The Association Between the *STAT1* g.3141C>T Polymorphism and Reproductive Performance in High-yielding Holstein-Friesian Dairy Cows

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SUMMARY

In dairy cattle, selection programs have mainly focused on high milk production which led to significant improvements in yield. However, it has also caused serious problems in bovine fertility. Reproductive performance is increasing in popularity worldwide. Therefore, this study aimed to evaluate the effects of g.3141C>T polymorphism of bovine *STAT1* gene on reproductive traits in high-yielding Holstein-Friesian cows. The data of 4800 cows were used and the initial experimental population consisted of 500 purebred cows housed in three free-stall barns. All animals were fed the same diets and had the same management procedures. The phenotypic traits analyzed in this study were total milk yield, 305-day milk yield, days open, the number of inseminations, and culling rates based on repeat-breeding. Body condition scores, lactation season, and lactation rank were also evaluated in statistical models. Initially, all of the cows were ranked by a selection index based on individual milk yield records and health traits. Next, a total of 75 cows were selected and genotyped for the *STAT1* marker located in 3'UTR by the PCR-RFLP method. Genotype-phenotype association analysis was carried out by the least-squares method as applied in a general linear model (GLM) procedure with Tukey's test as a post-hoc comparison. The association between the cull rates and the genotypes was evaluated by Pearson's chi-square test. Population genetics parameters including heterozygosity (He), homozygosity (Ho), number of effective alleles (Ne), and the polymorphic information content (PIC) were evaluated and the deviation from Hardy-Weinberg Equilibrium (HWE) was tested. Results revealed that g.3141C>T polymorphism exhibited admissible levels of population parameters (He=0.4801; Ne=1.9231) indicating that this marker is moderately informative for the selected population (PIC=0.3648). There was a deviation from HWE ($P<0.001$). In GLM, the association between the *STAT1* marker and the number of inseminations was found to be statistically significant ($P<0.05$). The TT animals were characterized by the highest number of inseminations (3.71±0.73). On the other hand, heterozygous animals were shown to be associated with desirable reproduction performance. This is a critical result because the *STAT1* g.3141C>T marker is included in many SNP-panels or SNP-chips for its previously reported effects on milk yield. To the best of our knowledge, this study has shown a novel effect of this *STAT1* marker on the number of inseminations per conception. Considering the TT genotype has a frequency of 26.67%, ignoring this association can lead to a significant reproduction performance decrease on a herd basis. Moreover, there was a significant association between the *STAT1* and cull rates ($P<0.01$). There was no association between the *STAT1* and any other traits analyzed. This study demonstrates novel effects of the *STAT1* gene, and hence, may contribute to the adequate genotypic evaluation of dairy cattle reproduction performance.

KEY WORDS

Fertility, high-yielding dairy cow, Holstein-Friesian, *STAT1*, PCR-RFLP.

INTRODUCTION

The large improvement in milk production traits, especially milk yield, over the last 40 years has resulted in a decrease in fertility trait[1]. It is well known that there is an antagonism between high yield and fertility[2]. Selection programs mainly focused on milk yield have led to significantly high milk yield accompanied by remarkable decreases in fertility and health traits. Recently, lifetime productivity and longevity are increasing in popularity day-to-day and current selection programs are gradually adopting this approach more commonly compared to conventional high yield-oriented management systems[3,4]. This novel scheme contributes to profitability by increasing the profit from an individual cow and decreasing the heifer replacement costs[5].

Genetic evaluation has enabled effective dairy cattle breeding and selection schemes that offer options according to different breeding purposes. In this context, numerous candidate genes associated with functional traits have been defined in dairy

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cattle. Among them, the signal transducer and activator of the transcription 1 gene (*STAT1*) is one of the most important genes associated with improved milk yield and content[6]. It is located on bovine chromosome 2 (BTA2) and encodes a cytoplasmic transcription factor that plays a major role in the regulation of cytokine signaling pathways and cellular functions[7-9]. STAT proteins are phosphorylated by Janus kinases (JAK) and thus they regulate the transcriptions of various genes[10]. These proteins comprise a family of seven structurally and functionally related proteins based on a cell- and tissue-specific distribution as follows: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6[10,11]. It is important to note that the JAK-STAT pathway regulates the lactation and phosphatidylinositol-3 kinases (PI3K/Akt) within the JAK-STAT overexpress in lactating cows[12]. The corresponding mechanism regulates many gene expressions and pathways related to important cellular pathways involving proliferation, differentiation, and apoptosis. The bovine *STAT1* gene maps to BTA2 at intervals 60 to 63 cM[6]. This genomic region has been associated with production traits by whole-genome scans[13,14].

The current knowledge on the genetic background of reproductive efficiency is rather limited compared to yield traits. Many important genetic markers have been evaluated focusing only on their production effects in dairy cattle. Notably, improvement applications in the reproductive status of cows, along with profitability in production, are preferred trends in sustainable dairy cattle breeding programs[3]. The *STAT1* has been studied in dairy cattle because of its relationship with improved milk yield and composition traits[6,9,10,15]. However, the effects of this gene on reproductive traits are insufficient in high-yielding dairy cows. Therefore, this study was aimed to evaluate the effects of a C/T single nucleotide polymorphism located in the 3' UTR region of bovine *STAT1* on certain reproductive traits in elite high-yielding Holstein-Friesian cows.

MATERIALS AND METHODS

Ethical considerations

All procedures performed complied with worldwide ethical considerations. Blood samples were taken from the animals only once, and no invasive procedure was applied other than this application. The study was approved by Bursa Uludag University Local Ethics Committee for Animal Research (approval number: 2022-02/04).

Animals and management

A total of 500 purebred Holstein-Friesian cows raised in the same commercial farm, located in the east part of the Aegean region of Turkey (Atasancak Acıpayam Dairy Farm, Acipayam/Denizli), were used in this study. The total herd size was 4800 cows. All animals were fed the same diets and were raised in free-stall barns with sand bedding. They had full access to water throughout the experiment. Automatic dipping and flushing systems were used in barns. All animals were milked three times per day in a parlor where 200 cows can be milked at the same time. On the farm, 950 cows can be milked per hour and 187 tons of milk is obtained per day. Blood samples (~4 mL obtained) were obtained from the *vena jugularis* of each cow.

Phenotypic traits

The phenotypic traits analyzed in this study were total milk yield, 305-d milk yield, days open, and the number of inseminations. A large dataset of 4800 animals was evaluated in this study. Cull rates, body condition scores, and lactation season were also evaluated in statistical models. Initially, all of the cows were ranked (G1-G100) by a selection index based on individual milk yield records (rate in the index: 40%), health traits (rate in the index: 45%), and feed conversion (15%). 305-d milk yield was calculated based on the dataset obtained from individual daily milk yield records. The herd-management software (Delpro, DeLaval) were used to record data. A total of 500 cows were selected by using this category system and high-value cows were determined based on their records. The number of inseminations was determined as the number of inseminations required for conception[3]. A cow was considered a repeat breeder if she had at least three artificial inseminations and no subsequent calving[16]. Some different causes of culling with relatively low incidences, including laminitis and enterotoxemia were excluded from the analysis. From the cows with the highest milk yields in the herd, 75 cows were selected (11408±130 kg, 305-d milk yield) for the *STAT1* genotyping.

Genomic DNA extraction and the genotyping

DNA extraction from blood samples was performed using the phenol-chloroform method as described by Green and Sambrook[17] with some minor modifications applied by the authors. The concentrations and the purity of DNA were determined using a NanoDrop 2000c spectrometer (Thermo Scientific, Wilmington, DE, USA). The genotyping of the SNP located in the 3' UTR region of bovine *STAT1* (GenBank Acc. No: AW289395) was performed by the PCR-RFLP method.

The primer sequences (from 5' to 3') were as follows:

F: 5'GCCTCAAGTTTGCCAGTGGC3'
R: 5'GGCTCCCTTGATAGAACTGT3'

PCR reaction mixtures consisted of 1 μL of forward and reverse primers-each (0.5 μM) based on the study by Cobanoglu et al.[6], 12.50 μL PCR master mix (OneTaq Quick-Load 2x MM, New England BioLabs Inc., Ipswich, MA, USA), ~2.5 μL of total purified DNA, 8 μL DNase and RNase-free molecular grade water (Thermo Fisher Scientific) were mixed to make a total volume up to 25 μL. MyGenie 96 thermal block (Bioneer Corporation, South Korea) was used for the DNA amplification reactions. The PCR condition was as follows: 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, touchdown annealing from 65 to 50°C for 45 s (−2°C/cycle), 72°C for 45 s, and a final extension at 72°C for 7 min.

The fragments of the PCR product (314 bp) were digested by the *Bsp*HI endonuclease. PCR and restriction products were controlled using 2% and 3%, respectively, agarose gel electrophoresis (migration for ~1 h at 100 V) and were visualized by a gel documentation system with UV transillumination (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). SafeView Classic (Applied Biological Materials Inc., Richmond, Canada) was used as a DNA-intercalating dye (~7 μL). To obtain the fragment size, a 100-1000 bp DNA ladder (Biomatik Co., Canada) was used in gels.

Statistical analysis

Genotype and allele frequencies were estimated according to Falconer and Mackay[18]. The deviation from Hardy-Weinberg

Equilibrium (HWE) was tested using a standard chi-squared goodness-of-fit. Population genetics parameters including heterozygosity (He), homozygosity (Ho), number of effective alleles (Ne), and the polymorphic information content (PIC) were calculated by the formulas demonstrated by Botstein et al.[19] and Nei and Roychoudhury[20]. Anderson-Darling test was used to evaluate the normality of data. Genotype-phenotype association analysis was carried out by the least-squares method as applied in a general linear model (GLM) procedure of Minitab software (Minitab Inc., Pennsylvania, USA, v17.1.0). Body condition scores, milk yield, lactation season, and lactation rank were included in the models when appropriate. Tukey's test was used as a post-hoc comparison. The association between the cull rates and the genotypes was evaluated by Pearson's chi-square test.

RESULTS

The electrophoresis pattern of *STAT1* PCR amplification of the 314 bp fragment is shown in Figure 1. Concerning the results of *Bsp*HI enzyme digestion, the amplicon was cleaved into three fragments (314 bp, 201 bp, and 113 bp). Two bands (314 bp and 201 bp) were distinctive for genotype determination as shown in Figure 2. Undigested fragment of the 314 bp was diagnostic for the TT genotype in the *STAT1* assay. Heterozygous genotype was indicated by two distinctive bands of 314 bp and 201 bp while 201 bp fragment was diagnostic for the CC genotype (Figure 2).

Two alleles and three genotypes for the *STAT1* g.3141C>T marker were found in the present study. Table 1 shows the genotype and allele frequencies. The predominant genotype was the CC (~47%). Nevertheless, the genotypic distribution seemed to be balanced with 20 cows each for the TT and heterozygous genotypes. Concerning population genetics parameters, admissible variability results were observed for the *STAT1* marker (Table 1). Results indicated that the Ne value approached 2.00. The *STAT1* g.3141C>T polymorphism is a moderately informative marker for the tested population. In the chi-square test, a deviation from HWE was observed in the studied population ($P<0.001$).

The least-squares means and their respective standard errors obtained for the effects of *STAT1* marker on reproductive traits in Holstein-Friesian cows are presented in Table 2. The marker *STAT1* g.3141C>T affected the number of inseminations ($P<0.05$). The TT animals were characterized by the highest number of inseminations (3.71±0.73) which is indicating a potential negative effect of this genotype on reproductive performance. Moreover, there was a significant association between the *STAT1* marker and cull-rates ($P<0.01$). There was no association between the *STAT1* and any other traits analyzed.

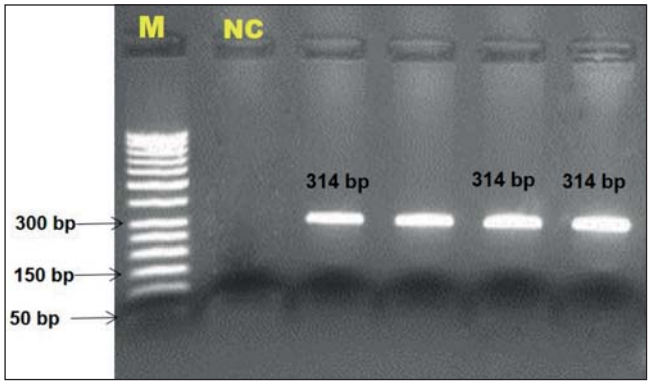

Figure 1 - The electrophoresis pattern of PCR amplification for the g.3141C>T polymorphism within the bovine *STAT1* gene.
M: Marker; NC: Negative control.

Table 1 - Genotypic, allelic frequencies (%), population genetics parameters, and Hardy-Weinberg Equilibrium (HWE) test results in the *STAT1* g.3141C>T polymorphism.

Locus	*STAT1*		
Genotype	CC	CT	TT
n	35	20	20
Genotypic frequency (%)	46.66	26.67	26.67
Allele	C	T	
Allelic frequency	0.60	0.40	
Theoretical Heterozygosity (H_{the})[1]	0.4801		
Number of effective alleles (Ne)	1.9231		
Polymorphism information content (PIC)	0.3648		
χ^2(HWE)	14.8146***		

[1]In a diallelic locus, 1 - theoretical heterozygosity (H_{the}) = locus homozygosity (Ho).
***$P<0.001$; not consistent with HWE.

DISCUSSION

The remarkable dominance of milk yield in current selection indexes has been started to be depleted gradually by non-production traits, including reproduction performance and health characteristics. The selective breeding for economically important traits was traditionally based on phenotypic recordings and it has been quite successful to some extent. From the 1990s, molecular genetics and its wide applications in animal breeding have led to more genetic improvement than using only phenotypic records. The detection and fine mapping of genes underlying the traits of interest, which can be termed quantitative traits loci (QTL), combination of QTL information and the best linear unbiased prediction-estimates of breeding values (BLUP-EBV), and marker-assisted selection (MAS) had provided promising improvement in information at the DNA level[21]. But the quantitative traits are much more complex than expected. In this context, numerous genotypic interactions make the genotype-phenotype association considerably hard to predict[22]. Antagonistic relationships among particular phenotypic traits generally cause failure to achieve the initially anticipated progress. Breeding strategies that mainly focus on the high production of dairy cattle have resulted in ignoring reproductive traits. Thus, the evaluation of the effects of widely used genetic markers on the reproductive performance of cows is a critical subject.

Here, we present a potential negative effect of the *STAT1* g.3141C>T polymorphism on the number of inseminations per conception in high-yielding Holstein-Friesian cows. Furthermore, the favorable genotype seemed to be the heterozygous genotype with the lowest number of inseminations (1.15±0.08). In this respect, the TT genotype was characterized by a higher number of inseminations compared to alternative genotypes ($P<0.05$). And more important, TT animals had +2.56 and +1.44 higher means for the number of inseminations compared to heterozygous and the CC animals, respectively. The geno-

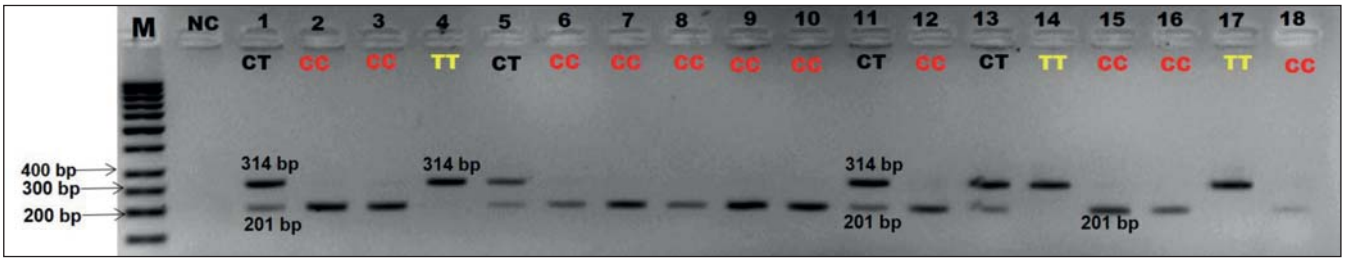

Figure 2 - The electrophoresis pattern of *Bsp*HI restriction enzyme digestion of PCR products the g.3141C>T polymorphism within the bovine *STAT1* gene.
M: Marker; NC: Negative control.

types CC and CT were associated with significant increases in milk, fat, and protein yields as demonstrated by Cobanoglu et al.[6]. These authors indicated that the C allele of the *STAT1* marker was also associated with an increase in milk protein and fat percentages. Similarly, Rychtářová et al.[9] have shown that significant associations were observed for the CC and CT genotypes in estimated breeding value for protein and fat percentages. In their study, animals with the TT genotype showed the lowest values for fat and protein percentage (although not statistically significant, $P>0.05$). Concerning the Jersey breed, the TT genotype was characterized by significantly higher means for test day milk yield (+2.07 kg and +1.29 kg), fat yield (+0.13 kg and +0.09 kg), and protein yield (+0.07 kg and +0.05) compared to those with CC and heterozygous genotypes, respectively, in contrast to Holstein cows[23]. On the other hand, Ardicli et al.[3] found no significant association between the *STAT1* g.3141C>T polymorphism and any of the reproductive performance traits. The frequency of the genotype TT in Holstein-Friesian cows has been reported in the range of 2.15-18.19% in various studies on the *STAT1* g.3141C>T marker[6,9,10,15,23]. This genotype seems to be rare in Jersey cattle[23]. In this study, the TT genotype frequency was remarkably high (26.67%) in high-yielding Holstein-Friesians. It is important to note that the *STAT1*-TT genotype has highly undesirable properties for both milk production and reproduction performance traits based on previously published papers and the present study. From another point of view, the heterozygous genotype has been characterized by the higher milk production trait means (based on previous association studies as discussed above) and the lowest number of inseminations (this study). It is conceivable to interpret that it is a positive and beneficial approach to decrease the frequency of the TT genotype and increase the number of CT animals at the herd level regarding the studied *STAT1* marker. However, as mentioned before, economically important quantitative traits are very complex. For instance, Khatib et al.[10] reported that the interaction between *STAT1* g.3141C>T and *STAT3* SNP19069 (as they designated) was highly significant for early embryonic survival rate. Furthermore, Cobanoglu et al.[6] indicated that the C allele seems to be associated with an increase in somatic cell counts compared with the T allele. There is plenty of room for a better understanding of the genotypic background of complex traits, such as bovine reproduction performance. But first, the genetics studies should focus more on non-production characteristics because the recent knowledge on the effects of many genetic markers on reproductive traits is rather limited compared to production traits in dairy cattle. Although MAS results can provide limited efficiency on the traits of interest in livestock production[21], the present results may be useful in the evaluation of popular genetic markers, such as *STAT1*, influences on non-production traits in dairy cattle. Notably, further studies are needed to confirm these findings and to discuss the other novel effects in different Holstein populations.

The most important problem caused by insufficient reproduction performance is the early culling of dairy cows. This leads to significant economic losses and prevents sustainability in dairy cattle farms. In this context, repeat breeding is defined as failure to conceive from three or more regularly spaced services in the absence of detectable abnormalities and is a substantial problem in cattle breeding resulting in increased calving interval and increased culling rates[16]. In this study, the relationship between the culling status of cows and the *STAT1* g.3141C>T genotypes was evaluated based on repeat-breeding. Consistent with the results in ANOVA, the TT cows had higher culling rates compared to CC and CT genotype carriers ($P<0.05$). It is important to note that TT cows were characterized by higher days open (250.81±44.70 d) compared to the heterozygous (130.62±46.20 d) and CC (180.01±31.10 d)

Table 2 - Least-square means and their corresponding standard errors for the effects of the *STAT1* g.3141C>T polymorphism on the phenotypic traits analyzed.

Traits analyzed	Genotypes			Significance
	CC	CT	TT	
305-d milk yield (kg)	10958±296	11291±440	10879±441	NS
Average daily milk yield (last 7d)[1]	39.57±0.95	40.93±1.40	39.39±1.36	NS
Days open (d)	180.00±31.10	130.60±46.20	250.80±44.70	NS[3]
Number of inseminations[2]	2.27±0.52[b]	1.15±0.08[b]	3.71±0.73[a]	$P<0.05$

[a,b]Different superscripts within a raw indicate a statistical significance in Tukey's post hoc comparison.
[1]Milk yield average in the week of sampling.
[2]Number of inseminations per conception.
[3]$P<0.1$

animals. But this effect was not substantiated in statistical analysis (Table 2). Since the *STAT1* TT is not a preferred genotype for milk yield, its effect on reproductive traits about this genotype has not been reported in the literature. Indeed, the effects of the *STAT1* gene on bovine reproduction are interestingly low. Taken together, we suggest that the TT is an undesirable genotype and the heterozygous genotype is significantly associated with superior characteristics in reproduction traits. It was also observed that most of the cows conceived by single artificial insemination are the CT genotype carriers (data not shown). In the present statistical analyses, the significant effects of lactation season and body condition score on some reproduction traits were observed but these are widely studied environmental factors in previously published papers, and hence, these factors will not be discussed further.

It is well known that an increase in milk yield negatively affects dairy cow fertility[24]. We thus think that evaluation of the *STAT1* gene effects on the selected reproduction traits in high-yielding Holstein-Friesians can provide more confidential and consistent interpretations from an applicable perspective at the herd level. The mean of 305-d milk yield ranges from 6608 kg to 7871.51 kg in the most of previously published papers regarding the *STAT1* g.3141C>T polymorphism[9,15,23]. In the present study, the selected cows had 11408±130 kg of 305-d milk yield (min: 9092 kg; max: 14358.52 kg). In this respect, the results demonstrated in this paper may reveal critical points in dairy cattle management concerning reproduction traits. The JAK-STAT pathway plays a major role in controlling cytokine signals and has an association with mammary gland development and milk production. Moreover, JAK-STAT signaling along with the lactogenic hormones regulates the processes of lactation and reproduction in mammals[12]. Hence, the *STAT1* g.3141C>T marker deserves a higher level of focus on bovine fertility.

CONCLUSIONS

This paper focuses on the effects of the *STAT1* g.3141C>T marker on certain reproduction traits in high-yielding Holstein-Friesian cows. Novel significant differences were found among the genotypes of the *STAT1* locus. In this context, the TT genotype was characterized by the highest number of inseminations and high values for the culling rates related to repeat-breeding. On the other hand, heterozygous animals were shown to be associated with desirable reproduction performance. The broadening of selection aims with the fertility traits may be more useful than conventional production-focused approaches to achieve sustainable and profitable dairy cattle management.

ACKNOWLEDGEMENTS

The authors gratefully thank Atasancak Acıpayam Dairy Farm (Acipayam/Denizli).

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