doi: 10.17221/8459-CJAS

Allele frequency for *c.335 A>C* polymorphisms in porcine ghrelin/obestatin prepropeptide gene and association analysis with performance traits in various pig breeds

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ABSTRACT: The allelic frequency of *c.335A>C* polymorphisms in the porcine ghrelin/obestatin prepropeptide (*GHRL*) gene was surveyed among six pig breeds and two subspecies of wild boars. The *c.335C* was the most frequent allele in Berkshire, Landrace, Large White, Yorkshire, and Clawn miniature pigs and Ryukyu wild boars (*Sus scrofa riukiuanus*), whereas *c.335A* was the most frequent allele in Duroc and Meishan pigs and Japanese wild boars (*S. s. leucomystax*). The association of *c.335A>C* with performance traits was analyzed in Duroc, Landrace, Large White, and (Landrace × Large White) × Duroc (LWD) cross-bred pigs. No associations between *c.335A>C* genotype and average daily weight gain, backfat thickness, or intramuscular fat were detected. However, an association was observed between loin eye muscle area (EMA) and *c.335A>C* genotype in Duroc gilts. The *AA* genotype group had larger EMA than the *AC* genotype group in Duroc gilts; however, this association was not significant in Duroc boars and barrows or the other pig populations investigated. These results demonstrate that *GHRL c.335A>C* is not a major quantitative trait loci candidate on pig chromosome 13 affecting fat deposition.

Keywords: quantitative trait locus; *GHRL*; pigs; polymorphism; genetic marker

INTRODUCTION

The ghrelin/obestatin prepropeptide gene (*GHRL*), also known as the preproghrelin gene, encodes a protein precursor which generates ghrelin and obestatin. Ghrelin is a 28-amino acid peptide that results from the post-translational processing of the N-terminal domain of the prepropeptide (Kojima et al. 1999). It is an endogenous ligand for the growth hormone secretagogue receptor and is involved in regulating growth hormone release (Kojima et al. 1999; Peino et al. 2000, Matsumoto et al. 2001). Although the biologic role remains controversial, a 23-amino acid peptide derived from the C-terminal domain of preproghrelin, which is known as obestatin, may exhibit physiological activity (Zhang et al. 2005; Chartrel et al. 2007; Gourcerol et al. 2007; Zhang et al. 2007; Tang et al. 2008).

Partially supported by the Japan Society for the Promotion of Sciences, Grants-in-Aid for Scientific Research (Grant No. 23580393).

doi: 10.17221/8459-CJAS

In humans, dbSNP:rs696217 in *GHRL* gene that causes a Leu72Met variation in preproghrelin protein is associated with body mass index and insulin secretion in children, and the *Met72* allele was found to contribute to the early onset of obesity (Korbonits et al. 2002; Miraglia del Giudice et al. 2004).

The porcine *GHRL* gene is located on chromosome 13 (SSC13) between 73475202 and 73480181 nucleotide positions based on the genomic assembly Sscrofa10.2 (GenBank Accession No. NC_010455.4). In earlier studies, QTLs affecting the intramuscular fat (IMF) content were mapped on SSC13 in the F_2 generation of Meishan × Duroc cross (Sato et al. 2003) and in the F_2 generation of Duroc × Large White cross (Sanchez et al. 2007). In addition, a QTL affecting backfat thickness (BFT) was also mapped to a similar region in Iberian × Meishan F_2 sows (Tomas et al. 2011).

Kim et al. (2004) determined partial protein coding sequences of the porcine *GHRL* gene and found a missense mutation (*c.335 A>C*) in exon 3 (it is exon 4 in the gene ID 396728). This polymorphism leads the Asn112Thr amino acid substation in the preproghrelin molecule. Kim et al. (2004) also reported that *c.335 A>C* was associated with IMF marbling, BFT, and bone and muscle composition in Berkshire pigs. Thus, the *GHRL c.335 A>C* polymorphism has been thought to be a potential candidate gene for the QTLs affecting IMF, BFT, or both on SSC13. Recently, the effect of this polymorphism was investigated in Polis Landrace pigs (Wojtysiak and Kaczor 2011). However, the detected effects of this *GHRL* polymorphism on porcine economic traits greatly varied between the two reports. Therefore, the precise effects of the *GHRL* polymorphism must be determined before this gene can be utilized as a genetic marker for pig selection.

In this study, we sought to contribute to information related to the association between the nucleotide polymorphisms of *GHRL* and meat production traits in pigs. We investigated the relationship between the porcine *GHRL c.335 A>C* polymorphism and economic traits in Duroc, Landrace, Large White, and commercial (Landrace × Large White) × Duroc (LWD) cross-bred pigs.

MATERIAL AND METHODS

Pig DNA samples. We collected DNA samples from a total of 1228 domestic pigs consisting of the following seven breeds and a commercial three-way cross-breed: Berkshire (50), Duroc (471), Landrace (250), Large White (292), Yorkshire (26), Meishan (6), Clawn miniature (52), and LWD (81). We also evaluated DNA samples from 64 Japanese wild boars (*Sus scrofa leucomystax*) and 12 Ryukyu wild boars (*S. s. riukiuanus*).

Total DNA was extracted from blood or tissue by a conventional phenol/chloroform method after treatment with proteinase K (Sambrook et al. 1989) or by the use of commercially available DNA isolation kits (mainly by using DNA Extractor WB Kit (Nippon Gene, Tokyo, Japan) and QuickGene DNA tissue Kit (FUJIFILM, Tokyo, Japan)).

Genotyping of the GHRL c.335 A>C missense substitution. To determine genotypes and examine the distribution of the *GHRL c.335 A>C* missense substitution, the PCR-RFLP method was used. DNA fragments (217 bp), including exon 4 of porcine *GHRL*, were amplified by PCR using primer sets of *Sus-GHRL*-typingF1 (TGTGATGTTGGGATCAAGTTGTCA) and *Sus-GHRL*-typingR1 (CACAGAAGCTCTTTAGAGAGGCAG). The PCR was performed according to the instructions of manufacturers of KAPA Taq DNA Polymerase (Kapa Biosystems, Woburn, USA) with the following program: the first denaturation at 64°C for 2 min, 33 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 15 s, extension at 72°C for 40 s, and final extension at 72°C for 5 min. The PCR products were incubated with *Tsp*45I (NEB, Ipswich, USA) under optimum conditions and then analyzed by 2% agarose gel electrophoresis.

Associations between GHRL c.335 A>C polymorphisms and meat production traits. Associations between *c.335 A>C GHRL* polymorphisms and meat production traits were tested in Duroc, Landrace, and Large White pigs kept at ZEN-NOH's pig-breeding farm in Kamishihoro laboratory (Hokkaido, Japan) and LWD hybrid pigs kept at ZEN-NOH Central Research Institute for Feed and Livestock in Tsukuba (Ibaraki, Japan). For the Duroc, Landrace, and Large White pigs, the average daily gain (ADG) was calculated as weight gained divided by the days elapsed during the test period, from approximately 30 to 90 kg live weight. At approximately 90 kg live weight, BFT and loin eye muscle area (EMA) were measured halfway along the body using a real-time B-mode ultrasound scanner SSD-500 (Aloka, Tokyo, Japan). SigmaScan Pro 5.0 software (2007) was used for EMA determination. IMF was measured in Duroc pigs (128 gilts and 88 boars). Biopsy samples (about

doi: 10.17221/8459-CJAS

1.0 g) were taken from loin muscle at a position halfway along the body and approximately 6.5 cm from the vertebral centerline. The percentage of ether extractable fat (i.e. crude fat) content in biopsy samples was used as IMF. For the LWD pigs, body composition traits were evaluated after slaughter at approximately 115 kg body weight. ADG was calculated as the weight gained divided by days elapsed during the test period from approximately 30 to 115 kg live weight. Carcass BFT was measured at three different sites: shoulder, mid-back, and lumbar. EMA was measured at the cutting plane between the fifth and sixth ribs. IMF in loin muscle was measured using the ether extract method. All animals were provided unlimited access to feed and water during the experimental period, and all experiments were performed in accordance with ZEN-NOH institutional guidelines for animal management.

The GLM procedure of MINITAB software (Version 14, 2004) was used to obtain the Least Squares Means of ADG, BF, EMA, and IMF to account for the fixed effects of the *GHRL* genotype on each sex and to test the significance of the results. The linear model used to analyze the data was as follows:

$$Y_{ijk} = \mu + G_j + CW_{ijk} + e_{ijk}$$

where:

Y_{ijk} = phenotypic value of each trait
μ = overall mean for each trait
G_j = effect of *GHRL* genotype
W_{ijk} = weight measurement
C = covariate of the weight measurement
e_{ijk} = random residual effect

Phenotypic differences between genotypic groups were tested by the Tukey's method.

RESULTS AND DISCUSSION

Distribution of GHRL c.335 A>C variation among domestic pig breeds. Digestion of the 217-bp PCR product with *Tsp*45I produced fragments of the following sizes: only 217 bp in the *c.335A* homozygote; 89, 128, and 217 bps in the *c.335A/C* heterozygote; and 89 and 128 bps in the *c.335C* homozygote.

A total of 1228 domestic pigs and 76 wild boars were genotyped (Table 1). The allelic frequency of *c.335C* was higher than that of *c.335A* in the Berkshire, Landrace, Large White, Yorkshire, Clawn miniature pigs, and Ryukyu wild boars. In contrast, the frequency of the *c.335A* allele was very

Table 1. Genotypic and allelic frequencies of *GHRL c.335 A>C* variants among pig populations

Breeds and populations	*n*	Genotypic frequency			Allelic frequency	
		c.335 A>C			*c.335 A>C*	
		AA	*AC*	*CC*	*A*	*C*
Berkshire	50	4	17	29	0.250	0.750
Duroc (commercial)	10	4	5	1	0.650	0.350
Duroc[b]	461	333	122	6	0.855	0.153
Landrace (commercial)	78	12	35	31	0.378	0.622
Landrace[b]	172	35	84	53	0.448	0.552
Large White (commercial)	10	0	4	6	0.200	0.800
Large White[b]	282	16	132	134	0.291	0.709
Yorkshire (Middle White)	26	2	17	7	0.404	0.596
Meishan	6	6	0	0	1.000	0.000
LWD[ab]	81	17	48	16	0.506	0.494
Clawn miniature	52	0	0	52	0.000	1.000
Japanese wild boar	64	64	0	0	1.000	0.000
Ryukyu wild boar	12	3	3	6	0.375	0.625

[a](Landrace × Large White) × Duroc three-way cross-bred
[b]populations used for association analysis

Czech J. Anim. Sci., 60, 2015 (9): 411–416

doi: 10.17221/8459-CJAS

Table 2. Association results between the *GHRL c.335A>C* genotype and Least Squares Means ± standard errors of average daily gain (ADG), eye muscle area (EMA), backfat thickness (BFT), and intramuscular fat (IMF) in pigs

Genotype *AA*		Gilt			P^a	Boar			P^a	Barrow			P
		AA	*AC*	*CC*		*AA*	*AC*	*CC*		*AA*	*AC*	*CC*	
Duroc	*n*	154	71	3		142	40	3		37	11	0	
	ADG (g/day)	944 ± 7	932 ± 7	930 ± 47	0.271	1012 ± 7	1015 ± 13	1093 ± 49	0.887	1027 ± 17	1027 ± 31		0.276
	BFT (cm)	1.64 ± 0.03	1.74 ± 0.04	1.74 ± 0.19	0.126	1.45 ± 0.03	1.54 ± 0.07	1.68 ± 0.19	0.574	1.78 ± 0.07	1.76 ± 0.12		0.866
	EMA (cm^2)	39.3 ± 0.3	37.7 ± 0.5	36.1 ± 2.5	0.014	37.9 ± 0.3	38.1 ± 0.6	30.9 ± 2.3	0.850	38.6 ± 0.9	37.8 ± 1.6		0.652
	IMF (%) (*n*)	4.28 ± 0.14 (88)	4.75 ± 0.21 (38)	3.34 ± 0.91 (2)	0.064	4.07 ± 0.19 (67)	4.81 ± 0.36 (19)	4.13 ± 1.11 (2)	0.072				
Landrace	*n*	19	54	36	*P*	16	30	17	*P*				
	ADG (g/day)	857 ± 23	917 ± 15	912 ± 19	0.078	964 ± 28	944 ± 24	922 ± 30	0.558				
	BFT (cm)	1.39 ± 0.05	1.44 ± 0.03	1.52 ± 0.04	0.070	1.21 ± 0.04	1.16 ± 0.03	1.22 ± 0.04	0.376				
	EMA (cm^2)	30.64 ± 0.87	31.65 ± 0.57	32.65 ± 0.69	0.162	32.36 ± 0.96	33.70 ± 0.82	34.43 ± 1.02	0.285				
Large White	*n*	9	52	51	*P*	3	25	33	P^b				
	ADG (g/day)	889 ± 32	869 ± 13	889 ± 14	0.558	855 ± 55	902 ± 19	918 ± 18	0.588				
	BFT (cm)	1.35 ± 0.09	1.42 ± 0.04	1.47 ± 0.04	0.459	0.93 ± 0.12	1.25 ± 0.04	1.26 ± 0.04	0.876				
	EMA (cm^2)	31.80 ± 1.21	34.18 ± 0.50	33.96 ± 0.52	0.193	29.96 ± 2.59	32.57 ± 0.89	33.16 ± 0.82	0.691				
(Landrace × Large White) × Duroc	*n*	6	29	7	*P*					11	19	9	*P*
	ADG (g/day)	637 ± 20	642 ± 8	667 ± 20	0.390					691 ± 14	680 ± 12	684 ± 19	0.836
	BFT[c] (cm)	1.70 ± 0.13	1.57 ± 0.06	1.79 ± 0.14	0.272					2.08 ± 0.13	1.98 ± 0.11	1.82 ± 0.18	0.415
	EMA (cm^2)	26.6 ± 1.5	28.5 ± 0.6	26.2 ± 1.5	0.233					26.3 ± 0.8	26.4 ± 0.7	25.0 ± 1.1	0.386
	IMF (%)	3.44 ± 0.42	3.00 ± 0.18	3.16 ± 0.43	0.590					2.53 ± 0.31	2.65 ± 0.26	2.76 ± 0.42	0.883

[a]rare *CC* genotype was factored out for statistical analysis

[b]rare *AA* genotype was factored out for statistical analysis

[c]backfat thickness at mid-back

doi: 10.17221/8459-CJAS

high in Duroc, Meishan pigs and Japanese wild boars. In particular, the *c.335A* allele was fixed in Japanese wild boars and Meishan pigs. This result suggested that the *c.335A* originated in East Asian pig populations. On the other hand, the *c.335C* allele was abundant in Ryukyu wild boars. These results are in line with previous studies that indicated a large genetic difference between Japanese wild boars and Ryukyu wild boars (Watanobe et al. 1999). The Clawn miniature pig is an experimental animal breed raised from crossing among Gottingen, Chinese native, Landrace, and Large White pigs (Oishi et al. 1991). In a small population, the genetic frequency may easily be changed by a random genetic drift. Thus, monotypic allele in Clawn miniature pig might result from the founder effect of this breed. In the Euro-American breeds, Duroc had very different feature in allelic frequency. This might be a result of the differences in selections for fat-related traits because Berkshire, Landrace, and Large White breeds were selected for leanness whereas Duroc was selected for fatness (Jones 1998). The genotypic and allelic frequencies in commercial LWD cross-bred pigs were consistent with those predicted on the basis of the allelic frequencies in the parental breeds.

Association between GHRL c.335 A>C variation and economic traits. Table 2 shows the Least Squares Means of economic traits in Duroc, Landrace, Large White, and LWD pigs depending on *GHRL c.335 A>C* genotypes. Although the *CC* homozygous had a tendency toward higher ADG and BFT than the *AA* homozygous in Landrace gilt ($P = 0.071$ and $P = 0.080$, respectively), we could not detect statistically significant difference in ADG and BFT between any different genotype pairs within the four populations examined. On the other hand, noticeable genotypic effects of *c.335 A>C* on BFT have been reported in Berkshire, Polis Landrace, and Italian Large White breeds (Kim et al. 2004; Wojtysiak and Kaczor 2011; Fontanesi et al. 2012). However, the reported genotypic effects of *c.335 A>C* on BFT greatly varied among studies. Kim et al. (2004) reported that the *AC* heterozygous had the highest average BFT, whereas Wojtysiak and Kaczor (2011) reported that the *AC* heterozygous had the lowest average BFT. Furthermore, we could not detect significant association between IMF and *c.335 A>C* in Duroc and LWD pigs, although pigs with the *AC* genotype showed a trend to have higher IMF than the *AA* homozygous in Duroc ($P = 0.064$ in gilt, $P = 0.072$ in boar). Kim et al. (2004) reported that the *AA* homozygous pigs had higher IMF marbling score than the *CC* homozygous in a Berkshire population. However, Wojtysiak and Kaczor (2011) reported that there was no association between IMF and *c.335 A>C* in Polish Landrace. A possible cause of these opposing results is that the *GHRL c.335 A>C* polymorphism itself may have insignificant impact on fat accumulation but may have weak-to-moderate linkages with IMF and BFT QTLs that differ among breeds and populations.

In this study, the genotypic effect of *c.335 A>C* was significant only on EMA in Duroc gilts (Table 2). The *AA* homozygous had higher average EMA than the *AC* heterozygous in Duroc gilts ($P = 0.014$). Wojtysiak and Kaczor (2011) also detected an association between this SNP and EMA; however, the genotypic effect was different because they indicated that the *AC* heterozygous animals had the largest average EMA. Moreover, we could not detect any associations between this SNP and EMA in Landrace, Large White, or LWD populations (Table 2). Thus, these results suggest that the genotypic effect of *c.335 A>C* on EMA is of little significance.

CONCLUSION

Summarizing, our study shows that the genotypic effects of porcine *GHRL c.335 A>C* on fat deposition traits were very small and unstable among breeds and populations. Therefore, *GHRL c.335 A>C* polymorphism cannot be a major genetic marker for fat deposition traits in pig selective breeding.

Acknowledgement. The authors would like to thank M. Nakamura, M. Kooda, M. Koseki, and S. Inoue for their valuable technical assistance on this project. We would like to thank Enago (www.enago.jp) for the English language revision.

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doi: 10.17221/8459-CJAS

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Received: 2014–06–12
Accepted after corrections: 2015–03–10

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