Animal Science Journal (2009) **80,** 486–489 doi: 10.1111/j.1740-0929.2009.00665.x

RAPID COMMUNICATION

Novel SNP in 5′ flanking region of *EDG1* associated with marbling in Japanese Black beef cattle

Takahisa YAMADA,[1] Seiki SASAKI,[2] Shin SUKEGAWA,[3] Takeshi MIYAKE,[1] Tatsuo FUJITA,[4] Hiroyuki KOSE,[5] Mitsuo MORITA,[2] Youichi TAKAHAGI,[3] Hiroshi MURAKAMI,[3] Fumiki MORIMATSU[3] and Yoshiyuki SASAKI[6]

[1]*Laboratory of Animal Breeding and Genetics, Graduate School of Agriculture, Kyoto University, Sakyo, Kyoto,* [2]*Maebashi Institute of Animal Science, Livestock Improvement Association of Japan, Maebashi,* [3]*Research and Development Center, Nippon Meat Packers, Inc., Tsukuba,* [4]*Oita Prefectural Institute of Animal Industry, Takeda,* [5]*Department of Life Science, Division of Natural Sciences, International Christian University, Mitaka, and* [6]*Beef Information & Genetics Institute, Inc., Otsu, Japan*

ABSTRACT

Marbling, defined by the amount and distribution of intramuscular fat, is an economically important trait of beef cattle in Japan. The *endothelial differentiation*, *sphingolipid G-protein-coupled receptor*, *1* (*EDG1*) gene has been considered as a positional functional candidate for the gene responsible for marbling. We have recently reported that 2 single nucleotide polymorphisms (SNPs), *c.-312A>G* in the 5′ untranslated region (UTR) and *c.***446G>A* in the 3′ UTR in *EDG1* were associated with marbling in Japanese Black beef cattle, but this was not functional and a causal mutation for marbling. In the present study, we detected 2 novel SNPs, referred to as *g.1475435G>A* and *g.1471620G>T*, in the 5′ flanking region of the *EDG1* between low-marbled and high-marbled steer groups, which were previously shown to have *EDG1* expression differences in *musculus longissimus* muscle. The *g.1475435G>A* SNP seemed not to segregate in Japanese Black beef cattle. The *g.1471620G>T* SNP was associated with the predicted breeding value for beef marbling standard number by the analyses using Japanese Black beef cattle population. Based on these findings, we hypothesized that the *g.1471620G>T* SNP might have an impact on *EDG1* expression and also marbling.

Key words: *association, beef cattle, EDG1, marbling, SNP.*

INTRODUCTION

Marbling is characterized by the amount and distribution of intramuscular fat. Increased marbling improves the palatability and acceptability of the meat (Busboom *et al.* 1993; Boylston *et al.* 1995; Matsuishi *et al.* 2001), and is an economically important trait of beef cattle in Japan (JMGA 1988). A better knowledge of the molecular architecture of marbling is important as it may generate new opportunities for more effective marker-assisted breeding, leading to economic benefits to the beef production industry.

The *endothelial differentiation*, *sphingolipid G-protein-coupled receptor*, *1* (*EDG1*) gene, involved in blood vessel formation (Liu *et al.* 2000), has been previously shown to possess higher expression levels in a high-marbled steer group than in a low-marbled steer group in *musculus longissimus* muscle across all ages (Sasaki *et al.* 2006a,b). *EDG1* is located within genomic region of a quantitative trait locus for marbling (Sasaki *et al.* 2006b; Yamada *et al.* 2006), and thus has been regarded as a positional functional candidate for the gene responsible for marbling (Yamada *et al.* 2009). We have recently reported that 2 single nucleotide polymorphisms (SNPs), *c.-312A>G* in the 5′ untranslated

Correspondence: Takahisa Yamada, Laboratory of Animal Breeding and Genetics, Graduate School of Agriculture, Kyoto University, Kitashirakawa, Sakyo, Kyoto 606-8502, Japan. (Email: tyamada@kais.kyoto-u.ac.jp)
Received 18 December 2008; accepted for publication 12 February 2009.

region (UTR) and *c.*446G>A* in the 3′ UTR, in the *EDG1* were detected between the 2 steer groups, and associated with marbling in Japanese Black beef cattle (Yamada *et al.* 2009). However, the SNPs were not regarded as a causal functional mutation for marbling (Yamada *et al.* 2009).

We have now detected novel SNPs in the 5′ flanking region of the *EDG1* and analyzed association of the SNPs with marbling 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), were assigned to low-marbled and high-marbled steer groups, respectively, and were used for SNP detection in this study. The details of these steers have been described previously (Sasaki *et al.* 2006a).

We performed 3 experiments for the association study. We used 96 Japanese Black sires, 1019 paternal half-sib Japanese Black progeny steers (57 to 545 steers per sire) produced from 6 sires heterozygous for newly discovered SNP, and 672 paternal half-sib Japanese Black progeny steers (217 and 455 steers per sire) produced from 2 sires homozygous for the SNP, respectively, in experiments 1, 2, and 3. The details of these sires and progeny steers have been described previously (Yamada *et al.* 2009). The predicted breeding values of the sires and the progeny steers for beef marbling standard number were obtained from the Oita recording system for beef cattle reported by Sasaki *et al.* (2006c), as described previously (Yamada *et al.* 2009).

DNA samples were prepared according to standard protocols as described previously (Yamada *et al.* 2009). This study conformed to the guidelines for animal experimentation of the Graduate School of Agriculture, Kyoto University (Kyoto, Japan).

SNP detection

PCR primers were designed to target a ~8-kb proximal 5′ flanking region, to screen polymorphisms in the *EDG1* 5′ flanking region from 2 low-marbled Holstein steers and 2 high-marbled cloned steers. PCR amplifications and DNA sequencing of PCR-amplified products were performed as described previously (Yamada *et al.* 2009). 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.

SNP genotyping

Two SNPs, *g.1475435G>A* and *g.1471620G>T*, detected in this study were genotyped using a PCR-restriction fragment length polymorphism (RFLP) method. PCR primers used for PCR-RFLP were 5′-CAACAGCACAGGGTTATATAAAACTG AAGT-3′ and 5′-CGGACACGGCTAAGTAACTAAACAG-3′ (Nucleotide positions relative to the transcription initiation site of the *EDG1* gene were −5581 to −5552 and −5383 to −5407, respectively.) for the *g.1475435G>A*, and 5′-GTG TTAATATGTATGAAGCTTGATAGTCAGGAAATAAAT-3′ and 5′-CCACTGTATCGCTGAGCTAGGT-3′ (Nucleotide positions relative to the transcription initiation site of the *EDG1* gene were −1775 to −1737 and −1621 to −1642, respectively.) for the *g.1471620G>T*. PCR amplifications were carried out as described previously (Yamada *et al.* 2009), using the annealing temperature of 60°C. An aliquot of PCR-amplified products was digested at 37°C for 1 h with restriction enzyme *Sca*I for the *g.1475435G>A*, and 65°C for 1 h with restriction enzyme *Tsp*509I for the *g.1471620G>T*, and electrophoresed on a 3.0% agarose gel. Agarose gels were stained with ethidium bromide and photographed under an ultraviolet light. Using this method, for the *g.1475435G>A*, 199-bp PCR fragments containing the SNP site were amplified, and *Sca*I-digested into 30- and 169-bp fragments at the *A* allele, but not at the *G* allele: the *AA* homozygotes, the *GG* homozygotes, and the *GA* heterozygotes resulted in 2 bands (30 and 169 bp), 1 band (199 bp), and 3 bands (30, 169, and 199 bp), respectively. For the *g.1471620G>T*, 155-bp PCR fragments containing the SNP site were amplified, and *Tsp*509I-digested into 38- and 117-bp fragments at the *T* allele, but not at the *G* allele: the *TT* homozygotes, the *GG* homozygotes, and the *GT* heterozygotes resulted in 2 bands (38 and 117 bp), 1 band (155 bp), and 3 bands (38, 117, and 155 bp), respectively.

Statistical analyses

The populations of the 3 experiments were separately analyzed. The effect of genotypes at the SNP on the predicted breeding values for beef marbling standard number 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 and the sire as the fixed effects and their interaction in experiments 2 and 3. Subsequently, in cases where the interaction effect was not statistically significant ($P > 0.05$), the interaction effect was excluded from the model in order to test significance of the SNP genotype effect. 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 AND DISCUSSION

SNP detection

We sequenced the *EDG1* 5′ flanking region from 2 low-marbled Holstein steers and 2 high-marbled cloned steers, which were shown to have different *EDG1* gene expression patterns in a previous study (Sasaki *et al.* 2006a). This sequence analysis revealed 2 SNPs in the *EDG1* 5′ flanking region: a G to A transition located 5551 bp upstream of the transcription ini-

tiation site (*g.1475435G>A*) and a G to T substitution located 1736 bp upstream of the initiation site (*g.1471620G>T*). The 2 Holstein steers were heterozygous for the *G* allele and the *A* allele at the *g.1475435G>A* site and homozygous for the *G* allele at the *g.1471620G>T* site, whereas the 2 cloned steers homozygous for the *A* allele at the *g.1475435G>A* site and for the *T* allele at the *g.1471620G>T* site.

Association study

The populations of the 3 experiments were separately analyzed for association of the SNPs with marbling.

Experiment 1

Genotyping 96 sires for the *g.1475435G>A* SNP showed no detection of the *G* allele, suggesting that the SNP seems to fix at the *A* allele and not to segregate in Japanese Black beef cattle. Genotyping 96 sires for the *g.1471620G>T* SNP revealed 30 animals homozygous for the *T* allele, 51 animals heterozygous for the *T* allele and the *G* allele, and 15 animals homozygous for the *G* allele. Statistically significant differences among the genotypes at the SNP were detected in the predicted breeding values for beef marbling standard number ($P = 0.025$) (Table 1). The predicted breeding value for beef marbling standard number was significantly higher in the *TT* homozygotes than in the *GG* homozygotes, and that of the heterozygotes intermediate between those of the 2 homozygotes (Table 1).

Experiment 2

To better estimate the effect of genotypes at the *g.1471620G>T* SNP on marbling, we used 1019 progeny steers from 6 sires heterozygous for the SNP. The interaction between the SNP genotype and the sire was not statistically significant ($P = 0.194$), and was excluded from our statistical model. In the model without the interaction effect, the SNP genotype had the statistically significant effect on the predicted breeding values for beef marbling standard number ($P = 0.001$) (Table 1). Consistent with the result obtained by using the 96 sires, the predicted breeding value for beef marbling standard number was significantly higher in the *TT* homozygotes than in the *GG* homozygotes, and that of the heterozygotes intermediate between those of the 2 homozygotes (Table 1).

Experiment 3

To further verify the association of the *g.1471620G>T* SNP with marbling, we used 672 progeny steers from 2 sires homozygous for 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 estimate of the effect of the SNP. The interaction between the SNP genotype and the sire was not statistically significant ($P = 0.525$), and was excluded from our statistical model. In the model without the interaction effect, the SNP genotype effect reached statistical significance ($P = 0.018$) for the predicted breeding values for beef marbling standard number (Table 1). Genotypic profiles of the predicted breeding value for beef marbling standard number were consistent with the results obtained by experiments 1 and 2 (Table 1).

Based on 3 experiments in this study, we showed that a novel SNP, *g.1471620G>T*, in the 5′ flanking region of the *EDG1*, is associated with marbling in Japanese Black beef cattle, with the *T* allele resulting in high levels of marbling. Especially, in experiments 2 and 3, because the dams can be considered to represent a random sample of the Japanese Black population, the association is likely to be true. Based on the association of the *g.1471620G>T* with marbling, together with *EDG1* expression difference between low-marbled (with *g.1471620G>T G* allele) and high-marbled steer groups (with *g.1471620G>T T* allele), we hypothesized that the SNP might have an impact on

Table 1 Effect of the SNP genotypes at the *g.1471620G>T* on the breeding values for beef marbling standard number in experiments 1, 2, and 3

Genotype	Breeding value for beef marbling standard number†		
	Experiment 1	Experiment 2	Experiment 3
TT	$2.95^{a} \pm 0.36$ (30)‡	$2.98^{e} \pm 0.05$ (319)	$3.96^{a} \pm 0.05$ (387)
GT	$2.36^{a,b} \pm 0.31$ (51)	$2.83^{e,f} \pm 0.05$ (507)	$3.78^{b} \pm 0.06$ (285)
GG	$2.05^{b} \pm 0.31$ (15)	$2.69^{f} \pm 0.06$ (193)	

†The breeding values are given as estimates ± SE (experiment 1) and least squares means ± SE (experiments 2 and 3). ‡The number of animals is shown in parentheses. [a,b]Means at different genotypes without a common letter in their superscripts significantly differ ($P < 0.05$). [e,f]Means at different genotypes without a common letter in their superscripts significantly differ ($P < 0.01$).

EDG1 expression and also marbling by affecting *EDG1* promoter activity.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research (B) (no. 14360166) from the Ministry of Education, Science, Sports, and Culture of Japan, and by the research funds of Japanese Livestock Technology Association.

REFERENCES

Boylston TD, Morgan SA, Johnson KA, Busboom JR, Wright RW Jr, Reeves JJ. 1995. Lipid content and composition of Wagyu and domestic breeds of beef. *Journal of Agricultural and Food Chemistry* **43,** 1202–1207.

Busboom JR, Jeremiah LE, Gibson LL, Johnson KA, Gaskins CT, Reeves JJ, Wright RW Jr. 1993. Effects of biological source on cooking and palatability attributes of beef produced for the Japanese market. *Meat Science* **35,** 241–258.

JMGA. 1988. *New Beef Carcass Grading Standards*. Japan Meat Grading Association, Tokyo.

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *Journal of Clinical Investigation* **106,** 951–961.

Matsuishi M, Fujimori M, Okitani A. 2001. Wagyu beef aroma in Wagyu (Japanese Black cattle) beef preferred by the Japanese over imported beef. *Animal Science Journal* **72,** 498–504.

Oita Prefectural Institute of Animal Industry. 1999. *Sire Summary*. Oita Prefectural Institute of Animal Industry, Oita.

Sasaki Y, Miyake T, Gaillard C, Oguni T, Matsumoto M, Ito M, Kurahara T, Sasae Y, Fujinaka K, Ohtagaki S, Dougo T. 2006c. Comparison of genetic gains per year for carcass traits among breeding programs in the Japanese Brown and the Japanese Black cattle. *Journal of Animal Science* **84,** 317–323.

Sasaki Y, Nagai K, Nagata Y, Doronbekov K, Nishimura S, Yoshioka S, Fujita T, Shiga K, Miyake T, Taniguchi Y, Yamada T. 2006a. Exploration of genes showing intramuscular fat deposition-associated changes in musculus longissimus muscle. *Animal Genetics* **37,** 40–46.

Sasaki Y, Yamada T, Miyake T. 2006b. Quantitative and molecular genetic approaches for the improvement of carcass traits in the Wagyu cattle. In: *8th World Congress on Genetics Applied to Livestock Production*, Session no. 13–01. MinasCentro Convention Center, Belo Horizonte, Minas Gerais, Brazil.

Shiga K, Fujita T, Hirose K, Sasae Y, Nagai T. 1999. Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese Black bulls. *Theriogenology* **52,** 527–535.

Yamada T, Itoh M, Nishimura S, Taniguchi Y, Miyake T, Sasaki S, Yoshioka S, Fujita T, Shiga K, Morita M, Sasaki Y. 2009. Association of single nucleotide polymorphisms in the endothelial differentiation sphingolipid G-protein-coupled receptor 1 gene with marbling in Japanese Black beef cattle. *Animal Genetics* **40,** 209–216.

Yamada T, Taniguchi Y, Nishimura S, Yoshioka S, Takasuga A, Sugimoto Y, Sasaki Y. 2006. Radiation hybrid mapping of genes showing intramuscular fat deposition-associated expression changes in bovine musculus longissimus muscle. *Animal Genetics* **37,** 184–185.