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ORIGINAL ARTICLE

Genetic association between *GHSR1a* 5′UTR-microsatellite and nt-7(C>A) loci and growth and carcass traits in Japanese Black cattle

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ABSTRACT

We carried out a genetic association study between five nucleotide polymorphisms (5′UTR microsatellite ($(TG)_n$), nt-7(C>A), L24V, DelR242 and Intron 1 microsatellite) of the *GHSR1a* gene and growth and carcass traits in 1285 steers sired by 117 Japanese Black bulls in a progeny testing program. We report herein, a significant association between the 5′UTR microsatellite and nt-7(C>A) loci and growth and carcass traits. We also propose a translational hypothesis that the association is due to differences in the secondary structure of *GHSR1b* mRNA (the non-spliced type with the 5′UTR microsatellite) among the *GHSR1a* gene haplotypes. Furthermore, we predicted the potential increase in profitability due to increased carcass weight in cow-calf fattening enterprises through planned matings based on DNA testing of the 5′UTR microsatellite. Statistical analysis revealed that the 5′UTR microsatellite locus had a significant additive effect on carcass weight (CW) and average daily gain (ADG), but not on beef marbling score (BMS). One of the four major microsatellite alleles (*19-TG* allele) with an allele frequency of 0.145, had a significantly ($P < 0.0007$) desirable effect on CW and ADG. We concluded that the *19-TG* allele could potentially be economically useful nucleotide markers for growth and carcass traits in Japanese Black cattle.

Key words: *5′ UTR-microsatellite, GHSR, growth and carcass traits, Japanese Black cattle, mRNA secondary structure.*

INTRODUCTION

The growth hormone secretagogue receptor (GHSR) is expressed in several animal tissues and appears to mediate the various actions of its endogenous ligand (ghrelin) and synthetic growth hormone secretagogue (GHS) (Howard *et al.* 1996; Petersenn 2002). It has also been demonstrated that ghrelin–GHSR functions are involved in growth hormone (GH) secretion, food intake and several other important functions (Cruz & Smith 2008). Recently, nucleotide polymorphisms and transcriptional analyses of the bovine *GHSR1a* gene were reported by Komatsu *et al.* (2010) in which five nucleotide polymorphic loci (5′UTR microsatellite $(TG)_n$, Intron 1 microsatellite $(GTTT)_n$, nt-7(C>A), L24V and DelR242) were recommended as nucleotide markers of choice for investigating genetic association between the *GHSR1a* gene and growth and carcass traits in the Wagyu breeds. Therefore our objectives in this study were:

1. to investigate the genetic association between the five nucleotide polymorphisms of the *GHSR1a* gene and growth and carcass traits in Japanese Black cattle;
2. to test and provide evidence that supports the hypothesis that any significant genetic association with growth and carcass traits is attributable to differences in the secondary structure of *GHSR1b* mRNA (the non-spliced type with the 5′UTR microsatellite) among the *GHSR1a* gene haplotypes; and
3. to predict the potential increase in profitability due to increased carcass weight in cow-calf fattening

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farms through planned matings based on DNA testing of the 5′UTR microsatellite ($(TG)_n$).

MATERIALS AND METHODS

Animals and care

The management of cattle and all livestock handling procedures were performed in accordance with the National Institute of Livestock and Grassland Science (NILGS)'s guidelines for animal care. We used a population of 1285 Japanese Black steers sired by 117 bulls in a progeny-testing program of the Livestock Improvement Association of Japan (LIAJ) from 2001 to 2003.

Sire-progeny-testing program in Japanese Black cattle

Average and above average-performing Japanese Black dams were randomly mated with 117 young Japanese Black sires in a progeny-testing program conducted by LIAJ in seven different regions. The resulting progeny during the calving months of July–October within the 2001–2003 calving years comprised of a total of 1285 half-sib steer calves (ranging from 10 to 14 progeny per sire). They were allowed to suckle their dams in addition to being fed concentrates and corn silage until weaning. After weaning, they were moved to the grower's barn and reared until the attainment of 7–8 months of age when the steer calves were divided into two groups and transported to two progeny-testing stations in Japan. They were fed with the conventional grower ration with an allowance of 20 days for adjustment and acclimatization followed by the standard 364 days (52 weeks) progeny-testing duration prior to slaughter. Routine management of the animals involved the recording of body weight, body shape, concentrate intake and grass-silage intake every 4 weeks. Steers were weighed at the beginning and end (WT) of the testing period so that average daily gain (ADG) could be computed. Carcass data collected included slaughter weight (WS), cold carcass weight (CW), ADG, rib eye area (REA), rib thickness (RT), carcass yield estimate (YE), subcutaneous fat thickness (SFT), inter-muscular fat thickness (IFT) and beef marbling score (BMS).

Analyses for the microsatellite and nucleotide polymorphisms

Genomic DNA was extracted from the ear and semen using the standard phenol extraction protocol. Analyses of the two microsatellites and DelR242 fragments were carried out as previously described (Komatsu *et al.* 2010). The primers for nucleotide polymorphism analyses are shown in Table 1.

Fragment analysis for the 5′ UTR microsatellite

1 PCR conditions: PCR using MS-F2 and MS-R2 primers was carried out in a total volume of 10 μL containing 10 ng genomic DNA, 5 pmol of each primer, 2 nmol of dNTP, 1 μL of 10 X Buffer ($MgCl_2$) and 0.5 μL (0.375 units) of AmpliTaq DNA polymerase (3/20 dilution with glycerol; Life Technologies Corporation, Foster City, CA, USA). After initial denaturation for 3 min at 94°C, PCR was continued for 1 min at 94°C, 1 min at 62°C and 1 min at 72°C (30 cycles in total), with a last extension for 1 min at 72°C.

2 Fragment analysis: The PCR products were run with the internal size standard on an ABI PRISM™ 3730x1 DNA Sequencer (Life Technologies Corporation). The size of

Table 1 Primers for DNA amplification, fragment analyses of microsatellites and nucleotide polymorphisms

Items	Description	Sequence	Amplified length (bp)	Primer positions†
(1) Microsatellite				
1) 5′UTR ($(TG)_n$)				
MS-F2	Fragment	5′-GGGCTGTGGGTCACTCTGTCC-3′	~180 bp	−307
MS-R2	analysis	5′-GAGGATGCTTGGAAAGGAAA-3′		−128
2) Intron 1 ($(GTTT)_n$)				
MS_Intron _FP	Fragment	5′-AATTTTACAATTCCTGAAATCCTGT-3′	128 bp (or	+1906
MS_Intron _RP	analysis	5′-ACCAGCAGATTTCAATCTAGTCATA-3′	132 bp)	+2033
(2) Indel				
DelR242				
GHSR_FP_672_3 bp_Del	Fragment	5′-TGTTTTCTGCCTCACTGTGC-3′	168 bp (or	+672
GHSR_RP_839_3 bp_Del	analysis	5′-AAGACTCCGGGAGAGGAGAG-3′	165 bp)	+839
(3) SNPs				
nt-7(C>A) and L24V (nt70(C>G))	A modified method of SSPCE‡			
1st PCR				
GHSR_SNP-7_F1	PCR	5′-ACTCTTTTGCGCCTAACTAAGGA-3′	198 bp	−99
GHSR_SNP-7_R1		5′-CTCGTCAGTCAGCGAGTCATT-3′		+99
2nd PCR for nt-7(C>A)	PCR-SSPCE			
GHSR_SNP-7_A_R2		5′-(FAM)-TCCACATGCTGCCT-3′	106 bp	+7
GHSR_SNP-7_C_R2		5′-(FAM)-CATTCCACATGCTGCCG-3′	109 bp	+10
2nd PCR for L24V (nt70(C>G))	PCR-SSPCE			
GHSR_SNP+70_C_F2		5′-(FAM)-CGGACCTGGGTTGGGACGCTC-3′	49 bp	+50
GHSR_SNP+70_G_F2		5′-(FAM)-CCTGGGTTGGGACGCTG-3′	55 bp	+54

†Position of the first 5′ nucleotide from the translation start. ‡Sequence-specific primer cycle elongation.

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the fragments was analyzed using the GeneMapper (Version 4.0) program (Life Technologies Corporation). Alleles were designated according to PCR product size, and allelic frequencies were calculated directly from the observed genotypes.

Fragment analysis for the Intron 1 microsatellite ((GTTT)$_n$)

The PCR was carried out using MS_Intron_FP and MS_Intron_RP primers. PCR and fragment analysis were carried out under the same conditions as previously described above for 5′UTR microsatellite fragment analysis.

Fragment analysis for DelR242

The PCR was carried out in a total volume of 10 μL containing 10 ng genomic DNA, 5 pmol of each primer (FP_672-3 bp_Del and RP_839-3 bp_Del), 5 μL of AmpliTaq gold PCR Master Mix (Life Technologies Corporation). After an initial denaturation for 4 min at 94°C, PCR was continued for 30 s at 94°C, 30 s at 5°C and 30 s at 72°C (35 cycles in total), with a last extension for 5 min at 72°C. The PCR products were run with the internal size standard on an ABI 3130x1 DNA Sequencer (Life Technologies Corporation). Alleles were designated according to PCR product size, and allelic frequencies were calculated directly from observed genotypes.

Modified Sequence-Specific Primer Cycle Elongation (SSPCE) for nt-7(C>A) and L24V(nt70(C>G))

1. The first PCR (using the first PCR primer set) was carried out in a total volume of 11 μL containing 10 ng genomic DNA, 5 pmol of each primer and 5 μL of the QIAGEN multiplex PCR Kit (QIAGEN GmbH, Hilden, Germany). After an initial denaturation for 15 min at 94°C, PCR was continued for 15 s at 94°C, 30 s at 60°C and 90 s at 72°C (35 cycles in total), with a last extension for 30 min at 72°C.
2. The second PCR (using the second PCR sets for the different loci and alleles) was carried out in a total volume of 10 μL containing 1 μL of the first PCR product (11/81 dilution with distilled H_2O), 12 pmol of GHSR_SNP-7_A_R2, 4 pmol of GHSR_SNP-7_C_R2, 1 pmol of GHSR_SNP+70°C_F2, 5 pmol of GHSR_SNP+70_G_F2, and 5 μL of the QIAGEN multiplex PCR Kit (QIAGEN GmbH). After an initial denaturation for 15 min at 94°C, PCR was continued for 15 s at 94°C, 30 s at 60°C and 90 s at 72°C (35 cycles in total), with a last extension for 30 min at 72°C. The PCR-SSPCE products were run with the internal size standard on an ABI 3730x1 DNA Analyzer (Life Technologies Corporation). The size of the fragments was analyzed using the GeneMapper (Version 4.0) program (Life Technologies Corporation). Alleles were designated according to PCR product size, and allelic frequencies were calculated directly from observed genotypes.

Statistical analysis

A preliminary statistical analysis was carried out using Scheffe's one-way analysis of variance with the following model: $y = \mu + \text{DNA marker} + e$, where y is phenotypic value of the animals; μis the mean of the animal population and e is the stochastic error.

Genetic association analysis between DNA markers and growth and carcass traits was carried out using a univariate model within the framework of a derivative-free restricted maximum likelihood algorithm as applied in the MTDFREML (Boldman *et al.* 1993). The following linear mixed animal model was used: $y = X_1b + X_2g + Zu + e$, where y is a vector of phenotypic observations; b is a vector of fixed effects and includes the year and month of birth, place of birth, location of test stations and the linear covariate of age at the beginning of the progeny test; g is a vector of the fixed additive effect of the *GHSR1a* microsatellite or SNP alleles or haplotypes of the microsatellite and SNP; u is a vector of random additive genetic effect of the polygene, which is assumed to be distributed across $N(0, A\sigma_a^2)$, where A is the additive relationship matrix among animals andσ_a^2 is the additive genetic variance of polygene; e is a vector of random residual effects, which is assumed to be distributed across $N(0, I\sigma_e^2)$; and X_1, X_2 and Z are the corresponding incidence matrices.

RNA secondary structure analysis

The Vienna RNA secondary structure server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; RNA fold web server) (Hofacker 2003) was used to predict the optimal secondary structure for *GHSR1b* and *GHSR1a* mRNAs. The mRNA sequences of the six haplotypes (haplotypes frequency: > 0.04) observed in this study were analyzed as follows: [1] the *GHSR1a* mRNAs, 1-2 type (the most abundant type) with the 3′UTR – A type (the longest 3′UTR with poly(A)$^+$ additional signal sequence (AAUAAA) (1) *A* (nt-7(C>A)), Hap02 and 07; (2) *A* (nt-7(C>A)), Hap08; (3) *C*, Hap03 and 04; (4) *C*, Hap25. [2] the ***GHSR1b*** mRNAs, 2-1 type (a major type) with the 3′UTR-C type (the most abundant and the shortest 3′UTR without AAUAAA) (1) [*19-TG*] – [*A*] (the [5′UTR microsatellite] – [nt-7(C>A)] haplotype), Hap07; (2) [*22-TG*] – [*A*], Hap02; (3) [*29-TG*] – [*A*], Hap08; (4) [*23-TG*] – [*C*], Hap03; (5) [*24-TG*] – [*C*], Hap04; (6) [*33-TG*] – [*C*], Hap25. Fold algorithms and basic options used were as follows: (1) minimum free energy (MFE) and partition function; (2) no GU pairs at the end of helices; and (3) no isolated base pairs.

Prediction of potential increase in profitability of cow-calf fattening farms

We predicted the potential increase in profitability (ΔV_{ind}) due to increase in carcass weight (CW) of Japanese Black cattle cow-calf fattening farms using planned mating based on DNA testing of the *19-TG* allele for 5′UTR microsatellite.

The *BeefIncome* Program (http://nilgs.naro.affrc.go.jp/prog/BeefIncome.html) (Komatsu *et al.* 2009) was used for the prediction of profitability.

The assumptions and explored diverse scenarios:

1. Initial frequency of the*19-TG* allele in Japanese Black cattle (p) = 0.145.
2. The *19-TG* (excellent) to *non-19-TG* (ordinary) allele additive substitution effect for CW (ΔCW_{QTL}), 8.125 kg (= 6.5 kg × (440/352)).
3. Carcass weight (*CW*), 440 kg.
4. Per-kg unit price for CW of Japanese Black cattle (CW_{PU}), ¥1900/kg.
5. Charge for DNA typing (C_{TYP}), ¥0 or ¥5000.
6. Semen price difference between sires selected and unselected by DNA typing through reproduction cycles (*SEM*), ¥0 or ¥10 000.

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7. The DNA typed sires (the [*19-TG*] homozygote) were used to breed dams (Hardy-Weinberg population).
8. Sire/dam ratio (*R(s/day)*), 1/30 or 1/300.
9. Number of dams (number of cows in the population) (*N*), 30 or 1200.
10. The age at first calving and the productive lifetime of dams (*N*: number of dams) were 3 and 8 years, respectively.
11. The replacement rate for cows was $\frac{1}{6}$. Calves not for replacement ($\frac{5}{6}N$) and cows over 8 years ($\frac{1}{6}N$) were moved for fattening and slaughter.
12. The planned time horizon (*T*) was 1 or 6 years.
13. Priority sequence of dams for slaughter: [*non-19-TG*] homozygote → [*19-TG/non-19-TG*] heterozygote → [*19-TG*] homozygote.
14. [Scenario 1]: Sires were known for DNA type (the *19-TG* homozygote): C_{TYP} = ¥0, *SEM* = ¥0 or ¥10 000.
    [Scenario 2]: Sires were DNA typed and selected (the *19-TG* homozygote): C_{TYP} = ¥5000, *SEM* = ¥0 or ¥10 000.

RESULTS AND DISCUSSION

Allele frequencies for the two microsatellites, nt-7(C>A), L24V and DelR242

5′ UTR microsatellite ((TG)ₙ)

A total of nine alleles (*15-TG, 19-TG, 21-TG, 22-TG,* 23-TG, *24-TG, 26-TG, 29-TG* and *33-TG*) and four major alleles (*19-TG, 22-TG, 23-TG*, and *24-TG*) were detected for the *GHSR1a* microsatellite in the population (Table 2). In addition to the four major alleles and frequencies previously described by Komatsu *et al.* (2010), two other minor alleles (*15-TG* and *26-TG*) were discovered in Japanese Black cattle in this present study.

L24V(nt70(C>G)), DelR242 and the Intron 1 microsatellite ((GTTT)ₙ)

The major alleles and allele frequencies of these loci were also the same as described previously (Komatsu *et al.* 2010). The *C* allele frequency of the *L24V* locus was more than 0.98. However, the *DelR242* allele was not found in Japanese Black cattle in this study. The allele *$(GTTT)_6$* of the Intron 1 microsatellite locus is characterized as a good marker for distinguishing between Group 1 and Group 2 haplotypes of the *GHSR1a* gene in *Bos taurus* breeds (Komatsu *et al.* 2010). The *GHSR1a* gene haplotypes are divided into two major groups (Group1 and Group2) by phylogenetic analysis (neighbor-joining (NJ) tree) in *Bos taurus* breeds. The allele *$(GTTT)_6$* formed the [*23-TG* or *24-TG* or *33-TG*] – [*C(nt-7(C>A))*] – [*C(L24V)*] – [*$(GTTT)_6$*] or the [*23-TG*] – [*C(nt-7(C>A))*] – [*G(L24V)*] – [*$(GTTT)_6$*] haplotypes.

The frequency of 0.047 of the *$(GTTT)_6$* allele demonstrates that Group 2 haplotype has a very low frequency of occurrence in Japanese Black cattle. In addition, the *$(GTTT)_8$* allele was found in a specific sire line of Japanese Black cattle as Mishima Island cattle. Furthermore, the allele formed the following haplotypes which are the same as those described in this breed and the Jersey breed: [*21-TG*] – [*A(nt-7(C>A))*] – [*C(L24V)*] – [*$(GTTT)_8$*]. It is possible that the allele *$(GTTT)_8$* found in Japanese Black cattle could have been derived from Mishima Island cattle, the oldest breed of native Japanese cattle (Nagamine *et al.* 2008). A total of 11 haplotypes with the following four major haplotypes, [*19-TG*] – *A*, [*22-TG*] – *A*, [*23-TG*] – *C* and [*24-TG*] – *C* were detected in this population.

Table 2 Allele frequencies for the two microsatellites, nt-7(C>A), L24V, DelR242 and haplotype frequency for [5′UTR microsatellite] – [nt-7(C>A)] in a population of 1285 steers of Japanese Black cattle

Locus	Allele†	Frequency†
5′UTR microsatellite ((TG)ₙ)	*15-TG‡ (166)§*	0.000¶
	19-TG (174)	**0.145**
	21-TG (178)	0.018
	22-TG (180)	**0.149**
	23-TG (182)	**0.265**
	24-TG (184)	**0.321**
	26-TG (188)	0.015
	29-TG (194)	0.045
	33-TG (202)	0.042
nt-7(C>A)	***C***	**0.628**
	A	**0.372**
L24V (nt70(C>G))	***C***	**0.987**
	G††	0.013
DelR242	***AGG***	**1.000**
	DelR242	0.000
Intron 1 microsatellite ((GTTT)ₙ)	***$(GTTT)_5$***	**0.946**
	$(GTTT)_6$‡‡	0.047
	$(GTTT)_8$§§	0.007
Haplotype ([microsatellite *(TG)ₙ*] – [nt-7(C>A)]	*15-TG‡ – A*	0.000¶
	19-TG – A	**0.145**
	21-TG – A	0.018
	22-TG – A	**0.149**
	22-TG – C	0.000¶
	23-TG – C	**0.265**
	24-TG – A	0.000¶
	24-TG – C	**0.321**
	26-TG – A	0.015
	29-TG – A	0.045
	33-TG – C	0.042

†A bold font means a major allele and allele frequency. ‡TG repeat number (5′ → 3′): ***15-TG*** = *TG(6)TC(1)TG(8);* ***19-TG*** = *TG(10)TC(1)TG(8);* ***21-TG*** = *TG(12)TC(1)TG(8);* ***22-TG*** = *TG(13)TC(1)TG(8);* ***23-TG*** = *TG(14)TC(1)TG(8);* ***24-TG*** = *TG(15)TC(1)TG(8);* ***26-TG*** = *TG(17)TC(1)TG(8);* ***29-TG*** = *TG(20)TC(1)TG(8);* ***33-TG*** = *TG(24)TC(1)TG(8)*. §Fragment size; ¶0.0004. ††The ***G*** allele forms the [*23-TG*] – [*C(nt-7(C>A))*] – [*G(L24V)*] – [*$(GTTT)_6$*] haplotype only. ‡‡The ***$(GTTT)_6$*** allele forms the [*23-TG* or *24-TG* or *33-TG*] – [*C(nt-7(C>A))*] – [*C(L24V)*] – [*$(GTTT)_6$*] or the [*23-TG*] – [*C(nt-7(C>A))*] -[*G(L24V)*] – [*$(GTTT)_6$*] haplotypes. §§The ***$(GTTT)_8$*** allele forms the [*21-TG*] – [*A(nt-7(C>A))*] – [*C(L24V)*] – [*$(GTTT)_8$*] haplotype only.

Nucleotide polymorphism association with growth and carcass traits

Although *L24V* and Intron 1 microsatellite loci were major alleles with frequencies over 0.94, we chose

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Table 3 Mean values of the four traits of each *GHSR1a* 5′UTR microsatellite genotype and allele additive substitution effect (α) of *19-TG* for *non-19-TG*

5′UTR microsatellite genotype†	No. of animals	WT‡ (kg)	WS (kg)	CW (kg)	ADG (kg)
19-TG/19-TG	24	618.67[d]§	614.00[d]	366.83[d]	0.961[b]
19-TG/non-19-TG	323	610.61[a]	605.28[a]	360.70[a]	0.938[a]
Non-19-TG/non-19-TG	935	593.38[c]	589.01[c]	349.15[c]	0.902[c]
Total mean (SD)	1282	598.20 (57.48)	593.52 (57.42)	352.39 (36.84)	0.912 (0.112)
	α	10.87	10.67	6.48	0.022
	SE	3.21	3.18	2.02	0.006
	P	< 0.0004	< 0.0004	< 0.0007	< 0.0002

†*19-TG; non-TG-19: 15-TG, 21-TG, 22-TG, 23-TG, 24-TG, 26-TG, 29-TG, 33-TG* (see Table 2 footnote in detail). ‡WT, weight at the termination of the test; WS, weight at slaughtering time; CW, cold carcass weight; ADG, average daily gain. §a,c: $P < 0.0001$; b,c: $P < 0.05$; c,d: $P < 0.10$.

Table 4 Mean values of the four traits in three [nt-7(C>A)] genotypes and six [5′UTR mirosatellite] – [nt-7(C>A)] haplotypes

Locus, genotype and haplotype†	No. of animals	Trait			
		WT‡ (kg)	WS (kg)	CW (kg)	ADG (kg)
(1) nt-7(C>A)					
AA	174	604.24[a]§	598.96[a]	355.40[a]	0.924[d]
AC	609	602.39[ac]	597.72[ac]	355.19[ac]	0.910[df]
CC	502	590.77[b]	586.27[b]	347.80[b]	0.902[e]
		P < 0.001	< 0.001	< 0.002	< 0.02
(2) haplotype ([5′UTR mirosatellite] – [nt-7(C>A)])					
19-TG-A/19-TG-A	24	618.67	614.00	366.83	0.960
19-TG-A/non19-TG-A	96	614.79[a]¶	608.88[a]	360.95[c]	0.940[c]
19-TG-A/non19-TG-C	228	608.78[c]	603.67[c]	360.49[a]	0.936[f]
non19-TG-A/non19-TG-A	54	579.09[be]	574.67[be]	340.46[be]	0.879[eg]
non19-TG-A/non19-TG-C	381	598.56	594.17	352.03	0.906[g]
non19-TG-C/non19-TG-C	502	590.78[bd]	586.27[bd]	347.8[ef]	0.902[eh]
Total mean (SD)	1285	598.10 (57.47)	593.42 (57.43)	352.33 (36.84)	0.912 (0.110)
		P < 1.0E-5	< 1.0E-5	< 1.0E-5	< 5.0E-5

†*19-TG; non-TG-19: 15-TG, 21-TG, 22-TG, 23-TG, 24-TG, 26-TG, 29-TG, 33-TG* (see Table 2 footnote in detail). ‡WT, weight at the termination of the test; WS, weight at slaughter; CW, cold carcass weight; ADG, average daily gain. §a,b: $P < 0.05$; b,c: $P < 0.005$; d,e: $P < 0.10$; e,f: $P < 0.10$. ¶a,b: $P < 0.05$; a,f: $P < 0.005$; c,d: $P < 0.05$; c,e: $P < 0.10$; f,g: $P < 0.10$; f,h: $P < 0.05$.

rather to focus on the 5′UTR microsatellite and *nt-7(C>A)* loci for the association study with growth and carcass traits because the effects of *L24V* and Intron 1 microsatellite loci on these traits did not reach statistical significance during the preliminary analysis. Furthermore, the allele *DelR242* was not detected in this population. However, preliminary statistical analysis revealed that the 5′UTR microsatellite locus had a highly significant additive effect on WT ($P < 0.005$), WS ($P < 0.005$), CW ($P < 0.001$), ADG ($P < 0.005$), RT ($P < 0.05$), SFT ($P < 0.05$) and YE ($P < 0.05$), but not REA ($P < 0.09$), IFT ($P < 0.58$) and BMS ($P < 0.61$).

Above all, the *19-TG* allele, one of the four major microsatellite alleles, had the most desirable effect on these traits. Based on the genotype results, the individuals could be classified into three groups: the [*19-TG*] homozygote, the [*19-TG*/*non-19-TG*] heterozygote and the [*non-19-TG*] homozygote. As portrayed in Table 3, MTDFREML statistical analysis clearly revealed that the *19-TG* allele had a very significant additive substitution effect on WT ($P < 0.0004$), WS ($P < 0.0004$), CW ($P < 0.0007$), and ADG ($P < 0.0002$). Further, it is apparent from Table 4 that nt-7(C>A) had a significant additive effect on WT ($P < 0.001$), WS ($P < 0.001$), CW ($P < 0.002$) and ADG ($P < 0.05$), but not on the other traits. The *A* allele of the nt-7(C>A) also had a significant effect on these four traits.

To further investigate the combined effect of both the 5′UTR microsatellite ($(TG)_n$) and nt-7(C>A) on these traits, the haplotypes of the 5′UTR microsatellite and the nt-7(C>A) were constructed and statistically analyzed. The results in Table 4 demonstrate that the [*19-TG*] – [*A*] haplotype had the most significant additive effect on these growth and carcass traits. Interestingly, the mean values of these traits for the [*non-19-TG*] – [*A*] homozygote were the lowest among these haplotype combinations.

Zhang *et al.* (2009) reported nucleotide polymorphisms of the coding regions of the *GHSR1a* gene and demonstrated a significant association between the nt456(*G*>A) and nt667(C>T) haplotypes and body weight and ADG at 6 months of age in Nanyang cattle. However, these two SNPs are synonymous mutations and the 5′UTR microsatellite was investigated herein.

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Table 5 Comparison of a number of Kozak sequence bases bound in secondary structure and structural elements of the *GHSR 1a* and *GHSR 1b* mRNAs

Splice variant	mRNA type†	Haplotype number‡	5′UTR type of mRNA‡	3′UTR type of mRNA‡	No. of poly(A)$^+$ additional signal§	No. of start codons in 5′UTR	Length of mRNA (bases)§	Length of 5′UTR (bases)	Secondary strucute type for 5′UTR (Putative)¶	No. of Kozak sequence bases bound in secondary structure††	Minimum free energy prediction (***ΔG***) (kcal/mol)
1a											
	A	Hap02, 07	1-2	A	1	5	2585	742	F	2	−861
	A	Hap08	1-2	A	1	5	2585	742	F	2	−859
	C	Hap03, 04	1-2	A	1	5	2585	742	F	2	−867
	C	Hap25	1-2	A	1	5	2585	742	F	2	−866
1b	***[19-TG] – A***	Hap07	2-1	C	0	2	4269	755	S – V	5	−1261
	[22-TG] – A	Hap02	2-1	C	0	2	4269	761	B – V	5	−1262
	[29-TG] – A	Hap08	2-1	C	0	2	4289	775	B – V	5	−1259
	[23-TG] – C	Hap03	2-1	C	0	2	4277	763	B – T	2	−1271
	[24-TG] – C	Hap04	2-1	C	0	2	4279	765	B – T	2	−1267
	[33-TG] – C	Hap25	2-1	C	0	2	4300	783	B – T	2	−1271

†SNP nt-7(C>A) for the *1a* mRNA and the [5′UTR microsatellite] – [SNP nt-7(C>A)] haplotype for the *1b* mRNA. ‡Haplotype number, the 5′UTR and 3-UTR types of the mRNAs: see Komatsu *et al*. (2010). §The 3′UTR poly(A)$^+$ additional signal, AATAAA; the lenth of an additional poly(A)$^+$, 250 bases. ¶*1a*: **F**, a **F**oot – like ; *1b*, the 5′UTR type – nt-7(C>A) type: **S – V**, a **S**traight and **V** configuration – like; **B – T**, a **B**end and **T**riangle – like; **B – V**, a **B**end and **V** configuration – like; see Figure 1. ††The concensus Kozak sequence is 'ACC**AUG**G' (**AUG**: the translation start codon). The sequence of this region of the bovine *GHSR1a* gene is 'AGC**AUG**T'. A bold font means the four major haplotypes.

Table 6 Hypothesis explaining the association bewteen the 5′UTR microsatellite(*(TG)$_n$*) and *nt-7(C>A)* haplotypes and functional Ghrelin receptor (GHSR1a) level based on the secondary structure of *GHSR 1a* and *GHSR 1b* mRNAs

[5′UTR microsatellite (*(TG)$_n$*)] and *nt-7(C>A)* types	*1a* mRNA scondary structure type†	*1a* mRNA translation efficiency	1a protein level	*1b* mRNA scondary structure type†§	*1b* mRNA translation efficiency	1b protein level	Functional Ghrelin receptor (GHSR1a) level¶
(1) 5′UTR microsatellite type							
19-TG	F	(+++++)‡	(+++++)	**S**	(+)	(+)	–
non-19-TG	F	(+++++)	(+++++)	**B**	(+++)	(+++)	–
(2) nt-7(C>A) type							–
C	F	(+++++)	(+++++)	**T**	(+++)	(+++)	–
A	F	(+++++)	(+++++)	**V**	(++)	(++)	–
(3) Haplotype of *1b* mRNA							
[19-TG] – C	F	+++++	+++++	**S – T**	++	++	+++
[19-TG] – A	F	+++++	+++++	**S – V**	+	+	++++
[non-19-TG] – C	F	+++++	+++++	**B – T**	+++	+++	++
[non-19-TG] – A	F	+++++	+++++	**B – V**	++++	++++	+

†mRNA secondary structure, see Figure 1A for *1a* mRNA; Figure 1B,C for *1b* mRNA. The *1a* type mRNA is a 5′UTR microsatellite region splice-out type (Komatsu *et al*. 2010). ‡+ means predicted relative levels for translation efficiency, 1a or 1b proteins and the functional Ghrelin receptor (GHSR1a). The order of the levels: +++++ > ++++ > +++ > ++ > +. §The [***19 – TG***] (S type) of the 5′UTR microsatellite region of the *GHSR1b* mRNA is assumed to have a small negative dominnant effect on translation. The *nt-7(C>A)* locus is assumed to affect the *GHSR 1b* mRNA scondary structure and the *C* type (nt-7(C>A)) of the 1b mRNA (**T**) is assumed to have a more effective for translation than the A type (**V**). However, the [***non-19-TG***] – A (**B – V** type) mRNA is assumed to have a specific effective interaction to translation machinary. ¶Predicted functional Ghrelin receptor (GHSR1a) level of each homozygote : [the 1a protein level] minus [the 1b protein level].

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Figure 1 (A) Optimal secondary structure of the *GHSR1a* mRNAs. Left: *A* (nt-7(C>A)), haplotype number, Hap02; Right: *C* (nt-7(C>A)), Hap03. The square box shows a translation start region. The square dotted arrow shows a Kozak sequence of the *GHSR1a* mRNA (AGC**AUG**U) and the **AUG** is the translation start codon. The underlined bases in this sequence are bound in a secondary structure of the *GHSR1a* mRNA. The solid arrow shows the 5′ end of the transcript. (B) Optimal secondary structure of the *GHSR1b* mRNAs. Left: *[19-TG] – [A]* (the [5′UTR microsatellite] – [nt-7(C>A)] haplotypes), the haplotype number, Hap07; Right: *[24-TG] – [C]*, Hap04. The circle dotted oval box shows the 5′UTR microsatellite ($(TG)_n$) region. The square box, the square dotted arrow, the **AUG**, the underlined bases and the solid arrow mean as referred to (A). A putative secondary structure type for a translation start region: V type, *A* (nt-7(C>A); T type, *C* (nt-7(C>A)). (C) Optimal secondary structure of the 5′UTR microsatellite region of the 6 haplotypes of the *GHSR1b* mRNAs. The circle dotted line shows the 5′UTR microsatellite ($(TG)_n$) region. A putative secondary structure type for the 5′UTR region: S type, *[19-TG]*; B type, *[non – 19-TG]* (*[22-TG], [23-TG], [24-TG], [29-TG], [33-TG]*). A putative secondary structure of the *1b* mRNAs: S-V type, *[19-TG]-A*; B-V type, *[22-TG]-A, [29-TG]-A*; B-T type, *[23-TG]-C, [24-TG]-C, [33-TG]-C*.

It has been reported that GT repeat polymorphisms in the *Tilapia* prolactin 1 (*prl 1*) 5′UTR promoter are associated with differences in *prl 1* gene expression and the growth response of salt-challenged fishes (Streelman & Kocher 2002). The length of the TG-repeat in the P1 promoter region of the growth hormone receptor (GHR) gene was significantly related to growth and carcass traits in beef cattle (Hale *et al.* 2000; Curi *et al.* 2005). Stepwise increases in repeat numbers from 0 to 21 for a CA-microsatellite located in the promoter of the human matrix metalloproteinase-9 gene has been reported to produce incremental surges in transcription rates (Shimajiri *et al.* 1999). Furthermore, a review of the simple sequence repeats (SSRs) in the 5′-UTR by Li *et al.* (2004) revealed that the regulation of gene expression is affected by both transcription and translation. Therefore, a logical explanation for the association between 5′UTR microsatellite ($(TG)_n$) and nt-7(C>A) polymorphisms and growth traits is through differences in transcriptional or translational levels of the *GHSR1a* gene. The first, a 'transcriptional hypothesis' is that the differences in DNA structure around the 5′UTR microsatellite region between [*19-TG*] and [*non-19-TG*] simultaneously affect transcriptional levels of both the *1a* and *1b* mRNAs from the *1a* gene. However, this hypothesis is hard to explain in the light of known GHSR1b function because it has been reported that the GHSR1b (the truncated receptor polypeptide) acts as a dominant-negative mutant of the GHSR1a (functional ghrelin receptor) due to the formation of GHSR1a/GHSR1b heterodimer (Leung *et al.* 2007).

The second, a 'translational hypothesis' is more intriguing and interesting as it relates to the differences in RNA secondary structure around the 5′UTR region of the *1b* mRNAs between the [*19-TG*] and [*non-19-TG*] or between the *A* and *C* (nt-7(C>A)), thus affecting translational levels of the *1b* mRNAs but not of the *1a* mRNAs. McClelland *et al.* (2009) reported that translational efficiency is inversely correlated with the stability of the mRNA secondary structure, the presence of base-pairing in the consensus Kozak sequence, the number of start codons in the 5′UTR and the length of the 5′UTR.

In order to test this 'translational hypothesis', we predicted the optimal RNA secondary structure, the number of Kozak sequence bases bound in the secondary structure (Kozak 2005) and minimum free energy (ΔG) of the *GHSR1a* and *1b* mRNAs using the Vienna RNA secondary structure server (Hofacker 2003). It is evident from Figure 1A and Table 5 that the optimal RNA secondary structures of the *1a* mRNAs appeared to be almost the same among haplotypes or between the *A* and *C* types (nt-7(C>A). Thus, the number of Kozak sequence bases ('AGCAUGU') bound in secondary structure of these types was the same as 2 regardless of the haplotypes (Fig. 1A, Table 5). On the other hand, the optimal RNA secondary structure of the 5′UTR regions of the *1b* mRNAs appeared to be different between the [*19-TG*] and [*non-19-TG*] types or between the *A* and the *C* types (nt-7(C>A)) (Fig. 1B,C, Table 5). A secondary structure of 5′UTR microsatellite region for the [*non-19-TG*] *1b* mRNAs seems to have a bending knot but not in the [*19-TG*] type (Fig. 1C). It would seem that the secondary structure of the 5′UTR microsatellite region of the [*19-TG*]-*1b* mRNAs had a small dominant negative effect on the translation efficiency of the *GHSR1b* mRNA due to its unique structure.

There were five Kozak sequence bases bound in the secondary structures for the *A* type (nt-7(C>A)) and two for the *C* type (Fig. 1B). The *A* type of the *1b* mRNAs seemed to have a lower effect on translation efficiency than that of the *C* type because of the different number of Kozak sequence bases bound in the secondary structure. In addition, there seemed to be no fundamental difference in the minimum free energy (ΔG) among the haplotypes of the *GHSR1a* gene (Table 5).

On the basis of these results, we propose herein a translational hypothesis that the differences in the RNA secondary structure of the *GHSR1*b mRNAs among the 5′UTR microsatellite – the nt-7(C>A) haplotypes affect the functional ghrelin receptor (GHSR1a) level (Table 6). The estimated functional ghrelin receptor levels of each homozygote are as

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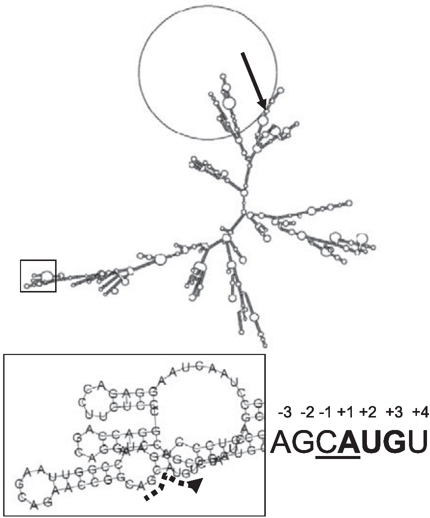
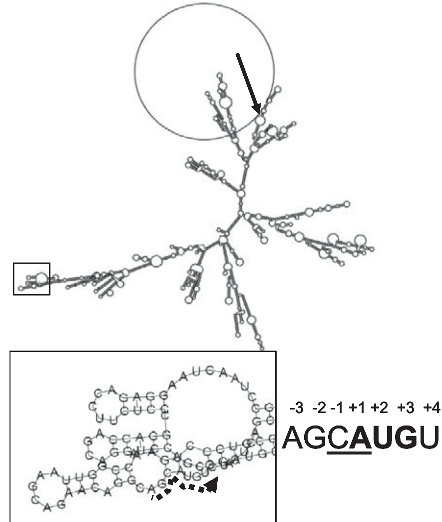
(A) *1a* mRNAs

A (nt-7(C>A)) (Hap02)

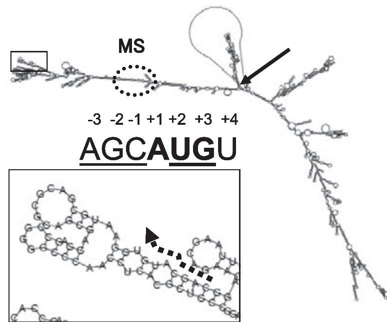


C (nt-7(C>A)) (Hap03)

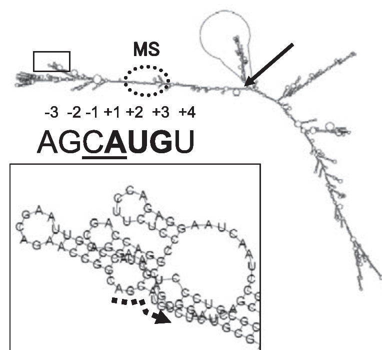


(B) *1b* mRNAs

[19-TG] – A (Hap07)

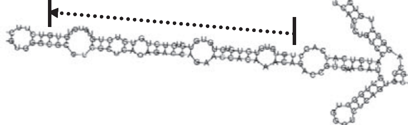


[24-TG] – C (Hap04)

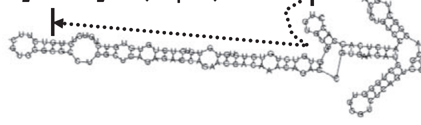


(C) *1b* mRNA 5'UTR microsatellite region

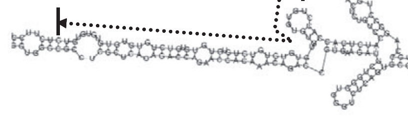
[19-TG]-A (Hap07)



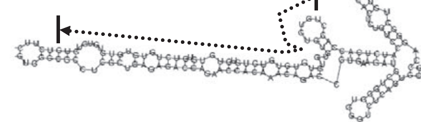
[22-TG]-A (Hap02)



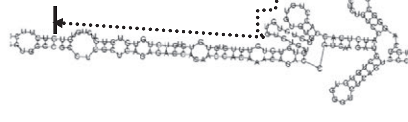
[23-TG]-C (Hap03)



[24-TG]-C (Hap04)



[29-TG]-A (Hap08)



[33-TG]-C (Hap25)

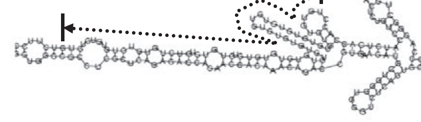


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Table 7 Increase in total profit in CW (¥/head) (ΔV_{ind}) predicted by DNA typing for the *19-TG* allele through 1 or 6-year planned time horizon under the two scenarios†

Case (Scenario)/Item	ΔV_{ind} (¥/head)			
Number of dams (N)	30	1 200	30	1 200
Sire/dam ratio (R(s/d))	(1/30)	(1/300)	(1/30)	(1/300)
Planned time horizon (years) (T)	1	1	6	6
Scenario (1)				
(1-1) C_{TYP} = ¥ 0; SEM = ¥ 0	10 608	10 972	11 193	11 424
(1-2) C_{TYP} = ¥ 0; SEM = ¥ 10 000	−503	972	217	1 417
Scenario (2)				
(2-1) C_{TYP} = ¥ 5000; SEM = ¥ 0	10 423	10 955	11 010	11 407
(2-2) C_{TYP} = ¥ 5000; SEM = ¥ 10 000	−688	955	34	1 400
Scenario (0): Hardy-Weinberg Population	3 080	4 466	3 080	4 466

†Assumptions, see below; Scenarios and equations, see Komatsu *et al.* (2009). Assumptions: (1) Initial frequency of the*19-TG* allele in Japanese Black cattle (p) = 0.145. (2) Carcass weight (*CW*),440 kg/head. (3) The 19-TG allele additive effect on CW (ΔCW_{QTL}), 8.125 kg (= 6.5 kg × (440/352)). (4) Per-kg unit price for CW of Japanese Black cattle (CW_{PU}), 1900 ¥/kg. (5) Charge for DNA typing (C_{TYP}), ¥0 or ¥5000. (6) Semen price difference between sires selected and unselected by DNA typing through reproduction cycles (*SEM*), ¥0 or ¥10 000. (7) Sire/dam ratio (*R(s/d)*), 1/30 or 1/300. (8) Number of dams (Number of cows in the population) (*N*), 30 or 1200. (9) Planned time horizon (years) (*T*), 1 or 6.

follows: [*19-TG*] − *A* > [*19-TG*] − *C* > [*non-19-TG*] − *C* > [*non-19-TG*] − *A*. The differences in the RNA secondary structure around the 5′UTR region of the *1b* mRNAs between the [*19-TG*] and [*non-19-TG*] or between the *A* and *C* (nt-7(C>A)) affect translational levels of the *1b* mRNAs and the functional ghrelin receptor level, and finally growth traits in cattle. This hypothesis should be validated by a molecular biological study in the future.

We predicted the potential increase in profitability in both small- and large-scale cow-calf fattening farms utilizing Japanese Black cattle through the use of planned mating based on DNA testing of the *19-TG* allele for 5′UTR microsatellite (usage of the*19-TG* homozygote sire) to increase CW (Table 7). It was demonstrated that it was possible to achieve approximately ¥10 000-worth profitability increase (ΔV_{ind}) provided that the cost of DNA diagnosis was ¥5000/head or less and that the semen price differential was ¥0/head in both a small-scale and large-scale cow-calf fattening farms utilizing Japanese Black cattle within a 1-year time frame. This result indicates that the *19-TG* allele for 5′UTR microsatellite is potentially an economically useful DNA marker for Japanese Black cow-calf fattening enterprises.

Conclusion

We have provided conclusive evidence that the *19-TG* allele of the 5′UTR microsatellite of the bovine *GHSR1a* gene affects growth and carcass traits in Japanese Black cattle. The *19-TG* allele of the 5′UTR microsatellite is potentially an economically useful DNA marker for Japanese Black cow-calf fattening farms.

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