Health and production traits in bovine are associated with single nucleotide polymorphisms in the *NOD2* gene

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ABSTRACT. The *NOD2* gene plays a fundamental role in initiating the inflammatory and subsequent immune response. *NOD2* was previously identified as a susceptibility locus for inflammatory bowel diseases in humans. In this study, we detected 2 mutations in exon 12 (A→T, G→A) among 5 cattle breeds (N = 315) and analyzed their associations with production traits and genetic resistance against bovine mastitis in Chinese Holstein and Chinese Simmental breeds (N = 218). The transitions (A→T) at position 114 bp were associated with somatic cell score (P < 0.01). The G→A at position 1594 bp plays a critical role in increasing 305-day milk yields. In Chinese Holstein and Chinese Simmental breeds, the BB genotype may contribute to disease susceptibility. Compared to all genotypic combinations, the A, B, and

FF genotypes are beneficial not only for reducing somatic cell score but also for increasing production.

Key words: Bovine mastitis; *NOD2* gene; Somatic cell score

INTRODUCTION

Inflammatory diseases such as bovine mastitis, is one of the main causes of the losses in the dairy industry, either clinical, or subclinical. Economic consequences include decreased milk production, alteration of cheese-making properties, and increased production costs (Pellegrini, 1994). Bovine mastitis is usually due to a microbial infection (Watts, 1988). The bacteria responsible for bovine mastitis can be classified as environmental (*Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*) or contagious (*Staphylococcus aureus* and *Streptococcus agalactiae*) (Bramley, 1996). The predisposition of mastitis has been proposed to be genetically determined in epidemiological studies with average heritability of 0.04 (Nash et al., 2004; Mark et al., 2005). However, the relative candidate genes involved in this process are unknown.

The innate immune response represents the first defense line for preventing systemic infection with bacteria. Nod2 is a key component in the innate immune system, and may be involved in the innate immune response to peptidoglycans (Bonen et al., 2003) and detection of bacterial products to induce the activation of proinflammatory signaling pathways, such as the nuclear factor-κB pathway (Girardin et al., 2001; Inohara et al., 2001). Lipopolysaccharides, as the main endotoxins derived from Gram-positive and Gram-negative bacteria, have been suggested to play a pivotal role in the pathogenesis of a variety of infectious and allergic diseases (Hugot et al., 2001). The *NOD2* gene, located on chromosome 16q21, has 12 exons and encodes a protein of 1040 amino acids (Ogura et al., 2001). The Nod2 protein contains a carboxy (C)-terminal leucine rich repeat domain, a nucleotide-binding site domain, and 2 amino (N)-terminal domains (CARDs) (Girardin et al., 2002; Inohara et al., 2002). The leucine rich repeat domain is involved primarily in the recognition of bacterial peptidoglycans, and the CARD domains are known to interact with CARD containing serine/threonine kinase Rip2 via homophilic CARD-CARD interactions; this leads to the activation of nuclear factor-κB (Ogura et al., 2001). The *NOD2* gene has been widely adopted in studies of the human immune system (Uehara et al., 2002) and swine immune system (Shimosato et al., 2005a,b; Tohno et al., 2008). Recently, the *NOD2* gene was detected in bovine mammary glands following intramammary infection with *S. aureus* (Cormac et al., 2011). However, the association between mutations in genetic polymorphism of *NOD2* gene and genetic resistance against bovine mastitis has not been examined.

The objective of this study was to identify the single-nucleotide polymorphisms in 8, 11, and 12 exons of the *NOD2* gene in 5 cattle breeds and to analyze the relationship between gene variation and bovine mastitis, 305-day milk yields, and constituents of milk, which will be beneficial for cattle breeding and genetics.

MATERIAL AND METHODS

Animal source

Tissue and milk samples from 218 cattle were randomly obtained from 2 breeds

(Chinese Holstein and Chinese Simmental). To obtain more information on the genetic structure of *NOD2* among different cattle populations, 86 cattle were randomly sampled from 3 Chinese indigenous breeds (Dechang buffalo, Tongjiang cattle, and Maiwa yak). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Provincial Key Laboratory of Animal Breeding and Genetics, Sichuan Animal Science Academy.

Milk sampling and analysis

Quarter milk samples were collected aseptically by dairy personnel according to International Dairy Federation standards (IDF, 1985). Milk samples were examined for fat, lactose, and total protein contents. The milk samples were screened for their somatic cell count (SCC) according to the National Mastitis Council (NMC, 2004). Milk SCC data were normalized by logarithmic transformation into somatic cell score (SCS) as SCS = log base 2 (SCC/100) + 3, where SCC is cells/µL (Schukken et al., 2003).

DNA isolation and mutation screening

Genomic DNA was isolated from ear tissue and stored at -80°C following standard procedures (Hirschhorn et al., 2002). Eight primer pairs (Table 1) were designed to amplify 3 exons of the cattle *NOD2* gene according to published gene sequences (GenBank accession numbers: AY518755, AY518758, AY518759). Polymerase chain reaction (PCR) was performed in a 25 µL volume containing 4 µL DNA template (20 ng/µL), 25 µL 2X Taq PCR MasterMix (TIANGEN, Beijing, China), 1.5 µL of each primer (10 µM), and 18 µL ddH$_2$O. The PCR protocol for the *NOD2* gene was 5 min at 95°C, 35 cycles of 94°C for 30 s, 46-54°C annealing for 30 s, 72°C for 40 s, and a final extension at 72°C for 10 min. For the single-strand conformation polymorphism, 1.5 µL PCR products was added to 6 µL loading buffer (20 mM EDTA, 0.025% bromophenol blue, and 0.025% xylenecyanol FF) denatured at 98°C for 10 min, and placed on ice for 10 min. The denatured PCR products were analyzed on a 10% polyacrylamide gel in 1X Tris/borate/EDTA buffer and electrophoresed at constant voltage (8 V/cm) for 12-14 h. DNA bands on the gel were detected by silver staining.

Table 1. Primers used for PCR.

Primers	Primer sequence (5'-3')	Position (bp)	Annealing temperature (°C)
Seq 1	F: 5'-CTCTTTGCTTTATCCCTTTC-3'	42	53.2
	R: 5'-GATGTGCTTTCCTGTGCC-3'	296	
Seq 2	F: 5'-AAAGCACATCAGTGCCTTGT-3'	287	53.7
	R: 5'-TACTTCCCGAGCATCCCT-3'	506	
Seq 3	F: 5'-ATGGATGCCACTGAGTATTT-3'	446	51.8
	R: 5'-CAGGGTACAAGGGAAAGAT-3'	649	
Seq 4	F: 5'-GGCATTCTCACCTTTGAT-3'	615	50.7
	R: 5'-TTTGGATACGGAGTTGTT-3'	893	
Seq 5	F: 5'-CTCAGTGTCCCAGCAGAA-3'	1538	52
	R: 5'-ATCCACCTTGATGAGCAG-3'	1747	
Seq 6	F: 5'-ATTAGCAAGGACTCAGGT-3'	1789	46.6
	R: 5'-ATGTATAGCACAAGGAAC-3'	2003	
Seq 7	F: 5'-CACTTTGCTGGGACCTGA-3'	63	56
	R: 5'-AACTGCGGGACGAACACT-3'	319	
Seq 8	F: 5'-ATCTGGTGAATGGAGGAG-3'	90	50.1
	R: 5'-CTAGGGAGCTGATGTGGT-3'	307	

Sequencing of polymorphism fragment

PCR products were purified using spin columns (Watson BioTechnologies, Shanghai, China) and sequenced in both directions using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in a 3700 DNA sequencer (Applied Biosystems) according to manufacturer instructions.

Statistical analysis

Gene frequencies were determined for each breed by direct counting. v2 tests were used to determine whether the individual variant was in Hardy-Weinberg equilibrium. The genotype and allele frequency distributions were compared using the Chi square test. The traits were compared between genotypes. The relationship between genotypes of the *NOD2* gene and bovine mastitis and milk quality were analyzed using one-way analysis (Wang et al., 2010; Ren et al., 2009) with the following model:

$$Y_{ijkl} = \mu + A_i + B_j + S_k + G_l + E_{ijkl} \qquad \text{(Equation)}$$

Yijkl = observed value; μ = overall mean for each trait; *BFi* = fixed effect of ith breed and farm; *Mj* = fixed effect of jth month of slaughtering; *G* = fixed effect of kth single SNP marker genotype; *Eijkl* = random error.

RESULTS AND DISCUSSION

Mutational analysis of the cattle *NOD2* gene

Investigation of 3 coding exons of the *NOD2* gene revealed 2 mutations in exon 12. The 2 transitions, A→T at position 114 base pairs and G→A at position 1594 base pairs were non-synonymous substitutions (Figure 1). Six genotypes, including AA, AB, BB, EE, EF, and EF, were determined by 4 alleles, A, B, E, and F, which were observed in exon 12 within the 5 breeds (Figure 2). The χ^2-test showed that the genotype distributions of AA, AB, and BB in Chinese Holstein, Chinese Simmental, Tongjiang cattle, and Maiwa yak were not in Hardy-Weinberg equilibrium (P < 0.05), while for the Dechang buffalo, the null hypothesis was not rejected (P > 0.05); the genotype distributions of EE, EF, and FF in Chinese Simmental, Dechang buffalo, and Maiwa yak were not in Hardy-Weinberg equilibrium (P < 0.05), while Chinese Holstein and Tongjiang cattle were in Hardy-Weinberg equilibrium (P < 0.05) (Table 2).

In all population, alleles A (0.6459) and E (0.6590) were more frequent than alleles B (0.3541) and F (0.3410), indicating that alleles A and E were advantage alleles (Table 2). Among the 87 cattle randomly sampled from 3 Chinese indigenous breeds, genotypes BB and FF were not detected, while the average frequencies of genotypes AA, AB, EE, and EF were

0.4253, 0.5747, 0.4483, and 0.5517, which were not significantly different from that in Chinese Holstein and Chinese Simmental breeds (0.4128, 0.4128, 0.3945, and 0.4771) (Table 2). Several studies reported that individuals of Dechang buffalo, Tongjiang cattle, and Maiwa yak were not predisposed to mastitis (Chu, 1999; Tohno et al., 2008). This phenomenon revealed that alleles B and F might increase the chance of mastitis infection.
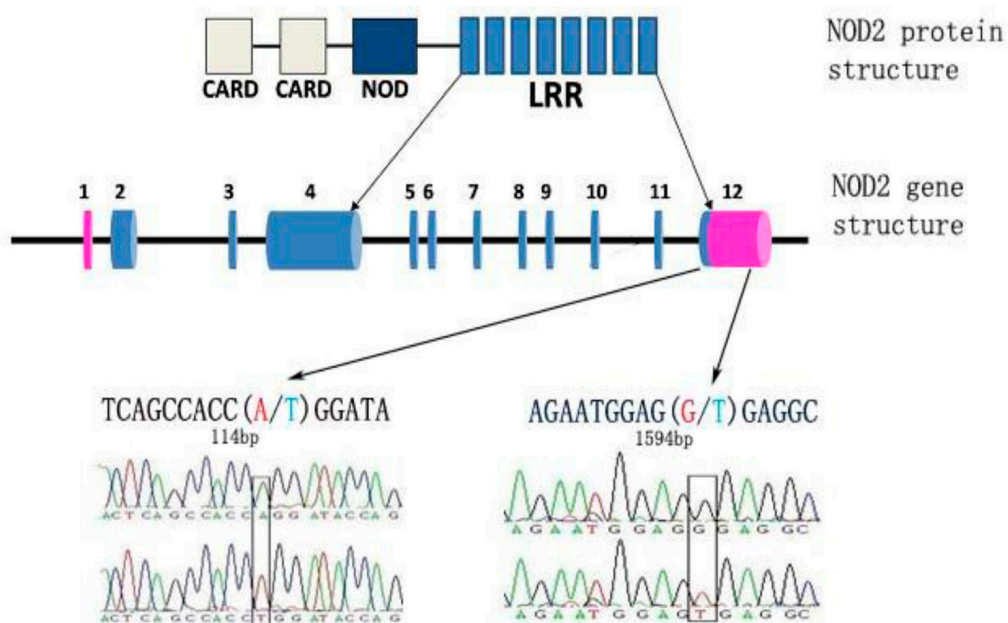

Figure 1. *NOD2* structure and location of single nucleotide polymorphisms.
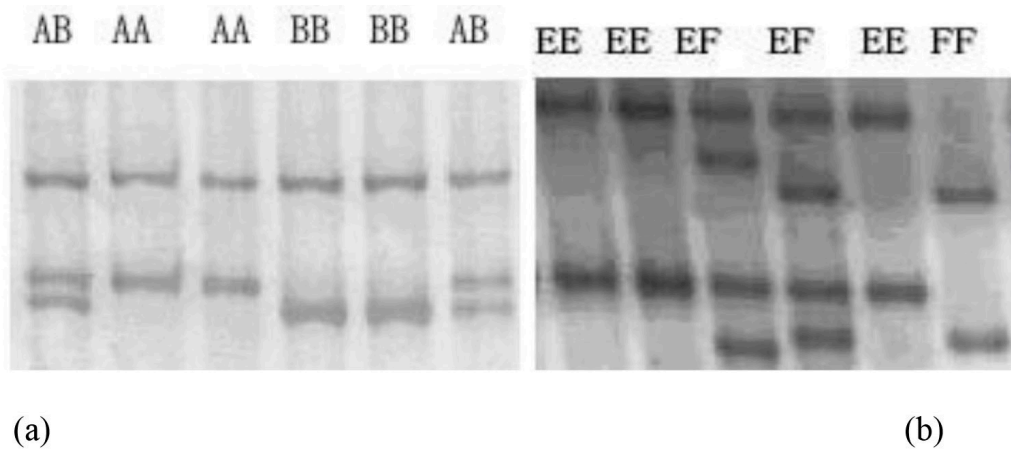

Figure 2. Polymorphisms in exon 12 of NOD2 gene. **a)** Electrophoresis of PCR-SSCP, AA, BB, and AB. **b)** Electrophoresis of PCR-SSCP, EE, FF, and EF.

Table 2. *NOD2* single nucleotide polymorphism frequencies and genotype frequencies in cattle.

	Allele frequencies				Genotype frequencies						χ^2	
	A	B	E	F	AA	AB	BB	EE	EF	FF	Primer 1	Primer 5
Chinese Holstein (N = 160)	0.6031	0.3969	0.6438	0.3562	0.4000 (64)	0.4063 (65)	0.1938 (31)	0.4125 (66)	0.4625 (74)	0.125 (20)	28.311**	0.0116[ns]
Chinese Simmental (N = 58)	0.6638	0.3362	0.6034	0.3966	0.4483 (26)	0.4310 (25)	0.1207 (7)	0.3448 (20)	0.5172 (30)	0.1379 (8)	9.9736**	6.9231*
Dechang buffalo (N = 34)	0.7059	0.2941	0.6912	0.3088	0.4118 (14)	0.5882 (20)	-	0.3824 (13)	0.6176 (21)	-	5.9028[ns]	6.7877*
Tongjiang cattle (N = 23)	0.5652	0.4348	0.6957	0.3043	0.1304 (3)	0.8697 (20)	-	0.3913 (9)	0.6087 (14)	-	12.211*	4.4023[ns]
Maiwa yak (N = 30)	0.8333	0.1667	0.7833	0.2167	0.6667 (20)	0.3333 (10)	-	0.5667 (17)	0.4333 (13)	-	33.2042**	6.4427*
Total cattle (N = 305)	0.6459	0.3541	0.659	0.341	0.4164 (127)	0.4590 (140)	0.1246 (38)	0.4098 (125)	0.4984 (152)	0.0918 (28)	11.3251**	1.0236[ns]
Genetic diversity analyses in Chinese Holstein and Chinese Simmental breeds Total (N = 218)	0.6193	0.3807	0.6331	0.367	0.4128 (90)	0.4128 (90)	0.1743 (38)	0.3945 (86)	0.4771 (104)	0.1284 (28)	-	-
Genetic diversity analyses in three Chinese indigenous breeds Total (N = 87) 174	0.8506	0.2874	0.7241	0.2759	0.4253 (37)	0.5747 (50)	-	0.4483 (39)	0.5517 (48)	-	-	-

*Values differ significantly at P < 0.05. **Values differ significantly at P < 0.01. [ns]Not significant.

Association between *NOD2* and mastitis in cattle

Statistical analyses revealed associations between transitions A→T and G→A and SCS. The generalized linear model was found to be useful, and the transitions A→T showed a significant association with SCS (P < 0.01) (Table 3). This transition has been described to play a critical role in increasing SCS in Canadian Holsteins (Sameer et al., 2007). Among the 218 individuals of Chinese Holstein and Chinese Simmental breeds groups, the animals with genotype BB had significantly higher SCS compared with genotypes AA and AB (P < 0.01) (Table 3). The genotypic combination BBFF had a higher SCS than AAFF and ABFF (P < 0.05) and was significantly higher than other genotypic combinations (P < 0.01) (Table 4). This indicates that the BB genotype may contribute to disease susceptibility; therefore, the *NOD2* gene may affect SCS. The association between the *NOD2* gene with protein, fat, and 305-day milk yields in Chinese Holstein and Chinese Simmental breeds groups (N = 218) were analyzed (Table 3). The allele F harboring the mutation G→A also contributed to the high protein and 305-day milk yields. The genotypic combination BBFF and AAFF showed higher protein and 305-day milk yields, respectively, compared to other genotypic combinations. Interestingly, the genotypic combinations simultaneously carrying allele A, B, and genotype FF were beneficial not only for reducing SCS, but also for increasing production. Thus, selection for these genotypic combinations in the population appears to be promising.

In summary, we detected significant associations between *NOD2* polymorphisms and the incidence of mastitis, protein, and 305-day milk yields. Allele B increased SCS, while allele F increased protein and 305-day milk yields in cattle.

Table 3. Healthy and production performance comparison of individuals with different genotypes and effects (P value) of polymorphism of NOD2.

	Genotypes						P Value	
	AA	AB	BB	EE	EF	FF	Primer1	Primer2
SCS	3.9661 ± 0.1816^A	3.7663 ± 0.1789^A	4.98 ± 0.2612^B	3.9927 ± 0.1801	3.8927 ± 0.1640	4.1465 ± 0.2902	<0.0001**	0.7159^{ns}
Fat yield (%)	3.3146 ± 0.1318^a	3.4771 ± 0.1298^a	2.6335 ± 0.1896^b	3.1975 ± 0.1307	3.3511 ± 0.1190	2.9372 ± 0.2106		
Protein yield (%)	3.2530 ± 0.3795^A	3.1886 ± 0.3737^A	4.3422 ± 0.5458^B	3.3991 ± 0.3394^A	3.269 ± 0.3213^A	4.0166 ± 0.4585^B		
305-day milk yields	6582.2805 ± 153.2662^a	6631.3063 ± 150.4436^a	7102.0215 ± 224.4428^b	6330.955 ± 149.2562^A	6777.5437 ± 154.3373^a	$7461.012 \pm 231.0142^{Bb}$		

Values with different superscripts within the same line differ significantly at P < 0.01 (A, B) and P < 0.05 (a, b), SE standard error.

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Table 4. Healthy and production performance comparison of individuals with different pyramiding genotypes.

	Genotypic combination								
	AAEE (46)	AAEF (31)	AAFF (12)	ABEE (29)	ABEF (52)	ABFF (10)	BBEE (10)	BBEF (22)	BBFF (6)
SCS	3.8661 ± 0.1916[A]	3.7663 ± 0.2089[A]	4.0863 ± 0.2159[a]	3.9537 ± 0.1801[A]	3.8927 ± 0.1541[A]	4.2765 ± 0.2902[a]	4.0431 ± 0.2194[A]	3.9531 ± 0.2059[A]	4.98 ± 0.2612[B]
Fat yield (%)	3.1949 ± 0.0598	3.1553 ± 0.0330	2.9327 ± 0.0447	3.3084 ± 0.0672	2.8750 ± 0.0553	3.0546 ± 0.2930	3.3245 ± 0.2930	3.0089 ± 0.1813	3.0635 ± 0.2930
Protein yield (%)	3.4274 ± 0.1548[B]	3.0451 ± 0.2910[B]	2.9078 ± 0.1540[B]	3.2798 ± 0.1374[B]	3.6340 ± 0.1743[b]	3.5938 ± 0.2264[B]	3.5839 ± 0.1940[B]	3.7682 ± 0.1032[b]	4.3215 ± 0.2319[Aa]
305-day milk yields	6681.1939 ± 153.2662[B]	7062.012 ± 150.4436[c]	7713.543 ± 224.4428[ab]	6658.435 ± 220.3947[B]	6443.129 ± 138.2673[B]	6564.368 ± 156.4014[B]	6490.179 ± 148.0387[B]	6502.151 ± 176.3467[B]	6339.902 ± 192.4892[ab]

Values with different superscripts within the same line differ significantly at $P < 0.01$ (A, B) and $P < 0.05$ (a, b), SE standard error.

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