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Associations of Single Nucleotide Polymorphisms in the Bovine *FADS6* Gene with Fatty Acid Composition in Hanwoo (Korean Cattle)

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Abstract

The bovine fatty acid desaturase (*FADS*) gene cluster consists of *FADS*1, *FADS*2, *FADS*3, and *FADS*6, which acts as key enzymes in fatty acid metabolism. Of these, the genetics effects of variants in *FADS*1, *FADS*2 and *FADS*3 have been previously studied. However, the genetic effects of variants of *FADS*6 gene have not been studied. The aim of this study was to identify genetic variants in the bovine fatty acid desaturase 6 (*FADS*6) gene and study their association with fatty acid composition in Hanwoo cattle. Six genetic variants were observed, three each in intron 2 and exon 6 by DNA sequencing analyses. The association of genetic variants with fatty acid composition was evaluated in 90 Hanwoo steers. The variants were confirmed and the animals were genotyped by RFLP (Restriction Fragment Length Polymorphism) and AS-PCR (Allele Specific PCR) analyses. The analysis revealed that palmitoleic acid (C16:1n7) was associated with g.3391G > A, g.3660A > C and g.15657C > T, and stearic acid (C18:0) showed highly significant association with g.3660A > C segments. Both g.3391G > A, g.3660A > C also had strong additive and dominance effect for Palmitoleic acid, while g.3660A > C also had a strong dominance effect for stearic acid. These results could be useful for modulating fatty acid composition in beef and produce meat with higher monounsaturated fatty acid to saturated fatty acid ratio (MUFA/SFA), which had been shown to have positive health effect in humans.

Keywords

Fatty Acid Composition, Beef, *FADS*6, Genetic Association, SNP

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1. Introduction

The quantity and distribution of Fatty acids (FA) in beef are directly associated with its quality and value [1]. The major FAs in cattle are myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), vaccenic (C18:1), oleic (C18:1), and linoleic (C18:2) acids. Out of this palmitic, stearic and oleic acids make up 80% of the FAs in beef. Oleic, palmitic, stearic, linoleic, palmitoleic and myristic acids are related to beef marbling. The composition of FA in the beef is of importance as it has a direct bearing on human health. The composition of FAs has more impact on human health than the amount of fat in the diet [2] [3]. The intake of saturated fatty acid (SFA) has a positive impact on cardio vascular diseases like atheroscleorosis whereas monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) positively affects human health [4]-[6]. Stearic acid is considered to be neutral in its effect on plasma cholesterol in humans [7]. FA composition has become an important issue in beef industry as a major factor for determining meat quality traits. Recently, the quality of the fat has been issued as a critical factor for beef flavor, marbling degree, and colors [7] [8]. Moreover, an increased ratio of MUFA to SFA can improve the texture and taste of the meet [9]. Due to increased consumer awareness towards the implication of red meat associated fat intake on health, FA composition has become an important economic trait in the beef industry [10]. FA composition of beef is greatly influenced by age, feeding regime, and genotype [11] [12].

Animal producers have tried to change FA compositions in meat products through feeding systems that contain either high or low FA composition for their breeding goals [13] [14]. But significant difference in FA composition has been observed even when animals are fed with the same diet [15] showing genetics to play an important role in the control of FA composition. Polyunsaturated fatty acid (PUFA) is known to be involved in a variety of physiological functions [16]. The availability of PUFA in mammalian cells greatly depends on the activity of enzymes involved in FA metabolism. In mammals, the delta 5 and 6-desaturases are the pivotal enzymes introducing de novo unsaturation in the carbon chain of precursors leading to the synthesis of long-chain PUFA [17]. Bovine fatty acid desaturase (*FADS*) gene cluster includes *FADS*1, *FADS*2, *FADS*3, and *FADS*6, and they act as key enzymes in FA metabolism. The expression of *FADS* genes is correlated with eicosatetraenoic acid (C30:5n-3) and arachidonic acid (C20:4n-6) in cell culture models of cystic fibrosis. In humans, polymorphism's on *FADS* are known to affect omega-3 and -6 FAs concentration in plasma and tissues [18]. Genome wide association studies (GWAS) have highlighted the influence of variations in the *FADS* gene cluster on lipid metabolism, glucose metabolism, total cholesterol level and low-density lipoprotein in humans [19]-[21]. This is the first study to report about genetic polymorphism in *FADS*6 on bovine FA composition.

The aim of this study is to discover genetic variants in the entire *FADS*6 gene that have strong associations with FA composition in Hanwoo cattle.

2. Materials and Methods

2.1. Animals and Sample Preparation

This experiment was approved by the ethics and welfare committee of the National Institute of Animal Science (NIAS) in Korea. A total of 90 Hanwoo cattle, which were registered in the national database under the guidelines provided from NIAS, were used with average weight (167.2 ± 13.4 kg) and age (206 ± 12 day). The animals were slaughtered at the packing facility of NIAS, and the meat samples between 12^{th} & 13^{th} ribs were stored in −70˚C until FA composition was measured.

Extraction and methylation of lipids using chloroform-methanol (2:1, v/v) were performed by the procedure of Folch and Morrison [22] [23], respectively. Fatty acid methyl esters were analyzed by a gas chromatograph (Star 3600; Varian Technologies, CA, USA) fitted with a fused silica capillary column, omega wax 205 (30 m × 0.32 mm i.d., 0.25 um film thickness. The injection port and detector were maintained at 250˚C and 300˚C, respectively, and results were presented as percentages of FAs based on the total peak area. BFT and MAR were measured between the 12^{th} and 13^{th} rib, and between the last back bone and 1^{st} hipbone for 3 times, respectively.

For DNA isolation, 2 g of muscle was chopped into 0.2 cm cubes and extracted with the genomic DNA E-prep kit according to the manufacturer's guideline (genomic DNA E-prep, Prepgene, Korea). To assess DNA concentration and purity, NanoDrop 1000 spectrophotometer was used (Thermo Scientific, Waltham, MA, USA).

2.2. PCR Amplification

To amplify the *FADS*6 gene, 15 primer sets were designed based on the *FADS*6 genomic sequences from the UCSC genomic region (chr19:57766830 - 57782480) in **Table 1**. Amplifications were conducted with 10 X reaction buffer, 2.5 mM dNTP, 50 ng of genomic DNA, and 0.2 U of Taq DNA polymerase, in a final volume of 20 ul. An initial denaturation at 94˚C for 3 min was followed by 35 cycles of denaturation at 94˚C/45 sec, annealing at 55˚C - 59˚C/1 min, extension at 72˚C/1 min and a final extension at 72˚C/6 min. The primers were designed to amplify sizes between 1000 and 1200 bp and have a GC content of 60% using PrimerSelect program of DNAStar package (version 6.1). After verification of sequences for the target segments, AS-PCR (allele-specific PCR) primers were designed to amplify approximately 500 bp segments that contain the SNP positions.

2.3. Genotyping

The amplified DNA fragments were purified using the PCR purification Kit (Nucleogen, Korea) and sequenced with the ABI3730 XL Genetic Analyzer (Applied Biosystems, USA) at NIAS. To confirm that the acquired sequences were from the bovine *FADS*6 gene, the sequence was compared with the nucleotide database through NCBI BLAST. Individual sequences were aligned with the SEQMAN program of DNAStar Package (version 6.1) to verify the SNPs. For the individual genotyping, RFLP (restriction fragment length polymorphism) and AS-PCR (Allele Specific-Polymerase chain reaction) analyses were performed. RFLP was used to detect three polymorphic sites in exon 6 with two primer sets (F1-CAGACCCCTCCCATCACAGAGC, R1-CCCCAGCG GTGGCCAGCACAG, F2-GGGCCCAACCCTGGCTCTCC, R2-AAAAAGCAAGCAGGGCAGGTGAT). The amplification reactions followed the following program, initial denaturation at 94˚C/3 min, followed by 35 cycles of 94˚C/45 sec, 57˚C/30 sec, 72˚C/20 sec and final extension at 72˚C/6 min. The digestions with restriction enzymes (*Alu I* and *MSP I*) were performed with 2 ul of PCR products mixed with three units of the appropriate restriction enzyme and then were incubated at 37˚C for 2 h. Polymorphisms in intron 2 were confirmed by AS-PCR with three primer sets (**Table 2**). The PCR amplification condition for the AS-PCR was an initial denaturation at 94˚C/3 min, followed by 35 cycles of 94˚C/45 sec, 59˚C/30 sec, 72˚C/20 sec and final extension at 72˚C/6 min.

Table 1. Primer sequence for the bovine fatty acid desaturase 6.

ID	Primer sequence		Location		Size (bp)
	Forward	Reverse	start	end	
*FADS*6_1	CGGCGGGGTCGGGTAGAT	AGGAGGTGGGGGCTGTTATGGA	383	1602	1219
*FADS*6_2	GCAGTTGGGGTTTGGGTTGTG	GGGGTGGCGGCGTCTGA	1504	2808	1304
*FADS*6_3	CCACCTGCGCACGTTTCTATTTT	GGTGGTCGGGGAGCTTGTTACT	2248	3646	1398
*FADS*6_4	GGCATGAGTAGGGCGAGTGGATT	GGAGCAGGCAGGCAAGGGTAGT	3378	4768	1390
*FADS*6_5	CCCTTGCCCACGGACTACCC	ACAGACTAAATGCCCATCAACACG	4734	5805	1071
*FADS*6_6	CCACACCTTCTTTATTCATTCACG	ACGGGGGCCAAGCTTTTAGAGAC	5760	6944	1184
*FADS*6_7	AAATGTACTTGCGCAGGGTCGTCT	TCCATGGGGTCACAAAAAGTCAAA	6878	8103	1225
*FADS*6_8	CATTGCAGGCAGATTCTTTACCAT	AGGCGCTCACCACGACTA	7926	9135	1209
*FADS*6_9	TGGCTGCTGGTGGAGGTCTGA	GCGGGCTTCGGGAGTCGT	8974	10,331	1357
*FADS*6_10	AGCAGAAGTGTGAGGAAGGAAAAC	GCGGGAGGCCAGCAAGTC	10,168	11,288	1120
*FADS*6_11	CCTGCGCTCGGGAACAAGA	TGCAGTGAAGAGCCAGTGAGC	10,343	11,669	1326
*FADS*6_12	TATTTGGTTGCATCAGGTCTTAGG	GGCCCCAGCTCCACAGTTCTT	11,355	12,381	1026
*FADS*6_13	TCGTGGCTGTGGGTGAGTAAG	GGGAGAAGGGCAGAAGGTAAGAGT	12,195	13,282	1087
*FADS*6_14	ATCTCCCTGGGCCTTTATTCTCAA	GTGGGTCCTGTGTCCGTCTCA	13,011	14,275	1264
*FADS*6_15	GAGGCTCAGCACAGGACACAGAAC	GCCCCGGGAAGGACAGC	14,187	15,533	1346

The sequences were based on the genomic regions (UCSC chr19:57766830 - 57782480).

Table 2. Least squared means and standard errors for fatty acid composition with each genotype from 3 *FADS*6 segments.

NP	Genotype (LSM ± SE)			Allele frequency	χ^2 (HW)	Fatty acid	P	Effect			
	GG(31)	GA (53)	AA (2)					Additive	P	Dominance	P
g.3391G > A	4925 ± 0.10	5.100 ± 0.13	3.745 ± 0.52	G = 0.668 A = 0.3314	13.1272*	Palmitoleic acid (C16:1n7)	0.044	1.180 ± 0.53	0.03	1.528 ± 0.60	0.012
	AA (2)	AC(65)	CC (19)								
g.3660A > C	3.742 ± 0.52	5.062 ± 0.09	4.750 ± 0.17	A = 0.401	27.4836*	Palmitoleic acid (C16:1n7)	0.021	−1.007 ± 0.55	0.07	1.632 ± 0.58	0.006
	12.019 ± 0.74	10.429 ± 0.13	11.208 ± 0.24	C = 0.598		Stearic acid (C18:0)	0.004	0.811 ± 0.77	0.30	−2.368 ± 0.82	0.005
g.15657C > T	CC (28)	CT (48)	TT (10)	C = 0.6046	2.410	Palmitoleic acid (C16:1n7)	0.051	0.346 ± 0.27	0.21	0.854 ± 0.34	0.016
	4.859 ± 0.14	5.113 ± 0.10	4.513 ± 0.23	T = 0.3953							

Parentheses are the numbers of animals. HW = Hardy Weinberg Equilibrium; *Indicates significance.

2.4. Statistical Analysis

The FA measurements were tested for normal distributions. 10 out of 17 FAs were used for the association tests due to no measurements for the remaining FAs. The statistical analysis of associations between SNP and FA composition was performed with the GLM procedure on SAS 9.2 with a model that has a fixed effect for genotype, a covariate for age, and a random effect for sire. The genotyping frequencies and minor allele frequencies and HWE (Hardy-Weinberg Equilibrium) were calculated with Arlequin version 3.5.

3. Results

3.1. Analysis of Genetic Variants

For the analysis of genetic variants of the *FADS*6 gene in Hanwoo, the targeted fragments were successfully amplified with 15 primer sets, and sizes were confirmed by direct sequencing analysis. A total of 6 genetic variants were found, with 3 each at the intron number 2 (nucleotide positions 3391, 3660, and 4655) and the exon number 6 (nucleotide positions 15,527, 15,590, and 15,657) based on a reference sequence (UCSC genomic regions chr19:57766830 - 57782480) by PCR-RFLP and AS-PCR (**Figure 1**). The PCR-RFLP analysis (**Figure 2**) verified unique restriction patterns, showing DNA fragments of different mobilities by agarose gel electrophoresis. The g.15527C > T SNP in exon 6 was digested with Alu I, and g.15590T > C, and g.15657C > T SNPs in exon 6 were digested with MSP I, while the polymorphisms (g.3391G > A, g.3660A > C, and g.4655G > A) were confirmed with AS-PCR analysis. Only g.3391G > A, g.3660A > C and g.15527C > T were found to have significant association with FAs. The allele frequency and Hardy Weinberg equilibrium (HWE) were calculated for these three SNPs. The SNP g.3391G > A presented allele frequency for G (0.6686) and A (0.3313), resulting in a genotype frequency of 36.05% (GG), 61.62% (GA) and 2.32% (AA) respectively. The genotype frequency at g.3660A > C was 2.32% (AA), 74.42 (AC) and 22.09% (CC), with allele frequencies for A being 0.4 and for C being 0.6. Allele frequency at g.15527C > T was C (0.6046) and T (0.3953), resulting in a genotype frequency of 32.55% (CC), 55.81% (CT) and 11.62% (TT) respectively .The HWE for g.3391G > A, g.3660A > C were found to be highly significant ($P > 0.0001$) (**Table 3**).

3.2. Association Analyses

As shown in **Table 3**, the analyses observed marginal associations for palmitoleic acid (C16:1n7) with g.3391G/A ($p = 0.044$) and g.15657C/T ($p = 0.051$) whereas, genotypes of g.3660A/C showed significant associations with palmitoleic acid (C16:1n7) and stearic acid (C18:0). At g.3391G > A, the GA genotype had higher association (5.100 ± 0.13) with Palmitoleic acid followed by GG and AA genotypes. The SNP g.3660A > C had significant association with Palmitoleic acid and Stearic acid, with the AC genotype being strongly associated

Table 3. The targeted SNP locations, amplification sizes, and primer sequences for Allele Specific PCR.

ID	Primer sequence	Size		SNP location
		Start	End	
Uni 1F	ACAGCCTGGAGGTGGGTGAG	3150	3169	3392
Uni 1R	TTGGAAGCAGGCATTGAAGAGTC	3603	3625	
ASPCR 1-1F	GCCACTGGCATGAGTAGGGCG	3372	3392	
ASPCR 1-2F	GCCACTGGCATGAGTAGGGCA	3372	3392	
Uni 2F	TCAACCAGGCAGCGGACAGT	3412	3431	3661
Uni 2R	GCAACAGGAGAAGCCACCACAG	3898	3919	
ASPCR 2-1F	GACCACCCCCATCACCTTTTTA	3640	3661	
ASPCR 2-2F	GACCACCCCCATCACCTTTTTC	3640	3661	
Uni 3F	CAGCATCACCATAGAAGAATC	4399	4419	4565
Uni 3R	CACCCCAGCCTGCCCCTACAT	4834	4854	
ASPCR 3-1F	ATATACCCCACTCACGCCAG	4637	4656	
ASPCR 3-2F	ATATACCCCACTCACGCCAA	4637	4656	

Uni: Unique primers to amplify approximately 500 bp; Primer sequences were based on the genomic region (chr19:57766830 - 57782480, UCSC).

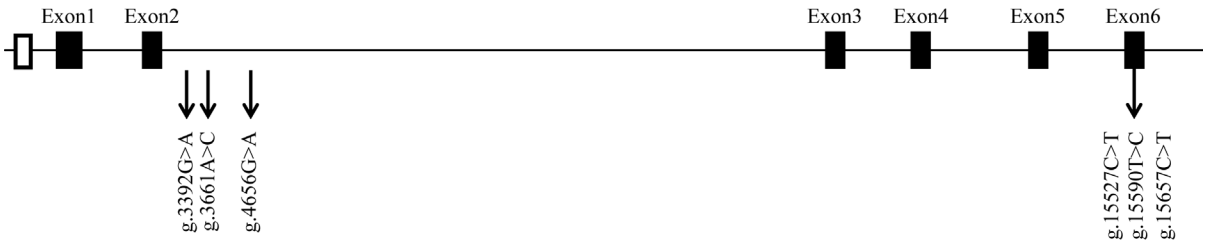

Figure 1. Gene Structure. Genetic variants have been confirmed using RFLP and AS-PCR analyses. Nucleotide positions 3392, 3661 and 4656 were located in intron 2 and 15,527, 15,590 and 15,657 were located in exon 6 based on a reference sequence (UCSC a genomic region of chr 19:57766830 - 57782480).

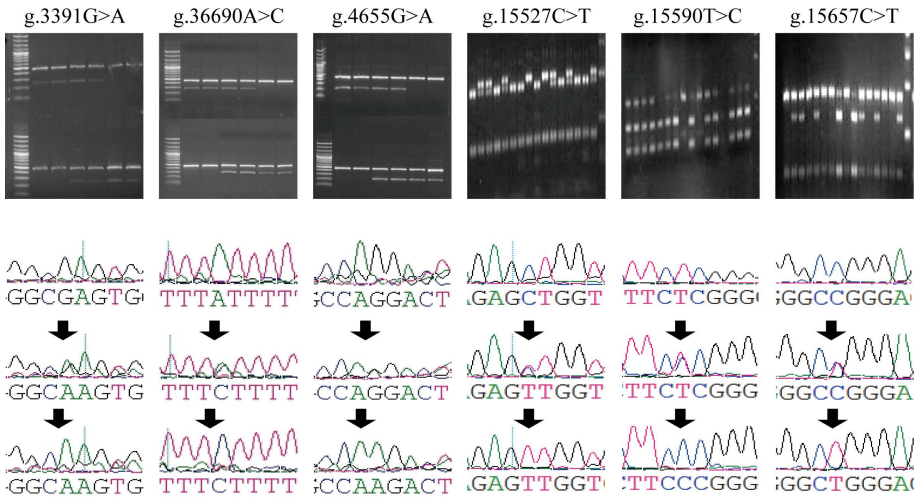

Figure 2. Detection of single nucleotide polymorphisms with RFLP and AS-PCR analyses. The segments (g.3391G > A, g.3660A > C, and g.4655G > A) were analyzed with AS-PCR and the segments (g.15527C > T, g15590T > C, and g.15657C > T) were analyzed by RFLP analyses with Alu I and MSP I restriction enzymes.

with the former and AA being significantly associated with the later. The SNP g.15657C > T had strong association with Palmitoleic acid, with the strongest association for genotype CT followed by CC and TT. Whereas, the genotypes of g.4655G > A, g.15527C > T, and g.15590T > C were not significantly associated with FA composition. Significant dominance genetic effects were detected for all segments, additionally, additive genetic effects were found between the genotypes of g.3391G > A and Palmitoleic acid (C16:1n7).

4. Discussion

Increased consumption of food rich in fat particularly saturated fatty acid predisposes man to cardio vascular disease [24]. Consumers are increasingly aware about the correlation between food and health. Beef is a highly nutritious and valued food. In fact, fat like conjugated linoleic acid (CLA) which is present in beef is considered to be beneficial for human health [25]. But the high concentration of saturated fatty acid in beef leads to negative effects in human health. Several studies have tried to manipulate fatty acid composition in beef [24]-[26]. Improving the polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) ratio is a way to produce healthier meet. PUFA and MUFA (monounsaturated fatty acids) are beneficial to human health [27]. Moreover, increasing the ratio of MUFA to SFA also leads to increase in texture and taste [9]. Studies have showed the association of polymorphism in fatty acid biosynthetic genes like SREBP01, LXRα, *FADS*1, *FADS*2, *FADS*4, FASN etc. with fatty acid composition and also beef quality traits [1] [10] [28]. *FADS*6 (Fatty acid desaturase 6) is a member of the fatty acid desaturase family, and is involved in the fatty acid biosynthesis pathaway. No functional study of bovine *FADS*6 has been reported, but they are found to be highly similar to human *FADS*6. The *FADS*6 gene in humans is homologous to *FADS*2 [29] [30]. *FADS*2 synthesizes Delta-6-desaturase (D6D). D6D catalyzes the first step in the synthesis of highly unsaturated fatty acid (HUFA) or LC-PUFA (long chain-poly unsaturated fatty acid) [29] [31]. This is the first study to report the effect of genetic variants in *FADS*6 on fatty acid composition in beef (Hanwoo cattle). Six genetic variations were found within *FADS*6 out of which 3 were in the 2nd intron (nucleotide positions 3391, 3660, and 4655) and 3 were in the 6th exon (nucleotide positions 15,527, 15,590, and 15,657). Out of them, the variation at g.3391G > A and g.15657C > T was found to have significant association with the composition of palmitoleic acid. Palmitoleic acid, a MUFA is known to have a positive effect on hepatic lipid accumulation and insulin resistance [9] [32]. The SNP g.3660A > C was found to have strong association ($P > 0.21$, $P > 0.004$) with additive and dominance effect ($P > 0.07$, $P > 0.006$) for palmitoleic acid and strong additive effect ($P > 0.004$) for stearic acid respectively. Stearic acid unlike other unsaturated fatty acid (UFA) has a neutral effect on human blood cholesterol level [33] [34]. Moreover, the firmness of carcass fat and the melting point of lipids in beef are closely related to the concentration of stearic acid therby influencing the quality of the meat [35]. Bovine *FADS*2 was homologous to human *FADS*6, and SNPs in bovine *FADS*6 were found to have an effect on several fat related traits, and SNP *FADS*2 g.-823G > A was proposed as a genetic marker for beef advancement [28]. The significant associations with dominance effects for genotypes of *FADS*6 g.15657C > T located in exon 6 may be an important factor and could be used as genetic markers for Palmitoleic acid (C16:1n7).

5. Conclusion

In conclusion, the identified SNPs are the first report to help in understanding the genetic structures of *FADS*6 on fatty acid compositions in cattle populations. The SNPs in *FADS*6 were found to have a strong association with palmitoleic and stearic acid composition. These variations might help in producing healthy beef, high in MUFA and stearic acid. With stearic acid influencing both taste and texture of meat, these SNPs might also be associated with beef quality traits. This study provides useful genetic information regarding the relation between *FADS*6 and fatty acid composition.

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