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REGULAR ARTICLES

Genetic association between SNPs in the *DGAT1* gene and milk production traits in Murrah buffaloes

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Abstract This study identified polymorphisms in the *DGAT1* gene in Murrah buffaloes and investigated the associations to milk production and quality traits (milk, fat and protein yields and percentages, somatic cell count). Genomic DNA was extracted from hair follicles collected from the tail of 196 females. Three SNPs were identified in *DGAT1* gene by sequencing. Statistical analyses were performed to verify the linkage and the association between polymorphisms and traits. The estimated value of r^2 between two SNPs in exon 17 (g.11,783G > A and g.11,785 T > C) was 0.029. SNP g.11,785 T > C was significantly associated ($P < 0.05$) to fat and protein percentage. Dominance effect was significant for milk and fat yields and protein percentage ($P < 0.05$). The additive effect of the SNP g.11,785 T > C was significant for protein production and somatic cell count ($P < 0.05$). It indicates that assisted marker selection might be done with considerations to balance production and udder health.

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Introduction

In several countries, the buffalo (*Bubalus bubalis*) has been raised for milk production due to the financial return of the dairy products. The buffalo milk has specific physiochemical and taste characteristics. Its flavor is slightly sweeter and whiter than cow's milk due to the almost total carotene absence in the fat. Moreover, buffalo milk is more concentrated. It has a higher protein, fat, and minerals content than cattle milk (Salman et al. 2014).

Some breeding programs evaluate the inclusion molecular genetic tools to assist selection. There are several studies with candidate genes associated to buffalo milk composition, such as leptin (Zetouni et al. 2013), ghrelin (Gil et al. 2013), alpha(s1)-, alpha(s2)-, beta- and kappa-caseins, beta-lactoglobulin, and alpha-lactalbumin (Ramesha et al. 2008; Riaz et al. 2008; Meignanalakshmi and Nainar 2009; El Nahas et al. 2013).

The *DGAT1* gene (diacylglycerol O-acyltransferase1) is a candidate gene due to its physiological function. It is related to fat metabolism in milk (Raut et al. 2012). The buffalo *DGAT1* gene has a size of 10,733 bp organized into 17 exons, and located on chromosome 15 (Amaral et al. 2008). This gene encodes an enzyme, consisting of 489 amino acids, related to the biosynthesis of triglycerides, and it is expressed in many tissues, particularly in the intestine, testis, adipose, mammary gland, and epithelial tissues (Cases et al. 1998). The identification of polymorphisms that explain a high variance of a trait might assist selection and gives a better idea of the physiology behind the expression of the trait.

Two pathways have been described for triglyceride biosynthesis (glycerol-3-phosphate and monoacylglycerol pathways) in mammals. In both pathways, diacylglycerol is synthesized and subsequently converted into triacylglycerol by the activity of the enzyme diacylglycerol O-acyltransferase1 (Coleman et al. 2000). The enzyme participates in intestinal fat absorption processes, regulation of triacylglycerol concentration in plasma, and fat storage in adipocytes (Cases et al. 1998).

Several studies reported single nucleotide polymorphisms (SNPs) in the buffalo *DGAT1* gene (Tantia et al. 2006; Mishra et al. 2007; Raut et al. 2012; Venkatachalapathy et al. 2013, Cardoso et al. 2015). Some studies showed the influence of SNPs in production traits in cattle. They increased the fat content of the milk in Holstein cows (Spelman et al. 2002) and decreased protein content and milk yield in Jersey cows (Weller et al. 2003). Cardoso et al. (2015) found that the a variable nucleotide repeat (VNRT) in the promoter region of *DGAT1* that explains 32 % of additive genetic variance of fat percentage and Yuan et al. (2007) found a nonsynonymous nucleotide substitution in *DGAT1* gene (Ala484Val) in Murrah buffaloes not described in cattle, but without association studies.

The aim of the present study was to identify polymorphisms in the *DGAT1* gene and verify their associations with milk production traits in Murrah buffaloes.

Material and methods

Animals

The Murrah buffaloes belong to Tapuio Farm, located in Taipu, RN, Brazil. The grazing system used was the Voisin rotational grazing system, with all animals raised on pasture. Annually, tests to detect some zoonoses, such as brucellosis and tuberculosis are carried out as well as the vaccination and parasites control programs.

The herd has approximately 1000 heads. It is composed by 468 dams with a mean milk production of 3500 L per day. The farm begins to dairy buffalo milk-recording program maintained by the Department of Animal Science, São Paulo State University (Unesp), Jaboticabal-SP, Brazil. Milk-recording and sampling are performed periodically to determine fat and protein content and somatic cell count.

Biological material sampling and DNA extraction

Hair follicle samples from 196 Murrah buffaloes were collected and stored at 4 °C until DNA extraction. Genomic DNA was extracted by the phenol-chloroform-isoamyl alcohol method (Sambrook et al. 1989). The DNA samples were quantified using a NanoDrop® 1000 spectrophotometer (Thermo Scientific, USA), and the concentrations were adjusted to 70 ng/μL.

PCR and sequencing

PCR primers used to amplify exon 17 of *DGAT1* gene were described by Yuan et al. 2007. The genomic DNA samples were amplified by PCR in a final volume reaction of 25 μL containing 70 ng DNA, 15 pM each primer, and 7.5 μL GoTaq ® Colorless Master Mix (Promega, USA).

The annealing temperature was selected for a PCR performed in aT100™ Thermal Cycler (Bio-Rad, USA) according to the following steps: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min (denaturation), 62 °C for 1 min (annealing), 72 °C for 1 min (extension), and followed by a final extension at 72 °C for 5 min.

After PCR amplification, 2 μL of each PCR product was electrophoresed on 1 % agarose gels stained with GelRed™ (Biotium, USA) in 1X TBE buffer at 90 V, for 50 min. PCR products were visualized in ultraviolet light, and the gel photographed by the Gel-Doc™ apparatus (BioRad, USA), and analyzed with the Image Analysis software (Kodak, USA) in order to check the amplification of the desired fragment.

The PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA). The samples were sequenced using the ABI PRISM® BigDye® Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) in an ABI 3730xl automatic sequencer (Applied Biosystems, USA). Each sequencing reaction was carried out using 2 μL Save Money buffer (200 mM Tris, pH 9.0, 0.5 mM $MgCl_2$), 1 μL primer (2.5 pmol/μL), 2 μL BigDye, and 70 ng DNA. Sequencing reaction comprised denaturation at 95 °C for 20 s, annealing at 62 °C for 15 s, and extension at 60 °C for 1 min, in a total of 25 cycles.

For polymorphism identification, DNA sequences obtained from *DGAT1* gene were analyzed by the CodonCode Aligner program (http://www.codoncode.com/aligner/download.htm).

Statistical analysis

Allelic and genotypic frequency

Allelic frequencies of the genotyped SNPs were determined by simple allele counting (Falconer and Mackay 1996) using the PROC FREQ in SAS (SAS 9.2, SAS Institute, Cary, NC, USA). Possible deviations from the genotypic and allelic frequencies were tested using the chi-square test, in order to verify whether the genotyped population was in Hardy-Weinberg equilibrium.

Linkage disequilibrium

Linkage disequilibrium (r^2) was estimated using the Plink software (http://pngu.mgh.harvard.edu/~purcell/plink/) to check which SNPs segregated together. Considering two loci with two alleles for each locus (A1/A2 and B1/B2), we have the following equation (Hill and Robertson 1966):

$$r^2 = \frac{D^2}{[\text{freq}(A_1)*\text{freq}(A_2)*\text{freq}(B_1)*\text{freq}(B_2)*]}$$

where (Hill 1981):

$$D = \text{freq}(A_1\_B_1)*\text{freq}(A_2\_B_2) - \text{freq}(A_1\_B_2)*\text{freq}(A_2\_B_1)$$

Association analysis

In order to proceed the association analyses of each SNP to the five production traits (milk, fat and protein yields, fat and protein percentages), the statistical model was used:

$$Y_{ijkl} = \mu + GC_i + S_j + M_k + \beta_1\left(I_{ijkl} - \overline{I}\right) + \beta_2\left(I_{ijkl} - \overline{I}\right) + e_{ijkl}$$

where Y_{ijkl} is the milk yield, milk fat and protein percentage at 305 days; μ is the constant inherent to all observations; GC_i is the fixed effect of contemporary group; S_j is the random effect of sire, with mean 0 and variance σ_s^2; M_k is the fixed effect of genotype for SNP; β_1 is the linear regression coefficient of trait Y_{ijkl} in relation of cow age at measurement; β_2 is the quadratic regression coefficient of trait Y_{ijkl} in relation of cow age at measurement; I_{ijkl} is the cow age evaluated at measurement; $\overline{I}$ is the cows average age evaluated at measurement; and e_{ijkl} is the random residual effect associated with trait Y_{ijkl} with mean 0 and variance σ_e^2.

The contemporary group was considered as the concatenation of year and calving season. Analysis of variance was performed using the PROC MIXED (SAS 9.2, SAS Institute, Cary, NC, USA). Somatic cell count (SCC) distribution was not normal and, therefore, was transformed to a logarithmic scale (CCST), using the function described by Dabdoub and Shook (1984):

$$\text{CCS}t = \left(\log_2\left(\frac{\text{CCs}}{100,000}\right)\right) + 3$$

The mean fat and protein yields, fat and protein percentages, and somatic cell count for the different genotypes were analyzed using the Tukey test ($P < 0.05$), and after, Bonferroni correction was applied at 5 % of significance for all traits. After analyzing the associations between SNPs and milk traits, the additive and dominance effects of the significant SNPs were tested. These analyses were performed by orthogonal contrasts using the PROC GLM in SAS (SAS 9.2, SAS Institute, Cary, NC, USA).

Results

A PCR fragment of 231 bp, containing part of intron 16, exon 17, and a portion of 3′UTR of the *DGAT1* gene, was amplified. The less related Murrah buffaloes were identified, and subsequently, a total of ten animals with the highest and ten animals with the lowest milk yields were used to investigate the presence of SNPs using the CodonCode Aligner software. The DNA sequence obtained was deposited on GenBank database (http://www.ncbi.nlm.nih.gov/nuccore) under accession number KF982856.

A total of three SNPs were identified in the sequenced region: one in intron 16 (g.11,644C > T) and two in exon 17 (g.11,783G > A and g.11,785 T > C). The nonsynonymous SNP g.11,785 T > C results from the change of alanine to valine at position 484 (Ala484Val).

The comparison of the 231 bp amplicon with the corresponding region in cattle allowed the identification of four inter-species nucleotide substitutions, one in exon 17 (g.11,726C > T), and three in the 3′UTR (g.11,807G > C, g.11,849G > T, and g.11,851G > A).

For statistical analysis, we considered only the SNPs identified in the coding region g.11,783G > A and g.11,785 T > C, which were submitted for dbSNP (http://www.ncbi.nlm.nih.gov/snp) under accession numbers ss2016683599 and ss2016683603, respectively.

Most allele frequencies have values greater than 0.10; it contributes to the association analyses, since they will not be affected by the sharp discrepancy in frequency distribution. The chi-square test result was 3.84 ($P > 0.05$); so all the SNPs analyzed had expected frequencies (Table 1). Therefore, the population is in Hardy-Weinberg equilibrium.

The estimated r^2 between SNPs g.11,783G > A and g.11,785 T > C, genotyped for the whole population, was 0.029. Thus, the SNPs are not linked. The nominal P values ($P < 0.05$) for the mean production traits showed no

Table 1 SNPs description according 196 animals genotyped: allele frequency (*AF*), genotype frequencies observed (*OGF*) and genotype frequencies expected for the Hardy-Weinberg equilibrium (*EGF-HW*), and observed chi-square test (χ^2 obs) for the SNPs identified

SNP	AF	OFG	EFG-HW	χ^2 obs[a]
g.11,783G > A	G = 0.93 A = 0.07	GG = 0.86 GA = 0.14 AA = 0	GG = 0.865 GA = 0.13 AA = 0.005	1.24
g.11,785 T > C	C = 0.40 T = 0.60	TT = 0.13 CT = 0.53 CC = 0.34	TT = 0.16 CT = 0.48 CC = 0.36	1.94

[a] χ^2 *obs* observed chi-square, values above 3.84 are significant ($P < 0.05$)

association ($P > 0.05$) of the SNP g.11,783G > A with milk yield and quality traits (Table 2).

The SNP g.11,785 T > C was associated to fat and protein percentages ($P < 0.05$). The TC genotype had the highest mean for fat percentage, followed by CC and TT genotypes. The highest mean protein percentage was observed for the TT genotype, whereas TC and CC genotypes were not statistically different ($P > 0.05$) (Table 2). The polymorphism remained significant after Bonferroni correction.

As previously described (Table 2), the SNP g.11,783G > A had no significant association ($P > 0.05$) to milk yield and quality traits. As consequence, the additive and dominance genetic effects for this SNP were not estimated. The magnitudes of the additive and dominance effects (Table 3), expressed in trait units, represent the average change expected by substitution of T by C allele (SNP g.11,785 T > C). The dominance effect was significant for milk and fat productions and protein percentage (Table 3), presenting decreasing values of 180, 0.40, and 0.40 kg, respectively. For protein production, the SNP g.11,785 T > C was significant for additive effect ($P < 0.05$), with an increase of 0.09 kg.

Table 3 Additive and dominance variance effects of significant SNP g.11,785 T > C for milk production (*MP*), fat (*FP*) and protein (*PP*) production, fat (*%FP*) and protein (*%PP*) percentages, and somatic cell count (*SCC*)

Trait	Additive	*P* value	Dominance	*P* value
MP (kg)	−30 ± 55	0.58	−180 ± 70	0.01
FP (kg)	−0.02 ± 0.12	0.89	−0.4 ± 0.16	0.01
PP (kg)	0.09 ± 0.03	0.007	0.07 ± 0.04	0.08
% F	−1.87 ± 3.88	0.63	−4.9 ± 5.0	0.33
% P	−0.02 ± 0.12	0.89	−0.4 ± 0.16	0.01
SCC	0.17 ± 0.07	0.03	0.08 ± 0.1	0.42

Additive: additive genetic effect. Dominance: dominance deviations effect

Discussion

The estimated linkage disequilibrium between the SNPs revealed that they segregated independently (r^2 lower than 0.33, Ardlie et al. 2002).

There are some SNP association studies of *DGAT1* gene and milk traits. Cardoso et al. (2015) found that the a variable number of tandem repeats (VNTR) in the promoter region of *DGAT1* was significant for fat percentage, and Meng et al. 2013 observed that the SNP c.1,350C > G (exon 17) was not associated to lactose, fat, and protein percentages in buffaloes. Grisart et al. 2002 identified a nonconservative substitution Lys232Ala that affected milk fat content and other traits of economic importance in dairy cows.

In the present study, SNP g.11,785 T > C was significant ($P < 0.05$) to fat and protein percentages. The observed amino acid substitution (Ala484Val) could influence the activity and function of the diacylglycerol O-acyltransferase1 protein and, as consequence, it affects milk composition. Yuan et al. 2007 suggested that the region where the SNP is located may be conserved across mammals, such as cattle, pigs, rats, and humans. It indicates potential further studies in other species for this region.

In the buffalo population herein studied, the SNP g.11,785 T > C has a significant dominance effect ($P < 0.05$). It indicates a better performance of the heterozygous. However, the dominance deviation effect is not inheritable, and it will not affect subsequent generations. The additive effect of the SNP g.11,785 T > C was significant for protein production ($P < 0.05$). Animals with TT genotypes have approximately 0.09 kg more milk protein than CC genotype. Additive effects respond to selection (Vitezica et al. 2013). However, it should be taken into account that it also presents additive effect for somatic cell count (0.17 ± 0.07) and depending on the selection pressure; it might be detrimental to udder health.

Table 2 Mean values for milk yield, fat and protein, fat and protein percentages and somatic cell count, and nominal values of *P*, to SNPs g.11,783G > A and g.11,785 T > C

SNP g.11,783G > A						
Genotype	MP (kg) ($P = 0.534$)	FP (kg) ($P = 0.487$)	PP (kg) ($P = 0.582$)	% F ($P = 0.301$)	% P ($P = 0.776$)	SCC ($P = 0.339$)
GG	1970	125	85	6.97	4.29	4.48
GA	2020	130	86	6.74	4.31	4.35
SNP g.11,785 T > C						
Genotype	MP (kg) ($P = 0.138$)	FP (kg) ($P = 0.839$)	PP (kg) ($P = 0.205$)	% F[a] ($P = 0.039$)	% P[a] ($P = 0.007$)	SCC ($P = 0.105$)
TT	1870	123	83	6.72b	4.45a	4.71
TC	2020	127	87	7.13a	4.27b	4.44
CC	1940	126	83	6.76ab	4.24b	4.41

MP milk production, *FP* fat production, *PP* protein production, *%F* fat percentage, *%P* protein percentage, *SCC* somatic cell count

[a] Means followed by the same letter are not significantly different (Tukey's test, 5 %)

According to Kim et al. (2011), Bonferroni correction is conservative and presents a high level of restrictions, since it limits the identification of markers associated to productive traits. SNP g.11,785 T > C in the exon 17 of the *DGAT1* gene was adjusted by Bonferroni correction and maintained its significance for fat and protein percentages. The strong association indicates the potential use of the marker in future-assisted selection, especially because the gene was already considered with major effect in other close species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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