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The chemokine receptor 1 gene polymorphism and its association with Somatic Cell Score and milk production traits in dairy cattle*

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CXC chemokine receptors 1 (CXCR1) play a key role in inflammatory response and eventual activation of the innate immunity. Mastitis is a big problem in dairy industry. In the presented study the bovine CXCR1 gene was considered a candidate gene of anti-*mastitis* defense in Chinese Holsteins. Polymorphism of bovine *CXCR1* was investigated and its associations with Somatic Cell Scores (SCS) and milk production traits (milk yield, milk fat and milk protein content) analysed. Six single nucleotide polymorphisms (SNP) were identified within a 311 bp segment of the bovine *CXCR1 locus* at positions +570 (G>A), +642 (G>A), +735 (G>C), +816 (C>A), +819 (A>G) and +820 (G>A) relative to the available mRNA sequence, of which *CXCR1c.*+820 G>A was revealed for the first time. The SNP *CXCR1c.*+735G>C resulted in the mutation of amino acid, but it showed no significant association with the SCS, milk yield, milk fat and milk protein percentage of Chinese Holsteins (P>0.05). Fifteen haplotypes were reconstructed by the six SNPs in the sample of Chinese Holstein population. The most common haplotype was Haplo1 carrying alleles AAGCAG equal to 0.37, followed by 7 haplotypes (from 2 to 7) with frequencies equal to 0.13, 0.11, 0.08, 0.08, 0.07, 0.07 and 0.06, respectively. Haplotype analysis revealed no significant associations with SCS and other milk production traits.

KEY WORDS: *CXCR1* gene / genetic resistance / haplotype / Holstein cattle / polymorphism / SNP

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Mastitis is an inflammation of the mammary gland predominantly caused by pathogens invading through the teat canal and is the most economically devastating disease affecting the dairy industry [Fetrow *et al.* 2000]. Phagocytosis by neutrophils is a crucial defense of the mammary gland and the prompt recruitment of these phagocytes from blood to milk compartments of the udder is essential for the outcome of the infection [Hill 1981, Lahouassa *et al.* 2008]. The ability of neutrophils to migrate into infected tissues depends upon recognition of inflammatory mediators by neutrophil cytokine, chemokine, and complement receptors [Burvenich *et al.* 1994]. Two chemokine receptors expressed on neutrophil surfaces, *CXCR1* and *CXCR2*, play key role in inducing neutrophil activation, chemotaxis, and eventual phagocytosis of pathogens [Peveri *et al.* 1988, Podolin *et al.* 2002]. The CXCR1 receptor interacts primarily with CXCL8 (IL-8), the most potent chemoattractant for neutrophils [Mitchell *et al.* 2003, Ahuja *et al.* 1996]. The activity of *CXCR1* is strongly associated with the inflammatory response to Gram-negative bacteria infections, and consequently is a key player activating the innate immune response [Oviedo-Boyso *et al.* 2006, Rainard and Riollet 2006].

The *CXCR1* gene has been mapped approximately 90.3 cM from the centromere of bovine chromosome (BTA) 2. These *loci* are approximately 1.3 cM from the natural resistance-associated macrophage protein (NRAMP)-1, a polymorphic gene related to immune function in cattle, humans and mice, indicating that this region of BTA 2 may be associated with immune function and disease resistance [Grosse *et al.* 1999]. The annotation of *CXCR1* was recently corrected by Pighetti and Rambeaud [2006] and their correction was confirmed through functional study by Lahouassa *et al*. [2008], indicating that some of the previous associations and functional studies investigated polymorphisms in *CXCR1* rather than *CXCR2* [Youngerman *et al.* 2004ab, Rambeaud and Pighetti 2005, Rambeaud *et al.* 2006]. The *CXCR1* has been considered as a candidate gene for bovine *mastitis* previous studies, but their results were not consistent [Youngerman *et al.* 2004, Leyva-Baca *et al.* 2008ab]. Thus, in the present study we investigated polymorphisms within the CXCR1 gene in Chinese Holsteins, and searched for its possible associations with SCS and milk production traits.

Materials and methods

Animals

The Chinese Holstein cattle with known pedigree from Xuzhou Dairy Farm were considered for the present study. The 205 cows, born in 1999-2000, were daughters of 25 sires. Mean number of daughters per sire was 8; each of the sire group contained no less than six cows. Cows were fed to appetite with a complete ration of artificially dried grass (mixed pasture containing mainly rye grasses), corn silage and concentrates.

Somatic cell scores (SCSs) and projected 305-day standardized milk yields, as well as percentages of milk fat and milk protein of the second lactation were obtained from the farm records. Test-day records of milk yield, fat percentage, fat yield, protein

percentage, protein yield, and SCC were collected at approximately monthly intervals. All cows were milked twice daily, and the milk of both milkings was analysed. Fat and protein percentages and SCCs were calculated as the weighted means of the morning and evening milking. Test-day SCCs were converted to SCSs using a base 2 logarithmic function: $SCS = \log_2(SCC/100) + 3$ [Ali and Shook 1980]. The milk yield was recorded for 305 milking days. Blood samples of cows were withdrawn from jugular vein and DNA was extracted from isolated leukocytes by a proteinase K and following the phenol-chloroform protocol [Sambrook and Russell 2001).

PCR amplification

Forward (5′-CTTCCGTGAGGCCTATCAAC-3′) and reverse (5′-AGGTCT-CAGCAATC ACATGG-3′) primers were designed from the bovine IL-8 receptor A (*CXCR1*; GenBank Accession no. EF597244) sequence to amplify a 311 bp region that contained five SNPs as determined in a beef cattle population [Youngerman *et al.* 2004b]. Polymerase chain reaction (PCR) was carried out in a total volume of 15 µl reaction mixture containing 50 ng of template DNA, 0.20 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa, Dalian, China). Applied were the following PCR conditions: initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 40 s, 60.6°C for 40 s and 72°C for 30 s, with a final extension at 72°C for 5 min.

Detection of CXCR1 polymorphism

PCR products were analysed for single-strand conformation polymorphisms (SSCP). Five µl aliquots of the above PCR products were mixed with 5 µl of the denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice. Denatured DNA was subjected to 10% PAGE (80×73×0.75 mm) analysis which was run with 1×TBE buffer (89 mM Tris–Borate, 2 mM EDTA, pH 8.3) for 3.5 h at room temperature under a constant voltage (150 v). The gel was stained with silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde) - Zhang *et al.* [2007].

The PCR products, which represented different banding patterns on gel, were purified with the GenElute PCR DNA Purification Kit (SIGMA-ALDRICH Corporation, USA) and sequenced using the ABI 377 sequencer (APPLIED BIOSYSTEMS, USA). The PCR products sequenced for each banding pattern were from five different individuals. Sequences were aligned using web-based CLUSTAL-W (http://www.ebi.ac.uk/clustalw/index.html) programme and SNPs were identified based on the sequence in GenBank (Accession no. EF597244).

Statistical

The HAPSTAT algorithm and software were implemented to reconstruct haplotype probabilities (http://www.bios.unc.edu/-lin/hapstat/documentation). The genetic effects were analysed by an allele substitution model using the PROC GLM function of SAS

[SAS Institute, 1999]. For single SNP analysis, the model was as follows:

$$y_{ijk} = \mu + s_i + b_i + c_{ij} + e_{ijk} \quad (1),$$

and for analysis of gene haplotypes was

$$y_{ijk} = \mu + \Sigma_{a=1,h} \beta_a P_{a:ij} + s_i + c_{ij} + e_{ijk} \quad (2),$$

where:

y_{ijk} – the k-th phenotypic record (preadjusted effects of age, parity, herd-year-season and deviated from the population mean) for the j-th daughter of the i-th sire;

b – regression coefficient for the effect of nucleotide substitution;

h – total number of haplotypes evaluated,

β_a – regression coefficient for the effect of a single haplotype a,

$P_{a:ij}$ – probability that daughter j of i-th sire carried haplotype a;

s_i – random effect of sire i;

c_{ij} – random effect of j-th daughter of i-th sire (nested within sire i);

e_{ijk} – random residual.

Only haplotypes with >10% frequency were assessed and haplotype I was used as the base in association analysis.

Results and discussion

Recently, three novel SNPs in the 5' upstream region of the bovine CXCR1 were identified: *CXCR1c.* – 344T>C, *CXCR1c.* – 1768T>A and *CXCR1c.* – 1830A>G. [Leyva-Baca *et al.* 2008a] indicating that the bovine *CXCR1* is highly polymorphic. Because of its highly polymorphic nature and implications for immune function, *CXCR1* may be an informative chromosomal region for association with disease phenotypes.

Based on the PCR-SSCP, eight banding patterns (A-H) were identified (Fig. 1). A total of six SNPs were detected by sequencing the PCR representing the eight SSCP patterns (Tab. 1). The SNPs +570 (G>A), +642 (G>A), +735 (G>C), +816 (C>A), +819 (A>G) corresponded to +612 (G>A), +684 (G>A), +777 (G>C), +858 (C>A) and +861(A>G) identified by Youngerman *et al.* [2004a], respectively. Table 1 shows the allele frequencies observed for all six SNPs detected in Chinese Holsteins. The SNPs of these cattle are all in accordance with Hardy-Weinberg equilibrium. Of the six SNPs, *CXCR1c.*+642 G>A was not reported in beef cattle derived from the U.S. Meat Animal Research Center reference population [Grosse *et al.* 1999]. This may be due to the limited sample size, because only 26 beef cattle were used in their study. In contrast, a SNP (CXCR1c.+741 C>A) detected in beef cattle [Grosse *et al.* 1999] was not found in an American Holstein sample population [Youngerman *et al.* 2004a] or Chinese Holsteins in the present study. Youngerman *et al.* [2004a] attributed this

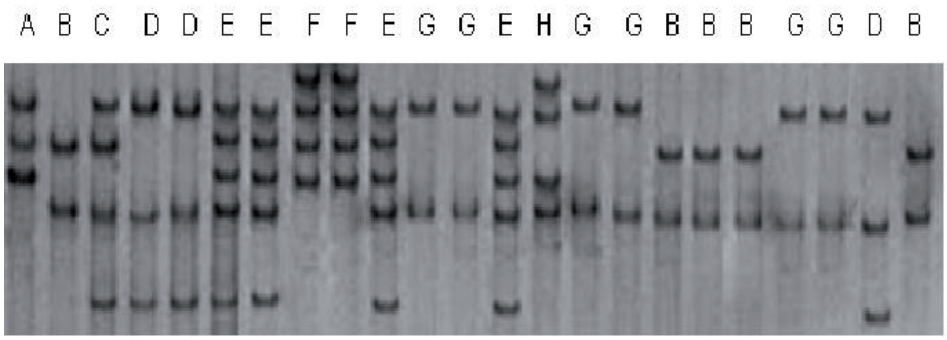

Fig 1. The *CXCR1* gene SSCP patterns of electrophoresis in Chinese Holsteins. A total of eight SNPs (+570 (G>A), +642 (G>A), +735 (G>C), +816 (C>A), +819 (A>G) and +820 (G>A)) were detected by sequencing the PCR representing the eight SSCP patterns. Denatured DNA was subjected to 10% PAGE (80×73×0.75 mm) analysis which was run with 1×TBE buffer (89 mM Tris–Borate, 2 mM EDTA, pH 8.3) for 3.5 h at room temperature under a constant voltage of 150v.

Table 1. Single nucleotide polymorphism (SNP) allele frequencies within the *CXCR1 locus* of Holstein and Jersey dairy cattle

Downstream of coding sequence	Allele of SNP	[1]Chinese Holstein allele frequency (n=410)	[2]American Holstein allele frequency (n=74)	[2]Jersey allele frequency (n=84)
570	A	0.69 (±0.02)	0.58 (±0.05)	0.92 (±0.03)
	G	0.31 (±0.02)	0.42 (±0.05)	0.08 (±0.03)
			P>0.05	[3]P<0.01
642	G	0.46 (±0.03)	0.68 (±0.05)	0.65 (±0.05)
	A	0.54 (±0.03)	0.32 (±0.05)	0.35 (±0.05)
			P<0.01	P<0.01
735	G	0.64 (±0.03)	0.57 (±0.05)	0.87 (±0.04)
	C	0.36 (±0.03)	0.43 (±0.05)	0.13 (±0.04)
			P>0.05	P<0.01
816	C	0.67 (±0.02)	0.74 (±0.05)	0.93 (±0.03)
	A	0.33 (±0.02)	0.26 (±0.05)	0.07 (±0.03)
			P>0.05	P<0.01
819	A	0.59 (±0.03)	0.57 (±0.05)	0.86 (±0.04)
	G	0.41 (±0.03)	0.43 (±0.05)	0.14 (±0.04)
			P>0.05	P<0.01
[4]820	G	0.88 (±0.02)	not determined	not determined
	A	0.12 (±0.02)		

[1]Present study.
[2]Youngerman *et al*. [2004a].
[3]*P* the Chi-square test of allelic type distribution between Chinese Holsteins (present study) and the results reported by Youngerman *et al*. [2004a].
[4]The novel SNP detected in the present study.

difference to limited sample sizes for the minor allele was found in only 12% of the beef cattle population. Alternative interpretation is that these polymorphisms arose as a result of dairy cattle breeding and selection.

The allele distribution of the five earlier identified SNPs does not differ between American Holsteins and Chinese Holsteins except that of SNP *CXCR1c.* +642 G>A (Tab. 1). A recent report on SNP *CXCR1c.* +735 G>C in Canadian Holsteins also confirms these results [Leyva-Baca *et al.* 2008a]. However, there is significant difference between Holstein and Jersey cattle ($P<0.01$) in allelic distribution of five previously identified SNPs (*CXCR1c.*+570 G>A, *CXCR1c.*+642 G>A, *CXCR1c.*+735 G>C, *CXCR1c.*+816C>A and *CXCR1c.*+819A>G) – Youngerman *et al.* [2004a]. This suggests that there is interbreed difference in allele distribution, which might be due to selection.

All six SNPs were used in the haplotype reconstruction. Table 2 shows the estimated haplotype frequencies for the 15 haplotypes reconstructed in the sample of Chinese Holstein population. The most common haplotype was designated as Haplo1 carrying alleles AAGCAG. The haplotype frequencies for Haplo9 to Haplo15 decreased from 0.002 to 0.005, and were therefore pooled into a single haplotype designed Haplo9. Haplo1 corresponds to haplotype AAGCA in previous report with similar frequencies (0.30-0.37) – Grosse *et al*. [1999], Youngerman *et al*. [2004a]. Compared to American Holsteins, two novel haplotypes (AAGAGA and GGGAAG) with frequency of more than 7% were detected [Youngerman *et al.* 2004a]. The haplotypes identified in the present study were five more than that of American Holstein population. This may due to different sample sizes. The lower haplotype frequencies in the present study result from the increased number of haplotypes.

Table 2. Estimated *CXCR1* haplotype frequencies for Chinese Holsteins (±SE)

Haplotype	570	642	735	816	819	820	[1]Haplotype frequency
Haplo1	A	A	G	C	A	G	0.37 (±0.03)
Haplo 2	G	G	C	A	G	G	0.13 (±0.02)
[2]Haplo 3	A	A	G	A	G	A	0.11 (±0.02)
Haplo 4	G	G	C	C	G	G	0.08 (±0.01)
Haplo 5	A	G	G	C	A	G	0.08 (±0.01)
[2]Haplo 6	G	G	G	A	A	G	0.07 (±0.01)
Haplo 7	A	G	C	C	G	G	0.07 (±0.01)
Haplo 8	A	A	C	C	A	G	0.06 (±0.01)
[3]Haplo 9							0.03 (±0.01)

[1]The standard errors are given in parentheses.
[2]Haplotypes 3 and 6 were detected for the first time.
[3]The rare haplotypes were pooled into Haplo9.

The transversion G>C at +735 results in the mutation of amino acid (Gln>His). Because this SNP is located in the region encoding the third intracellular loop of the receptor, an important region for the G-protein coupling activation, it may play

a role in receptor-ligand interaction between CXCR1 and IL-8. Cows with the CC or GC genotype at *CXCR1*+735 showed significantly lower neutrophil migration to recombinant human IL-8 (rhIL-8) than cows with the GG genotype [Rambeaud and Pighetti 2005]. A significant association was revealed between *CXCR1c.*+735G>C genotype and percentage of subclinical *mastitis* cases in Holsteins [Youngerman *et al.* 2004b]. However, such an association occurred neither in the present study nor (Tab. 3) in a recent report on the Holsteins of Canada [Leyva-Baca *et al.* 2008a]. Reasons for the discrepancy between the results from previous reports [Youngerman *et al.* 2004b, Leyva-Baca *et al.* 2008a] and this study are not clear. A possible explanation might be sample sizes. There are also differences between US, Canadian and Chinese Holsteins. And Chinese Holstein cattle are the descendants of Holstein and native cattle. So another possible explanation is the breed or variety specificity.

Table 3. Association of the single nucleotide polymorphisms (SNP) *CXCR1c.* +735 G>C with the SCS, milk yield (kg), milk fat (%) and milk protein (%) in Chinese Holsteins

Trait	Mean allele substitution effect ±SE*	P-Value
SCS	0.19±0.12	0.09
Milk yield (kg)	1952±2803	0.48
Milk fat (%)	0.09±0.15	0.54
Milk protein (%)	-0.05±0.07	0.46

*The allele +735 G served as the base, and the base values of SCS, milk yield, fat content and protein content were 3.75, 5994, 3.56, 3.21, respectively.

Haplotype analysis revealed no significant associations with SCS and other milk production traits (Tab. 4). These were in accordance with the results of allele substitution effects. The SNP *CXCR1c.* +735G>C was not associated with any of the traits. The other five SNPs were synonymous, thus they may not affect the function of *CXCR1* peptide.

Table 4. Estimated haplotype effects (means±SE with P-value in parentheses) on SCS, milk yield, milk fat and milk protein content in Chinese Holsteins

Trait	Haplo1	Haplo2	Haplo3
SCS	0.00	0.00±0.44 (0.99)	0.31±0.45 (0.50)
Milk yield (kg)	0.00	2230±2295 (0.33)	2869±2445 (0.24)
Fat (%)	0.00	0.06±0.13 (0.64)	0.05±0.14 (0.73)
Protein (%)	0.00	-0.08±0.11 (0.49)	-0.16±0.11 (0.14)

In summary, no significant associations of the detected SNP with SCS and other milk production traits were identified in the present study. However, this could not deny the defensive function of *CXCR1*. A further work should include another regions of the gene, for example a regulatory region (e.g. promoter). Such mutations could affect gene expression level and a level of *CXCR1* protein. An expression level of the gene might be a more important factor for *mastistis* resistance than some mutations within coding sequences, especially those not changing the amino acid sequence.

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