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Haritha B Pillai
Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Naicy Thomas
Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Elizabeth Kurian
Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

TV Aravindakshan
Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

V Babitha
Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Sreeja R Nair
Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Corresponding Author:
Haritha B Pillai
Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Association analysis between novel polymorphism in A4GALT gene and milk production traits in crossbred cattle of Kerala

Haritha B Pillai, Naicy Thomas, Elizabeth Kurian, TV Aravindakshan, V Babitha and Sreeja R Nair

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Abstract
Alpha 1, 4-Galactosyltransferase (A4GALT) is a protein coding gene which is related to pathways such as pathway of glycosphingolipids biosynthesis and sphingolipid pathway. The A4GALT gene was studied as a candidate gene for milk production and quality traits (305-day milk yield, peak yield, average daily yield, fat per cent and SNF per cent) in Crossbred cows of Kerala. According to the sequence of bovine A4GALT gene, primers were designed to detect polymorphisms of A4GALT gene in 180 crossbred cows by High resolution melt curve analysis. Allele frequencies of G and A alleles were 0.28 and 0.72 respectively. The homozygous GG genotype was not identified in the screened population. The genotypes frequencies were heterozygous genotype GA (0.45) and homozygous genotype AA (0.55). The impact of the genotypes of the c.721A>G polymorphism in the A4GALT gene on milk production traits in crossbred animals was studied. The genotypes of c.721A>G polymorphism in A4GALT gene had significant ($p \leq 0.05$) influence on 305-day milk yield, peak yield and average daily yield in crossbred animals. The GA genotypes showed significantly superior values for 305-day milk yield, peak yield and average daily yield ($p \leq 0.05$) compared to AA genotypes. The association study between genotypes and other traits was shown to be statistically insignificant ($p > 0.05$). The findings indicate that A4GALT can serve as a potential gene for milk production traits.

Keywords: Crossbred, A4GALT, milk, polymorphism

Introduction
Milk and milk products are widely consumed around the world. The continual challenge of enhancing milk quality and manufacturing characteristics has led to a continuous study emphasis on identifying candidate genes that can increase milk quality, specifically in terms of fat content. The milk production traits have a vital role in livestock production and its related economy (Caroli *et al.*, 2010) [2]. The ongoing objective has been to boost milk production, therefore, the identification of potential genes for improved milk production features. In order to increase efficiency, the livestock industry has devised a breeding scheme that prioritises the selection and utilisation of animals with exceptional milk production characteristics. The identification and confirmation of genetic markers linked with milk production traits are essential and critical steps in establishing a marker-assisted selection (MAS) method. Therefore, the broad objective in numerous animal breeding initiatives globally is to enhance production through genetic selection (Meredith *et al.*, 2012; Narayana *et al.*, 2017; Heimes *et al.*, 2019) [6, 7, 4].

The A4GALT gene is associated with glycosphingolipid biosynthetic process. The A4GALT gene encodes for α1,4-galactosyltransferase, also known as P1/Pk synthase. This enzyme plays a essential role in the production of Pk antigen (Steffensen *et al.*, 2000) [11]. The A4GALT gene is significant in transfusion medicine, obstetrics, and pathogen susceptibility because it is encoded by a glycosyltransferase that synthesises terminal Gal1-4 Gal of Pk (Gb3/CD77) glycosphingolipid (Thuresson *et al.*, 2011) [10]. Additionally, it has been found that the A4GALT gene is linked to the enrichment of pathways that are connected with the persistence

of lactation in Canadian Holstein cattle (Do *et al.,* 2017) [3]. According to genome wide association study conducted in Thai dairy cattle, A4GALT gene may cause changes in milk production traits as well as somatic cell score (Buaban *et al.,* 2022) [1]. Since the A4GALT gene has a role in lactogenesis, it could be a candidate gene for dairy cattle production traits. Thus, the aim of this study was to explore the potential association between the novel polymorphism in exon 1 of the A4GALT gene and milk production parameters, as well as quality traits, in Crossbred cattle of Kerala.

Materials and Methods

Experimental animals

The experimental procedures were carried out in compliance with the standards and regulations set by the institute ethics committee of the Kerala Veterinary and Animal Sciences University in Kerala, India. Data and samples were collected from 180 crossbred cattle at University Livestock Farm, Mannuthy and Cattle Breeding Farm, Thumburmuzhy for the purpose of association study.

Sample and data collection

Blood samples (2 mL) were collected from jugular veins in EDTA vials and were stored at 4 ºC. The genomic DNA was isolated from the obtained blood samples using the phenol chloroform procedure, as described by Sambrook and Russell in 2001. The DNA content, purity, and quality were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). High-quality DNA samples, characterised by their purity and integrity, were utilised for subsequent analysis. Representative milk samples from 180 crossbred cattle during morning and evening were collected once in a month for ten months and brought to laboratory at refrigerated conditions. Data on test day milk yield, average daily milk yield collected from farm records for these animals. The samples were tested for fat per cent and SNF per cent using automatic milk analyser (MRC instruments). Estimation of 305-day milk yield and corresponding milk parameters was carried out using Test Interval Method (TIM) method based on guidelines by International Committee for Animal Recording (ICAR) was used (ICAR, 2020) [5].

Identification of Single Nucleotide Polymorphism (SNP)

The data obtained from transcriptome analysis was examined to identify SNPs in genes. Subsequently, a SNP was identified at NC_037332.1:113536361 location in *Bos Taurus* genome (ARS-UCD1.2) by an allele variation of G to A. This SNP (c.2049C>T) was chosen from A4GALT gene for association analysis.

High resolution melt (HRM) curve analysis

The population was screened for SNP (c.721A>G) in the exon 1 which was previously identified from RNA sequencing data by High resolution melt (HRM) curve analysis. The conditions for HRM were optimised initially by conducting conventional PCR at a temperature range of 58 ºC to 65 ºC, based on which an HRM protocol was designed, for screening the target population for the double nucleotide substitution. The primers were custom developed for purpose of amplification of a short fragment (130 bp) with the polymorphism from the *Bos taurus* Genome downloaded from NCBI using Primer 3 (V.0.4.1) web application. The forward and reverse primers have the following sequences: 5′-CCTTCCTGGCCTTCGAGC -3′ and 5′-CACCATTTCTTGAAGACCCGC -3′, respectively. The PCR was conducted using a reaction volume of 25 µL, consisting of 50 ng of genomic DNA, 12.5 µL of DNA Emerald Amp® GT PCR Master mix (Takara), and 10 picomoles of each primer (Sigma-Aldrich). The thermal cycling parameters consisted of an initial step at 95 °C for 5 minutes, followed by 35 cycles. Each cycle consists of three steps: 95 °C for 30 seconds, 58-65 °C for 30 seconds, and 72 °C for 30 seconds. Finally, there was a final step at 72 °C for 5 minutes. The annealing temperature was standardised to 63 °C.

DNA samples obtained from Crossbred cattle were screened for polymorphism located in exon 1 of A4GALT gene, by using a new methodology called High-resolution melt (HRM) curve analysis. The reactions were performed in the Eco Real-Time PCR System (Illumina) employing Eco 48-well plates that were sealed with Eco adhesive seals. The reference sample was selected from one of the homozygous samples that were validated using agarose gel electrophoresis and sequencing. The experiment involved utilising SSO Fast EVA green super mix, primers, and template DNA to carry out the reaction. The thermal cycling parameters consist of an initial step at 95 °C for 5 minutes, followed by 35 cycles. Each cycle consists of the following steps: 95 °C for 1 minute, 60 °C for 30 seconds, 72 °C for 30 seconds, and finally 72 °C for 5 minutes. Following that, melt curve analysis was carried out. PCR products displaying distinct melt curves were chosen for each fragment and sent to sequencing using corresponding forward and reverse primers to identify any potential nucleotide changes. The sequencing was conducted utilising an automated sequencer at Agri Genome Labs Pvt. Ltd. in Cochin.

Association analysis

The association of genotypes of SNP with milk production traits such as 305-day milk yield, average daily yield, peak yield, fat per cent and SNF per cent were analysed using SPSS V.26 (fixed General Linear Model). A significance level of $p \leq 0.05$ was used to determine if there was a significant difference between the means. The model used for milk production traits was

$$Y_{ijklm} = \mu + H_i + S_j + P_k + G_l + e_{ijklm}$$

Where, *Yijklm* is the trait of m^{th} cow in i^{th} herd, j^{th} season, k^{th} parity and belonging to l^{th} genotype, μ is population mean of trait, H_i is effect of i^{th} herd (i = 1 or 2), S_j is effect of j^{th} season (j = 1 to 3), P_k is effect of k^{th} parity (k = 1 to 4), G_l is effect of l^{th} genotype (l = 1 or 2) and e_{ijklm} is random error.

3. Results and discussion

The 130 bp long region of A4GALT which had the SNP (c.721A>G) (rs478884695) in exon 1 was amplified under gradient PCR for standardising the conditions for HRM analysis. The polymorphism was analysed using a novel HRM protocol in crossbred cattle. The amplicon was 130 bp long fragment, which gave two pronounced melt curves indicating two different genotypes in the target population (Fig. 1 and Fig. 2). The representative amplicons exhibiting different melt curves were sequenced to confirm the genotype as GA (T_m 87.7 °C) and AA (T_m 88.1 °C) and heterozygote as depicted in Fig. 3, Fig 4 and Fig 5. Sequencing of PCR products from each category identified an SNP at 721^{th} (A→G transition) position of CDS that was a nonsynonymous SNP with a codon change of ACC to GCC resulted in an amino acid substitution of Threonine to Alanine with a SIFT score of 0.57 and was predicted to be benign. The SNP

c.721A>G was found to be a missense variant resulted in amino acid substitution p. Thr241Ala. and are predicted to be tolerated (benign). The PolyPhen-2 tool was used to predict the effect of an amino acid substitution on the function of the protein. The programme indicated that the substitution of threonine with alanine at position 241 of the A4GALT protein (p.Thr241Ala) was likely to be harmless, as it received a score of 0.00 (Fig. 6). In cattle there no published data is available regarding this SNP, thus making it novel.

In the studied population allele A (0.72) was found to have higher frequency compare to allele G (0.28). The homozygous GG genotype was not identified in the screened population. The genotypes frequencies were as follows: homozygous genotype GG (0), heterozygous genotype GA (0.45) and homozygous genotype AA (0.55). The genotype and allele frequencies are mentioned in Table 1.

The impact of the genotypes of the c.721A>G polymorphism in A4GALT gene on milk production traits in crossbred animals was studied. The genotypes of c.721A>G polymorphism in A4GALT gene had significant ($p \leq 0.05$) influence on 305-day milk yield, peak yield and average daily yield in crossbred animals. The GA genotypes showed significantly higher values for 305-day milk yield, peak yield and average daily yield ($p \leq 0.05$) compared to AA genotypes. The Table 2 provides the mean values and standard errors for milk production traits associated with various genotypes of the c.721A>G polymorphism in the A4GALT gene. Previous research have indicated that the A4GALT gene has the potential to influence milk production features in dairy cattle (Do *et al.*, 2017; Buaban *et al.*, 2022) [3, 1]. But there were no association studies have been documented to be associated with the A4GALT gene in cattle when it comes to milk production traits.

In conclusion, a novel SNP, a A/G transition, was detected in exon 1 of A4GALT gene with significant impact on 305-day milk yield, peak yield and average daily yield in crossbred cattle of Kerala. The polymorphism had no significant effect on milk quality parameter. The results suggest that A4GALT can be used as a candidate gene for milk production traits. Also, additional research should be conducted on a larger sample size of animals to validate the polymorphisms and their associations before employing the A4GALT gene as a marker for selection in dairy cattle.

Table 1: Genotype and allele frequencies for SNP (c.721A>G)

Gene	SNP	Genotype frequencies			Allele frequencies	
A4GALT	c.721A>G (n=180)	GG/0 (0)	GA/0.45 (99)	AA/0.55 (81)	G/0.28	A/0.72

Figures in parenthesis represent number of observations

Table 2: Effect of c.721A>G polymorphism in A4GALT gene on milk production traits in crossbred cattle of Kerala

Sl. No.	Trait	Mean ± SE		p-value
		GA (81)	AA (99)	
1	305-day milk yield (kg)	2947.43 ± 92.8[a]	2558.34 ± 83.94 [b]	$p \leq 0.05$
2	Peak yield (kg)	13.42 ± 0.39 [a]	12.37 ± 0.35 [b]	$p \leq 0.05$
3	Average daily yield (kg)	9.66 ± 0.3 [a]	8.39 ± 0.28 [b]	$p \leq 0.05$
4	Fat per cent	3.94 ± 0.05	4.03 ± 0.04	$p > 0.05$
5	SNF per cent	7.76 ± 0.03	7.79 ± 0.03	$p > 0.05$

Means with different superscripts in the same row differ significantly, Figures in parenthesis represent number of observations

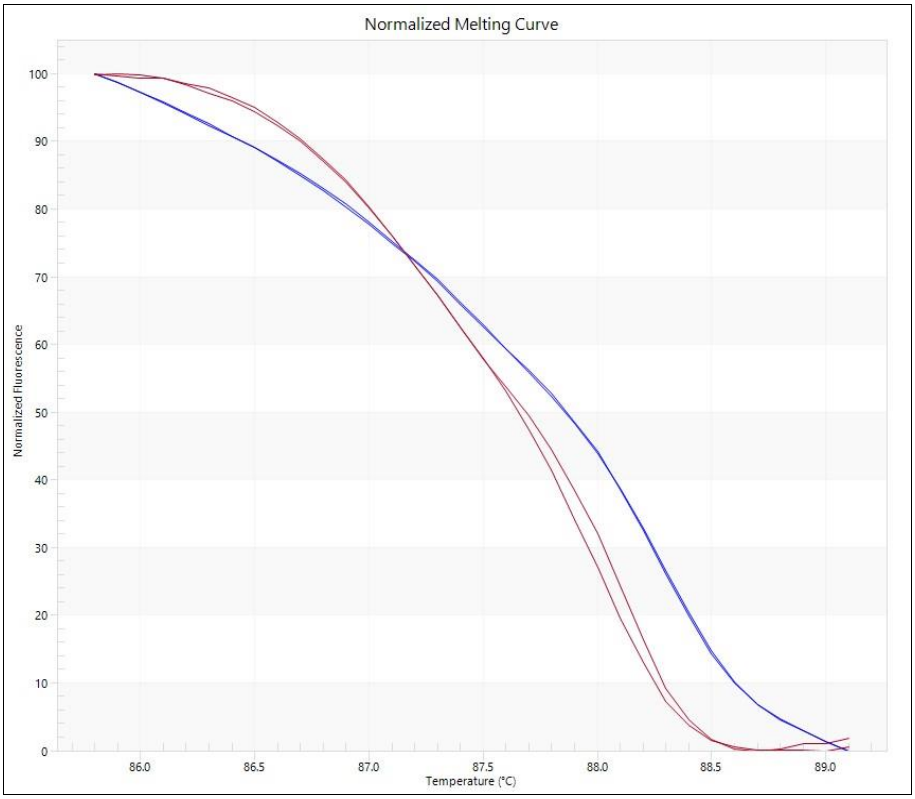

Fig 1: Normalised melt curve of HRM analysis, depicting homozygote AA (red) and heterozygote GA (blue)

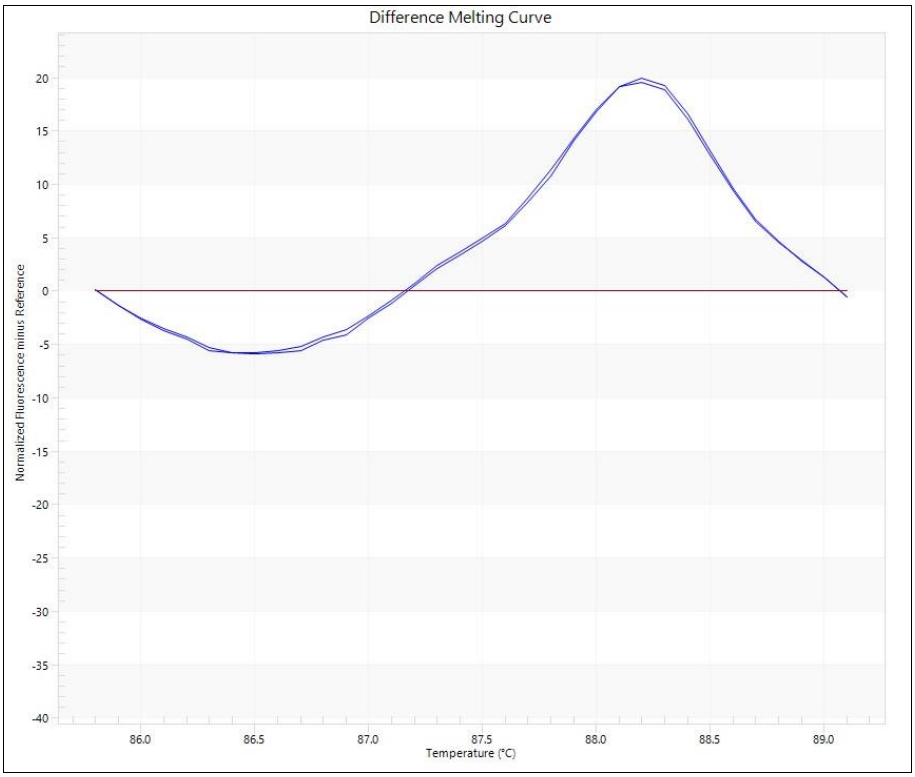

Fig 2: Difference melt curve of HRM analysis, depicting homozygote AA (red) and heterozygote GA (blue)

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1    CACCATTTCTTGAAGACCCGCGTGAGCAGCTGCGGGCCCTGGTGACCCCAGATCCAGCCG  60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1    CACCATTTCTTGAAGACCCGCGTGAGCAGCTGCGGGCCCTGGTGACCCCAGATCCAGCCG  60

61   TTGTAGTGGGTCACGAAGTCACGCATGCACTGCGCCATGAACTCGTGGTGCTGCTCGAAG  120
     |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
61   TTGTAGTGGGCCACGAAGTCACGCATGCACTGCGCCATGAACTCGTGGTGCTGCTCGAAG  120

121  GCCAGGAAGG  130
     ||||||||||
121  GCCAGGAAGG  130
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Fig 3: Sequence analysis using BLASTn showing C to A variation

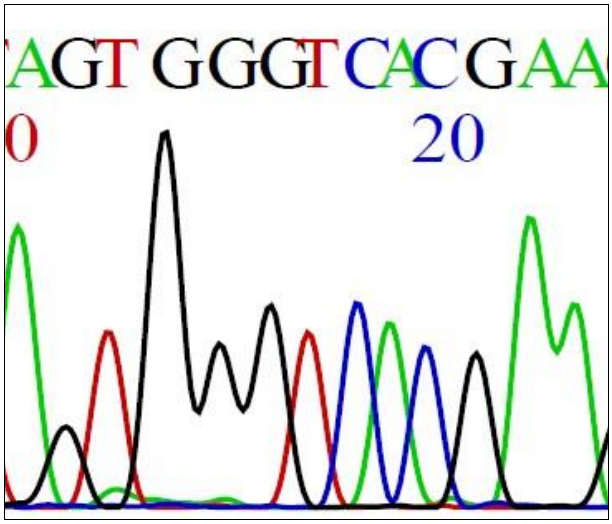

Fig 4: Chromatogram of AA genotype

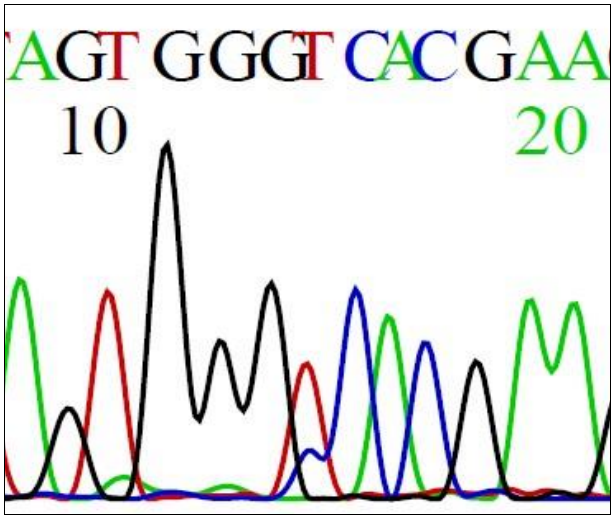

Fig 5: Chromatogram of GA genotype

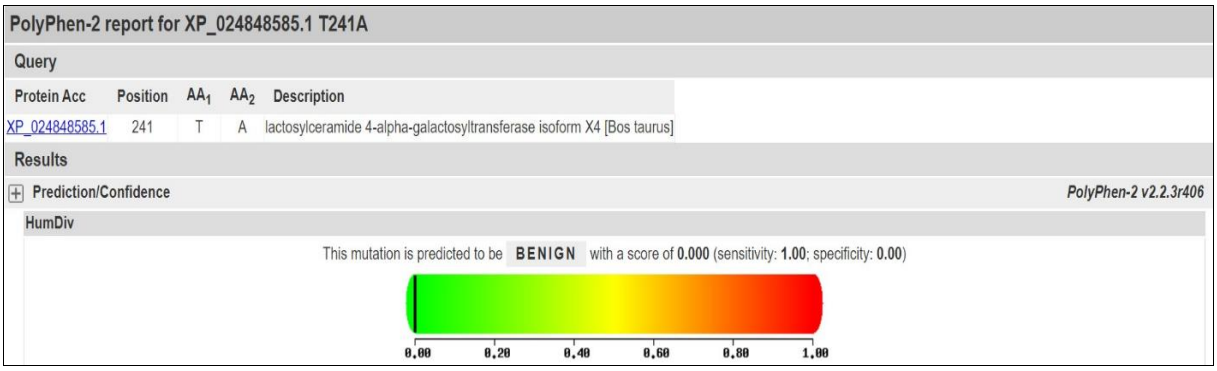

Fig 6: Results of the PolyPhen-2 analysis for single variant query (p.Thr241Ala).

Conflict of interest statement

The authors declare that there is no actual or potential conflict of interest that could inappropriately influence in this work.

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