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The *Pancreatic lipase* Gene is Associated with Marbling in Japanese Black Beef Cattle

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Abstract: Marbling defined by the amount and distribution of intramuscular fat so-called Shimofuri is an economically important trait of beef cattle in Japan. The *Pancreatic lipase* (Pnlip) gene involved in energy income and fat regulation has been previously shown to be regarded as possible candidate for a rat Quantitative Trait Locus (QTL) responsible for intramuscular fat content. It is located within the genomic region of a bovine QTL for marbling and thus was considered as a positional functional candidate for the gene responsible for marbling. In this study, we showed that Single Nucleotide Polymorphisms (SNPs) in the bovine PNLIP were associated with the predicted breeding value for beef marbling standard number by analyses using a population of Japanese Black beef cattle. The effect of genotypes at each of the SNPs on the predicted breeding value for subcutaneous fat thickness was not statistically significant. The findings suggest that PNLIP SNPs may be useful for effective marker-assisted selection to increase the levels of marbling in Japanese Black beef cattle.

Key words: Association, beef cattle, Japanese black breed, marbling, PNLIP, single nucleotide polymorphism

INTRODUCTION

Marbling is characterized as the amount and distribution of intramuscular fat in a cross section of musculus longissimus muscle and is called Shimofuri. Marbling has an important effect on the economics of beef production (Busboom *et al*., 1993; Boylston *et al*., 1995; Matsuishi *et al*., 2001). Thus, it is greatly interesting to obtain better knowledge on the molecular architecture of marbling and to generate new opportunities for more effective marker-assisted selection.

Marbling involves a series of events that initiate and maintain preadipocyte proliferation, differentiation of preadipocytes into adipocytes and adipocyte maturation throughout musculus longissimus muscle (Smith *et al*., 2000). The physiological or anatomical environment surrounding adipocyte-lineage cells as well as intramuscular adipocyte-lineage cells are thought to contribute to specific adipogenic events involved in marbling (Smith *et al*., 2000). The environment can promote proliferation, differentiation or maturation of adipocyte-lineage cells throughout the muscle by many mechanisms including controlling energy balance, controlling the structural integrity of the sarcomere and affecting intramuscular vascularization.

We previously detected a Quantitative Trait Locus (QTL) responsible for intramuscular fat content on rat chromosome 1 by whole genome screening using the Otsuka Long-Evans Tokushima Fatty (OLETF) rat which exhibits higher levels of intramuscular fat content in musculus longissimus muscle than other rat strains (Tanomura *et al*., 2002). Further, we demonstrated that Pnlip gene encoding pancreatic lipase is possible candidate for the intramuscular fat content QTL, using a congenic strain (Tanomura *et al*., 2003) trapping the OLETF allele of the intramuscular fat content QTL genomic region on the genetic background of normal rat strain in a monogenic context (Tanomura *et al*., 2011). Pancreatic lipase encoded by PNLIP is known to be involved in energy income and fat regulation. The

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hydrolysis of dietary triglycerides to fatty acids by pancreatic lipase is essential for the intestinal absorption of long-chain triglyceride fatty acids (Lowe, 2002) indicating an involvement of pancreatic lipase in energy income. Further, the treatment with Orlistat that blocks predominantly pancreatic lipase activity in the small intestine promotes significant fat loss and prevents fat regain in obese patients (Sjostrom *et al*., 1998; Hill *et al*., 1999) indicating an involvement of pancreatic lipase in fat regulation. Thus, an increase in pancreatic lipase amount or activity is likely to result in excess energy income and then increase of intramuscular fat content leading to high levels of marbling.

It has been reported that a marbling QTL was mapped to genomic region containing PNLIP on bovine chromosome 26, using a half-sib family of Japanese Black beef cattle (Takasuga *et al*., 2007). Thus, the PNLIP was considered as a positional functional candidate for the gene responsible for marbling.

We herein analyzed association of the Single Nucleotide Polymorphisms (SNPs) in the bovine PNLIP with marbling and subcutaneous fat thickness in Japanese Black beef cattle.

MATERIALS AND METHODS

Samples and data: We performed two experiments for the association study. We used 101 Japanese Black sires in experiment 1. The sires were used either at present or previously at 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 experiment, 2, 367 paternal half-sib Japanese Black progeny steers produced from a sire homozygous for the SNP in the PNLIP with dams considered to represent a random sample of the female population were used. These progeny steers were fattened and shipped to a carcass market in the Oita prefecture. Semen or blood from each 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 previously reported by Sasaki *et al*. (2006a). In the recording system, the breeding values were predicted from carcass records of Japanese Black steers and heifers, fattened in the Oita prefecture. The fattened animals were shipped to various carcass markets from 1988-2003 where they were slaughtered and their carcasses evaluated. The data were edited to connect across subclasses such that each market-year subclass had ≥50 animals and each farm had ≥10 animals. The final number of animals was 48,045 and there were 89 market-year subclasses, 332 farms and 228 sires.

Marbling and subcutaneous fat thickness were measured on carcasses dissected at the 6th and 7th rib section according to the Japanese meat grading system by certified graders from the Japan Meat Grading Association (Tokyo, Japan) (JMGA, 1998). Marbling was scored from 1-12 (beef marbling standard number) with a standard model panel in which higher scores correspond to more marbling. Subcutaneous fat thickness was measured at the rib interface perpendicular to the outside surface at a point three-fourths along the length of the longissimus muscle from its chine bone end.

Data were analyzed by the REML method, using the MTDFREML programs (Boldman *et al*., 1995) and genetic and environmental variances were estimated. The BLUP option in the programs using the estimated variance components was chosen to predict the breeding values of animals with a single trait model. Sex, market-year and farm were considered fixed effects. Fattening period and slaughter age were also considered as up to quadratic covariates. The fattening period denotes the period from the start of fattening to shipping to market for each animal. These fixed effects were all significant ($p<0.001$). Random effects included the additive genetic effect of the individuals that is the animal model was adopted to predict the breeding values.

This study conformed to the guidelines for animal experimentation of the Graduate School of Science and Technology, Niigata University (Niigata, Japan).

SNP genotyping: We genotyped three SNPs, rs41648172, rs41648176 and rs41648178 in dbSNP which are located in the intron 6, exon 7 and intron 7 regions of the PNLIP, respectively. These SNPs exhibited significantly different allele frequency distribution between extremely high-marbled and extremely low-marbled sire groups. The SNPs were genotyped using PCR-Restriction Fragment Length Polymorphism (RFLP) method. PCR primers used for PCR-RFLP were 5'-CAGTGCTATCTCCCGGAGTC-3' and 5'-GAAATCTAGGTGGCCCACAA-3' (nucleotide positions relative to the transcription initiation site of the PNLIP were 10935-10954 and 11480-11461, respectively) for the three SNPs. PCR amplifications were performed using 25 ng of the prepared DNA as template in a final volume of 25 μL containing 0.5 μM of each primer, 0.2 mM of each dNTP, 0.625 U of Ex Taq polymerase (Takara, Shiga, Japan) and 1 X Ex Taq buffer (Takara). The PCR conditions were as follows: 94°C for 3 min, 35 cycles

of 94°C for 50, 66°C for 50 and 72°C for 50 sec followed by a further 5-min extension at 72°C. An aliquot of PCR-amplified product was digested at 37°C for 1 h with restriction enzyme *HpyCH4IV* for the rs41648172 SNP and *MslI* for the rs41648176 SNP and at 65°C for 1 h with restriction enzyme *TaqI* for the rs41648178 SNP and electrophoresed on a 3.0% agarose gel. Agarose gels were stained with ethidium bromide and photographed under an ultraviolet light. Using this method, 546 bp PCR fragments containing the three SNP sites were amplified and for the rs41648172 SNP, the TT homozygotes, the CC homozygotes and the CT heterozygotes resulted in 1 band (546 bp), 2 bands (131 and 415 bp) and 3 bands (131, 415 and 546 bp), respectively.

For the rs41648176 SNP, the CC homozygotes, the TT homozygotes and the CT heterozygotes resulted in 3 bands (53, 223 and 270 bp), 4 bands (53, 116, 154 and 223 bp) and 5 bands (53, 116, 154, 223 and 270 bp), respectively. For the rs41648178 SNP, the TT homozygotes, the CC homozygotes and the CT heterozygotes resulted in 1 band (546 bp), 2 bands (74 and 472 bp) and 3 bands (74, 472 and 546 bp), respectively. For experiment 2, the rs41648172 SNP was only genotyped.

Statistical analyses: The populations of the two experiments were separately analyzed. The effect of genotypes at each 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 only the SNP genotype as the fixed effect in experiment 2. Statistical analysis was performed by the MIXED (experiment 1) and GLM procedures (experiment 2) of the SAS program (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

The populations of the two experiments were separately analyzed for association of the PNLIP SNPs with marbling and subcutaneous fat thickness.

Experiment 1: Genotyping, the 101 sires for the three PNLIP SNPs revealed 15 animals homozygous for the C allele, 56 animals heterozygous for the C allele and the T allele and 30 animals homozygous for the T allele for the rs41648172 SNP, 12 TT homozygotes, 55 heterozygotes and 30 CC homozygotes for the rs41648176 SNP and 21 CC homozygotes, 56 heterozygotes and 24 TT homozygotes for the rs41648178 SNP. The SNP genotype effect reached marginal significance at the rs41648172 SNP ($p = 0.054$) for the predicted breeding values for beef marbling standard number but not for subcutaneous fat thickness ($p = 0.952$) (Table 1). The predicted breeding value for beef marbling standard number was higher in the TT homozygotes at the rs41648172 SNP than in the CC homozygotes at the SNP and that of the heterozygotes intermediate between those of the two homozygotes (Table 1). The SNP genotype effect was not statistically significant at the rs41648176 SNP and the rs41648178 SNP for the predicted breeding values for beef marbling standard number and subcutaneous fat thickness (Table 1).

Table 1: Effect of the SNP genotypes of each of the rs41648172, rs41648176 and rs41648178 SNPs on the breeding values for beef marbling standard number and subcutaneous fat thickness in experiment 1

			Breeding value[1]	
SNP	Genotypes	No. of animals	Beef marbling standard number	Subcutaneous fat thickness (mm)
rs41648172	TT	30	2.99±0.29	-2.73±0.94
	CT	56	2.39±0.28	-2.99±0.95
	CC	15	2.09±0.41	-3.10±1.36
rs41648176	CC	30	2.92±0.43	-2.58±1.41
	CT	55	2.46±0.38	-3.22±1.26
	TT	12	2.53±0.37	-4.37±1.19
rs41648178	TT	24	2.91±0.32	-2.08±1.03
	CT	56	2.51±0.31	-3.16±1.02
	CC	21	2.09±0.39	-3.10±1.30

[1]The breeding values are given as estimates±SE

Table 2: Effect of the SNP genotypes of the rs41648172 SNP on the breeding values for beef marbling standard number and subcutaneous fat thickness in experiment 2

		Breeding value[1]	
SNP genotypes	No. of animals	Beef marbling standard number	Subcutaneous fat thickness (mm)
CT	204	2.22^{a}±0.06	-2.14±0.24
CC	163	1.95^{b}±0.07	-2.61±0.26

[1]The breeding values are given as least squares means±SE. Mean values at different genotypes without a common superscript letter significantly differ ($p<0.01$)

Experiment 2: To verify further, the effect of genotype at the rs41648172 SNP on marbling and subcutaneous fat thickness, we used 367 progeny steers from a sire homozygous for the C allele at the rs41648172 SNP. These steers could be grouped according to the alleles that they received from their dams, allowing an estimation of the linkage disequilibrium estimate of the effect of the SNP to be made. The SNP genotype had the statistically significant effect on the predicted breeding values for beef marbling standard number ($p = 0.003$) but not for subcutaneous fat thickness ($p = 0.185$) (Table 2). Consistent with the result obtained by using the 101 sires, the predicted breeding value for beef marbling standard number was significantly higher in the CT heterozygotes

at the rs41648172 SNP than in the CC homozygotes at the SNP (Table 2). On the basis of two experiments, using the 101 sires and the 367 progeny steers from a sire homozygous for the C allele at the rs41648172 SNP, we showed that the rs41648172 SNP is associated with marbling in Japanese Black beef cattle with the T allele at the rs41648172 SNP resulting in high levels of marbling. This was especially evident in experiment 2 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.

On the basis of the association of the rs41648172 SNP with marbling, we can hypothesize that the SNP might have a direct impact on marbling by affecting pancreatic lipase amount or activity or both. However, the rs41648172 SNP is intron SNP and thus a more likely event is that the rs41648172 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 QTL corresponding to the chromosomal position of the PNLIP did not show a statistically significant effect on subcutaneous fat thickness (Takasuga *et al*., 2007). Thus, it is likely that the rs41648172 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 (Sasaki *et al*., 2006b).

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 endothelial differentiation, sphingolipid G-protein-coupled receptor, 1 (EDG1) gene were associated with marbling in Japanese Black breed (Yamada *et al*., 2009). Thus, the present study seems to be an additional report to show polymorphisms associated with marbling using Japanese Black breed. The information on the rs41648172 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.

CONCLUSION

In this study, we show that the SNP in PNLIP encoding pancreatic lipase is associated with marbling in Japanese Black beef cattle. This study will provide an useful information for the establishment of effective marker-assisted selection to increase the levels of marbling in Japanese Black beef cattle.

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