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Effect of *DGAT1* variants on milk composition traits in Iranian Holstein cattle population*

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The results from a number of QTL mapping studies recently provided the evidence for quantitative trait *loci* affecting milk yield and milk fat content localized on bovine chromosome 14 (BTA14) close to *DGAT1* gene. *DGAT1* gene codes for diacylglycerol-acyltransferase enzyme which plays a main role in the triglyceride synthesis and subsequently the milk composition. A transition mutation in this gene results in substitution of guanine by adenine leading to the substitution of lysine by alanine in diacylglycerol-acyltransferase enzyme. In this study, 398 blood samples were collected from the Holstein dairy farms in Iran. A 411 bp fragment of exon 8 in *DGAT1* gene was amplified and the animals were genotyped using RFLP-PCR technique. Three genotypes including KK (36 animals), AA (136 animals) and KA (226 animals) were identified. Frequencies of K and A (mutant type) alleles were estimated to be 0.37 and 0.63, respectively. The effect of *DGAT1* gene on FAT_{2x} (milk fat yield adjusted for two milkings per day, kg), FAT_{P2X} (fat content of milk adjusted for two milkings per day, %), EBV_{FP} (estimated breeding value for fat content of milk, %) and PRO_{PER305} (milk protein content adjusted for 305 days, %) occurred highly significant (P<0.001)

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The first evidence for chromosomal locations responsible for variation in important economic traits in farm animals has been reported by Gelderman [1975]. Many genomic studies on dairy cattle showed quantitative trait *loci* (QTL) with major effect on milk production located in centromeric end of chromosome 14 (BTA14) – [Looft *et al.* 2001, Heyen *et al.* 1999]. In addition, it has been reported that QTL on BTA6, 20, and 26 have major effects on milk production traits [Coppieters *et al.* 1998, Ashwell *et al.* 2004, Chen *et al.* 2006, Gautier *et al.* 2006]. Diacylglycerol acyltransferase 1 (*DGAT1*) gene located on BTA14, codes for diacylglicerol acyltransferase enzyme, a microsomal enzyme that catalyzes the final step of triglyceride synthesis [Ripoli *et al.* 2006]. Milk fat contains approximately 98% triglycerides. Characteristic of ruminant milk fat is the occurrence of short-chain fatty acids comprising four to six carbon atoms. When triglycerides are synthesized, fatty acids are first attached to positions sn-1 and sn-2 (according to the stereospecific numbering) on the glycerol molecule under the catalyzing effects of enzymes. When a fatty acid is attached to the third position, the enzyme acyl-CoA:diacylglycerol acyltransferase 1 (*DGAT1*) acts as a catalyst [Marshall and Knudesn 1977]. Recent studies indicated a significant association between *DGAT1* gene and milk production traits [Grisart *et al.* 2002, Spelman *et al.* 2002, Winter *et al.* 2002]. Fat and protein are economically important components of cows' milk and the current breeding programmes aim at counteracting a further decrease in these components [Naslund *et al.* 2008]. Milk proteins are synthesized in the mammary gland, but 60% of amino acids used to build the proteins are obtained from the animal's diet. Both the total protein and amino acid pool of milk vary with cow breed and animal's individual genetic structure [Naslund *et al.* 2008].

A transition mutation leads to the substitution of guanine by adenine in *DGAT1* gene resulting in the substitution of lysine by alanine in diacylglycerol-acyltransferase enzyme [Grisart *et al.* 2002, Winter *et al.* 2002].

Studies of polymorphism of *DGAT1* in *Bos taurus* and *Bos indicus* breeds show that K allele is a wild type and the A allele substitution probably occurred after the divergence of *Bos taurus* and *Bos indicus* [Kaupe *et al.* 2004]. Allele K of *DGAT1* gene increases fat yield of milk and fat and protein content, whereas allele A increases both milk and protein yield [Kaupe *et al.* 2007, Thaller *et al.* 2003]. The $DGAT^{K}$ allele elevates the $DGAT^{A}$ by 0.34 per cent points (pp) in fat, 0.08 pp in protein and 10.6 kg in fat yield, while milk and protein yields decrease by 316 and 5.64 kg, respectively [Grisart *et al.* 2002]. The objective of this study was to investigate the association of *DGAT1* variants with milk production traits in Iranian Holstein cattle.

Material and methods

Sampling and genotyping

The study included 398 Iranian Holstein-Friesian cows from the active dairy population. The first lactation records of the cows born in 2003 to 2006 were considered. The cows were distributed in ten Holstein dairy herds in Tehran and Isfahan provinces

of Iran. DNA was extracted from blood samples using standard salting out protocol [Miler 1988] and *DGAT1* gene was amplified with standard PCR (Thermo cycler, BIOMETRA, Germany). PCR reaction was performed in a 15 µl volume using 100 ng genomic DNA, PCR buffer (1X), 1.5 mM $Mgcl_2$, 0.2 mM dNTPs, 0.6 Pm of each primer, 5% DMSO and 2U Taq polymerase enzyme. The addition of DMSO to the PCR reactions allowed an equal amplification of both alleles. The sequences of primers [Kaupe *et al.* 2004] were as follows:

Forward: 5'-GCACCATCCTCTTCCTCAAG-3'
Reverse: 5'-GCACCATCCTCTTCCTCAAG-3'

DGAT1 was sequenced and registered in NCBI gene bank with AY065621 accession number. PCR reaction conditions were: 10 min at 95°C, 35 cycles of 60 s at 95°C, 60 s at 60°C, 60 s at 72°C and final extension in 7 min at 72°C. The PCR reaction amplified the 411 bp fragment of exon 8 in *DGAT1* gene. The PCR products (5 µl) were digested using 2 units of the *Cfr*I (FERMENTAS, Lithuania) restriction enzyme for 3 hrs at 37°C and separated on a 2% agarose gel. Gels were stained with ethidium bromide and visualized under UV light. Finally, genotyping of the animals was performed using the RFLP-PCR technique.

The studied milk fat traits were:

– FAT_{2X} (milk fat yield adjusted for two-milkings per day, kg);
– $FATP_{2X}$ (milk fat content adjusted for two milkings per day, %);
– EBV_F (estimated breeding value for milk fat yield, kg);
– EBV_{FP} (estimated breeding value for milk fat content, %).

In total, 228 records were obtained for FAT_{2X}, FAT_{P2X} traits and 372 records for EBV_F and EBV_{FP} traits.

The studied milk protein traits were:

– PRO_{305} (milk protein yield adjusted for 305 days, kg);
– PRO_{ME} (milk protein yield adjusted for mature body weight, kg);
– PRO_{PER305} (milk protein content adjusted for 305 days, %).

In total, 194 records were obtained for milk protein traits.

Statistical

The Hardy-Weinberg test, gene and genotype frequencies, observed and expected homozygosity and heterozygosity and average heterozygosity were estimated using the Pop Gene software version 3.1d [Nei 1977].

Effects of *DGAT1* variant. The effect of *DGAT1* genotypes on milk composition traits was tested using the GLM procedure of the SAS package [SAS, Version 8] implementing the following statistical model:

$$y_{ijkmn} = \mu + G_i + S_j + M_k + N_m + e_{ijkmn}$$

where:

y_{ijkmnr} – an observation on the milk components (fat and protein);
μ – overall mean;

G_i – fixed effect of genotypes (3 levels);
S_j – fixed effect of herd (10 levels);
M_k – fixed effect of year (3 levels);
N_m – fixed effect of calving seasons (4 levels);
e_{ijkm} – residual effect to each observation.

Tukey test was used for pair wise comparisons of the means.

Additive and dominance effects. Covariates were 0, 1 and 2 to account for number of variant *DGAT1* alleles for the KK, KA and AA genotypes, respectively. To test for dominance, an additional regression covariate was added with value of 0 for homozygous and 1 for heterozygous animals. A significant result for this covariate was intercepted as evidence of dominance effects [Esmailizadeh *et al.* 2008]. Additive and dominance effects were estimated using the ASReml software [Gilmour *et al.* 2006].

DGAT1 gene variance was estimated using the $(1\text{-}MS_{full\ model}/MS_{reduced\ model})$ formula [Knott *et al.* 1996] where: $MS_{full\ model}$ and $MS_{reduced\ model}$ – residual mean squares of the model with the *DGAT1* effect fitted and the model without the *DGAT1* effect, respectively.

Results and discussion

Among the 398 animals studied, 36 genotypes were *KK*, 226 *KA* and 136 were *AA*. Frequencies for *K* and *A* alleles were 0.37 and 0.63, respectively. Comparable values of 0.64 for lysine and 0.36 for alanine were reported by Gautier *et al.* [2007]. Study on the New Zealand dairy cattle population has shown that frequencies of K allele in Holstein, Jersey and Ayrshire cattle were 0.60, 0.88 and 0.23, respectively [Spelman *et al*. 2002]. Results from this and other studies imply that the frequencies of DGAT1 alleles in different breeds are diverse worldwide [Ripoli *et al*. 2006, Lacorte *et al*. 2006]. In this study, frequency of A allele was greater than of allele K (0.63 versus 0.37). It has been reported that in Holstein cattle the frequency of A allele is greater than of allele KK [Hori-Oshima *et al*. 2003, Lacorte *et al*. 2006]. However, Spelman *et al*. [2002], Thaller *et al*. [2003], Chandra *et al*. [2005] and Kaupe *et al.* [2007] reported that A allele frequency is less than that of K allele in Holstein breed. In this study, the following DNA restriction fragments were obtained for *DGAT1* gene using the *Cfr*I enzyme. The fragments were 411 bp (no digestion) for the KK genotype, 203 and 208 bp for the AA, 411 and 203 or 208 bp for the KA (Fig. 1). The KA genotype showed the highest frequency (0.56), whereas the KK had the least frequent genotype (0.09). A comparable value of the most frequent genotype was reported by Thaller *et al.* [2003]. In this study the Hardy Weinberg equilibrium was estimated with chi-square test and likelihood ratio test. The value of chi-square was 17.71, greater than the critical value. The likelihood ratio test was estimated as 18.30, greater than

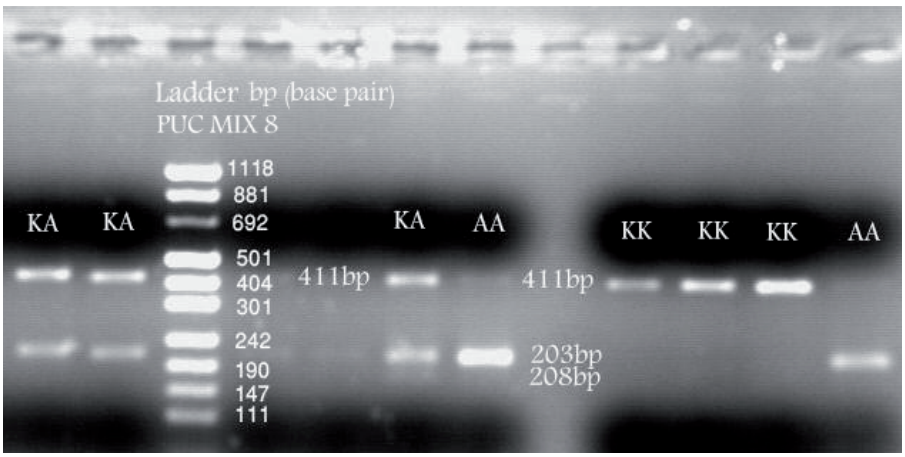

Fig. 1. Electrophoretic separation of *DGAT1* gene PCR products digested with *Cfr*I.

the critical value. The population under this study was not found to be in a Hardy-Weinberg equilibrium, as for years it has been under selection for milk production traits. The values of chi-square and likelihood ratio test are shown in Table 1, while the observed and expected homozygosity, heterozygosity and average heterozygosity of *DGAT1* gene are given in Table 2.

Table 1. Chi-square values and likelihood ratio test

Test	Degree of freedom	Value	P-value
Chi-square	1	17.71	0.000026
Likelihood ratio	1	18.30	0.000019

Table 2. Summary of homozygosity and heterozygosity

Gene	Sample size	Observed homozygosity	Observed hetrozygosity	Expected homozygosity	Expected heterozygosity	Average hetrozygosity
DGAT1	796	0.43	0.47	0.53	0.57	0.47

Table 3 shows the explored effects of *DGAT1* genotypes upon milk production traits. Many studies showed significant association between *DGAT1* polymorphism and milk yield and composition. E.g., Ripoli *et al.* [2006] and Grisart *et al.* [2002] investigated the effect of *DGAT1* polymorphism on milk composition in Holstein cattle. They reported significant effects of *DGAT1* genotypes on milk composition. A pronounced influence of *DGAT1* on milk production traits, especially on milk fat yield, has been reported in many cattle populations, including the Jersey [Spelman *et*

Table 3. Genotype effects of *DGAT1* on milk fat and milk protein production traits

Trait	F value	P value	Genotype effect
FAT_{2X}	7.07	0.0011	***
FAT_{P2X}	17.51	0.0001	***
EBV_F	1.39	0.2501	ns
EBV_{FP}	9.30	0.0001	***
PRO_{305}	1.49	0.2276	ns
PRO_{ME}	1.61	0.2062	ns
PRO_{PER305}	7.30	0.0009	***

***P<0.001; ns – not significant genotype effect.

al. 2002], Fleckvieh [Thaller *et al.* 2003], Normande [Gautier *et al.* 2007] and Angeln [Sanders *et al.* 2006] breeds, as well as German [Thaller *et al.* 2003], Dutch [Grisart *et al.* 2002], French [Gautier *et al*. 2007], Polish [Szyda and Komisarek 2007] and New Zealand Holstein-Friesians [Grisart *et al.* 2002]. However, Spelman *et al.* [2002] reported no association between *DGAT1* gene and fat milk in Ayrshires. The strong effects of the *DGAT1* variants on milk composition become evident when comparing them with other single-gene effects. For example, the genotypes of the osteopontin gene *(OPN)* have been reported by various authors to be associated predominantly with milk fat yield, milk fat content as well as protein content and yield [Khatib *et al.* 2007]. Although the size of the estimated effects of *OPN* gene on these traits are mostly within the range of *DGAT1* effects on fat traits, these results are inconsistent across studies. Therefore, it might be concluded that the linked genes are responsible, and thus *OPN* gene does not possess the same candidate status as *DGAT1*. We conclude that, in the analysed samples, the *DGAT1* variant is the one that showed the most consistent effect on milk fat and protein. The least square means (LSM) results showed that the KA genotype group yielded more milk fat compared with the other

Table 4. The least squares means and their standard errors of milk production traits for AA, KA and KK genotypes

Genotype/trait	KK	KA	AA
FAT_{2X}	224.5 ± 11.78^{AB}	238.1 ± 8.46^{B}	223.78 ± 8.42^{A}
FAT_{P2X}	3.16 ± 0.11^{AB}	3.27 ± 0.08^{B}	3.09 ± 0.08^{A}
EBV_F	3.35 ± 1.36	2.05 ± 3.96	2.28 ± 2.42
EBV_{FP}	0.015 ± 0.066^{A}	0.025 ± 0.039^{AB}	0.07 ± 0.016^{B}
PRO_{305}	215.68 ± 9.94	225.1 ± 7.28	219.12 ± 7.23
PRO_{ME}	250.11 ± 11.35	$261.3\pm7\pm7.31$	253.68 ± 8.26
PRO_{PER305}	3.10 ± 0.050^{A}	3.07 ± 0.030^{B}	3.06 ± 0.030^{AB}

^{AB}Means in rows bearing different superscripts differ significantly at P<0.01.

groups. The LSM of milk fat yield of AA, KA and KK genotypes are presented in Table 4. In lactation 1 (305-days), the cows of the KA genotype yielded more milk fat than did the KK and AA individuals. However, KK cows produced more milk protein than the KA and AA individuals. Different results were obtained by Komisarek *et al.* [2004] who reported that cows of KK genotype produced milk with higher fat content and yielded more fat than other individuals of Holstein-Friesian and Jersey cattle. As for milk protein, the PRO_{PER305} KK genotype cows yielded milk with higher protein content than did the other Holstein-Friesians.

Table 5. The additive and dominance effects of *DGAT1* gene

Trait	Additive	Significant effect	Dominance	Significant effect
FAT_{2X}	-0.08±5.0	ns	16.12±6.13	*
FAT_{P2X}	0.03±0.05	ns	0.14±0.06	*
EBV_F	-0.32±1.57	ns	1.95±1.95	ns
EBV_{FP}	0.02±0.01	*	-0.01±0.02	ns
PRO_{305}	-1.68±4.42	ns	8.37±5.52	ns
PRO_{ME}	-1.77±5.0	ns	9.98±6.31	ns
PRO_{PER305}	0.01±0.02	ns	0.01±0.03	ns

*P<0.05; ns – not significant effect.

Table 5 gives the additive and dominance effects of the alleles. The additive effect is the important component contributing to the variance of breeding values and is the chief cause of resemblance. It is, therefore, the main determinant of the observable genetic properties of the population and of the population response to selection [Falconer *et al.* 1995]. The results indicate that the K allele increases the EBV_{FP} trait (by +0.02). The dominance effect arises from the property of dominance among alleles at a *locus*. Statistically, it is the effect between alleles, or within *locus.* Also, it depends on gene frequency and thus is partly a property of the population and is not simply a measure of the degree of dominance [Falconer *et al.* 1995]. In this study, dominance effects were significant for FAT_{2X} and FAT_{P2X} traits. The results indicate that KA genotype was related to higher fat yield as compared to KK and AA genotypes. The variance due to a single gene incorporates both additive and dominance variance. *DGAT1* variance for significant traits such as FAT2x FAT_{P2X}, EBV_{FP} and PRO_{PER305} were estimated as 2.32, 3.07, 0.89 and 0.77, respectively. These values confirm that *DGAT1* gene plays the main role in phenotypic variation of traits in question making the variant in the gene a valuable tool for gene-assisted selection to improve the milk production traits.

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