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Original Article

Identification of leptin gene polymorphisms associated with carcass traits and fatty acid composition in Japanese Black cattle

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Running head: Leptin gene polymorphisms in cattle

ABSTRACT

Previous studies have indicated that some leptin gene polymorphisms were associated with economically important traits in cattle breeds. However, polymorphisms in the leptin gene have not been reported thus far in Japanese Black cattle. Here, we aimed to identify the leptin gene polymorphisms which are associated with carcass traits and fatty acid composition in Japanese Black cattle. We sequenced the full-length CDS of leptin gene for eight Japanese Black cattle. Sequence comparison revealed eight SNPs. Three of these were predicted to cause amino acid substitutions: Y7F, R25C, and A80V. Then, we genotyped these SNPs in two populations (JB1 with 560 animals and JB2 with 450 animals) and investigated the effects on the traits. Y7F in JB1 and A80V in JB2 were excluded from statistical analysis because the minor allele frequencies were low (<0.1). Association analysis revealed that Y7F had a significant effect on the dressed carcass weight in JB2; R25C had a significant effect on the C18:0 and C14:1 in JB1 and JB2, respectively; and A80V had a significant effect on the C16:0, C16:1, C18:1, MUFA, and SFA in JB1. The results suggested that these SNPs could be used as an effective marker for the improvement of Japanese Black cattle.

Keywords: *fatty acid composition, Japanese Black cattle, leptin.*

1 INTRODUCTION

2 Japanese Black cattle is the main beef cattle breed in Japan and is appreciated
3 worldwide for the quality of its beef, including its high marbling and good flavor
4 (Wheeler *et al.* 2004). Some effective DNA markers for the improvement of this breed
5 have been previously identified. For example, Sukegawa *et al.* (2010) reported an
6 association between a SNP in the *EDG1* gene and the beef marbling score, and
7 Hayakawa *et al.* (2015) identified a *FASN* gene polymorphism in the promoter region
8 noted to be associated with fatty acid composition. However, because beef-quality
9 traits are regulated by polygenes, additional DNA markers in other genes are required
10 for a more effective marker-assisted selection in this breed.

11 Leptin, an adipocyte-derived circulating protein, is a central factor for
12 decreasing food intake and virtually stopping body weight gain (Chen *et al.* 1996).
13 Fantuzzi and Faggioni (2000) reported that leptin alters the balance of T cell-derived
14 cytokines and acts as an inhibitor of apoptosis in T lymphocytes. Ingvarsen and
15 Boisclair (2001) concluded that leptin has a direct effect on not only food intake and
16 energy homeostasis but also immunity. Furthermore, leptin regulates the expression of
17 some genes and the activation of enzymes (Siegrist-Kaiser *et al.* 1997; Minokoshi *et al.*
18 2002). Thus, polymorphisms in the leptin gene coding sequence may affect some
19 important traits in Japanese Black cattle.

20 Previous studies have identified polymorphisms in the leptin gene coding
21 sequence and have reported that non-synonymous substitutions in the leptin gene are
22 associated with many economically important traits in other breeds, including carcass

traits (Corva *et al.* 2009; Tian *et al.* 2013; Silva *et al.* 2014), milk production traits (Buchanan *et al.* 2003; Chebel *et al.* 2008; Glantz *et al.* 2012), and fertility traits (Giblin *et al.* 2010). However, polymorphisms in the leptin gene have not been reported thus far in Japanese Black cattle. Therefore, we aimed to identify the leptin gene polymorphisms associated with carcass traits and fatty acid composition in Japanese Black cattle.

MATERIALS AND METHODS

Animals

We used two Japanese Black cattle populations (JB1 and JB2) to evaluate the association of leptin genotypes with carcass traits and fatty acid composition. JB1 comprised the commercial cattle population produced in Miyazaki Prefecture, Japan from April 2006 to October 2007 and included 560 cattle (506 steers and 54 heifers), whereas JB2 comprised the cattle population fattened by the Wagyu Registry Association across Japan for field progeny testing from 2002 to 2011 and included 450 cattle (288 steers and 162 heifers). On fattening farms, a group of animals (usually a few to several individuals) are managed together in a pen. They have restricted access to roughage and *ad libitum* access to concentrate, which consists of corn, barley, and wheat bran. The average age at slaughter in JB1 and JB2 was 29.10 ± 1.62 and 28.54 ± 1.34 months, respectively.

DNA polymorphism identification

To detect DNA polymorphisms, we sequenced the full-length CDS of the bovine leptin gene using genomic DNA from eight Japanese Black cattle, which were selected from JB2 population based on the pedigree information. Primer sets for the PCR amplification and sequencing analysis were designed based on GenBank sequences [NC_007302.5] using OLIGO 7.41 (Table 1). Following purification of the PCR product using ExoSAP-IT® (Affymetrix, Inc., CA, USA), we performed standard double-stranded DNA cycle sequencing with approximately 20 ng of amplified product using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Genotyping

We applied PCR-RFLP to genotype three nucleotide substitutions (Y7F, R25C, and A80V) in the bovine leptin gene. Three PCR primer sets were designed for DNA fragments flanking these polymorphisms (Table 1). PCR was performed with 20 ng of genomic DNA and TaKaRa Ex Taq™ polymerase (TaKaRa, Kyoto, Japan) using the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, CA, USA) with the following thermo-cycling protocol: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 63°C (Y7F) or 62°C (R25C and A80V) for 30 s, and 72°C for 1 min (Y7F and R25C) or 30 s (A80V), with a final extension step of 72°C for 7 min. The fragments flanking Y7F, R25C, and A80V were digestible with *BspDI*, *Acil*, and *BclI*, respectively. We performed the digestions in 20 µL reaction mixture, with approximately 5 µg of PCR products and 2.5 units of each restriction enzyme. The digested PCR products were

confirmed by agarose gel electrophoresis of the undigested products and sequenced homozygous and heterozygous samples.

Traits

The carcass weight (kg), rib eye area (cm²), rib thickness (cm), subcutaneous fat thickness (cm), yield estimate (%), and beef marbling standard were measured by official graders of the Japan Meat Grading Association. Cervical muscles were obtained for genomic DNA extraction and genotyping; and adipose tissues were collected from the perirenal fat (JB1) and intramuscular fat of the longissimus muscle (JB2) to measure fatty acid composition. All samples were stored at –20°C prior to DNA extraction or fatty acid purification. The extraction and methylation of fatty acid were performed following the methods described by Folch *et al.* (1957) and O’Keefe *et al.* (1968), respectively. We analyzed the fatty acid methyl esters C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, and C18:2 and calculated the percentage composition of each (Table 2).

Statistical analysis

The effects of the polymorphisms on each trait were statistically tested by analysis of variance (ANOVA) using a model that accounted for age at slaughter, sire, sex, and genotype without interactions. Factors that were found to be significant were then further tested using Tukey’s honestly significant difference (HSD) test.

RESULTS

Polymorphism identification

We sequenced the CDS of the leptin gene from the genomic DNA of eight Japanese Black cattle. Sequence comparison revealed eight SNPs (20A/T, 73T/C, 150C/G, 239C/T, 396C/T, 399T/C, 411T/C, and 495C/T, with the translation initiation site assigned as +1) in the CDS. Three of these were predicted to cause amino acid substitutions: tyrosine to phenylalanine at 20A/T (Y7F; rs29004487), arginine to cysteine at 73T/C (R25C; rs29004488), and alanine to valine at 239C/T (A80V; rs29004508).

Genotyping

We genotyped three polymorphisms (Y7F, R25C, and A80V) in two Japanese Black cattle populations (JB1 and JB2) (Table 3). In Y7F, the allele A frequency was 0.98 and 0.89 in JB1 and JB2, respectively. In R25C, the allele C frequencies were 0.84 and 0.70 in JB1 and JB2, respectively. In A80V, the allele C frequency was 0.87 and 0.96 in JB1 and JB2, respectively. Y7F in JB1 and A80V in JB2 were excluded from statistical analysis because the minor allele frequencies (MAFs) of these SNPs were <0.1 in each group. Homozygous cattle for minor alleles of Y7F in JB2, R25C in JB1, and A80V in JB1 were also excluded because of the small sample sizes.

Effect of leptin gene polymorphisms on traits

We investigated the effects of three polymorphisms in the leptin gene on carcass traits and fatty acid composition using ANOVA (Table 4). Y7F had a significant effect on the dressed carcass weight in JB2; R25C had a significant effect on the C18:0 and C14:1

contents in JB1 and JB2, respectively; and A80V had a significant effect on the C16:0, C16:1, C18:1, SFA, and MUFA contents in JB1.

Tukey–Kramer HSD test was conducted to investigate the effects of Y7F, R25C, and A80V on traits in JB1 and JB2 in more detail. Table 5 presents the mean values for each trait and the significant differences among genotypes in JB1 and JB2. The AA type in Y7F exhibited a significantly higher dressed carcass weight than the AT type in JB2. R25C had a significant effect on the C14:1 content in JB2, with an additive effect among genotypes being observed; animals with the C allele exhibited a higher percentage of C14:1 than those with the T allele. In addition, the CC type in R25C contained a significantly lower percentage of C18:0 than the CT type in JB1. The CC type in A80V had a significantly greater effect on C16:0 and SFA but a smaller effect on C16:1, C18:1, and MUFA than the CT type.

DISCUSSION

Previous studies have demonstrated an association between leptin gene polymorphisms and economically important traits in cattle. However, this is the first study to investigate leptin gene polymorphisms in Japanese Black cattle.

We found three non-synonymous SNPs in the leptin gene (Y7F, R25C, and A80V). These SNPs have previously been found in other breeds, with MAFs of 0.02 and 0.025 in Simmental (Orrù *et al.* 2011) and Holstein Friesian (Clempton *et al.* 2011) cattle, respectively, in Y7F; MAFs of 0.42, 0.34, 0.45, and 0.32 in Angus, Charolais, Hereford, and Simmental cattle, respectively, in R25C (Buchanan *et al.* 2002); and an

MAF of 0.33 in Jersey cow in A80V (Komisarek & Antkowiak 2007). The Japanese Black cattle investigated in the present study exhibited lower MAFs than these breeds, ranging from 0.02 to 0.30. This is possibly due to the strong selection for beef quality in Japanese Black cattle. In addition, Gutiérrez-Gil *et al.* (2010) reported a QTL that influenced fatty acid composition at 93.5–98.6 Mbp on BTA4 near the leptin gene. Therefore, given the low MAF and the detection of this QTL, the region around the leptin gene may be a possible candidate for improving beef quality.

Y7F was predicted to cause the substitution tyrosine with phenylalanine in leptin. However, this position is not so important in mature leptin because a signal peptide (1st to 21st amino acids) is cleaved off before leptin is excreted from the adipose tissue (Zhang *et al.* 1994; Buchanan *et al.* 2002). In the present study, we found an association between Y7F and dressed carcass weight in JB2, with the A allele (Y type) being detected as economically favorable because it increased dressed carcass weight. However, this association was not strong ($0.01 < p < 0.05$) because Y7F is not included in mature leptin and may be due to a linkage disequilibrium. In addition, the genotyping results of JB1 and JB2 suggested that the A allele frequency is very high in Japanese Black cattle as well as in Simmental and Holstein Friesian cattle (Clempton *et al.* 2011; Orrù *et al.* 2011). Therefore, it would be difficult for Y7F to be used as a selective DNA marker in these breeds.

R25C was predicted to cause the substitution arginine with cysteine in leptin. This substitution may affect the tertiary structure of leptin because it adds an unpaired cysteine (Liefers *et al.* 2003). In the present study, R25C was associated with the C18:0

1 and C14:1 contents in JB1 and JB2, respectively; and a previous study reported that
2 R25C was associated with the C14:0, C16:0, C17:1, and C18:0 contents in Chinese
3 Simmental cattle (Tian *et al.* 2013). This SNP has also been associated with many other
4 traits, such as dressed carcass weight, rib eye area, loin thickness, milk yield, milk fat
5 and protein content, somatic cell count linear scores, calving difficulty, and length of
6 gestation (Buchanan *et al.* 2003; Chebel *et al.* 2008; Giblin *et al.* 2010; Tian *et al.* 2013).
7 Buchanan *et al.* (2002) demonstrated that the T allele in R25C was associated with
8 fatter carcasses, whereas the C allele was associated with leaner carcasses in Angus,
9 Charolais, Hereford, and Simmental cattle, suggesting that the T allele, which adds an
10 extra cysteine to the protein, imparts a partial loss of biological function and thus
11 could be the causative mutation for carcass fat content. Therefore, considering the
12 effect that R25C has on the tertiary structure of leptin and its association with many
13 carcass traits, it is possible that this polymorphism would directly affect fatty acid
14 composition and thus could be used as an effective marker in Japanese Black cattle.

15 A80V was predicted to cause the substitution alanine with valine in leptin. In
16 the present study, this SNP was significantly associated with the C16:0, C16:1, C18:1,
17 SFA, and MUFA contents in JB1. An association between A80V and fatty acid
18 composition has also been reported in Chinese Simmental cattle (Orrù *et al.* 2011). In
19 addition, Reicher *et al.* (2012) reported that the A80V mutation affects leptin
20 functioning by decreasing its affinity toward the leptin receptor. These findings suggest
21 that A80V is a prospective DNA marker for fatty acid composition in Japanese Black
22 cattle. Furthermore, although the presence of the T allele in A80V had a positive effect

on fatty acid composition, its frequency was low in our study populations (0.13 and 0.04 in JB1 and JB2, respectively), which matches the previous finding of Orrù *et al.* (2011), for Chinese Simmental cattle (0.30). Therefore, A80V may have a large effect on the improvement of fatty acid composition in Japanese Black cattle.

In conclusion, we found that three of the eight SNPs (Y7F, R25C, and A80V) in the coding region of the leptin gene in Japanese Black cattle were non-synonymous and associated with carcass traits or fatty acid composition, and two of these (R25C and A80V) could be used as effective DNA markers for improving fatty acid composition in this breed.

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Table 1 Oligonucleotide primers in bovine leptin gene

primer	sequence	product size (bp)	annealing temperature
sequencing Exon 2, Genotyping c.20A>T	F 5'- TTTCTTGATTCCGCCGCACCTCT -3' R 5'- GCTCAGTTACCAGGCAGGAAGAA -3'	721	63
sequencing Exon 3	F 5'- CAGAAAGATAGGAGCCCAGGAGA -3' R 5'- GCTTCCATCGTATGTTGTGTGGG -3'	974	62
Genotyping c.73C>T	F 5'- GGGGAGTGCCTTTCATTACTGTC -3' R 5'- GGAGGAGAGGAGCTGTCTTTATG -3'	495	62
Genotyping c.239C>T	F 5'- CACCTCTACGCTCTAGGGAAAG -3' R 5'- AGGCAGACTGGTGAGGATCTGTTGGTTGAT -3'	221	65

Exon 1 in bovine leptin gene is a non-coding exon.

Table 2 Carcass traits and fatty acid composition in Japanese Black populations

	JB1			JB2		
	Mean		SD	Mean		SD
Carcass traits						
Dressed carcass weight (kg)	432.09	±	3.94	444.17	±	3.64
Rib-eye area (cm ²)	56.28	±	0.64	55.77	±	0.64
Rib thickness (cm)	7.60	±	0.07	7.73	±	0.06
Subcutaneous fat thickness (cm)	2.46	±	0.06	2.87	±	0.06
Yield estimate (%)	74.22	±	0.11	73.69	±	0.11
Beef marbling standard	6.17	±	0.17	6.42	±	0.17
Fatty acid composition (%)						
C14:0	2.48	±	0.05	2.63	±	0.04
C14:1	0.87	±	0.03	0.87	±	0.02
C16:0	23.69	±	0.22	26.77	±	0.15
C16:1	2.72	±	0.05	3.91	±	0.06
C18:0	19.65	±	0.25	11.45	±	0.12
C18:1	48.59	±	0.38	51.98	±	0.21
C18:2	1.99	±	0.04	2.33	±	0.05
SFA	45.83	±	0.39	40.85	±	0.22
MUFA	52.18	±	0.39	56.75	±	0.22

MUFA, monounsaturated fatty acid; SD, standard deviation; SFA, saturated fatty acid.

JB1: the commercial cattle population produced in Miyazaki Prefecture.

JB2: the cattle population fattened across Japan for field progeny testing from 2002 to 2011.

Table 3 Genotype and allele frequency of leptin gene polymorphisms in JB1 and JB2

Population			JB1	JB2
Y7F	Genotype	AA	0.97 (118)	0.79 (356)
	frequency	AT	0.03 (4)	0.19 (86)
	(n)	TT	0.00 (0)	0.02 (7)
	Allele frequency	A	0.98	0.89
		T	0.02	0.11
R25C	Genotype	CC	0.70 (387)	0.48 (212)
	frequency	CT	0.28 (158)	0.44 (197)
	(n)	TT	0.02 (11)	0.08 (37)
	Allele frequency	C	0.84	0.70
		T	0.16	0.30
A80V	Genotype	CC	0.76 (424)	0.96 (172)
	frequency	CT	0.23 (129)	0.04 (8)
	(n)	TT	0.01 (7)	0.00 (0)
	Allele frequency	C	0.87	0.96
		T	0.13	0.04

JB1: the commercial cattle population produced in Miyazaki Prefecture.

JB2: the cattle population fattened across Japan for field progeny testing from 2002 to 2011.

Table 4 Significant test of leptin gene polymorphisms for carcass traits and fatty acid composition using ANOVA

	Y7F	R25C		A80V
	JB2	JB1	JB2	JB1
Carcass traits				
Dressed carcass weight (kg)	*	ns	ns	ns
Rib-eye area (cm ²)	ns	ns	ns	ns
Rib thickness (cm)	ns	ns	ns	ns
Subcutaneous fat thickness (cm)	ns	ns	ns	ns
Yield estimate (%)	ns	ns	ns	ns
Beef marbling standard	ns	ns	ns	ns
Fatty acid composition (%)				
C14:0	ns	ns	ns	ns
C14:1	ns	ns	**	ns
C16:0	ns	ns	ns	**
C16:1	ns	ns	ns	*
C18:0	ns	*	ns	ns
C18:1	ns	ns	ns	**
C18:2	ns	ns	ns	ns
SFA	ns	ns	ns	**
MUFA	ns	ns	ns	**

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid

* $p < 0.05$, ** $p < 0.01$, ns non-significant

JB1: the commercial cattle population produced in Miyazaki Prefecture.

JB2: the cattle population fattened across Japan for field progeny testing from 2002 to 2011.

Table 5 Effect of leptin gene polymorphisms on carcass traits and fatty acid composition in JB1 and JB2

Population	SNP	Trait	Genotype	Mean	SD
JB1	R25C	C18:0 (%)	CC	19.27 ^B	± 0.29
			CT	20.14 ^A	± 0.34
	A80V	C16:0 (%)	CC	24.02 ^A	± 0.25
			CT	22.70 ^B	± 0.36
		C16:1 (%)	CC	2.66 ^B	± 0.06
			CT	2.84 ^A	± 0.09
		C18:1 (%)	CC	48.34 ^B	± 0.39
			CT	49.78 ^A	± 0.57
		SFA (%)	CC	46.16 ^A	± 0.40
			CT	44.49 ^B	± 0.59
		MUFA (%)	CC	51.87 ^B	± 0.40
			CT	53.48 ^A	± 0.59
JB2	Y7F	Dressed carcass weight (kg)	AA	444.41 ^A	± 2.70
			AT	431.17 ^B	± 5.19
	R25C	C14:1 (%)	CC	0.92 ^A	± 0.02
			CT	0.86 ^{AB}	± 0.02
			TT	0.80 ^B	± 0.05

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid

A, B means with different superscripts within the same trait differ significantly at $p < 0.05$ (Tukey's HSD analysis).

JB1: the commercial cattle population produced in Miyazaki Prefecture.

JB2: the cattle population fattened across Japan for field progeny testing from 2002 to 2011.