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Full Length Research Paper

Novel SNPs polymorphism of bovine CACNA2D1 gene and their association with somatic cell score

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Mastitis is a major cause of economic loss in dairy cattle. In this study, the bovine CACNA2D1 gene was taken as a candidate gene for mastitis resistance. The objective of this study was to identify single nucleotide polymorphisms (SNPs) in the bovine CACNA2D1 gene and evaluate the association of these SNPs with mastitis in cattle. Through DNA sequencing and PCR-RFLP analysis, three mutations C367400T, A496561G and G519663A were detected in the cattle CACNA2D1 gene. Altogether 240 dairy cattle of three breeds (Holstein, Simmental, and Sanhe cattle) were genotyped and allele frequencies were determined. The effects of CACNA2D1 polymorphisms on somatic cell score (SCS) were analyzed and a significant association was found between G519663A and SCS. The mean of genotype GG was significantly lower than those of genotypes AG and AA. The results of this research will be useful in further studies to determine the role of the CACNA2D1 gene in mastitis resistance and further work will be necessary to investigate whether the CACNA2D1 gene play a role in defending the host from mastitis.

Key words: Association analysis, CACNA2D1 gene, dairy breeds, mastitis, somatic cell score.

INTRODUCTION

Mastitis is the most frequent disease in dairy cattle with large economic consequences (Janzen, 1970; Lescourret and Coulon 1994; Schukken et al., 1997; Kossaibati et al., 1998; Nash et al., 2003; Ruegg, 2003). Recent advances in molecular biotechnology provided great opportunities to incorporate molecular information into the traditional genetic evaluation models and to improve selection accuracies in livestock populations. These advancements have enabled the detection of some of the genes that contribute to genetic variation in economically important quantitative traits. Classical detection and mapping of genes, genetic markers and is easier to record and used as an indicator trait for CM as the genetic correlation is around 0.7 (Lund et al., 1999; Carle´n et al., 2004; Heringstad et al., 2006). Since it is difficult to measure the mastitis phenotype using a direct index, milk SCS has been most widely used as an indirect way to evaluate mastitis. Milk SCS is a $\log_2$ score of the milk somatic cell count (SCC) and has a positive correlation with clinical mastitis (Rupp and Boichard, 1999). Dairy cattle were less resistant to mastitis than dual-purpose breeds, Holstein cows were more easier and have mastitis than Sanhe and Sim- mental cows (Zhang et al., 2009; Wang et al., 2007). The calcium channel, voltage-dependent, alpha-2/delta subunit 1 (CACNA2D1) gene encodes for a member of the alpha-2/delta subunit family, a protein in the voltage-dependent calcium channel complex. The cattle CACNA2D1 gene contains 39 exons and 38 introns and has been mapped to BTA 4q18 (Buitkamp et al., 2003). It is located within the genomic region of SCS QTL (Zhang et al., 1998;

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Abbreviations: SNPs, Single nucleotide polymorphisms; **SCS,** somatic cell score; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism.

Table 1. Primer pairs designed for the bovine CACNA2D1 gene.

Name		Primer sequence	Ampicon length (bp)	Annealing temperature (°C)	Region
Frag 1	FOR:	5′-TGAAAGGGTTGTCCTGCCATC-3′	322	61.0	Intron 5
	REV:	5′-GTGCTTGTTGTTCCCATTGCC-3′			
Frag 2	FOR:	5′-CCATATCTGTGTCCTGTGTCT-3′	386	59.0	Intron 15
	REV:	5′-GGTAAAGTAAAGTGGAAGTCG-3′			
Frag 3	FOR:	5′-TCTAACGCCTTATTGACATC-3′	269	54.6	Intron23-exon24-intron24
	REV:	5′-CTTACTGTTTCCTTTGGTTC-3′			

Rupp and Boichard, 2003) and nearby the QTL of SCC (Daetwyler et al., 2008; Longeri et al., 2006) (http://www.animalgenome.org/cgi-bin/QTLdb/BT/draw_chromap?opt qtl =,chromos = 4,orderqtl = QTL_symbol, scale = 4, density = 10, submit = GO). Previous work indicated that the mutation of calcium channel genes would lead to a series of hereditary diseases in human beings and animals (Robinson et al. 2000). For instance, the mutations of CACNA2D1 and ryanodine receptor gene (RYR1) would cause human malignant hyperthermia syndrome (MHS) (Loke and Maclennan, 1998; Robinson et al. 2000), central core disease (CCD) (Robinson et al. 2002) and porcine stress syndrome (PSS) (Fujii et al. 1991). Recent research on calcium channel gene was mainly in human beings and model animals, and there are few researches in livestock such as cattle, pig and sheep. Therefore, the CACNA2D1 gene is considered to be one of the potential candidate genes influencing SCS and mastitis. As very little is known about the genetic variability of this gene among cattle breeds with SCS, the present study was undertaken in order to identify polymorphisms in the cattle CACNA2D1 gene and to evaluate the effects of identified polymorphisms on SCS, as indicator trait to mastitis.

MATERIALS AND METHODS

Cattle resource population and measure of mastitis

The cattle resource population of this study consisted of 73 Holstein (Caotan Dairy Farm, Xi'an, Shaanxi Province), 78 Sanhe (Xiertala Breeding Farm, Hailar, Inner Mongolia Auto- nomous Region) and 89 Simmental cows (Gaolintun Breeding Farm, Tongliao, Inner Mongolia Autonomous Region). Genomic DNA was extracted from whole blood by the standard phenol/ chloroform/isoamyl alcohol extraction protocol (Mullenbach et al., 1989) then dissolved in TE buffer (10 mmol/l Tris-HCl and 1 mmol/l EDTA, pH 8.0), and kept at -20°C.

Measure of mastitis

The milk samples, including an antiseptic, were collected and sent to Beijing Dairy Cattle Centre for somatic cell count (SCC) detection and converted into SCS, using formula: SCS = log 2 (SCC/100000) + 3. All experimental protocols and care of the animals were performed according to authorization granted by the Chinese Ministry of Agriculture.

Primer design and PCR amplification

The mRNA (GenBank NO. XM_609993.4) and DNA sequences (GenBank NO. NC_007302.4) of the bovine *CACNA2D1* gene were used for primers design using Primer Premier5.0 software (Premier Biosoft International, Palo Alto, CA). Primer sequences and their corresponding amplified fragment sizes and region are shown in Table 1. The polymerase chain reactions (PCR) were carried out in a total volume of 20 µl solution containing 50 ng template DNA, 1 × buffer (Tris-HCl 100 mmol/l, pH 8.3; KCl 500 mmol/l), 0.25 µmol/l primers, 2.0 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs, and 0.5U *Taq* DNA polymerase (Promega, Madison, WI, USA). The polymerase chain reaction (PCR) protocol was 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at the corresponding temperature (shown in Table 1) for 30 s and 72°C for 30 s, and a final extension at 72°Cfor 8 min. The PCR products were separated on 1.5% agarose gel (Promega) including 0.5 µg/ml of ethidium bromide, photographed under UV light.

Genotype determination of PCR-RFLP and DNA sequencing analysis

The PCR amplified products were purified using a Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology, P. R. China) and sequenced by an ABI 377 sequencer (Beijing Aolaibo Biotechnology, P. R. China; Applied Biosystems 377 DNA sequencer, Foster city, CA, USA). After sequence analyses and subsequently blast alignment, new finding SNPs finally designing RFLP. For the PCR-RFLP assays, aliquots of 5 µl PCR amplified products were digested with 2U restriction enzyme at 37°C for 10 h following the supplier's manual. The digested products were detected by electrophoresis for 1 h within 100 V in 2.5% agarose gel stained with ethidium bromide in 1 × TAE buffer.

Statistical analysis

Differences in genotypic and allelic frequencies at bovine CACNA2D1 gene among three populations were analyzed by Popgene32 (Yeh et al. 1997). The Hardy-Weinberg equilibrium of the mutation was determined by $\chi 2$ test. Analysis of associations between the genotypes of single nucleotide polymorphisms (SNPs) and SCS that reflects mastitis traits was carried out with the GLM procedure, using SAS software (Statistical Analysis System 9.1, SAS Institute Inc.) by the following formula:

$$y_{ijklm} = \mu + b_i + f_j + a_k + p_l + g_m + h_n + i_k + e_{ijklmn},$$

Table 2. Genotype and allelic frequencies of CACNA2D1 SNPs and their association with SCS.

Breed	Number of cattle	Frequency	C367400T			A496561G			G519663A		
			TT	CT	CC	AA	AG	GG	AA	AG	GG
Holstein	73	Genotype	0.52 (38)	0.19 (14)	0.29 (21)	0.42 (31)	0.16 (12)	0.42 (30)	0.38 (28)	0.36 (26)	0.26 (19)
		Allele	0.62		0.38	0.51		0.49	0.56		0.44
Sanhe	78	Genotype	0.38 (30)	0.24 (19)	0.38 (29)	0.44 (34)	0.22 (17)	0.34 (27)	0.50 (39)	0.32 (25)	0.18 (14)
		Allele	0.51		0.49	0.54		0.46	0.66		0.34
Simmental	89	Genotype	0.38 (34)	0.29 (26)	0.33 (29)	0.45 (40)	0.17 (15)	0.38 (34)	0.35 (31)	0.36 (32)	0.29 (26)
		Allele	0.53		0.47	0.53		0.47	0.53		0.47
Total	240	Genotype	0.43 (102)	0.24 (59)	0.33 (79)	0.44 (105)	0.18 (44)	0.38 2 (91)	0.41(98)	0.35 (83)	0.24 (59)
		Allele	0.55		0.45	0.53		0.47	0.58		0.42
SCS (LSM ± SE)			3.35 ± 0.77	3.33 ± 0.82	3.46 ± 0.81	3.47 ± 0.78	3.28 ± 0.80	3.27 ± 0.78	3.62 ± 0.78a	3.30 ± 0.76a	2.54 ± 0.80b

The numbers in parentheses are the genotype individuals.

Where y_{ijklm} = lactation average SCS, μ = global mean, b_i = breed effect, f_j = calving number effect, a_k = age effect, p_l = lactation month effect, g_m = genotype effect, h_n = farm effect, i_k = season, and e_{ijklmn} = residual.

RESULTS

Identification and genotyping of SNPs

Three single nucleotide polymorphisms (SNPs) were identified by the PCR-RFLP method, including C367400T, A496561G and G519663A, which were located in position 367400, 496561 and 519663 of the cattle CACNA2D1 gene, respectively in intron 5, intron 15 and exon 24. These mutations created new *Rsa I*, *Taq I* and *Hpa* II restriction sites. The PCR product of Frag1 was digested with the *Rsa I* enzyme. The three possible genotypes were defined by three distinct banding patterns: TT (322 bp fragment), CT (322, 236 and 86 bp fragments), and CC (236 and 86 bp fragments). The PCR product of Frag2 was digested with the *Taq I* enzyme, the genotype AA represents the occurrence of one band of 386 bp, genotype AG represents three restriction fragment bands of 386 bp, 229 bp and 157 bp, and genotype GG represents two bands of 229 bp and 157 bp. The PCR product of Frag3 was digested with *Hpa*II enzyme, and divided into three genotypes, AA (269 bp fragments), AG (269, 175 and 94 bp fragments) and GG (175 and 94 bp fragments). DNA sequencing analysis confirmed that C→T mutations at position 367400, A→G mutations at position 496561 and G→A mutations at position 519663 of the bovine CACNA2D1 gene, occurred. The geno- typic and allelic frequencies of the CACNA2D1 gene in 240 cattle are presented in Table 2. The result of Hardy-Weinberg equilibrium for the three SNPs in the studied populations indicated that the polymorphism site in the three populations fitted with Hardy-Weinberg equilibrium ($P > 0.05$).

Association of the CACNA2D1 gene polymorphisms with SCS

The analysis of variance on somatic cell score (SCS) was calculated using the model with genetic marker effect (Table 2). The relationship between genotypes and SCS was evaluated and shown in Table 2. Only Polymorphism G519663A was significantly associated with SCS. Cows of genotypes AA and AG had higher SCS than those of the GG genotype. Polymorphisms C367400T and A496561G were not significantly associated with SCS (Table 2).

The effect of different breed types within the three polymorphisms on SCS was analyzed and are shown in Table 3. SCS of Holstein cows was significantly higher than that of the Sanhe and Simmental cows, while SCS of the Sanhe cows were significantly higher than that of the Simmental cows for all three polymorphisms (Table 3).

Table 3. Effects of different breeds on SCS.

Single nucleotide polymorphism	Breed	Somatic cell score (LSM ± SE)
C367400T	Holstein	4.93 ± 0.81a
	Sanhe	3.28 ± 0.80b
	Simmental	2.21 ± 0.81c
A496561G	Holstein	4.88 ± 0.79a
	Sanhe	3.23 ± 0.79b
	Simmental	1.98 ± 0.80c
G519663A	Holstein	4.72 ± 0.79a
	Sanhe	2.89 ± 0.78b
	Simmental	1.85 ± 0.79c

Least squares means (LSMs) with the different superscripts differ significantly ($P < 0.05$); SE, standard error.

DISCUSSION

For many years, breeding goals for dairy cattle had focused mainly on increasing the productivity and had ignored health traits such as disease resistance. Higher yielding cows tend to have higher health costs. For instance, mastitis is the most prevalent production disease in dairy herds world-wide and is responsible for several effects on production. Milk production losses, drugs, discarded milk, veterinarian service, labor, milk quality impairment and culling of cows are the economic damage of mastitis. Prospects for the development of an effective vaccine are limited by the variety of microorganisms causing mastitis and by a lack of information on the genetic factors that influence disease resistance. The interest in selection for resistance to health problems in the dairy industry, as well as the selection for improvement of the health of live- stock for consumers, are internationally of increasing importance (Stear et al., 2001). But unfortunately, health traits usually have low heritabilities and limited amounts of data, which hamper the potential for genetic improvement by traditional selection methods. Consequently, there has been considerable interest in defining genetic and immunological markers that could be used to select for improved disease resistance (Park et al., 2004).The candidate gene approach may provide a more direct understanding of the genetic basis for the expression of quantitative differences bet- ween individuals (Noguera et al., 2003), and revealing genomic regions and specific markers that are associated with traits. In the present study, CACNA2D1 was considered to be a potential candidate gene influencing mastitis, because the cattle CACNA2D1 gene has been mapped to BTA 4q18 (Buitkamp et al., 2003) and located within the genomic region of QTL for SCS (Zhang et al., 1998; Rupp and Boichard, 2003) and nearby SCC (Longeri et al., 2006). Three novel SNPs of the CACNA2D1 gene, which were located in intron 5, intron 15 and exon 24, were preliminarily identified by PCR-RFLP and sequenced. Sequence analysis showed that these new alleles were caused by C to T, A to G and G to A mutations at positions 367400, 496561 and 519663, respectively. These mutations were detectable by digestion with restriction enzymes *Rsa I, Taq I and Hpa* II, respectively. In the three researched populations, nucleotide T of C367400T, nucleotide A of A496561G and nucleotide A of G519663A were the predominant nucleotides and the frequencies of the TT Genotype of C367400T, AA of A496561G and AA of G519663A were highest in all populations except for the AG Genotype of A496561G, which was highest in the Simmental population. The effects of the PCR-RFLP polymorphism genotypes and the associations between the genotypes and SCS were analyzed. Significant association between the G519663A locus and SCS was found. Individuals of genotype GG had significant lower SCS than those of genotype AG and AA. However, the CT and AG genotypes of the C367400T and A496561G SNPs, respectively, were also associated with lower SCS than their homozygotes, but it was not significantly different. Furthermore, the SCS of Holstein cows being significantly higher than that of the Sanhe and Simmental cows, and the SCS of the Sanhe cows being significantly higher than that of Simmental cows, lead to the conclusion that dairy cattle were less resistant to mastitis than dual-purpose breeds. Zhang et al (2009) and Wang et al (2007) had reported the similar result, respectively (Zhang et al., 2009; Wang et al., 2007). Results from this study indicate that that the *CACNA2D1* gene has potential effects on SCS and mastitis resistance.

In conclusion, the CACNA2D1 gene was found to be associated with SCS. This study provides preliminary information that the CACNA2D1 gene has potential effects on mastitis resistance. Since the detection of this association is based on a relatively small sample size, further work are necessary to study these SNPs in larger populations and other breeds to better clarify the role of these SNPs on SCS in cattle and investigate whether the CACNA2D1 gene play a role in defending the host from

mastitis.

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