Association of polymorphism in Exon 3 of toll-like receptor 4 gene with somatic cell score and milk production traits in Holstein dairy cows of Iran

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Abstract

Mastitis is a complex inflammatory disease of the mammary gland that is caused by the invasion of pathogens. This leads to reduced synthetic capacity, compositional changes and increased somatic cell counts (SCC) in milk. Toll-like receptor 4 *(TLR4)* is a cell surface receptor that recognizes lipopolysaccharides (LPS) of gram-negative bacteria. Its role in pathogen recognition and the subsequent immune response and differential expression of the gene during mastitis have prompted the investigation of *TLR4* gene as a candidate to improve mastitis resistance in dairy cattle. The aim of this study was to analyse the possible association of a single nucleotide polymorphism (SNP) in putative co-receptor-binding region 2 (T4CRBR2) of the *TLR4* gene with somatic cell score (SCS) and milk-related traits in 408 Iranian Holstein cows. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was performed for genotyping. The effect of the genotype on the traits of interest was analysed using the general linear model (GLM) procedure of SAS software. The B allele frequency was 0.634 and the distribution of genotypes was not in Hardy-Weinberg equilibrium in the overall population. The B allele of the SNP was associated with higher 305-day milk yield and breeding value for milk yield, and lower fat percentage and lower SCS, as compared with allele A. The association between *TLR4* polymorphism with SCS and milk production traits reported in this study suggests that this SNP has the potential to be used as a marker for selection, based on molecular information.

Introduction

Mastitis is a complex disease, which can be defined as inflammation of the mammary gland. This disease is caused by the invasion and multiplication of pathogenic microorganisms in the mammary gland, leading to reduced synthetic capacity and compositional changes (Harmon *et al.*, 1994). This is followed by an influx of leucocytes into the milk and as a result an increase of the milk somatic cell counts (SCC) (Auldist & Hubble, 1998). Owing to high genetic correlations found between clinical mastitis and somatic cell score (SCS) (Hinrichs *et al.*, 2005; Heringstad *et al.*, 2006) and relatively easy recording of SCC compared with mastitis, SCC is used as an indicator of mastitis and as a tool for selection for mastitis resistance (Koivula *et al.*, 2005). Heringstad *et al.* (2003) suggested that mastitis resistance has a significant genetic component, and it is possible to gain noticeable selection response for clinical mastitis, and that the unfavourable genetic correlation between mastitis and milk production traits would lead to a further increase in mastitis occurrence if mastitis is ignored in breeding programmes.

The innate immune response is the first line of defence against microbial pathogens (Janeway & Medzhitov, 2002). Genes encoding immune factors that detect and eliminate pathogens are strong potential candidates for enhancing resistance against infectious diseases like mastitis. Toll-like receptor 4 *(TLR4)* is a cell

surface receptor that recognizes lipopolysaccharides (LPS) of gram-negative bacteria (Akira & Takeda, 2004). Gene expression patterns indicate the innate immune response of the mammary gland, which is characterized by production of a large amount of important mediators of innate immunity following activation of the *TLR4*. Toll-like receptor 4 has evolved with its accessory proteins (LBP, CD14, and MD-2/Ly96) to recognize lipopolysaccharides (LPS). The detection of the LPS molecule by *TLR4* leads to the activation of intracellular signalling molecules (Zheng *et al*., 2006). Mastitis significantly enhances the expression of the *TLR4* gene (Goldammer *et al*., 2004).

Based on the above studies on the role of *TLR4* in pathogen recognition and subsequent initiation of the inflammatory and immune response, and on differential expression of the gene during mastitis, *TLR4* has been proposed as a strong candidate for increasing mastitis resistance in breeding programmes (Ogorevc *et al*., 2009).

Several polymorphisms have been identified in bovine *TLR4* gene (White al., 2003), some of which are reported to affect SCS. One single nucleotide polymorphism (SNP) in a putative promoter region (P-226) and two SNPs in exon 3 (E3+1656 and E3+2021) of *TLR4* have been found to be associated with estimated breeding values for lactation persistency, and SCS in the Canadian Holstein bull population (Sharma *et al*., 2006). In addition, Wang *et al*. (2007) found an association between polymorphism in putative co-receptor-binding region 1 (T4CRBR1) of the gene and SCS in Chinese Holstein, Sanhe and Chinese Simmental cattle. No significant association was revealed between the genotypes of locus T4CRBR2 and SCS for these cattle. Another SNP (g.8664C>T) in the toll IL-1 receptor (TIR) domain of the bovine *TLR4* was shown to be associated with SCS (Wang *et al*., 2008). Beecher *et al*. (2010) also revealed an association of E3+2021 with milk fat and protein percentage in late lactation in Holstein-Friesian, Jersey, Norwegian Red, Montbeliarde, and Holstein-Friesian x Jersey cows, but not in Holstein-Friesian bulls.

Owing to the effect of mastitis on milk yield and composition (Shuster *et al*., 1991; Auldist & Hubble, 1998; Leitner *et al*., 2004) and the existence of a genetic correlation between mastitis and milk yield and composition (Hinrichs *et al*., 2005), it is possible that *TLR4,* as a candidate gene influencing mastitis resistance, also affects milk production and components. Literature that addresses the association of this gene with milk-related traits is limited (Beecher *et al*., 2010). The present study was designed to investigate the possible association of SNP at the 1397-bp position in putative co-receptor-binding region 2 (T4CRBR2) of the *TLR4* gene with SCS and milk-related traits in Iranian Holstein cows.

Materials and Methods

Blood samples were collected from 408 Iranian Holstein cows randomly selected from five dairy herds in Isfahan province, using vacuum venoject tubes containing EDTA, and stored at –20 °C for DNA extraction. Accurate phenotypic data for 305-day milk yield, milk fat and protein percentage, SCS, and breeding value data for fat and milk yield were obtained from the Vahdat Industrial Agriculturists & Dairymen Cooperative of Isfahan.

Genomic DNA was extracted from blood samples by the salting out method (Miller *et al*., 1988). Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. PCR was used to amplify the 382-bp DNA fragments containing the polymorphism of interest. Each PCR reaction was carried out in a total volume of 20 μL containing 50 ng genomic DNA, 10 pmoL each primer, 2 μL 10X PCR buffer, 2 mM MgCl2, 200 μMdNTPs and 2.5 units Taq DNA polymerase. Sequences of the primers used in the PCR were reported previously by Wang *et al*. (2007). The sequences of the forward and reverse primers were 5' AGACAGCATTTCACTCCCTC 3′ and 5′ ACCACCGACACACTGATGAT 3′, respectively. PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, 59 °C annealing temperature for 30 s, extension at 72 °C for 40 s, and a final extension at 72 ºC for 10 min. The PCR products were digested with 5 U of *Alu*I enzyme (Fermentas, St. Leon-Rot, Germany) in 20 μL of reaction volume at 37 °C for 10 h. The digested products were separated by horizontal electrophoresis on 2.5% agarose gel in 0.5 x TBE (90 v for 1.5 h) stained with ethidium bromide and visualized under UV light. The A allele was indicated by two bands at 300 bp and 82 bp and the B allele resulted in three fragments of 160, 140 and 82 bp.

The allele and genotype frequencies were estimated by direct counting, and deviation from Hardy-Weinberg equilibrium was examined using the POPGENE v. 1.31 (Yeh *et al*., 1997). For the association studies, the traits of interest were analysed using the least square method of the general linear model (GLM) procedure of the SAS software (SAS, 1994) according to the following model:

$$Y_{ijk} = \mu + G_i + HYS_j + + b_1(X_{ijk} - X) + b_2(W_{ijk} - W) + e_{ijk}$$

where Y_{ijk} is value for each trait, μ is overall mean, G_i is the effect of the ith genotype (AA, AB and BB), HYSj is the combined effect of herd (1, 2, 3, 4, and 5), year and season of parturition, b_1 is the linear regression coefficient of milk yield, X_{ijkl} is milk yield, X is mean milk yield, b_2 is the linear regression coefficient of open days, W_{ijkl} is open days, W is mean open days and e_{ijk} is the random residual effect.

For the analysis of 305-day milk yield, milk yield as a covariate was omitted from the model. Also, wherever breeding value was used instead of phenotypic value for a trait, effects other than genotype were excluded from the model.

Results

Both alleles (A and B) and three genotypes (AA, AB and BB) of the T4CRBR2 locus were observed in all five herds. The genotype and allele frequencies of T4CRBR2 in Iranian Holstein cows are listed in Table 1. The result of the test for Hardy-Weinberg equilibrium for this locus indicated that the genotypes in herds three, four and five did not show any significant deviation from Hardy-Weinberg equilibrium, while herds one, two and the overall population were not distributed according to Hardy-Weinberg equilibrium (Table 1).

Table 1 Allele and genotype frequencies of the *TLR4* polymorphism in Iranian Holstein cows

Herd	Number	Allele		Genotype			Chi-square
		A	B	AA	AB	BB	
1	96	0.453	0.547	0.115	0.677	0.208	12.51[***]
2	79	0.342	0.658	0.038	0.607	0.355	9.38[**]
3	85	0.324	0.676	0.118	0.412	0.470	0.36[ns]
4	65	0.354	0.646	0.077	0.554	0.369	2.70[ns]
5	83	0.343	0.657	0.096	0.494	0.410	0.67[ns]
Total	408	0.366	0.634	0.091	0.551	0.358	14.19[***]

Ns: not significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$.

Least square means of the three *TLR4* genotypes for the traits of interest are presented in Table 2. Genotypes of T4CRBR2 were significantly associated with 305-day milk yield, breeding value for milk yield, fat percentage and SCS ($P < 0.05$). Genotype AA had the lowest and BB the highest 305-day milk yield, while

Table 2 Least square means for traits of interest in cows with different *TLR4* genotypes

Trait	Genotype (mean ± SE)		
	AA	AB	BB
305-day milk yield (kg)	8 905[a] ± 233	9 402[b] ± 130	10 537[c] ± 151
BV for milk yield (kg)	265.7[a] ± 101.7	508.6[b] ± 42.7	509.4[b] ± 52.0
BV for fat yield (kg)	9.13[a] ± 3.57	12.06[a] ± 1.50	11.04[a] ± 1.86
Fat percentage	3.41[c] ± 0.08	3.21[b] ± 0.05	3.05[a] ± 0.05
Protein percentage	2.98[a] ± 0.03	2.96[a] ± 0.02	2.92[a] ± 0.02
Somatic cell score	3.11[b] ± 0.32	2.98[b] ± 0.14	1.82[a] ± 0.19

Different superscripts in each row indicate significant differences at $P < 0.05$.

AB was intermediate (P <0.05). Furthermore, cows with genotype AA had significantly lower breeding value for milk yield compared to AB and BB genotypes (P <0.05).

Cows homozygous for the A allele had significantly higher milk fat percentage than those of AB genotype (P <0.05) and cows of AB genotype had a higher fat percentage than those homozygous for the B allele (P <0.05). Animals carrying genotype BB showed lower SCS in comparison with genotypes AB and AA (P <0.05). No significant association was evident between the genotypes of the SNP and protein percentage and breeding value for fat yield in our study (P >0.05).

Discussion

The allele frequencies obtained in the present study were in agreement with the findings of Wang *et al.* (2007), who reported frequencies of 0.42, 0.44 and 0.45 for allele A and 0.58, 0.56 and 0.55 for allele B in Chinese Holstein, Sanhe and Chinese Simmental populations, respectively.

In our study, the B allele of T4CRBR2 SNP was found to be associated with lower SCS. However, Wang *et al.* (2007) did not show any relationship between this polymorphism and SCS. Since the SNP in the T4CRBR2 locus is a synonymous one in coding region of the gene, it will not change the amino acid sequence and the function of *TLR4*. Thus, the SNP is not a functional mutation, but may be in linkage disequilibrium with a functional polymorphic site in *TLR4* or other closely linked gene(s) influencing this trait. *TLR4* has been mapped to the distal end of bovine chromosome 8 (White *et al.*, 2003) and Quantitative Trail Loci (QTL) affecting SCS, and clinical mastitis have been found on this chromosome. Klungland *et al.* (2001) mapped a single QTL affecting SCC at position 54 cM, and a QTL for clinical mastitis at position 46 cM in a Norwegian sire family.

The allele B of the SNP was associated with higher 305-day milk yield and breeding value for milk yield. The association of the B allele with higher 305-day milk yield and breeding value for milk yield observed in the current study may be attributed to the negative effect that increased SCS and mastitis have on milk production. Although QTL for milk yield have not been detected on BTA8 in the vicinity of *TLR4*, unfavourable genetic correlation has been found between mastitis and milk yield (Hinrichs *et al.*, 2005). Clinical mastitis can induce mammary cell death and affect milk secretion detrimentally (Capuco *et al.*, 1986; Long *et al.*, 2001).

In the present study, the allele B of the SNP was associated with lower fat percentage. However, no association was found between the polymorphism and protein percentage and breeding value for fat yield. A study by Beecher *et al.* (2010) indicated an association of E3+2021 polymorphism in *TLR4* gene with milk fat and protein percentage in late lactation in Holstein-Friesian, Jersey, Norwegian Red, Montbeliarde, and Holstein-Friesian x Jersey cows, but not in Holstein-Friesian bulls. The association of the *TLR4* polymorphism with fat percentage in our study can simply be attributed to negative genetic correlation between fat percentage and milk yield (Chauhan & Hayes, 1991; Rosati & Van Vleck, 2002). Our observation that the B allele was associated with higher milk yield and lower fat percentage supports this hypothesis. On the other hand, our association of allele A with higher SCS and higher fat percentage is in agreement with findings of other studies that have reported an increase in fat content as a result of mastitis (Shuster *et al.*, 1991; Pyorala *et al.*, 2003; Bruckmaier *et al.*, 2004).

Conclusions

An association of T4CRBR2 polymorphism in the *TLR4* gene with SCS, 305-day milk yield, breeding value for milk yield and fat percentage was evident in this study. Our results suggest that this SNP may be of use as a potential genetic marker in selection programmes for these traits in dairy cattle. Further association studies of various polymorphisms located in different regions of the TLR4 with these traits in other breeds and populations of cattle are needed in order to verify the obtained results.

Acknowledgment

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