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# Investigation of the promoter polymorphisms of the growth hormone (*GH1*), growth hormone receptor (*GHR*), insulin-like growth factor (*IGF-I*), and prolactin (*PRL*) genes and the correlation between gene expression and milk yields in Holstein cattle raised in Central Anatolia

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**Abstract:** The aim of this study was to investigate the promoter polymorphisms of the growth hormone (*GH1*), growth hormone receptor (*GHR*), insulin-like growth factor (*IGF-I*), and prolactin (*PRL*) genes, and the correlation of the expression levels of these genes with lactation and daily milk yields in Holstein cattle. A total of 154 lactating Holstein cows raised in the Central Anatolia Region of Turkey, with a mean age of 5.4 years and a mean body weight of 571.7 kg, constituted the study material. Each blood sample was examined by real-time PCR for mRNA expression levels and the promoter polymorphisms of the *GH1*, *GHR*, *IGF-I*, and *PRL* genes. The only statistically significant difference (P = 0.029) was observed in the daily milk yield between the *GH1* genotypes, where the TT genotype cows had higher daily milk yields than cows of the CC and CT genotypes. Moderate negative correlations were found between the expression levels of *GHR* (r = –0.490, P < 0.001), *IGF-1* (r = –0.481, P < 0.001), *PRL* (r = –0.383, P < 0.029), and the lactation milk yield. There were also low negative correlations between the expression levels of the *GHR* (r = –0.262, P = 0.007), *IGF-1* (r = –0.264, P = 0.006), *PRL* (r = –0.215, P = 0.029), and the daily milk yield. However, these relationships did not exist between the expression levels of *GH1* and lactation (r = –0.084, P = 0.386) and the daily milk yields (r = –0.043, P = 0.656). Based on the results, it is suggested that *GH1* could be used as a genetic marker in the selection of breeder animals for daily milk yield.

**Key words:** Dairy cattle, marker genes, milk yield, single nucleotide polymorphism, gene expression

## 1. Introduction

Best animal husbandry practices entail selecting animals of high genetic merit for the targeted yield traits as the parents of the next generation (1). In animal species with long generation intervals, including cattle, genetic research aimed at the improvement of yield traits using conventional selection methods can be challenging, costly, and time-consuming. However, the marker-assisted selection method, based on the use of conventional selection methods in combination with genetic markers known or considered to be associated with certain yield traits, overcomes these disadvantages (2).

It has been reported that, in cattle, the allelic structures of the growth hormone (*GH1*), growth hormone receptor (*GHR*), insulin-like growth factor (*IGF-I*), and prolactin (*PRL*) genes could be used as genetic markers for the estimation of the lactation performance of potential breeders (3).

The gene encoding *GH1*, a single-chain polypeptide of 22 kDa size secreted from the anterior hypophyseal gland, is composed of five exons and four introns and is located on the 19th chromosome of the bovine karyotype, and is approximately 1800 bp in length (3). This gene is considered a good candidate for use in genetic improvement efforts aimed at the increase of milk and meat yields (3).

The growth hormone acts by binding to growth hormone receptors found in the cells of the tissues it affects (4). Therefore, the *GHR* gene, located on the 20th chromosome in cattle, is suggested to be used as a genetic marker for the improvement of phenotypes affected by the growth hormone (4).

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IGF-I, also referred to as somatomedin, plays a key role in several physiological and metabolic processes in vertebrates (5). IGF-I contributes either directly or indirectly to the manifestation of the effect of the growth hormone (5). Owing to their significant role in the regulation of postnatal growth, both the growth hormone and IGF-I, which are found in the circulatory system, play a critical role in the control of lactation, development of the mammary gland, and the emergence of important yield traits, such as growth and fertility (5). Thus, it has been reported that the gene encoding IGF-I, which is located on the 5th chromosome of the bovine karyotype, could be used as a genetic marker in efforts aimed at the improvement of milk yield (5).

In mammals, prolactin is involved in more than a hundred physiological processes, including the development of the mammary gland, and the start and continuation of lactation by influencing lactogenesis. Prolactin is responsible for the synthesis of the proteins, lactose, and lipids found in milk (6–8). Owing to these effects, the *PRL* gene found on the 23rd chromosome of the bovine karyotype has been reported to be a potential genetic marker for milk yield in cattle (6,8,9).

A literature search revealed that a study investigating the relationship between milk production traits and the promoter regions of *GH1*, *GHR*, *IGF-I*, and *PRL* genes had yet to be conducted. Therefore, the present study could be the first to investigate the nearest transcription binding sites, single nucleotide polymorphisms (SNPs), and polymorphisms in promoter regions of the *GH1*, *GHR*, *IGF-I*, and *PRL* genes and the correlation between gene expression levels lactation and daily milk yield in Holstein dairy cattle raised in the Central Anatolia Region of Turkey.

## 2. Materials and methods

### 2.1. Animal material

A total of 154 lactating Holstein cows with a mean age of 5.4 years and a mean body weight of 571.7 kg with no inbreeding in a farm in Central Anatolia were used in the study. Blood samples taken from the tail vein of the cows were used for the DNA and RNA analysis. All milk yield records of prior periods were kept for those cows.

### 2.2. Expression studies by quantitative real-time PCR

Total RNA was isolated from the cells of venous blood samples using TRIzol reagent (Roche, Mannheim, Germany). Concentration and purity of RNA for each sample were confirmed using the Nanodrop 1000 (Thermo Scientific, San Jose, CA, USA). RNA samples were stored at −80 °C until used. Total RNA (1 µg) served as a template for first-strand cDNA synthesis in a 20-µL reaction using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNA was amplified for 30 min at 55 °C and then heated for 5 min at 85 °C. Quantitative real-time PCR (qPCR) was performed using the Roche 480 Light Cycler for the *GH1*, *GHR*, *IGF-1*, and *PRL* genes. All samples were analyzed in duplicate. The qPCR data were analyzed using the delta cycle threshold method. The cycle threshold for each sample was calculated and relative mRNA abundance was determined based on that of the β-actin control (10). Normalizations of expression data were carried out using the comparative Cq method (11).

### 2.3. DNA extraction and genotyping

Genomic DNA was tested from with a High Pure PCR Template Preparation Kit (Roche). The final DNA concentration was determined with a Nanodrop 1000 (Thermo Scientific). Each PCR reaction used 100 ng of DNA. SNPs were selected in the promoter region of the nearest point on or in these regions of the *GH1*, *GHR*, *IGF1*, and *PRL* genes transcription binding sites according to the National Center for Biotechnology Information (NCBI) database (dbSNP, NCBI). Selected SNPs, rs numbers, and probe sequences are given in Table 1. Appropriate probes were designed (Roche) and used for SNP analysis of *GH*, *GHR*, *IGF1*, and *PRL* genes and the study was performed in a LightCycler 480 (Roche) device.

### 2.4. Statistical analyses

The study group was tested for suitability of all the genes identified genotype frequencies of the Hardy–Weinberg equilibrium (HWE) with the chi-squared test. Differences in genotypes and gene expression levels with the lactation and daily milk yield levels were compared with one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The Pearson correlation coefficients of the logarithm (log10) of the expression levels of the selected *GH1*, *GHR*, *IGF-I*, and *PRL* genes with the lactation milk yield and average daily milk yield of the dairy cows were also calculated. In statistical analysis, SPSS 14:01 (SPSS Inc., Chicago, IL, USA) was used.

## 3. Results

### 3.1. Allelic and genotypic frequencies of the *GH1, GHR, IGF-I,* and *PRL* genes

The population examined in this study using the PCR technique was found to be in the HWE for these four genes (Table 2).

The PCR results indicated a higher genotype CC frequency of 72.7% and an allele C frequency of 84.7% for the *GH1* gene; a higher genotype AG frequency of 49.3% and an allele G frequency of 60.7% for the *GHR* gene; a higher genotype CT frequency of 53.2% and an allele C frequency of 51.3% for the *IGF-I* gene; and a higher genotype AA frequency of 87.6% and an allele A frequency of 93.3% for the *PRL* gene. No statistically significant difference was determined between the *GH1*, *GHR*, *IGF-I*, and *PRL* genes for genotype and expression level (Table 3).

**Table 1.** Accession numbers of the SNP mutation types of *GH1*, *GHR*, *IGF-I*, and *PRL* genes and nucleotide sequence of probes.

| Gene | Accession Number | Mutation | Nucleotide sequences of probes |
|---|---|---|---|
| *GH1* | rs135233632 | C/T single-nucleotide variation | F-CACCTGTGTGCTCTATACATTTATGC |
| | | | R-TGACAAGCCTGCGGGACAT |
| *GHR* | rs136797458 | A/G single-nucleotide variation | F-GCCAGAGATCCATACCATACCTGTAGGAC |
| | | | R-GGGCAAGAATGTGGAAATCTAGTG |
| *IGF1* | rs109763947 | C/T single-nucleotide variation | F-CCTCACTTGGCAACCAGG |
| | | | R-AAAATACTGATGCTGTCCTCTGATT |
| *PRL* | rs110790201 | A/G single-nucleotide variation | F-AAATCTTGACTTCAGCCAGCAAT |
| | | | R-ACCAGAAATGAACATCTAGGAAGGAT |

**Table 2.** Allelic and genotypic frequencies of *GH1*, *GHR*, *IGF-I*, and *PRL* genes.

| Genes | | Genotypes | | | Allele frequency (%) | | Statistical significance (chi-squared HWE) |
|---|---|---|---|---|---|---|---|
| GH1 | | CC | CT | TT | C | T | $X^2 = 0.78$ P = 0.37 (df = 1) |
| | Observed | 112 | 37 | 5 | 84.7 | 15.3 | |
| | Expected | 110.6 | 39.8 | 3.6 | | | |
| *GHR* | | AA | AG | GG | A | G | $X^2 = 0.10$ P = 0.74 (df = 1) |
| | Observed | 24 | 76 | 54 | 39.3 | 60.7 | |
| | Expected | 24.9 | 74.8 | 54.9 | | | |
| *PRL* | | AA | AG | GG | A | G | $X^2 = 0.21$ P = 0.64 (df = 1) |
| | Observed | 135 | 18 | 1 | 93.3 | 6.7 | |
| | Expected | 134.6 | 18.7 | 0.6 | | | |
| *IGF-1* | | CC | CT | TT | C | T | $X^2 = 0.41$ P = 0.66 (df = 1) |
| | Observed | 38 | 82 | 34 | 51.3 | 48.7 | |
| | Expected | 40.5 | 76.9 | 36.5 | | | |

HWE: Hardy–Weinberg equilibrium; $X^2$: chi-square value; df: degree of freedom.

### 3.2. Comparison of milk yields for the *GH1, GHR, IGF-I,* and *PRL* genotypes

In the present study, no statistically significant difference was determined between the *GH1*, *GHR*, *IGF-I*, and *PRL* genes for genotype and lactation and daily milk yields, except that the *GH1* gene had influence on the daily milk yield of the cows. Among the cows examined in this study, those with a *GH1*-TT genotype had an average daily milk yield higher than those of the cows with the CC and CT genotypes (P = 0.029) (Table 4).

### 3.3. Correlation between the expression levels of the *GH1, GHR, IGF-I,* and *PRL* genes and milk yields

The correlations of the logarithm (log10) of the expression levels of the selected *GHR*, *IGF-I*, and *PRL* genes with the lactation and daily milk yields of the cows in this study were found to be statistically significant. The correlations were all negative and ranged from low (daily milk yield) to moderate (lactation milk yield). However, these relationships did not exist between the expression levels of the *GH1* and the lactation and the daily milk yields (Table 5).

**Table 3.** Comparison of the results according to genotype and expression (log10).

| Genes | Genotype | N | Expression (log10) $\overline{X} \pm S\overline{x}$ |
|---|---|---|---|
| *GH1* | CC | 112 | –0.186 ± 0.099 |
| | CT | 37 | –0.071 ± 0.076 |
| | TT | 5 | 0.020 ± 0.482 |
| Statistical significance control (ANOVA) | | | F: 0.576 P = 0.563 |
| *GHR* | AA | 24 | –3.464 ± 0.165 |
| | AG | 76 | –3.387 ± 0.083 |
| | GG | 54 | –3.346 ± 0.101 |
| Statistical significance control (ANOVA) | | | F: 0.205 P = 0.815 |
| *IGF-I* | CC | 38 | –3.778 ± 0.124 |
| | CT | 82 | –3.905 ± 0.085 |
| | TT | 34 | –3.779 ± 0.144 |
| Statistical significance control (ANOVA) | | | F: 0.498 P = 0.609 |
| *PRL* | AA | 135 | –5.858 ± 0.094 |
| | AG | 18 | –5.588 ± 0.311 |
| | GG | 1 | –5.281 |
| Statistical significance control (ANOVA) | | | F: 0.595 P = 0.553 |

$\overline{X} \pm S\overline{x}$ : mean ± standard error

**Table 4.** A comparison of lactation and daily milk yields by genotype.

| Genes | Genotype | Lactation milk yield (L) $\overline{X} \pm S\overline{x}$ | Daily milk yield (L) $\overline{X} \pm S\overline{x}$ |
|---|---|---|---|
| *GH1* | CC | 6218.7 ± 226.9 | 22.0 ± 0.8[a] |
| | CT | 6098.1 ± 347.9 | 19.6 ± 1.5[a] |
| | TT | 5791.5 ± 139.5 | 33.0 ± 3.0[b] |
| Statistical significance control (ANOVA) | | F: 0.079 P = 0.924 | F: 3.65 P = 0.029 |
| *GHR* | AA | 7113.6 ± 561.9 | 25.4 ± 1.4 |
| | AG | 6105.5 ± 282.3 | 22.1 ± 1.1 |
| | GG | 5964.5 ± 282.8 | 19.9 ± 1.1 |
| Statistical significance control (ANOVA) | | F: 1.55 P = 0.218 | F: 2.64 P = 0.076 |
| *PRL* | AA | 6178.9 ± 192.5 | 21.9 ±0.8 |
| | AG | 6630.5 ± 638.4 | 21.0 ± 1.7 |
| | GG | 6480 | 23.0 |
| Statistical significance control (ANOVA) | | F: 3.33 P = 0.716 | F: 0.92 P = 0.912 |
| *IGF-1* | CC | 6184.8 ± 430.5 | 20.5 ± 1.3 |
| | CT | 6320.4 ± 246.1 | 21.3 ±1.0 |
| | TT | 5738.4 ± 379.7 | 23.4 ± 1.6 |
| Statistical significance control (ANOVA) | | F: 0.72 P = 0.488 | F: 1.03 P = 0.361 |

$\overline{X} \pm S\overline{x}$ : mean ± standard error; [a,b]: the difference between the averages in the same column with different letters are statistically significant.

**Table 5.** The expression of genes (log10) correlates with the results of lactation and daily milk yields.

| Expression (log10) | Lactation milk yield (L) | | Daily milk yield (L) | |
|---|---|---|---|---|
| | r | P-value | r | P-value |
| *GH1* | –0.084 | 0.386 | –0.043 | 0.656 |
| *GHR* | –0.490 | <0.001 | –0.262 | 0.007 |
| *IGF-1* | –0.481 | <0.001 | –0.264 | 0.006 |
| *PRL* | –0.383 | <0.001 | –0.215 | 0.029 |

L: liter, r: Pearson correlation coefficient.

## 4. Discussion

All livestock breeders aim at improving the yields of their herds and increasing the income of their holdings. However, only considering individual phenotype in selection aimed at the improvement of quantitative traits would reduce the efficacy and success of selection efforts. The hypothesis that the variations in certain genes involved in physiological processes that affect yield traits in sexes could be related to yield traits (1) introduced a novel approach to genetic improvement research in livestock. In the present study, owing to their serving as control points in the regulation of gene transcription, the *GH1*, *GHR*, *IGF-I*, and *PRL* genes were investigated for promoter polymorphisms. The correlations between the expression levels of these genes and the lactation and daily milk yields of the cows were also investigated.

In a study conducted by Kovács et al. (3), on the basis of the determination of their having no effect on milk yield traits in Holstein cattle, it was suggested that *GH1-Alu*I polymorphisms could not be used for genetic improvement. Similarly, Khatami et al. (9) ascertained that, in the Holstein and Yaroslavl breeds, *GH1-Alu*I polymorphisms were not related to milk yield traits. However, these researchers determined that the percentage of animals with a high milk yield (producing 6000 kg of milk in each lactation period) among the individuals with a VV genotype was 1.75 times greater than the percentage of animals with a high milk yield among the individuals with a LL genotype and 2.5 times greater than the same percentage of animals among the individuals with a LV genotype.

Furthermore, Grochowska et al. (12) reported that, in Holstein and Simmental cattle, the *GH1-Alu*I-LV genotype was positively related to milk yield. On the other hand, Heidari et al. (13) demonstrated that individuals with a VV genotype had milk yields higher than those of individuals of other genotypes, but also indicated that this difference was statistically insignificant. In their study, in which they investigated the correlation between the *GH1-Msp*I polymorphism and milk yield in Holstein cattle, Zhou et al. (14) determined that individuals with an AA genotype had a higher milk yield than individuals with an AB genotype, and suggested that the *GH1-Msp*I polymorphism could be used for the improvement of milk yield traits.

In the present study, it was determined that, when evaluated for the SNP detected in the promoter region of the *GH1* gene, the daily milk yield of individuals with a TT genotype was higher than that of individuals of the other genotypes. Therefore, the results obtained in the present study are in support of previous research reporting the *GH1* gene to only be statistically significant with milk yield traits in cattle. Thus, it is suggested that the *GH1* locus could be used for the genetic improvement of dairy cattle herds for milk yield. Nevertheless, in the present study, the correlation between the expression level of the *GH1* gene and milk yields was statistically insignificant.

Some literature reports suggest that, in cattle, *GHR-Alu*I polymorphism is related to milk yield and milk composition (15). Similarly, several studies carried out in different cattle breeds have demonstrated that the *GHR*-F279Y polymorphism detected in the exon region of the *GHR* gene is also related with milk yield and milk composition (16).

Neither was a statistically significant correlation found between gene expression levels and milk yield. In view of the role of the receptor encoded by this gene in the lactation process, further research is needed to elucidate the correlation of the polymorphisms of this gene with milk yield and other traits.

Owing to its localization to the somatotropic axis and its direct or indirect contribution to the emergence of the effects of *GH1*, the *IGF-1* gene has been reported to be able to be used as a genetic marker for milk yield (12) and meat yield (17) in cattle.

In a study carried out in Holstein cattle on the correlation of adenine/cytosine substitution in the promoter region of the *IGF-1* gene with milk yield traits,

it was ascertained that milk yield varied with the different genotypes (18).

However, in an investigation into the correlation of the 10 SNPs of the *IGF-1* gene with milk yield in Holstein dairy cows, of the SNPs detected, only the *IGF1i3* SNP was found to be related to milk yield increase (5). On the other hand, Siadkowska et al. (17) reported that, in Holstein cattle, no correlation existed between the *IGF-1-Sna*BI genotype and daily milk yield. In the present study, it was also ascertained that the *IGF-1-Sna*BI polymorphism was not statistically significant with lactation and daily milk yields. Furthermore, a statistically significant correlation existed between the expression level of the *IGF-1* gene and milk yields.

The potential of using the gene encoding prolactin as a marker gene for milk yield in mammals has led to the investigation of the correlation of this gene with milk yield. However, studies on the correlation between the *PRL* gene and milk yield have produced varying results. Khatami et al. (9) reported that in cows with high milk yield (>6000 kg), the percentage of individuals with a BB genotype was 9% to 10% lower than the percentages of individuals with the AA and AB genotypes. Furthermore, Alipanah et al. (8) reported that the milk yield of individuals with a BB genotype was higher than those of individuals of the AA and AB genotypes.

Dybus et al. (7) suggested that the *PRL* gene was not related to milk yield traits. Furthermore, Brym et al. (6) indicated that, while the *PRL-Rsa*I genotype was related to milk yield in the first lactation period in Holstein cattle, this was not true for Jersey cattle. On the other hand, Chrenek et al. (19) reported that, in the Brown Swiss, different *PRL-Rsa*I genotypes were not related to milk yield traits.

In the present study, it was also determined that, in Holstein cattle, the comparisons of the *PRL* genotypes and the expression level of the *PRL* gene with lactation and daily milk yields were statistically insignificant.

The present study demonstrated that in 154 Holstein cattle raised in Central Anatolia, of the SNPs detected in the promoter regions of the gene encoding four different proteins influential on milk yield, only the SNP located in the promoter region of the *GH1* gene was compared with milk yield. This result is in agreement with previous studies reporting that the *Alu*I and *Msp*I polymorphisms of the *GH1* gene are related to milk yield and milk yield traits in various cattle breeds. Furthermore, the present study demonstrated that a correlation existed between the expression levels of the *GHR*, *IGF-I*, and *PRL* genes and milk yields.

As a result, we determined that there is no statistically significant importance between the promoter polymorphisms of the *GH1*, *GHR*, *IGF-I*, and *PRL* genes and gene expression levels. On the other hand, of the *GH1*, *GHR*, *IGF-I*, and *PRL* genes investigated in the present study due to their potential of being used as marker genes for milk yields in view of their role in the development of the mammary gland, the start and continuation of lactation, and the growth and development processes, only the *GH1* gene (the TT genotype) was found to not be statistically significant with average milk yield increase. Previous research demonstrated that, in cattle, an *Msp*I polymorphism in the 3rd intron of the *GH1* gene (3) and an *Alu*I polymorphism in the 5th exon of the same gene (7) were related to milk yield traits. Similarly, in Holstein cattle raised in Turkey, the promoter polymorphisms of the *GH1* gene were ascertained to have a statistically significant relationship with daily milk yields.

In conclusion, based on the results obtained for the *GH1* gene in cattle, both in the present study and in previous research, the *GH1* gene can be used in the selection of breeder animals for milk yield traits. However, we have determined that the promoter polymorphisms of the *GHR*, *IGF-I*, and *PRL* genes and milk yields are not statistically significant. Further research is needed to fully elucidate the relation between these genes and milk yield traits.

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