Association of CAST Gene Polymorphisms with Carcass and Meat Quality Traits in Chinese Commercial Cattle Herds*

Jiao Li, Lu-Pei Zhang, Qian-Fu Gan, Jun-Ya Li, Hui-Jiang Gao, Zheng-Rong Yuan,
Xue Gao, Jin-Bao Chen and Shang-Zhong Xu**

Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

ABSTRACT : Calpastatin (CAST), an endogenous inhibitor of the calpains, plays an important role in post-mortem tenderization of meat. The objectives of this study were to investigate single nucleotide polymorphisms (SNPs) in the bovine *CAST* gene and association with carcass and meat quality traits. A total of 212 cattle from commercial herds were tested in this study including 2 pure introduced breeds, 4 cross populations, and 3 pure Chinese native breeds. Five SNPs were identified at position 2959 (A/G), 2870 (G/A), 3088 (C/T), 3029 (G/A) and 2857 (C/T) in the *CAST* gene (GenBank Accession No. AF159246). Allele frequencies of SNP2959 and SNP2870 were 0.701 (A) and 0.462 (A), respectively. A general linear model was used to evaluate the associations between the two markers and 7 traits. The results showed that both SNP2959 and SNP2870 were significantly (p<0.01) associated with the Warner-Bratzler shear force (WBSF), while they had no significant association with the other 6 traits in the whole population. However, in Chinese native pure breeds, only SNP2870 had significant association with WBSF (p<0.05). The simultaneous analysis of two-marker genotype effects indicated animals containing the A/G haplotype (A for SNP2959 and G for SNP2870) tended to have lower shear force than those containing the G/A haplotype, and, especially, animals homozygous for the A/G haplotype had approximately 2 kg lower shear force than those homozygous for the G/A haplotype (p<0.01). These results suggested that both markers may be effective for the marker-assisted selection of meat quality traits in Chinese commercial herds, especially SNP2870 which can be used for Chinese native cattle. (**Key Words :** Calpastatin Gene, Carcass and Meat Quality Traits, Beef Cattle, SNPs)

INTRODUCTION

Calpastatin (CAST), initially identified in skeletal muscle by Busch et al. (1972), is the endogenous inhibitor of the calpains (Goll et al., 2003), which can remove the Z-disc from the skeletal muscle myofibrils in the presence of Ca^{2+} and play an important role in post-mortem tenderization of meat (Goll, 1974; Richard et al., 1995). Thus, the calpastatin functions as a regulator of the rate and extent of post-mortem tenderization (Koohmaraie, 1994; Koohmaraie et al., 1995). The bovine *CAST* gene, mapped

to BTA 7 (Bishop et al., 1993), has a relationship with a QTL for shear force and has been proposed as a positional and functional candidate gene for this QTL (Choi et al., 2006; Drinkwater et al., 2006; Davis et al., 2008).

Several studies have reported the genetic variation which occurred in the bovine *CAST* gene, such as in coding regions (Chung et al., 1999) and non-coding regions (Schenkel et al., 2006; Juszczuk-Kubiak et al., 2008). The SNP2959 in the 3' untranslated region (UTR) of the *CAST* gene was identified by Barendse (2002), Morris (2006), Casas (2006), and Curi (2008), while SNP2870, also in the 3' UTR of the *CAST* gene, was first identified by Corva (2007). In recent years, some companies have performed genetic tests for meat tenderness in beef cattle by utilizing these genetic polymorphisms in the *CAST* gene. For examples: UoG-CAST was applied in the Igenity TenderGENE test panel (Merial Ltd., Atlanta, GA) and SNP2959 was applied in the GeneSTAR Tenderness test panel (Genetic Solutions Pty. Ltd., Albion, Australia). However, no similar studies were reported on these markers in Chinese commercial herds.

The objectives of the present study were to investigate SNPs in the bovine *CAST* gene and evaluate the association between SNPs and meat tenderness and other important carcass and meat quality traits in Chinese commercials herds.

MATERIALS AND METHODS

Animals and management

For studying associations between gene markers and beef cattle traits, a total of 212 commercially fed steers whose age at the start of the experiment ranged from 20 to 35 months were randomly selected from three beef feedlots (Dachang, Sanyuan and Baolongshan). These animals consisted of eight cattle breeds: Angus (N = 33), Hereford (N = 18), Limousin×Luxi (N = 18), Charolias×Fuzhou (N = 23), Simmental×Jinnan (N = 48), Simmental×Mongolian (N = 26), Luxi (N = 15), Jinnan (N = 17) and Qinchuan (N = 14). All cross animals were F1 calves that came from native breeds of Fuzhou, Luxi, Jinnan and Mongolian sired respectively by bulls of Charolais, Limousin Simmental and Simmental. Charolais and Limousin bulls were progenies of pure breed animals introduced from France, and Simmental bulls were progenies of pure breed Fleckvieh introduced from Germany. Angus steers and Hereford were progenies of pure bred cattle introduced from Australia and England, respectively. Luxi, Jinnan and Qinchuan were pure Chinese native breeds.

The diets in the three beef feedlots had different components, but were mainly composed of 50% corn grain, 18-20% cotton seed cake, 10-11% distiller's grains, 11% wheat bran plus 4-5% vitamins and mineral supplements. A pretest adjustment period of 20 d was allowed for the animals to adapt to the diets and environment. Test animals were raised in feedlots for 201 d including the adjustment period of 20 d. In the feedlot, feeding stalls were housed in a shed with the long (north and south) sides closed and the 2 short (east and west) sides open to form a large pen in which animals could move freely. A feeding regime of three times a day was followed during the experiment period. Just before the feeding time, every animal was kept in the feeding stall marked with the same number and released to the pens after feeding. Residual feed intake was collected, weighed and recorded daily.

Phenotypic information

Carcass and meat quality traits were measured according to the criterion GB/T 17238-1998 Cutting Standard of Fresh and Chilled Beef in China (China Standard Publishing House). Slaughter body weight (SBW) was measured just before slaughter after a 24 h period of fasting. The rib area (REA, cm²) was measured at the 12th

and 13th rib interface. The Warner-Bratzler shear force (WBSF) was measured after 7 days post-mortem. Carcass weight (CW) was measured just after slaughter. Other carcass traits (dressing percentage (DP), marbling score (MBS) and back fat thickness (BFT) measurements were carried out at 4 days post-mortem. Meat samples for measuring WBSF were taken from the interface between the 12th and 13th rib. The evaluation of MBS in China is a 6 point regime, the numbers 1 to 6 correspond to traces, slight, small, modest, moderate and abundant. The details are shown in Table 1.

SNP identification and genotyping

DNA samples were extracted from blood samples by phenol/chloro extraction. Based on the 3' UTR of the bovine *CAST* gene sequence (GenBank accession AF159246), one pair of primers (F: 5'-ACATTCTCCCCACAGTGCC-3' and R: 5'-GACAGA GTCTGCGTTTTGCTC-3') was designed. Polymerase chain reaction (PCR) amplifications were performed in a 20-μl volume containing 50 ng of DNA template, 10 pM each primer, 0.25 mM dNTP, 2.5mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (TaKaRa, Dalian, China). The PCR protocol was 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 40 s, and a final extension at 72°C for 10 min. The PCR products were 375 bp fragments, which were purified by using a Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology, P.R. China) and sequenced (Beijing Aolaibo Biotechnology, P.R. China; Applied Biosystems 3730×1 DNA sequencer, Foster city, CA, USA).

Genotyping was performed using PCR-RFLP (restriction fragment length polymorphism). 4 μl of the PCR product was digested for 8 h with 10 U of the corresponding restriction enzyme, Dde I at 37°C for SNP2959 and TspE I at 65°C for SNP2870.

Statistical analyses

The linkage disequilibrium (LD) and haplotype analysis was performed by SHEsis software (Shi and He, 2005; Li et al., 2009) and other analysis was performed using SAS

Table 1. Number of phenotypic records, means and standard deviations

Traits	Number	Mean	SD
Slaughter body weight (kg)	212	566.17	74.72
Carcass weight (kg)	212	315.53	48.81
Dressing percentage (%)	212	55.51	3.25
Rib area (cm²)	212	73.42	13.46
Marbling score (1-5)	212	2.16	1.02
Back fat thickness (cm)	212	1.09	0.48
Warner-Bratzler Shear force (kg)	212	4.26	1.53

software (Veraion 9.1.2, SAS Institute, Inc, Cary, NC). Descriptive characteristics of quantitative traits were obtained using PROC MEANS, allele frequencies were tabulated and compared by Fisher's Exact Test among genetic groups, and PROC FREQ was used to compute Monte Carlo estimates of the exact p-values, PROC ALLELE was employed to provide tests of linkage disequilibrium between each pair of markers. To keep reasonable probability values for Type I errors, a modified Bonferroni correction was performed to test each of the individual tests of multiple comparisons at a significance level α/n, and keep the overall significance level of tests to be α. The value of n was equal to the number of genotypes for each SNP tested (n = 3).

Associations between genotype and BSW, REA, HCW, DP, MBS, BFT and TS were evaluated using PROC GLM, fitting the following model:

$$Y_{ijkl} = \mu + BF_i + Month_j + G_k + e_{ijkl},$$

Where Y_{ijkl} = observed value; μ = overall mean for each trait; BF_i = fixed effect of ith breed and farm, i = 1,2,…11; $Month_j$ = fixed effect of jth month of slaughtering, j = 1,2,…13; G_k = fixed effect of kth single SNP marker genotype, k = 1,2,3, e_{ijkl} = random error, l = 1,2,…213.

RESULTS

Genotype and allele frequencies

Five SNPs, A2959G, A2870G, C3088T, G3029A and C2857T, were detected in 375-bp DNA fragments by sequencing. SNP3088, SNP3029 and SNP2857 were not reported before detection in this study, but their polymorphism information content (PIC) was less than 0.25

(data not shown). The genotype and allelic frequencies of SNP2959 and SNP2870 for each breed, combined genetic group and the whole populations are presented in Table 2.

For SNP2959, the GG genotype was absent in the Qinchuan population, and the A allele frequencies found in introduced breeds, crossbreed, Chinese native breeds and the whole populations were 0.677, 0.717, 0.656 and 0.701, respectively. Moreover, the Simmental×Mongolian group had a higher frequency of the A allele (0.841) than other breeds. For SNP2870, the AA genotype was absent in the Charolias×Fuzhou population, and the A allele frequencies found in introduced breeds, crossbreed, Chinese native breeds and the whole population were 0.373, 0.474, 0.533 and 0.462, respectively. Qinchuan tended to have a higher frequency of the A allele (0.679) than other breeds.

The LD analysis indicated that the SNP2959 and SNP2870 were weakly linked (D' = 0.348). Four haplotypes were found in all 212 steers, and the frequency of haplotype A/A, A/G, G/A and G/G was 0.27, 0.43, 0.20, 0.10, respectively.

Association analysis

Least squares means (LSM), standard errors (SE) and levels of significance are presented in Table 3. The gene-specific SNP association analysis showed that both the SNP A2959G and G2870A had significant association with WBSF (p<0.01). No significant associations were observed between the two markers and the other six traits.

Association analysis on SNP2959 in the 212 cattle showed that animals with genotype GG (5.13±0.28 kg) had significantly higher WBSF than those with genotype GA (4.01±0.22 kg) or AA (3.98±0.19 kg) (p<0.01). The LSM difference between genotype GG and GA, GG and AA, GA and AA was 1.12 kg, 1.15 kg and 0.03 kg, respectively. For

Table 2. Genotype frequencies and allelic frequencies within breed groups, combined genetic groups and in entire beef steer population for the 2 SNPs in the *CAST* gene

| SNP | Genotype | Breed group | | | | | | | | | Combined genetic group[2] | | | Overall N = 212 |
		Limousin ×Luxi N[1] = 18	Charolais ×Fuzhou N = 23	Simmental ×Jinnan N = 48	Simmental ×Mongolian N = 26	Qinchuan N = 14	Luxi N = 15	Jinnan N = 17	Angus N = 33	Hereford N = 18	Introduced breeds N = 51	Crossbreeds N = 115	Native breeds N = 46	
2959	GG	0.167	0.087	0.073	0.091	0	0.133	0.235	0.242	0.111	0.196	0.096	0.130	0.127
	GA	0.333	0.609	0.382	0.136	0.5	0.467	0.177	0.212	0.333	0.255	0.374	0.370	0.344
	AA	0.500	0.304	0.546	0.773	0.5	0.4	0.588	0.546	0.556	0.549	0.530	0.5	0.528
	Frequency of A	0.667	0.609	0.737	0.841	0.75	0.633	0.677	0.652	0.722	0.677	0.717	0.656	0.701
2870	GG	0.222	0.348	0.255	0.455	0.143	0.333	0.412	0.333	0.5	0.392	0.252	0.304	0.297
	GA	0.667	0.652	0.527	0.364	0.357	0.4	0.235	0.515	0.389	0.471	0.548	0.326	0.481
	AA	0.111	0	0.218	0.182	0.5	0.267	0.353	0.152	0.111	0.137	0.2	0.370	0.222
	Frequency of A	0.444	0.326	0.482	0.364	0.679	0.467	0.471	0.409	0.306	0.373	0.474	0.533	0.462

[1] Number of animals.

[2] Introduced breeds represent pure breed Angus and Hereford, Crossbreds represent Limousin×Luxi, Charolais×Fuzhou, Simmental× Jinnan and Simmental×Mongolian, Native breeds represent pure breed Luxi, Jinnan and Qinchuan.

Table 3. Associations between 2 SNPs and 7 traits in commercial beef steer population (Values represent least squares mean±standard error)

Marker	N	SBW[1] (kg)	CW[1] (kg)	REA[1] (cm^2)	DP[1] (%)	MBS[1] (1-5)	BFT[1] (cm)	WBSF[1] (kg)
G2959A								
GG	27	558.27±12.01	307.15±7.99	72.58±2.28	54.54±0.60	2.02±0.19	1.07±0.09	5.13 [a]±0.28
GA	73	564.74±9.10	318.39±6.06	72.20±1.72	55.91±0.45	2.09±0.14	1.19±0.07	4.01 [b]±0.22
AA	112	564.90±7.86	313.44±5.23	72.46±1.49	55.24±0.39	2.28±0.12	1.17±0.06	3.98 [b]±0.19
p-value		0.864	0.422	0.983	0.094	0.241	0.561	0.0005*
G2870A								
GG	63	563.16±9.45	313.96±6.32	71.71±1.79	55.37±0.48	2.20±0.15	1.14±0.07	3.94 [b]±0.23
GA	102	566.39±7.70	314.88±5.15	72.32±1.46	55.19±0.39	2.12±0.12	1.21±0.06	4.10 [b]±0.18
AA	47	556.22±10.54	310.39±7.05	73.49±2.00	55.57±0.53	2.31±0.17	1.04±0.08	4.81 [a]±0.25
p-value		0.63	0.82	0.71	0.77	0.54	0.097	0.005*

[1] Slaughter body weight (SBW), carcass weight (CW), rib area (REA), dressing percentage (DP), marbling score (MBS), back fat thickness (BFT), Warner-Bratzler Shear force (WBSF).
[a,b] Within a column, means with different superscripts were significantly different, p<0.05.
* Significance level, p<0.01.

SNP G2870A, the WBSF value for the animals with genotype AA (4.81±0.25 kg) was significantly higher than the WBSF value of those with genotype GG (3.94±0.23 kg) or GA (4.10±0.18 kg) (p<0.01). The LSM difference between genotype AA and GA and between AA and GG was 0.71 kg and 0.87 kg, respectively. The LSM difference between genotype GA and GG was also only 0.16 kg.

Considering the uniform genetic background across the animals, the results might be inaccurate. So, further association between the two markers and WBSF was analyzed in three groups with different genetic background

separately, as shown in Table 4. The results showed that marker A2959G was significantly associated with WBSF in IB group (introduced breeds) (p<0.05) and CB (cross breeds) group (p<0.05), but had no significant correlation with WBSF in NB (native breeds) group. On the contrary, the marker G2870A had a significant effect on WBSF in native breeds (p<0.05), with a 1.56 kg difference in WBSF between genotype AA and genotype GG.

The two-marker genotype data presented in Table 5 were used to determine two-marker haplotype frequencies unambiguously for the 170 steers which were homozygous

Table 4. Associations between 2 SNPs and Warner-Bratzler Shear Force (WBSF) values in different genetic group (Values represent Least squares means±standard error)

Marker	Genetic group	Genotype			Average	p-value
		GG (No.)	GA (No.)	AA (No.)		
G2959A	IB[1]	3.91±0.53[a] (10)	2.87±0.45[b] (13)	2.73±0.38[b] (28)	3.68±0.24	0.041**
	CB[1]	5.29±0.43[a] (11)	4.15±0.27[b] (43)	4.12±0.24[b] (61)	4.40±0.13	0.029**
	NB[1]	5.70±0.69 (6)	4.54±0.42 (17)	4.54±0.36 (23)	4.72±0.22	0.334
G2870A	IB[1]	2.19±0.47 [a] (20)	3.08±0.37 [b] (24)	2.89±0.66 [ab] (7)	3.68±0.24	0.083*
	CB[1]	4.44±0.31 (29)	4.12±0.22 (63)	4.87±0.36 (23)	4.40±0.13	0.088*
	NB[1]	3.74±0.44 [b] (14)	4.86±0.46 [b] (15)	5.30±0.36 [a] (17)	4.72±0.22	0.032**

[1] Introduced breeds (IB), Crossed breeds (CB), and Native breeds (NB).
[a,b] Within a row, means with different superscripts were significantly different, p<0.1.
* Significance level, p<0.1. ** Significance level, p<0 .05.

Table 5. Combined 2-marker genotypic effects on Warner-Bratzler shear force (kg) and frequencies from 212 cattle

G2870A	G2959A					
	GG		GA		AA	
	WBSF (kg)[1]	No.	WBSF (kg)	No.	WBSF (kg)	No.
GG	4.58±0.68	4	4.19±0.41	12	3.70±0.24	47
GA	4.82±0.38	14	3.67±0.25	42	4.08±0.23	46
AA	5.78±0.47	9	4.49±0.34	19	4.48±0.35	19

for at least one of the two markers. For example, if an animal's genotype at the two markers was GG and GA, then it must carry one G/G haplotype and one G/A haplotype. However, the remaining 42 calves were double heterozygotes (GA genotype for SNP2959 and SNP2870), and could not be definitively assigned haplotypes because they could result either from a combination of G/A and G/A haplotypes or G/G and A/A haplotypes. Comparing the LSM of WBSF, it was not difficult to find that beef from animals containing A/G haplotype tended to have lower shear force than beef which containing G/A haplotype, and, especially, animals homozygous AA for SNP2959 and homozygous GG for SNP2870 (i.e., homozygous for A/G haplotype) had shear force approximately 2 kg lower than homozygous GG for SNP2959 and homozygous AA for SNP2870 (i.e., homozygous for the G/A haplotype; p<0.01). For homozygous G/G haplotype and A/A haplotype animals, the shear force was intermediate.

DISCUSSION

The polymorphisms of the *CAST* gene have been reported in foreign breeds, such as Hereford (Van Eenennaam et al., 2007), Angus (GeneNOTE 4), Brahman (Casas et al., 2006) and Sanga (Burrow, 2003). So, it was valuable to note that the sample population in this study consisted of 9 breeds, including 4 crossbreeds, 3 Chinese native breeds and 2 introduced breeds. So these results will provide useful information in selecting animals with better carcass and meat quality traits in Chinese commercial herds.

We have characterized variation in the bovine *CAST* gene to identify markers affecting meat quality and found 5 polymorphisms in a 375-bp DNA fragment by sequencing. Three of these were firstly identified, caused by G to A, C to T and C to T substitution at positions 3029, 3088 and 2857 (according to the GenBank sequence AF 159246), respectively. However, the mutant allele frequencies of the 3 novel SNPs were low, and have not been researched deeply here. In the current study, allele A of SNP2959 and allele G of SNP2870 were associated with increased tenderness. The frequency of meat-tenderness favorable allele A of SNP2959 was 0.609 (Charolias×Fuzhou) to 0.841 (Simmental×Mongolian) which agreed with Curi (2008), who genotyped 147 *Bos indicus* and *Bos Taurus×Bos indicus*, and observed that the A allele frequency was 0.42 to 0.85. For SNP2870, the A allele frequency varied from 0.306 to 0.679, obviously higher than documented by Casas (2006), which varied from 0.24 to 0.53 in 313 steers from crosses between Angus, Hereford and Limousin cattle.

In the current work, SNP2959 and SNP2870 showed highly significant correlation with WBSF and no significant correlations with slaughter body weight, rib area, carcass weight, dressing percentage, marbling score and back fat thickness. Here, WBSF is the force required to shear a cooked steak after 7 days post-mortem, and the WBSF values are negatively inter-correlated with *tenderness* values (Moon, 2006). Casas (2006) indicated animals with AA genotype for SNP2870 had higher average shear force than animals with GG genotype, but no significant effect on WBSF was detected. Obviously, these results were not completely consistent with ours, which might arise due to different size and composition of the test animals. Furthermore, the LSM difference of WBSF between genotype GA and AA on SNP2959 (0.03 kg) and difference between GA and GG on SNP2870 (0.16 kg) were significantly lower than others. These results indicated that the allele A of SNP2959 and the allele G of SNP2870 have dominance effects over their allelic genes.

However, this paper also indicated that animals inheriting the GG genotype at position 2959 tended to have lower slaughter body weight, carcass weight, dressing percentage, marbling score, back fat thickness, and higher rib area than animals inheriting the GA or AA genotype. Likewise, beef from steers with the AA genotype at position 2870 tended to have lower slaughter body weight, carcass weight, back fat thickness and higher rib area and dressing percentage than beef from steers with the GA and GG genotype.

The results of association analysis between the 2 markers and WBSF in different genetic groups accorded with the foregoing results, and suggested that SNP2959 had significant effect on WBSF in IB and CB groups, while SNP2870 had more significant association in NB groups. Besides, there was an obvious trend for animals from IB group (3.68±0.24 kg) to have a lower shear force compared with animals from CB (4.40±0.13 kg) or NB groups (4.72±0.22 kg).

In previous studies, researchers independently evaluated the two markers, but they have not simultaneously assessed their effects on meat tenderization. The combined 2-marker genotypic effect analysis showed that animals containing the A/G haplotype tended to have lower shear force than those containing the G/A haplotype. Moreover, animals homozygous for A/G haplotype had shear force approximately 2 kg lower than those homozygous for the G/A haplotype. Overall, this gave a favorable indication that the A/G haplotype might reduce the ability of CAST to inhibit CNPN1 and increase meat tenderness.

Meat tenderness, one of the most important factors leading to consumer satisfaction, mainly depends on the post-mortem meat tenderization process (Koohmaraie, 1994) and CAST can regulate proteolysis by inhibiting μ- and m-calpain activity (Pringle et al., 1997). In this work, SNP2959 and SNP2870 in the *CAST* gene were genotyped

by PCR-RFLP, the corresponding restriction enzymes were DdeI and TspEI, respectively. The method was easy to operate and inexpensive compared to mass spectrograph. In order to facilitate the experiments, one pair of primers was designed to amplify a 375-bp PCR product, which contained the two restriction sites. So only one PCR was needed and the product could be used to genotype the two markers. The 3'UTR has gained attention due to its importance in determination of gene expression by influencing mRNA stability and translational efficiency (Hughes, 2006). For these reasons, it was considered worthwhile to validate the effects of the two polymorphisms on beef tenderness.

Summing up, SNP2959 and SNP2870, located at the 3' UTR of the bovine *CAST* gene, showed a detectable effect on tenderness, and neither of the two markers tested showed any effects on slaughter body weight, rib area, carcass weight, dressing percentage, marbling score and back fat thickness. Specially, SNP2870 might be more effective than SNP2959 in future marker-assisted selection in Chinese native cattle populations. Therefore, these results provide evidence that the *CAST* gene has potential effects on meat tenderness and expands the possibilities for using genetic markers in CAST to improve meat tenderness in Chinese commercial herds, especially native yellow cattle and/or crossbreed descent. Further work will be necessary to study these SNPs in a larger population and investigate association of CAST gene polymorphisms with meat tenderness in Chinese commercial cattle herds.

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