



Detection of Single-Nucleotide Polymorphism in *AGPAT6* Gene, Associated with Milk Fat Content, using Tetra-Primer ARMS PCR-Based Assay, in Karan Fries Breeding Bulls

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Background: The bovine *AGPAT6* gene is one of the potential candidate genes governing milk fat synthesis.

Objectives: Identification of single nucleotide polymorphisms (SNP) in the targeted region of *AGPAT6* gene and their effect on expected breeding values (EBV) of first lactation milk production traits viz. fat %, fat yield and 305 days milk yield in Karan Fries (KF) breeding bulls were sought.

Materials and Methods: A tetra-primer ARMS PCR technique was adapted to genotype an SNP, g.36,175,805C>T located on 5' flanking region of *AGPAT6* gene. The relationship between EBV of milk production traits and polymorphic locus of *AGPAT6* gene was assessed.

Results: Three kinds of genotype (CC, CT, and TT) with respect to g.36,175,805C>T SNP locus were observed. The identified SNP had significant ($P < 0.05$) influence on EBV of fat % (EBV-FP). The KF bulls with CC and CT genotype had comparatively higher EBV-FP than the bulls with the TT genotype. The substitution of "C" allele by "T" allele led to a decrease of 0.0045 % in the EBV-FP.

Conclusion: The identified SNP was significantly associated with EBV-FP, thus it may be utilized as a molecular marker for developing marker-assisted selection strategy to enhance the milk fat content in KF cattle population.

Keywords: *AGPAT6* gene, Allele substitution effect, Karan Fries bulls, Milk fat content, SNP, T-ARMS PCR

1. Background

Milk fat content is an important economic trait and has received major emphasis among the milk constituents in dairy breed improvement programs. It follows the quantitative inheritance pattern and is governed by a large number of genes at the molecular level. The single nucleotide polymorphisms (SNPs) in candidate genes governing the milk fat synthesis have largely been studied as they may be utilized as molecular markers for developing marker-assisted selection strategy for early evaluation of animals. Milk fatty acid synthesis is a complex process and involves sequential esterification of the glycerol chain catalyzed by a variety of acyltransferases (1). The sn-1-acylglycerol-3-phosphate-O-acyltransferases (AGPATs) enzymes play a crucial role in milk fat synthesis by catalyzing an intermediary step of esterification at the sn-2 position of glycerol-3-phosphate (1-3). A total of

11 isoforms of AGPATs are known in human beings, each of which is encoded by independent genes (4, 5). However, the *AGPAT6* is the most abundant isoform in bovine mammary glands (6). The *AGPAT6* has also been recognized as microsomal glycerol-3-phosphate acyltransferase (GPAT) and renamed as *GPAT4* (7, 8). Bovine *AGPAT6* gene is located on *Bos taurus* autosome 27 (BTA-27), comprising 14 exons and spans about 30965 base pair in length (<https://www.ncbi.nlm.nih.gov/gene/511614>). At the cellular level, the expression of *AGPAT6* was found to be strongly up-regulated during lactation (3, 6). Knockout of the *AGPAT6* gene in mice produces animals with underdeveloped alveoli and ducts of mammary glands, and the milk from double knockout animals is markedly depleted in diacylglycerols and triacylglycerols (3). In few studies conducted in taurine cattle, the *AGPAT6* gene has been reported as one of the potential candidate genes, and

SNPs located especially in 5' flanking and promoter regions of *AGPAT6* gene were highly significantly associated with milk fat content (9-12). However, reports on genetic polymorphisms in *AGPAT6* gene are largely limited and no previous report is available on *AGPAT6* gene polymorphism in indigenous cattle breeds.

2. Objectives

The present study was conducted to identify SNPs in 5' flanking region of *AGPAT6* gene in Karan Fries (KF) breeding bulls and to assess their effect on breeding values of first lactation milk production traits viz. fat %, fat yield and 305 days milk yield.

3. Materials and Methods

3.1. Data Source

The present study was carried out on KF cattle kept under progeny testing program at National Dairy Research Institute (NDRI), Karnal, Haryana, India. The KF is an Inter se mating is the mating of individuals with same level of genetic inheritance. Such as mating of individuals of first filial generation (F1) that produces second filial generation (F2) is inter se mating. crossbred population developed as a milch breed by crossing Holstein Friesian (*B. taurus*) and Tharparkar (*B. indicus*) cattle at NDRI farm. The geographic location of NDRI livestock farm is at an altitude of 250 meters above mean sea level in the Indo-Gangetic Alluvial Plains on 29.7014° N latitude and 76.9848° E longitude. Cows were provided with ad libitum feeding refers to offering a feed to animals as much as they desire, rather than putting any restriction on feeding. seasonal green fodder and roughages, and an additional amount of 1.0 kg concentrate mixture (20 % CP and 3400 kcal/kg DE) for every 2.5 kg milk produced above 5.0 kg daily milk yield.

3.2. Phenotypic Information

11,569 first lactation monthly test day milk production records of 1394 KF cows, sired by 120 KF bulls and calved between 1989 and 2014 were analyzed in the present study. Milk production data on 305 days milk yield and monthly test day milk fat % were collected. Cows those had produced milk for at least 100 days and more than 500 kg, and had first test day record before 65 days after calving in their first lactation, were included in the study. All the test day fat % records of each cow were averaged (weighted arithmetic mean) to obtain average first lactation milk fat %. First lactation fat yield was estimated by the formulae, first lactation fat yield (kg) = (average fat % × 305 days milk yield in kg)/100. Expected breeding values (EBVs) of all 120 KF bulls

for first lactation traits viz. fat %, fat yield and 305 days milk yield (symbolized as EBV-FP, EBV-FY, and EBV-305dMY, respectively) were estimated based on daughters performance by a multivariate BLUP-Animal model using Wombat software package (13). The animal model used for the analysis was as follows:

$$Y_{ijklm} = \mu + Yr_i + S_j + AG_k + SL_l + A_m + e_{ijklm}$$
 where, Y_{ijklm} : observation on m^{th} animal; μ : overall mean; Yr_i : fixed effect of i^{th} year of calving (1989 to 2014); S_j : fixed effect of j^{th} season of calving (winter, summer, rainy and autumn); AG_k : fixed effect of k^{th} age at first calving group (≤ 30 , 31-36 and ≥ 37 months); SL_l : fixed effect of l^{th} stage of lactation (< 90 , 91-180, > 180 days); A_m : random effect of m^{th} animal; and e_{ijklm} : random error, NID (0, σ_e^2).

3.3. DNA Extraction and Genotyping

Genomic DNA was isolated from the frozen semen straws of 56 KF bulls, amongst the 120 bulls whose breeding values were estimated. Three mini semen straws (0.25 mL) from each bull were used for DNA isolation and the protocol used for DNA isolation involved two steps: Lysis and extraction. Lysis of spermatozoa was done as per Hossain *et al.* (14) with some modifications while the extraction step was done using a standard Phenol Chloroform extraction. The SNP, g.36,175,805C>T (db SNP id: rs110878096), located on 5' flanking region of *AGPAT6* gene (NCBI Reference Sequence: AC_000184.1, https://www.ncbi.nlm.nih.gov/nuccore/AC_000184.1?report=genbank) was genotyped using Tetra-Primer Amplification Refractory Mutation System Polymerase Chain Reaction (T-ARMS PCR) technique. Primers were designed using Primer 1 software. The sequence of primers used in the present study was as follows: outer forward, 5'-AATCAGGCTCTAGAAGCACAGGTGAATT-3'; outer reverse, 5'-AGGCTAGAACTCACACAACTGCAAAGAG-3'; inner forward, 5'-TGGTCCTGAAAATTTCAAATTTGTACAC-3'; and inner reverse, 5'-CAGGTGCCTGGATTGCTTTAATCGAA -3'. The expected polymerase chain reaction (PCR) fragment size for g.36,175,805 C>T mutation were 168 bp for C allele, 131 bp for T allele, and 246 bp for the common outer fragment. The PCR was performed in a total volume of 25 μ L containing approximately 100 ng DNA, 2.5 μ L of 10X buffer, 2 mM of $MgCl_2$, 0.2mM dNTPs, 10 pM of each of outer and inner pair of primer and 1 U of Taq Polymerase (Sigma-Aldrich, USA). The amplification conditions (Touchdown reactions) were: Initial denaturation at 95 °C for 2 minutes, 5 cycles of denaturation at 95 °C for 15 seconds, with annealing temperature of 60 °C for the first cycle, decreasing by 1

°C per cycle until annealing temperature of 56 °C was reached for 15 seconds and extension at 72 °C for 20 seconds, followed by 25 cycles of denaturation at 95 °C for 15 seconds, with annealing temperature of 55 °C for 15 seconds and extension at 72 °C for 20 seconds, and final extension at 72 °C for 5 minutes. The accuracy and efficiency of the T-ARMS PCR assay were evaluated by DNA sequencing (M/s. First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia) of 246 bp outer band product of three samples for each genotype.

3.4. Statistical Analysis

The allelic and genotypic frequencies and their accordance with Hardy-Weinberg equilibrium were studied using POPGENE software package (15). The relationship between EBV of milk production traits and polymorphic locus of *AGPAT6* gene was analyzed by GLM procedure of SAS 9.3 (Statistical Analysis System Institute Inc., Cary, NC, USA) using the following model:

$$Y_{ijk} = \mu + G_i + Sr_j + e_{ijk}$$

where, Y_{ijk} : EBV-FP, EBV-FY and EBV-305dMY of k^{th} bull; μ : overall mean; G_i : fixed effect of i^{th} genotype; Sr_j : fixed effect of j^{th} sire; and e_{ijk} : random error, NID (0, σ_e^2). The allele substitution effect of polymorphic locus of *AGPAT6* genes was estimated by GLM procedure of SAS 9.3 using the following model:

$$Y_{ij} = \mu + \beta SNP_i + e_{ij}$$

where, Y_{ij} : EBV of j^{th} bull; μ : overall mean; SNP_i : number of copies of 1 allele of SNP (corresponding to 0, 1 and 2 copies) carried by j^{th} bull; β : regression coefficient for the SNP considered (also known as allele substitution effect); and e_{ij} : random error, NID (0, σ_e^2)

4. Results

4.1. SNP Genotyping

Genotype profiling of KF breeding bulls revealed three kinds of genotype viz. CC (168 and 246 bp), CT (168, 131 and 246 bp) and TT (131 and 246 bp) with respect to SNP locus, g.36,175,805C>T (Fig. 1). The presence of SNP scored using T-ARMS PCR assay was further confirmed by sequencing the outer band product (246

bp) of each genotype. It indicated that the genotypes scored by the assay were 100 % similar to the DNA sequencing. The chromatogram representing respective genotypes is depicted in Figure 2.

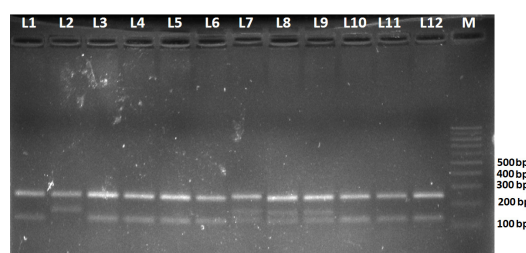


Figure 1. Agarose gel electrophoresis of the tetra-primer ARMS PCR based assay products of identified SNP, g.36,175,805C>T in Karan Fries bulls Lane 1, 3,4,5,6,10,11 and 12, TT genotype (131 and 246 bp); lane 2, CC genotype (168 and 246 bp); lane 7-9, CT genotype (168, 131 and 246 bp); lane M 100-bp marker ladder

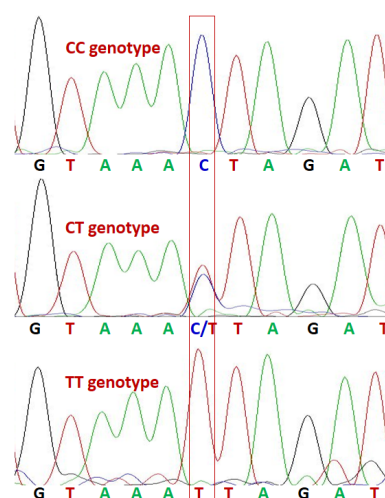


Figure 2. Chromatogram showing CC, CT and TT genotypes with respect to the SNP locus, g.36,175,805C>T in *AGPAT6* gene in Karan Fries bulls

4.2. Test of Hardy-Weinberg Equilibrium

The allele and genotype frequencies and the chi-square test for Hardy-Weinberg equilibrium for the SNP locus, g.36,175,805C>T of the *AGPAT6* gene are presented in Table 1. The frequencies of C and T alleles were 0.2 and 0.8; and the frequencies of CC, CT, and TT genotypes were 0.07, 0.25 and 0.68, respectively. The results of the chi-square test revealed that the KF bull population was in Hardy-Weinberg equilibrium.

Table 1. Allele and genotype frequency, and chi-square test for Hardy-Weinberg equilibrium for the SNP locus, g.36,175,805C>T of the *AGPAT6* gene in Karan Fries bulls

Allele frequency	Genotype frequency	Chi-square (χ^2) value	P-value	Interpretation
C 0.2	CC 0.07 (4)	2.70	0.10NS	H-W Equilibrium
T 0.8	CT 0.25 (14)			
	TT 0.68 (38)			
Total	56			

Figures in parenthesis indicate number of observations

4.3. Association Analysis

The EBV-FP, EBV-FY, and EBV-305dMY of KF bulls with respect to different genotypes are presented in **Table 2**. It revealed that SNP locus, g.36,175,805C>T had significant ($P<0.05$) influence on EBV-FP. The KF bulls with CT genotype followed by CC genotype had comparatively higher EBV-FP than the bulls with the TT genotype. However, the EBV-FY and EBV-305dMY

didn't vary significantly with respect to different genotypes (**Table 2**).

The allele substitution effect of SNP locus, g.36, 175, 805C>T was also analyzed and is depicted in Table 3. It revealed that substitution of "C" allele of *AGPAT6* by "T" allele led to a significant ($P<0.05$) decrease of 0.0045 % of in the EBV-FP. The allele substitution effect was not significant for EBV-FY and EBV-305dMY.

Table 2. Mean expected breeding values of milk production traits with respect to different genotypes of SNP locus, g.36,175,805C>T of *AGPAT6* gene in Karan Fries bulls

Effect	N	EBV-FP (%)	EBV-FY (kg)	EBV-305dMY (kg)
Overall	56	4.197 ± 0.005	129.216 ± 3.175	3083.049 ± 64.013
CC	4	4.196 ± 0.014 ^a	127.214 ± 9.319	3042.865 ± 187.872
CT	14	4.207 ± 0.007 ^a	131.022 ± 4.273	3127.117 ± 86.141
TT	38	4.187 ± 0.003 ^b	129.412 ± 2.131	3079.165 ± 42.956

EBV-FP: expected breeding value of milk fat %, EBV-FY: expected breeding value of fat yield, EBV-305dMY: expected breeding value of 305 days milk yield

^{ab}Means bearing different superscripts between rows within column differ significantly ($P<0.05$)

5. Discussion

Identification of genotypes significantly associated with milk fat content will help in the early evaluation of bulls at a younger age. In the present study, we have detected and screened the KF breeding bull population for the SNP locus viz. g.36,175,805C>T located on 5' flanking region of *AGPAT6* gene using T-ARMS PCR assay. As per the *Bos_taurus_UMD_3.1.1* whole genome shotgun sequence data available on NCBI (NCBI Reference Sequence: AC_000184.1), the SNP locus, g.36,175,805C>T is mapped around 16.7 kbp downstream of *GINS4* and 22.2 kbp upstream of *AGPAT6* gene on BTA- 27 (https://www.ncbi.nlm.nih.gov/nucore/AC_000184.1?report=genbank). A genome-wide association study conducted earlier in exotic crossbred cattle populations, also reported g.36,175,805C>T SNP (10). However, no report is available on genetic polymorphism in *AGPAT6* gene in indigenous cattle breeds.

The association analysis of identified genotypes was carried out with the breeding values of bulls for various milk production traits as the breeding value is the inheritable part of an individual's genotypic value that is transmitted from parents to offspring. The results revealed that the EBV-FP differed significantly ($P<0.05$) among

different genotypes with respect to the targeted SNP. The heterozygous genotypes were found superior for all the three traits studied. Between the homozygous genotypes, CC genotype was found superior to the TT genotype for the EBV-FP. On the other hand, the bulls with the TT genotype had higher EBV-FY and EBV-305dMY than the bulls with the CC genotype, although the differences were not statistically significant (**Table 2**).

The allele substitution effect analysis of identified SNP revealed that the substitution of "C" allele of *AGPAT6* by "T" allele led to a significant ($P<0.05$) decrease of 0.0045 % of in the EBV-FP. However, Littlejohn *et al.* (10) reported comparatively higher estimates of allele substitution effect (-0.0252 %) on milk fat content for the same SNP. The findings of the present study suggest that the identified SNP can be used as a molecular marker for developing marker-assisted selection strategy to enhance milk fat content in KF cattle population. The "C" allele of SNP locus, g.36,175,805C>T may be propagated by mating bulls with "CC" genotype in the herd. However, further research needs to be done to identify more genetic variants accounting large proportion of genetic variance of milk fat content on large bull population for developing more efficient and accurate genomic selection strategy.

Table 3. Allele substitution effect (α) of g.36,175,805C>T SNP locus of *AGPAT6* gene on expected breeding values of milk production traits in Karan Fries bulls

Trait	α	SE	P-value
EBV-FP (%)	-0.0045	0.0035	0.048
EBV-FY (kg)	1.896	2.720	0.282
EBV-305dMY (kg)	37.916	53.829	0.288

EBV-FP: expected breeding value of milk fat %, EBV-FY: expected breeding value of fat yield, EBV-305dMY: expected breeding value of 305 days milk yield

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