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ORIGINAL ARTICLE

Association of a single nucleotide polymorphism in ribosomal protein L27a gene with marbling in Japanese Black beef cattle

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ABSTRACT

Marbling, defined by the amount and distribution of intramuscular fat, is an economically important trait of beef cattle in Japan. The c2-11#2 expressed sequence tag (EST) has been previously shown to possess expression difference in *musculus longissimus* muscle between low-marbled and high-marbled steer groups, and to be located within genomic region of a quantitative trait locus for marbling. Thus, the *ribosomal protein L27a* (*RPL27A*) gene containing the c2-11#2 EST sequence was considered as a positional candidate for the gene responsible for marbling. In the present study, a single nucleotide polymorphism (SNP) in the promoter region of the *RPL27A*, referred to as *g.3109537C>T*, was detected between the 2 steer groups. The SNP was associated with the predicted breeding value for beef marbling standard number by the analyses using Japanese Black beef cattle population. The effect of genotypes of the SNP on the predicted breeding value for subcutaneous fat thickness was not statistically significant. These findings suggest that the *RPL27A* SNP may be useful for effective marker-assisted selection to increase the levels of marbling in Japanese Black beef cattle.

Key words: *association, beef cattle, marbling, RPL27A, SNP.*

INTRODUCTION

Marbling characterized by the amount and distribution of intramuscular fat, of which the increase improves the palatability and acceptability of the meat (Busboom *et al.* 1993; Boylston *et al.* 1995; Matsuishi *et al.* 2001), is an economically important trait of beef cattle in Japan (JMGA 1988). It is greatly interesting to obtain better knowledge on the molecular architecture of marbling and to generate new opportunities for more effective marker-assisted breeding, from the important effect of marbling on the economics of beef production.

We have recently showed that single nucleotide polymorphisms (SNPs) in the *endothelial differentiation, sphingolipid G-protein-coupled receptor, 1* (*EDG1*) gene, which is involved in blood vessel formation (Liu *et al.* 2000) and shows higher expression levels in high-marbled steer group than in low-marbled steer group in *musculus longissimus* muscle across all ages of the test period (Sasaki *et al.* 2006a), were associated with marbling in Japanese Black beef cattle (Yamada *et al.* in press).

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SNP genotyping

A SNP, *g.3109537C>T*, detected in the promoter region of the *RPL27A* gene was genotyped using PCR-restriction fragment length polymorphism (RFLP) method. PCR primers used for PCR-RFLP were 5′-CCTGTTTCAGAGAATCTAAGTCC-3′ and 5′-CTGAT CAGTTTCACTTCTAGTTCAG-3′ (Nucleotide positions relative to the transcription initiation site of the *RPL27A* gene were −7345 to −7323 and −7154 to −7178, respectively.). PCR amplifications were carried out as described in SNP detection section, using a final volume of 20 mL and the annealing temperature of 57°C. An aliquot of PCR-amplified products was digested at 37°C for 1 h with restriction enzyme *Bsu*36I, and electrophoresed on a 3.0% agarose gel. Agarose gels were stained with ethidium bromide and photographed under an ultraviolet light. Using this method, 192-bp PCR fragments containing the SNP site were amplified, and *Bsu*36I-digested into 73- and 119-bp fragments at the *T* allele, but not at the *C* allele: the *TT* homozygotes, the *CC* homozygotes, and the *CT* heterozygotes resulted in 2 bands (73 and 119 bp), 1 band (192 bp), and 3 bands (73, 119, and 192 bp), respectively.

Statistical analyses

The populations of the 3 experiments were analyzed separately. The effect of genotypes of the SNP on the predicted breeding values for beef marbling standard number and subcutaneous fat thickness was analyzed with the model that included the SNP genotype as the fixed effect and the sire (father of the sire) as the random effect in experiment 1. The SNP genotype effect was analyzed with the model that included the SNP genotype as the fixed effect in experiments 2 and 3. Statistical analysis was performed by the MIXED (experiment 1) and GLM procedures (experiments 2 and 3) of SAS program (SAS Institute, Inc., Cary, NC, USA).

RESULTS

SNP detection

The bovine *RPL27A* gene comprises 559 bp of 1 exon including 31 bp of 5′ untranslated region (UTR) sequence and 81 bp of 3′ UTR sequence, based on genomic sequence from the NCBI database. We sequenced the *RPL27A* gene from 2 low to marbled Holstein steers and 2 high-marbled cloned steers, which were previously shown to have different *RPL27A* gene expression patterns in ddPCR analysis (Sasaki *et al.* 2006a). This sequence analysis revealed 1 SNP in the *RPL27A* gene: a C to T transition located 7272 bp upstream of the transcription initiation site in the promoter region (*g.3109537C>T*). The 2 Holstein steers were homozygous for the *C* allele at the *g.3109537C>T* site, whereas the 2 cloned steers homozygous for the *T* allele.

Association study

The populations of the 3 experiments were separately analyzed for association of the *g.3109537C>T* SNP with marbling and subcutaneous fat thickness.

Experiment 1

Genotyping 101 sires for the SNP revealed 37 animals homozygous for the *C* allele, 45 animals heterozygous for the *C* allele and the *T* allele, and 19 animals homozygous for the *T* allele. Statistically significant differences among the genotypes of the SNP were detected in the predicted breeding values for beef marbling standard number ($P = 0.022$), but not for subcutaneous fat thickness ($P = 0.098$) (Table 1). The predicted breeding value for beef marbling standard number was significantly higher in the *TT* homozygotes than in the *CC* homozygotes, and that of the heterozygotes intermediate between those of the 2 homozygotes (Table 1).

Experiments 2 and 3

To better estimate the effect of genotype of the SNP on marbling and subcutaneous fat thickness, in experiments 2 and 3, respectively, we used 195 and 217 progeny steers from a sire homozygous for the C allele and the T allele at the SNP. These steers could be grouped according to the alleles that they received from their dams, allowing a linkage disequilibrium

Table 1 Effect of the SNP genotypes of the *g.3109537C>T* on the breeding values for beef marbling standard number and subcutaneous fat thickness in experiment 1

SNP genotype	No. of animals	Breeding value†	
		Beef marbling standard number	Subcutaneous fat thickness (mm)
TT	19	$3.08^{a} \pm 0.31$	-3.51 ± 0.97
CT	45	$2.58^{a} \pm 0.30$	-3.79 ± 1.02
CC	37	$2.08^{b} \pm 0.35$	-1.74 ± 1.16

†The breeding values are given as estimates ± SE. [a,b]Means at different genotypes without a common letter in their superscripts significantly differ ($P < 0.05$).

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From the result of our previous differential-display PCR (ddPCR) analysis in *musculus longissimus* muscle (Sasaki *et al.* 2006a), as well as the *EDG1*, the c2-11#2 expressed sequence tag (EST) exhibited higher expression levels in a high-marbled steer group than in a low-marbled steer group in the early stage of the test period. The c2-11#2 EST is also located within genomic region of a quantitative trait locus for marbling (Takasuga *et al.* 2007), which is mapped in a half-sib family of Japanese Black cattle to bovine chromosome 5 region.

The c2-11#2 EST sequence corresponds to a portion of the *ribosomal protein L27a* (*RPL27A*) gene. Thus, the *RPL27A* gene was regarded as a positional candidate for the gene responsible for marbling.

We herein explored SNP in the *RPL27A* gene and analyzed association of the SNP with marbling and subcutaneous fat thickness in Japanese Black beef cattle.

MATERIALS AND METHODS

Samples and data

Two Holstein steers and 2 somatic nuclear-derived cloned steers (Shiga *et al.* 1999) from a Japanese Black Itofuku sire with a very high estimated breeding value for marbling (Oita Prefectural Institute of Animal Industry 1999), which were assigned for low-marbled and high-marbled steer groups, respectively, in our previous ddPCR analysis (Sasaki *et al.* 2006a), were used for SNP detection in this study. The details of these steers are described previously (Sasaki *et al.* 2006a). *Musculus longissimus* muscle tissues were obtained from these steers as described previously (Sasaki *et al.* 2006a). We used 2 high-marbled cloned steers to confirm the authenticity of newly discovered SNP in the *RPL27A* gene.

We performed 3 experiments for the association study. We used 101 Japanese Black sires in experiment 1. The sires used to be or are used in the Oita Prefectural Institute of Animal Industry (Oita, Japan). There was no strong bias for a specific father or a specific maternal grandfather of the sires, and the sire panel likely represents a variety of the sire lines. In experiments 2 and 3, respectively, 195 and 217 paternal half-sib Japanese Black progeny steers produced from a sire homozygous for one and the other alleles at newly discovered SNP in the *RPL27A* gene, with dams considered to represent a random sample of the female population, were used. These progeny steers were fattened, and shipped to the carcass market in the Oita prefecture. Semen or blood of the sires and adipose tissues of the progeny steers were collected for SNP genotyping. DNA samples were prepared from the materials according to standard protocols. The predicted breeding values of the sires and the progeny steers for beef marbling standard number and subcutaneous fat thickness were obtained from the Oita recording system for beef cattle reported by Sasaki *et al.* (2006b), as described previously (Yamada *et al.* in press).

This study conformed to the guidelines for animal experimentation of the Graduate School of Agriculture, Kyoto University (Kyoto, Japan).

SNP detection

We screened the NCBI bovine genome sequence database (National Center for Biotechnology Information, Bethesda, MD, USA) with the c2-11#2 EST sequence, and obtained 3484,171-bp bovine genomic sequence (NW_001495105) containing the *RPL27A* gene, which contains the c2-11#2 EST sequence. Using this genomic sequence, PCR primers were designed to target ~8-kb proximal promoter and exon regions, to screen polymorphisms in the *RPL27A* gene from 2 low to marbled Holstein steers and 2 high-marbled cloned steers. PCR amplifications were performed using 25 ng of the prepared DNA as template in a final volume of 100 mL containing 1 mmol/L of each primer, 0.25 mmol/L of each dNTP, 2.5 U of Go *Taq* polymerase (Promega, Madison, WI, USA), and 1 X Go *Taq* buffer (Promega). The PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, the appropriate annealing temperature for 30 s, and 72°C for 1 min, followed by a further 5-min extension at 72°C. PCR products were examined by electrophoresis through a 1.0% agarose gel to determine the quality and quantity for DNA sequencing. DNA sequencing of PCR-amplified products was performed by direct sequencing with an ABI3730 sequencer (ABI, Foster City, CA, USA) following standard Big Dye protocols (ABI). Primers used for PCR amplifications and obtained from primer walking were used as sequencing primers. Nucleotide polymorphisms were identified by comparison of sequence traces among the 4 DNA samples, and designated according to nomenclature for the description of sequence variations in the HGVS (Human Genome Variation Society, Fitzroy, VIC, Australia) (http://www.hgvs.org/mutnomen/). Primer sequences will be available on request.

Table 2 Effect of the SNP genotypes of the *g.3109537C>T* on the breeding values for beef marbling standard number and subcutaneous fat thickness in experiment 2

SNP genotype	No. of animals	Breeding value†	
		Beef marbling standard number	Subcutaneous fat thickness (mm)
CT	66	3.31[a] ± 0.09	−0.59 ± 0.36
CC	129	3.06[b] ± 0.09	−0.13 ± 0.37

†The breeding values are given as least squares means ± SE.
[a,b]Means at different genotypes without a common letter in their superscripts significantly differ ($P < 0.05$).

Table 3 Effect of the SNP genotypes of the *g.3109537C>T* on the breeding values for beef marbling standard number and subcutaneous fat thickness in experiment 3

SNP genotype	No. of animals	Breeding value†	
		Beef marbling standard number	Subcutaneous fat thickness (mm)
TT	67	3.58[a] ± 0.12	0.81 ± 0.51
CT	150	3.19[b] ± 0.08	1.36 ± 0.34

†The breeding values are given as least squares means ± SE.
[a,b]Means at different genotypes without a common letter in their superscripts significantly differ ($P < 0.05$).

estimate of the effect of the SNP. The SNP genotype had the significant effect on the predicted breeding values for beef marbling standard number ($P = 0.040$), but not for subcutaneous fat thickness ($P = 0.380$) in experiment 2 (Table 2). The SNP genotype effect also reached statistical significance ($P = 0.010$) for the predicted breeding values for beef marbling standard number, but not for subcutaneous fat thickness ($P = 0.369$) in experiment 3 (Table 3). Genotypic profiles of the predicted breeding value for beef marbling standard number in experiments 2 and 3 were consistent with the result obtained by experiment 1 (Tables 2,3).

DISCUSSION

We screened the *RPL27A* gene in low-marbled and high-marbled steer groups for SNP and found 1 SNP, *g.3109537C>T*, to possess different genotypes between the 2 steer groups. Based on 3 experiments using the 101 sires, the 195 progeny steers from a sire homozygous for the *C* allele at the SNP, and the 217 progeny steers from a sire homozygous for the *T* allele at the SNP, we showed that the SNP is associated with marbling in Japanese Black beef cattle, with the *T* allele resulting in high levels of marbling. This was especially evident in experiments 2 and 3, because the dams can be considered to represent a random sample of the Japanese Black population and thus the association is likely to be true. Furthermore, the association of the *RPL27A* SNP with marbling was corroborated by an independent study using 104 paternal half-sib Japanese Black families with a total of 821 progeny steers (S. Sukegawa, unpublished data).

Based on the association of the *g.3109537C>T* with marbling, together with *RPL27A* expression difference between low-marbled (with *g.3109537C>T C* allele) and high-marbled steer groups (with *g.3109537C>T T* allele), we can hypothesize that the SNP in the promoter region might have an impact on *RPL27A* expression and also marbling by affecting *RPL27A* promoter activity. However, from a view of common biological significance of *RPL27A* as structural constituent of ribosome, a more likely event is that the *RPL27A* SNP is in linkage disequilibrium with an unidentified and truly relevant mutation, rather than a functional and a causal mutation for marbling.

The effect of genotypes of the SNP was not statistically significant ($P > 0.05$) for subcutaneous fat thickness. Furthermore, the marbling quantitative trait locus corresponding to the chromosomal position of the *RPL27A* did not show a statistically significant effect on subcutaneous fat thickness (Takasuga *et al.* 2007). Thus, it is likely that the *RPL27A* SNP is not associated with subcutaneous fat thickness in Japanese Black beef cattle. This might be supported by the fact that Japanese Black breed exhibits low genetic correlation between marbling and subcutaneous fat thickness.

Several previous studies have reported polymorphisms associated with beef marbling using beef cattle breeds other than Japanese Black (Barendse 1999; Hale *et al.* 2000; Buchanan *et al.* 2002; Thaller *et al.* 2003; Casas *et al.* 2004; Jiang *et al.* 2005; Nkrumah *et al.* 2005; Barendse *et al.* 2006, 2007; Michal *et al.* 2006). We have recently reported that SNPs in the *EDG1* gene were associated with marbling in Japanese Black breed (Yamada *et al.* in press). Thus, our present study seems to be an additional report to show polymorphisms associated with marbling using Japanese Black breed.

The information on the *RPL27A* SNP obtained in this study, as well as the *EDG1* SNPs, may be applied to effective marker-assisted selection to increase the levels of marbling in Japanese Black beef cattle.