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RESEARCH ARTICLE

In vivo evidence on the functional variation within fatty acid synthase gene associated with lipid metabolism in bovine *longissimus dorsi* muscle tissue

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Abstract

In Korean cattle, intramuscular fat (IMF), or marbling, of the *longissimus dorsi* muscle (LM) cross section is one of the most important indicators of beef quality and are influenced by environmental and genetic factors. This study was to evaluate the effect of SNPs on the beef quality in Korean cattle for functional studies, such as site-directed mutagenesis based on bovine adipocytes. The fatty acid synthase (*FASN*) gene plays an important role in lipogenesis. FASN is an essential metabolic and multifunctional enzyme in fatty acid synthesis. Several studies have reported that SNPs g.841G, g.16024A, g.16039T, and g.17924G have a significant impact on marbling scores in Korean cattle and Japanese Black cattle population. These SNPs are located in transcription factor binding sites, the beta-ketoacyl reductase, and thioesterase domains. Our results revealed that the g.17924 A>G SNP is located in the thioesterase domain of the FASN protein, and changes from polar, neutral, and hydrophilic to nonpolar, aliphatic, and hydrophobic, respectively. In in vivo LM tissue of Korean cattle, the g.17924A>G SNP has an effect on increasing fat deposition. Therefore, g.17924A>G SNP could be a causal mutation for increasing fat deposition in Korean cattle LM tissue.

Keywords Functional SNP · FASN · In vivo LM tissue · Intramuscular fat · Korean cattle

Introduction

Several molecular genetic researchers have identified single nucleotide polymorphisms (SNPs) related to lipid synthesis for improving intramuscular fat (IMF) deposition in the *longissimus dorsi* muscle (LM) of Korean cattle (Cheong et al. 2008; Lee et al. 2014; Oh et al. 2012b; Shin et al. 2007). Although these SNPs were statistically identified, the protein function of each SNP allele has not been verified by site-directed mutagenesis, based on bovine adipocytes derived from intramuscular fat.

In Korean cattle, IMF, or marbling, of the LM cross section is one of the most important indicators of beef quality, as well as juiciness, tenderness, and taste (Hausman et al. 2009). Beef quality of Korean cattle is classified into five grades (1++, 1+, 1, 2, and 3) by the Korean beef carcass grade system of the Korea Institute for Animal Products Quality Evaluation (KAPE). Deposition of IMF is influenced by several factors such as breed, lipid metabolism genes, and nutrition (Maltin et al. 2003). Furthermore, fat deposition is mainly determined by the expression of related genes during lipogenesis and glycerol-3-phosphate pathways (Jeong et al. 2012).

The fatty acid synthase (*FASN*) gene plays an important role in lipogenesis. FASN is an essential metabolic and multifunctional enzyme in fatty acid synthesis. This cytosolic enzyme catalyzes the synthesis of palmitate from acetyl-coenzyme A and malonyl-coenzyme A in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). The functional FASN enzyme is a homodimer of 250 kDa subunits, and contains seven catalytic activities and the acyl carrier protein (ACP) (Roy et al. 2005). Several studies have reported that SNPs g.841G, g.16024A, g.16039T, and g.17924G have a significant impact on marbling scores in

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Korean cattle and Japanese Black cattle population (Abe et al. 2009; Bhuiyan et al. 2009; Hayakawa et al. 2015; Li et al. 2012; Oh et al. 2012a; Zhang et al. 2008). These SNPs are nonsynonymous, except for g.841G, and are located in transcription factor binding sites (g.841G), the beta-ketoacyl reductase (g.16024A and g.16039T), and thioesterase (g.17924G) domains. Therefore, the aim of this study is to evaluate the effect of SNPs on the beef quality in Korean cattle for functional studies, such as site-directed mutagenesis based on bovine adipocytes.

Materials and methods

Animals and phenotype

Korean cattle steers (n = 192) raised in Pyeongchang-gun, Gangwon Province, were used in this study. All the steers were maintained under constant environmental conditions at six feedlots. The beef quality data of the steers were collected from the Korean beef carcass grade system of the KAPE. Genomic DNA was extracted from the LM using a LaboPass™ Tissue Mini kit (Cosmo Genetech, Seoul, Korea).

Mapping of functional SNP candidates and SNP genotyping

The bovine *FASN* sequences used in this study were obtained from the NCBI database. The resource numbers of DNA, mRNA, and protein sequences were as follows: DNA (AC_000176), mRNA (NM_001012669), and protein (NP_001012687). To align the functional SNPs to DNA, mRNA, and protein sequences, the alignment was analyzed using the Graphical View Legend on the NCBI database.

SNP genotyping, using the Fluidigm® SNP™ Type assay, was undertaken as follows: 20–40 mg of tissue was subjected to crude lysis using Proteinase K (Qiagen, Hilden, Germany) and Chelex 100 (Sigma-Aldrich, St Louis, USA). SNP genotyping was carried out using Fluidigm 96.96 Dynamic Array™ integrated fluidic circuits (IFC) and EP1™ system platform. Genomic DNA (30 ng) was used as a template for the pre-amplification step, which was performed using a combination of a locus-specific primer (LSP) and a specific target amplification (STA) primer. The pre-amplified product was diluted to 1:100 in distilled water, and subjected to a second round of PCR amplification using the LSP and a set of fluorescently labeled allele-specific primers (ASPs). SNPs were called using pre-defined algorithms implemented with the Fluidigm SNP Genotyping Analysis software (ver. 4.3.2). The clustering setting automatic confidence threshold was set at 65, the data normalization method was SNP Type normalization, and genotypes were manually confirmed for each SNP.

Statistical analysis and 3D protein structure prediction

For the 192 Korean cattle steers, the genotype frequency of functional SNPs was calculated using the 'ftable' function in R program. The correlation coefficients between pairwise SNPs were measured using Haploview. In order to ascertain whether nonsynonymous SNPs influenced phenotypic variation, the relationship between the SNPs and beef quality were analyzed by cross-tabulation using Fisher's exact tests. Whether the mutations identified in the FASN coding regions could potentially lead to structural alterations of the FASN protein, we analyzed protein sequences using homology modelling software (Swiss-PDB-Viewer) to predict 3D protein structure.

Results and discussion

Characterization of potential functional SNPs from the *FASN* gene

The *FASN* gene plays an important role in lipid metabolism; non-synonymous SNPs in the exon region and SNPs in the 5′ and 3′ untranslated regions (UTRs) have an impact on the regulation of gene expression for increasing IMF deposition in the LM tissue of cattle. The location of potential functional SNPs in the *FASN* sequence, obtained from the NCBI database (Accession no. AC_000176), and alignment of SNPs in RNA and protein sequence are shown in Fig. 1. Information and characterization of potential functional SNPs within the *FASN* gene identified in previous studies (Bhuiyan et al. 2009; Hayakawa et al. 2015; Matsuhashi et al. 2011) are shown in Table 1.

Hayakawa et al. (2015) reported that the g.841G>C SNP is located within a GC-rich region in the 5′ UTR that consists of multiple binding elements for transcription factors belonging to the Sp family. The Sp family are involved in cellular processes including cell differentiation, cell growth, apoptosis, and immune response. Especially, two members of Sp family, Sp1 and Sp3, bind to GC-rich motifs of promoters and regulate transcription of *FASN* gene (Goto et al. 2006; Ordovas et al. 2008; Suske 1999; Wolf et al. 2001). In a previous study, a gene reporter assay using cell lines showed a significant difference between two alleles of the g.841G>C SNP related to fatty acid synthesis (Hayakawa et al. 2015). As shown in Fig. 1 and Table 1, g.16024A>G and g.16039T>C SNPs are located in the exon 34 region, which belong to beta-ketoacyl reductase of the FASN protein domain. Beta-ketoacyl reductase catalyzes the reduction

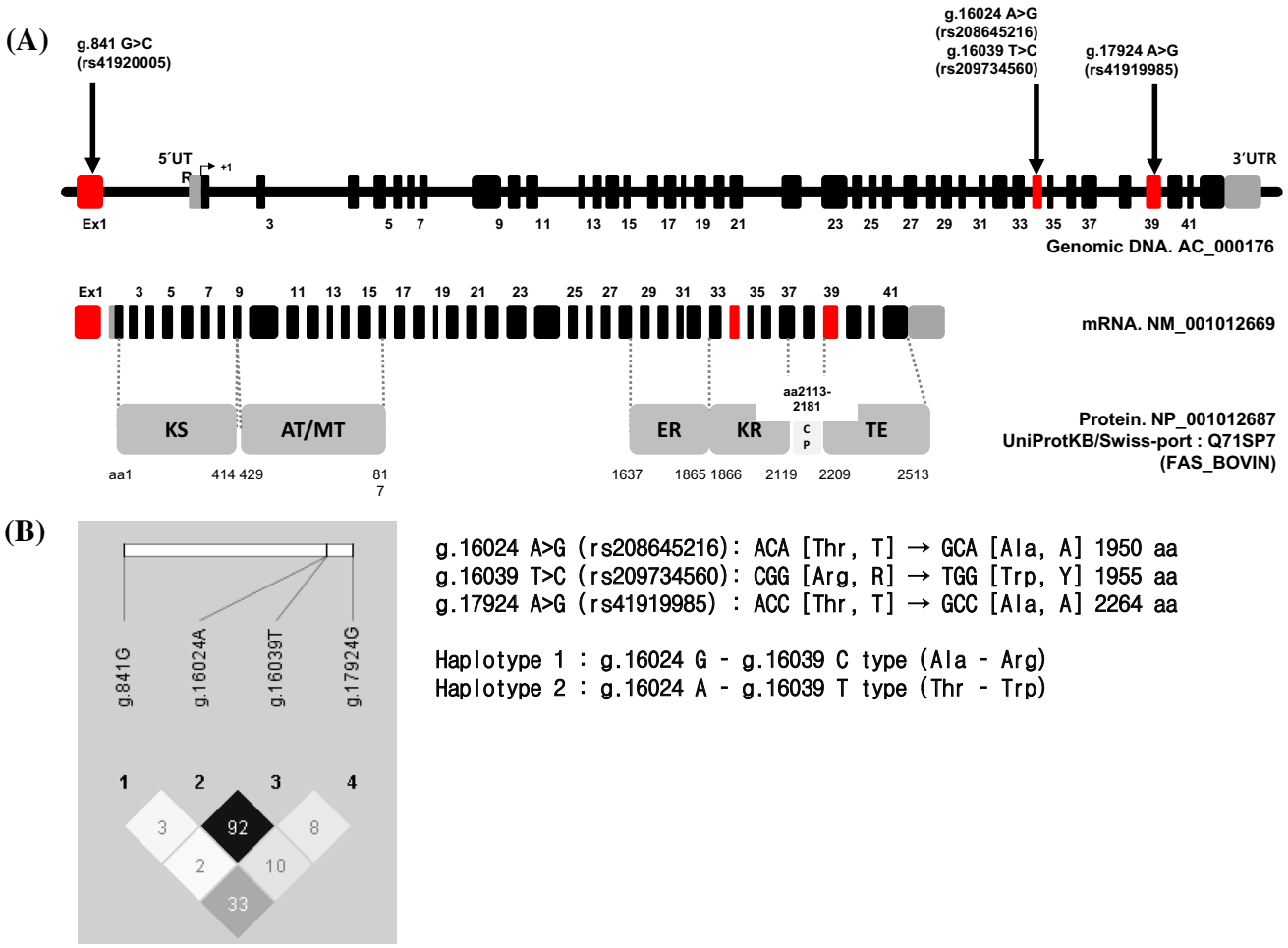

Fig. 1 Map of functional SNP candidates located in the DNA, mRNA, and protein sequences of the *FASN* gene and the pairwise correlation between SNP. **a** The diagram shows four potential functional SNPs that were aligned with DNA, RNA, and protein sequences of the FASN. Red boxes indicate the position of functional SNP candidates. Gray and black boxes indicate UTR and exon regions, respectively. **b** This figure shows the result of correlation between potential functional SNPs using Haploview program

Table 1 Information and functional characterization of SNPs in the fatty acid synthase genes identified in previous studies

SNP	dbSNP no.	Region	Breed	Amino acid substitution	Protein domain	References
g.841G>C	rs41920005	Exon 1	Japanese black cattle	–	Transcription factor binding site	Hayakawa et al. (2015)
g.16024A>G	rs208645216	Exon 34	Japanese black cattle	Thr/Ala, 1950 aa	Beta-ketoacyl reductase	Matsuhashi et al. (2011)
g.16039T>C	rs209734560	Exon 34	Japanese black cattle	Arg/Trp, 1955 aa	Beta-ketoacyl reductase	Matsuhashi et al. (2011)
g.17924A>G	rs41919985	Exon 39	Hanwoo	Thr/Ala, 2264 aa	Thioesterase	Bhuiyan et al. (2009)

Thr threonine, polar and neutral R group, hydrophilic; *Ala* alanine, nonpolar and aliphatic R group, hydrophobic; *Arg* arginine, polar and charged R group, hydrophilic; *Trp* tryptophan, nonpolar and aromatic R group, hydrophobic

reaction of hydroxylacyl ACP with ketoacyl ACP. Mutation of the g.16024A>G SNP changed the polar, neutral, and hydrophilic features of threonine residues to the nonpolar, aliphatic, and hydrophobic features of alanine residues, respectively. Furthermore, mutation of the g.16039T>C SNP changed the polar, charged, and hydrophilic characteristics of arginine residues to the nonpolar, aromatic, and hydrophobic characteristics of tryptophan residues, respectively. The g.17924A>G SNP is located in the exon 39 region included in the thioesterase domain of the FASN protein. Mutations of the g.17924A>G SNP altered the nonpolar, aromatic, and hydrophobic characteristics of tryptophan

residues to the nonpolar, aliphatic, and hydrophobic features of alanine residues, respectively.

In order to evaluate correlation among four SNPs, we performed analysis of linkage disequilibrium (LD) between pairwise SNPs (Fig. 1b). Among those SNPs in the *FASN* gene, the LD coefficient between g.16024A>G and g.16039T>C SNPs was $r^2 = 0.92$, which was higher than that between any other pairwise SNPs in the *FASN* gene.

In vivo evidence of the genotype effect of functional SNPs on beef quality

Beef quality of Korean cattle is classified into five grades (1++, 1+, 1, 2, and 3) depending on IMF deposition, by the Korean beef carcass grade system of the Korea Institute for Animal Products Quality Evaluation (KAPE) (Fig. 2b). Distribution of IMF deposition differs between beef grades 1 and 2 in in vivo LM tissue. We used Fisher's exact test to evaluate the effect of increasing fat deposition from in vivo LM tissue for four potential functional SNPs (Table 2).

Although the genotype frequency of the g.17924A>G SNP was not statistically different ($P = 0.149$), the type frequency of combination of farm and g.17924A>G SNP was significantly different among the beef grades ($P = 0.041$). The results suggested that the g.17924A>G SNP had an effect on increasing fat deposition in Korean cattle LM tissue and was influenced by the farm's feeding and environment.

In vivo evidence of the genotype effect of functional SNPs within the *FASN* gene on the beef quality in Korean cattle is shown in Fig. 2. As shown in Fig. 2b, the highest grades of beef (1++ and 1+) showed higher IMF deposition than the lowest grades (2 and 3). On the basis of the difference in IMF deposition between beef grades 1++ and 3, we analyzed the effect of each genotype of the g.17924A>G SNP (Fig. 2a).

As shown in Fig. 2a, animals with the AA genotype of g.17924A>G SNP had a higher appearance ratio in grade 2 than the other genotypes of the g.17924A>G SNP. However, animals with the GG genotype of the g.17924A>G SNP had a higher appearance ratio in the highest grade (1++) than those with the other genotypes.

In previous studies, g.17924A>G SNPs have monomorphic loci, and are fixed to the G allele in Japanese Black cattle (Abe et al. 2009; Hayakawa et al. 2015). In addition, (Schennink et al. 2009; Zhang et al. 2008) reported that the g.17924A>G SNP has a significant effect on the fatty acid

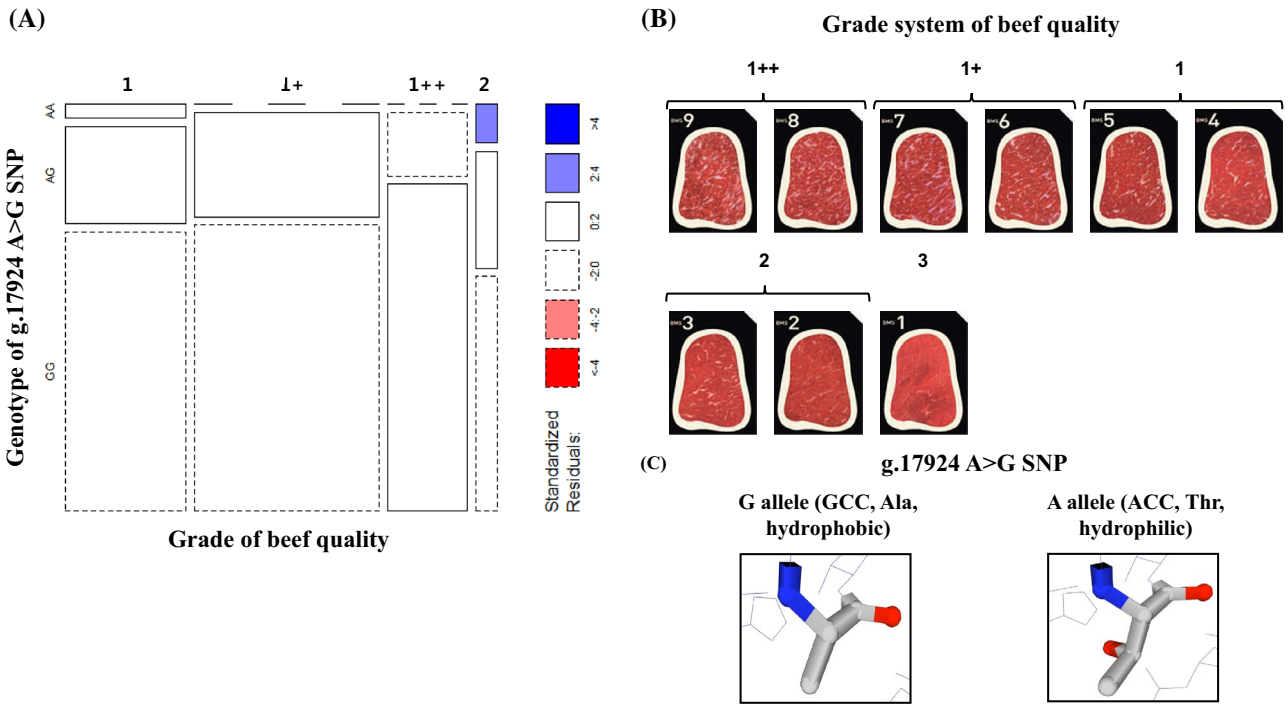

Fig. 2 In vivo evidence of genotypic effect of functional SNPs within the *FASN* gene on the beef quality in a Korean cattle population. **a** This figure shows the association between beef quality grade and genotype of g.17924A>G SNP analyzed using a 2×2 contingency table. The box in the diagram shows standardized residuals of the difference between observed and expected values of beef grade and the g.17924A>G SNP. **b** The beef grade of Korean cattle depending on IMF deposition in the LM tissue after slaughter. Beef quality of Korean cattle is classified into five grades (1++, 1+, 1, 2, and 3) by the Korean beef carcass grade system of the KAPE. **c** Blue position indicates nitrogen from amino group. Red position indicates oxygen from carboxyl group and threonine residue. Each figure shows the change in amino acid residue by each allele of the g.17924A>G SNP

Table 2 Significant levels of Fisher's exact test between beef quality and the combination of functional SNPs and feeding environment

																			Farm *	
												Farm *	Farm *	Farm *	g.841 G>C *				g.841 G>C *	
										Farm *	Farm *	g.841 G>C *	g.841 G>C *	g.841 G>C *		g.16024 A>G *	g.16024 A>G *	g.16024 A>G *		g.16024 A>G *
						Farm *	Farm *	g.841 G>C *	g.841 G>C *	g.16024 A>G *	g.16024 A>G *	g.16024 A>G *	g.16024 A>G *	g.16024 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *
				Farm *	Farm *	g.841 G>C *	g.841 G>C *	g.16024 A>G *	g.16024 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	g.17924 A>G *	NA	NA	NA	NA	NA	NA
	Farm	g.841 G>C	g.16024 A>G	g.17924 A>G	g.841 G>C	g.16024 A>G	g.17924 A>G	g.16024 A>G	g.17924 A>G	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Beef quality	NA	NA	NA	0.149	NA	NA	0.041	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA not significant

composition of rib steaks in purebred American Angus bulls. The GG genotype frequency of the g.17924A>G SNP was low in purebred American Angus bulls and dairy cattle compared to Korean cattle and Japanese Black cattle.

In conclusion, our results revealed that the g.17924A>G SNP is located in the thioesterase domain of the FASN protein, and changes from polar, neutral, and hydrophilic to nonpolar, aliphatic, and hydrophobic, respectively. In in vivo LM tissue of Korean cattle, the g.17924A>G SNP have an effect on increasing fat deposition. Taken together, g.17924A>G SNP could be a causal mutation for increasing fat deposition in Korean cattle LM tissue.

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Compliance with ethical standards

Conflict of interest Dong-yep Oh declares that he has no conflict of interest. Insik Nam declares that he has no conflict of interest. Sehwan Hwang declares that he has no conflict of interest. Hongsik Kong declares that he has no conflict of interest. Honggu Lee declares that he has no conflict of interest. Jaejung Ha declares that he has no conflict of interest. Myunggi Baik declares that he has no conflict of interest. Man Hwan Oh declares that he has no conflict of interest. Songmi Kim declares that she has no conflict of interest. Kyudong Han declares that he has no conflict of interest. Yoonseok Lee declares that he has no conflict of interest.

Ethical approval All research protocols and animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in Gyeongsangbuk-do, Republic of Korea (Gyeongbuk IACUC-87).

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