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Polymorphisms in lipogenic genes and milk fatty acid composition in Holstein dairy cattle

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ABSTRACT

Changing bovine milk fatty acid (FA) composition through selection can decrease saturated FA (SFA) consumption, improve human health and provide a means for manipulating processing properties of milk. Our study determined associations between milk FA composition and genes from triacylglycerol (TAG) biosynthesis pathway. The GC dinucleotide allele of diacylglycerol O-acyltransferase 1:g.10433-10434AA >GC was associated with lower palmitic acid (16:0) concentration but higher oleic (18:1 *cis*-9), linoleic (18:2 *cis*-9, *cis*-12) acid concentrations, and elongation index. Accordingly, the GC dinucleotide allele was associated with lower milk fat percentage and SFA concentrations but higher monounsaturated FA and polyunsaturated FA (PUFA) concentrations. The glycerol-3-phosphate acyltransferase, mitochondrial haplotypes were associated with higher myristoleic acid (14:1 *cis*-9) concentration and C14 desaturation index. The 1-acylglycerol-3-phosphate acyltransferase 1 haplotypes were associated with higher PUFA and linoleic acid concentrations. The results of this study provide information for developing genetic tools to modify milk FA composition in dairy cattle.

1. Introduction

Regulation of bovine milk fatty acid (FA) composition by dietary and genetic means has received much attention in recent years [23,32,33,41] because of the potentially adverse effects of certain dietary saturated FA (SFA) on human health [24,25,30]. The objective of our study was to develop strategies for selection of animals producing milk with higher concentration of unsaturated FA (UFA) and lower concentration of SFA. Moderate to high heritabilities of milk FA [1,32,46] and low conversion efficiency of dietary UFA into milk UFA caused by ruminal biohydrogenation of dietary FA [15] prompted us to choose a genetic approach to develop strategies for modifying bovine milk FA composition.

Biosynthesis of milk triacylglycerols (TAG) occurs in mammary epithelial cells through stepwise addition of activated fatty-acyl groups to glycerol-3-phosphate by different acyltransferases [13]. The first and rate-limiting step in the TAG biosynthesis pathway is catalyzed by glycerol-3-phosphate acyltransferases (GPAT) that add fatty-acyl groups to the sn-1 position of glycerol-3-phosphate, leading to the production of monoacylglycerols (MAG). The major *GPAT* isoforms known to be expressed in bovine mammary gland are glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*) and 1-acylglycerol-3-phosphate acyltransferase 6, microsomal (*AGPAT6*; [5]). The second step in the TAG biosynthesis pathway is catalyzed by AGPAT which add fatty-acyl groups to the sn-2 position on the glycerol backbone, leading to the conversion of MAG to diacylglycerol (DAG), also called phosphatidic acid. The second most abundant *AGPAT* isoform expressed in bovine mammary gland [5] is *AGPAT1*, which seems responsible for catalyzing this step of the pathway in ruminants. The DAG has to be dephosphorylated before advancing to the final step of TAG biosynthesis. The enzyme catalyzing the removal of a phosphate group from DAG is called phosphatidate phosphatase or lipin (LPIN; [36]) with its major isoform expressed in bovine mammary gland being *LPIN1* [5]. The final step of TAG biosynthesis pathway is catalyzed by diacylglycerol acyltransferases (DGAT) that add fatty-acyl groups to the sn-3 position on the glycerol backbone, leading to the production of TAG.

Positional distribution of FA in milk TAG is not random, but influenced by specificity of different acyltransferases for particular FA [12] implying that mutations in acyltransferase genes might change their specificity for particular FA, leading to changes in milk FA composition. We therefore hypothesize that genetic polymorphisms in *GPAM*, *AGPAT1*, *AGPAT6*, *LPIN1*, and *DGAT1* might change milk FA composition. Animals were genotyped for SNP discovered in *GPAM*, *AGPAT1*, *AGPAT6*, and *LPIN1* to reconstruct intragenic haplotypes that were tested for

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associations with milk FA composition within each gene separately. The association of the previously known *DGAT1*:g.10433–10434AA > GC mutation with milk FA composition was simultaneously quantified.

2. Materials and methods

2.1. Animals and milk fatty acid composition

Collection of milk samples was performed once a month during morning milking throughout a 305-day lactation period with subsequent storage of the samples at −20 °C until further analyses. Association tests between *GPAM*, *AGPAT1*, *AGPAT6*, *LPIN1*, and *DGAT1* polymorphisms and milk FA composition were performed using 3,437, 3,505, 3,391, 3,687, and 2,360 milk samples collected both from 299, 298, 292, 285, and 168 Holstein cows, the daughters of 86, 83, 82, 85, and 65 sires, respectively, in the Iowa State University herd, and from 122, 132, 123, 171, and 123 Holstein cows, the daughters of 84, 89, 82, 110, and 84 sires, respectively, represented in the Kansas State University dairy herd. Cows were fed different diets in these herds but the potential feed effects on milk FA composition were accounted for in statistical analysis. There were 8, 8, 8, 9, and 7 sires common to both herds. All the animals were treated in accordance with guidelines established by Iowa and Kansas State University Committees on Animal Care. Milk FA composition was analyzed by gas chromatography as in Nafikov et al. [32]. The procedure was based on milk total lipid extraction with hexane:isopropanol (3:2, vol/vol) and subsequent FA methyl ester production and chromatographic analysis with a Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) equipped with CP-8400 auto-sampler, CP-8410 auto-injector, CP-1177 split/splitless injector, and flame ionization detector (FID) with helium as a carrier gas. A separation of FA methyl esters in hexane was performed with a fused silica capillary column (Supelco™-2560 Capillary Column, 100 m × 0.25 mm i.d., with 0.2 μm film thickness) using the temperature programs for injector and column as in Nafikov et al. [32]. Individual FA methyl ester standards (Matreya LLC, Pleasant Gap, PA) were used to determine retention times for all FA analyzed, and peak area measurements calculated with Star Chromatography Workstation Version 6 (Varian Analytical Instruments) were used to quantify FA. Altogether, 39 milk FA were identified and used in calculating weight percentages of the 16 most abundant milk FA. Additional traits such as concentrations of SFA, UFA, MUFA, PUFA, SFA:UFA ratio, C14 (desaturation index = 14:1/ (14:0 + 14:1)), C16 (desaturation index = 16:1/ (16:0 + 16:1)), and C18 (desaturation index = 18:1/ (18:0 + 18:1)) desaturation indices, and elongation index (elongation index = (18:0 + 18:1)/ (16:0 + 16:1 + 18:0 + 18:1)) were calculated from all 39 milk FA analyzed and used to test for associations with genetic polymorphisms.

2.2. SNP discovery and genotyping

Genomic DNA was extracted with DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA) from white blood cells of the dairy cows. SNP discovery was performed by sequencing *GPAM*, *AGPAT1*, *AGPAT6*, and *LPIN1* (Table S1) in exonic and flanking intronic regions using genomic DNA samples from 12 cows, daughters of 12 different sires. Primers were designed with Primer3 (version 0.4.0) software [39]. The PCR reactions were performed with either conventional Taq or HotStar Taq DNA polymerases in a DNA Engine thermal cycler (Bio-Rad Laboratories, Hercules, CA) with a total reaction volume of 25 μL containing 50 ng of genomic DNA. The HotStar Taq DNA polymerase was used to improve the amplification of GC-rich DNA regions. The PCR temperature gradient procedure was used to determine optimal annealing temperature for a particular primer set (Table S2). After PCR amplification, unused primers and dNTPs were removed with ExoSAP-IT® (USB Corporation, Cleveland, OH) prior to sequencing. Using bidirectional genomic DNA sequencing for higher resolution, novel SNP discovery were performed by aligning DNA sequences with Vector NTI Advance™ 10 (Life Technologies, Grand Island, NY). Genotyping the discovered SNP was performed with Sequenom MassARRAY platform using 10 ng of genomic DNA dissolved in DNase-free water [17]. Intragenic haplotype reconstructions and their frequency estimations were performed with PHASE (version 2.1) program [47,48]. Samples having the best haplotype pairs with probabilities <0.9 were excluded from further analyses, and only haplotypes with population frequency >0.05 were used in association analyses with minor frequency haplotypes pooled into the "other" category. Genotyping the *DGAT1*:g.10433–10434AA >GC polymorphism was performed with a RFLP assay. Genomic DNA was amplified by PCR reaction performed using HotStar Taq DNA polymerase and TGGGCTCCGTGCTGGCCCTGATGGTCTA and TTGAGCTCGT AGCACAGGGTGGGGGCGA as forward and reverse primers with annealing temperature of 63 °C. The PCR product was digested with EaeI restriction enzyme (New England Biolabs Inc., Ipswich, MA) for 1 hr at 37 °C and run on 2% agarose gel to reveal DNA fragments. Homozygous animals for AA dinucleotide allele of *DGAT1*:g.10433–10434AA >GC had only one DNA fragment of 405 bp long whereas homozygous animals for GC dinucleotide allele had two DNA fragments of lengths 230 and 175 bp (Fig. S1).

2.3. Statistical analysis

Associations between intragenic haplotypes and milk FA composition were tested by fitting the following linear mixed model for longitudinal data with ASReml software [18]:

$$Y_{ijkn} = \mu + dim_i + dim^2{}_i + dim^3{}_i + cg_j + \left(dim_i \times cg_j\right) + \left(dim^2{}_i \times cg_j\right) + \left(dim^3{}_i \times cg_j\right) + \sum b_k H_k + A_n + \varepsilon_{ijkn}$$

where Y_{ijkn} is a response variable; μ is a general mean; dim_i, $dim^2{}_i$, and $dim^3{}_i$ are covariates describing linear, quadratic, and cubic effects of days in milk on the response variable; cg_j is a fixed effect of contemporary group ($cg_j = 24$ classes) representing the combination of herd (two herds), season of calving (four seasons: December to February, March to May, June to August, and September to November), and age of calving (three age groups: group 1 < 2.2 years old, group 2 between 2.2 and 4 years old, and group 3 > 4 years old); H_k is a haplotype or dinucleotide allele effect fitted as a covariate coded as 0, 1, or 2 for the number of copies present in an animal; b_k is a partial regression coefficient representing the substitution effect for the k^{th} haplotype or dinucleotide allele as a deviation from the effect of the most frequent haplotype or dinucleotide allele set to zero; A_n is the random additive genetic effect of the n^{th} animal; and ε_{ijkn} is a residual error. A first order autoregressive covariance structure was used in the model to account for correlations between repeated milk sampling performed on the same animal throughout the 305-day lactation. The covariance structure was selected based on the biology of the experiment, values of Akaike's and Bayesian Information Criteria, and the residual log likelihood [40] and assumes equal variances but correlations that decline exponentially with an increase in interval between monthly time points [27]. Pedigree information regarding sire, dam, paternal and maternal grandsires and granddams of each cow was used to fit the random additive animal genetic effects into the statistical model by forming the inverse additive genetic relationship matrix for the cows. The significance of haplotype and dinucleotide allele associations was tested for each gene separately by F-test and declared significant if the adjusted *P*-value was <0.05. The adjusted *P*-value was calculated with Bioconductor "multtest" package in R [35] using the Benjamini and Hochberg [3] stepup false discovery rate-controlling procedure to account for multiple testing of associations in five genes. If haplotype or dinucleotide allele associations for a particular gene were significant, pair-wise comparisons between intragenic haplotypes or dinucleotide alleles were performed and based on the Bonferroni adjustment to control type I error rate during multiple