrium observed.

It has been well established in laboratory mouse strains that the *MX1* gene exhibits polymorphisms, including indel mutations and nonsense muta-

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tions (Jin et al. 1998). Results have shown that mice carrying the *MX1* gene with a large deletion or a nonsense mutation are influenza virus-susceptible, have decreased metabolic stability of *MX1* mRNA, and lack the ability to synthesize the MX1 protein (Staeheli et al. 1988). A single amino acid substitution between Asn and Ser at position 631 in the chicken MX1 protein has been reported to determine resistant and sensitive antiviral activity (Seyama et al. 2006; Wang et al. 2012). In our study, the g. 143196228 G>A mutation leads to a substitution of arginine to histidine at position 419 in predicted N-terminal GTPase domain, which might alter the MX1 function and regulate the activity of GTPase. However, further confirmation of this is needed.

Bovine mastitis is the most costly and most common disease in the dairy industry worldwide (Nash et al. 2003). Therefore, mastitis resistance is an important breeding objective. It is likely that non-bovine associated viruses, such as Newcastle disease virus, can also play an indirect role in the etiology of bovine mastitis. So mastitis resistance is an important breeding objective. However, genetic progress by traditional breeding alone is difficult, because the incidence of mastitis is lowly heritable, difficult to record, and antagonistically correlated with production traits (Carlen et al. 2004). Breeding schemes often use SCS because it shows a moderate to high genetic correlation with CM (Lund et al. 2007). Our association analysis of *MX1* SNPs and SCS showed that g.143181370 T>C and g.143182088 C>T were significantly associated with the mean SCS (Table 6). Combined genotype analysis of the four loci showed that animals carrying *TTAACCTT* and *CTAGCCCT* combined genotypes had a higher mean SCS than those carrying *CCAGCCCC*, *CTAGCTCT*, and *CCGGCTCC* genotypes, and this result is consistent with the higher mean SCS recorded in the g.143181370 T>C *TT* and g.143182088 C>T *CC* genotypes ($P < 0.05$) (Table 7). Thus, the identification of these two SNPs (g.143181370 T>C and g.143182088 C>T) of the bovine *MX1* gene might facilitate the implementation of genetic selection programs aimed at improving the innate resistance of cattle to diseases. But investigation of specific gene functions and their interactions is very important, because innate resistance is a very complex trait that is controlled by several genes.

Mastitis is controlled by numerous genes, many of which have been mapped to QTL regions, and some of which could have strong effects on a particular trait. The bovine *MX1* has been mapped to the BTA (*Bos taurus* autosome) 1 (from approximately 143.16 to 143.20 Mb). Alignment of BTA 1 on the radiation hybrid (RH) map of the USDA-MARC cattle database indicated within a specific region (DIK4116), QTLs associated with mastitis and production, such as SCS (Rodriguez-Zas et al. 2002; Rupp and Boichard 2003; Daetwyler et al. 2008), milk profitability index (Chamberlain et al. 2012), and milk protein yield (Heyen et al. 1999). Our association analysis showed that g.143181370 T>C and g.143182088 C>T were significantly associated with the mean SCS. All the evidence along with the known biological function and the fact that bovine *MX1* maps closely to mastitis and production QTLs make *MX1* a promising candidate gene for mastitis resistance. The SNPs described here may be used as genetic markers linking QTLs with effects on these traits.

CONCLUSION

In conclusion, the results of the association study suggested that the two SNPs in bovine *MX1* could be used as genetic markers of mastitis resistance. The identification of these SNPs would likely facilitate the implementation of genetic selection programs aimed at improving disease resistance in cattle. The significant effects of *MX1* on mastitis confirmed the potential benefits of a marker-assisted selection (MAS) program for dairy cattle breeding in China.

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