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Short communication

Polymorphism in the promoter of *TLR4* gene by PCR-RFLP and its association with somatic cell score in Chinese Holstein

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Abstract

The objective of this study was to examine the effect of the polymorphism in the promoter of toll-like receptor 4 (*TLR4*) on mastitis and milk production traits. Two novel single nucleotide polymorphisms (SNPs) G-1539A before 5′-upstream region and G+265C in the 5′-UTR of *TLR4* gene were detected, and genetic association analysis was applied on 421 Chinese Holstein cows. The statistical analysis indicated that two SNPs was significantly associated with 305 Days milk yield ($P<0.05$) and somatic cell score (SCS) respectively ($P<0.01$). Our results provide evidence that polymorphisms in the promoter of *TLR4* are associated with milk production traits and SCS, and may be used as a possible candidate for marker-assisted selection and management in Holstein cattle breeding program.

Keywords: promoter, SNP, PCR-RFLP, Chinese Holstein, *TLR4*

Abbreviations: He: heterozygosity, Ne: effective allele numbers, PCR: polymerase chain reaction, PIC: polymorphism information content, RFLP: restriction fragment length polymorphism, SCS: somatic cell score, SNPs: single nucleotide polymorphisms, *TLR4*: toll-like receptor 4

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Introduction

Bovine mastitis, defined as »an inflammation of the mammary gland«, is generally considered the most frequent and costly disease of dairy cattle. Financial losses due to mastitis occur for animals experiencing both subclinical and clinical disease. Losses resulting from clinical mastitis are usually apparent and consist of discarded milk and transient reductions in milk yield (Ruegg 2003). Additional losses include the risk of antibiotic residues and culling or death of affected animals (Ruegg 2003). Subclinical mastitis is the most economically important form of mastitis because of long-term reductions in milk yield (Seegers *et al.* 2003). The disease cost to the dairy industry was 1.5 billion € annually in the Europe (Seegers *et al.* 2003). This is due to increased involuntary culling, therapeutic costs, reduced milk yield and changes in milk composition.

Bovine *TLR4* gene was discovered in 2003 and mapped to the distal end of BAT8 (White *et al.* 2003). *TLR4* has been testified as a candidate gene for resistance to mastitis using quantitative real-time polymerase chain reaction (PCR) and the association analysis between single nucleotide polymorphisms (SNP) and traits (Goldammer *et al.* 2004, Werling *et al.* 2003). The objective of the present study was to explore genetic variants in the promoter of *TLR4* gene and analyze the association between SNP and milk production traits and mastitis.

Material and methods

Animal samples

The blood samples of 421 multiparous Chinese Holstein cattle at Xi'an Dairy Farm were collected from the jugular vein. All experimental protocols and animal care were performed according to authorization granted by the Chinese Ministry of Agriculture. Each blood sample was placed in a tube with acid-citrate-dextrose anticoagulant for genomic DNA extraction following the standard procedures. The DNA content was estimated spectrophotometrically, and the genomic DNA was diluted to 50 ng/µl. DNA samples were stored at –20 °C for subsequent analysis.

Detection of SNPs and genotyping

Primers used to amplify cattle the promoter of bovine *TLR4* gene were designed by Primer5 software, according to the published gene sequence (GenBank acc. no. AC_000165). Primers, restriction enzymes selected (ABI, Foster City, CA USA) and fragment sizes were listed in Table 1. The detection results of allelic variation were based on the electrophoretic pattern of the restriction enzyme-treated PCR products.

PCR amplifications were performed in a total volume of 20 µL, where the volume mixture contained: 50 ng of genomic DNA as template, 2 µL 10 × PCR buffer (Mg^{2+} plus), 0.25 µM of each primer, 0.25 µM of each dNTP and 2.0 U Tap DNA polymerase (MBI Fermentas, Amherst, NY, USA). PCR conditions were as follows: after an initial denaturation of 5 min at 95 °C amplicons were generated for 35 cycles of 30 s at 94 °C, 30 s at an optimal annealing temperature, and 45 s at 72 °C, followed by a 10 min final extension at 72 °C.

Restriction fragment length polymorphism analysis was used to identify the genotypes of SNPs. The PCR products were digested in a total volume of 10 µL containing: 5 µL of PCR product, three units of restriction enzyme (0.3 µL), 1 µL of reaction buffer and 3.7 µL of ddH_2O. The mixture was incubated for 10 h at specific temperature (*Bgl I* and *Msp I* restriction enzymes were digested

at 37 °C, respectively). Digested PCR products were mixed with 10× loading buffer and subjected to 2.5 % agarose gel electrophoresis in 1× TBE at constant voltage (110 V) for 1.0 h at room temperature. Individuals were then genotyped based on different electrophoresis patterns. Fragments displaying different PCR-RFLP patterns were purified with Qiaquick spin columns (Qiagen, Hilden, Germany) and sequenced with the ABI PRISM 3730 sequencer (ABI, Foster City, CA, USA) and sequences were analyzed with BioXM software v2.6 (Biomax Informatics AG, Munich, Germany). The sequences obtained were named with letters of the alphabet.

Statistical analysis

To investigate the effects of the promoter of *TLR4* gene genotypes on SCS and milk production traits, the general linear models (GLM) was used with the procedure of SPSS software v16 (SPSS Inc., Chicago, IL, USA) (Holzer & Precht 1992, Chen *et al.* 2013). The following linear model was applied:

$$Y_{ijklmn} = \mu + F_i + G_j + S_k + E_l + H_m + e_{ijklmn} \quad (1)$$

where Y_{ijklmn} is the observed value, μ is the overall mean, F_i is the fixed effect of the farm, G_j is the fixed effect of the genotype, S_k is the fixed effect of sire, E_l is the fixed effect of the season, H_m is the fixed effect of parity, and e_{ijklmn} is the random residual effect. A value of $P<0.05$ was regarded as significant after Bonferroni correction for multiple testing.

Results

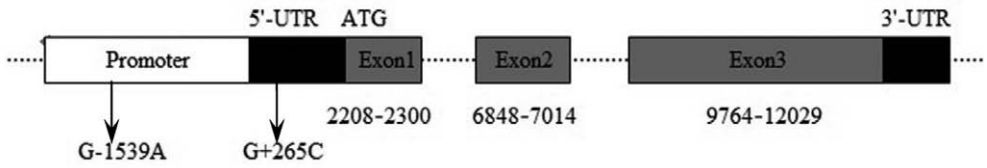
In the present study, Genomic DNA of Chinese Holstein breed was successfully amplified using primer pairs for the promoter of *TLR4* gene (Table 1).

Table 1
Primers and annealing temperatures used for PCR-RFLP analysis of the *TLR4* promoter

Primers	Sequences of primers and Position	AT, °C	SAF, bp	Methods	Restriction enzyme
SNP1	5'- TTC TTC AAC CCA ACC CAC CT -3' 5'- GCC CTG GCT CAC CAC AAC TA -3'	56	546	PCR-RFLP	*Bgl I*
SNP2	5'- GGG TAT TTT GTT ATG GCT GG -3' 5'- CCA TCA TCC TGG CAT TTT -3'	54.5	477	PCR-RFLP	*Msp I*

AT: annealing temperature, SAF: size of amplification fragment

In total, two variants were identified in the study (Figure 1 and Table 2). PCR-RFLP was used to genotype the individuals. According to the sequence mutations, the PCR products could be digested with *BglI* and *MspI* restriction enzymes. The genotypes observed for the two SNPs were illustrated in Figure 2. Genetics diversity parameters including allele and genotype frequencies, Chi-square test, effective number of allele (Ne), heterozygosity (He), homozygosity and polymorphism information content (PIC) were calculated and summarized in Table 2. According to Table 2, SNP1 agreed with Hardy-Weinberg disequilibrium ($P>0.05$), SNP2 did not agree with Hardy-Weinberg disequilibrium ($P<0.05$). According to the genetic diversity classification of PIC (PIC value <0.25, low polymorphism; 0.25< PIC value <0.5, intermediate polymorphism and PIC value <0.5, high polymorphism), Two SNPs were moderate polymorphic loci which inferred the selection potential of the two loci were abundant.



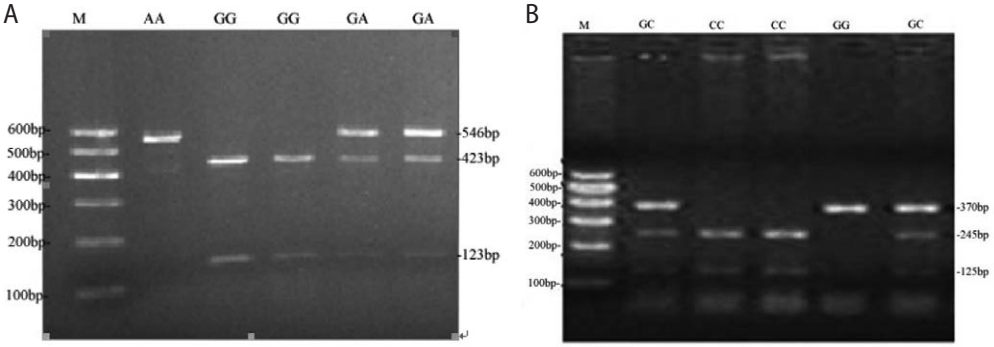
Gray boxes represent the exons. Black boxes represent the untranslated regions. Blank box represents the promoter. Dotted lines represent the introns. Number represents the nucleotide sequence position relative to the reference sequence. G-1539A and G+265C are two SNPs found in the bovine *TLR4* gene.

Figure 1
Genomic sequences of the bovine *TLR4* gene (AC_000165)

Table 2
Genotypic and allelic frequencies (%), value of χ2 test and diversity parameter of the promoter of bovine *TLR4* gene

Loci	Genotype	Genotype no.	GF	Allele	AF	χ^2 (HWE)	He	Ne	PIC
G-1539A	GG	219	52.12	G	71.45	$P>0.05$	0.4080	1.6891	0.3248
	GA	163	38.65	A	28.55				
	AA	39	9.23						
G+265C	GG	146	34.58	G	61.45	$P<0.05$	0.4738	1.9003	0.3615
	GC	226	53.74	C	38.55				
	CC	49	11.68						

GF: genotypic frequency, AF: allelic frequency, χ^2(HWE): Hardy-Weinberg equilibrium χ^2 value, He: gene heterozygosity, Ne: effective allele, PIC: polymorphism information content



(A) The 2.5 % agarose gels electrophoretic patterns of the promoter of *TLR4* gene.
M=DNA molecular weight marker is Marker I; G-1539A genotype: AA=546 bp, GG=423 bp+123 bp, GA=546 bp+423 bp+123 bp.

(B) The 2.5 % agarose gels electrophoretic patterns of the promoter of *TLR4* gene.
M=DNA molecular weight marker is Marker I; G+265C genotype: GC=370 bp+245 bp+125 bp+65 bp+42 bp, CC=245 bp+125 bp+65 bp+42 bp, GG=370 bp+65 bp+42 bp. It is difficult to see the 65bp and 42bp DNA fragment on 2 % agarose gel.

Figure 2
PCR-RFLP detection results of the promoter of *TLR4* gene PCR product

The results of the association analyses between two mutations in the promoter of *TLR4* gene and milk production traits and SCS were shown in Table 3. According to Table 3, at locus G-1539A, the animals with the GG genotype had much higher 305 days milk yield than those with the AA genotype ($P<0.01$). At locus G+265C, the animals with the GC genotype had a smaller SCS than those with the CC genotype ($P<0.05$). The rest of the records of milk production traits and SCS had no significant association ($P>0.05$). The result suggested that the SNP could be functional affecting the level of *TLR4* mRNA and protein expression as previously reported (Pottier *et al.* 2007).

Table 3
Milk production traits and SCS for different genotypes in Chinese Holstein cows (n=421)

Loci	Genotypes	Genotype no.	305 days milk yield, kg (mean±SE)	Fat rate, % (mean±SE)	Protein rate, % (mean±SE)	SCS (mean±SE)
G-1539A	GG	219	8 147.37±159.68^{A}	3.69±0.05	2.94±0.03	3.94±0.30
	GA	163	7 960.64±202.80^{AB}	3.54±0.57	2.99±0.04	3.91±0.37
	AA	39	7 635.22±386.72^{B}	3.72±0.57	2.96±0.11	3.61±0.68
	P		0.009	0.601	0.840	0.188
G+265C	GG	146	7 886.72±210.11	3.70±0.06	2.97±0.05	3.58±0.32^{ab}
	GC	226	7 818.65±203.89	3.62±0.05	2.97±0.03	3.39±0.30^{b}
	CC	49	7 663.42±373.33	3.48±0.13	2.83±0.03	3.83±0.61^{a}
	P		0.175	0.515	0.230	0.038

Values with different superscripts within the same column differ significantly at $^{a,b}P<0.05$ and $^{AB}P<0.01$.

Discussion

This study suggests significant association of the promoter of *TLR4* gene genetic variation in determining bovine's SCS and milk production traits. The genetic variation we found could be used as molecular marker for the selection of animals with optimizing production traits. Moreover, further work is needed in the large population or other cattle breeds to confirm the associations we found along with functional analysis such as RNA interference (RNAi) and overexpression study to understand the regulation mechanisms of the promoter of *TLR4* gene on animal production traits. Consider overall, this work provides the insights that variations in production traits could be used as molecular marker for the selecting breeding of Chinese Holstein cattle in China.

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