Genotype of stearoyl-CoA desaturase is associated with fatty acid composition in Japanese Black cattle

Masaaki Taniguchi,[1] Takeshi Utsugi,[1] Kenji Oyama,[2] Hideyuki Mannen,[2] Masato Kobayashi,[3] Yoshihiro Tanabe,[4] Atsushi Ogino,[4] Soichi Tsuji[2]

[1]Laboratory of Animal Breeding & Genetics, Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe 657-8501, Japan
[2]Laboratory of Animal Breeding & Genetics, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657-8501, Japan
[3]Yamagata Agricultural Research and Training Center, Shinzyo, 996-0041, Japan
[4]Maebashi Institute of Animal Science, Livestock Improvement Association of Japan Inc., Maebashi 371-0121, Japan

Abstract

To investigate the genetic factors that affect fatty acid composition of beef, we compared the full-length bovine stearoyl-CoA desaturase (*SCD*) cDNA from 20 Japanese Black steers. Two types of the *SCD* gene with single nucleotide polymorphisms (SNPs) were observed in the ORF of *SCD* cDNA, in which an amino acid replacement from valine (type V) to alanine (type A) was predicted. We developed a method for genotyping these two *SCD* genes based on PCR-RFLP. We have classified 1003 Japanese Black carcasses into three genotypes, VV, VA, and AA, and compared fatty acid composition among them. The *SCD* type A gene contributed to higher MUFA percentage and lower melting point in intramuscular fat. The *SCD* genotype was not the only genetic factor contributing to fatty acid composition of Japanese Black steers, but the *SCD* genotype was considered one of the causes of genetic variation in fatty acid composition of Japanese Black steers. Transcription factors such as sterol regulatory element binding protein-1c (SREBP-1c) may account for the remaining part of the genetic variation in fatty acid composition.

The Japanese Black breed of cattle is valued for its highly marbled meat and fat melting points that are lower than in other breeds. The lower melting points reflect the higher percentage of unsaturated fatty acids in fat (Yang et al. 1999a). As well as contributing to the softness of bovine fat, such fatty acid profiles may also contribute positively to beef flavor (Melton et al. 1982). Stearoyl-CoA desaturase (SCD) is the enzyme responsible for conversion of saturated fatty acids into mono-unsaturated fatty acids (MUFA) in mammalian adipocytes. In the case of ruminants, fatty acids in the feed are chemically reduced by microorganisms in the rumen and absorbed as saturated fatty acids. The composition of fatty acids stored in the fat depots reflects the previous action of SCD on substrates such as stearic acid or palmitic acid (Kim and Ntambi 1999). While nutrition can be clearly shown to contribute to the fatty acid profile of subcutaneous fat and intramuscular fat, the genetic factors in determining the fatty acid profile of its fat are being defined. Given its determinant role in fatty acid oxidation, *SCD* is a candidate for genetic variation in fatty acid composition. There have been no studies that describe either mutation in the *SCD* gene or its relationship to fatty acid composition, though Yang et al. (1999b) have presented interesting correlations between SCD enzyme activity and fatty acid composition in a survey of bovine adipose tissues, and our previous study revealed that the *SCD* mRNA expression level was related to MUFA percentage (Taniguchi et al. 2003).

In this study, we have compared sequences of the full-length bovine *SCD* cDNA and the 5'-upstream region and found two types of the gene with single nucleotide polymorphisms (SNPs) in the ORF. The *SCD* genotypes may affect fatty acid composition, because one of those SNPs found in the ORF was predicted to cause an amino acid substitution. Therefore, we have investigated relationships between *SCD* genotypes and fatty acid compositions or

Correspondence to: S. Tsuji; E-mail: tsuji@ans.kobe-u.ac.jp

melting points of carcass fat of fattened Japanese Black steers, utilizing a developed method for genotyping at the *SCD* locus.

Materials and methods

Animals. For cDNA synthesis, muscle samples were collected from 20 Japanese Black cattle within 5 min of slaughter at an abattoir in Gifu City, Japan. The samples were immediately transferred into liquid nitrogen and stored at –80°C prior to RNA preparation.

For genomic DNA extraction, the blood samples of 1003 Japanese Black steers from two testing stations, Hokkaido and Hiroshima, were collected by the Livestock Improvement Association of Japan (LIAJ) as part of their progeny testing in 2000 and 2001. These individual steers were derived from 64 sires. Their feeding conditions, i.e., slaughter age, concentration, and forage intake, and fattening period were tightly controlled under the progeny testing protocols (Oikawa et al. 2000). The intramuscular fats of these steers were also collected from the *M. trapezius* muscle immediately after slaughter to analyze fatty acid profiles.

Fatty acid analysis. Total lipids were extracted from approximately 300 mg of *M. trapezius* with chloroform:methanol (2:1, vol/vol) according to the method of Folch et al. (1957). The lipids were methylated by the method of O'Keefe et al. (1968) with sodium methylate. Methyl esters were analyzed with a gas chromatograph-equipped flame ionization detector (Hitachi G-3000, Tokyo, Japan). The fat melting points were determined by a flow-through method (Japanese Pharmacopoeia, 14th edition; http://jpdb.nihs.go.jp/jp14e).

Comparisons of DNA sequences between individuals. Total RNA extraction and cDNA synthesis were performed according to our previous study (Taniguchi et al. 2003). In the previous study, we determined the full-length sequence of bovine *SCD* cDNA, and the data were deposited on DDBJ (Accession No. AB075020). To amplify 5351 bp full-length *SCD* cDNA, seven primer sets were designed, as shown in Fig. 1. To detect the 5′-upstream region of the bovine *SCD* gene, we screened a bovine cosmid library constructed with pWE15 vector (Stratagene, CA). Reverse primers designed at a nearby translation initiation site were applied to the cosmid clone to determine the sequence of transcription regulation site for the *SCD* gene. The sequences of DNA fragments were analyzed by using the Sequi-

Therm EXCEL II DNA sequencing kit-LC (Epicentre Technologies, Madison, WI) and an automated sequencer DNA 4200 (Li-Cor, NE).

Genotyping. We applied PCR-RFLP methods to detect three nucleotide substitutions in the ORF of *SCD* cDNA. Three primer sets were synthesized with DNA fragments flanking 702 bp, 762 bp, and 878 bp mutation sites counted from the translation initiation site. The PCR amplification was performed with 20 ng of genomic DNA, each with three primer sets and TaKaRa Ex Taq™ polymerase (TaKaRa, Kyoto, Japan). Amplifications were performed with a thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems, CA), with the following thermo-cycling protocol: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, with a final extension step, 72°C for 7 min. Primer sets used for PCR amplification were as follows: primer names correspond to nucleotide substitution sites (F, sense primer; R, antisense primer), *SCD*-F702, 5′-GTGTCCTGTTGTTGTGCTTCATC CTGCC-3′; *SCD*-R702, 5′-AATATTCTCTCGGGG GTTGATGGTCT TG-3′; *SCD*-F762, 5′-GTTTTTTG CCACCTTATTCCGGTA-3′; *SCD*-R762, 5′-AAT ATTCTCTCGGGGGTTGATGGTCTTG-3′; *SCD*-F878, 5′-ATGTATGGATACCGCCCTTATGAC-3′; *SCD*-R878, 5′-TTCTGGCACGTAACCTAATACCC TAAGC-3′. The first fragment (702 bp) was digestible with *Nco*I, and the last one (878 bp) with *Fhu*4H1. No suitable restriction enzyme was found to detect the second mutation at 762 bp. Then the Mismatch PCR-RFLP (Haliassos et al. 1989) was adopted to introduce an *Rsa*I recognition site so that we could detect the genotype at this position. The digestions with restriction enzymes were performed with 20 μL of PCR products mixed with three units of the appropriate restriction enzyme and then were incubated at 37°C for 3 h.

Statistical analysis. In order to clarify the effects of the *SCD* genotype, the 64 sires of steers were grouped by using a dendrogram drawn by the UPGMA method. The genetic distance between sires i and j (d_{ij}) was defined as $d_{ij} = 1 - r_{ij}$, where r_{ij} denotes the relationship coefficient calculated by all known pedigree information. Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2.1 (Kumar et al. 2001). Then the MUFA percentage and melting point of intramuscular fat were analyzed by ANOVA with a model that accounted for a fixed sire group and *SCD* genotype without interaction. Differences of these fat traits between *SCD* genotypes were tested by using Fisher's Least Square Difference (LSD). Regression anal-
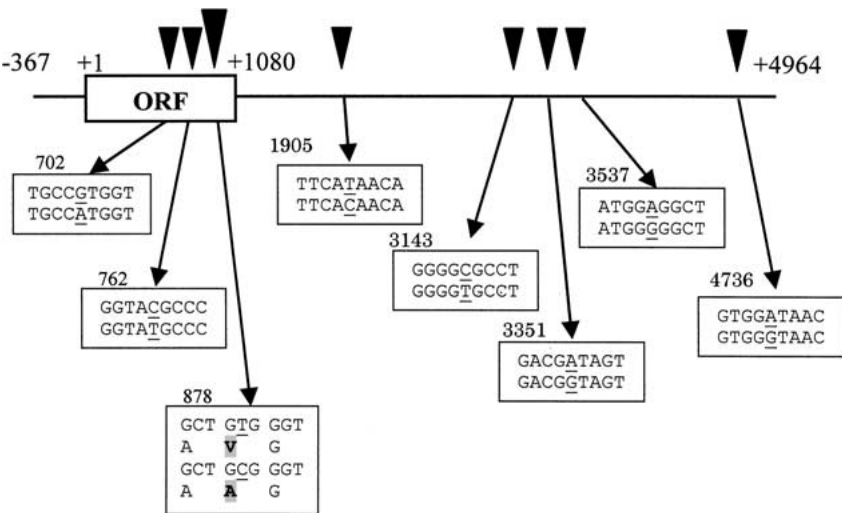
Fig. 1. Schematic illustration of full-length bovine SCD cDNA. The full length of bovine *SCD* was 5331 bp long. The ORF has 1080 nucleotides coding 359 amino acids, and the 3'UTR has 3884 nucleotides. Triangles show the positions of 8 SNPs, and the boxes indicate mutation sites of SNPs. Underlines and hatched boxes show nucleotide substitutions and amino acid replacement, respectively. The large triangle shows the site of amino acid replacement. The numbers show the nucleotide number on both ends of primers, with the translation initiation site assigned as +1.

ysis was used to investigate the association between MUFA percentages, melting point of intramuscular fat, and *SCD* genotypes. The average effect of the gene substitution of *SCD* genotypes to the MUFA percentage and melting point of intramuscular fat was calculated by the standard formula (Falconer 1981).

Results

Eight nucleotide substitutions in bovine SCD gene. We compared the full-length bovine *SCD* cDNA sequence among 20 Japanese Black individuals. In a previous study, we found that the 3'UTR sequence of bovine SCD cDNA included four "AT-TTA" motifs that affect mRNA stability in mouse and human (Sessler et al. 1996; Zhang et al. 1999). However, there was no repeat variation in the region. There were eight SNPs in bovine *SCD* cDNA sequences (Fig. 1). In this study, we discovered nucleotide substitutions at 702 bp (G to A), 762 bp (C to T), and 878 bp (T to C) in ORF, and 1905 bp (T to C),

3143 bp (C to T), 3351 bp (A to G), 3537 bp (A to G), and 4736 bp (A to G) in 3'UTR (Fig. 1). The SNP at 878 bp was predicted to cause substitution of the amino acid valine to alanine in the SCD protein. For the present, three SNPs observed in the ORF were confirmed to link together to constitute two types of *SCD*: type V (valine type) and type A (alanine type). After making primer sets at a flanking region of each SNP, genotypes were determined with the PCR-RFLP. One of the examples of genotyping is shown in Fig. 2.

The amino acid substitution site at the 293 position is shown with a hatched column in Table 1. The sires of 1003 experimental individuals were classified into two groups (Group I and Group II), based on relationship coefficient. Forty-five of 64 sires were classified to Group I, and 19 of 64 sires were classified to Group II. The averages of relationship coefficient of Group I and Group II were 0.241 and 0.182, respectively, while it was 0.049 between the two groups. The 1003 animals were classified according to their sire groups, and *SCD*
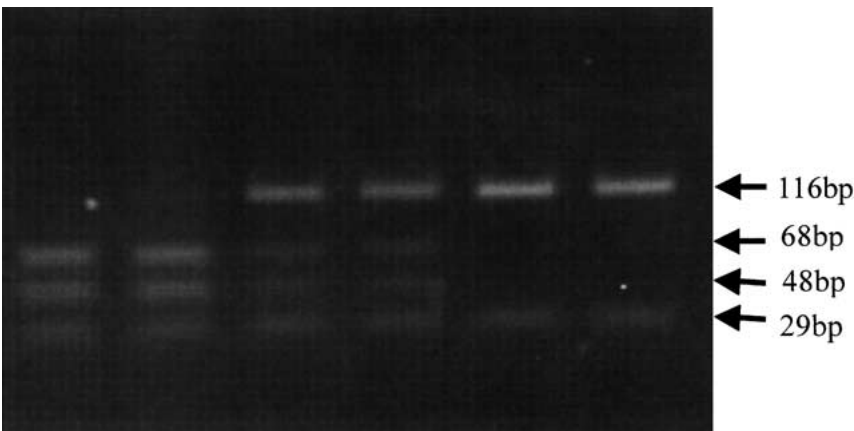

Fig. 2. Genotyping of SCD at the 878-bp polymorphic position. Digestion of an amplified fragment, including 878-bp polymorphic position by restriction enzyme *Fnu*4HI, shows genotypes AA, VA, and VV. The arrowheads show the size of DNA fragment (bp). These DNA fragments were size fractionated by using 1% agarose gel.

Table 1. SNPs in *SCD* cDNA ORF region and the gene frequency of two types of *SCD* defined for 1003 Japanese Black steers. Bold column shows the SNP that is likely to cause amino acid replacement.

Type	Nucleotide substitutions			Gene frequency		
	702	762	**878**	Group I	Group II	Total
A	A	T	**C**	0.65	0.47	0.59
V	G	C	**T**	0.35	0.53	0.41

genotyping was performed. Table 1 shows the gene frequency for these two types of *SCD* gene. In both groups, the gene frequencies were not in Hardy-Weinberg equilibrium and may reflect selection pressure for other carcass traits such as beef marbling.

Sequence of SCD gene 5′upstream resign. In this study, we determined the sequence, including the transcription regulatory site of the *SCD* gene, and deposited it to DDBJ (Accession No. AB106355). Transcription factors binding sites such as the sterol regulatory element (SRE), nuclear factor Y (NF-Y), and CCAAT enhancer binding protein alpha (C/EBPα) were found in the 5′-upstream region as well as the human *SCD* gene promoter (Bene et al. 2001; Zhang et al. 2001). We also compared the 5′-upstream region of 1011 bp from translation initiation site among 20 Japanese Black individuals. However, no mutations were observed in the region.

SCD amino acid alignment among mammalian species. The putative bovine SCD amino acid sequence from the ORF sequence demonstrated high homology with that of goat (93.9%, AF325499; Bernard et al. 2001), sheep (93.6%, AJ001048; Ward et al. 1998), human (87.2%, HM_005063; Zhang et al. 1999) and mouse (80.5%, NM_009127; Ntambi et al. 1988). Three histidine-rich regions were found in bovine SCD as well as in other species' SCD and in other desaturases, as shown in Fig. 3 (Shanklin et al. 1994; Cho et al. 1999). The second amino acid in the third box of the histidine-rich region was highly conserved across the mammalian species (Fig. 3).

Fatty acid composition and melting point of intramuscular fat. Table 2 shows the means of unsaturated fatty acid proportion and melting point of intramuscular fat between the three *SCD* genotypes and also between the groups. Differences were significant in MUFA percentage (high in type AA, middle in type VA, low in type VV). Melting points of intramuscular fat tissue were also significantly different between *SCD* genotypes (high in type VV, middle in type VA, low in type AA). Significant differences were also observed between the groups ($P < 0.001$). In Table 2, Group I showed higher MUFA percentage (58.6%) and lower melting point of intramuscular fat (25.7°C) than those (57.5%, 26.8°C) of Group II.

Contribution of SCD genotype to MUFA percentage and melting point of intramuscular fat. The regression analysis of MUFA percentage on genotypes showed a positive and significant correlation (R = 0.186, $P < 0.001$), as shown in Fig. 4A. However, the MUFA percentage was quite variable within a genotype, and the genotypes explained 4% of total variation of MUFA ($R^2 = 0.04$). The regression analysis of melting point of intramuscular fat on genotypes showed a negative and significant correlation (R = 0.163, $P < 0.001$), as shown in Fig. 4B. The melting point of intramuscular fat was also variable within the genotype, and the genotypes explained 3% of the total variation of the melting point of intramuscular fat ($R^2 = 0.03$).

The average effects of the gene substitution of the *SCD* type A gene on MUFA percentage and melting point of intramuscular fat were +0.805 and −1.03, respectively.

Discussion

The enzyme SCD produces MUFAs from saturated fatty acids such as stearic acid and palmitic acid, introducing a double bond at the Δ^9-position of the saturated acids. The breed differences in fatty acid composition of bovine fat were well defined (Yoshimura and Namikawa 1985; Zembayashi et al. 1995). Indeed, Yang et al. (1999b) found that SCD enzyme activity correlates positively with unsaturated fatty acid content in bovine fat tissue. Fatty acid profile is important to the global beef industry, because it has an impact on the visual manifestation of marbling during processing (Smith et al. 1998), the softness of the fat, and the flavor of the meat on the consumers' plate (Melton et al. 1982). The fatty acid composition of bovine tissues is affected by some factors such as animal sex (Clemens et al. 1973), diet (Mandell et al. 1998), and genetic factors (Perry et al.
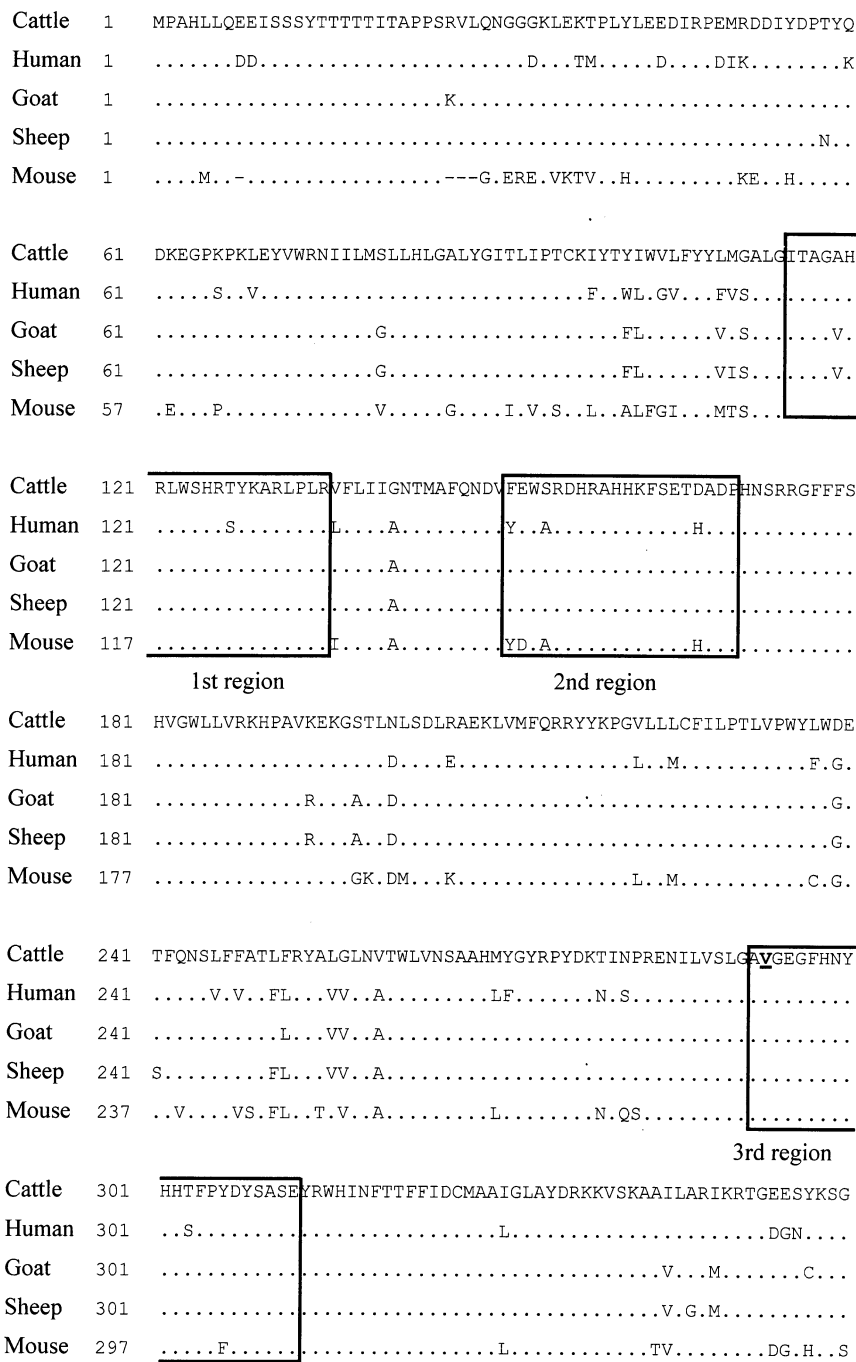
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Cattle   1    MPAHLLQEEISSSYTTTTTITAPPSRVLQNGGGKLEKTPLYLEEDIRPEMRDDIYDPTYQ
Human    1    .......DD....................D...TM.....D....DIK........K
Goat     1    .......................K..............................
Sheep    1    .....................................................N..
Mouse    1    ....M..-.................---G.ERE.VKTV..H.........KE..H.....

Cattle   61   DKEGPKPKLEYVWRNIILMSLLHLGALYGITLIPTCKIYTYIWVLFYYLMGALG|ITAGAH
Human    61   .....S..V....................F..WL.GV...FVS...|.......
Goat     61   ................G...........FL......V.S...|....V.
Sheep    61   ................G...........FL......VIS...|....V.
Mouse    57   .E...P............V.....G....I.V.S..L..ALFGI...MTS...|

Cattle   121  |RLWSHRTYKARLPLR|VFLIIGNTMAFQNDV|FEWSRDHRAHHKFSETDADF|HNSRRGFFFS
Human    121  ......S........|L....A.........|Y..A.........H..|.........
Goat     121  ...............|.....A.........|..............|.........
Sheep    121  ...............|.....A.........|..............|.........
Mouse    117  |..............|I....A.........|YD.A.........H..|.........
                   1st region                2nd region

Cattle   181  HVGWLLVRKHPAVKEKGSTLNLSDLRAEKLVMFQRRYYKPGVLLLCFILPTLVPWYLWDE
Human    181  ...................D....E.............L..M.........F.G.
Goat     181  ...........R...A..D.....................G.
Sheep    181  ...........R...A..D.....................G.
Mouse    177  ..............GK.DM...K..............L..M..........C.G.

Cattle   241  TFQNSLFFATLFRYALGLNVTWLVNSAAHMYGYRPYDKTINPRENILVSLGA|V|GEGFHNY
Human    241  .....V.V..FL...VV..A.........LF.......N.S...........
Goat     241  .........L...VV..A..................................
Sheep    241  S.........FL...VV..A................................
Mouse    237  ..V....VS.FL..T.V..A.........L.......N.QS........|.........
                                                                 3rd region

Cattle   301  |HHTFPYDYSASE|YRWHINFTTFFIDCMAAIGLAYDRKKVSKAAILARIKRTGEESYKSG
Human    301  ..S.........|.....................L.................DGN....
Goat     301  ............|.......................V...M.......C...
Sheep    301  ............|.......................V.G.M..........
Mouse    297  .....F......|..................L...........TV........DG.H..S
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Fig. 3. Comparison of deduced amino acid sequence between different species. Three histidine-rich domains are boxed. Bovine SCD amino acid sequence shows high homology with that of other mammalian species. The amino acid replacement from valine to alanine, shown with a solid underline, is observed in the third histidine-rich region.

Table 2. Comparison of MUFA content and melting point in fat tissue between two SCD genotypes and sire groups.

Effect	n	MUFA (%)	Melting point (°C)
Genotype			
AA	278	58.8 ± 0.1^a	25.4 ± 0.2^a
VA	635	58.2 ± 0.1^b	26.1 ± 0.1^b
VV	90	57.1 ± 0.3^c	27.6 ± 0.3^c
Sire group			
I	709	58.6 ± 0.1^a	25.7 ± 0.1^a
II	294	57.5 ± 0.1^b	26.8 ± 0.2^b

Mean values with different superscripts in the same column differ significantly ($P < 0.001$).
MUFA indicates the percentage of mono-unsaturated fatty acids including C14:1, C16:1, and C18:1.
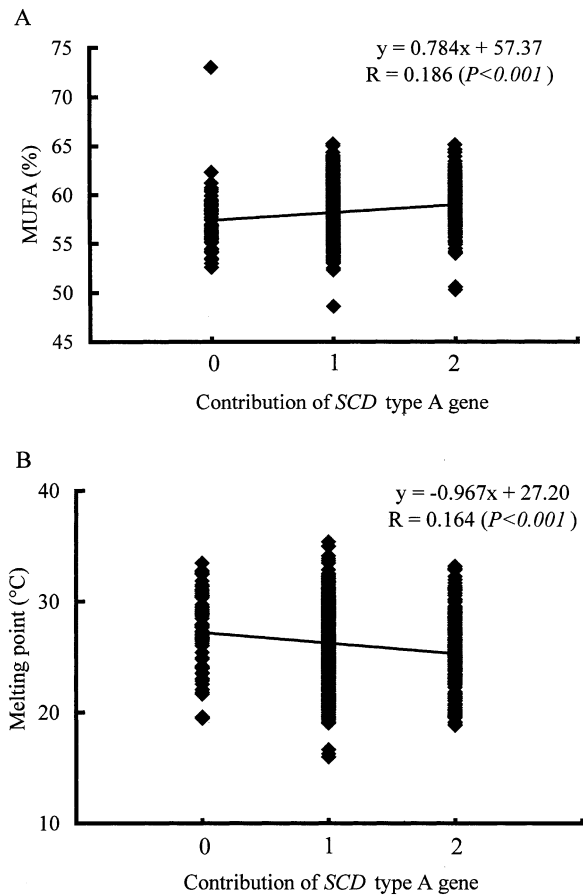
A

$$y = 0.784x + 57.37$$
$$R = 0.186\ (P<0.001)$$

B

$$y = -0.967x + 27.20$$
$$R = 0.164\ (P<0.001)$$

Fig. 4. Effects of *SCD* type A gene on MUFA content and melting point of intramuscular fat tissue. Numbers on X-bar correspond to numbers of *SCD* type A gene, where 2 means AA homozygote, 1 means VA heterozygote, and 0 means VV homozygote. The relationship between genetic contributions of SCD types A gene and MUFA percentage (A), melting point of intramuscular fat (B). The coefficient of determination of type A gene (R^2) was 0.04 in MUFA and 0.03 in melting point, both calculated from regression analysis.

1998). Oka et al. (2002) recently found that sire groups in Japanese Black also had a significant effect on fatty acid composition.

In this study, we compared the full-length sequence of *SCD* cDNA of 20 Japanese Black cattle and identified eight SNPs, including one which may change the enzymes catalytic activity, although Chung et al. (2000) also determined partial *SCD* cDNA sequence of Korean cattle and reported the differences in the mRNA level between sexes.

As described in Fig. 3, an amino acid substitution from valine to alanine was observed in the third histidine-rich region. In this study, the valine at the site of 293 amino acids was highly conserved across the mammalian species, which means that the valine is an ancestral amino acid at this position of SCD (Strittmatter et al. 1988; Shanklin 1994).

This amino acid substitution may have a key role on the enzyme function. Thus, it is possible that genetic variation in SCD may account for some of the observed variation of fatty acid composition between Japanese Black steers.

We then determined to what extent the *SCD* genotype was associated with MUFA percentage in the intramuscular fat of M. trapezius. The *SCD* type A gene had the average effect of gene substitution of 0.805% on MUFA percentage. Actually, the genotype AA showed 1.7% higher MUFA percentage than the genotype VV (Table 2). However, the contribution of the *SCD* genotype to MUFA variation was not so high, explaining only 4% of the total variation ($R^2 = 0.04$). In addition, there were significant differences in MUFA percentage and melting point between Groups I and II ($P < 0.001$). Therefore, the *SCD* genotype is not the only cause of genetic variation in fatty acid composition of Japanese Black carcasses. Other genetic factors must contribute to the variation of MUFA percentage in adipose tissue.

We attempted to find variation in the "ATTTA" motif repeat in the 3′UTR sequence which has previously been shown to affect mRNA stability in human and mouse (Sessler et al. 1996; Zhang et al. 1999). However, no such variation was observed in that region. Furthermore, we compared 1011 bp genomic DNA sequence of the *SCD* gene, including the sequence of the 5′-upstream region. Likewise, no SNPs were found in the 5′ upstream putative regulatory motifs. We have, however, found differences in the level of *SCD* mRNA expression in muscle and fat tissues between Japanese Black and Holstein steers (Taniguchi et al. 2003), so it is speculated that one of the other factors for MUFA variation may be a transcriptional regulator; e.g., a plausible candidate is sterol responsible-element binding protein 1c.

The results of our study suggested that the genotyping of the *SCD* gene is a useful tool for selection of favorable flavored beef carcasses, and the other genetic factors for MUFA variation are being focused on the transcription factors of the *SCD* gene.

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