Novel polymorphisms in bovine *CD4* and *LAG-3* genes associated with somatic cell counts of clinical mastitis cows

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Genet. Mol. Res. 17 (1): gmr16039859

Received October 14, 2017

Accepted November 08, 2017

Published December 26, 2017

DOI http://dx.doi.org/10.4238/gmr16039859

ABSTRACT. Clinical mastitis cows normally produce clotted milk, thus the much higher somatic cells in milk are unable to be counted by routine FOSS machine. The proteins coded by *CD4* and *LAG-3* genes can bind to MHC class II molecules and play important roles in inflammatory diseases. The present study was designed to investigate the effects of single nucleotide polymorphisms (SNPs) in bovine *CD4* and *LAG-3* genes on the somatic cell counts (SCCs) of clinical mastitis Holstein cows. For the first time, we detected SCCs in the clinical mastitis cows' milk by Newman's staining combined with microscope assays. Our association results showed that two novel SNPs (T104010752C and C104028410T) identified in bovine *CD4* and *LAG-3* genes respectively were significantly associated with SCCs of clinical mastitis cows ($P<0.05$). In addition, the combined genotypic effect of both the SNPs was also significant on SCCs ($P<0.05$). The results imply that the novel SNPs in *CD4* and *LAG-3* genes could be significant candidate markers against Clinical mastitis in Holstein cattle.

Key words: Dairy cattle; CD4; LAG3; SCCs; Clinical mastitis

INTRODUCTION

Mastitis is an inflammatory disease of mammary gland associated with dairy cattle health and welfare concern. This disease causes huge economic losses to the dairy farmers and the dairy industry worldwide (Viguier et al. 2009; Well et al. 1998). Strong genetic correlations ranging from 0.7 to 0.84 between mastitis and somatic cell counts (SCCs) were reported by many studies (Hinrichs et al. 2005; Koivula et al. 2005). SCC and its log transformed score (somatic cell score, SCS) are widely used as indirect indicators of mastitis and are commonly used in modern dairy management. However, clotted milk in clinical mastitis cows causes drastic increases in SCCs. Therefore, making the SCCs is unable to be checked by a FOSS machine, a routine test machine used in dairy herd improvement. *LAG-3* and *CD4* molecules are known to bind to MHC class II molecules and play a vital role in inflammatory progress in many species (Baixeras et al. 1992; Huard et al. 1994). Both *CD4* and *LAG-3* genes have been revealed to have a role in anti-cancer (Lee et al. 2010). Polymorphism in *LAG-3* gene has been reported to be significantly associated with multiple sclerosis (Zhang et al. 2005). *In vitro* studies in mice revealed that *LAG-3* establishes effective protection against mammary carcinogenesis (Cappello et al. 2003; Corthay et al. 2005).

In bovine genome, *CD4* and *LAG-3* are neighbour genes on BTA5 and share high genomic similarity (Bruniquel et al. 1997), which have remarkable structural resemblances and are, therefore, regarded as evolutionary first cousins of Ig superfamily (Workman et al. 2002; Triebel et al. 1990). *CD4* exerts a crucial role in the immune response of pathogen-induced mastitis in dairy cows (Zhao et al. 2015). An influx of activated *CD4*+ T lymphocytes in the mammary gland is a distinct characteristic of mastitis. Meanwhile, our previous research reported that a significant effect of SNP in *CD4* gene with somatic cell score (SCC) in Chinese Holstein cows (He et al. 2011).

It was thought that *LAG-3* might act as a negative regulator and *CD4* might act as a positive regulator of T cell activation (Workman et al. 2002). The present study was, therefore, designed with the objectives to evaluate the SCC and SCS for the clotted milk samples from clinical mastitis cases by microscope when they are undetectable by routine dairy herd improvement test (DHI), and to evaluate the association of novel SNPs in *CD4* and *LAG-3* genes with mastitis indicator traits (SCC and its log transformed indicator, SCS) in clinical mastitis cows' samples.

MATERIALS AND METHODS

Resource population

Blood and milk samples of sixty clinical mastitis cases of Chinese Holstein cattle were collected from two dairy farms located in North West of China. The mastitis was confirmed by the clinical signs showed by the cattle, including swollen and inflamed udder, high temperature, and presence of flakes or blood in the milk. The mastitis cattle were separated from the rest of the healthy herd and were under treatment. The cows were in different parities (parity 1 - 4) and at different stages of lactation. Blood samples were collected in 9 mL tubes from the caudal vein of cattle for DNA extraction. Milk samples were collected from all the four teats of the same cow at morning milking in 50 mL tubes, placed on ice boxes and immediately sent to Beijing Dairy Cattle Centre for routine dairy herd improvement test (DHI), in which SCC was detected via FOSS machine (Fossomatic™ FC, Foss, Denmark). As the samples were from clinical mastitis cows and the SCC was considerably high, it was unable to detect SCC from these samples by routine test. Then, SCC in these clinical mastitis samples was checked and calculated using Newman dye under direct microscopic observation. Somatic cell score (SCS) was calculated from SCC (SCS = log2 [SCC/100] + 3, the unit of SCC is 1,000 cells/ml) (Rupp & Boichard, 1999).

Newman's staining solution configuration method

The following reagents were used for preparing Newman's staining solution: 1-1.2 g methylene blue, 60 mL ethanol (95%), 40 mL tetrachloroethane (or xylene) and 6 mL glacial acetic acid. First, ethanol was put into tetrachloroethane and kept in the water bath to be heated to 60°C and then the Meilan powder mixture was added. It was kept on mixing till the dye was completely dissolved. After cooling, the glacial acetic acid was added, and then rotated slowly and constantly. Finally, it was filtered through a coarse filter and preserved in a well-stopper bottle.

Calculating SCC under the Microscope

To calculate SCC in the milk of the clinical mastitis cows, first, 0.01 mL milk was put on a glass slide and spread it equally on 1cm square area, the milk could dry naturally on the glass slide. Then, the glass slide was passed a few times on the flame to fix the milk. When the milk became completely dry, then 1-3 drops of the dye were poured directly on 1 cm square area of the slide. The slide was washed with distilled water in order to get rid of the dye. Care was taken to avoid washing off the dye completely. The washing step was very crucial because the dye was not firmly attached to the slide. Gently a drop of water was put on the slide to soak the dye, and the water was slowly removed. The slide could dry for few minutes and then it was checked with the oil immersion microscopy and the number of somatic cells was record in 1 cm square area. The size of the view was decided by the SCC, if there were many cells seen in every view then several spots (10-15 spots) were checked; if rare or no cells were seen on slide then more than 50 spots were checked and finally calculated the somatic cell count in 1 mL of milk. The average number of cells in every view (spot) × 1/each visual field area × 100 = somatic cell count in 1 mL of milk. For example: Each visual field area = πr^2, i.e. the diameter of the oil microscope's lens was 1.6 mm, and the eyepiece's was 10 mm, so oil immersion lens area was 0.16 mm (0.016 cm) in diameter and thus = $3.1416 \times (0.008)^2$, visual field area = 0.0002 square cm. If the average cells counted were 4 in each field, then the SCC in 1 mL of milk was: $4 \times 1 / 0.0002 \times 100 = 2{,}000{,}000$

DNA extraction and SNPs investigation

Genomic DNA from the whole blood was extracted with Tiangen Blood DNA Kit following the manufacturer's instructions (Tiangen Biotech Co., China). The quantity and quality of extracted DNA were measured by NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Inc.). A DNA pool was constructed from 10 randomly selected cattle samples (50 ng/μl per sample). Two pairs of primer were designed to amplify 5' and 3' flanking region of *CD4* and *LAG-3* genes, respectively, using the software of Primer 3 web Program (v.0.4.0) and Oligo6.0.

CD4-F: 5'-CAAGAACAGGTGCCTAAGAG-3'

CD4-R: 5'-CCAGGTCATGAAGGTCCCAG-3'

LAG3-F: 5'-CTTTGCAACAACCCACATCT-3'

LAG3-R: 5'-GGGGTGTCTCTCAACCTTTC -3'

PCR reaction was performed in a final volume of 25 μL containing 1.0 μl genomic DNA, 12.5 μL Taq Master Mix solution (SinoBio, Shanghai, China), 10 μmoL/L 1.0 μl each primer, and 9.5 μL dd H2O. After initial denaturation at 95°C for 7 min, PCR was followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were evaluated by electrophoresis on 2.0% agarose gel with ethidium bromide. PCR products of pooled DNA were then sent to HuaDa gene sequencing company for SNP detection. After successful identification of SNPs by pool DNA, these SNPs were employed for screening in a population of 60 clinical mastitis samples by Snapshot technique.

Statistical analysis

Fixed effect model was used to analyze the association of SNPs and phenotypic traits using GLM procedure in SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA):

$$Y\_ijklm = \mu+\alpha\_i+\beta\_j+\gamma\_k+\delta\_l+\lambda\_m+e \qquad \text{(Model 1)}$$

Where, Y_ijklm represent the phenotype of SCC and SCS, μ is overall mean; α_i is fixed effect of genotype; β_j is fixed effects of herd, γ_k is fixed effects of parity; δ_l is fixed effect of season of calving; λ_m is fixed effect of year of calving and e is the random residual error.

In model 1, the estimated genotype effect was further divided into additive effect (A) and dominant effect (D). The additive effect was the mean deviation of two homozygous genotypes (Formula 1), and the dominant effect was calculated by the deviation of heterozygous genotype from the mean deviation of two homozygous genotypes (Formula 2) (He et al. 2011).

$$A=(AA-BB)/2 \quad \text{(Formula 1)}$$

$$D=AB-(AA+BB)/2 \quad \text{(Formula 2)}$$

Where, AA, AB and BB were least square means of genotype AA, AB and BB, respectively.

The combination effects of SNPs in *LAG-3* and *CD4* genes were analyzed as follows:

$$Y_{ijklm}=\mu+\eta_i+\beta_j+\gamma_k+\delta_l+\lambda_m+e \quad \text{(Model 2)}$$

Where, Y_{ijklm},μ,β_j,γ_k,δ_l,λ_m and e are the same as in (Model 1) and η_i represents combination genotype effect.

RESULTS

Routine somatic cell count (SCC) test was unable to perform for the clotted milk of clinical mastitis cows, therefore, SCCs of the milk samples were checked directly under microscope using Newman dye assay in the present study (Figure 1).

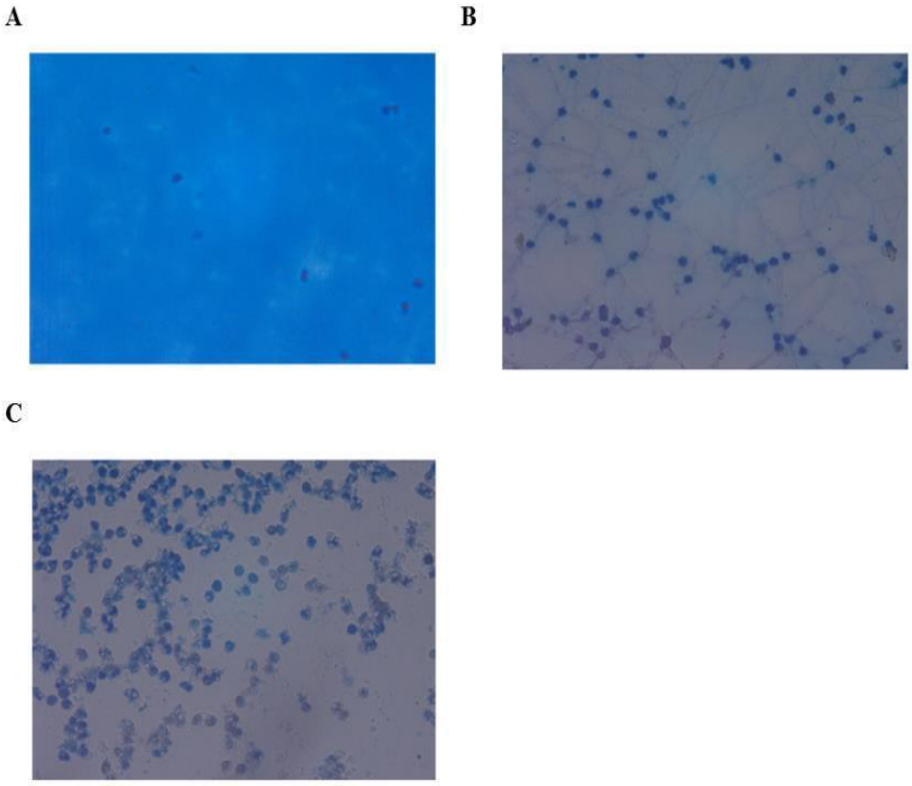

Figure 1. Images of direct microscopic examination of SCC of clinical mastitis samples. (A) Inflammatory condition, (B) Sever inflammation, and (C) Extremely sever inflammation.

Two novel SNPs were identified in 5' flanking region of *CD4* (SNP1 T104010752C) and 3' flanking region of *LAG-3* (SNP2 C104028410T) gene by screening pool DNA of randomly selected 10 head of cattle, respectively (Table 1 and Figure 2).

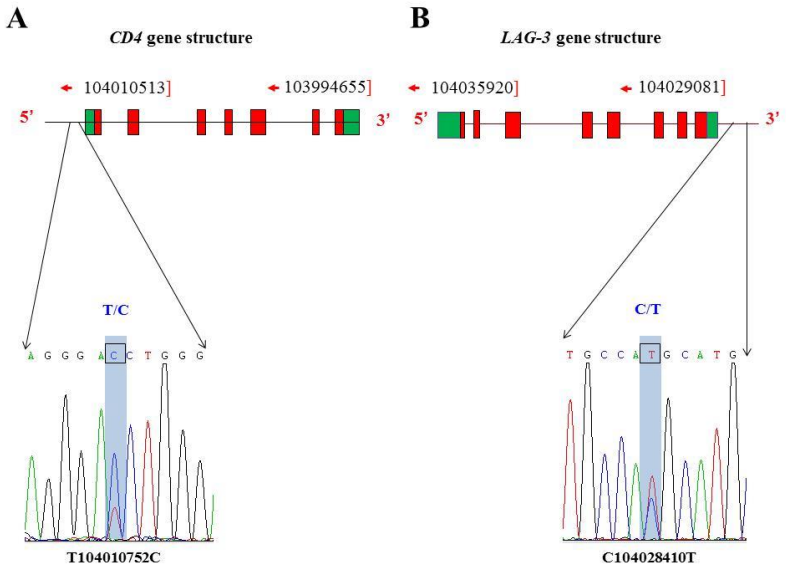

Figure 2. Sequencing and genotyping figures of the two SNPs in bovine *CD4* and LAG-3. (A) SNP T104010752C in 5' flanking region of *CD4*, (B) SNP C104028410T in 3' flanking region of *LAG-3* on BTA5.

Table 1. Information of the 2 SNPs found in *CD4* and *LAG-3*genes in the study.

SNP	Gene	Location	Position	Mutation	Reference
1	*CD4*	5' Flanking region	5chr 104010752	T>C	Novel
2	*LAG-3*	3' Flanking region	5chr 104028410	C>T	Novel

These two SNPs were then genotyped in 60 Holstein cows with clinical mastitis. Allele and genotype frequencies and Chi square test $\chi 2$ results are summarized in Table 2. Chi square test ($\chi 2$) showed that genotypic frequency of SNP2 was in Hardy-Weinberg equilibrium (P>0.05), whereas, SNP1 was not in HWE (P<0.05) in the population (Table 2).

Table 2. Genotypic and allelic frequencies and χ^2 test of SNPs in *CD4* and *LAG-3* gene.

SNP	Genotype frequency		Allele frequency*		*P*-value
SNP1/*CD4*	TT	CT	T	C	< 0.05
	0.25 (n§ = 15)	0.75 (n = 45)	0.63	0.37	
SNP2/*LAG-3*	CC	TC	C	T	> 0.05
	0.61 (n = 37)	0.38 (n = 23)	0.81	0.19	

*The wild-type alleles were in the left. § n = number of cow.

The effects of the two SNPs on phenotypes (SCC and SCS) are presented in Table 3. Statistical analysis revealed that although both the SNPs were not significantly associated with SCS, they were all significantly associated with SCC (P<0.05). Heterozygous genotypes CT in SNP1 (*CD4*) and wild type homozygous genotype CC in SNP2 (LAG-3) were associated with lower level of SCC. The results of combination genotypes

of the two genes revealed that combination genotypes of SNP1-SNP2 were significantly associated with SCC (P<0.05). The lowest SCC was showed by genotype CTCC (Table 4).

Table 3. Effect of SNPs in *CD4* and *LAG-3* on SCC and SCS traits in Holstein cattle.

Marker	Gene	Genotype	SCC	SCS
SNP1	*CD4*	TT	9783.62 ± 3476.02[a]	7.56 ± 0.79
		CT	3948.91 ± 3124.14[b]	7.26 ± 0.67
		P value	< 0.05	0.61
SNP2	*LAG-3*	CC	3514.52 ± 2816.03[b]	6.97 ± 0.69
		CT	7684.40 ± 3175.93[a]	7.71 ± 0.76
		P value	< 0.05	0.18

*The Bonferroni *t* test was used for pair comparison in the study. Mean within a column with no common superscript letters differ at *P*<0.05. (SCC = somatic cell count, SCS = somatic cell score).

Table 4. Combination genotypes effect of SNPs in *CD4* and *LAG-3* on SCC and SCS traits in Holstein cattle.

Markers	Genes	Genotypes	SCC	SCS
SNP1-SNP2	*CD4-LAG-3*	TTCC	6423.83 ± 6101.38[ab]	7.09±1.02
		TTCT	9946.26 ± 6212.37[a]	7.93±1.04
		CTCC	1043.69 ± 4337.23[b]	6.92±0.73
		CTCT	4303.39 ± 4801.43[ab]	7.62±0.81
		P value	< 0.05	0.58

*Means differ at *P*<0.05. (SCC = somatic cell count, SCS = somatic cell score).

DISCUSSION

Somatic cell count (SCC) in cow milk is widely used as a predictor of mastitis and indicator in dairy industry worldwide. However, clinical mastitis cows produce clotted milk, thus the much higher somatic cells in milk are unable to be counted by routine FOSS machine. In the present study, our direct microscopic check for high SCC samples of clinical mastitis cattle can transform a threshold trait (case or control) to a consistent trait (SCC) which can be used for genetic association study.

Detecting SCC related genes in bovine genome will play important roles in prevention of mastitis incidence and those genes will be used as molecular markers to select mastitis resistant cows. *CD4* and *LAG-3* are powerful candidate genes in many inflammatory diseases in different species (Wang et al. 2017, Okagawa et al. 2016). In the present study, association of two novel SNPs in 5' and 3' flanking region of bovine *CD4* and *LAG-3* genes, respectively, with mastitis indicator traits was evaluated. We found that both the SNPs in *CD4* and LAG-3, either individually or in combination, showed significant association with SCC (P<0.05) in clinical mastitis Holstein cows. These data indicate that both genes might be not only useful candidate genes for clinical mastitis but also the significant SNPs in these two genes could be important molecular markers in mastitis susceptibility prevention.

Genetic mutations responsible for phenotypic difference are the most effective choice of markers assisted selection in dairy cattle breeding programs (Dekkers & Hospital 2002). Different studies have reported that the important functional role of enhancer and flanking region in transcription initiation and regulation (Dekkers & Hospital 2002; Hussein et al. 2012; Lee et al. 2010; Lomvardas et al. 2006). Thus, genetic variation in the

flanking regions of a gene can significantly influence a phenotypic trait (Bulger & Groudin 2011; Zhang et al. 2005). Therefore, polymorphisms in flanking region or intron may have significant effects although they are not translated. In the present study, the significant association of SNPs in flanking region of *CD4* and *LAG-3* with mastitis traits is promising to consider these SNPs as important markers in mastitis susceptibility.

More noticeably, genome-wide SNPs are being used in genomic selection for mastitis resistance in dairy cattle, which can be used to predict a cow's genetic merit at birth before any phenotypic information is available (accuracy is 0.47) (Raadsma et al. 2008). To improve the accuracy of SCC selection, the significant related SNPs should be added in the genomic selection strategy. Both *CD4* and *LAG-3* genes are located adjacent on chromosome (BTA) 5 which is within quantitative trait locus (QTL) for mastitis susceptibility (Meredith et al. 2013). Bovine *CD4* plays a vital role in the immune response of pathogen-induced mastitis in dairy cows (Cao et al. 2012). Recently, our colleague He et al. (2011) reported a significant association of SNP in *CD4* gene with SCS. *LAG-3* plays a pivotal role in the suppression of EBV-specific cell-mediated immunity in Hodgkin's Lymphoma (Gandhi et al. 2006). *CD4* and *LAG-3* genes were reported to be significantly associated with the risk of multiple myeloma and multiple sclerosis (Lee et al. 2010; Zhang et al. 2005). In the present study, we found that the mutation type homozygous genotype was missing in both the SNPs which showed that wild type genotype was relatively stable. In addition, the combination genotype CTCC of the SNP1 and SNP2 showed lower SCC compared to the other genotypes, which suggest that the cows with CTCC relatively resistant to mastitis. Since CC in SNP2 is homozygous, SNP2 in *LAG-3* is strongly suggested to be considered in the bovine genomic selection to improve clinical mastitis resistance in Holsteins.

Although this is the first association study of clinical mastitis samples' SCC with *CD4* and *LAG-3* polymorphisms, the microscopic examination method for SCC detection has several shortcomings, i.e., the method needs preparation of the slides, dying the slide and then observing it under microscope in the laboratory. Hence, an onsite detection method for clinical mastitis SCC and the potential molecular markers explored in the current study should be improved and used in the future.

CONCLUSION

The results imply that direct microscopic check for high SCC samples of clinical mastitis cattle can transform a threshold trait (case or control) to a consistent trait (SCC) that can be used for genetic association study. *CD4* and *LAG-3* could be potential candidate genes and the SNP2 in *LAG-3* might be useful clinical mastitis markers integrated in Holstein cattle genomic selection.

ACKNOWLEDGMENTS

Authors are grateful to the manager and staff of the farm for providing data. The present work was financially supported by Beijing Dairy Industry Innovation Team (BAIC06), Modern Agro-industry Technology Research System (CARS-37), National Natural Science Foundation of China (31272420) and the Program for Changjiang Scholar and Innovation Research Team in University (IRT-15R62).

DECLARATION OF CONFLICTING INTEREST

The authors of the manuscript declare that they have no conflict of interest.

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