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Significant associations of the mitochondrial transcription factor A promoter polymorphisms with marbling and subcutaneous fat depth in Wagyu x Limousin F_2 crosses

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Abstract

Mitochondrial transcription factor A (TFAM), a nucleus-encoded protein, regulates the initiation of transcription and replication of mitochondrial DNA (mtDNA). Decreased expression of nuclear-encoded mitochondrial genes has been associated with onset of obesity in mice. Therefore, we hypothesized genetic variants in *TFAM* gene influence mitochondrial biogenesis consequently affecting body fat deposition and energy metabolism. In the present study, both cDNA (2259 bp) and genomic DNA (16,666 bp) sequences were generated for the bovine *TFAM* gene using a combination of *in silico* cloning with targeted region PCR amplification. Alignment of both cDNA and genomic sequences led to the determination of genomic organization and characterization of the promoter region of the bovine *TFAM* gene. Two closely linked A/C and C/T single nucleotide polymorphisms (SNPs) were found in the bovine *TFAM* promoter and then genotyped on 237 Wagyu x Limousin F_2 animals with recorded phenotypes for marbling and subcutaneous fat depth (SFD). Statistical analysis demonstrated that both SNPs and their haplotypes were associated with marbling ($P = 0.0153$ for A/C, $P = 0.0026$ for C/T, and $P = 0.0004$ for haplotype) and SFD ($P = 0.0200$ for A/C, $P = 0.0039$ for C/T, and $P = 0.0029$ for haplotype), respectively. A search for transcriptional regulatory elements using MatInspector indicated that both SNPs lead to a gain/loss of six putative-binding sites for transcription factors relevant to fat deposition and energy metabolism. Our results suggest for the first time that *TFAM* gene plays an important role in lipid metabolism and may be a strong candidate gene for obesity in mammals.

Keywords: *TFAM*; Nuclear-encoded mitochondrial gene; Promoter polymorphisms; Fat deposition; Obesity.

Mitochondrial transcription factor A (TFAM), a member of a high mobility group protein family and the first-identified mitochondrial transcription factor [1], is essential for maintenance and biogenesis of mtDNA. First, TFAM plays a histone-like role in mitochondria, as it is tightly associated with mtDNA as a main component of the nucleoid [2]. Evidence has shown that one molecule of mtDNA is packed with ~900 molecules of TFAM on average [3], which makes mtDNA no longer naked. Second, TFAM regulates mtDNA copy number in mammals. Investigation using a combination of mice with TFAM overexpression and TFAM knockout demonstrated that mtDNA copy number is directly proportional to the total TFAM protein level in mouse embryos [4]. RNA interference of the endogenous TFAM expression in HeLa cells also indicated that the mtDNA amount is correlated in parallel with the amount of TFAM [5]. Third, TFAM stimulates

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transcription of mtDNA. The TFAM protein possesses two tandem high mobility group domains, which makes TFAM bind, unwind, and bend DNA without sequence specificity and thus facilitate transcription initiation of mtDNA [6]. Evidence has shown that import of wt-TFAM into liver mitochondria from hypothyroid rats increased RNA synthesis up to 4-fold [7].

It has been known for many years that adipose tissue plays a central role in regulation and manipulation of energy metabolism through the storage and turnover of triglycerides and through the secretion of factors that affect satiety and fuel utilization. However, many key aspects of adipogenesis are accompanied by stimulation of mitochondrial biogenesis [8]. For example, the major site of fatty acid β-oxidation occurs in mitochondria [9], which may provide key intermediates for the synthesis of triglycerides via the action of pyruvate carboxylase [10]. In addition, a relatively large mitochondrial mass is needed to generate acetyl-CoA for fatty acid activation prior to esterification into triglycerides. All these studies demonstrated the essential role and function of mitochondria in lipid metabolism.

To further explore the mechanism of mitochondrial involvement in adipogenesis, Wilson-Fritch et al. [8,11] studied the 3T3-L1 cell (representative of white adipocytes) differentiation by using both proteomic and genomic approaches. Proteomic analysis revealed a 20- to 30-fold increase in the concentration of numerous mitochondrial proteins, while genomic analysis with gene expression profiling using Affymetrix GeneChips detected a statistically significant increase in the expression of many nucleus-encoded mitochondrial genes during adipogenesis. In particular, the authors found a profound decrease of approximately 50% in the levels of transcripts for nuclear-encoded mitochondrial genes accompanying the onset of obesity [11]. As *TFAM* is one of the nuclear-encoded mitochondrial genes, we hypothesize that this gene plays an important role in lipogenesis or fat deposition via its role in mitochondrial biogenesis. Here, we present for the first time evidence to support this hypothesis, because significant associations between the bovine *TFAM* promoter polymorphisms and marbling scores and subcutaneous fat depth (SFD) were observed in a F_2 population of Waygu x Limousin crosses.

Materials and methods

Development of Waygu x Limousin reference population. A F_1 generation of a Wagyu x Limousin cross was developed at Washington State University and transferred to the Fort Keogh Livestock and Range Research Laboratory, ARS, USDA in the autumn of 1998, including 6 F_1 bulls and 113 F_1 dams. Inter se mating of these F_1 animals produced 71 F_2 progeny in 2000, 90 in 2001, and 109 in 2002, respectively. Each calf was weighed within 24 h after birth and again at weaning when the calves averaged approximately 180 days of age. After weaning, the calves were returned to native range pastures and were supplemented with 0.7 kg of both barley cake and alfalfa pellets per calf per day. In mid-January, the calves were moved from the range and were fed silage and chopped hay to achieve anticipated gains of 0.5–0.8 kg per day. They were then placed on a finishing diet for approximately 150 days followed by slaughter. Growth rate, carcass, and meat quality data were collected on all F_2 calves. Marbling scores varied from $4 = \text{Slight}^0$ to $9.5 = \text{Moderately Abundant}^{50}$ (SD = 1.00) and SFD measurements ranged from 0.1 to 1.3 in. (SD = 0.18) in this F_2 population. Marbling was a subjective measure of the amount of intramuscular fat in the *longissimus* muscle based on USDA standards (http://www.ams.usda.gov/). SFD was measured at the 12–13th rib interface perpendicular to the outside surface at a point three-fourths the length of the longissimus muscle from its chine bone end. DNA was extracted from blood samples. Based on the availability of both data and DNA samples, 246 observations were used in the current study.

Cloning of the bovine TFAM gene. Unfortunately, both cDNA and genomic DNA sequences were not available for the bovine *TFAM* gene when we started the project. However, the bovine genome mapping project has advanced significantly in recent years. In particular, more than 500,000 bovine expressed sequence tags (ESTs) (http://www.ncbi.nlm.nih.gov/) and 3X bovine genome sequences (http://www.hgsc.bcm.tmc.edu/projects/bovine/) have been released to the public databases. Therefore, a combination of an in silico comparative cloning with a PCR target cloning approach was developed and used to determine both cDNA and genomic DNA sequences of the bovine gene (Fig. 1). The procedure included three steps: (1) BLAST searches against the public databases using a full-length cDNA sequence of the human *TFAM* gene as a reference to retrieve all bovine sequences that are orthologous to the human gene; (2) annotation of both ESTs and genomic DNA sequences in order to design primers for the target region amplification to close gaps if present; and (3) alignment of cDNA sequences and genomic DNA sequences to determine the full-length cDNA sequence and genomic organization of the bovine *TFAM* gene.

Two pairs of primers were designed to close two gaps for the genomic DNA sequence of the bovine *TFAM* gene (Table 1). PCRs were performed using 25 ng of bovine genomic DNA as template in a final volume of 10 μL containing 12.5 ng of each primer, 200 μM dNTPs, 1.5–3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl, and 0.2 U of Platinum Taq polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were carried out as follows: 94 °C for 2 min, 32 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s, followed by a further 5 min extension at 72 °C. PCR products were examined by electrophoresis through a 1.5% agarose gel with 1× TBE buffer to determine the quality and quantity for DNA sequencing. Sequencing was performed on ABI 3730 sequencer in the Laboratory for Biotechnology and Bioanalysis (Washington State University). Sequences of these two PCR-amplified products spanning the gap regions and three contigs of genome sequences derived from the cattle genome sequence project were then assembled to form a complete genomic DNA sequence for the bovine *TFAM* gene.

Genetic polymorphisms and association analysis. Primers were designed to target the promoter region and all coding regions in order to screen genetic polymorphisms in the bovine *TFAM* gene (Table 1). Four DNA pools were formed, one from all six F_1 bulls, one from 30 randomly selected F_1 dams, one from 30 F_2 high marbling progeny, and one from 30 F_2 low marbling progeny. PCR products for each pair of primers were amplified on these four DNA pools and directly sequenced on an ABI 3730 sequencer following standard Big Dye protocols. Nucleotide polymorphisms were identified by comparison of sequence patterns among these four DNA pools. Unfortunately, no polymorphisms were detected in the coding sequences, but two SNPs, i.e., C/A transversion and C/T transition, were found in the bovine *TFAM* promoter region.

These two SNPs in the bovine promoter region were then genotyped in Wagyu x Limousin F_2 animals that have both DNA samples