Genetics and Molecular Research

Association of *CD4* SNPs with fat percentage of Holstein cattle

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Genet. Mol. Res. 15 (3): gmr.15038697
Received April 6, 2016
Accepted July 25, 2016
Published September 16, 2016
DOI http://dx.doi.org/10.4238/gmr.15038697

ABSTRACT. Cluster of differentiation 4 gene (*CD4*) is well known for its role in immunity, but its effects on production traits remain to be elucidated. The present study was designed to explore single nucleotide polymorphisms (SNPs) in the exons, flanking introns, and promoter of *CD4*, as well as to analyze their effects on milk production traits (percentage of protein, fat, and lactose; mastitis indicator traits somatic cell count; and somatic cell score). A total of 10 SNPs, including eight in the exon and two in the intron regions, were identified using pooled DNA sequencing. These SNPs were screened in a population of 258 Chinese Holstein using the SNaPshot technique. We analyzed the effects

of SNPs, parity, herd, year, and season of calving on the production and mastitis indicator traits. Our analysis revealed two haplotypes and strong linkage disequilibrium (D' > 0.97) among all SNPs. All 10 SNPs were significantly associated with fat percentage ($P < 0.01$). Cows homozygous for the wild-type genotypes had higher fat percentages than those with the other genotypes. The dominant and additive effects were also significant for fat percentage ($P < 0.05$). These results suggest that *CD4* plays a role in production traits as well as in immune function. The identified SNPs could be used as genetic markers for selection of dairy cows with improved fat percentage. We propose further studies of these SNPs in a larger population as well as further investigations of the function of this gene.

Key words: *CD4*; Holstein; Association study; Production traits; Single nucleotide polymorphism; Milk fat

INTRODUCTION

Bovine milk is a rich source of high value components, e.g., protein, fat, and lactose. In dairy cattle, the cellular mechanisms of milk synthesis are well understood. However, the molecular mechanisms underlying differences in milk components require further study for a more comprehensive understanding (Raven et al., 2013). Milk fat percentage is highly heritable and ranges from 0.23 to 0.42 (Toghiani, 2012). The higher the heritability, the greater the genetic control of a trait, which means that selection can result in faster genetic progress. Recent advances in molecular biology for identification of specific regions on chromosomes affecting economically important traits have opened new insights into improvement of production traits (Alim et al., 2012). Marker-assisted selection is used for genetic improvement of breeding stocks using mapping and tagging of genes, genetic markers, and quantitative trait loci (QTL) (Kolbehdari et al., 2009). The use of functional polymorphisms directly related to phenotypic variation are the most efficient and best choice for marker-assisted selection in breeding programs (Dekkers and Hospital, 2002). A number of single nucleotide polymorphism (SNPs) exist across the genome of many species and are preferred in selection program over other molecular markers due to their comparatively lower mutation rate and easy and cost effective genotyping (Hinds et al., 2005).

Genes contributing in polygenic effects are known as QTL and are efficiently used worldwide in marker-assisted breeding programs. With the advancement of molecular techniques, identification of QTL is being effectively used in the development of pharmacological and medical approaches to genetically improve production and disease resistance traits (Heyen et al., 1999). Variation in QTL can contribute considerably to the desired phenotypes. SNPs in many genes, such as *SCD*, *FTO*, *EPS8*, and *GPAT4*, have been identified to be associated with milk fat percentage (Alim et al., 2012; Wang et al., 2013a; Zetouni et al., 2013; Zielke et al., 2013). The cluster of differentiation 4 gene (*CD4*) is known to play an essential role in inflammation-related conditions in many species. A point mutation in *CD4* (C868T) was found to be strongly correlated with increased susceptibility to HIV-1 infection in African populations (Oyugi et al., 2009; Hennig et al., 2011). An invasion of activated $CD4^+$ T cells in the udder of cattle is a typical characteristic of mastitis (He et al., 2011). *CD4* plays an important role in the immune response to mastitis in dairy animals (Cao et al., 2012).

He et al. (2011) studied the association of SNPs in *CD4* and *STAT5B* with mastitis and

production traits in dairy cattle. They observed significant associations of SNPs in *STAT5B* with production traits and in *CD4* with somatic cell score (SCS), in addition to a significant combined genotype effect of SNPs in *STAT5B* and *CD4* with production traits (He et al., 2011). Considering the important role of *CD4* primarily during inflammation as well as in production traits, we aimed to analyze the effects of SNPs in *CD4* on milk production traits (protein, fat, and lactose percentages) and the mastitis indicator traits somatic cell count (SCC) and SCS in Chinese Holstein cattle.

MATERIAL AND METHODS

Experimental animals and phenotypic measurements

All experimental procedures were carried out by professional veterinarians according to the Chinese animal care guidelines and were approved and supervised by the relevant authorities of the Ethics Committee of the China Agriculture University Beijing, China. Blood and milk samples from 258 randomly selected Chinese Holstein cows were collected from three dairy farms (one located in a suburb of Beijing and two located in a suburb of Harbin) in the north of China. The cows were in different lactation (1-4), ranging in age from 3 to 7 years, that had been fed the same lactation diet recommended for lactating Chinese Holstein cattle. Fresh milk samples were collected in 50-mL tubes placed in an ice box and instantly transferred to Beijing Dairy Cattle Centre for a Dairy Herd Improvement test, to determine the protein, fat, and lactose percentages and SCC values. Log transformed SCS was calculated from SCC using the formula [SCS - $\log_2$(SCC/100) + 3; the unit of SCC is 1000 cells/mL] (Rupp and Boichard, 1999). Blood was collected from the caudal vein in 9-mL tubes for DNA isolation. Genomic DNA was extracted from whole blood samples of the cows using a TIANamp Genomic DNA kit (TianGen, Beijing, China) following the manufacturer protocols.

Detection and genotyping of the polymorphisms

DNA was pooled (50 ng/μL per sample) from 30 randomly selected cattle samples. To identify SNPs, primers were designed to amplify the exons, flanking introns, and the 2-kbp promoter region of *CD4* using Primer 3 v.0.4.0. A polymerase chain reaction (PCR) of the pooled DNA was run in a final reaction volume of 25 μL containing 50 ng genomic DNA, 2.5 μL 10X PCR buffer, 0.5 μL each primer, 1 U Taq DNA polymerase, and 2.5 mM each dNTP (TaKaRa, Dalian, China). The PCR protocol was set to 10 min at 95°C for initial denaturing, followed by 34 cycles at 95°C for 30 s; 56°C for 30 s; 72°C for 30 s; and a final extension at 72°C for 10 min for all the primers. The PCR products from the pooled DNA were sequenced by Huada Gene-Sequencing Company. The identified SNPs were then genotyped in all 258 sampled individuals of Chinese Holstein cows using the SNaPshot assay.

Statistical analysis

The chi-square test was used to analyze the allele and genotype frequency of the identified SNPs. Association analysis of the SNPs with production traits and mastitis indicator traits (SCC and SCS) was performed using the general linear model procedure in SAS program (SAS v. 9.1.3; SAS Institute Inc., 2002) using the following model:

$$Y_{ijklmn} = \mu + G_i + H_j + P_k + S_l + a_m + e_{ijklmn} \quad \text{(Equation 1)}$$

in which, Y_{ijklm} represents the phenotypes percentage of fat, protein, and lactose, SCC and SCS; μ is the overall mean; the following five effects were included as fixed effects: G_i: genotype, H_j: herd, P_k: parity, S_i: season of calving, α_m: year of calving; and e is the random residual error.

In Equation 1, the estimated genotype effect was further divided into additive (A) (Equation 2) and dominant effects (D) (Equation 3) (Falconer and Mackay, 1996).

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

$$D = AB - (AA + BB)/2 \quad \text{(Equation 3)}$$

where, AA, AB, and BB were the least square means of genotypes AA, AB, and BB, respectively.

RESULTS

A total of 10 SNPs were identified in *CD4* in the pooled DNA sample, including eight SNPs in the exons and two SNPs in the introns (Table 1). The SNPs in intron 2 and exon 4 were novel, whereas the rest were already listed in the NCBI database. The seven SNPs in exon 2 were located in close vicinity, within approximately 100 bp of the exon region.

Table 1. Information of the 10 SNPs found in the *CD4* gene on chromosome 5 in the present study.

SNP	Location	Position	Mutation	Reference	Amino acid change
1	Exon 2	104010282	A>C	rs110955838	Lys by Gln
2	Exon 2	104010197	A>G	rs134689546	Lys by Arg
3	Exon 2	104010196	G>T	rs13674294	Lys by Asn
4	Exon 2	104010194	G>A	rs135920682	Arg by His
5	Exon 2	104010183	T>C	rs134722546	Phe by Lys
6	Exon 2	104010181	T>G	rs133342397	Phe by Leu
7	Exon 2	104010180	T>C	rs135440143	Tyr by His
8	Intron 2	104010171	A>G	Novel	
9	Exon 4	104001608	G>A	Novel	Asp by Asn
10	Intron 6	103996918	C>T	rs110421401	

The allele and genotype frequencies are summarized in Table 2. A chi-square test indicated that the genotype frequencies of all SNPs in the population were in Hardy-Weinberg equilibrium ($P > 0.05$) (Table 2). All SNPs were found to be in strong linkage disequilibrium (LD, Table 3 and Figure 1). The association analysis showed that all SNPs were highly significantly associated with fat percentage ($P < 0.01$) and non-significantly associated with SCC and SCS ($P > 0.05$) (Table 4). Cows with the homozygous wild type genotype showed 0.91% higher fat percentage than the heterozygous genotype. Furthermore, we found that the wild type genotypes were associated with higher protein and lactose percentage compared to the other genotypes, although these differences were not statistically significant. Moreover, both the dominant (-0.51%) and additive effects (0.40%) of the SNPs were found to be significant for fat percentage ($P < 0.05$) (Table

3). The results of the haplotype analysis revealed two haplotypes (AAGGGTTAGC and CGTATGCGAT) in all the SNPs at a frequency of 0.55 and 0.45.

Table 2. Genotype and allele frequencies and χ^2 test of the 10 SNPs in the *CD4* gene.

SNP	Genotype frequency			Allele frequency*		χ^2 test
SNP1	AA	AC	CC	A	C*	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP2	AA	AC	CC	A	C	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP3	AA	AG	GG	A	G	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP4	GG	GT	TT	G	T	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP5	GG	AG	AA	G	A	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP6	GG	GT	TT	G	T	P > 0.05
	0.17 (N = 45)	0.55 (N = 140)	0.28 (N = 73)	0.44	0.56	
SNP7	TT	GT	GG	T	G	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP8	TT	CT	CC	T	C	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP9	TT	CT	CC	T	C	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	
SNP10	CC	CT	TT	C	T	P > 0.05
	0.28 (N = 73)	0.55 (N = 140)	0.17 (N = 45)	0.56	0.44	

N = number of cows; *wild-type alleles are in the left column.

Table 3. Haplotype distribution of polymorphisms in the *CD4* gene.

Haplotype	SNPs										Frequency
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	
1	A	A	G	G	T	T	T	A	G	C	0.55
2	C	G	T	A	G	G	C	G	A	T	0.45

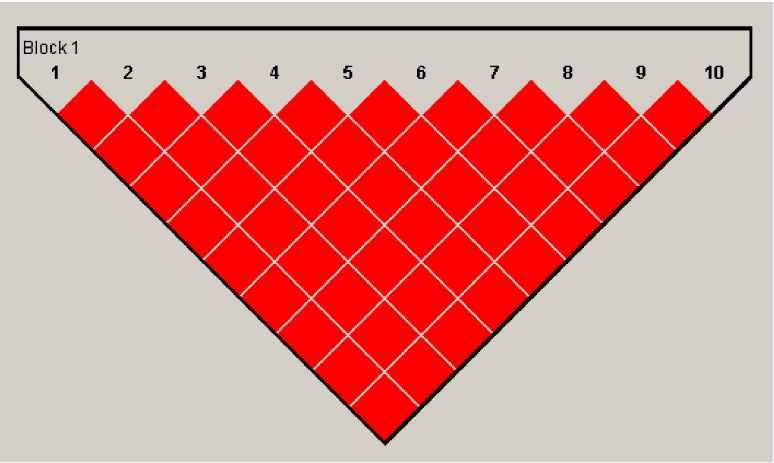

Figure 1. Pairwise linkage disequilibrium (LD) of SNPs in the bovine *CD4* gene. Red color indicates that all SNPs are in strong LD (D' > 0.97).

Table 4. Effect of SNPs in the *CD4* gene and their additive and dominant effects on fat, protein, and lactose percentages (means ± SE) in Holstein cattle.

Genotype	Fat	Protein	Lactose	SCC	SCS
Wild type genotype	3.60 ± 0.41^{A}	3.66 ± 0.27	4.53 ± 0.29	3782.61 ± 2942.73	6.75 ± 1.15
Heterozygous genotype	2.69 ± 0.36^{B}	3.56 ± 0.24	4.43 ± 0.26	4562.08 ± 3214.54	6.91 ± 1.08
Mutation type genotype	2.80 ± 0.45^{AB}	3.49 ± 0.30	4.36 ± 0.33	3637.46 ± 2721.76	6.65 ± 1.04
P value	<0.01	0.79	0.81	0.76	0.64
Additive	0.40 ± 0.19	0.08 ± 0.13	0.08 ± 0.14	-827.73 ± 1573.70	0.41 ± 0.33
P value	<0.05	0.51	0.53	0.75	0.69
Dominant	-0.51 ± 0.25	-0.01 ± 0.17	-0.02 ± 0.18	-1713.89 ± 2102.31	0.16 ± 0.45
P value	<0.05	0.92	0.92	0.81	0.73

The Bonferroni *t*-test was used for the pair-wise comparisons of fat, protein, and lactose percentages in the study. Different superscripted letters indicated a statistical difference at $P < 0.01$ within the column means; the values which are significant ($P < 0.05$) are highlighted in bold.

DISCUSSION

We analyzed the association of SNPs in *CD4* with milk production traits in a Chinese Holstein population. The present study was a continuation of a previous study by He et al. (2011), who reported a significant association of SNPs in exon 16 of *STAT5B* with production traits and in intron 6 of *CD4* with SCS, in addition to a significant additive effect on protein yield. The results of the present study revealed that all SNPs in exons 2 and 4 were missense mutations, and the seven SNPs in exon 2 were all closely associated with each other (located in a 100-bp gene region). All SNPs were in strong LD and highly significantly associated with fat percentage. In the present study, the results of the association analysis of *CD4* were in partial agreement with those of a previous study in which a significant association of SNPs (103996918C/T) with SCS was reported (He et al., 2011). Although we found that the SNPs were non-significantly associated with SCC and SCS, the trend was in line with the results of this previous study. The possible reasons for these differences could be due to the samples used in the previous study included a larger population of healthy lactating cows from a different environment. *CD4* encodes a co-receptor molecule found on $CD4^+$ T cells along with the T cell receptor. Any variation in the *CD4* receptor could potentially cause improper activation and proliferation of $CD4^+$ T cells (Zamani et al., 2010). A significant association of SNPs in *CD4* with HIV-2 infection has been reported (Hennig et al., 2011). Increased $CD4^+$ T lymphocytes have been reported by many studies at the initial stages of mastitis (Taylor et al., 1997; Soltys and Quinn, 1999; Rivas et al., 2007). Moreover, *CD4* and *STAT5B* have cascading effects during inflammation, although the genes are present on different chromosomes (Wilson et al., 2009). The role of *CD4* gene during inflammation is well documented but little is known about its possible association with production traits, which was the aim of the present study. The significant association of closely linked missense SNPs in *CD4* with fat percentage observed in the present study opens up new insights to consider this gene as a potential candidate for milk production trait improvement. We observed a significant association between the wild type genotypes of the 10 SNPs in LD, with fat percentage, as well as trends towards associations with increased protein and lactose percentages. This finding suggests that these SNPs could be of considerable importance in the selection for high milk production traits in dairy cattle.

Bovine milk fat percentage is known to be influenced by genetic variation in many

genes located on different chromosomes, e.g., chromosomes 1, 3, 4, 5, 6, 7, 8, and 18 (Boichard et al., 2003; Viitala et al., 2003; Ashwell et al., 2004; Schnabel et al., 2005; Hayes et al., 2010; Wang et al., 2013b; Wang et al., 2015). Milk fat percentage is a polygenic trait, controlled by many genes. A significant association of a combined effect of *CD4*/*STAT5B* on both mastitis and production traits has been documented (He et al., 2011), and the association of aberrant CD4 methylation with mastitis traits has also been documented (Wang et al., 2013b). Moreover, polymorphisms (rs41652648 and rs41591894) in ITPR2 and SNP rs41592942 in *GUCY2C* on chromosome 5 were found to be significantly associated with fat yield in dairy cattle (Kolbehdari et al., 2009). Several SNPs on chromosome 5 show large additive effects on fat yield (Sun et al., 2014). *CD4* is also located on bovine chromosome 5 and, therefore, the results of the present study suggest that polymorphisms in *CD4* may have effects on fat percentage in combination with other genes. The present study used 258 Chinese Holstein cattle from three dairy farms located in the north west of china. We propose to further elaborate the identified SNPs using a bigger cohort of Chinese Holstein, including other cattle breeds, as well as cattle from different regions of China and other parts of the world. Moreover, only SNPs in the exons, flanking introns, and the 2-kbp promoter region were screened in the present study. We suggest that future studies should explore the whole *CD4* for SNPs and their associations with fat percentage and mastitis indicator traits, to perform an in-depth functional analysis of the SNPs. In the present study, the significant association of the strongly linked 10 SNPs in *CD4* suggested that they can potentially increase fat percentage individually or in association with loci located on other chromosomes. Thus, the present study suggests that *CD4* could be a candidate gene for milk production traits and that the identified SNPs could be used as potential genetic markers to improve fat percentage in Chinese Holstein cattle.

Conflicts of intereset

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Earmarked Fund for Modern Agro-industry Technology Research System (#CARS-37), the National Natural Science Foundation of China (#31272420), the Fund for Basic Research from the Ministry of Education of the People's Republic of China (#2011JS006), the National Key Technologies R & D Program (#2011BAD28B02), and the Program for Changjiang Scholar and InnovationResearch Team in University (#IRT1191).

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