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ADFP promoter polymorphism associated with marbling score in Korean cattle

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Marbling score (MS) is the major trait that affects carcass quality in beef cattle. In this study, we investigated the association between genetic polymorphisms of the adipose differentiation-related protein gene (*ADFP*) and carcass traits in Korean cattle (also known as Hanwoo). Using direct DNA sequencing in 24 unrelated Korean cattle, 25 novel polymorphisms were identified within all exons and their flanking regions of *ADFP*, including the promoter region (1.5 kb). Among them, 21 polymorphic sites were selected for genotyping in the beef cattle (n = 425). Statistical analyses revealed that one promoter polymorphism (c.*-56-18A* > G) was associated with MS (P = 0.009). The "A" allele of c.*-56-18A* > G exerted a lowering effect on MS, e.g., the lowest MS was found in "A/A" (MS = 2.09 ± 1.23), intermediate in "A/G" (MS = 2.11 ± 1.31), and the highest in "G/G" (MS = 2.47 ± 1.47). Our findings suggest that these polymorphisms in *ADFP* might be important genetic factors involved in carcass quality in beef cattle. [BMB reports 2009; 42(8): 529-534]

INTRODUCTION

The successful application of genetic marker-assisted selection of commercial animals will depend on the identification of genes, including those underlying quantitative traits. Exploration of genetic polymorphisms involved in different phenotypes of quantitative traits, and understanding how these genes/polymorphisms interact with the environment or with other genes affecting economic traits are necessary.

Intramuscular or marbling fat has been considered important in beef production because it is a major factor in consumer satisfaction and directly or indirectly affects human obesity (1, 2). Therefore, a study of marbling depotsition in cattle is important as marbling is the primary determinant of price or beef meat quality. It is also of scientific interest as major economic traits of cattle are affected by multifactorial polygenic qualities in which both genetic and environmental factors play a role (3, 4).

Fat deposition is influenced by the number of fat cells or adipocytes found in muscle. Among them, the adipose differentiation-related protein (*ADFP*) is a likely candidate gene for fat deposition traits. The expression of *ADFP* was first identified in the early differentiation stage of adipocyte cells (5). *ADFP* is also deeply involved in lipid metabolism. Abundant *ADFP* stimulates fatty acid uptake and triglyceride formation, facilitating the receptor-mediated uptake of very low density lipoproteins (6-8). Its ubiquitous distribution (9), and the functional role of *ADFP* as a fatty acid?binding protein (10) in lipid droplet formation (7) suggest that the genetic variation of this gene might be associated with carcass traits. In a recent study, polymorphisms in the *ADFP* gene showed significant associations with carcass traits and intramuscular fat content in chickens (11). In spite of the functional importance of *ADFP* in the regulation of fat deposition and the genetic study of chickens (11) to the best of our knowledge there have been no genetic association studies of *ADFP* and carcass traits in beef cattle.

In this study, we investigated *ADFP* as one of the major candidate genes affecting meat quality. Extensive screening of *ADFP* was performed by direct sequencing to detect polymorphisms, and examined their genetic association with carcass traits. Here, we present 25 novel polymorphisms identified in *ADFP* and results of an association study with the marbling score (MS) in Korean cattle (Hanwoo).

RESULTS AND DISCUSSION

By direct DNA sequencing, 25 polymorphisms were identified in *ADFP*; 4 in the promoter region, 6 in coding exons, 3 in an untranslated region, and 12 in introns. The location and allele frequencies of these polymorphisms are shown in Table 1 and Fig. 1A. Using pairwise linkage analysis with DNA from 24 unrelated Korean cattle, two sets of polymorphisms (c.*-56-67G* > *A*:c.*-56-1G* > *C* and c.*309+211G* > *A*: c.*462C* > T (*S154S*))

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Table 1. Genotypes and allele frequencies of 25 polymorphisms in *ADFP*

Polymorphism	Position	AA change	Genotypes (number of animals)			Total number of animal	Minor allele frequency	Hetero-zygosity	HWE
c.-56-418T > G*	Promoter	–	T (244)	GT (90)	G (77)	411	0.297	0.417	6.7×10^{-21}
c.-56-67G > A*	Promoter	–	G (400)	AG (5)	A (6)	411	0.021	0.041	2.0×10^{-44}
c.-56-18A > G*	Promoter	–	A (171)	AG (130)	G (114)	415	0.431	0.491	1.7×10^{-12}
c.-56-1G > C	Promoter	–	G (18)	CG (0)	C (1)	19	0.053	0.100	7.5×10^{-5}
c.-39G > C*	5'UTR	–	G (337)	CG (56)	C (22)	415	0.120	0.212	1.3×10^{-12}
c.-26+128C > G*	Intron1	–	C (349)	CG (57)	G (4)	410	0.079	0.146	0.629
c.-26+149G > A*	Intron1	–	G (171)	AG (195)	A (55)	421	0.362	0.462	0.999
c.-26+163T > C*	Intron1	–	T (163)	CT (177)	C (70)	410	0.387	0.474	0.192
c.-26+175C > T*	Intron1	–	C (279)	CT (121)	T (21)	421	0.194	0.312	0.265
c.-26+321G > C*	Intron1	–	G (348)	CG (62)	C (3)	413	0.082	0.151	0.991
c.17T > C (V6A)*	Exon2	V6A	T (290)	CT (117)	C (12)	419	0.168	0.280	0.999
c.30+27T > C*	Intron2	–	T (173)	CT (183)	C (65)	421	0.372	0.467	0.363
c.106A > G (R36G)*	Exon3	R36G	A (406)	AG (15)	G (0)	421	0.018	0.035	0.933
c.309+211G > A	Intron4	–	G (19)	AG (5)	A (0)	24	0.104	0.187	0.850
c.462C > T (S154S)*	Exon5	S154S	C (361)	CT (37)	T (2)	400	0.051	0.097	0.621
c.553C > T (L185L)*	Exon5	L185L	C (392)	CT (7)	T (1)	400	0.011	0.022	1.1×10^{-4}
c.595+106Ginsdel	Intron5	–	ins (23)	insdel (0)	del (1)	24	0.042	0.080	6.1×10^{-6}
c.596-11T > A*	Intron5	–	T (342)	AT (76)	A (7)	425	0.106	0.189	0.519
c.777+64C > T*	Intron6	–	C (204)	CT (179)	T (42)	425	0.309	0.427	0.957
c.777+135A > G*	Intron6	–	A (323)	AG (95)	G (7)	425	0.128	0.224	1.000
c.913-1G > A	Intron7	–	G (22)	AG (2)	A (0)	24	0.042	0.080	0.978
c.1065Tinsdel (S355S)*	Exon8	S355S	ins (414)	insdel (2)	del (0)	416	0.002	0.005	0.999
c.1217Tinsdel (Y406L)*	Exon8	Y406L	del (330)	insdel (76)	ins (8)	414	0.111	0.198	0.356
c.1353*235T > C*	3'UTR	–	T (124)	CT (199)	C (92)	415	0.461	0.497	0.773
c.1353*302C > T*	3'UTR	–	C (220)	CT (151)	T (36)	407	0.274	0.398	0.397

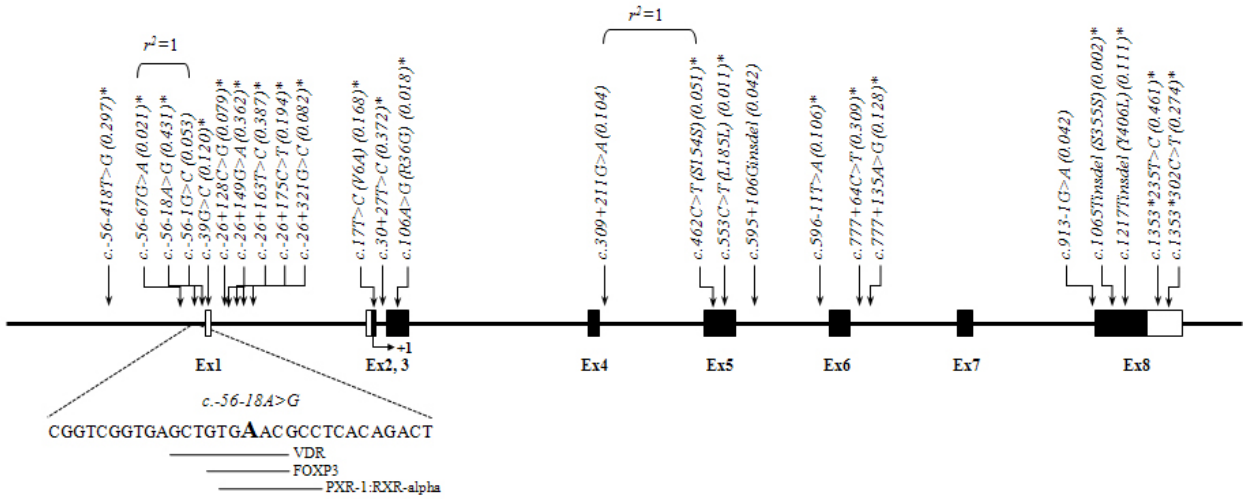
Asterisks (*) indicate polymorphisms genotyped in a larger Korean cattle cohort (n = 425).

were found in absolute LD ($|D'| = 1$ and $r^2 = 1$) (Fig. 1A). Among these polymorphisms, *c.-56-418T > G, c.-56-67G > A, c.-56-18A > G, c.-56-1G > C, c.-39G > C, c.553C > T (L185L)*, and *c.595+106Ginsdel* showed very low Hardy-Weinberg Equilibrium (HWE) probabilities (Table 1). This suggests that the selected animals have been endogamous, which could be a result of the intensive selection process of the NLRI progeny testing program in order to fulfill beef industry needs.

Among the 25 polymorphisms, 21 of them (*c.-56-418T > G, c.-56-67G > A, c.-56-18A > G, c.-39G > C, c.-26+128C > G, c.-26+149G > A, c.-26+163T > C, c.-26+175C > T, c.-6+321G > C, c.17T > C (V6A), c.30+27T > C, c.106A > G (R36G), c.462C > T (S154S), c.553C > T (L185L), c.596-11T > A, c.777+64C > T, c.777+135A > G, c.1065Tinsdel (S355S), c.1217Tinsdel (Y406L), c.1353*235T > C*, and *c.1353*302C > T*) were selected for larger-scale genotyping based on location (polymorphisms in exons were preferred), minor allele frequency exceeding 0.05, and LD (a polymorphism was chosen if it was in absolute LD [$r^2 = 1$] with one or more other polymorphisms). Minor allele frequency, heterozygosity, and HWE values are shown in Table 1. Among the 21 genotyped polymorphisms, no common haplotypes (freq. > 0.1) were constructed and they also showed weak LDs (Fig. 1B and 1C).

Associations of *ADFP* polymorphisms with MS were analyzed using the mixed-effect model with sire and age as covariates. Sire was treated as a random effect and age as a fixed effect. No positive associations were detected with CW (Table 2). However, one polymorphism (*c.-56-18A > G*) in the promoter region was significantly associated with MS (P = 0.009). The "A" allele of *c.-56-18A > G* was shown to be correlated with the lowest MS, e.g., the lowest MS was found in "A/A" (MS = 2.09 ± 1.23), intermediate in "A/G" (MS = 2.11 ± 1.31), and the highest in "G/G" (MS = 2.47 ± 1.47) (Table 2). However, when Bonferroni corrections were strictly adopted, the associated P-value showed that it was not significant, using the threshold of significance would be 0.0012 (21 polymorphisms and 2 phenotypes-CW and MS-analyzed). However, although there is a chance of type 1 error due to multiple comparisons when considering that the comparisons were not totally independent of each other due to tight LDs among SNPs/haplotypes and related phenotypes, the sig-

A. Map of *ADFP* (adipose differentiation-related protein) on chromosome 8 (8.7 kb)



B. Haplotypes in *ADFP*

Hap.	c.-56-418T>G	c.-56-67G>A	c.-56-18A>G	c.-30G>C	c.-26+128C>G	c.-26+149G>A	c.-26+163T>C	c.-26+175C>T	c.-26+321G>C	c.177>C (V6A)	c.301+27T>C	c.106a>G (R36G)	c.462C>T (S154S)	c.553C>T (L185L)	c.596-11T>A	c.777+64C>T	c.777+135A>G	c.1065Tinsdel (S355S)	c.1217Tinsdel (Y406L)	c.1353*235T>C	c.1353*302C>T	Freq.
ht1	T	G	G	G	C	G	C	C	G	T	T	A	C	C	T	T	A	ins	ins	C	T	0.061
ht2	T	G	A	G	C	G	C	C	G	T	C	A	C	C	T	C	A	ins	del	T	C	0.041
ht3	T	G	A	G	C	G	C	C	G	T	T	A	C	C	T	C	A	ins	del	C	C	0.039
ht4	G	G	A	C	C	A	C	C	G	T	C	A	C	C	T	C	A	ins	del	T	C	0.038
ht5	T	G	A	G	C	A	T	C	G	T	T	A	C	C	T	C	A	ins	del	T	C	0.037
ht6	T	G	A	G	G	G	T	C	G	C	T	A	C	C	A	T	G	ins	del	C	T	0.036
ht7	T	G	G	G	C	G	C	C	G	T	C	A	C	C	T	C	A	ins	del	T	C	0.032
ht8	G	G	G	G	C	G	T	T	G	T	C	A	C	C	T	C	A	ins	del	T	C	0.030
ht9	G	G	G	G	C	G	T	T	G	T	T	A	T	C	T	C	A	ins	del	C	C	0.030
Others.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.656

C. LDs among *ADFP* polymorphisms

Fig. 1. Gene map, haplotypes, and LD coefficients in *ADFP* (A) Gene map and polymorphisms in *ADFP* on chromosome 8. The coding exon is marked by black blocks and 5′ and 3′ UTRs by white blocks. The first base of the transcriptional site is denoted as nucleotide +1. Asterisks (*) indicate polymorphisms genotyped in a larger Korean cattle cohort (n = 425). Putative transcription factor sites (VDR, FOXP3, and PXR-1:RXR-alpha) are indicated (PROMO, http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). (B) Haplotypes of *ADFP*. Haplotypes with frequency >0.03 are presented. Others contain rare haplotypes. (C). Linkage disequilibrium coefficient ($|D'|$ and r^2) among *ADFP* polymorphisms.

nificance of the association might be worthy of a follow-up in future studies.

ADFP is known to be a lipid droplet-associated protein that is expressed early during adipose differentiation (7) and transcriptionally activated when adipocyte precursors differentiate into mature adipocytes (10). Therefore, *ADFP* might be involved in MS variation related to fat deposition. Although the mechanisms involved in the association of alternative polymorphisms of *ADFP* with MS are not currently understood, our study suggests that *ADFP* polymorphisms may affect MS in beef cattle.

The polymorphism within the promoter region, *c.-56-18A > G*, was investigated for a putative transcription factor binding site using PROMO software, which revealed that it is located in the consensus sequence of VDR (vitamine D receptor), FOXP3 (forkhead box P3), and PXR-1:RXR-alpha (Pregnane X Receptor 1: Retinoid x receptor alpha) binding sites. The nucleotide change from "A" to "G" of this polymorphism could disrupt the transcription binding site (Fig. 1).

In addition, one intronic polymorphism (*c.913-1G > A*) activating a cryptic splice site leading to alternative splicing, and two exonic polymorphisms (*c.1065Tinsdel [S355S]* and *c.1217Tinsdel [Y406L]*) inducing frame shift mutations were identified. Although *c.1065Tinsdel (S355S)* and *c.1217Tinsdel*

Table 2. Association analyses of *ADFP* polymorphisms with carcass weight and marbling score among Korean cattle

Traits	Polymorphism	Position	AA change	Genotype			P value
				C/C	C/R	R/R	
				n (mean ± SD)	n (mean ± SD)	n (mean ± SD)	
Carcass weight	c.-56-418T > G	Promoter	–	244 (312.60 ± 31.32)	90 (308.07 ± 35.27)	77 (312.26 ± 40.35)	0.51
	c.-56-67G > A	Promoter	–	400 (311.13 ± 33.44)	5 (301.20 ± 31.96)	6 (332.33 ± 52.29)	0.53
	c.-56-18A > G	Promoter	–	171 (306.77 ± 34.01)	130 (310.35 ± 33.76)	114 (318.95 ± 32.70)	0.14
	c.-39G > C	5'UTR	–	337 (313.63 ± 32.12)	56 (299.70 ± 37.98)	22 (304.00 ± 42.75)	0.88
	c.-26+128C > G	Intron1	–	349 (311.49 ± 34.49)	57 (310.37 ± 26.07)	4 (340.75 ± 29.80)	0.36
	c.-26+149G > A	Intron1	–	171 (313.32 ± 31.78)	195 (309.68 ± 33.10)	55 (312.67 ± 41.40)	0.56
	c.-26+163T > C	Intron1	–	163 (314.00 ± 33.55)	177 (309.08 ± 33.82)	70 (312.73 ± 32.16)	0.82
	c.-26+175C > T	Intron1	–	279 (309.65 ± 34.03)	121 (314.85 ± 32.50)	21 (317.71 ± 36.27)	0.70
	c.-26+321G > C	Intron1	–	348 (311.57 ± 33.62)	62 (312.02 ± 33.05)	3 (321.67 ± 27.59)	0.71
	c.17T > C (V6A)	Exon2	V6A	290 (312.82 ± 33.70)	117 (308.38 ± 34.28)	12 (305.17 ± 34.28)	0.22
	c.30+27T > C	Intron2	–	173 (310.97 ± 34.82)	183 (311.88 ± 30.93)	65 (310.18 ± 39.33)	0.11
	c.106A > G (R36G)	Exon3	R36G	406 (311.47 ± 33.84)	15 (305.07 ± 35.34)	–	0.53
	c.462C > T (S154S)	Exon5	S154S	361 (311.50 ± 34.14)	37 (312.08 ± 31.29)	2 (295.50 ± 23.33)	0.15
	c.553C > T (L185L)	Exon5	L185L	392 (311.19 ± 33.84)	7 (313.43 ± 28.80)	1 (334.00)	0.57
	c.596-11T > A	Intron5	–	342 (311.38 ± 34.20)	76 (311.57 ± 32.99)	7 (306.57 ± 19.50)	0.85
	c.777+64C > T	Intron6	–	204 (313.49 ± 35.35)	179 (308.53 ± 31.62)	42 (312.83 ± 34.52)	0.24
	c.777+135A > G	Intron6	–	323 (312.02 ± 34.12)	95 (309.89 ± 32.90)	7 (299.43 ± 29.23)	0.30
	c.1065Tinsdel (S355S)	Exon8	S355S	414 (311.27 ± 33.66)	2 (307.00 ± 74.95)	–	0.61
	c.1217Tinsdel (Y406L)	Exon8	Y406L	330 (311.42 ± 34.78)	76 (309.50 ± 30.75)	8 (318.00 ± 23.65)	0.84
	c.1353*235T > C	3'UTR	–	124 (312.42 ± 37.58)	199 (311.01 ± 32.66)	92 (309.23 ± 32.08)	0.08
	c.1353*302C > T	3'UTR	–	220 (313.64 ± 35.33)	151 (307.23 ± 31.69)	36 (311.14 ± 36.71)	0.31
Marbling score	c.-56-418T > G	Promoter	–	244 (2.25 ± 1.34)	90 (2.14 ± 1.40)	77 (2.19 ± 1.27)	0.46
	c.-56-67G > A	Promoter	–	400 (2.21 ± 1.36)	5 (2.00 ± 0.71)	6 (2.00 ± 0.63)	0.40
	c.-56-18A > G	Promoter	–	171 (2.09 ± 1.23)	130 (2.11 ± 1.31)	114 (2.47 ± 1.47)	0.009
	c.-39G > C	5'UTR	–	337 (2.25 ± 1.36)	56 (1.96 ± 1.22)	22 (2.09 ± 1.19)	0.43
	c.-26+128C > G	Intron1	–	349 (2.20 ± 1.33)	57 (2.33 ± 1.43)	4 (2.25 ± 0.96)	0.19
	c.-26+149G > A	Intron1	–	171 (2.23 ± 1.30)	195 (2.16 ± 1.34)	55 (2.27 ± 1.43)	0.26
	c.-26+163T > C	Intron1	–	163 (2.36 ± 1.44)	177 (2.14 ± 1.26)	70 (2.04 ± 1.29)	0.06
	c.-26+175C > T	Intron1	–	279 (2.11 ± 1.30)	121 (2.43 ± 1.38)	21 (2.14 ± 1.42)	0.44
	c.-26+321G > C	Intron1	–	348 (2.22 ± 1.35)	62 (2.21 ± 1.29)	3 (2.00 ± 1.00)	0.44
	c.17T > C (V6A)	Exon2	V6A	290 (2.22 ± 1.34)	117 (2.08 ± 1.29)	12 (2.83 ± 1.47)	0.19
	c.30+27T > C	Intron2	–	173 (2.16 ± 1.23)	183 (2.26 ± 1.41)	65 (2.12 ± 1.40)	0.19
	c.106A > G (R36G)	Exon3	R36G	406 (2.17 ± 1.31)	15 (2.87 ± 1.68)	–	0.31
	c.462C > T (S154S)	Exon5	S154S	361 (2.20 ± 1.33)	37 (2.49 ± 1.52)	2 (2.00 ± 1.41)	0.07
	c.553C > T (L185L)	Exon5	L185L	392 (2.23 ± 1.35)	7 (1.86 ± 0.69)	1 (1.00)	0.15
	c.596-11T > A	Intron5	–	342 (2.20 ± 1.35)	76 (2.14 ± 1.24)	7 (3.00 ± 1.41)	0.22
	c.777+64C > T	Intron6	–	204 (2.27 ± 1.35)	179 (2.15 ± 1.32)	42 (2.12 ± 1.25)	0.91
	c.777+135A > G	Intron6	–	323 (2.21 ± 1.35)	95 (2.09 ± 1.23)	7 (3.29 ± 1.50)	0.26
	c.1065Tinsdel (S355S)	Exon8	S355S	414 (2.21 ± 1.34)	2 (2.00 ± 1.41)	–	0.72
	c.1217Tinsdel (Y406L)	Exon8	Y406L	330 (2.25 ± 1.37)	76 (2.11 ± 1.24)	8 (1.88 ± 0.99)	0.67
	c.1353*235T > C	3'UTR	–	124 (2.21 ± 1.33)	199 (2.23 ± 1.38)	92 (2.12 ± 1.20)	0.41
	c.1353*302C > T	3'UTR	–	220 (2.28 ± 1.39)	151 (2.09 ± 1.26)	36 (2.17 ± 1.32)	0.93

Genotype and haplotype distributions and P values controlling for sire and age at slaughter as covariates are shown. C/C, C/R, and R/R represent the common allele, and heterozygotes and homozygotes for the rare allele, respectively. n (mean ± SD): number of animals (mean of values ± standard deviations). Significant associations are shown in boldface.

(Y406L) were not associated with MS, further biological and/or functional studies are needed for these polymorphisms.

In summary, we have identified 25 novel polymorphisms in *ADFP* in Korean cattle, and 21 common polymorphic sites were selected for genotyping. Statistical analysis revealed that one polymorphism (c.*-56-18A* > *G*) in the promoter region showed a significant association with the marbling score (MS). The polymorphism and association information identified in this study would be useful for future genetic studies of cattle.

MATERIALS AND METHODS

Animals and phenotypic data

Korean native cattle genomic DNA samples were obtained from 425 steers produced from 74 sires used in the progeny testing program of the National Livestock Research Institute (NLRI) of Korea. All steers were fed for 731.34 ± 16.49 days under a tightly controlled feeding program in the Daekwanryeong and Namwon branches. The average birth date was May 10 [range: April 16-June 3]. After two years, animals were slaughtered between May 3 and May 17. They had been weaned at a mean age of 3 months and fed with 30% concentrates and 70% roughage until they were 6 month old. After 6 months of age, they were fed with concentrates consisting of 15% crude protein (CP)/71% totally digestible nutrients (TDN) until 14 months; 13% CP/72% TDN until 20 months; and 11% CP/73% TDN until they were 24 months of age. Roughage was offered *ad libitum*, and steers had free access to fresh water during the entire period. Live weights were determined before slaughter. The mean of the live weights was 539.78 ± 51.62 kg. Yield grades for carcasses were determined by cold carcass weight (CW). After a 24-h chill, CW was measured, and then the left side of each carcass was cut between the last rib and the first lumbar vertebra to determine MS. The mean of the CW was 311.17 ± 33.50 kg. MS was determined by assessing the degree of marbling in the cut surface of the ribeye. The degree of marbling was evaluated according to the Korean Beef Marbling Standard (1 = trace, 7 = very abundant) (12). The mean of the MS was 2.25 ± 1.42.

Sequencing analysis of *ADFP*

All exons of *ADFP* and their flanking regions were sequenced, including promoter regions (1.5 kb), to discover variants in 24 unrelated Korean cattle using the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Eleven primer sets for the amplification and sequencing analysis were designed based on GenBank sequences (Ref. Genome seq.: AF239708 released on 2 Jan. 2002). Primer information is available in Ad. Table 1. Sequence variants were verified by chromatograms (Ad. Fig. 1).

Genotyping by single-base extension (SBE) and electrophoresis

For genotyping of polymorphic sites, amplifying and extension primers were designed for single-base extension (SBE) (13). Primer extension reactions were performed with the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems). To stop the primer extension reaction, one unit of shrimp alkaline phosphatase (SAP) was added to the reaction mixture, and the mixture was incubated at 37°C for 1 hour, followed by 15 min at 72°C for enzyme inactivation. The DNA samples, containing extension products, and Genescan 120 Liz size standard solution were added to Hi-Di formamide (Applied Biosystems) according to the recommendation of the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then electrophoresis was performed using the ABI Prism 3100 Genetic Analyzer. The results were analyzed using the programs ABI Prism GeneScan and Genotyper (Applied Biosystems). Probe information is available in Ad. Table 2.

Statistical analysis

χ^2-tests were used to determine whether the individual variant was in equilibrium at each locus in the population (Hardy-Weinberg equilibrium). Associations between individual SNPs and MS were determined using the mixed-effect model, treating "sire" as a random effect, and "age" at slaughter included in the model as a covariate using the library (nlme) in the R statistical package (http://www.r-project.org). Other covariates were not available for this analysis. For the haplotype analyses, the model was fit with the same covariates in a similar manner. A widely used measure of linkage disequilibrium (LD) was examined between all pairs of biallelic loci, D' (the correlation coefficient [Dalta $|D'|$]) and r^2, and strength of LD between pairs of SNPs was measured as D' using Haploview (14, 15). Haplotypes and their frequencies were inferred using the algorithm developed by Stephens *et al*. (16). Phase probabilities of each site were calculated for each individual using PHASE software (input option: ignoring families, http://depts.washington.edu/ventures/UW_Technology/Express_Licenses/PHASEv2.php) (16). Phase probabilities of all polymorphic sites for haplotypes were calculated for each individual using this software. Single-base changes in promoter sequences may alter the regulation of gene expression, particularly if the variant affects a transcription factor binding site, and were therefore included in our analyses. Putative transcription factor binding sites were identified using the PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) database.

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