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Variants and Gene Expression of the *TLR2* Gene and Susceptibility to Mastitis in Cattle

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ABSTRACT

Toll-like receptor 2 (*TLR2*), a key component of the innate immune system, plays an important role in the initiation of the inflammatory response to foreign pathogens. This study was carried out to investigate the *TLR2* gene expression difference in mammary gland tissues of the cows infected with mastitis and healthy cows and to search for variants of the bovine *TLR2* gene for further clarifying the relationship between *TLR2* gene and mastitis in cattle. The quantitative real-time polymerase chain reaction (qPCR) revealed that clinical mastitis caused by *Staphylococcus aureus* could significantly increased the *LTR2 mRNA* abundance ($p<0.05$). Seven novel single nucleotide polymorphisms (SNPs) in the 5' upstream of the *TLR2* gene in six cattle breeds (Hereford, Simmental, Limousin, Chinese indigenous Luxi Yellow cattle, Bohai Black cattle and Chinese Holstein cattle) were identified by the direct sequencing method. Four SNPs are located at the putative promoter region and their variants can alter the transcriptional factor binding sites. Genetic diversity showed that the SNP (4bp deletion) in the promoter was unique and two distinct haplotypes were found in Hereford cattle. Six SNPs in the *TLR2* gene exon2 and 3'UTR were genotyped by PCR-RFLP and CRS-PCR methods in 398 Chinese Holstein cattle. Case-control study and associated analysis revealed that the cow with the genotype TT (3.15±0.52) in c.+189T>G has significantly lower ($p<0.05$) somatic cell score (SCS) than that with genotype TG (4.84±0.21) or GG (5.31±0.27) in Chinese Holstein cattle. In conclusion, haplotype analysis and linkage disequilibrium findings showed that the hapoltype TG of two loci (c.+189T>G and c.+631A>G) in the coding region may be used as a tolerance haplotype for the bovine mastitis.

Key words: Cattle, *TLR2* gene, promoter, mastitis, gene expression, haplotype

INTRODUCTION

Mastitis is one of the most frequently occurring and complex diseases of the mammary gland in dairy cows and it causes a great economic loss. The prevalence of mastitis ranges from 10 to 50% (Neelesh, 2007). Epidemiologic analyses show the most prevalent agents associated with this mastitis infection are *Staphylococcus aureus* (*S. aureus*), *Streptococcus strains* and *Escherichia coli* (*E. coli*) pathogens (Chaneton *et al*., 2008).

Toll-like receptors (TLRs), key components of the innate immune system, are responsible for the initiation of the inflammatory response to foreign pathogens such as bacteria, fungi and viruses

(Stevens *et al.*, 2008). Toll-like receptor 2 (TLR2) is a member of TLRs and uniquely capable of recognizing the peptidoglycan and lipoteichoic acid from *S. aureus* and other gram-positive bacteria (Bannerman *et al.*, 2004). Bovine *TLR2* can properly transduce signals from *S. aureus* and *E. coli*. However, *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce the expressions of *TNFalpha and interleukin-8* (CXCL8) in the udder (Yang *et al.*, 2008). The TLR2 gene was strongly expressed during mastitis caused by *S. aureus* (Goldammer *et al.*, 2004) and *Strep. uberis* (Swanson *et al.*, 2009). The previous reports suggested that *TLR2* may play a role in the host response to intramammary infections. Taken together, *TLR2* appears to present an attractive candidate susceptibility gene for mastitis.

Bovine *TLR2* gene, located to the proximal end of BTA 17, contains two exons and encodes 784 amino acids (White *et al.*, 2003). Polymorphisms in the *LTR2* gene encoding receptor associated with the innate immune system are likely to contribute to the overall variation in the resistance or susceptibility to mastitis in dairy cattle. Somatic cell score (SCS) represents a log score of the milk somatic cell count (SCC) and has been genetically correlated with clinical mastitis (Shook and Schutz, 1994). Several studies have identified some ploymorphic sites in the bovine *TLR2* gene in the *Bos indicus* and *Bos taurus* cattle breeds (Jann *et al.*, 2008; Opsal *et al.*, 2008; Mariotti *et al.*, 2009; Seabury *et al.*, 2010) . Of which, three SNPs of *TLR2* gene were localised at positions shaped by positive selection in the ruminant dataset (Leu227Phe, His305Pro, His326Gln) and in domains involved in the recognition of ligands, should be considered as candidate SNPs for immune related traits in cattle (Jann *et al.*, 2008). However, no SNPs were found in Canadian Holstein bulls (Pant *et al.*, 2008). Similarly, no significant association was found between the chromosomal regions surrounding *TLR2* and mastitis in Norwegian Red cattle (Opsal *et al.*, 2008). To data, there is no report on the polymorphisms of the bovine *TLR2* gene promoter.

Therefore, the objectives of this study were: (1) to determine the *TLR2* mRNA expressions in healthy and mastitis infected cow udders; (2) to clone and characterize the bovine *TLR2* gene promoter region; (3) to investigate the genetic variations of the promoter of the bovine *TLR2* gene in six cattle breeds (Hereford, Simmental, Limousin, Chinese indigenous Luxi Yellow cattle, Bohai Black cattle and Chinese Holstein cattle) and (4) to evaluate the associations between variations within the *TLR2* gene with SCS in Chinese Holstein cattle.

MATERIALS AND METHODS

Animals and experiment designs

Experiment 1. Expressions of *TLR2* mRNA of mammary tissues in healthy and clinical mastitis cows: Twelve udder tissue samples were collected aseptically from 6 slaughtered cows within 10 min after slaughtering in the slaughter house at Jinan, Shandong Province on February 2009. Base on the clinical symptoms and bacteriological test after culture with blood agar. The glands were categorized as infected by *S. aureus* and healthy groups. Two samples (infected and healthy) were collected from each cow. The udder tissues were collected from the deepest udder quarter and frozen in liquid nitrogen for RNA analysis. qPCR experiment was carried out from July 2009, to October, 2009, at the laboratory of the College of Animal Science and Technology, China Agricultural University.

Experiment 2. Polymorphisms of the promoter of the *TLR2* gene among six cattle breeds: Six cattle breeds (26 Hereford, 30 Simmental, 156 Limousin, 46 Chinese indigenous Luxi Yellow cattle, 42 Bohai Black cattle and 225 Chinese Holstein cattle) were included in this experiment.

Samples were obtained from July, 2007 to May, 2009 in Beijing and Shandong Province, China. The promoter region of the *TLR2* was amplificated among six breeds. Then, their amplificated fragments were sequenced for the SNPs identification. The experiment was performed in 2009.

Experiment 3. Polymorphisms of the coding region and untranslated regions (UTR) of the *TLR2* gene in Chinese Holstein cattle: A total of 398 cows, aged 3-6 years, were selected randomly from 8 dairy farms (Shandong, China) including in the Dairy Herd Improvement test scheme for the case-control study. Animals were milked three times daily by machine in farms. The animals with 5526±145.2 kg (mean±SE) 305-day milk yield per lactation. The DNA was extracted from blood samples following the standard phenol-chloroform method with minor modifications (Moller *et al*., 2004). Blood samples were collected from 2006-2008. Genotyping and data analysis were carried out in 2008-2009, at the laboratory of the Center of Dairy Cattle Research, Shandong Academy of Agricultural Sciences.

Milk samples were collected three times for one day and one time per month of lactation. SCC was measured by the Fossomatic cell counter (Foss Electric, FOSSMATIC 5000, Denmark). The control group was defined as the SCC below 1×10^5 cells mL^{-1} and without the record of clinical mastitis. The subclinical mastitis group (Case) was considered that SCC above 3×10^6 cells mL^{-1} in the milk of the cows.

Gene expression analysis: Total RNA was extracted from approximate 150 mg of mammary tissue using a TRNzol Kit (Tiangen, China) and further purified with the RNase-free DNase I (Promega, Madison, WI). RNA purity and concentration were measured with the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA). Next, the ImProm-II™ Reverse Transcription (Promega, USA) was used to convert approximately 1 μg of RNA from each sample to cDNA according to the modified protocol. A volume of 5 μL each reaction, containing 1 μg RNA, 4 μM Oligo(dT)15 (Tiangen, China) and nuclease-free water, was incubated at 70°C for 5 min, then chilled quickly at 4°C for 5 min and held on ice. Above reaction component was added to the 15 μL RTMix and annealed at 25°C for 5 min, extended the first strand for 1 h at 42°C, incubated for 15 min at 70°C. RTMix combined the 3.1 μL nuclease-free water, 4 μL ImProm-II™ 5×buffer, 3 mM MgCl 2,0.5 mMdNTP (Tiangen, China), 20U Rnasin (Tiangen, China) and 1 μL ImProm-II™ Reverse Transcription (Promega, USA). qPCR was carried out using cDNA templates and the SYBRGreen PCR Master Mix (Tiangen, China) in ABI PRISM 7900 Sequence Detector System (Applied Biosystem, Foster City, CA) according to manufacture's protocol. Each sample was run in triplicate. qPCR primers of *TLR2* (F:5'-TGCGTTGGTTTGGATAGTGA-3' and R: 5'-AGACCAGAGGGGATGGAGTT-3'; Size = 231 bp) and the internal control *GAPDH* (F:5'-GGGTCATCATCTCTGCACCT -3' and R:5'- GGTCATAAGTCCCTCCACGA-3'; Size = 176 bp) were used for qPCR. The protocol was as follows: initial hold at 50°C for 2 min, then denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 30 sec denaturation, 60.0°C for 30 sec annealing and 68°C for 20 sec extension. The last stage for the dissociation curve was as follows, 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The relative quantification of *TLR2* gene expression was calculated by the standard curve-based method for relative real time PCR (Larionov *et al*., 2005). The relative quantity of *TLR2* mRNA was expressed by 100 fold of the mean.

Cloning and bioinformatics of the 5' flanking region of the *TLR2* gene: Primers *TLR2*-r-F and LTR2-r-R (Table 1) were designed to amplify the 925 bp fragment of the 5' upstream of the

Table 1: The primers used in the amplification of *TLR2* gene in cows and other PCR conditions

SNPs	Primers 5' to 3'	Amplicon size (bp)	Annealing temperature (°C)	Restriction enzyme	Genotype
g.-840C>T	F:GTGGGGGGGATTTTGAAGTAA	925	58.0	*Bgl*II	CC:925 bp
(*TLR2*-r)	R:GAGCAGATGAGGATGAGACGG				TT:736+189 bp
g.-439C>T	The same as g.-840C>T	925	58.0	*Alw*NI	CC:593+332 bp;TT:925 bp
c.+189T>G	F:CTCTGTCTTGTGACCCAACT	295	55.1	*Eco*R v	TT:295 bp; GG:186+109 bp
(p. Glu>Asp)	R:ACATAAAGGGACCTGAACC				
c.+202G>A	F:CCCAACTGGTGTCTGCGATGG	130	66.6	*Hae*III	GG:90+21+19 bp
	R: ACCTCTGCAGGTCTCTGTGGC				AA:111+19 bp
c.+454G>A	F:ATCGCTTATCTAACTTATCATCCTC	221	56.6	*Pst*I	GG:221 bp
	R:AGCACTGATCTCAAGCTCCTC				AG:221+138+93 bp
c.+631A>G	F: GATTCTACATCTGAAGCAGCAT	194	54.4	*Hin*1 II	AA:194 bp
(p.Ile>Val)	R: CAACAAAACTTTCATCGGTG				AG:194+172+22 bp
c.+1631C>T	F: CAACTTGATTCTTTTCAGCAACTG	101	56.7	*Dra* I	CC:81+20 bp
	R:AGTGCCTGCTGTCCCTGTTT				TT:101 bp
c.+2812T>C	F:GGTGGTCCTTCTATCACTAGTTATA	444	51.0	*Hha*I	TT:444 bp
	R:ATGTTTTTCCAATTTGGGC				CC:426+18 bp

TLR2 gene. The promoter and transcriptional factor binding sites were predicted with the MatInspector Professional (http://www.genomatix.de/).

Construction of DNA pools for SNP detection in the coding region and 3'-UTR: Ten cows with high SCS and 10 with low SCS were selected for creating DNA pools. Two DNA pools were constructed by aliquoting an equal amount of DNA from each selected cows in each group. These pools were used for the amplification of the promoter, exon2 and 3'-UTR of the *TLR2* gene to detect SNPs. The PCR products were sequenced for SNPs searching using DNAMAN.

Genotyping: PCR-restriction fragment length polymorphism (PCR-RFLP) and Created Restriction Site PCR (CRS-PCR) methods (Huang *et al.*, 2010) were used to genotype SNPs. Seven primer pairs (Table 1) were designed with primer 5.0 software based on reference sequence (GenBank No. NM.174197.2). PCR reaction was carried out in a total volume of 25 μL containing 50 ng DNA, 0.25 mM dNTPs, 2.0 mM $MgCl_2$, 10 μM of each primer, 2 units Taq DNA polymerase (TaKaRa, China) and 10x Taq buffer. The PCR conditions were 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, at annealing temperature for 30 sec (Table 1), 72°C for 40 sec and a final elongation for 5 min at 72°C, with the PTC-200™ thermal cycler (MJ Research, Inc., Watertown, MA, USA). The PCR products were digested corresponding to the restriction enzyme (Table 1), following to the detection using 12% polyacryolamide gel electrophoresis and 3% agrose gel. The genotype was identified by the electrophoretic banding pattern.

Statistical analysis: The difference of the gene expressions between healthy and mastitis-infected groups was tested by paired T-test in SPSS version 11 (SPSS Inc, USA). The gene diversity, heterozygosity, polymorphism information content (PIC) and haplotype were analyzed by the powerMarker V3.25 software (Liu and Muse, 2005). Allele frequencies, haplotype and linkage disequilibrium (LD) analyses were determined by SNPAlyze version 3.2 software (http://www.dynacom.co.jp) in case-control study. Hardy-Weinberg equilibrium was assessed by Chi-Square analysis. The association analysis between the SNPs markers genotypes of the *TLR2*

gene and SCS were analyzed by the least squares method as applied in the GLM procedure of SAS(SAS Institute Inc,Cary, NC, USA). The applied linear model was as follows:

$$Y_{ijklmn} = \mu + F_i + G_j + S_k + E_l + H_m + e_{ijklmn}$$

where, Y_{ijklmn} was the observed value; μ was the overall mean; F_i was the fixed effect of farm; G_j was the fixed effect of genotype; S_k was the fixed effect of sire; E_l was the fixed effect of season; H_m was the fixed effect of parity and e_{ijklmn} was the random residual effect.

The p-value below 0.05 is regarded as significant.

RESULTS

Relative expression of *TLR2* mRNA of the mammary glands in healthy and mastitis-infected cows: The relative expression of *TLR2* mRNA in the infected mammary gland was significantly ($p<0.05$) higher than that in the healthy group (68.13±8.92 vs. 27.25±5.46).

Bioinformatics and polymorphism analysis of the promoter of *TLR2* gene: The 925 bp (Simmental, Limousin, Luxi Yellow cattle, Bohai Black cattle, Holstein) and 921 bp (Hereford) fragments were obtained. The putative promoter of the bovine *TLR2* gene locates at the position between -696 to -35 bp. Analysis of an approximately 661 sequence (-696 to -35) revealed 154 putative binding sites for various transcription factors (date not shown). No canonical TATA or CAAT boxes are found in the sequence analyzed. Seven novel SNPs were identified by the sequence multiple alignment. Four SNPs (g.-454 GAATdel, g.-439C>T, g.-221T>C and g.-211A>C) locates at the putative promoter region, whereas, another three SNPs (g.-840C>T, g.-760T>C, g.-758T>C) were not present in this region. In the 5' regulatory region between the nucleotides -838 and -35, the putative SIXF [Sine oculis (SIX) homeodomain factors] binding site was found. Because of the mutation from C to T at the nucleotide -840, the SIXF was not found, whereas, GATA binding factors was appear. The 4bp deletion (GAAT) eliminated two SORY (SOX/SRY-sex/testis determining and related HMG box factors) and one HOXF (Paralog hox genes 1-8 from the four hox clusters A, B, C, D) transcriptional factor binding sites. However, in the locus g.-439C>T, the mutation results in an additional Zinc Finger Transcriptional Repressor (ZFTR) binding site.

Three SNPs in the 5' flanking region of *TLR2* gene were genotyped among six cattle breed populations. The genetic parameters of the three loci were as shown in Table 2 and 3. In the locus g.-840C>T, it was fixed in Limousin cattle population and was polymorphic among five breed populations (Table 3). In the locus g.-454 GAATdel, it was fixed among five cattle breeds and was polymorphic only in the Hereford cattle. All Hereford individuals are all heterozygote. The sequences alignment compared with the reference sequence (GenBank No. NM.174197.2) showed that 4 bp was deleted among five cattle breeds and the 4 bp (GAAT) of *TLR2* is Hereford breed specific. In the locus g.-439C>T, it was polymorphic in five cattle breeds except for in the Simmental cattle. The number of haplotype of three SNPs ranged from 2 to 6 (Table 5). Haplotype analysis result revealed that there were two specific haplotypes (TAC and TAT) in Hereford cattle population.

Table 2: The genetic patterns of the 5' flanking region of the bovine *TLR2* gene among six cattle breeds*

	Genotype of g.-840C>T (SNP-1)			Genotype of g.-454 GAATdel (SNP-2)			Genotype of g.-439C>T (SNP-3)		
Breed	CC	CT	TT	AA	AB	BB	CC	CT	TT
Hereford (n = 26)	0.15	0.62	0.23	0	1	0	0.85	0.15	0.00
Simmental (n = 30)	0.40	0.60	0.00	1	0	0	1.00	0.00	0.00
Limousin (n = 156)	1.0	0.00	0.00	1	0	0	0.25	0.63	0.12
LX (n = 46)	0.25	0.57	0.18	1	0	0	0.76	0.18	0.06
BHB (n = 42)	0.83	0.11	0.06	1	0	0	0.21	0.38	0.51
CH (n = 225)	0.73	0.18	0.09	1	0	0	0.62	0.24	0.14

*LX: Luxi yellow cattle; BHB: Bohai black cattle; CH: Chinese holstein

Table 3: Diversity parameters of LTR2 gene among six cattle populations

	Gene diversity			Heterozygosity			PIC		
Breed	SNP-1	SNP-2	SNP-3	SNP-1	SNP-2	SNP-3	SNP-1	SNP-2	SNP-3
Hereford (n = 26)	0.50	0.50	0.14	0.62	1	0.15	0.37	0.38	0.13
Simmental (n = 30)	0.42	0.00	0.00	0.60	0	0.00	0.33	0.00	0.00
Limousin (n = 156)	0.00	0.00	0.53	0.00	0	0.62	0.00	0.00	0.42
LX (n = 46)	0.50	0.00	0.27	0.50	0	0.21	0.37	0.00	0.23
BHB (n = 42)	0.19	0.00	0.49	0.11	0	0.37	0.17	0.00	0.37
CH (n = 225)	0.32	0.00	0.40	0.17	0	0.24	0.27	0.00	0.33

LX: Luxi yellow cattle; BHB: Bohai black cattle; CH: Chinese holstein

The genetic variants of the coding and 3'UTR regions of the bovine *TLR2* gene in Case-control study: Table 4 shows the distribution of genotypic and allelic frequencies of the 6 SNPs in each group. Except for the c.+1631C>T ($p = 0.001$) in the case group, the observed and expected genotypic frequencies of the another five SNPs in the control and case groups were in good agreement with the predicted Hardy-Weinberg equilibrium values (data not shown). The genotypic and allelic frequency of c.+189T>G and the allelic frequency of c.+631A>G were significantly different between the control and case subjects ($p<0.05$). These data indicate that the distribution of c.+189T>G in cows can affect the overall analysis in the total subject group. Therefore, this could be used as a genetic marker of subclinical mastitis in cows, because the probability value for the allelic distribution of this SNP was significant.

In order to further assess the relationship of the two SNPs (c.+189T>G and c.+631A>G) and mastitis, the association analysis between two SNPs and SCS was performed. The results also revealed that genotype TT (3.15±0.52) in c.+189T>G has significant lower SCS ($p<0.05$) than that of genotypes TG (4.84±0.21) and GG (5.31±0.27). Because of the rarity of the T allele of the c.+189 locus in the present samples detected, a more large population is needed to confirm the result.

Possible Linkage Disequilibrium (LD) was evaluated by D' and R^2 values among six *TLR2* gene polymorphisms (five in the exon2 and one in 3'-UTR) in subclinical and control cows (Table 6). LD analysis showed that c.+189T>G and c.+631A>G was not located in one haplotype block in the case and control groups. Therefore, the two SNPs were used to isolate the susceptibility haplotypes. Base on the results, the haplotype of the two SNPs were estimated and four haplotypes (GA, GG, TA, TG) were present. The distributions of 3 haplotypes (GA, GG and TA) showed no significant

Table 4: Genotype distribution in healthy and subclinical mastitis cows of screening analysis

Parameters	Control	Subclinical mastitis	p-value
c.+189T>G			
Genotype	n = 245	n = 208	
GG	137	136	
GT	96	69	
TT	12	3	0.0326*
Allele	n = 489	n = 419	
G	370	341	
T	120	75	0.0184*
c.+202G>A			
Genotype			
AA	166	161	
AG	50	40	
GG	3	0	0.1806
Allele			
A	382	362	
G	56	40	0.197
c.+454G>A			
Genotype			
GG	258	151	
AG	36	19	0.7316
Allele			
G	552	321	
A	36	19	0.7398
c.+631A>G			
Genotype			
AA	175	120	
AG	106	45	
GG	13	5	0.0572
Allele			
A	456	285	
G	132	55	0.0217*
c.+1631C>T			
Genotype			
TT	160	178	
TC	6	4	0.4295
Allele			
T	326	360	
C	6	4	0.4328
c.+2812T>C			
Genotype			
TT	242	150	
TC	50	19	0.0881
Allele			
T	534	319	
C	50	19	0.102

*Significant difference in distribution

Table 5: Haplotypical frequencies of three SNPs in the 5 flanking region of *TLR2* gene among six breed populations

Breed	CBC	CBT	CAC	TBC	TBT	TAC	TAT
Hereford	0.23	0.00	0.23	0.23	0.04	0.23	0.04
Simmental	0.70	0.00	0.00	0.3	0.00	0.00	0.00
Limousin	0.58	0.42	0.00	0.00	0.00	0.00	0.00
LX	0.49	0.05	0.00	0.34	0.11	0.00	0.00
BHB	0.39	0.51	0.00	0.03	0.07	0.00	0.00
CH	0.55	0.25	0.00	0.20	0.00	0.00	0.00

Table 6: Linkage disequilibrium (LD) analyses of the control and case groups in Chinese Holstein cattle

Groups		c.+189 T>G	c.+202 G>A	c.+454 G>A	c.+631 A>G	c.+1631 C>T	c.+2812 T>C
(a) Control gronp							
c.+189	D'		0.729	0.692	0.455	1.00	0.606
T>G	R^2		0.259	0.107	0.199	0.064	0.115
c.+202				0.295	0.459	1.00	0.450
G>A				0.044	0.104	0.003	0.161
c.+454					0.601	0.234	0.347
G>A					0.081	0.016	0.085
c.+631						0.742	0.237
A>G						0.05	0.019
c.+1631							0.3852
C>T							0.028
(b) Case gronp							
c.+189	D'		0.645	0.650	0.456	0.609	0.722
T>G	R^2		0.219	0.108	0.152	0.019	0.125
c.+202				0.315	0.393	1.00	0.424
G>A				0.064	0.093	0.001	0.108
c.+454					0.351	1.00	0.423
G>A					0.038	0.001	0.179
c.+631						0.588	0.349
A>G						0.001	0.037
c.+1631							0.125
C>T							0.003

differences ($p>0.05$) in control and case groups. However, the haplotype TG was significantly different ($p<0.05$). Therefore, the TG maybe used as a tolerance haplotype for the bovine mastitis.

DISCUSSION

Swanson *et al.* (2009) reported that *TLR2* mRNA expressions were increased by the mastitis infected by the *Strep. uberis*, *S. aureus* and *E. coil* in udder tissues. However, no change was observed in the bovine mammary epithelial cells challenged by the same *Strep.uberis*. Our data is also consistent with the previous report by Goldammer *et al.* (2004). Heat-killed *S. aureus* and *E. coli* can activate equally well bovine *TLR2* to induce NF-kappaB activation (Yang *et al.*, 2008). The results suggest that mastitis increases the expression of *TLR2* contributing to the innate immune defense system in the cow udder. It is suggested that the *TLR2* gene may be considered as a candidate gene for mastitis resistance in Chinese Holstein cattle population.

The mutational SNPs in the 5' regulatory region can alter the transcript factor binding sites, subsequently, resulting in the change of *TLR2* gene expression. For example, SIX as a regulator of development and disease, is involved in the muscular development and can inactive the immune

related gene *Slc12a2* (Ando *et al*., 2005; Kumar, 2009). GATA binding factors was caused by the mutation from C to T at the nucleotide -840. It is interesting that *GATA* is a part of a multi-pathogen defense pathway that regulates innate immunity independently of the DAF-2/DAF-16 signaling pathway (Kerry *et al*., 2006). Furthermore, *GATA* is involved in the regulation of diverse processes including the development of the heart, haematopoietic system and sex gonads (He *et al*., 2007). *HOXF* can regulates numerous pathways during developmental and normal cellular processes (Svingen and Tonissen, 2006). Zinc Finger Transcriptional Repressor (ZFTR) binding site which promotes immortalization of human mammary epithelial cells (Nonet *et al*., 2001). These results can provide some basic information to better understanding the regulation of the *TLR2* gene expression. To directly evaluate the functional relevance of the three SNPs polymorphisms, the transcriptional promoter activity using the luciferase reporter system are being conducted.

Haplotype analysis result showed that two haplotypes (TAC and TAT) were specific for Hereford cattle population. The difference may be caused by different selected purpose and their selected history of cattle.

TLR2 is critical in the immune response against gram positive bacteria. Three SNPs can alter the putative transcriptional factor binding sites and have different gene diversities and haplotypes among cattle breed populations, which suggests the polymorphisms in the promoter of *TLR2* may affect the ability of the protein to recognise its related ligands. To our knowledge, this is the first report on the polymorphism of promoter of *TLR2* gene in cattle.

Since, *TLR2* as a major receptor can respond to a variety of peptides derived from gram-positive organisms, a mutation in *TLR2* can affect the immune response to various bacterial stimuli (Brightbill *et al*., 1999). In human, the Arg753Gln mutation in *TLR2* may predispose individuals to life-threatening staphylococcal infections. The mutation is located at the C terminus of human *TLR2*, it likely affects the signaling function of the molecule, rather than ligand binding (Lorenz *et al*., 2000). To date, several studies have identified some mutations in the coding and non-coding regions of *TLR2* gene in *Bos indicus* and *Bos taurus* cattle breeds (Seabury and Womack, 2008; Mariotti *et al*., 2009; Zhang *et al*., 2009; Seabury *et al*., 2010). Furthermore, they demonstrated that they can enhance the ability of resistance for infection disease. Whereas, no significant association between the chromosomal regions surrounding *TLR2* and mastitis in Norwegian Red cattle was found by a combined linkage and linkage disequilibrium methods (Opsal *et al*., 2008).

TLR2 have a common domain architecture: an extra-cellular domain containing 20 Leucine Rich Repeats (AA54 -AA584), a transmembrane domain (AA585-AA607) and an intracellular TIR domain at AA633-AA783 (Jann *et al*., 2008). In the present study, the SNP (c.+189T>G) is located at the extra-cellular domain region, can alter amino acid (Glu to Asp), which further possibly affect the function of the *TLR2* gene in part.

CONCLUSION

In conclusion, the present study reconfirmed the finding that the clinical mastitis caused by the *S. aureus* could significantly enhance *TLR2* mRNA in mammary gland in Chinese Holstein cattle. The bovine *TLR2* promoter was firstly cloned and characterized. Seven novel SNPs were revealed and two distinct hapolotypes (TAC and TAT)) of the g.-840C>T, g.-454 GAATdel and g.-439C>T loci in the 5' regulatory region were found in Hereford cattle. The haplotype TG of two SNPs (c.+189T>G and c.+631A>G) in the coding region maybe used as a tolerance haplotype for the bovine mastitis.

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