Association of *MAP4K4 gene* single nucleotide polymorphism with mastitis and milk traits in Chinese Holstein cattle

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The objective of the studies presented in this Research Communication was to investigate the association of single nucleotide polymorphisms present in the *MAP4K4* gene with different milk traits in dairy cows. Based on previous QTL fine mapping results on bovine chromosome 11, the *MAP4K4* gene was selected as a candidate gene to evaluate its effect on somatic cell count and milk traits in ChineseHolstein cows. Milk production traits including milk yield, fat percentage, and protein percentage of each cow were collected using 305 d lactation records. Association between *MAP4K4* genotype and different traits and Somatic Cell Score (SCS) was performed using General Linear Regression Model of R. Two SNPs at exon 18 (c.2061T > G and c.2196T > C) with genotype TT in both SNPs were found significantly higher for somatic SCS. We found the significant effect of exon 18 (c.2061T > G) on protein percentage, milk yield and SCS. We identified SNPs at different location of *MAP4K4* gene of the cattle and several of them were significantly associated with the somatic cell score and other different milk traits. Thus, *MAP4K4* gene could be a useful candidate gene for selection of dairy cattle against mastitis and the identified polymorphisms might potentially be strong genetic markers.

Keywords: MAP4K4, somatic cell scores, polymorphism, mastitis.

Mastitis is the most common disease in the bovine dairy industry. Many breeding programmes are affected while there is a lack of phenotypic data especially in mastitis selection where lowered somatic cell count (SCC) is taken as a surrogate of clinical mastitis (Rupp & Boichard, 1999). Single nucleotide polymorphisms (SNPs) within genes of the local mammary innate immune response are attracting particular importance as potential DNA markers for mastitis susceptibility (Chen et al. 2011). Mitogen activated protein kinase kinase kinase kinase (*MAP4K4*) is a member of the germinal centre kinase group related to *Saccharomyces cerevisiae* (Zohn et al. 2006; Delpire, 2009). Many experiments have been conducted to link the expression and functions of *MAP4K4* to various pathophysiological processes including metabolism, inflammation, neural degeneration and cancer. It has been suggested that inhibition of *MAP4K4* function may be beneficial in treating disease associated with these processes. *MAP4K4*

gene is responsible for the key processes in initiation of innate response (Dror et al. 2007). Since *MAP4K4* is an important gene that regulates inflammation, it would be of use to study the role of *MAP4K4* in mastitis resistance. *MAP4K4* is a good candidate gene since together with IL-17R and SOCS7 it plays a key role in the *NF-kB* signalling pathway directing transcriptional regulation of pro-inflammatory genes in macrophages (Karin & Greten, 2005; Dror et al. 2007). Identification of SNPs in bovine *MAP4K4* could yield useful information (Schulman et al. 2009) and we hypothesised that *MAP4K4* gene could have an important role in immunity and could be associated with mastitis resistance. To test this hypothesis, an association study investigating the relationship between bovine *MAP4K4* polymorphism and mastitis and different milk traits in dairy cows was performed.

Material and methods

All experiments were carried out according to the regulations for the Administration of Affairs Concerning

Experimental Animals published by the Ministry of Science and Technology, China in 2004 and approved by the Animal Care and Use Committee from the Dairy Cattle Research Center, Hubei Academy of Agricultural Sciences, Hubei, P. R. China.

Animal management and blood sampling

The dairy herd, located in Hubei province, China, belongs to the sub-humid tropical climates, where the annual average temperature is 16–17 °C and the annual rainfalls is 1100–1200 mm with 75% relative humidity. Cows were housed in free stall barns and fed with total mixed ration (TMR) thrice daily. 10 ml Blood samples from 500 Chinese Holstein cows with good DHI were collected in a tube with EDTA (pH = 8.0) anticoagulants and samples were kept in −20 °C until the genomic DNA was extracted. DNA was extracted according to the method described by Wang et al. (2011). After genomic DNA isolation, the quantity and purity of DNA were measured using NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Inc.)

Primer design, sequencing and genotyping

Information from Ensembl Genomic Browserser (http://www. ensembl.org), sequence of *MAP4K4* gene referring to Bos_taurus_UMD_3·1 assembly (ENSBTAG00000013023) exonic and intronic region of *MAP4K4* gene was annotated. We designed 34 pairs of primers (Supplementary File S1) to amplify 5′ upstream region, 3′ UTR, 3′ downstream region, exonic and adjacent intronic regions of bovine *MAP4K4* gene. All the primers were designed using the Primer Premier 5·0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesised by Sangon Biological Engineering Technology (Shanghai, China). Briefly, the total 25 μl volume of mixture contained 50 ng genomic DNA, 12·5 μl 2X reaction mix (including 500μM dNTP each; 20 mM Tris–HCl; pH 9; 100 mM KCl; 3 mM MgCl2), 0·5 μM of each primer, and 0·5 units of Taq DNA polymerase. The cycling protocol was as follows: 5 min at 95 °C, 35 cycles of denaturing at 94 °C for 30 s, annealing at T_m°C for 30 s, extending at 72 °C for 30 s, with a final extension at 72 °C for 10 min.

The PCR products with different electrophoretic patterns were directly sequenced in both directions using an ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) following the standard protocol. The DNAMAN software package (Version 6.0; Lynnon Biosoft, Quebec, Canada) was used to analyse the sequencing results and to determine the mutation position. Selection of enzymes for genotyping was done with software http:// watcut.uwaterloo.ca/template.php. The PCR products were digested with the corresponding restriction endonucleases (Supplementary File S3) at 37 °C for 8 h, according to manufacturer recommendations. The digested products were observed on a 2·5% agarose gel stained with ethidium bromide and final genotype of the animal was determined.

Statistical analysis

Exact test of Hardy–Weinberg Equilibrium was conducted before the association study using the R package 'genetics' SNPs were filtered for deviation from Hardy-Weinberg Equilibrium (P-value $\leq 0\cdot05$). The associations between the retained SNPs and the traits (fat percentage, protein percentage, SCS, 305 d milk yield) were analysed using the general linear model (GLM) procedure of R. The general linear model is as following:

$$y_{ijk} = \mu + F_i + P_j + SNP_k + e_{ijk}$$

Where y_{ijk} is the observed value, μ is the overall population mean, F_i is the effect of the ith farm, P_j is the effect of the jth parity, SNP_k is the effect of the kth single SNP. e_{ijk} is the random error. Tukey Honest Significant Differences (Tukey HSD) method was used for multiple comparison between different genotypes.

Results and Discussion

### Characterisation of boMAP4K4 SNPs and genotype allelic frequencies

A total of 19 SNPs were detected in the*MAP4K4* gene (ENSBTAG00000013023) of which 4 were located in 5′ upstream region, eight in exons and seven in introns. Seven SNPs were synonymous substitutions and one was non-synonymous substitution (c.1562C/T) that changed amino acid Ser into Phe (Supplementary File S2). Analysis of this 1200 bp sequence by UCSC/Ensembl showed the region in chr11: 6609650-11:6609699 where it was marked as a promoter. Similarly, we identified no miRNA binding sites at 3′UTR(ENSBTAG00000013023) *MAP4K4* gene. Analysis of these results with identified SNPs results revealed that none of the SNPs were found within these potential regions. Nine SNPs were selected for the genotyping, of which eight were within Hardy–Weinberg equilibrium (Supplementary Files S3 and S4). The 9th SNP (c.2115A/G) did not fit with Hardy–Weinberg equilibrium ($P < 0\cdot01$).

Analysis of boMAP4K4 SNPs with milk traits and SCS

We calculated the somatic cell score from somatic cell count to analyse the probable resistibility/ susceptibility of animals for mastitis. The analysis revealed SNP3 (C.2061T/ G) and SNP4 (C.2196T/C) to be significant for somatic cell scores (Figure 1). In both these SNPs the homozygote (TT) animals were found to have significantly higher ($P < 0\cdot01$) SCS than heterozygote (TG) and (TC) animals respectively (Table 1). Analysis of productivity traits within the genotyped animals revealed that the SNP6 (11:6646091G/A) and SNP8 (11:6646308A/G) at intron 21 were significantly associated with fat percentage. In SNP6, the AA genotype animals had significantly ($P < 0\cdot05$) higher fat percentage when compared with the heterozygote GA and homozygote

Table 1. Analysis of bo*MAP4K4* SNP genotype by SCC and milk productivity trail

SNP ID	SNP location	Gen.	Fat %	Protein %	SCS	305d Milk c Yield
SNP1	Exon 13	CC	3·63 ± 0·49	3·18 ± 0·25	8·343 ± 1·41	6931·98 ± 44·50
		CT	3·66 ± 0·46	3·18 ± 0·27	8·471 ± 1·64	6729·33 ± 85·32
		TT	3·69 ± 0·54	3·19 ± 0·27	8·504 ± 1·34	6742·27 ± 67·12
SNP2	Exon 14	CC	3·71 ± 0·51	3·17 ± 0·28	8·746 ± 1·37	6372·32 ± 67·89
		TC	3·68 ± 0·44	3·16 ± 0·24	8·472 ± 1·44	6945·33 ± 55·45
		TT	3·61 ± 0·48	3·18 ± 0·26	8·272 ± 1·41	6985·44 ± 81·28
SNP3	Exon 18	GG	3·68 ± 0·52	3·14 ± 0·25**	7·814 ± 1·57**	7114·26 ± 78·41*
		TG	3·63 ± 0·43	3·18 ± 0·24**	8·473 ± 1·36**	6922·35 ± 74·65
		TT	3·66 ± 0·49	3·22 ± 0·26**	8·989 ± 1·24**	6265·50 ± 64·30*
SNP4	Exon 18	CC	3·68 ± 0·53	3·21 ± 0·27	8·157 ± 1·58*	6919·66 ± 27·79
		TC	3·64 ± 0·44	3·16 ± 0·24	8·476 ± 1·32	6943·81 ± 57·67
		TT	3·62 ± 0·48	3·21 ± 0·27	8·657 ± 1·47*	6572·83 ± 81·09
SNP5	Intron 18	AA	3·61 ± 0·51	3·16 ± 0·27	8·283 ± 1·46	7137·54 ± 80·83
		GA	3·67 ± 0·46	3·18 ± 0·38	8·454 ± 1·51	6618·79 ± 22·32
		GG	3·67 ± 0·59	3·17 ± 0·23	8·601 ± 1·35	7063·53 ± 75·62
SNP6	Intron 21	AA	3·75 ± 0·50*	3·18 ± 0·25	8·401 ± 1·59	6594·59 ± 82·71
		GA	3·63 ± 0·43*	3·19 ± 0·37	8·325 ± 1·39	6976·36 ± 76·05
		GG	3·64 ± 0·48*	3·18 ± 0·49	8·634 ± 1·39	6841·72 ± 78·61
SNP7	Intron 21	CC	3·65 ± 0·39	3·14 ± 0·25	8·502 ± 1·33	6924·18 ± 82·27
		TC	3·71 ± 0·49	3·21 ± 0·27	8·495 ± 1·40	6790·99 ± 58·59
		TT	3·64 ± 0·51	3·14 ± 0·46	8·493 ± 1·51	6994·82 ± 77·45
SNP8	Intron 21	AA	3·64 ± 0·57**	3·14 ± 0·27	8·401 ± 1·51	7171·21 ± 82·5**
		AG	3·67 ± 0·40	3·17 ± 0·25	8·474 ± 1·44	7002·69 ± 71·73
		GG	3·81 ± 0·48**	3·21 ± 0·31	8·691 ± 1·24	6113·12 ± 80·41**

*$P < 0.05$
**$P < 0.01$
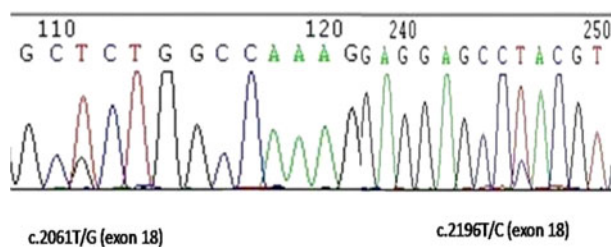
Fig. 1. Sequencing for the identified SNPs at exon 18 (c.2061T/G and c.2196T/C) of *MAP4K4* gene on cow chromosome 11.

GG had animals. A similar pattern was observed in SNP8, where the homozygote GG had significantly higher ($P < 0.01$) fat percentage compared with the homozygote CC (Table 1).

The *MAP4K4* gene plays a crucial role in the innate and acquired immune systems and a number of studies in humans showed that its genetic variation may alter the body's susceptibility to infectious diseases (Bouzakri et al. 2009). There have not been any reports concerning the characterisation of *MAP4K4* gene in cow genome and the identification of SNPs associated with milk traits. Results from human and other species shows that *MAP4K4* is a strong candidate gene for resistance to bacterial diseases in mastitis (Karin & Greten, 2005; Dror et al. 2007). It is important to screen the polymorphisms in*MAP4K4* gene

and elucidate its effect on immune cell functions which would help to facilitate breeding aimed at disease-resistance by marker-assisted selection method in mastitis. Analysis of bovine *MAP4K4* SNPs may contribute to the discovery of mastitis resistance related genetics traits because bacteria are the most common cause of mastitis in cattle. The SCS has been used as the criterion for improving mastitis resistance (Shook & Schutz, 1994). Statistical analysis revealed that cows with c.2061/G -GG and c.2061/G-TG genotypes had significantly lower SCSs than the TT subjects, whereas the cows with c.2196C/T-CC had significantly lower SCSs than the TT subjects, which indicates that genotypes GG, TG and CC might be associated with mastitis resistance. Allele T of SNPs c.2061/G and T of SNP c.2196C/T may predispose cows to clinical mastitis via increased neutrophil trafficking to mammary gland, because clinical mastitis is correlated with SCS, as reported by Rupp & Boichard (1999). Thus, cattle with genotypes GG, TA and CC could be used for breeding strategy.

In conclusion, our findings confirm that MAP4K4 c.2061/G and c.2196C/T could be regarded as novel candidate genetic molecular markers for mastitis resistance/susceptibility in Chinese Holstein. However, this does not imply that genetic selection for mastitis resistance should be based on only Bo-*MAP4K4* alleles. The possible associations of c.2061/G and c.2196C/T with mastitis resistance and the occurrence of preferable haplotypes within the *MAP4K4* gene are interesting topics for future studies.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029916000832

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