Recent achievements of candidate polymorphism detection for fatty acid composition in Japanese Black cattle

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INTRODUCTION
In Japanese Black cattle, beef quality, especially beef marbling, has been improved to add value to the beef for more than sixty years (Gotoh et al. 2014). As the results, its beef is appreciated worldwide for the high marbling (Wheeler et al. 2004). Breeders for Japanese Black cattle have, recently, focused on fat quality as one of factors for beef quality in addition to beef marbling. In particular, fatty acid composition is regarded as an important indicator for beef quality because a ratio of various fatty acids affects tenderness and flavor of beef (Yang et al. 1999; Smith et al. 2006). For instance, beef representing high oleic acid (C18:1) percentage would be evaluated as high quality because the beef is tender and has a good flavor. In addition, unsaturated fatty acids including C18:1 have a favorable effect on human health by reducing LDL-cholesterol (Rudel et al. 1995). For these reasons, beef representing higher percentage of unsaturated fatty acids is recognized as high quality.

Fatty acid composition is regulated by various genes related to fatty acid metabolism such as synthesis, oxidation, transport, and desaturation for fatty acids. In fact, some studies suggested that fatty acid composition would be able to be genetically improved since its heritability ranged moderate to high in Japanese Black cattle (Inoue et al. 2011; Nogi et al. 2011; Sakuma et al. 2017). Meanwhile, DNA markers for fatty acid composition have been developed. Taniguchi et al. (2004) reported that an amino acid substitution A293V in stearoyl-CoA desaturase (SCD) gene was significantly associated with the percentage of monounsaturated fatty acid (MUFA) and melting point of beef fat. In fatty acid synthase (FASN) gene, two amino acid substitutions (T1950A and W1955R), which were in almost complete linkage disequilibrium (LD), and a SNP in promoter region (g.841G>C) were significantly associated with fatty acid composition (Abe et al. 2009; Hayakawa et al. 2015). Since the SCD and FASN genes are involved in fatty acid desaturation and synthesis, respectively, these polymorphisms would be the promising candidates as responsible polymorphisms for fatty acid composition. However, the proportion of genetic variance explained by the SCD and FASN polymorphisms was reported to range from 3.29 to 24.71 in a Japanese Black population (Matsuhashi et al. 2011). In addition, promising candidate polymorphisms for fatty acid composition had never been identified except for SCD and FASN polymorphisms. These previous reports suggested that there would be some other genes which have effects on fatty acid composition. Then, we have tried to identify novel responsible genes and polymorphisms for fatty acid composition in Japanese Black cattle in the past few years.

1. Identification of candidate polymorphisms for fatty acid composition in leptin gene
As described in the introduction, polymorphisms in various genes have previously been reported to be significantly associated with fatty acid composition in Japanese Black cattle. However, other candidate polymorphisms would remain unknown. Hence, we firstly tried to identify other candidate polymorphisms in leptin (LEP) gene, which was a functional candidate gene (Kawaguchi et al. 2017).

LEP gene encodes an adipocyte-derived circulating protein. The protein has a central role for decreasing
food intake and virtually stopping body weight gain (Chen et al. 1996). In addition, it is known as a regulator of the expression of some genes and the activation of enzymes related to fat metabolism (Siegrist-Kaiser et al. 1997; Minokoshi et al. 2002). The gene might, therefore, have effects on fat related traits. In fact, some previous studies reported that polymorphisms in LEP gene were significantly associated with fat related traits including fatty acid composition in foreign cattle breeds (Corva et al. 2009; Tian et al. 2013; Silva et al. 2014). However, the association between LEP gene polymorphisms and fatty acid composition had not been investigated in Japanese Black cattle yet.

We conducted the first study on LEP gene polymorphisms in Japanese Black cattle. In the study, we identified a total of eight SNPs by sequencing and comparing the full-length coding sequence (CDS) among eight animals. Three of them were expected to substitute amino acids (Y7F, R25C, and A80V). We subsequently genotyped these amino acid substitutions in two Japanese Black populations (JB1: n = 560 and JB2: n = 450) to verify their effects on fatty acid composition using statistical analysis, ANOVA and Tukey-Kramer honestly significant difference (HSD) test. Y7F and A80V showed extremely low minor allele frequencies in JB1 and JB2, respectively. Therefore, their effects were verified only in one population, while R25C was verified in both populations. As the results, R25C and A80V were significantly associated with fatty acid composition (Table 1). In contrast, significant association was not observed between Y7F and fatty acid composition.

R25C would cause the substitution of arginine for cysteine in leptin protein. Since the substitution adds an unpaired cysteine, which is one of important factors to determine the protein structure and function (Bardwell & Bechwith 1993; Giles et al. 2003; Zhang et al. 2006), it might have a crucial effect on leptin activity. In previous studies, the SNP was also significantly associated with fat related traits such as a total of fatty acid contents of beef in Chinese Simmental (Tian et al. 2013) and milk fat content in Holstein (Chebel et al. 2008). Buchanan et al. (2002) particularly reported that animals having T allele (cysteine type) revealed fatter carcasses than animals having C allele (arginine type), suggesting that T allele might impart a partial loss of biological function by adding an extra cysteine to the leptin protein. The SNP might eventually affect fatty acid composition in Japanese Black cattle.

A80V was significantly associated with percentages of some fatty acids in JB1. Orrù et al. (2011) also reported the association between A80V and fatty acid composition in Chinese Simmental. In addition, Reicher et al. (2012) reported that A80V would have impact on an affinity of leptin for its receptor, suggesting that the polymorphism might be responsible for fatty acid composition. Furthermore, the frequency of the T allele (valine type) was low in both Japanese Black populations (0.13 and 0.04), while the T allele represented a positive effect on fatty acid composition in our study. A80V would conclusively be an effective marker to improve fatty acid composition in Japanese Black cattle.

In conclusion, two of three amino acid substitutions (R25C and A80V) would be the possible candidate polymorphisms for fatty acid composition in terms of the

| Population | SNP | Trait | Genotype | Mean ± SD |
|------------|-----|-------|----------|-----------|
| JB1 (n = 560) | R25C | C18:0 (%) | CC | 19.27±0.29 |
|             |     |       | CT | 20.14±0.34 |
|             | A80V | C16:0 (%) | CC | 24.02±0.25 |
|             |     |       | CT | 22.70±0.36 |
|             |     | C16:1 (%) | CC | 2.66±0.06 |
|             |     |       | CT | 2.84±0.09 |
|             |     | C18:1 (%) | CC | 48.34±0.39 |
|             |     |       | CT | 49.78±0.57 |
|             | SFA (%) |       | CC | 46.16±0.40 |
|             |     |       | CT | 44.49±0.59 |
|             | MUFA (%) |      | CC | 51.87±0.40 |
|             |     |       | CT | 53.48±0.59 |
| JB2 (n = 450) | R25C | C14:1 (%) | CC | 0.92±0.02 |
|             |     |       | CT | 0.86±0.02 |
|             |     |       | TT | 0.80±0.05 |

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid
A, B means with different superscripts within the same trait differ significantly at p < 0.05 (Tukey's HSD analysis).
Detection of candidate SNPs for C18:1

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significant associations with fatty acid composition in our study and the impacts on the leptin function according to the previous reports. Indeed, we succeeded to identify possible candidate polymorphisms within LEP gene. However, we should conduct more efficient methodology to comprehensively reveal candidate polymorphisms and genes, since we spend enormous time and effort for experiments to survey polymorphisms in each gene.

2. Genome-wide association study for oleic acid percentage

As the next approach to identify candidate genes for fatty acid composition, we conducted genome-wide association study (GWAS) for oleic acid percentage (C18:1) (Kawaguchi et al. 2018). In previous studies, GWAS for fatty acid composition has also been conducted in Japanese Black populations of Yamagata Prefecture (Uemoto et al. 2010) and Gifu Prefecture (Ishii et al. 2013). In both studies, a QTL was detected on BTA19, where FASN gene is located. Other QTLs were additionally detected on BTA23, 25, and 26 only in the Gifu Prefecture population, suggesting that QTL regions might differ from each population even in the same breed. Hence, we performed GWAS in Hyogo Prefecture population, which had not been investigated by GWAS analysis yet.

We conducted GWAS using two DNA pools composed of 100 animals (Kawaguchi et al. 2018). To select the total of 200 individuals from 1836 Japanese Black cattle bred in Hyogo Prefecture, we firstly calculated e-value (random residual effect) of C18:1 using some analytical models for each animal. The e-value is a sort of corrected phenotype (Uemoto et al. 2010) and we selected each 100 animal for DNA pools from the highest and lowest 15% of the whole e-value distribution. The number of progeny from each sire was limited to less than 10 for each group in order to avoid overrepresentation of a single sire. We estimated allele frequencies using Illumina BovineSNP50v2 BeadChip in each group. All 54,609 SNPs went through quality control that excluded SNPs with minor allele frequencies < 0.05 in either groups. A total of 26,857 SNPs was excluded by the quality control. Significant associated SNPs were not detected on BTA19 and 26 where FASN and SCD genes were located. The result would be due to extremely low minor allele frequencies of the candidate polymorphisms within these genes in this population. We subsequently genotyped the most significant SNPs in each region, Hapmap43702-BTA-84086 and BTB-00554873, in 899 animals randomly selected from Hyogo Prefecture population. The significant threshold of genome-wide significance at 5% accounting for multiple testing by Bonferroni correction was $p = 1.80 \times 10^{-6}$. The x-axis indicates the chromosome no., and the y-axis indicates -log10 ($p$-value). Dashed line indicates the threshold of Bonferroni 5% significance level.

Figure 1 Genome-wide plots -log10 ($p$-value) for an association of loci with C18:1 (Kawaguchi et al. 2019)

| Chr | Position (UMD3.1) | SNP | $p$-value† |
|-----|------------------|-----|------------|
| 9   | 65,738,419       | Hapmap34494-BES3_Contig286_1171 | $3.72 \times 10^{-7}$ |
|     | 69,956,436       | Hapmap43702-BTA-84086             | $1.17 \times 10^{-7}$ |
|     | 70,069,173       | Hapmap60557-rs29018515            | $7.67 \times 10^{-7}$ |
| 14  | 19,250,398       | BTB-00554873                      | $3.74 \times 10^{-7}$ |

† The significant threshold of genome-wide significance at 5% accounting for multiple testing by Bonferroni correction was $p = 1.80 \times 10^{-6}$.

Table 2 Significant SNPs in GWAS (Kawaguchi et al. 2018)
Prefecture population to validate the association between these SNPs and C18:1. As the result of ANOVA, they were significantly associated with C18:1 ($p = 0.0080$ and $0.0003$), suggesting that the associations in GWAS would not false positive. In addition, Tukey-Kramer HSD test resulted in the significantly differences between the least square means for e-values of each genotype (Table 3). These results suggested that these SNPs would be DNA markers to improve fatty acid composition in the Hyogo Prefecture population. In particular, BTB-00554873 would be a more effective marker, since its minor allele showed the positive effect on C18:1. Meanwhile, QTL for fatty acid composition have been identified through GWAS in Japanese Black cattle in previous studies (Uemoto et al. 2010; Ishii et al. 2013). However, this was the first report of QTL on BTA9 and BTA14, suggesting that novel responsible genes would be located on these regions.

We subsequently surveyed candidate genes in the regions 5 Mbp upstream and downstream of the most significant SNP on BTA9 and BTA14. The KEGG pathway annotation was confirmed using DAVID v6.8 (https://david.ncifcrf.gov/) to select putative candidate genes from a total of 114 and 104 genes located in the candidate region on BTA9 and BTA14, respectively. The DAVID search revealed that 37 genes on BTA9 and 15 genes on BTA14 are involved in a total of 36 and 34 pathways in KEGG pathway database, respectively. Among them, we focused on three pathways, pantothenate and CoA biosynthesis (vanin1, vanin2, ectonucleotide pyrophosphatase/phosphodiesterase 1, and ectonucleotide pyrophosphatase/phosphodiesterase 2 genes on BTA9), glycerophospholipid metabolism (lysophospholipase 1 gene on BTA14), and steroid biosynthesis (squalene epoxidase gene on BTA14).

We additionally researched the gene functions of these six genes through NCBI database and previous reports. According to the functional information, vanin1 (VNN1) gene required for fatty acid synthesis (Pitari et al. 2000) and lysophospholipase 1 (LYPLA1) gene involved in fat metabolism through hormonal regulation (Wren et al. 2001; De Vriese & Delporte 2008; Satou et al. 2010) were selected as the most probable candidate for each QTL on BTA9 and BTA14.

To identify the candidate polymorphisms within VNN1 and LYPLA1 genes, we sequenced the full-length CDS of these genes in eight animals in high and low groups used in the GWAS. As the results, we identified only one candidate polymorphism on exon 1 of VNN1 gene. The candidate polymorphism caused an amino acid substitution T66M, which showed significant differences in allele frequencies between the high and low groups. We genotyped the candidate SNP, VNN1 T66M, to investigate its effect on C18:1 in the Hyogo Prefecture population (n = 899) using ANOVA. The statistical analysis revealed that VNN1 T66M was significantly associated with C18:1 ($p = 0.0162$) in the population. However, its $p$-value was higher than that of the most significant SNP in GWAS (Hapmap43702-BTA-84086) ($p = 0.0080$), suggesting that this SNP might not be responsible for the QTL on BTA9. In this study, we selected candidate gene according to KEGG pathway annotation. Since the information shows biological process in which each gene is involved, it is useful to learn gene functions. In fact, function of 37 genes on BTA9 and 15 genes on BTA14 were revealed according to the KEGG pathway annotation. On the other hand, KEGG pathway was not annotated on 77 and 89 genes on BTA9 and BTA14, respectively. Therefore, we will need to research on these genes based on other information to identify more promising candidate polymorphisms.

3. Whole-genome resequencing to comprehensively identify candidate polymorphisms for the QTL on BTA9

| Polymorphism | Genotype frequency (n) | Allele frequency | e-values ± SE | ANOVA p-value |
|--------------|------------------------|------------------|--------------|---------------|
| Hapmap43702-BTA-84086 | TT | 0.528 | 0.528 | 0.032 $a$ | 0.0080 |
| | TC | 0.42 | 0.42 | -0.234 $ab$ | 0.739 |
| | CC | 0.051 | 0.051 | -0.736 $b$ | 0.261 |
| | T | 0.739 | 0.739 | ± 0.085 | 0.005 |
| | C | 0.261 | 0.261 | ± 0.273 | 0.032 |
| BTB-00554873 | AA | 0.177 | 0.177 | 0.339 $a$ | 0.0003 |
| | AC | 0.577 | 0.577 | -0.122 $b$ | 0.081 |
| | CC | 0.246 | 0.246 | -0.441 $b$ | 0.124 |
| | A | 0.466 | 0.466 | ± 0.146 | 0.339 |
| | C | 0.534 | 0.534 | ± 0.081 | 0.081 |

* e-values: the mean of e-values for C18:1 for each genotype
* a, b: means with different superscript are significantly different between genotypes

| Table 3 Genotype and allele frequencies of Hapmap43702-BTA-84086 and BTB-00554873 and their effects on C18:1 in the Japanese Black cattle population (n = 899) (Kawaguchi et al. 2018) |
Recently, whole-genome resequencing analysis has been used for detecting polymorphisms that might be responsible for some economic traits in livestock animals. For instance, Jiang et al. (2016) and Li et al. (2015) used the technology to identify possible candidate polymorphisms for milk composition traits in cow and pH value of chicken meat, respectively. Briefly, they performed whole-genome resequencing analysis using two groups composed of 4 or 5 animals, which showed favorable phenotype or unfavorable phenotype. Through comparison of genotypes between the groups, confirmation of polymorphisms’ location, and researching for gene function, they conclusively identified possible candidate polymorphisms. These studies suggested that whole-genome resequencing data would be informative to identify candidate polymorphisms. Thus, we tried to comprehensively detect candidate polymorphisms for the QTL on BTA9 and validate the effect on C18:1 in Japanese Black population of Hyogo Prefecture (Kawaguchi et al. 2019).

Eight animals were selected from a total of 200 animals used in the above GWAS based on sires and genotypes of the most significant SNP (Hapmap43702-BTA-84086) (Table 4). Four animals from high C18:1 group were TT homozygous and four animals from low C18:1 group were CC homozygous. They were the progenies of different sires among the four animals in each group. In these eight animals, the whole-genome resequencing analysis was conducted using a HiSeq X Five Sequencing System (Illumina Inc., San Diego, CA, USA). Reads were mapped to the cattle reference genome assembly (UCSC bosTau8). Polymorphisms were called by comparing nine genome sequences, including the reference sequence. The called polymorphisms were annotated to the gene reference (NCBI RefSeq).

We determined the region 5 Mbp upstream and downstream of Hapmap43702-BTA-84086 (about 64.9 – 74.9 Mbp) on BTA9 as a candidate region for C18:1. We focused on polymorphisms detected within the candidate region. To identify more possible candidate polymorphisms, intergenic variants were firstly excluded. We subsequently confirmed genotypes to expect degree of linkage disequilibrium with Hapmap43702-BTA-84086. Since the genotypes of Hapmap43702-BTA-84086 completely differed between the high and low groups, polymorphisms with a large number of allele differences between high and low groups were expected to be in LD with the SNP. As the results, we detected 1,993 candidate polymorphisms within a total of 23 genes.

Moreover, we researched the function of 23 genes using NCBI database and previous reports to determine candidate genes regarding fatty acid metabolism. As the results of the research, cytochrome b5 reductase 4 (CYB5R4), mediator complex subunit 23 (MED23), and VNN1 genes were determined as more possible candidate genes than the other genes. CYB5R4 is an electron donor for fatty acid desaturation by SCD (Zhu et al. 2004; Deng et al. 2010). In fact, C18 desaturation index (C18:1/C18:0) was markedly low in CYB5R4-knockout mice than in wild-type mice (Larade et al. 2008). MED23 is a subunit of Mediator complex, which would be one of the transcription factors related to fatty acid synthesis (Bastie et al. 2005; Knuesel & Taatjes 2011; Chu et al. 2014). VNN1 is an enzyme to synthesize pantethine (Pitari et al. 2000; Kavian et al. 2015), which is required to synthesize fatty acids. These genes would be essential for appropriate fatty acid metabolism. We finally selected three candidate polymorphisms, CYB5R4 c.*349G>T, MED23 c.3700G>A (V1234I), and VNN1 c.197C>T (T66M) in terms of their annotations (Table 5).

We genotyped the candidate polymorphisms and validated their effects on C18:1 in the Hyogo Prefecture

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Table 4 Japanese Black cattle used for whole-genome resequencing analysis (Kawaguchi et al. 2019)

| Group | Sample | Genotype | Sire | C18:1 | corrected C18:1 |
|-------|--------|----------|------|-------|----------------|
| high  | 1      | TT       | 1    | 63.05 | 58.44         |
|       | 2      | TT       | 2    | 59.81 | 56.57         |
|       | 3      | TT       | 3    | 59.25 | 56.13         |
|       | 4      | TT       | 4    | 57.89 | 56.37         |
| low   | 5      | CC       | 3    | 49.72 | 50.76         |
|       | 6      | CC       | 5    | 48.39 | 49.79         |
|       | 7      | CC       | 6    | 47.6  | 49.46         |
|       | 8      | CC       | 7    | 46.17 | 49.75         |

Genotype: the genotype of Hapmap43702-BTA-84086, which was the most significantly associated with C18:1 in the GWAS corrected C18:1: the sum of overall mean and e-value for each animal.
population (n = 899) by statistical analysis. All of them were in LD with Hapmap43702-BTA-84086 and significantly associated with C18:1, suggesting that they might be responsible for the QTL (Table 6). In particular, \textit{CYB5R4} c.*349G>T showed the lowest \( p \)-value and highest proportion of additive genetic variance (\%\( \text{VA} \)) of the four polymorphisms including Hapmap43702-BTA-84086, suggesting that the SNP would be the most likely candidate polymorphism. \textit{CYB5R4} c.*349G>T was located on 3\(^{'}\) UTR of \textit{CYB5R4} gene. SNPs within the region might affect the gene expression through the regulation of affinity of miRNA for the region. Therefore, we hypothesized that the SNP might be responsible for C18:1 through the alteration of the expression level of CYB5R4 protein, which is essential for fatty acid desaturation by SCD. We need further analysis to prove the hypothesis.

Meanwhile, the other two candidate polymorphisms, \textit{MED23} c.3700G>A and \textit{VNN1} c.197C>T, also had similar \( p \)-values to the most significant SNP in the GWAS (Hapmap43702-BTA-84086). In addition, whole-genome resequencing data included some other candidate polymorphisms although they were not as plausible as the three candidates in terms of the gene function. Since the results of our study cannot rule out the possibility that these candidates including \textit{MED23} c.3700G>A and \textit{VNN1} c.197C>T are responsible for the QTL, we need further verification to identify the responsible gene and polymorphism.

**CONCLUSION**

We have aimed to reveal the candidate polymorphisms for fatty acid composition in Japanese Black cattle in the past few years. As the major achievements, we succeeded to identify a novel candidate polymorphism \textit{CYB5R4} c.*349G>T, which was the most plausible according to the results of verification of its effect on C18:1 in Japanese Black population. Moreover, we demonstrated the availability of the methodology using whole-genome resequencing data to detect plausible candidate polymorphisms for fatty acid composition in cattle. We expect that our report would encourage researchers to use the methodology for various economic traits in livestock animals to efficiently identify candidate polymorphisms.

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### Table 5 Summary of candidate polymorphisms selected using whole-genome resequencing data (Kawaguchi et al. 2019)

| Polymorphism | Position (bosTau8) | Annotation | Amino acid substitution | Genotype | ANOVA p-value | %VA | LD |
|--------------|--------------------|------------|-------------------------|----------|---------------|-----|----|
| \textit{CYB5R4} c.*349G>T | 66,377,383 | 3\(^{'}\) UTR | - | GG, GG, GG, GT, TT, TT | 0.008 | 4.2 | 0.77 |
| \textit{MED23} c.3700G>A | 70,521,413 | missense | V1234I | GG, GG, GG, GG, AA, AA | 0.030 | 3.3 | 0.83 |
| \textit{VNN1} c.197C>T | 71,851,690 | missense | T66M | CT, CC, CC, TT, TT | 0.016 | 2.24 | 0.53 |

The position is based on the genome assembly, bosTau8 (UMD3.1.1). They are actually located on the same position in the previous assembly, UMD3.1.

### Table 6 Effect of the three candidate polymorphisms on C18:1 in the Japanese Black population (n = 899) (Kawaguchi et al. 2019)

| Polymorphism | e-values ± SE | ANOVA p-value | %VA | LD |
|--------------|---------------|---------------|-----|----|
| \textit{CYB5R4} c.*349G>T | GG (n = 454) | GT (n = 401) | TT (n = 44) | 0.008 | 4.2 | 0.77 |
| MED23 c.3700G>A | AA (n = 441) | AG (n = 412) | GG (n = 46) | 0.018 | 0.08 | -0.652 ± 0.274 |
| VNN1 c.197C>T | CC (n = 566) | CT (n = 304) | TT (n = 29) | 0.016 | 2.24 | 0.53 |

e-values: the mean of e-values for C18:1 for each genotype
%VA: the proportion of additive genetic variance
LD: the coefficient of linkage disequilibrium with Hapmap43702-BTA-84086

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

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