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Preliminary Association Study of SNPs in *MFN2* Gene Showing Marbling-Associated Expression Changes

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Abstract: Researchers have previously showed that the *mitofusin 2* (*MFN2*) gene involved in energy balance through mitochondrial fusion and expressed in slow-twitch oxidative fiber that is observed in high-marbled muscle, possesses expression differences in musculus longissimus muscle between low-marbled Holstein and high-marbled Japanese Black steer groups. In the present study, researchers found that a marker (IDVGA-49) close to the MFN2 was polymorphic between low-marbled Holstein and high-marbled Japanese Black steer groups and exhibited significantly different allelic distribution between Japanese Black sires with extremely high predicted breeding value for marbling and with extremely low one. Further, researchers detected Single Nucleotide Polymorphism (SNP) in the *MFN2* gene between low-marbled Holstein and high-marbled Japanese Black steer groups. The allelic distributions of the 6 SNPs in the MFN2 were indistinguishable between Japanese Black sires with extremely high predicted breeding value for marbling and with extremely low one. The findings suggest that an unidentified true causal mutation which is in linkage disequilibrium with the IDVGA-49 marker but not the 6 SNPs may be related to changes in *MFN2* gene expression and/or marbling. The IDVGA-49 marker may be useful for effective marker-assisted selection to increase the levels of marbling.

Key words: Allelic distribution, MFN2, close marker, marbling, single nucleotide polymorphism, Japan

INTRODUCTION

Marbling characterized by the amount and distribution of intramuscular fat in a cross section of musculus longissimus muscle is one of the economically important traits of beef cattle (JMGA, 1988). High levels of marbling improve the palatability and acceptability of beef by affecting the taste and tenderness of the meat (Busboom *et al*., 1993; Boylston *et al*., 1995; Matsuishi *et al*., 2001). Because of the importance of marbling on the economics of beef production, there is great interest in gaining a better understanding of the molecular architecture of marbling and in generating new opportunities for more effective marker-assisted breeding. Researchers have previously undertaken differential-display PCR (ddPCR) in low-marbled and high-marbled steer groups at 8, 10, 12 and 14 months of age, encompassing the time that marbling starts to appear, to explore genes showing marbling-associated expression changes in musculus longissimus muscle (Sasaki *et al*., 2006b). Among the detected genes, the *mitofusin 2* (*MFN2*) gene which is known to be mitochondrial membrane protein that participates in mitochondrial fusion in mammalian cells and is crucial to the maintenance and operation of the mitochondrial network and in mitochondrial metabolism in muscle cells exhibited higher expression levels in high-marbled Japanese Black steer group than in low-marbled Holstein steer group across all ages of the test period (Sasaki *et al*., 2006b). Skeletal muscle contains two subpopulations of mitochondria, Subsarcolemmal (SS) and Intermyofibrillar (IMF) mitochondria (Hood, 2001; Cogswell *et al*., 1993). SS mitochondria may be important for driving processes at the cell surface including fatty acid oxidation and glucose

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transport (Hood, 2001). In contrast, IMF mitochondria are thought to provide energy for muscle contractions (Hood, 2001). Researchers suggested that the high level of MFN2 expression may promote energy provision and enhance glucose transport through mitochondrial fusion to contribute to development of marbling.

It has been reported that a marbling quantitative trait locus was mapped to genomic region containing the *MFN2* gene on bovine chromosome 16, using a half-sib family of Japanese Black beef cattle (Takasuga *et al*., 2007). Thus, the MFN2 was considered as a positional functional candidate for the gene responsible for marbling.

To examine whether polymorphism around the MFN2 is useful for effective marker-assisted selection to increase the levels of marbling, we herein analyzed the allele frequency distribution of a marker close to the MFN2 in high-marbled and low-marbled cattle. Researchers further explored polymorphism in the *MFN2* gene and examined allelic distribution in the polymorphism between high-and low-marbled cattle.

MATERIALS AND METHODS

Samples: 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 (OPIAI, 1999) which were assigned for low-marbled and high-marbled steer groups, respectively in the previous ddPCR analysis (Sasaki *et al*., 2006b) were used for microsatellite marker genotyping and polymorphism detection in this study. The details of these steers are described before (Sasaki *et al*., 2006b). Musculus longissimus muscle tissues were obtained from these steers as described previously (Sasaki *et al*., 2006b). We used 2 high-marbled cloned steers to confirm the authenticity of newly discovered Single Nucleotide Polymorphism (SNP) in the *MFN2* gene.

Further, researchers used 34 Japanese Black unrelated sires (17 sires with extremely high predicted breeding value for marbling and 17 sires with extremely low one) selected from 101 unrelated sires, a panel of that represent almost all of the lines within a Japanese Black beef cattle population, for microsatellite marker genotyping and SNP genotyping in this study. The predicted breeding values were obtained from the recording system for beef cattle reported by Sasaki *et al*. (2006a). The accuracy of the predicted breeding values in the 101 sires was 0.935±0.008 ranging from 0.770-0.990. Semen or blood were collected and DNA samples were prepared from the materials according to standard protocols. This study conformed to the guidelines for animal experimentation of the Faculty of Agriculture, Niigata University (Niigata, Japan).

Microsatellite marker genotyping: Researchers screened the cattle genome maps (NAGRP Cattle Genome Coordination Program) and obtained IDVGA-49 microsatellite marker as the close marker to the *MFN2* gene. PCR amplification of the IDVGA-49 was performed in a 10 μL volume containing 25 ng of template DNA, 0.2 mM of each dNTP, 0.1 μM of each primer, 0.5 U of Go Taq polymerase (Promega, Madison, WI) and 1 X Go Taq buffer (Promega). The amplification condition was as follows: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min followed by 72°C for 5 min. Forward primer was labeled with FAM fluorescent dye. Primer sequences were obtained from NAGRP Cattle Genome Coordination Program (http://www.animalgenome.org/cattle/). PCR products were electrophoresed in an ABI3730 sequencer (ABI, Foster City, CA). Allelic sizes were scored using software GeneMapper 4.0 (ABI).

Polymorphism detection: Researchers screened the NCBI databases (National Center for Biotechnology Information, Bethesda, MD) and obtained bovine genomic sequence (NC_007314) containing the *MFN2* gene. PCR primers were designed to target ~4 kb proximal promoter region, exon 1 and 2 regions and intron 1 region for the *MFN2* gene using this genomic sequence in order to screen polymorphisms in the gene between 2 low-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 μL containing 1 μM of each primer, 0.25 mM of each dNTP, 2.5 U of Go Taq polymerase (Promega) and 1 X Go Taq buffer (Promega). The PCR conditions were carried out as follows: 94°C for 2 min, 35 cycles of 94°C for 30 sec, the appropriate annealing temperature for 30 sec 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 the direct sequencing with an ABI3730 sequencer (ABI) 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.genomic.unimelb.edu.au/mdi/mutnomen/). Primer sequences will be available on request.

Table 1: Primers designed, restriction enzyme and incubation temperature used in PCR-RFLP

SNP	Forward and reverse primers (5'- 3')	Restriction enzyme	Incubation temperature (°C)
g.38438677A>G	F: ACTTTCAATCAAGGCTAAGTGAGGACA R: CTGAGCAACTATGGCTCATCAAGCA	SphI	37
g.38437771C>T	F: TTAAAGAGTAGAAGGGAACAGGTGA R: TTATGTGCCAGGCATTGGGA	Hpy99I	37
g.38436281A>G	F: TTGCCATCCCCAAGAGTCAG R: ACTCTCAATTCCACAGTGCAAAG	TspRI	65
g.38436013T>C	F: AAGCCTAGTCCTTGAACTGG R: AGTTTACTCACTGAGGTACTGG	BfaI	37
g.38435987A>T	F: CTGTGGTCCCTCTGGATGTG R: CAAACTCATCCCCGAGGGAA	PflMI	37
g.38435768T>C	F: TAAACTTCCCTCGGGGATGA R: CGATGGTGAGAGACTTTCACAG	AciI	37

SNP genotyping: The 6 SNPs, g.38438677A>G and g.38437771C>T, g.38436281A>G, g.38436013T>C and g.38435987A>T and g.38435768T>C site, respectively detected in the promoter, intron 1 and exon 2 regions of the *MFN2* gene were genotyped by PCR-Restriction Fragment Length Polymorphism (RFLP) Method. An aliquot of PCR primers used for PCR-RFLP were shown in Table 1. PCR amplifications were carried out as described in polymorphism d etection section using a final volume of 20 μL and the annealing temperature of 58°C. PCR-amplified products was digested at each temperature for 2 h with each restriction enzyme shown in Table 1 and electrophoresed on a 2.0% agarose gel. Agarose gels were stained with ethidium bromide and photographed under an ultraviolet light.

Statistical analysis: The allelic distributions of the IDVGA-49 and the detected SNP were compared between 17 sires with extremely high predicted breeding value for marbling and 17 sires with extremely low one by χ^2-test. Statistical analysis was performed by the FREQ procedure of SAS program (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Researchers first genotyped 2 low-marbled Holstein steers and 2 high-marbled Japanese black steers which were previously shown to have different *MFN2* gene expression patterns in ddPCR analysis (Sasaki *et al*., 2006b) for the IDVGA-49 microsatellite marker close to the *MFN2* gene. This genotyping analysis revealed polymorphism of the IDVGA-49 between the high-and low-marbled steer groups. The high-marbled steers were homozygous for 156 bp allele at the IDVGA-49 whereas the low-marbled steers homozygous for 164 bp allele. Researchers further genotyped 34 Japanese black unrelated sires consisting of 17 sires with extremely high predicted breeding value for marbling and 17 sires with extremely low one for the IDVGA-49. Statistically significant difference in the allelic distribution between 17 sires with extremely high breeding value and 17 sires with extremely low one was detected for the IDVGA-49 (Table 2). The frequency of the 156 bp allele at the IDVGA-49 was higher in animals with the high breeding value than with the low one and the 164 bp allele frequency in animals with the low one than with the high one (Table 2).

Table 2: Comparison of allelic distribution in IDVGA-49 between 17 sires with extremely high predicted breeding value for marbling and 17 sires with extremely low one

		Allele frequency		
Gene	Allele (bp)	Sires with high breeding value	Sires with low breeding value	p-value
MFN2	156	0.20	0.05	<0.05
	164	0.80	0.95	

Researchers second sequenced the ~4 kb proximal promoter region, exon 1 and 2 regions and intron 1 region of the *MFN2* gene from 2 low-marbled Holstein steers and 2 high-marbled Japanese black steers. This sequence analysis revealed 2 SNPs in the promoter region of the *MFN2* gene: g.38438677A>G SNP is a G to A substitution located 1587 bp upstream from the transcription initiation site; g.38437771C>T SNP is a C to T substitution located 681 bp upstream from the transcription initiation site. Further this analysis revealed 3 SNPs in the intron 1 region of the *MFN2* gene: g.38436281A>G SNP is a A to G substitution located 810 bp downstream from the transcription initiation site; g.38436013T>C SNP is a T to C substitution located 1078 bp downstream from the transcription initiation site; g.38435987A>T SNP is a A to T substitution located 1104 bp downstream from the transcription initiation site. Additionally, revealed 1 SNP in the exon 2 region of the *MFN2* gene: g.38435768T>C is T to C substitution located 1323 bp downstream from the transcription initiation site. The low-marbled steers were homozygous for A, T, G, C, T and C alleles at the SNPs whereas the high-marbled steers homozygous for G, C, A, T, A and T alleles at the g.38438677A>G, g.38437771C>T, g.38436281A>G, g.38436013T>C, g.38435987A>T and g.38435768T>C site, respectively. Using PCR-RFLP method, researchers genotyped 34 Japanese black unrelated sires consisting of 17 sires with extremely high predicted breeding value for marbling and 17 sires with

extremely low one. For the g.38438677A>G SNP, the AA homozygotes, the GG homozygotes and the AG heterozygotes resulted in 2 bands (23 and 204 bp), 1 band (227 bp) and 3 bands (23, 204 and 227 bp), respectively. For the g.38437771C>T SNP, the CC homozygotes, the TT homozygotes and the CT heterozygotes resulted in 2 bands (57 and 235 bp), 1 band (292 bp) and 3 bands (57, 235 and 292 bp), respectively. For the g.38436281A>G SNP, the AA homozygotes, the GG homozygotes and the AG heterozygotes resulted in 1 band (168 bp), 3 bands (17, 64 and 87 bp) and 4 bands (17, 64, 87 and 168 bp), respectively. For the g.38436013T>C SNP, the TT homozygotes, the CC homozygotes and the TC heterozygotes resulted in 3 bands (5, 54 and 133 bp), 1 band (192 bp) and 4 bands (5, 54, 133 and 192 bp), respectively. For the g.38435987A>T SNP, the AA homozygotes, the TT homozygotes and the AT heterozygotes resulted in 1 band (179 bp), 2 bands (46 and 133 bp) and 3 bands (46, 133 and 179 bp), respectively. For the g.38435768T>C SNP, the TT homozygotes, the CC homozygotes and the TC heterozygotes resulted in 1 bands (238 bp), 2 bands (118 and 120 bp) and 3 bands (118, 120 and 238 bp), respectively. The genotyping data suggested that g.38438677A>G and g.38435987A>T SNPs are in complete linkage disequilibrium. No significant difference in the allele frequency distribution between 17 sires with extremely high breeding value and 17 sires with extremely low one was detected for the all SNP (Table 3). Thus, researchers showed the higher frequency of the IDVGA-49 156 bp allele in high-marbled cattle as compared to low-marbled cattle, although the allelic distribution of the 6 SNPs detected in the *MFN2* gene was indistinguishable between high-marbled and low-marbled cattle. This study, together with the mapping of a marbling quantitative trait locus within genomic region containing the MFN2 on bovine chromosome 16.

Table 3: Comparison of allelic distribution in the detected SNP between 17 sires with extremely high predicted breeding value for marbling and 17 sires with extremely low one

		Allele frequency		
SNP	Allele	Sires with high breeding value	Sires with low breeding value	p-value
g.38438677A>G	A	0.389	0.611	0.272
	G	0.540	0.460	-
g.38437771C>T	C	0.520	0.480	0.582
	T	0.444	0.556	-
g.38436281A>G	A	0.411	0.589	0.401
	G	0.529	0.471	-
g.38436013T>C	T	0.389	0.611	0.272
	C	0.540	0.460	-
g.38435987A>T	A	0.531	0.469	0.417
	T	0.421	0.579	-
g.38435768T>C	T	0.531	0.469	0.417
	C	0.421	0.579	-

Takasuga *et al*. (2007) suggests that an unidentified true causal mutation which is in linkage disequilibrium with the IDVGA-49 marker but not the 6 SNPs, directly affects changes in *MFN2* gene expression and/or marbling. The IDVGA-49 microsatellite marker may be useful for effective marker-assisted selection to increase the levels of marbling.

CONCLUSION

In this study, researchers show that the frequency of the 156 bp allele at the IDVGA-49 microsatellite marker close to the *MFN2* gene is higher in high-marbled cattle than in low-marbled cattle although, the allelic distribution of the 6 SNPs detected in the *MFN2* gene is indistinguishable between high-marbled and low-marbled cattle. This study suggests that an unidentified true causal mutation which is in linkage disequilibrium with the IDVGA-49 marker but not the 6 SNPs may be related to changes in *MFN2* gene expression and/or marbling. The IDVGA-49 marker may be useful for effective marker-assisted breeding to increase the levels of marbling.

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