経済形質関連マーカーを用いた熊本系褐毛和種の遺伝的特性の解明

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Elucidating genetic characteristics of the Kumamoto sub-breed of Japanese Brown cattle with DNA markers for economically important traits

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ABSTRACT

The Kumamoto sub-breed of Japanese Brown cattle is famous for its heavy carcass weight, and increasing carcass weight is one of the most important breeding goals for this sub-breed. Genetic approaches may be especially useful for breeding this cattle and improving its traits. In this study, we elucidated the genetic characteristics of the Kumamoto sub-breed by analyzing the genotype and allele frequencies of DNA polymorphisms. Seven DNA polymorphisms were selected from the *SREBP1, SCD, FASN, EDG1, NCAPG, LYST,* and *F11* genes, which affect economically important traits. For genotyping, 112 offspring of 19 sires were collected. Our genotyping results show an extremely low allele frequency of the S allele of *SREBP1*, which affects fatty acid composition in intramuscular fat, in the Kumamoto sub-breed. Therefore, this polymorphism might not be useful for enhancing the breeding of this sub-breed. The causative mutation of *LYST* was not detected in our sampling population, suggesting a lower risk of Chediak-Higashi syndrome in the Kumamoto sub-breed. On the other hand, the Kumamoto sub-breed possessed high frequencies of desirable alleles of the *SCD* and *NCAPG* genes, which affect fatty acid composition and carcass weight, respectively, possibly because of artificial selection. Hence, these DNA markers could be used to improve economically important traits of the Kumamoto sub-breed. Our results also indicate that the causative mutation of factor XI deficiency might be common in the population because multiple sires were carriers of this disease. Since this disease may cause fetal losses, elimination of this disease from the Kumamoto sub-breed would be desirable.

Key words: economically important traits, genetic disease, Japanese Brown cattle, Kumamoto prefecture

Introduction

Wagyu, a variety of beef cattle of Japanese origin, includes four genetically distinct breeds: Japanese Black, Japanese Brown, Japanese Shorthorn, and Japanese Polled (Gotoh *et al.* 2018). Japanese Brown cattle, raised mainly in the Kumamoto and Kochi prefectures, are the second most popular breed among the Wagyu breeds. The coat color patterns of the sub-breeds found in these two prefectures are apparently different, suggesting genetic isolation between

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these two sub-breeds (Sasazaki *et al.* 2005; Matsumoto *et al.* 2020). The Kumamoto sub-breed is known to be easy to rear because of its calmer temperament and higher heat resistance (Ito *et al.* 2014; Imai *et al.* 2020). Moreover, weight gain is greater in this sub-breed, such that the average carcass weight of the Kumamoto sub-breed is heavier than that of Japanese Black cattle, which accounts for the majority of Wagyu breed cattle (Sasaki *et al.* 2006a). Genetic approaches may be useful for increasing carcass weight, which is an important breeding goal for the Kumamoto sub-breed, because economically important traits, including carcass weight, are highly heritable in this sub-breed (Sasaki *et al.* 2006a; Sumio 2007). However, information on their genetic characteristics is limited.

Various DNA polymorphisms that influence economically important traits in cattle have been identified, and some of them are utilized as DNA markers for marker-assisted selection. Taniguchi *et al.* (2004) identified a missense variant in the *SCD* (*stearoyl-CoA desaturase*) gene, which affects fatty acid composition of intramuscular fat of Japanese Black cattle. In this variant, the 293rd amino acid changes from Val to Ala. This gene influences the mono-unsaturated fatty acid (MUFA) content that contributes to the tenderness and flavor of the meat, which is regarded as one of the most economically important traits (Melton *et al.* 1982). The Ala allele of the *SCD* gene increases MUFA percentage in a dose-dependent manner. Furthermore, this single nucleotide polymorphism (SNP) does not negatively affect other economically important traits (Ohsaki *et al.* 2009). Subsequent studies using various beef cattle breeds have confirmed the effects of this SNP on fatty acid composition (Orrù *et al.* 2011; Li *et al.* 2012).

Because most economically important traits are quantitative traits controlled by various genes, development of additional DNA markers could help beef cattle breeding. Hence, many genes affecting economically important traits have been identified. For example, *fatty acid synthase* (*FASN*) and *sterol regulatory element binding protein 1* (*SREBP1*) are other genes associated with fatty acid composition (Hoashi *et al.* 2007; Abe *et al.* 2009). Similarly, the beef marbling standard (BMS), one of the most economically important traits, is influenced by the *sphingosine-1-phosphate receptor 1* (*EDG1*) gene (Yamada *et al.* 2009). The *non-SMC condensin-I complex, subunit G* (*NCAPG)* gene influences carcass weight (Setoguchi *et al.*

2009). In Wagyu populations, several genetic diseases, which may have negative impacts on beef cattle production, and their causative genes have been reported. These include *lysosomal trafficking regulator (LYST)* gene, which is the causative gene for Chediak-Higashi syndrome and *coagulation factor XI (F11)* gene, which is the causative gene for factor XI deficiency (Kunieda *et al.* 1999, Kunieda *et al.* 2005). The clinical symptoms of these diseases are relatively mild, suggesting that carriers of the causative mutations might be found in the population of the Kumamoto sub-breed of Japanese Brown cattle. Investigation of mutations in these genes will be useful for breeding the Kumamoto sub-breed.

In this study, to elucidate the genetic characteristics of the Kumamoto sub-breed of Japanese Brown cattle, we analyzed the genotype and allele frequencies of variants in seven genes: *SREBP1* g.101_185ins (rs133958066), *SCD* c.878T>C: p.Val293Ala (rs41255693), *FASN* c.5854A>G: p.Thr1952Ala (rs208645216), *EDG1* c.-312A>G (rs211101524), *NCAPG* c.1326T>G: p.Ile442Met (rs109570900), *LYST* c.6044A>G: p.His2015Arg (AB026620), and *F11* c.870_884ins (AB196307). Subsequently, we performed a chi-square test for deviation from Hardy-Weinberg equilibrium (HWE) to reveal the effects of each variant in this sub-breed. Our results might help determine an effective breeding strategy for this sub-breed.

Materials and Methods
Animals

In this study, 112 cattle (84 steers and 28 heifers), all of which belong to the Kumamoto sub-breeds of Japanese Brown cattle, were analyzed. Their meat was commercially purchased from Toyozumi shokuniku, a meat store in Kumamoto Prefecture, in 2018 and 2019. Genomic DNA samples were extracted using the standard phenol-chloroform method for genotyping. These cattle were derived from 19 sires, and 56 of them were offspring of a single sire (sire A). Hence, the results of genotyping were shown as 112 cattle (sampling population), offspring of 18 sires (Group I) and offspring of sire A (Group II). The genotyping results of Group I were compared with other breeds because these cattle were assumed to reflect the characteristics of Kumamoto sub-breed. The genotyping results of Group II were also shown because the genetic

characteristics of sire A may provide important information for breeding improvement of Kumamoto sub-breed.

Genotyping

Genotyping for the nucleotide substitutions in *SCD*, *FASN*, *EDG1*, *NCAPG,* and *LYST* was performed by the restriction fragment length polymorphism method. To detect the *SREBP1* and *F11* polymorphisms, PCR was carried out. We genotyped *SREBP1, SCD, FASN, EDG1, LYST* and *F11* genes according to Matsuhashi *et al*. (2011), Ookura *et al*. (2013), Siqintuya *et al*. (2014), Kaneda *et al*. (2011), Kunieda *et al*. (1999), and Kunieda *et al*. (2005), respectively. We designed the primer set to amplify the region including the *NCAPG* SNP based on the reference sequence (GenBank NC_037333.1) in Oligo7 (Molecular Biology Insights, Vondelpark, CO). Go-Taq® (Promega Corporation, Madison, WI) was used as the PCR enzyme and PCR was performed with the following conditions: 35 cycles at 95 °C for 30 sec, annealing temperatures given in Table 1 for 30 sec, and 72 °C for 30 sec. To amplify the region including *EDG1* c.-312A>G, an extension procedure was performed for 60 sec. For *F11* genotyping, the extension procedure was prolonged to 70 sec. Subsequent restriction enzyme reaction was performed at 37 °C for 15 min. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Primer sequences and restriction enzymes are listed in Table 1. After genotyping, the chi-square test (χ^2) was used to test deviation from HWE.

Results and Discussion

We carried out genotyping for seven genes, but two of them were not polymorphic in the sampling population (Table 2). One of them was *SREBP1*, g.101_185ins, which has an 84-bp in/del polymorphism in intron 5 (Hoashi *et al*. 2007). *SREBP1* is a transcription factor involved in fatty acid synthesis (Shimano 2001), and this polymorphism is thought to affect fatty acid composition. Hoashi *et al*. (2007) reported that the S allele (Short type) positively affects fatty acid composition. However, Ookura *et al*. (2013) reported that the L allele (long type) has a positive effect. The effect of *SREBP1* g.101_185ins on the Kumamoto sub-breed is unknown, but this in/del cannot be used for breeding, because most individuals in the Kumamoto sub-breed were assumed to possess the L allele. On the other hand, the S allele frequency was 0.42 in the Kochi sub-breed (Kaneda *et al*. 2011), suggesting that these sub-breeds have different breeding histories. The other SNP that we detected was *LYST* c.6044A>G. This SNP is a missense mutation (p. His2015Arg) in exon 18, and the Arg allele is the causative allele of Chediak-Higashi syndrome, an autosomal recessive bleeding disorder (Kunieda *et al*. 1999). This genetic disease was found in several breeds, including Japanese Black cattle. However, we did not detect any individuals with an Arg allele from this population, suggesting that the risk of this genetic disease might be low in the Kumamoto sub-breed.

The SCD enzyme converts saturated fatty acids into MUFAs in adipocytes (Kim and Ntambi 1999), and the Ala allele of p.Val293Ala has a positive effect on MUFA content (Taniguchi *et al*. 2004). Allele frequency of the Ala allele

Table 1. Primer sequences and restriction enzymes for genotyping

SNP	Sequence (5' → 3')	Tm (°C)*	Enzyme	Size (bp)
SREBP1	F: CCACAACGCCATCGAGAAACGCTAC	65	-	L: 432
g.101_185ins	R: GGCCTTCCCTGACCACCCAACTTAG			S: 348
SCD	F: GTGGAAAATCAGGTAGGTCTCAGCG	60	*Aci*I	T: 237, 370
c.878T>C	R: CAAGCAAGACTACCACCCAGATCAG			C: 237, 58, 312
FASN	F: CTCACTGTCTGTCCCACAGG	60	*Hha*I	A: 243
c.5854A>G	R: GCCTTTGGAGGGCTTCTTAG			G: 94, 149
EDG1	F: AAAGAAGCCACTCAGCCTCAGCG	62	*Msc*I	A: 77, 348
c.-312A>G	R: GTGAGCCCAGAACAATCCGATCC			G: 425
NCAPG	F: ACTCTACCTACCACACCAATATCCCT<u>C</u>AT[†]	60	*Nla*III	T: 177
c.1326T>G	R: AAGCCAAAAGCACTGAAAACA			G: 30, 147
LYST	F: GAAAATTACAGCGAGAAGTCCTTGG	60	*Fok*I	A: 66, 42
c.6044A>G	R: TGACAAACATAAGTATTAGTAGGAGG			G: 108
F11	F: TCACATCTCAATATGTGCTTCTGC	55	-	L: 110
c.870_884ins	R: TCTACGATGTCCAGTTCTTCTCC			S: 95

[†]Underline indicates a mismatch nucleotide to introduce the *Nla*III recognition site into the PCR product.
* Annealing temperature for the PCR reaction.

has been previously estimated in various cattle breeds: 0.76 in Japanese Black cattle, 0.83 in Angus cattle, and 0.49 in Simmental cattle (Kaneda *et al.* 2011; Orrù *et al.* 2011; Nishimaki *et al.* 2016). The frequencies of this allele in the Kumamoto sub-breed were 0.81 in the sampling population, 0.74 in Group I, and 0.89 in Group II (Table 2, 3). The high frequency of the Ala allele of 0.81 in the sampling population was contributed by Group II: offspring of sire A constituted half of our sample population, and 77% of them were homozygous for the Ala allele. Moreover, Group I showed deviation from the HWE (Table 3). Our data indicated that the genotype of sire A might be homozygous for the Ala allele and other sires might have more than one Ala allele. *SCD* c.878T>C (p.Val293Ala) has been reported to improve MUFA content in Japanese Black, Fleckvieh, Hanwoo, and Holstein cattle (Barton *et al.* 2010; Oh *et al.* 2011; Mannen 2012). Therefore, this SNP may also have a desirable effect on MUFA content of the Kumamoto sub-breed, and this effect may be the cause of the deviation from the HWE. However, the correlation between the polymorphism and fatty acid composition in the Kumamoto sub-breed remains to be elucidated.

The Ala allele of *FASN* p.Thr1952Ala, a missense mutation in exon 34, has been reported to have a positive effect on fatty acid composition (Abe *et al.* 2009). Frequency of this allele was 0.47 in Japanese Black cattle, 0.98 in Angus cattle, and 0.92 in Hereford cattle (Kaneda *et al.* 2011). The frequencies of this allele in the Kumamoto sub-breed were 0.43 in sampling population, 0.41 in Group I, and 0.46 in Group II (Table 2, 3), suggesting that this SNP

might improve the fatty acid composition of this sub-breed. However, further studies are required to fully understand the effect of this SNP in the Kumamoto sub-breed.

Expression of the *EDG1* gene, which is involved in blood vessel formation (Liu *et al.* 2000), is higher in high-marbled cattle than in low-marbled cattle (Sasaki *et al.* 2006b). An SNP in the untranslated region, c.-312A>G, might control *EDG1* expression, and cattle with the G allele show higher BMS (Yamada *et al.* 2009). The frequencies of this allele in the Kumamoto sub-breed were 0.05 in sampling population, 0.06 in Group I, and 0.05 in Group II (Table 2, 3). Our genotyping revealed that the G allele was quite rare in the Kumamoto sub-breed of Japanese Brown cattle. The frequency of this allele in other breeds, such as Angus and Hereford cattle, was also low (Kaneda *et al.* 2011). In contrast, Japanese Black cattle, known as high-marbled cattle, showed a higher G allele frequency of 0.41 (Watanabe *et al.* 2009).

The *NCAPG* gene encodes a condensin subunit that plays an important role in cell growth (Dej *et al.* 2004). Cattle with the Met allele of p.Ile442Met, a missense mutation in exon 9, show heavier carcass weight (Setoguchi *et al.* 2009). The frequencies of the Met allele in the Kumamoto sub-breed were 0.60 in sampling population, 0.43 in Group I, and 0.76 in Group II (Table 2, 3). The frequencies of this allele in the other breeds were as follows: 0.25 in Japanese Black cattle, 0.40 in Angus cattle, and 0.67 in Simmental cattle (Glenske *et al.* 2011; Nishimaki *et al.* 2016). This difference in the allele frequency might explain why the carcass weight of Japanese Brown and Simmental

Table 2. Genotype and allele frequency in Kumamoto sub‐breed of Japanese Brown cattle

SNP	Sampling population (n = 112)				
	Genotype frequency			Allele frequency	
SREBP1	S/S (n = 0)	S/L (n = 0)	L/L (n = 112)	S	L
	0.00	0.00	1.00	0.00	1.00
SCD	Ala/Ala (n = 70)	Ala/Val (n = 42)	Val/Val (n = 0)	Ala	Val
	0.63	0.37	0.00	0.81	0.19
FASN	Ala/Ala (n = 23)	Ala/Thr (n = 50)	Thr/Thr (n = 39)	Ala	Thr
	0.21	0.45	0.34	0.43	0.57
EDG1	G/G (n = 0)	G/A (n = 11)	A/A (n = 101)	G	A
	0.00	0.1	0.9	0.05	0.95
NCAPG	Met/Met (n = 42)	Met/Ile (n = 49)	Ile/Ile (n = 21)	Met	Ile
	0.38	0.44	0.18	0.6	0.4
LYST	His/His (n = 112)	His/Arg (n = 0)	Arg/Arg (n = 0)	His	Arg
	1.00	0.00	0.00	1.00	0.00
F11	S/S (n = 55)	S/L (n = 50)	L/L (n = 7)	S	L
	0.49	0.45	0.06	0.71	0.29

Table 3. Genetic characteristics of offspring of 18 sires (Group I) and offspring of sire A (Group II)

SNP	Group I (n = 56)					χ^{2*}	Group II (n = 56)				
	Genotype frequency			Allele frequency			Genotype frequency			Allele frequency	
SCD	Ala/Ala	Ala/Val	Val/Val	Ala	Val	6.84**	Ala/Ala	Ala/Val	Val/Val	Ala	Val
	(n = 27)	(n = 29)	(n = 0)				(n = 43)	(n = 13)	(n = 0)		
	0.48	0.52	0.00	0.74	0.26		0.77	0.23	0.00	0.89	0.11
FASN	Ala/Ala	Ala/Thr	Thr/Thr	Ala	Thr	0.28	Ala/Ala	Ala/Thr	Thr/Thr	Ala	Thr
	(n = 10)	(n = 25)	(n = 21)				(n = 13)	(n = 25)	(n = 18)		
	0.18	0.45	0.37	0.41	0.59		0.23	0.45	0.32	0.46	0.54
EDG1	G/G	G/A	A/A	G	A	0.18	G/G	G/A	A/A	G	A
	(n = 0)	(n = 6)	(n = 50)				(n = 0)	(n = 5)	(n = 51)		
	0.00	0.11	0.89	0.06	0.94		0	0.09	0.91	0.05	0.95
NCAPG	Met/Met	Met/Ile	Ile/Ile	Met	Ile	0.02	Met/Met	Met/Ile	Ile/Ile	Met	Ile
	(n = 10)	(n = 28)	(n = 18)				(n = 32)	(n = 21)	(n = 3)		
	0.18	0.5	0.32	0.43	0.57		0.57	0.38	0.05	0.76	0.24
F11	S/S	S/L	L/L	S	L	0.03	S/S	S/L	L/L	S	L
	(n = 31)	(n = 21)	(n = 4)				(n = 24)	(n = 29)	(n = 3)		
	0.55	0.38	0.07	0.74	0.26		0.43	0.52	0.05	0.69	0.31

* Chi-square test ($\chi 2$) with one degree of freedom (df = 1) was used to test deviation from HWE.
** significant at p < 0.05 ($\chi 2 > 3.84$, df = 1)

cattle is heavier than that of Japanese Black and Angus cattle (Sasaki *et al*. 2006a; Kause *et al*. 2015).

Factor XI deficiency is an autosomal recessive bleeding disorder. The causative mutation of this genetic disease is *F11* c.870_884ins, a 15-bp insertion in exon 9 (Kunieda *et al*. 2005). The phenotype of this disease is mild, and individuals with the causative L allele (insertion type) grow normally in most cases; hence, this mutation tends to escape artificial selection (Ohba *et al*. 2008). While the L allele frequency was 0.38 in Japanese Black cattle (Ookura *et al*. 2013), the frequencies of 0.29 in sampling population, 0.26 in Group I, and 0.31 in Group II were estimated in the Kumamoto sub-breed in the current study. This suggests that multiple sires, including sire A, were carriers of factor XI deficiency (Table 3). Although this disease does not influence carcass traits (Ookura *et al*. 2013), factor XI deficiency could have negative economic effects because of the association between fetal losses and the L allele (Ogata *et al*. 2014). Therefore, elimination of this allele from Japanese Brown cattle is desirable.

In conclusion, we analyzed the genotype and allele frequencies of seven polymorphisms in the Kumamoto sub-breed of Japanese Brown cattle in the present study. Our sample population showed polymorphisms in *SCD*, *FASN*, *EDG1*, *NCAPG*, and *F11*. The polymorphisms of *SCD*, *FASN*, and *NCAPG* might be useful for breeding the

Kumamoto sub-breed. The deviation from HWE in *SCD* suggests the necessity to elucidate the effect of this polymorphism on fatty acid composition of the Kumamoto sub-breed. The heavy carcass weight of the Kumamoto sub-breed may be due to the high allele frequency of the desirable *NCAPG* allele. In addition, the mutant allele causing factor XI deficiency was found to be widespread in the Kumamoto sub-breed.

Acknowledgement

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