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HUMAN GENETICS

Genetic Variation in Hypothalamic-Pituitary Axis Candidate Genes and Their Effects on Milk Production Traits in Iranian Holstein Cattle

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Abstract—This study investigated the polymorphism of hypothalamic-pituitary axis genes and their effects on milk production traits. To this end, we used genomic information of 134 animals. The studied genes were prolactin (PRL), growth hormone (GH), pituitary transcription factor (Pit-1), and signal transducers and activators of transcription (STAT5a). Genomic DNA was isolated from semen, and the existing genotypes for all studied genes were identified using PCR-RFLP assay. The frequencies of the genotypes AA, AG, and GG of PRL-*Rsa*I, LL, LV, and VV of GH-*Alu*I, AA, AB, and BB of Pit1-*Hin*fI, and TT, TC, and CC of STAT5a-*Ava*I were 0.754, 0.246, and 0, 0.873, 0.127, and 0, 0.142, 0.358, and 0.500, and 0, 0.261, and 0.739, respectively. The genotypes of PRL-*Rsa*I, GH-*Alu*I, and STAT5a-*Ava*I were in the Hardy–Weinberg equilibrium ($P > 0.05$), except for Pit1-*Hin*fI ($P < 0.05$). The association of Pit1-*Hin*fI and STAT5a-*Ava*lI gene polymorphisms with milk fat yield (MFY) and milk protein yield (MPY) was significant ($P < 0.05$). However, no effective association ($P < 0.05$) was observed between genotypes and other studied traits. The results suggest that Pit1-*Hin*fI and STAT5a-*Ava*lI polymorphisms can be used as possible candidates in selecting some milk protein production traits, such as MFY and MPY.

Keywords: hypothalamic-pituitary axis, PCR-RFLP, cattle, polymorphism, breeding value of bulls

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INTRODUCTION

The main goal of the dairy cattle industry is to improve milk quantity and quality through selection programs. In addition to conventional selection methods based on phenotype information, marker-assisted selection methods can be used as a fundamental strategy in animal breeding programs [1]. Although milk yield and composition traits are polygenic traits, the effects of some candidate genes, such as prolactin (PRL), growth hormone (GH), pituitary transcription factor (Pit-1), and signal transducer and activator of transcription 5A (STAT5A), on the physiological pathway of those traits have been identified [2–4]. Such genes are related to endocrine systems that have a physiological influence on milk yield and composition.

The PRL gene consists of 5 exons and 4 introns and encodes a polypeptide with 199 amino acids associated with reproduction [5, 6], mammary gland growth, lactation initiation, milk yield and content [6–11]. Then, PRL could be an outstanding candidate gene to affect milk production traits [9, 12, 13]. The GH gene contains 5 exons [14] and encodes a single chain polypeptide with 191 amino acids released from the pituitary gland. This gene is essential for growth, fertility, mammary gland development, and lactation process [2, 15–17]. The GH gene polymorphisms and their associations with production traits, especially milk yield characteristics, have been investigated by various researchers [8, 13, 18–27]. The Pit1 with 6 exons [28] encodes a protein with 291 amino acids containing DNA binding POU domain [29]. This gene activates bovine GH, thyrotrophin ß, and PRL genes [2] and is considered a candidate gene for regulating the expression of milk protein genes [30–33]. The STAT5A gene with 19 exons encodes a protein with 794 amino acids [34]. The STAT5A gene, as a member of the JAK-STAT signaling pathway, has a leading role in developing mammary glands, secreting milk, regulating lactation, and resisting infections, such as mastitis in bovine [35–38]. Moreover, the JAK-STAT pathway regulates the casein gene and balances GH and milk protein contents [39]. Additionally, STAT5A is used by the PRL gene as a mediator for the lactation process in mammals [40].

According to the abovementioned facts about the hypothalamic-pituitary axis candidate genes, in this study, we examine polymorphisms of the PRL-*Rsa*I, GH-*Alu*I, Pit1-*Hin*fI, and STAT5a-*Ava*I gene poly-

Table 1. Primer sequences used in PCR, product size and restriction enzymes

Gene	Primer sequence	Size, bp	Amplified region	Enzyme	Genomic position[1]	Genotypes and fragments sizes, bp	References
PRL	F: 5'GAGTCCTTATGAGCTTGATTCTT3' R: 5'CCTTCCAGAAGTCGTTTGTTTTC3'	156	Exon3	*Rsa*I	Chr: 23 35340238 (bp) (A→G)	AA:156 AG:156, 82, 74 GG:82, 74	[12]
GH	F: 5'GTGGGCTTGGGGAGACAGAT3' R: 5'GTCGTCACTGCGCATGTTTG3'	282	Intron4 and exon5	*Alu*I	Chr 19 48119373 (bp) (C→G) 48119473 (bp)	LL:150, 82, 50 LV:150, 132, 82, 50 VV[2]:150, 132	[46]
Pit-1	F: 5'AAACCATCATCTCCCTTCTT3' R: 5'AATGTACAATGTGCCTTCTGAG3'	451	Intron5 and exon6	*Hin*fI	Chr 1 35433311 (bp) (A→G)	AA:451 AB:451, 244, 207 BB[3]:244, 207	[47]
STAT5a	F: 5'CTGCAGGGCTGTTCTGAGAG3' R: 5'TGGTACCAGGACTGTAGCACAT3'	215	Intron6 and exon7	*Ava*I	Chr 19 42407081 (bp) (C→T)	TT:215 TC:215, 181, 34 CC:181, 34	[49]

1—Including chromosome number, A nucleotide substitution and genomic position on genome (bp) based on ARS-UCD1.2 reference genome release.
2—By substitution G with C (C→G), the amino acid leucine (Leucine/L) is converted to valine (Valine/V).
3—Molecular basis of this polymorphism was the silent mutation (A→G) located within the exon 6 of the Pit-1 gene. The A to G mutation (A→G) cause to create restriction site known as B allele in all previous studies on this genomic position.

morphisms and their associations with milk production characteristics, including milk yield (MY), milk fat yield (MFY), milk protein yield (MPY), milk fat percentage (MFP), and milk protein percentage (MPP).

MATERIALS AND METHODS

DNA Samples

In this study, 134 frozen semen related to proven Iranian Holstein and Milk Production characteristics records of 187,481 individuals from 1992 to 2015 were provided by the Animal Breeding Center of Iran. These animals were under the recording system and progeny test program of this center. DNA was extracted from frozen semen straws according to Zadworney and Kuhnlein's protocol [41]. The quality of the extracted DNA was evaluated by 1% agarose gel and spectrophotometer based on absorbance at 260 nm/280 nm.

DNA Amplification and PCR-RFLP

A PCR-RFLP method was used to determine the gene's polymorphisms. The detailed information about the target regions on the genome and their primers and enzymes for PCR reactions is presented in Table 1. A PCR for all studied genes was conducted in 25 μL containing 50 ng DNA, 10× reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20), $MgCl_2$ (2.5 mM), dNTP (200 μM), primers (5 pmol), and Taq polymerase (1 unit belonging Metabion, Germany). The amplification conditions of the studied sites are depicted in Table 2.

The digestion of PCR products was carried out in 15 μL consisting of PCR product (10 μL), Tango buffer (1.5 μL), and nuclease-free water (3.15 μL), which were similar for all genes plus *Rsa*I (5 units Biolabs, New England) for PRL, *Alu*I (4 units Metabion, Germany) for GH, *Hin*fI (10 units) for Pit-1, and *Ava*I (7 units Fermentase, Germany) for STAT5A. RFLP fragments were distinguished on 2.5% for PRL, Pit-1, and STAT5A and 3% for GH and were then visualized on an agarose gel.

Statistical Analysis

The studied loci's allele and genotype frequencies and HWE tests were calculated using GENALEX 6.4 software [42]. Table 3 shows the summary statistics for pedigree and milk production data sets. The production records of first were collected from 1992 to 2015 on 74,213 to 187,481 animals for different traits (Table 3). The breeding values (BV) of genotyped bulls for milk production traits, including MY and its MFY, MPY, MFP, and MPP contents of first lactation production period, were predicted based on a sire model using the AIREML procedure in MATVEC software [43]. The analysis model included herd-year-season as a fixed, animal as a random and calving age as a covariate effect. A fixed model was used to associate genes polymorphisms and estimated BV of the studied traits using the GLM procedure in SAS 9.4 [44]. The Tukey–Kramer's test was used to compare the least square mean results. The statistical fitted model was:

Table 2. The PCR protocols for the investigated genes

		PRL	GH	Pit-1	STAT5a
Initial denaturation		94°C_2 min			
34 cycles	Denaturation	94°C_30 s	94°C_45 s	94°C_45 s	94°C_60 s
	Annealing	59°C_45 s	62°C_60 s	56°C_60 s	61°C_60 s
	Extension	72°C_30 s	72°C_60 s	72°C_120 s	72°C_60 s
Final extension		72°C_3 min	72°C_5 min	72°C_7 min	72°C_3 min

The underlines between temperature and time means is "for."

Table 3. The phenotype and pedigree distribution for milk production traits

Traits	Number of animal in data file	Number of animal in pedigree file	Number of unknown parents in pedigree file	Means	CV, %
MY, kg	187481	274078	36663	6410	22.45
MFY, kg	175447	262244	35441	200.4	24.83
MPY, kg	74216	148709	17414	213.12	18.45
MFP, %	175417	262224	35439	3.16	16.19
MPP, %	74213	176092	26513	3.12	9.32

$$Y_{ijk} = \mu + \text{year}_i + \sum g_{j(k)} + e_{ijk},$$

where y_{ijk} is the dependent variable (Estimated BV), μ is the overall population mean for each trait, year_i is the ith birth-year, $g_{j(k)}$ is the jth genotype (j = 1, 2, 3) for kth gene (PRL, GH, Pit-1 and STAT5a), and e_{ijk} is the residual effects.

RESULTS AND DISCUSSION

A pedigree with 6 generations back was used in BV estimation with 42 to 3033 daughter records for each evaluated bull. The BV reliabilities ranged from 79.40 to 99.08 percent. The relationship among the evaluated bulls ranged from 0.0001 to 0.3900. All studied genotypes, except for Pit1-*Hin*fI, reached the Hardy–Weinberg equilibrium ($P > 0.05$).

Allele Frequency

The RFLP results showed that all studied sites were polymorphic. The allele frequencies of the genes are shown in Table 4. Based on the table, the frequencies of alleles A and G of PRL-*Rsa*I, L and V of GH-*Alu*I, A and B of Pit1-*Hin*fI, and T and C of STAT5a-*Ava*I are 0.877 and 0.123, 0.937 and 0.063, 0.321 and 0.679, and 0.131 and 0.869, respectively.

An allele frequency (0.87) for PRL-*Rsa*I was in the same range reported by Çitek et al. [45] from 0.81 to 0.90 for five studied cattle breeds, Alipanah et al. [19], and higher than 0.53 in Jersey crossbred [6], 0.65 in Holstein [9], and 0.6 in 1198 Indian dairy cows, which were calculated based on a meta-analysis of 15 published studies [26].

The L allele frequency of GH-*Alu*I was reported to be 0.85, 0.93, and 0.51 in Danish Red, Holstein, and Jersey, respectively [46], 0.884 in Holstein [4], and 0.52 in Jersey [20], which are in line with our findings (L allele frequency of 0.937) (Table 4).

The A allele frequency (0.321) was lower than the B allele for Pit1-*Hin*fI, which agrees with previous studies reporting from 0.05 in dairy Gyr breed to 0.41 in East Anatolian Red cattle [2, 47–50]. The frequency of STAT5a-*Ava*I alleles was in line with the one in previous studies [3, 51].

Polymorphisms Association with Milk Production Traits

The results of the association analysis between the genes and milk production traits are shown in Table 5.

PRL-RsaI Polymorphisms

No significant ($P > 0.05$) associations were found between the three genotypes of PRL-*Rsa*I with studied traits. Numerous studies were carried out on PRL-*Rsa*I polymorphisms and their association with milk production traits in different cattle breeds [9, 52, 53]. Our results contrast with the studies on PRL-*Rsa*I polymorphisms and their association with milk production traits (in all or at least in some studied traits) in different cow breeds [9, 52–54]. Dybus et al. [52] studied five milk production traits in three lactation periods of Jersey and Black-and-White cattle breeds. They found that Jersey cows with AA genotype in the

Table 4. Allele and genotype frequencies of PRL, GH, Pit-1 and STAT5a genes in Iranian proved bulls

Genes	Allele frequency		Genotypic frequency		
PRL	A	G	AA	AG	GG
	0.877	0.123	0.754	0.246	0
GH	L	V	LL	LV	VV
	0.937	0.067	0.873	0.127	0
Pit-1	A	B	AA	AB	BB
	0.321	0.679	0.142	0.358	0.5
STAT5a	T	C	TT	TC	CC
	0.131	0.869	0	0.261	0.739

first lactation had lower MFY and MFP. In another study, Dybus et al. [55] found a significant effect of PRL-*Rsa*I polymorphisms on MPY. Oğuzkan and Bozkurt [9] studied the association of MY, MFP, and MPP traits with PRL-*Rsa*I polymorphisms. They observed a higher MY for AA genotype in Holstein cattle [9], and similar results were obtained for MY [6, 55].

GH-AluI Polymorphisms

The GH-*Alu*I polymorphisms have been introduced as a potential candidate gene for milk yield and meat production traits. Previous studies have confirmed this fact for body weight [56, 57]. However, the association of GH-*Alu*I with milk production traits varied from no association [58–61] to significant association with higher milk yield to LL genotype [4] or higher performance for other genotypes [50, 62]. A comprehensive meta-analysis of GH-*Alu*I polymorphism effects on milk production traits by Akcay et al. [63] revealed no association between GH-*Alu*I polymorphisms and milk production traits. Our results agree with those of Akcay et al. [63] and other previous studies [26, 58–61].

Pit1-HinfI Polymorphisms

In this study, significant associations ($P < 0.05$) were detected between Pit1-*Hin*fI gene polymorphisms and MFY and MPY traits. For both traits (MFY and MPY), individuals with AB genotype yielded the highest performance, and the lowest performance for MFY and MPY was related to the AA genotype. The allele substitution means effect (B instead of A) was estimated to be 1.12 ± 0.99 kg for MFY. These confirm those of other studies that found significant associations of Pit I-*Hin*fI genotypes with MFY and MPY. Renaville et al. [48] showed the association of Pit I-*Hin*fI genotypes with MFY in Italian Holstein-Friesian bulls. De Mattos et al. [2] in Gyr cows detected two genotypes with significant differences between daughter MFY deviations of AB, BB, and AB genotypes. By contrast, Pozovnikova et al. [64] found no significant association for MFY. The association of Pit I-*Hin*fI genotypes with MPY in this study is consistent with the findings of Renaville et al. [48] and Dybus et al. [65] and is inconsistent with those of Pozovnikova et al. [64].

Furthermore, the associations of Pit I-*Hin*fI with MY, MFP, and MPP were not significant. This finding agrees with the conclusions made by Alejandra et al. [66], Pozovnikova et al. [64], and Anggraeni et al. [67] for MY, and Dybus et al. [65], Gorbani et al. [68], and Daniela et al. [69] for milk production traits. Nonetheless, Zabeel et al. [32] and Carşai et al. [70] were reported an association between Pit I-*Hin*fI and MY.

STAT5a-AvaI Polymorphisms

We detected significant associations ($P < 0.05$) between STAT5a-*Ava*I gene polymorphisms and MFY and MPY traits. More specifically, the individuals with TC genotype had a higher MFY and MPY than those with CC genotype for MFY and MPY traits. Nonetheless, no individuals were observed for TT genotype.

The allele substitution means effect (T instead of C) was 2.88 and 3.7 kg/year for MFY and MPY, respectively. These results are consistent with those reported by Selvaggi et al. [3] and Dario and Selvaggi [51], who found a significant difference between CC and TC genotypes for MFY ($P < 0.01$) and a higher MFY in CC compared to TC genotypes. Our results for MPY agree with those of Bao et al. [71], Cosier et al. [72], and Dario and Selvaggi et al. [51], who observed a higher MPY for CC than for CT genotype.

The observed difference between STAT5a-*Ava*I genotypes and MY was not significant ($P < 0.05$). This result is consistent with the findings of He et al. [73], Al-Azzawi and Al-Dulaimi [74], and Metin al. [23]. In contrast with our findings, Selvaggi et al. [3] found a significant difference ($P < 0.01$) between the CC and CT genotypes for MY. Another study by Dario and Selvaggi [51] demonstrated a higher MY for CC than

Table 5. Least square means and SE of BV for MY, MFY, MP, MFP and MPP in Iranian Holstein bulls for PRL, GH, Pit-1 and STAT5a genotypes

	Genotypes	MY, kg/year	MFY, kg/year	MPY, kg/year	MFP, %	MPP, %
PRL	AA ($n = 101$)	360.4 ± 43.4	7.53 ± 0.88	6.59 ± 0.96	–0.063 ± 0.016	–0.037 ± 0.01
	AG ($n = 33$)	372.7 ± 70.9	8.51 ± 1.44	7.88 ± 1.58	–0.051 ± 0.026	–0.030 ± 0.015
	GG ($n = 0$)	–	–	–	–	–
	P-value	0.323	0.125	0.191	0.516	0.451
GH	LL ($n = 117$)	364.4 ± 41.0	7.59 ± 0.84	7.07 ± 0.92	–0.064 ± 0.015	–0.028 ± 0.008
	LV ($n = 17$)	362.0 ± 99.6	8.65 ± 2.032	6.14 ± 2.23	–0.037 ± 0.038	–0.059 ± 0.020
	VV ($n = 0$)	–	–	–	–	–
	P-value	0.092	0.083	0.097	0.314	0.189
Pit-1	AA ($n = 19$)	232.2 ± 89.3	4.55 ± 1.81[b]	1.86 ± 1.95[b]	–0.041 ± 0.034	–0.036 ± 0.020
	AB ($n = 48$)	394.2 ± 58.0	8.89 ± 1.18[a]	8.55 ± 1.26[a]	–0.058 ± 0.022	–0.036 ± 0.013
	BB ($n = 67$)	384.3 ± 52.3	7.97 ± 1.06[a]	7.31 ± 1.11[a]	–0.068 ± 0.020	–0.036 ± 0.012
	P-value	0.194	0.047	0.034	0.182	0.328
STAT5a	TT ($n = 0$)	–	–	–	–	–
	TC ($n = 35$)	450.6 ± 69.585	9.895 ± 1.418[a]	10.112 ± 1.533[a]	−0.071 ± 0.027	−0.052 ± 0.015
	CC ($n = 99$)	333.3 ± 41.982	7.019 ± 0.856[b]	5.779 ± 0.925[b]	−0.056 ± 0.016	−0.031 ± 0.009
	P-value	0.178	0.041	0.023	0.588	0.244

CT genotypes. In the present study, while no significant differences were found between the genotypes of MFP and MPP, the individuals with TC genotype, instead of CC, had lower MFP and MPP. These results are in agreement with those of Selvaggi et al. [3], Dario and Selvaggi [51], Al-Azzawi and Al-Dulaimi [74], and Metin et al. [23]. However, He et al. [73] found significant associations between STAT5a-*Ava*I and MPP and introduced this site as a potential candidate gene for MPP selection.

CONCLUSIONS

In this study, the *loci,* including PRL-*Rsa*I, GH-*Alu*I, Pit1-*Hin*fI, and STAT5a-*Ava*I, were selected due to their importance in functional and economic traits in livestock. Although the effect of these genes on milk production traits in various cattle breeds has been studied previously, the results were inconclusive. Therefore, we examined the polymorphisms of genes and their associations with milk production characteristics. Based on the results, the association of Pit1-*Hin*fI and STAT5a-*Ava*lI gene polymorphisms with MFY and MPY was significant ($P < 0.05$). However, no effective association ($P < 0.05$) was observed between genotypes and other traits. According to our results, Pit1-*Hin*fI and STAT5a-*Ava*lI polymorphisms can be used as possible candidates in selecting some milk production traits, such as MFY and MPY. However, further associational studies and comprehensive Meta-analyses are warranted to understand the exact role of the studied genes in milk production traits.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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