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Original Article

Detection of candidate polymorphisms around the QTL for fat area ratio to rib eye area on BTA7 using whole-genome resequencing in Japanese Black cattle

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Running head: whole-genome resequencing for beef marbling

Abstract

In our previous study, we performed genome-wide association study (GWAS) to identify the genomic region associated with Fat area ratio to rib eye area (FAR) and detected a candidate in BTA7 at 10–30 Mbp. The present study aims to comprehensively detect all polymorphisms in the candidate region using whole-genome resequencing data. Based on whole-genome resequencing of eight animals, we detected 127,090 polymorphisms within the region. Of these, 31,945 were located within the genes. We further narrowed the polymorphisms to 6,044 with more than five allele differences between the high and low FAR groups that were located within 179 genes. We subsequently investigated the functions of these genes and selected 170 polymorphisms in eight genes as possible candidate polymorphisms. We focused on SLC27A6 K81M as a putative candidate polymorphism. We genotyped the SNP in a Japanese Black population (n = 904) to investigate the effect on FAR. Analysis of variance revealed that SLC27A6 K81M had a lower p-value (p = 0.0009) than the most significant SNP in GWAS (p = 0.0049). Although only SLC27A6 K81M was verified in the present study, subsequent verification of the remaining candidate genes and polymorphisms could lead to the identification of genes and polymorphisms responsible for FAR.

***Keywords**: beef marbling, Japanese Black cattle, whole-genome resequencing*

INTRODUCTION

Beef marbling is an economically important trait indicating beef quality. In recent years, researchers have identified genes and polymorphisms responsible for beef marbling in Japanese Black cattle. However, the gene primarily responsible for controlling beef marbling remains unknown. Meanwhile, as a novel indicator for marbling, fat area ratio to rib eye area (FAR), which is calculated using a computer image analysis of a carcass cross-section, has been developed to evaluate beef marbling in detail (Kuchida, Osawa, Hori, Kotaka, & Maruyama, 2006). Because this trait is measured by computer analysis, it can be considered a more objective trait. In fact, our previous study showed that the trait has recently been used to provide additional information for the accurate evaluation of beef marbling in Japan (Nakajima et al., 2018).

In our previous study, we performed a genome-wide association study (GWAS) for FAR in a Japanese Black population (Nakajima et al., 2018). Two significantly associated SNPs were detected on BTA7, suggesting that a polymorphism responsible for FAR would be located around these SNPs. Moreover, we searched a region 5-Mbp upstream and downstream of the most significant SNP and detected candidate genes, including ANGPTL4, PLIN4, and SIRT6, in terms of their function in fat metabolism. However, the polymorphisms within these genes have still not been determined.

In post-GWAS analysis, polymorphisms are regularly detected by sequencing each candidate gene to search for responsible polymorphisms (Pausch et al., 2014; Xie et al., 2014; Yang et al., 2017). In addition, using next-generation sequencing (NGS) technologies, methods to detect all polymorphisms in candidate regions have been developed and used in animal science. Li et al. (2015) performed whole-genome resequencing in ten birds to detect polymorphisms on a fine-mapped QTL for a pH value of chicken meat. Furthermore, Kawaguchi et al. (2019) performed whole-genome

resequencing in eight cattle to detect all polymorphisms within a QTL for oleic acid percentage in beef. They eventually identified additional candidates as responsible polymorphisms by narrowing down the polymorphisms. These studies suggest that whole-genome resequencing data can be a powerful tool, especially in cases where a functionally promising candidate gene is not found within the candidate region. The objective of the present study was to comprehensively detect candidate polymorphisms for FAR in the candidate region on BTA7 using whole-genome resequencing data.

MATERIALS AND METHODS

Sample selection for whole-genome resequencing

In our previous study, we used a Japanese Black cattle population comprising 1836 animals that had been bred in the Hyogo Prefecture (Nakajima et al., 2018). After correcting for the FAR phenotype of each animal using an analytical model, we selected 200 animals for pool-based GWAS. Of these, 100 animals had higher FAR (high group) and the other 100 had lower FAR (low group).

In the present study, eight animals were selected from the aforementioned 200 animals for whole-genome resequencing based on their sires and genotype of the most significant SNP in the GWAS results (ARS-BFGL-NGS-35463; Table 1). We selected four animals with the AA genotype from the 100 “high” animals and four animals with the GG genotype from the 100 “low” animals, which were the progenies of different sires among the four animals in the high and low groups. Genomic DNA was extracted from each 50-mg longissimus cervicis muscle sample following the standard phenol–chloroform method.

Sequencing, read mapping, and polymorphism calling

DNA degradation was monitored based on its concentration by spectrometry, fluorometry, and 1% agarose gel electrophoresis. A paired-end library was constructed using high-quality DNA for each individual, and its read length was 150 bp. Sequencing was performed using a HiSeq X Five Sequencing System (Illumina Inc., San Diego, CA, USA). Sequencing data were normalized by Genedata Expressionist 9.1.4a. We mapped the reads to the cattle reference genome assembly (UCSC bosTau8) downloaded from the UCSC Genome Browser assembly (https://genome-asia.ucsc.edu/cgi-bin/hgGateway) using BWA-MEM 0.7.12. and excluded PCR duplicates using Picard 2.2.4. GATK 3.6 (2016-12-08-g1c2527f) was used to call polymorphisms by comparing the genome sequences, including the reference sequence. The polymorphisms were annotated to the gene reference (NCBI RefSeq) based on their location (intron, exon, untranslated region, upstream, downstream, splice site, and intergenic region) and characteristics (synonymous/non-synonymous amino acid replacement, gain/loss of start/stop site, and frameshift mutations) using SnpEff v4.2 with the reference sequence (bosTau8). Sequencing and mapping summary was shown in Table S1.

Candidate polymorphisms detection

In our previous study, the GWAS analysis for FAR revealed two significant SNPs at 17.5 and 28.5 Mbp on BTA7 (Nakajima et al., 2018). In the present study, we determined a 20-Mbp region (10.0–30.0 Mbp) as the candidate region to include all previously

reported QTL for marbling score around this region considering that genetic correlation between FAR and marbling score was 0.99 (Nakajima et al., 2018) : a QTL analysis using microsatellite markers identified DIK079–DIK8044 (13.2–26.4 Mbp) in Japanese Black cattle (Hirano, Watanabe, Inoue, & Sugimoto, 2007); a QTL scan identified DIK2819–UWCA20 (23.4–27.4 Mbp) in commercial American Angus (McClure et al., 2010); and GWAS identified rs41588220–rs43501063 (20.0–22.0 Mbp) in 10 breeds (Saatchi, Schnabel, Taylor, & Garrick, 2014). We selected polymorphisms in the candidate region from all the polymorphisms detected by whole-genome resequencing.

We first excluded intergenic polymorphisms and then focused on the linkage disequilibrium (LD) between the polymorphisms and ARS-BFGL-NGS-35463 because the responsible polymorphism should be in LD with ARS-BFGL-NGS-35463. We compared the genotypes of four animals in the high group with those of four animals in the low group as an indicator of LD. The ARS-BFGL-NGS-35463 genotype completely differed between the high and low groups; eight allele differences were found between the high and low groups. Therefore, polymorphisms with several allele differences were expected to be in LD with the SNP. In the present study, we focused on the polymorphisms with five or more allele differences.

We investigated the function of genes containing these polymorphisms from the NCBI database and previous reports and determined candidate genes based on their function in fatty acid metabolism, such as synthesis, transport, desaturation, and oxidation.

Genotyping the candidate polymorphisms

Polymorphisms were prioritized as candidates based on their location and characteristics. We selected the candidate polymorphisms that were most likely to affect the function of each candidate gene.

To test the effect of candidate polymorphisms on FAR, we used a Japanese Black cattle population (n = 904) that had been randomly selected from 1836 individuals bred in the Hyogo Prefecture, Japan, and graded from 2010 to 2012. This population contained at least 10 offspring per sire. We genotyped the K81M polymorphism in the SLC27A6 gene and ARS-BFGL-NGS-35463 using TaqMAN assay and RFLP. Primer sequences, reaction conditions, and restriction enzymes are shown in Table 2.

Statistical analysis

We previously corrected phenotypes to select animals for pool-based GWAS (Nakajima et al. 2018). In the current study, statistical analysis was performed using the corrected values. As described in the previous study, a linear mixed model was applied to FAR as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z_1u_1} + \mathbf{Z_2u_2} + \mathbf{e}$$

where y is a vector of observations (FAR); **b** is a vector of fixed effects including overall mean, slaughter year, slaughter month, sex of animals, linear covariate for inbreeding coefficient, and linear and quadratic covariates for age at slaughter; $\mathbf{u_1}$ and $\mathbf{u_2}$ are vectors of random farm and animal genetic effects, respectively; **e** is a vector of random residual effects; and **X**, $\mathbf{Z_1}$, and $\mathbf{Z_2}$ are known incident matrices. Restricted maximum likelihood by the expectation-maximization algorithm (EM-REML) and best

linear unbiased prediction (BLUP) were employed to estimate variance components and all random effects in the model, respectively.

The e-values of 904 animals were analyzed by one-way analysis of variance (ANOVA) with genotype as a source of variation and Tukey-Kramer's honestly significant difference (HSD) test implemented in JMP13 (SAS institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Whole-genome resequencing

We performed whole-genome resequencing in eight Japanese Black cattle. A total of 127,090 polymorphisms were identified in the candidate region (Chr 7: 10–30 Mbp) by comparing genome sequences among nine animals, including the reference (bosTau8). Of all detected polymorphisms, 31,945 were located within the genes. In our previous study (Nakajima et al., 2018), we selected six candidate genes (GCDH, ANGPTL4, PLIN3, PLIN4, PLIN5, and SIRT6) from the genes located in the candidate region based on their function in fat metabolism. In the present study, we simultaneously detected all intragenic polymorphisms, including these six genes, rather than separately sequencing each gene, suggesting that whole-genome resequencing could be a powerful tool to rapidly and conveniently detect candidate polymorphisms.

We further narrowed the polymorphisms to 6,044 having more than five allele differences between the high and low FAR groups (Table 3) located in 179 genes (Table S2). We subsequently investigated the functions of 179 genes based on the information from the NCBI database and previous reports. Eight genes (TECR, GCDH, ANGPTL4, PLIN3, DPP9, SIRT6, ACSL6, and SLC27A6) were identified as candidate genes in terms of their function in fat metabolism (Table 4).

Selection of a candidate polymorphism

Overall 170 candidate polymorphisms were found within eight genes (Table S3). We subsequently confirmed their annotations to select a more likely candidate polymorphism. Some polymorphisms were located in the coding region 5' or 3' UTR upstream or downstream, and the rest were located in the introns. Because polymorphisms located in the 5' or 3' UTR upstream or downstream region might affect gene expression, they could be possible candidates. However, there was little information regarding the regions related to gene expression of each gene. Therefore, we primarily focused on polymorphisms in the coding region and an amino acid substitution within the SLC27A6 gene as a possible candidate polymorphism.

SLC27A6 K81M (BTA9:26329353_UMD3.1) (SNP ID: rs109305471) is part of the acetyl-CoA synthetase-like superfamily domain, which includes the FATP/VLACS signature motifs (Nafikov et al., 2013; Zou, DiRusso, Ctrnacta, & Black, 2002). Although the region of FATP/VLACS signature motifs within the SLC27A6 gene has still not been demonstrated, Zou et al. (2002) reported that a polymorphism in FATP/VLACS signature motifs in FAT1 gene affects the levels of fatty acid accumulation and long-chain fatty acyl-CoA synthetase activities. K81M might accordingly affect the gene function by structural regulation of the FATP/VLACS signature motifs. Therefore, we selected SLC27A6 K81M as a likely candidate polymorphism.

Effect of the candidate polymorphism on FAR

We genotyped SLC27A6 K81M and the most significant SNP in GWAS (ARS-BFGL-NGS-35463) in a Japanese Black cattle population (n = 904). The minor allele frequencies of SLC27A6 K81M and ARS-BFGL-NGS-35463 were 0.259 and 0.317, respectively (Table 5). ANOVA revealed that both SLC27A6 K81M and ARS-BFGL-NGS-35463 are significantly association with FAR (p = 0.0009 and 0.0049, respectively). SLC27A6 K81M showed a lower p-value than ARS-BFGL-NGS-35463, suggesting that SLC27A6 K81M might be the responsible polymorphism.

SLC27A6 gene belongs to the fatty acid transport protein family (FATP, SLC27A1-6) (Doege & Stahl, 2006). FATPs are 70–80KDa integral membrane proteins with an extracellular/luminal N-terminal and a cytosolic C-terminal domain and are involved in the translocation of long-chain fatty acids across the plasma membrane (Gimeno, 2007). Considering the function of the gene, it is possible that alteration of the protein structure caused by K81M polymorphism might affect lipid accumulation in the cells, leading to an increase in FAR. However, previous studies in humans and mice have reported that FATP6 (SLC27A6) is not expressed in tissues other than the heart and hair follicles in the skin (Schmuth, 2005), suggesting that SLC27A6 might be less likely to directly affect FAR. Further investigation on the mechanism of how bovine SLC27A6 affects FAR is necessary to determine whether SLC27A6 K81M is responsible for FAR. Especially, gene expression analysis such as RT-PCR, in situ hybridization and RNAseq data using intramuscular tissue would provide additional and useful information to effectively select candidate gene and polymorphisms.

Conclusion

In the present study, verification of SLC27A6 K81M is one of the initial steps. Subsequent verification of the remaining candidate genes and polymorphisms detected in the study is required and expected to lead to the identification of genes and polymorphisms responsible for FAR.

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TABLE 1 Japanese Black cattle used for whole-genome resequencing

Group	Sample	Genotype	Sire	FAR	corrected FAR
high	1	AA	1	0.590	0.428
	2	AA	2	0.580	0.438
	3	AA	3	0.576	0.440
	4	AA	4	0.562	0.415
low	5	GG	5	0.288	0.311
	6	GG	6	0.266	0.325
	7	GG	7	0.256	0.306
	8	GG	8	0.245	0.314

Genotype: the genotype of ARS-BFGL-NGS-35463, which was the most significantly associated with FAR in the GWAS (Nakajima *et al.* 2018)

FAR: Fat area ratio to rib eye area

corrected FAR: the corrected values of FAR using an analytical model

TABLE 2 Protocol summary for genotyping

Polymorphism	Method		Sequence	PL (bp)	RE
ARS-BFGL-NGS-35463	PCR-RFLP	primer	F: 5'- GCC TGT GTT CTA CAG CAA GAG CA -3'	253	*Alw*NI
			R: 5'- CTT CGT CAG GGT TTG CAT CCT TG -3'		
SLC27A6 K81M	TaqMan	primer	F: 5'- GAT GTC TCC CTC GTA GAT GAT G -3'	129	-
			R: 5'- GTT CGC TGT GGA CTT TGG -3'		
		prove	FAM- CCG CTT CAT GGC CTG GAT CAG -BHQ		
			HEX- CCG CTT CTT GGC CTG GAT CAG -BHQ		

PL: PCR product length, RE: restriction enzyme

TABLE 3 The number of intragenic polymorphisms detected within candidate region for FAR.

annotation	Allele differences*				total
	8	7	6	5	
intron	631	350	627	2690	4298
upstream	168	122	60	316	666
downstream	207	132	82	402	823
5'UTR	15	5	5	12	37
3'UTR	18	14	11	52	95
synonymous	24	3	2	45	74
missense	10	2	5	15	32
splice region	3	4	7	5	19
Total	1076	632	799	3537	6044

FAR: Fat area ratio to rib eye area

*Allele differences: the number of differences in alleles between high and low FAR groups

TABLE 4 Function of eight genes containing the candidate polymorphisms

Gene	Posittion (bp)	Function	reference
TECR	12329819..12357348	the long-chain fatty acids elongation	Moon and Horton (2002)
GCDH	13771152..13777998	glutaryl-CoA dehydrogenase activity	Hyman and Tanaka (1984)
ANGPTL4	18236517..18243587	inactivation of the lipoprotein lipase	Kersten et al. (2005)
PLIN3	20503752..20524341	lipid droplet formation	Buers et al. (2009)
DPP9	20640566..20679323	dipeptidyl aminopeptidase activity	Bjelke et al. (2006)
SIRT6	21079093..21087378	lipid and glucose metabolism	Feldman, Baeza, and Denu (2013)
ACSL6	23773498..23884958	formation of acyl-CoA from fatty acids	Soupene and Kuypers (2008)
SLC27A6	26237925..26330069	fatty acid transportation	Gimeno et al. (2007)

TABLE 5 Effect of ARS-BFGL-NGS-35463 and *SLC27A6* K81M on FAR in the Japanese Black population (n = 904)

Polymorphism	Position	Genotype frequency (n)			Allele frequency		e-values ± SE			*p*-value
ARS-BFGL-NGS-35463		AA	AG	GG	A	G	AA	AG	GG	
	17,490,481	0.100 (90)	0.435 (393)	0.466 (421)	0.317	0.683	0.0079 [a] ± 0.0031	-0.0021 [b] ± 0.0015	-0.0031 [b] ± 0.0014	0.0049
SLC27A6 K81M		KK	KM	MM	K	M	KK	KM	MM	
	26,329,353	0.059 (53)	0.402 (363)	0.540 (488)	0.259	0.741	0.0068 [a] ± 0.0035	0.0011 [a] ± 0.0015	-0.0047 [b] ± 0.0013	0.0009

FAR: Fat area ratio to rib eye area

e-values: the least square mean of e-values for FAR for each genotype

a, b: means with different superscript are significantly different between genotypes