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Joint Analysis of the *DGAT1*, *OPN* and *PPARGC1A* Genes Effects on Variation of Milk Production and Composition in Holstein Cattle Population

Research Article

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ABSTRACT

The aim of this study was to investigate effects of *DGAT1*, *OPN* and *PPARGC1A* candidate genes on milk production traits in Iranian Holstein cattle. Several papers have studied single nucleotide polymorphisms (SNPs) and their association with economic traits in dairy cows, but the combined effect of these genes has not been examined in Iranian Holstein cattle population. Blood samples were collected from 398 registered Holstein cows. Total DNA was extracted using the salting out protocol. The PCR-RFLP technique was used for SNPs genotyping. The largest genotype frequency was estimated as 0.65 for *PPARGC1A* (c.1892)^{CT} and the least frequency was estimated as 0.09 for *DGAT1*^{KK} genotype. The allele frequencies were in the range 0.36 to 0.64 for *PPARGC1A* (c.3359) A and C alleles, respectively. The allelic substitution effects were estimated using a multiple regression model. The effects of allelic substitution for *DGAT1*^{K} and *PPARGC1A* (c.1892)^{T} were significant on estimated breeding values for fat percentage (EBV_{FP}) ($P<0.01$). In addition, the results of multivariate analysis indicated the significant effect of *DGAT1* and *PPARGC1A* (c.1892) on EBV_{FP} ($P<0.05$). However, there were no association between *OPN* and *PPARGC1A* (c.3359) polymorphisms and studied traits.

KEY WORDS *DGAT1*, Holstein, *OPN*, *PPARGC1A*.

INTRODUCTION

Candidate gene approach and whole genome scans are two main strategies for QTL identification (Andersson, 2001). The candidate gene approach studies the relationship between the traits and known genes that may be associated with the physiological pathways underlying the trait (Liu *et al.* 2008). This approach has been successful to some extent. For example, several studies have identified QTLs for milk composition on chromosomes 6 and 14 (Riquet *et al.* 1999; Farnir *et al.* 2002; Olsen *et al.* 2005). The economic traits are polygenic. It means that they are controlled by many loci. Several studies indicated genetic variation in milk production traits cannot be explained by few candidate genes (Kaupe *et al.* 2007). Therefore, the effects of all candidate loci should be explored together in the same statistical model diacyl glycerol acyltransferase 1 (*DGAT1*) is located near the centromeric region of *Bos taurus* autosome 14 (BTA14). The first evidence for the effect of *DGAT1* variation on milk yield and composition in Holstein cattle

was reported by Grisart *et al.* (2002). *DGAT1* is considered as the key enzyme in controlling the synthesis rate of triglycerides in adipocytes. A non-conservative K232A substitution (conservation of alanine to lysine) in *DGAT1* was associated with milk production and compositions in Holstein cattle (Thaller *et al.* 2003). Spelman *et al.* (2002) and Banos *et al.* (2008) reported that the K232A substitution in exon 8 of the *DGAT1* gene was associated with increasing of milk fat yield and decreasing of milk production and protein yield. Some studies showed that there are significant associations between *DGAT1* and milk, fat yield and protein yield. Bovine chromosome six (BTA6) harbors at least six QTLs influencing milk production traits of dairy cattle.

The osteopontin (*OPN*) and peroxisome proliferator activated receptor gamma co-activator 1 Alpha (*PPARGC1A*) are about 6 Mb apart, which is about 12 cM for this region of chromosome 6 (Olsen *et al.* 2005). *OPN* is a strong functional candidate for milk production and it is a highly phosphorylated glycoprotein (Leonard *et al.* 2005).

Schnabel *et al.* (2005) reported an association between *OPN* and milk protein percentage in the North American Holstein population. *PPARGC1A* has main role in fat and glucose metabolism and plays a critical role in the activation of nuclear hormone receptors and transcription factors regulating energy homeostasis (Liang and Ward, 2006; Kowalewska-Luczak *et al.* 2010). Structure of *PPARGC1A* gene is made from 13 exons and expressed at different levels in a great number of tissues (Liang and Ward, 2006). Khatib *et al.* (2007) showed significant associations between *PPARGC1A* (*c.3359*) gene, milk yield, milk protein percentage, and somatic cell score in the North American Holstein population. The aim of this study was to investigate the joint effects of *OPN*, *PPARGC1A* and *DGAT1* candidate genes on milk production traits in Iranian Holstein cattle population.

MATERIALS AND METHODS

Animals and traits

Totally 398 blood samples were collected from Holstein-Friesian cows of Iran, which were distributed in ten dairy herds in two provinces of Iran. The cows were under official milk recording of Animal Breeding Center (Karaj-Iran).

Finally 372 records for estimated breeding values for milk production adjusted for 305 days (EBV_M), fat yield (EBV_F) (kg) and fat percentage (EBV_{FP}) were obtained from the Animal Breeding Center for analyzing association between genotypes and economics traits. The EBVs were estimated by random regression test day model.

DNA extraction, PCR amplification and SNPs genotyping

DNA extractions were performed using standard salting out protocol (Miller *et al.* 1988). PCR reactions were performed using standard PCR (Thermo cycler, Biometra, Germany). More details about primers are shown in Table 1 (Kaupe *et al.* 2004; Weikard *et al.* 2005; Khatib *et al.* 2007).

PCR reaction for *DGAT1* (GenBank: EU077528), *OPN* (GenBank: NW_255516) and *PPARGC1A* (GenBank: AY321517) loci were performed in a 25 µL volume using 100 ng genomic DNA, PCR buffer (1X), 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.6 pmol of each primer and Taq polymerase enzyme (2U). All accession numbers are available in NCBI site. For *DGAT1* gene, the addition of DMSO to the PCR reactions allowed an equal amplification of both alleles. The annealing temperature for *DGAT1*, *OPN* and *PPARGC1A* are considered as 60, 53 and 55 centigrade and finally 411, 290, 195 and 357 base pairs fragments were amplified for *DGAT1*, *OPN*, *PPARGC1A* (c.1892) and *PPARGC1A* (c.3359). The PCR products (5 µL) were digested using 2 units of the restriction enzymes (FERMENTAS, Lithuania) and separated on a 2% agarose gel. The gels were stained with ethidium bromide and visualized under UV light. Finally, SNPs were genotyped by PCR-RFLP technique. Table 2 shows more detail about restriction enzyme conditions.

Gene and genotype frequencies

The population genetic parameters including gene and genotypic frequencies, Hardy-Weinberg equilibrium (Chi-square test), Indices of genetic diversity in population (Nei (H) and Shannon (I)) were estimated using the PopGene software version 3.1d (Nei, 1977).

Joint analysis of *DGAT1*, *OPN* and *PPARGC1A* variants

The effects of genotypes were tested using the GLM procedure (Pillais trace test) of SPSS (2010) implementing the following fixed model:

$$y_{ijkmn} = \mu + P_i + A_j + D_k + O_m + e_{ijkmn}$$

Where:

y: observation for each trait.

µ: overall mean.

P_i, A_j, D_k and O_m: fixed effects of genotypes of *PPARGC1A* (c.1892), *PPARGC1A* (c.3359), *DGAT1* and *OPN* genes.

e_{ijkmn}: residual effect.

The mean comparisons were performed using the Tukey test for significant genotypes.

Table 1 Primer sequence for PCR reaction

Loci	Forward and revers primer
DGAT1	F 5′-GCACCATCCTCTTCCTCAAG-3′ R 5′-GGAAGCGCTTTCGGATG-3
OPN	F 5′-GCAAATCAGAAGTGTGATAGAC-3′ R 5′-CCAAGCCAAACGTATGAGTT-3
PPARGC1A (c.3359)	F 5′-GCGAGCACGGTGTTACATTACTAAGGAGAGTTGGCTAG-3′ R 5′-GTTGTGTTGCACTCAATGGAC-3′
PPARGC1A (c.1892)	F 5′-CATAGCCGGCGCCCCAGGTAAGATGCACGTTGGC-3′ R 5′-CTGGTACTCCTCGTAGCTGTC-3′

Table 2 Restriction enzymes and the digestion conditions

Locus	Position	Enzyme	Digestion temperature (˚C)	Digestion time
DGAT1	K232A	*CfrI*	37	3 h
OPN	c.8514	*BsrI*	65	5 h
PPARGC1A	c.3359	*NheI*	37	3 h
PPARGC1A	c.1892	*HaeIII*	37	5 h

The effects of allele substitutions on milk production traits were tested using the following multiple linear regression models (Knott *et al.* 1996):

$$y_{ijkl} = \mu + b_i x_i + b_j x_j + b_k x_k + b_l x_l + e_{ijkl}$$

Where:
y: observation for EBV_M, EBV_F and EBV_{FP} traits.
μ: overall mean.
b_i, b_j, b_k, b_l: regression coefficients representing the allelic substitutions for ($DGAT^K$, OPN^T, *PPARGC1A* (c.3359)^A, *PPARGC1A* (c.1892)^T.
x_i, x_j, x_k, x_l: indicator variables for genotypes of *DGAT1*, *OPN*, *PPARGC1A* (c.3359), *PPARGC1A* (c.1892) loci.
e_{ijkl}: residual effect.

RESULTS AND DISCUSSION

The most extreme genotypes frequencies were estimated as 0.65 and 0.09 for *PPARGC1A* (c.1892)^{CT} and $DGAT1^{KK}$ loci, respectively. Similar results were obtained about genotype frequencies in Holstein cattle population by Khatib *et al.* (2007), Thaller *et al.* (2003) and Komisarek and Dorynek (2009). In addition, the most and the least allele frequencies were calculated as 0.64 and 0.36 for A and C alleles of *PPARGC1A* (c.3359).

The joint testing of all of the candidate loci in the population under study indicated significant deviation from the Hardy-Weinberg equilibrium (P<0.05), which seems expectable due to the long time selection for milk production. The genetic diversity indices showed that the population has desirable genetic variation. More detail about values and frequencies are shown in Table 3. Figure 1 gives more information about the results of digestion. Similar results were obtained using the multivariate analysis and between subject test Table 4.

The results indicated that the *PPARGC1A* (c.1892) and *DGAT1* polymorphisms had significant association with EBV_{FP}. The summaries of statistical analysis are illustrated in Tables 5 and 6. The results obtained are supported from other studies. Weikard *et al.* (2005) reported significant association between SNP in intron 9 of the *PPARGC1A* (c.1892) gene and fat yield, which means that the *PPARGC1A* gene might be involved in genetic variation underlying the QTL for milk fat synthesis on BTA6. Schennink *et al.* (2009) showed that two SNPs in *PPARGC1A* (c.3359 and c.1892) had significant effects on fat yield. However, we found that the effect of *PPARGC1A* (c.3359) and *OPN* polymorphism were not significant, but different results reported by Zhang *et al.* (1998) and Mosig *et al.* (2001), who identified candidate gene affecting milk production traits close to *OPN* location.

Table 3 Summery of frequencies, H-W equilibrium and genetic variation indices

Locus	Allele frequency		Genotype frequency			H-W	Index	
DGAT1	K	A	KK	KA	AA	χ^{γ}	Shannon	Nei
	0.37	0.63	0.09	0.56	0.35	17.71**	0.66	0.46
OPN	C	T	CC	CT	TT			
	0.47	0.53	0.19	0.57	0.24	7.50**	0.69	0.49
PPARGC1A (c.3359)	A	C	AA	CA	CC			
	0.64	0.36	0.38	0.52	0.10	6.01*	0.65	0.46
PPARGC1A (c.1892)	C	T	CC	CT	CC			
	0.56	0.44	0.23	0.65	0.12	8.51**	0.68	0.49

* (P<0.05) and ** (P<0.01).

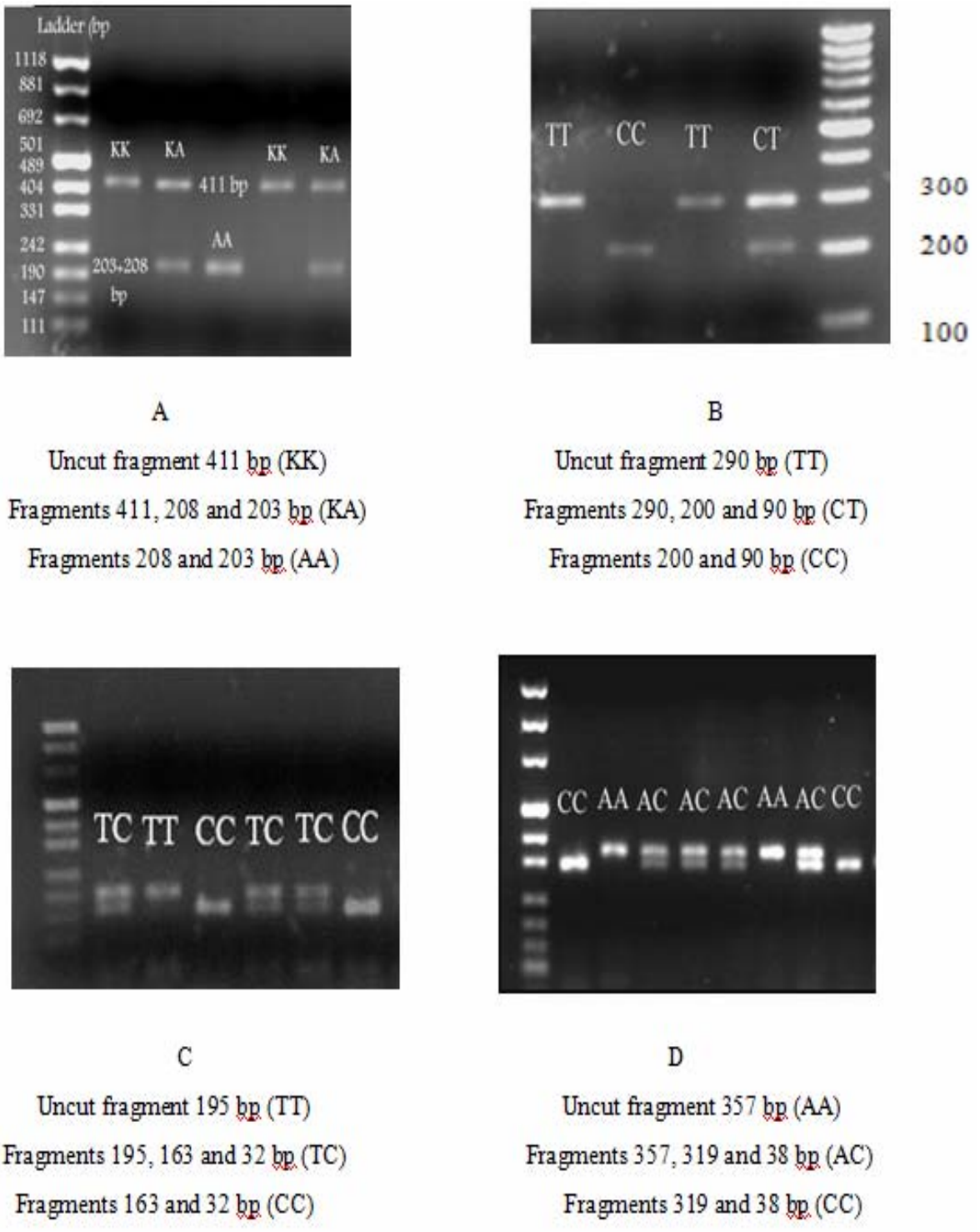

Figure 1 Electrophoretic separation of *DGAT1* (A), *OPN* (B), *PPARGC1A* (c.1892) (C) and *PPARGC1A* (c.3359) (D) genes PCR products
Figures a, c and d have a common ladder (PUC mix 8)
Figure b: ladder (gene ruler DNA ladder)

Table 4 The results of multivariate analysis (F-statistics) for EBV_{FP}

Locus	*DGAT1*	*OPN*	*PPARGC1A* (c.3359)	*PPARGC1A* (c.1892)
F-value	3.19*	1.52	1.93	2.41*

* (P<0.05).

Table 5 The analysis results of between subjects effects (F-values)

Locus/trait	*DGAT1*	*OPN*	*PPARGC1A* (c.3359)	*PPARGC1A* (c.1892)
EBV_M	2.12	1.18	0.44	1.89
EBV_F	1.40	1.20	0.14	0.10
EBV_{FP}	8.45**	1.98	2.07	4.79**

** (P<0.01).

Table 6 The results of mean comparisons for significant genes

DGAT1			*PPARGC1A* (c.1892)		
KK	KA	AA	CC	CT	TT
0.05±0.02[b]	0.04±0.01[b]	-0.01±0.001[a]	-0.008±0.001[a]	0.03±0.01[a]	0.09±0.02[b]

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 7 The effect of allele substitution for candidate genes

Locus/trait	$DGAT^K$	OPN^T	*PPARGC1A* (c.3359)^A	*PPARGC1A* (c.1892)^T
EBV_M	-76.75±42.54	37.25±108.05	-40.77±43.22	92.29±45.91
EBV_F	+0.75±1.32	1.97±1.26	-0.08±1.41	0.45±1.50
EBV_{FP}	+0.04±0.01**	0.01±0.01	0.02±0.01	-0.37±0.01**

** (P<0.01).

In addition, Leonard *et al.* (2005) showed significant association of *OPN* gene with milk protein percentage. Therefore, we suggest the further studies need to clarify the association between *OPN*, *PPARGC1A* (c.3359) and milk production traits in other populations. There are several possible reasons for different results of studies, including differences in allele frequency, the statistical models used to undertake the association analysis and genetic background of the animals in the study (Berry *et al.* 2010) and environmental circumstances where the animals were producing. The association between *DGAT1* gene and EBV_{FP} was significant which may be due to critical role of *DGAT1* gene in the synthesis rate of triglycerides (Grisart *et al.* 2002). Kadlecova *et al.* (2014) reported significant association between *DGAT1* genotypes and fat percentage in primiparous Holstein cows. Anton *et al.* (2012) indicated the significant effects of the *DGAT1* K232A polymorphism on milk yield, fat and protein percentage, as well. In addition, Fontanesi *et al.* (2015) illustrated that *DGAT1* polymorphism was highly associated with fat yield and fat percentage in Reggiania dairy cows (local breed in north of Italy). The results of mean comparisons illustrated that the genotypes of $DGAT1^{KK}$ and *PPARGC1A* $(c.1892)^{TT}$ had highest EBV_{FP}. More results of mean comparisons are shown in Table 6. Table 7 gives the additive effects (allele substitutions) of the alleles. The result indicated that and $DGAT^K$ allele increased the EBV_{FP} by +0.04 ± 0.01. Some standard errors were estimated more than their allele substitution effect. It can be due to low number of data, which are used in this study.

The results of allele substitutions were confirmed by other studies. Winter *et al.* (2002) and Strzałkowska *et al.* (2005) showed that the $DGAT1^K$ allele has a positive effect on milk fat content in different cattle breeds.

Naslund *et al.* (2008) reported that $DGAT1^K$ variant was associated with an increase in milk fat and protein percentages but decrease milk yield compared with the $DGAT^A$ variant. Similar results showed that the $DGAT^K$ allele exceeds of $DGAT^A$ allele, by (+0.34) percentage unit in fat (Grisart *et al.* 2002). The $DGAT1^K$ allele increases milk fat yield, whereas the $DGAT1^A$ allele increases both milk and protein yield (Kaupe *et al.* 2007; Thaller *et al.* 2003).

According to our finding *PPARGC1A* $(c.1892)^T$ allele decreased the EBV_{FP} by -0.37 ± 0.01. In addition, an association of the *PPARGC1A* $(c.1892)^T$ allele with higher fat yield has been suggested in German Holsteins (Weikard *et al.* 2005). Alim *et al.* (2012) indicated that *PPARGC1A* $(c.1892)^T$ allele increased protein yield and protein concentration but there was no association between *PPARGC1A* $(c.1892)^T$ allele and fat yield (%, kg).

CONCLUSION

Milk and its products are regarded as the most important nutritional resource, meeting the energy requirements and offering high quality protein and various vitamins and minerals. Earlier, most genetic improving programs of agriculturally important livestock population have been carried out through complete phenotypic and pedigree information. However, applying molecular genetic information in breeding stock may lead to a better understanding of quantitative traits. Briefly, the results show that there is significant association between *PPARGC1A*, *DGAT1* and EBV_{FP} trait. Generally, detection and estimation of associations of identified genes and genetic markers with economic traits are the basis of a successful application of marker-assisted selection (MAS) in breeding programs. The MAS strategies

can be used for pre-selection of young bulls prior to progeny test.

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