An isolation, polymorphism and trait association study of bovine *GAD1* and *GAL* genes

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Abstract

The type and frequency of bovine *GAD1* and *GAL* alleles have not yet been studied in beef cattle. Initial, partial characterization of several polymorphism sites of *GAD1* and *GAL* genes was reported, and association analyses with feed intake, growth and carcass traits were performed. There was a single nucleotide polymorphism (SNP) in the 533bp fragment of *GAD1* (Accession no. DQ139316), and this fragment revealed a polymorphism *Ssp I* restriction site caused by a G/A transition, located in 3'-UTR. A polymorphism *Pst I* restriction site at 102 (A/G transition) and a polymorphism *Bsh1236 I* restriction site at 837 (C/G transition) in the 2816bp PCR fragment (Accession no. DQ146944) corresponding to the bovine *GAL* gene were also identified. One hundred and thirty eight unrelated cattle from four breeds (Simmental, Angus, Hereford and Crossed Simmental) were genotyped and their allele frequencies were determined. In a preliminary trait association study, significant associations of *GAD1* SNP-409, *GAL* SNP-102 and SNP-837 were found with average daily feed intake (ADFI). *GAD1* and *GAL* SNPs were found to be good candidates to be used in marker-assisted selection (MAS) for ADFI.

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Introduction

Glutamate decarboxylase (*GAD*) is an inhibitory neurotransmitter that catalyzes glutamic acid decarboxy (Soghomonian & Martin, 1998). It can also improve the concentration of the inhibitory neurotransmitter of γ-Aminobutyric acid (*GABA*), which is formed by glutamic acid decarboxylase (Erlander *et al*., 1991), and makes animals become quiet and sleepy. It can induce the release of hormones associated with pepsin and insulin, which have been shown to regulate feed intake and metabolic rate, and stimulate appetite. *GAD* can advance animal growth and development. *GAD* exists as a 67-kDa soluble form (*GAD1*) (Kanaani *et al*., 1999). The neuropeptide galanin (*GAL*) is a bioactive neuropeptide and widely expressed in the central and peripheral nervous systems, neuroendocrine tissue, including adenohypophysis where it can attenuate neurochemical and behavioural effects related to opiate reinforcement (Rasmussen *et al*., 1990). *GAL* can regulate animal intake, sleeping and secretions of insulin and the pituitary hormone. *GAL* also inhibits mesolimbic dopamine neurotransmission, and has a critical effect on the animal's digestive, neuroendocrine and reproductive systems (Tsuda *et al*., 1998; Ericson *et al*., 1999). Galanin mediates its effects through activation of at least three receptors to modulate animal growth and development, reproduction and release of milk. It also stimulates the release of the growth hormone by inhibiting the secretion of the growth inhibitory hormone and mediates its effects on animal intake through noradrenalin (Bedecs *et al*., 1992; Branchek *et al*., 2000). In this study the bovine *GAD1* and *GAL* genes were isolated. Single nucleotide polymorphisms (SNPs) were identified, and allele frequencies in four bovine breeds, and the associations with feed intake, growth and partial carcass traits were investigated. This study is a first step to a further understanding of whether bovine *GAD1* and *GAL* alleles could serve as genetic markers for feed intake, growth and carcass traits in cattle.

Materials and Methods

Fifty one crossbred steers (Simmental crossed with indigenous female yellow cattle in China) (405 ± 50.5 kg; 30 ± 2 months of age) were selected randomly and housed individually in a concrete-floored cowshed. The pre-trial period (for adaptation to treatment) was 15 days and the test period 180 days. The steers were fed according to the NY/T 815-2004 feeding standards for beef cattle (Agricultural Department of People's Republic of China, 2004), and had free access to water and feed during the entire 195 d of the trial. All animals were weighed at 08:00 on days 1, 75 and 195, with prior removal of feed and water (12 h). Feed not consumed was weighed and recorded at 20:00 each day. At the end of the feeding period the steers were slaughtered at a processing facility (KeErQing Beef cattle Co., Ltd., P. R. China). Carcass measurements were taken according to criterion GB/T 17238-1998 of the Cutting Standard for Fresh and Chilled Beef in China (China Standard Publishing House). In addition, 84 animals including Simmental (n = 28), Angus (n = 28) and Hereford (n = 28) were randomly selected from commercial populations and used to determine *GAD1* and *GAL* allelic frequencies.

Blood samples were collected from all steers on day 75 of the experiment. The routine phenol chloroform extraction method was used to extract the genomic DNA, and was diluted to 50 ng/μL for PCR.

Traits recorded were: Dressing percentage (DP; %), backfat thickness (BF; cm), carcass weight (CW; kg), meat percentage (MP; %), average daily gain (ADG; kg), average daily feed intake (ADFI; kg), ratio of feed-to-meat (RFM), beginning average daily gain (BADG; kg), finishing average daily gain (FADG; kg), loin-muscle area (LMA; cm^2), carcass chest width (CCW; cm) and carcass length (CL; cm). All data on intake, growth and carcass traits were measured or calculated. The data were analyzed using the model procedure of SAS (SAS, 2001), according to the following model:

$$Y_{ij} = \mu + G_i + b_{ij}w_{ij} + \varepsilon_{ij}$$

where Y_{ij} stands for observed value; μ for population mean; G_i for the ith genotype; b_{ij} as the regression coefficient; w_{ij} the weight and ε_{ij} the random error.

The human mRNA sequence of *GAD1* obtained from NCBI (NM_000817) was employed to search for bovine expressed sequence tag (ESTs) in a NCBI database through standard BLAST (http://www.ncbi.nlm.nih.gov/blast/) algorithm (Wang *et al*., 2003; Yang *et al*., 2003; Zhu *et al*., 2005). The bovine ESTs that had more than 80% similarity with the corresponding human mRNA were identified as, and assembled into contigs, and used to design primer pairs using a primer design, software Primer 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 1) and synthesized by Shanghai Bioasia Biotechnology Co. Ltd in China.

As for the *GAL* gene, after alignment, the bovine mRNA sequence (NM_173914) with the human DNA sequence (NC_000011), primers to amplify across putative intron regions were designed based on the bovine *GAL* mRNA sequence (Table 1).

The PCR products were obtained in 20 μL reaction volume consisting of 50 ng of bovine genomic DNA, 1 × PCR buffer, 0.3 μm of each primer, 75 μm of each dNTP, 1.5 mM $MgCl_2$ and 2.0 units *Taq* DNA polymerase (Fermentas). The PCR parameters were: 5 min at 95 °C followed by 30 s at 94 °C, 30 s at the annealing temperature (Table 1) and 30 s at 72 °C for 34 cycles and a final extension of 72 °C at 10 min. PCRs were performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc, Hercules, CA, USA). The products were purified with the Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology Co., Ltd. P. R. China), cloned into the pGEM-T Easy Vector Kit (Promega, Madison, WI, USA) and sequenced (Shanghai Bioasia Biotechnology Co., Ltd. P. R. China) (Applied Biosystems 3730xl DNA Analyzer, Foster city, CA, USA). The sequenced fragments of these genes were then used in the BLAST searches against the "nr" databases at the NCBI to ensure they were the expected bovine sequences.

By comparing the sequences of the PCR fragments amplified from 10 individuals that represented five breeds (Simmental, Angus, Hereford, Gailouwei and Shanhe cattlle), we identified three single nucleotide polymorphisms (SNPs). Polymorphisms were genotyped with PCR-RFLP. The PCR product was digested with restriction endonucleases at 37 °C for 4 h. The restriction fragments were scored on 1.5% agarose gels. The allele frequency analysis included 135 (including 51 steers) unrelated animals from four breeds (Table 2). Analysis of *GAL* intron sequence revealed two mutations, at 102 (A/G transition) and 837 (C/G transition) of the targeted sequence (Accession no. DQ146944), which were located in the 3rd intron. Two pairs of primers were designed for genotyping (Table 1) and amplifying 463bp and 514bp fragments, respectively.

Table 1 Primers used for polymorphism search

Gene names	Primer names	Primer sequences	Tm (°C)	Size (bp)	Binding region
GAD1	(F5)	5'-GGAGTCACAGAGATTGGTCA-3'	60	553	3'UTR
	(R5)	5'-AGAGTCGTTGTTCCAGGTGT-3'			3'UTR
GAL[1]	(3F)	5'-AAGAGAGGCTGGACCCTGAA-3'	60	2815	3exon
	(3R)	5'-CGAGGCCATGCTTGTCTTGA-3'			4exon
	(3F)	5'-AAGAGAGGCTGGACCCTGAA-3'	65	465	3exon
	(R5)	5'-CAGTCTTGGCGTGAGCATGT-3'			3intron
	(F6)	5'-AAACATGCTCACGCCAAGAC-3'	62	514	3intron
	(R6)	5'-CTGATGGCTATCCTCAACGA-3'			3intron

[1] The primer pair 3F-3R was used to isolate the 2816bp length genomic DNA fragment of *GAL*. The primer pair 3F-R5 and the primer pair F6-R6 were used to isolate 463bp and 514bp which were selected from the exon-intron region and intron of 2816bp bovine fragment

Results and Discussion

In order to investigate allele frequencies of these SNPs in beef populations, we developed RFLP assays and genotyped 135 unrelated animals from four different breeds (Simmental, Cross Simmental, Angus and Hereford).

The *GAD1* 553bp fragment revealed a polymorphism *Ssp I* restriction site by a nucleotide substitution within 3-UTR at position 409(G/A transition). Three distinct genotypes were observed: (1) AA-fragment 553bp, (2) BB-fragment: two fragments of 410bp and 143bp, and (3) AB-three fragments. Genotyping results (Figure 1) revealed that the average frequency for allele A was 0.0663 (0 - 0.1154) and for allele B, 0.9337 (0.8846 -1.0000).

Table 2 The number of the genotypes in different breeds

Gene	Genotype	Breeds							
		Simmental	N	Angus	N	Hereford	N	Cross Simmental	N
GAD1	AA	0	28	1	26	0	27	1	51
	AB	4		4		0		6	
	BB	24		21		27		44	
*GAL*102	AA	11	26	12	27	21	27	21	51
	AB	12		14		5		20	
	BB	3		1		1		10	
*GAL*837	AA	4	27	4	28	4	28	8	51
	AB	13		20		10		23	
	BB	10		4		14		20	

Amplifying a 463bp fragment of *GAL* and containing a polymorphic *Pst I* restriction site caused by the substitution (A/G) at nucleotide 102 of the 463bp fragment, three distinct genotypes were observed: (1) AA-fragment 463bp, (2) BB-fragment: two fragments of 360 and 103bp, (3) AB-three fragments.

Genotyping results (Figure 1) revealed that the average frequency of allele A was 0.6771 (0.6324 - 0.8704), and for allele B, 0.3229 (0.1296 - 0.3658).

A 514bp fragment of the bovine *GAL* gene was amplified and a C/G substitution at nucleotide 396 was identified. After digestion of the 514bp fragment with *BSH1236 I*, three genotypes were identified: (1) AA-fragment 514bp, (2) BB-fragment: two fragments of 397 and 117bp, (3) AB-three fragments. Genotyping results (Figure 1) revealed that the average frequency for allele A was 0.4215 (0.3214 - 0.5000), and for allele B, 0.5785 (0.5000 - 0.6786). A summary of genotyping results of the three polymorphisms is included in Table 2.

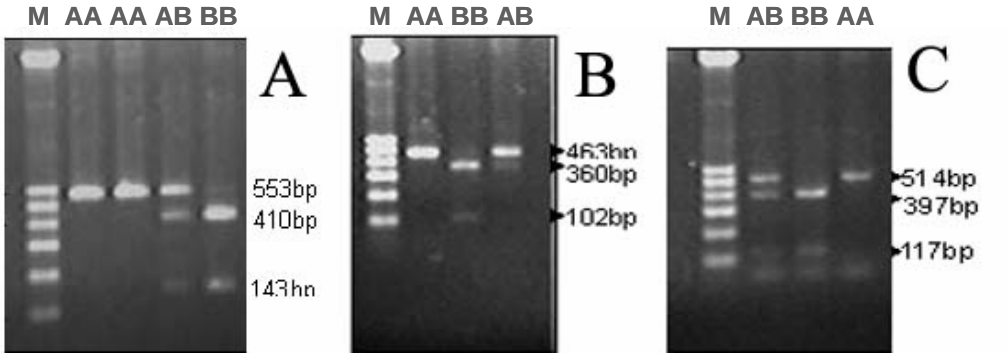

Figure 1 Agrose gel (2%) showing different genotypes of bovine *GAD1* and GAL genes. M:100-600bp ladder. A, B and C represent the *Ssp I*, *Pst I* and *Bsh1236 I* loci, respectively. The genotypes are given at the top of the columns

Table 3 Effect of *GAD1* and *GAL* genotype on feed intake, growth and carcass characteristics in the cattle

Genotypes	Traits (mean ± s.e.)*					
	ADG kg	CW kg	DP %	MP %	BF cm	BADG kg
GAD1						
AA	0.99 ± 0	290.20 ± 0	54.80 ± 0	52.07 ± 0	0.75 ± 0	0.93 ± 0
AB	0.75 ± 0.08	282.23 ± 10.24	53.76 ± 0.42	50.00 ± 0.72	1.39 ± 0.20	0.74 ± 0.14
BB	0.75 ± 0.03	273.63 ± 4.45	53.00 ± 0.20	50.18 ± 0.51	1.13 ± 0.05	0.68 ± 0.07
GAL (102 SNP)						
AA	0.70 ^{b} ± 0.04	269.63 ± 6.10	53.12 ± 0.26	51.57 ^{a} ± 0.85	1.07 ^{b} ± 0.08	0.55 ± 0.12
AB	0.74 ^{b} ± 0.04	276.94 ± 6.76	52.96 ± 0.32	49.41 ^{ab} ±0.44	1.25 ^{a} ± 0.08	0.80 ± 0.09
BB	0.89 ^{a} ± 0.04	284.48 ± 7.82	53.19 ± 0.51	48.90 ^{b} ± 0.72	1.15 ^{ab} ± 0.10	0.77 ± 0.08
GAL (837 SNP)						
AA	0.71 ± 0.09	271.44 ± 7.32	53.92 ± 0.07	52.17 ± 2.19	0.90 ± 0.12	0.61 ± 0.12
AB	0.73 ± 0.04	272.50 ± 6.85	52.92 ± 0.30	50.27 ± 0.73	1.13 ± 0.06	0.61 ± 0.12
BB	0.80 ± 0.04	277.02 ± 5.60	53.17 ± 0.31	49.46 ± 0.51	1.19 ± 0.09	0.79 ± 0.08

* Within columns means with different superscripts indicate significant differences at $P < 0.05$
ADG - average daily gain; CW - carcass weight; DP - dressing percentage; MP - meat percentage; BF - backfat thickness; BADG - beginning average daily gain

Association studies were carried out only on the 51 Simmental crossbred steers. As described in the Materials and Methods, a carefully designed 195 days feed intake and growth study was performed on the animals. After analyzing the relationship of the genotypes (AA, AB and BB) and their phenotypic data (Tables 3 and 4) for the 12 traits, we found that the SNP in GAL was significantly correlated to ADFI ($P < 0.01$). Animals with an AA (3.59 ^{c} ± 0) genotype had a significant lower (~40%) ADFI in comparison to the BB (6.24 ^{a} ± 0.36) or AB (5.53 ^{b} ± 0.85) genotypes. However, as the frequency of the recessive allele A is rather low (~ 0.0663) in the four beef populations investigated, this SNP deviates from the Hardy Weinberg

Law. Therefore, the feasibility of using this SNP for marker-assisted selection (MAS) needs to be further studied in larger populations of different beef breeds. The SNP（A/G^{102}）in GAL is significantly related to ADFI and RFM ($P < 0.01$). Animals with a BB ($5.19^{c} \pm 0.72$) genotype had a significant lower (~18%) ADFI in comparison to the AA ($6.15^{a} \pm 0.51$) and AB ($6.47^{b} \pm 0.50$) genotypes. The SNP was also related to growth traits (FADG) and carcass traits (MP, LMA, and CCW), but less pronounced ($P < 0.05$). Animals with the BB genotype had higher ($P < 0.05$) CCW, LMA and FADG, than the AB and AA genotypes, though effects of both AA and AB genotypes were not consistent. The BB genotypes had a significant higher ($P < 0.05$) ADG than the AA and AB genotypes. However, it is reasonable to attribute this to the small population employed in the study. Genotypes had no effect on other traits ($P > 0.05$) investigated (Tables 3 and 4). The SNP（C/G^{837}）in GAL is significantly related to ADFI and RFM ($P < 0.01$). Animals with an AA ($4.77^{c} \pm 1.10$) genotype had a lower (~21.8%) ($P < 0.05$) ADFI in comparison to the BB ($5.17^{b} \pm 0.44$) and AB ($7.27^{a} \pm 0.48$) genotypes. SNP was also correlated to CCW, but the differences were less pronounced ($P < 0.05$) than those of ADFI and RFM. It had no effect on the other traits ($P > 0.05$) investigated (Tables 3 and 4).

Table 4 Effect of *GAD1* and *GAL* genotype on feed intake, growth and carcass characteristics in the cattle

Genotypes	Traits (mean ± s.e.)*					
	LMA cm^2	CCW cm	CL cm	FADG kg	RFM	ADFI kg
GAD1						
AA	63.00 ± 0	73.00 ± 0	152.00 ± 0	0.83 ± 0	3.62 ± 0	$3.59^{c} \pm 0$
AB	65.00 ± 3.97	71.17 ± 0.75	148.17 ± 1.30	0.63 ± 0.09	8.38 ± 2.27	$5.53^{b} \pm 0.85$
BB	64.18 ± 1.67	70.50 ± 0.42	145.57 ± 0.94	0.62 ± 0.02	9.05 ± 0.69	$6.24^{a} \pm 0.36$
GAL (102 SNP)						
AA	$66.05^{ab} \pm 2.31$	$69.60^{b} \pm 0.68$	144.05 ± 1.55	$0.59^{b} \pm 0.04$	$10.08^{a} \pm 1.30$	$6.15^{a} \pm 0.51$
AB	$60.76^{b} \pm 2.53$	$71.33^{a} \pm 0.46$	147.48 ± 1.03	$0.60^{b} \pm 0.03$	$8.97^{a} \pm 0.74$	$6.47^{b} \pm 0.50$
BB	$68.70^{a} \pm 2.07$	$71.65^{a} \pm 0.75$	147.40 ± 1.46	$0.76^{a} \pm 0.04$	$5.87^{b} \pm 0.70$	$5.19^{c} \pm 0.72$
GAL (837 SNP)						
AA	64.80 ± 3.83	$67.60^{b} \pm 1.50$	143.80 ± 2.27	0.62 ± 0.06	$8.17^{b} \pm 3.42$	$4.77^{c} \pm 1.10$
AB	62.04 ± 2.74	$70.80^{a} \pm 0.56$	145.96 ± 1.39	0.61 ± 0.03	$10.75^{a} \pm 1.01$	$7.27^{a} \pm 0.48$
BB	67.20 ± 1.52	$71.08^{a} \pm 0.50$	146.70 ± 0.91	0.66 ± 0.04	$6.75^{b} \pm 0.60$	$5.17^{b} \pm 0.44$

* Within columns means with different superscripts indicate significant differences at $P < 0.05$
LMA - loin-muscle area; CCW - carcass chest width; CL – carcass length; FADG - finishing average daily gain; RFM - ratio of feed-to-meat; ADFI - average daily feed intake

Conclusion

It is believed that 3'-UTR and intron sequences are usually involved in regulatory roles of genes. The two SNPs located in the intron 3 of *GAL* gene and one in 3'-UTR of *GAD1* gene affected the feed intake, growth and carcass traits in the experimental population studied. For the *GAD1* gene, our experimental data showed that animals with a BB genotype have a significantly higher food intake. It is reasonable to predict that BB animals should have a faster growth rate because the higher ADFI, while the AA genotype would be low. For the *GAL* gene, our experimental data showed that animals with an AB genotype had a significantly lower food intake, faster growth rate, bigger carcass chest width, and hence, a significantly lower feed-to-meat ratio (RFM). This suggests that these *GAD1* and *GAL* SNPs could be good candidate markers for MAS. However, further investigations in larger bovine populations would be useful for a better understanding of the effects of *GAD1* and *GAL* polymorphisms. The association could be causal or the *GAD1* and *GAL* polymorphism could simply be acting as a genetic marker for a linked QTL with effects on ADFI.

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