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Full Length Research Paper

The Novel single nucleotide polymorphisms (SNPs) of the bovine STAT4 gene and their relationship with production attributes in Chinese Holstein Cattles

Mi Chan[1]*, Tsai Lin[2] and Andy Wong[3]

Department of Animal Science, Faculty of Agricultural Sciences, Peking University, Beijing, China.

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Signal transducer and activator of transcription 4 (STAT4) belongs to the STATs family which may play important roles in the activation of milk protein genes and the development of mammary glands. Four novel single nucleotide polymorphisms (SNPs) (g.2624A>C ss175327225, g.60330A>G ss175327226, g.63823G>C ss175327227, g.66912C>T ss175327228) of the *STAT4* gene were investigated in 966 cattle of three breeds (Chinese holstein, Luxi yellow and Bohai black cattle) in China by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), PCR-restriction fragment length polymorphism (RFLP) and DNA sequencing methods. The SNP (g.60330A>G ss175327226) could be genotyped into three genotypes (AA, AG and GG) with PCR-RFLP using *Msp* I and another SNP (g.66912C>T ss175327228) only into two genotypes (CC and TC) by PCR-SSCP. The allelic frequencies of the SNP (g.60330A>G) were different among the three different cattle breeds (P < 0.05). The associations between polymorphisms of the *STAT4* gene and dairy performance traits were analyzed in 793 Chinese holstein cows. The SNP (g.60330A>G) markedly affected the 305 d matured equivalency (P < 0.05) and fat content (P < 0.05). However, the SNP (g.66912C>T) had no significant relationship with the milk production traits (P > 0.05). Together, the SNP (g.60330A>G) of the *STAT4* gene may serve as a molecular marker for the differentiation of various cattle populations and selection of the milk yield and fat content in bovine breeding program.

Key words: Dairy cattle, *STAT4* gene, SNP, milk production traits.

INTRODUCTION

In dairy cattle, milk yield and milk composition traits, which are under the control of multiple genes, are economically important traits. It is of great significance to select cows with higher milk yield and better milk composition for breeders and consumers. Some progress in breed improvement has been made for selection of milk production traits, but they are expensive and time consuming. Contrary to this, marker assisted selection (MAS) can improve the accuracy of selection and therefore genetic progress can be obtained faster and at a lower cost. It is thus very useful to study the genetic variations of candidate genes and their association with milk production (Kuss et al., 2003; Khatib et al., 2007).

Bovine signal transducer and activator of transcription 4 (*STAT4*) may be one of the candidate genes, that is implied by two clues. First, one recent report showed that *STATs* were activated sequentially during a mammary developmental cycle in the following order, *STAT1-STAT4-STAT6-STAT5-STAT3-STAT1* (Watson *and* Neoh 2008), in addition to the main bovine *STATs* family

*Corresponding author. E-mail: Chanmi@gmail.com

Abbreviations: STAT4, Signal transducer and activator of transcription 4; **PCR-SSCP,** polymerase chain reaction-single-strand conformation polymorphism; **RFLP,** restriction fragment length polymorphism; **SNPs,** single nucleotide polymorphisms; **MAS,** marker assisted selection; ***PRLR*,** prolactin receptor*;* **PIC,** polymorphism information contents; **He,** heterozygosites; **Ea,** effective number of allele; **HWE,** Hardy-Weinberg equilibrium.

Table 1. Primers used for amplification and sequencing of the bovine *STAT4* gene.

Primers	Loci	Primer sequence 5'-3'	Annealing temperature (°C)	Product sizes (bp)
P 1	g.62624A>C (Intron1)	TGTTGTTTGCCTTCAAGTTGC AATGGATGAAACCAGAAAGGAC	54.6	746
P 2	g.60330A>G (Intron4)	CCCAAGACCTTGATTCCAAT ATGCCTTGCAACCTATAAACC	53.0	477
P 3	g.63823G>C (Intron5)	TCTTCCAGAGCCATTCAGTTTT AAGAAGAAACCCTAGTCATATCCCT	52.7	327
P 4	g.66912T>C (Intron7)	TCTTAGGATGGCAGGTTAC TGGTAGCTATAGTACAGGCAC	54.6	426

members *STAT1*, *STAT3* and *STAT5* were involved in prolactin receptor (*PRLR*) signaling by JAK/STAT pathway that finally activate the expression of milk protein genes (Bole-Feysot et al., 1998). Second, several studies on *STATs* revealed a correlation with milk production and it can be inferred naturally that *STAT4* probably has the same effect as its "partners". Mutations in the *STAT5A* gene were associated with embryonic survival and milk performance traits in cattle (Brym et al., 2004; Khatib et al., 2008). Polymorphisms in *STAT6* were associated with carcass and growth efficiency traits in feedlot cattle (Rincon et al., 2009). Moreover, in humans, *STAT4* genetic variations have been found to be associated with rheumatoid arthritis, systemic lupus erythematosus and primary Sjögren's syndrome (Li et al., 2009; Ji et al., 2010; Nordmark et al., 2009).

In mammals, seven members were found in the *STAT* gene family: *STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6* (Darnell, 1997). Bovine *STAT4*, containing eight exons and seven introns, is located on BTA2q34. To date, no polymorphism of bovine *STAT4* gene has been reported. Therefore, in the present study, we intend to investigate the polymorphisms of the *STAT4* gene in three Chinese indigenous cattle breeds, and assess their associations with production traits in Chinese holstein cows, which might be a genetic marker in production traits for cow breeding and genetics.

MATERIALS AND METHODS

Animal source

A total of 966 cattle of three breeds (Chinese holstein, n = 793; Luxi yellow, n = 136; Bohai black, n = 37) were used. The Luxi yellow and Bohai black (ages ranging from 1 to 3.5 years) were selected from their original conservation areas in Shandong Province. Chinese holstein individuals (ages ranging from 4 to 7 years, including first to fourth parity) from 23 sires were from seven farms in China and milk samples were taken from each cow once a month, in the course of routine control milking, during the whole lactation. Data of milk performance traits (305 d matured equivalency, fat percentage, protein percentage, fat content and protein content) were collected from the laboratory of dairy herd improvement (DHI) center, OX Biotechnology, Shandong, China,using the milk composition analyser (Foss Milk Scan FT 6000, Denmark) for statistical analysis. The mean and standard error of 305 d matured equivalency, fat percentage (%) and protein percentage (%) were 5642.44±221.89 kg, 3.37±0.08 and 3.07±0.08, respectively, in 793 Chinese holstein breed.

DNA samples

Genomic DNA of 966 animals was isolated from 3.8% sodium citrate-treated blood samples by phenol-chloroform method, respectively. The content of DNA was estimated spectrophotometrically and diluted to 50 ng/µl.

Polymerase chain reaction (PCR) amplification

PCR primers (Table 1) were designed using Primer 5.0 software to amplify and sequence the exons and the flanking regions of the bovine *STAT4* gene (GenBank accession. NC_007300). The 25 µl of PCR volume was consisted of 50 ng genomic DNA, 2.5 µl 10 × PCR Buffer, 1.8 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.28 µmol/L of each primer and 0.5 U of *Taq* DNA polymerase (TaKaRa, Dalian, China). After an initial denaturation at 94°C for 5 min, the PCR amplification was performed by 35 cycles of denaturing at 94°C for 30 s, annealing at Tm°C for 30 s (Table 1), and primer extension at 72°C for 30 s. The final extension was undertaken at 72°C for 8 min. PCR product was electrophoresed on 1% agarose gel.

PCR- restriction fragment length polymorphism (RFLP)

The transition (A > G) at position g.60330 of the *STAT4* gene, creating a new *Msp* I restriction site, can be genotyped by PCR-RFLP method. 3 µl of PCR products amplified by P 2 (Table 1) of the *STAT4* gene were digested with 5 U *Msp* I (TaKaRa, Dalian, China) at 37°C for 8 h following instructions of the manufacturer. The digested products were detected by electrophoresis in 10% polyacrylamide gels (29 acrylamide: 1 bisacrylamide) and stained with 0.1% silver nitrate.

PCR- single-strand conformation polymorphism (SSCP)

The PCR-SSCP method was used to scan mutations within the amplified regions. 4 µl of PCR products were mixed with 6 µl denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole, and 0.025% bromophenol blue), heated in boiling water for 10 min, and immediately chilled on ice. The denatured PCR products were separated for 16 h at 4 voltage/cm on 10% poly acrylamide gel (29

acrylamide: 1 bisacrylamide) in 1×TBE buffer at a constant temperature of 4°C. The gel was stained with 0.1% silver nitrate.

Statistical analysis

Genetic indices, namely, polymorphism information contents (PIC), heterozygosites (He) and effective number of allele (Ea) were calculated. Hardy-Weinberg equilibrium (HWE) was performed by POPGENE32 (ver.1.31). In order to determine the associations between SNPs of the *STAT4* gene and milk production traits, the least square means estimates (LSM) with standard errors as applied in the general linear model (GLM) procedure of the statistical analysis system (SAS) (SAS Institute Inc., Cary, NC, USA, 2002) was used. Fixed effects of farm, genotype, season of birth and parity were included as independent variables. The applied linear model was

$$Y_{ihjklm} = \mu + G_i + S_h + SE_j + H_k + F_l + e_{ihjklm}$$

Where, Y_{ijklm} was the milk yield or the observed number of milk on each of the ijklmth animal; μ was the overall mean; G_i was the fixed effect associated with genotype; S_h was the fixed effect of sire; SE_j was the fixed effect of season; H_k was the fixed effect of parity; F_l was the fixed effect of farm; e_{ijklm} was the random residual effect. A value of $P < 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Genotypic frequencies in three cattle breeds

In the present study, four novel SNPs of g.2624A>C (intron 1), g.60330A>G (intron 4), g.63823G>C (intron 5) and g.66912T>C (intron 7) were revealed from 80 samples (20 from Luxi yellow, 20 from Bohai black, and 40 Chinese holstein were selected randomly and averagely from different farms) by DNA direct sequencing and comparison with the reference sequence (GenBank accession. NC_007300). The SNPs were submitted to the National Centre for Biotechnology Information (submitted SNP numbers: g.2624A>C ss175327225, g.60330A>G ss175327226, g.63823G>C ss175327227, g.66912C>T ss175327228). Two SNPs (g.2624A>C and g.63823G>C) were not done in further genotyping after sequencing in the present studied populations, while the other two SNPs were found in the three cattle breeds.

The SNP (g.60330A>G) can be detected by *Msp* I endonuclease (CCGG). Therefore, digestion of the PCR fragment with *Msp* I resulted in fragment lengths of 477 bp (allele A) and/or 347+130 bp (allele G). Correspondingly, AA, AG, and GG genotypes have one band (477 bp), three bands (477, 347 and 130 bp), and two bands (347 and 130 bp), respectively (Figure 1A). The frequencies of the genotypes and alleles are shown in Table 2. In Chinese holstein cow, the genotypic frequency of AG was the highest and AA was the lowest, while genotype AA was dominant in Luxi yellow and Bohai black cattle. These findings imply the results of different breeding objectives in dairy cattle (Chinese holstein) and beef cattle (Luxi yellow and Bohai black) in a long-term artificial selection.

Two unique SSCP banding patterns (CC/TC) were detected in the locus (g.66912C>T) by PCR-SSCP (Figure 1B) and sequencing analysis (Figure 2). Of the 66912C>T mutation, it was only found in Chinese holstein (CC and TC, TC genotype frequency was higher), but not in Luxi yellow and Bohai black (only TC). The frequency of genotype CC was 0.265 in Chinese holstein, accordingly, the frequency of allelic C was 0.632 (Table 2).

This phenomenon could be due to the different breeding objective and artificial selection pressure between combination of two of Luxi yellow cattle, Bohai black cattle and Chinese holsteins. The Luxi yellow and Bohai black cattle are two of the most representative indigenous bovine breeds in China, which have been designated nationally as protected resources due to their good performance traits and fleshy characteristics. They have been bred as beef and draft dual-purpose cattle and selected for beef production for thousands of years, which might decrease the frequency of the variations, while Chinese holsteins belongs to dairy cattle, which was cultured through grading cross between native cow and pure-bred bull of holstein introduced to China (Qiu, 2002). The difference of genotypic frequencies between Luxi yellow and Bohai black cattle and Chinese holstein may result from the long-term breeding for different purpose and selection history (Zhang et al., 2010). Our results suggested that SNP may help to identify cattle breed.

Population genetic parameters of different polymorphisms of the STAT4 gene in three cattle breeds

PIC, He and Ea and the status of equilibrium assessed in the three populations are presented in Table 3. Genetic index showing the genetic polymorphisms of both loci were moderately polymorphic loci in Chinese holstein, but g.60330A>G loci was a low polymorphic loci in Luxi yellow and Bohai black according to the criterion of PIC (Vaiman et al., 1994). In both loci, the populations were not all in HWE ($P < 0.05$) except for Bohai black cattle at the g.60330A>G locus ($P > 0.05$). As there may be a continuous migration or the long-term artificial selection of animals in the population studied.

Associations between the SNPs in the STAT4 gene and milk production traits in Chinese holstein cattle

Associations between the SNPs (g.60330A>G and g.66912C>T) in 793 Chinese holstein cattle and milk production traits were analyzed (Table 4). The results showed that factors like season, parity and farm had no effect on milk production traits of the both polymorphisms. A g.60330A>G mutation had significant relationship between genotypes (AG and GG) and 305 d matured equivalency

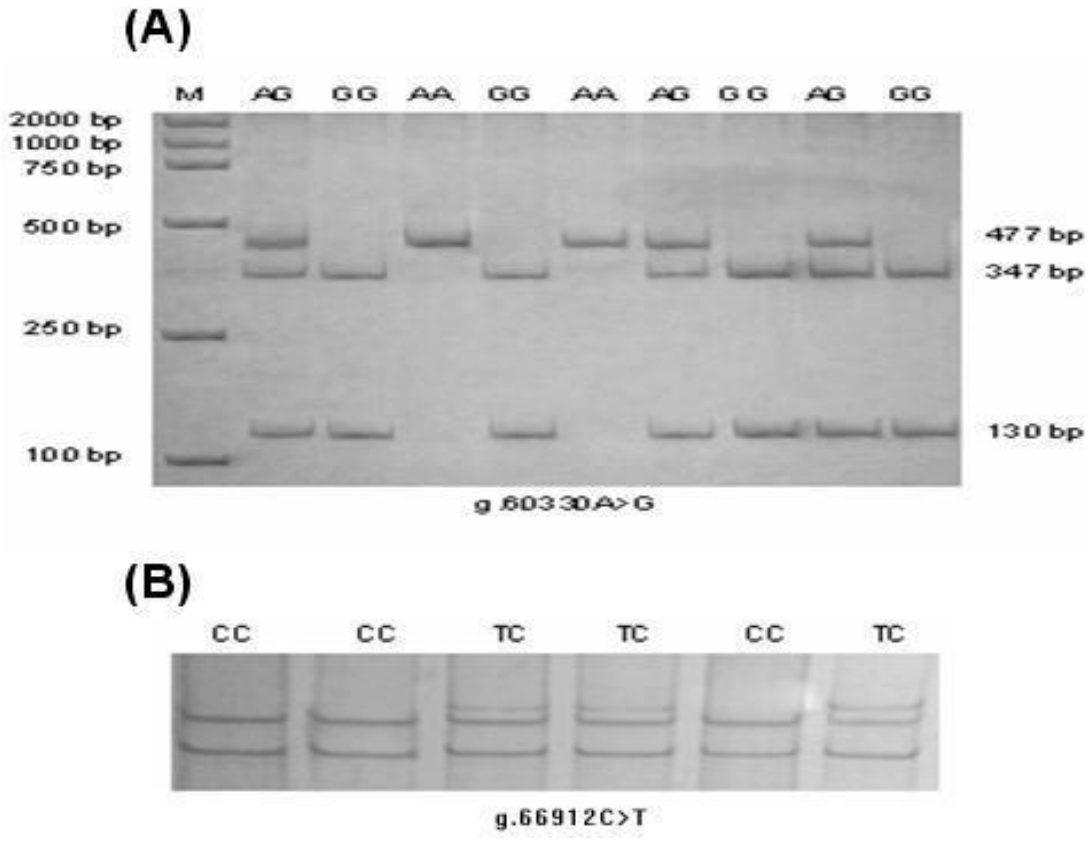

Figure 1. (A) 10% polyacrylamide gel electrophoresis of PCR fragment of the SNP (g.60330A>G) of the *STAT4* gene digested with *Msp* I; M, DL2000 (2000, 1000, 750, 500, 250, 100 bp) (B) PCR-SSCP patterns of the SNP (g.66912C>T) of the bovine *STAT4* gene.

Table 2. Genotypic and allelic frequencies of the bovine *STAT4* gene.

Breeds/samples	Genotypic frequencies/samples			Allelic frequencies	
g.60330A>G	AA	AG	GG	A	G
CH/793	0.150/119	0.565/448	0.285/226	0.432	0.568
LY/136	0.831/113	0.096/13	0.073/10	0.879	0.121
BB/37	0.730/27	0.243/9	0.027/1	0.851	0.149
g.66912C>T	CC	CT	TT	C	T
CH/793	0.265/210	0.735/583	0	0.632	0.368
LY/136	0	1/136	0	0.500	0.500
BB/37	0	1/37	0	0.500	0.500

CH: Chinese holstein, LY: Luxi yellow, BB: Bohai black.

($P < 0.05$), AG cow (5770.55± 271.05) producing more milk than GG (5400.95±80.97), and fat content of AA genotype cattle (3.47±0.10) was higher than that of AG (3.34±0.08) ($P < 0.05$), but no associations were found with protein content, fat yield and protein yield. However, there were no significant relationships between the g.66912C>T and milk production traits ($P > 0.05$).

The members of STAT family play various roles in mammary gland development (Watson and Neoh 2008). In the adult gland, *STAT1* and *STAT3* were constitutively expressed and *STAT3* had an essential function in the regulation of cell death and tissue remodeling in the post-lactational regression period (Selbert et al., 1998; Chapman et al., 1999; Schere-Levy et al., 2003; Nguyen and Pollard, 2000). *STAT4* was expressed in a reciprocal manner to STAT5. About the 10th day of gestation when substantial numbers of alveolar epithelial cells begin to appear, *STAT5A* was induced, while at this moment,

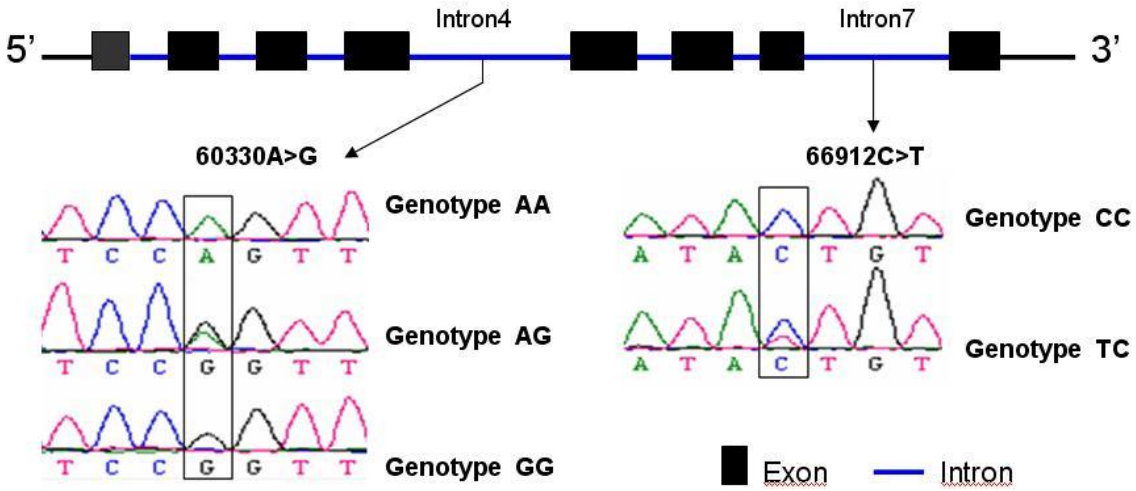

Figure 2. The structure sketch and sequencing maps of the SNPs (g.60330A>G and g.66912C>T) of the bovine *STAT4* gene.

Table 3. Population genetic parameters in two loci (g.60330A>G and g.66912C>T) of the bovine *STAT4* gene.

Loci	Breed	PIC	He	Ea	HWE (P value)
g.60330A>G	Chinese holstein	0.370	0.491	1.964	< 0.05
	Luxi yellow	0.190	0.213	1.271	< 0.05
	Bohai black	0.221	0.253	1.339	> 0.05
g.66912C>T	Chinese holstein	0.357	0.465	1.869	< 0.05

*PIC: polymorphism information contents; He: heterozygosities; Ea: effective number of allele; HWE: Hardy-Weinberg equilibrium.

STAT4 was becoming undetectable (Philp et al., 1996). *STAT4* may need to be suppressed to permit *STAT6* activity which was demanded for *STAT5* activity (Khaled et al., 2007; Watson and Neoh, 2008). Therefore, the variations of these genes may further affect the function of the mammary gland. According to Iavnilovitch et al. (2002), *STAT5* served as a multifunctional regulator of mammary cell proliferation, milk protein gene expression, and post-lactational apoptosis in mice. Khatib et al. (2008) found that allele G of SNP12195 (exon 8) in *STAT5A* was associated with a decrease in both protein and fat percentages. Schennink et al. (2009) reported that *STAT5A* (g.9501G>A) polymorphism had shown significant effects on milk-fat composition. Selvaggi et al. (2009) reported a genetic polymorphism of *STAT5A* protein, a substitution C>T at position 6853 within exon 7 in Italian Brown cattle, CC cows produced more milk than CT and protein content was higher from CC compared with CT genotypes.

In the present study, *STAT4* gene was for the first time selected in one livestock species for association with quantitative traits based on candidate gene approach. We have found four novel SNPs of the *STAT4* gene in three Chinese cattle with PCR-SSCP and/or sequencing. Though no significant relationship had been found between g.66912C>T polymorphism and milk performance traits in Chinese holstein, this novel SNP had extended the spectrum of genetic variation of dairy cattle *STAT4* gene, which may offer a better comprehension of genetic variation in animal resources.

The dominant genotype of g.60330A>G polymorphism was found in Chinese holstein contrary to Luxi yellow and Bohai black. The g.60330A>G mutation had strong effect on 305 d matured equivalency with the dominant genotype AG producing the most milk. These findings imply the results of different breeding objectives in dairy cattle (Chinese holstein) and beef cattle (Luxi yellow and Bohai black) in artificial selection for a long time. As little information about *STAT4* gene is known in cattle, it becomes immediately essential to further research on *STAT4* gene in the livestock.

In our current study, two novel SNPs were detected and located intron 4 and 7, respectively. Although intron was not the sequence for coding protein, the evidences were provided that intron played an important regulating role in gene expression, regulation (Nott et al., 2003), transcription and mRNA splicing (Zan et al., 2007). Although, whether the detected mutations affected gene

expression remain to be explored. *STAT4* gene possibly contributed to conducting association analysis and can be recognized as genetic marker in milk production traits and other performance for animal breeding and genetics.

In conclusion, we suggested g.60330A>G mutation of *STAT4* gene as a good DNA marker and *STAT4* as a candidate gene that could be used in breeding programs to select dairy cattle. Furthermore, milk yield and quality (fat or protein percentage) are all important economic traits, but are generally opposite in milk. In view of previous studies, the mutations of *STAT5A* had main effect on the fat and/or protein contents. Therefore, *STAT4* and *STAT5A* could be selected together to increase milk yield and quality in order to achieve a win-win situation in cow breeding.

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