Asian-Aust. J. Anim. Sci.
Vol. 19, No. 8 : 1085 - 1089
August 2006
www.ajas.info

Mapping of the Porcine Calpastatin Gene and Association Study of Its Variance with Economic Traits in Pigs

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ABSTRACT : The objectives of this study were to confirm a location of the calpastatin (CAST) gene in chromosome 2 and to detect associations of genetic variations with economic traits in the porcine CAST gene as a candidate gene for growth and meat quality traits in pigs. Calpastatin is a specific endogenous inhibitor of calpains. The calpain protease system is ubiquitous, and is involved in numerous growth and metabolic processes. Three single nucleotide variations were identified within a 1.6 kb fragment of the porcine CAST gene and these polymorphisms were used for genetic linkage mapping. Linkage and QTL mapping were performed with the National Livestock Research Institute (NLRI) reference families using eight microsatellites and SNP makers in the CAST gene. The porcine CAST gene was mapped adjacent to the markers, SW395 and SW1695 on SSC2 with LOD scores of 15.32 and 8.50, respectively. According to the QTL mapping, a significant association was detected at 82 cM between SW395 and CAST-*Hinf* I for weight at the age of 30 weeks. In addition, an association study was performed with the F_2 animals of NLRI reference families for *Hinf* I, *Msp* I and *Rsa* I polymorphisms in the CAST gene. Two polymorphisms, CAST-*Rsa* I and CAST-*Hinf* I, showed significant correlation for growth traits at $p<0.01$ and $p<0.05$, respectively. (**Key Words :** Calpastatin (CAST), Linkage Mapping, QTL Mapping, Korean Native Pig)

INTRODUCTION

The increase in national gross income influenced consumers to buy pork based on quality rather than quantity. Hence, researches on swine feeding have focused on improving the meat quality together with meat quantity. Also, the swine industry aimed to increase the production of quality meat to meet the consumers' increasing demand for high quality pork. To produce the needed quantity and quality of pork, molecular genetic techniques have been utilized in the meat industry.

Calpastatin (CAST) is a specific endogenous inhibitor of calpains which are intracellular calcium-dependent cysteine proteinases that are present in all mammalian cells. The calpain/capastatin protease system is ubiquitous and is involved in numerous growth and metabolic processes. In the skeletal muscles, the rate of growth is dependent on muscle protein degradation. An *in vitro* study has revealed that CAST levels were significantly decreased during the process of myoblast fusion (Barnoy et al., 1996). Also, this system has been implicated in the regulation of protein turnover and growth (Goll et al., 2003) and in meat texture development (Sensky et al., 1996).

Koohmaraie et al. (1991) reported CAST and μ-calpain activity to be significantly low in pork. Kretchmar et al. (1994) examined calpain and CAST activities in lean and obese lines of pigs at 2.5 and 7 months of age. They found that CAST activities decreased with age in both lines, but the activities were significantly higher in obese pigs than in lean pigs at both ages.

Andersson et al. (1994) reported initially that QTL for growth and fat deposition in pigs were detected on chromosome 4 using genome wide scan. After that, QTL for growth and fat deposition traits were detected using genome-wide scans (Knott et al., 1998; Paszek et al., 1999; Kim et al., 2005).

In this study, the porcine CAST gene was selected as a positional candidate underlying QTL on chromosome 2. The chromosomal location of the porcine CAST gene was

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Received August 24, 2005; Accepted February 23, 2006

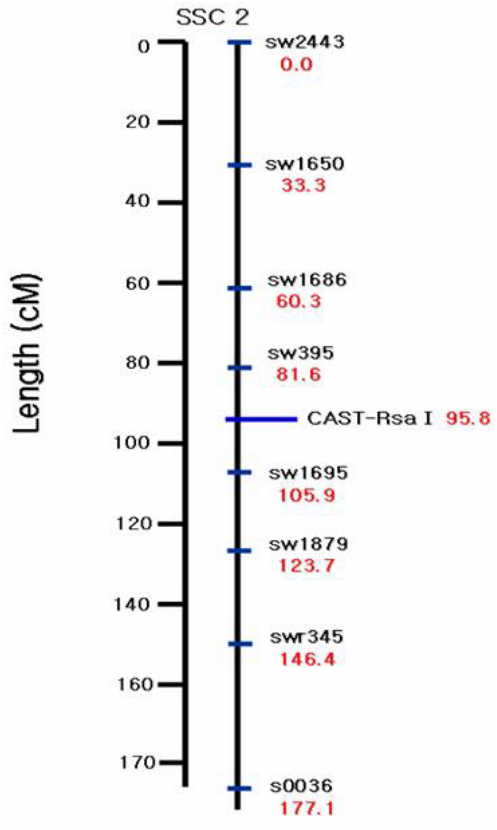

Figure 1. Microsatellite markers and their map distances (cM) with added SNPs of the *CAST* gene in SSC2.

identified, and QTL mapping was performed in the NLRI reference families generated from Korean native boars and Landrace sows using linkage map added SNPs of the CAST gene. In addition, association studies were performed with F_2 animals from the NLRI reference families for *Hinf* I, *Msp* I and *Rsa* I polymorphisms in the CAST gene.

MATERIALS AND METHODS

Experimental animals and phenotyping

The NLRI reference families were constructed from a cross between Korean native boars and Landrace sows. Five boars of Korean native pig and ten sows of Landrace were selected randomly from a herd at the National Livestock Research Institute (NLRI), Rural Development Administration (RDA), Korea. Each boar was mated with two or more different sows to produce the F_1 animals. Each F_1 sire was randomly selected from each litter, and mated with full-sib sows. Thus, 10 sires and 31 dams were used to produce 240 F_2 animals. Phenotypic data including birth weight, body weight at 3, 5, 12 and 30 weeks of age, carcass weight, backfat thickness, body fat, muscle pH, drip loss, cooking loss, water holding capacity (WHC), shear force and intramuscular fat (crude fat) content were collected and evaluated from the F_2 animals at the National Livestock Research Institute (NLRI).

DNA extraction, microsatellite genotyping and PCR-RFLP

Blood samples were collected from all the F_2 animals and their parents (F_1) and grandparents (F_0), and genomic DNA was extracted with Genomic DNA Purification Kit (Promega, USA). A total of eight polymorphic microsatellite markers were selected and used for genotyping the animals of the NLRI reference families. An average marker interval was approximately 21 cM based on the USDA-MARC Pig Map (Rohrer et al., 1996). The PCR was carried out in a GeneAmp PCR System 9600 (Applied Biosystems, USA).

For microsatellite genotyping, the PCR products of up to at least 3 markers were pooled, and analyzed simultaneously using an automated DNA sequencer (ABI 310 Genetic Analyzer, Applied Biosystems, USA). The fragment length of a PCR product was determined using Genescan software version 2.1 (Applied Biosystems, USA), and marker genotypes were assigned to the animals using the Genotyper software version 2.5 (Applied Biosystems, USA).

For PCR-RFLP of the CAST gene, the primer sequences were: forward primer, 5'-GCG TGC TCA TAA AGA AAA AGC-3'; and reverse primer, 5'-TGC AGA TAC ACC AGT AAC AG-3' (Ernst et al., 1998). The PCR was performed using 50 ng genomic DNA in a 50 μl reaction mixture containing 1×PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 μM each primer and 0.5 U *Taq* DNA polymerase (promega). The PCR profile included an initial denaturation of 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min and a final extension of 72°C for 10 min. The PCR products were digested with the restriction digestion enzymes, *Hinf* I, *Msp* I and *Rsa* I, and the fragment length polymorphisms separated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Statistical analysis

The GLM procedure of SAS (Version 8.01; SAS, Inst., Inc., Cary, NC) was used to analyze the association of SNP marker genotypes of the *CAST* candidate gene with traits for growth and meat quality. The linear model used was as follows:

$$Y_{ijk} = u + S_i + G_j + e_{ijk}$$

Where Y_{ijk} is the observation for each trait, u is the overall mean for each trait, S_i is the effect of sex, G_j is the effect of genotype and e_{ijk} is the random residual effect.

The linkage and QTL mapping including the porcine

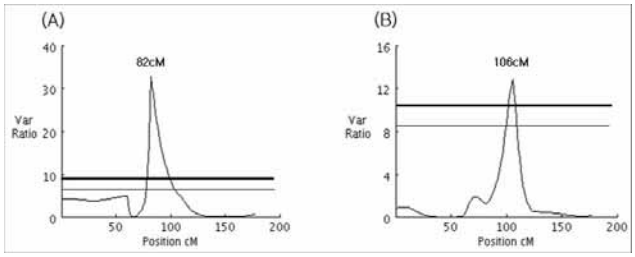

Figure 2. Test statistics profiles for chromosome 4 that show characteristics of QTL expression for the age of 3weeks (A) and 30weeks (B): the two lines present the 5% (─) and 1% (━) single position significance thresholds by permutation. The x-axis indicates the relative position on the linkage map and the y-axis represents the F-value.

CAST gene locus were performed using the data of the NLRI reference families. Linkage mapping with two-point linkage analysis was performed using the CRIMAP software version 2.4 (Green et al., 1990).

For the QTL analysis, the F_2 QTL Analysis Servlet of QTL express which is a web-based QTL mapping tool was used (http://qtl.cap.ed.ac.uk/) (Seaton et al., 2002). A single QTL was fitted by regressing on the additive and dominance coefficients for the QTL at each putative position of the QTL (every 1 cM).

RESULTS AND DISCUSSION

Linkage mapping analysis

Figure 1 shows the results of the linkage analysis using CRI-MAP, including microsatellite marker names and their map positions (cM) in Kosambi mapping function.

A linkage map was obtained with the eight microsatellites and the SNP of the CAST gene, CAST-*Rsa* I on chromosome 2. The map obtained allowed us to locate the CAST gene in the interval between the markers SW395 and SW1695 (SW395-11.8 cM-CAST-*Rsa* I-12.5 cM-SW1695). The porcine CAST gene was mapped adjacent to the markers, Sw395 and Sw1695 on SSC2 with LOD scores of 15.32 and 8.50, respectively. The distance covered with the eleven markers was 177 cM (sex averaged) (Figure 1). Although the same microsatellites were used in a previously study (Lee et al., 2003), the length of the total linkage map obtained in this study was longer than that of the previously one. It was assumed that the addition of CAST-*Rsa* I caused a weakening of marker informativeness for linkage mapping. Also, as is usually reported in pigs, there was a significant difference in the lengths of the maps for the two sexes (166 cM in females and 190 cM for males). Ernst et al. (1998) reported a revised map of porcine chromosome 2 (Zhang et al., 1995) which included the microsatellite

Table 1. Associations between genotype for *CAST-RFLP* and economic traits in pigs

Genotypes[1]	*CAST-Hinf* I				*CAST-Msp* I				*CAST-Rsa* I			
	AA	AB	BB	Prob.	AA	AB	BB	Prob.	AA	AB	BB	Prob.
Frequency[2]	10.0%	27.3%	62.7%		28.2%	42.9%	28.9%		53.8%	31.8%	14.4%	
Birth weight	1.24	1.30	1.22	*	1.24	1.25	1.24	-	1.26	1.25	1.18	-
	±0.03^{ab}	±0.02^{a}	±0.01^{b}		±0.02	±0.01	±0.02		±0.01	±0.02	±0.03	
Weight	23.19	24.97	23.53	-	24.09	24.40	22.99	-	22.79	25.16	24.64	**
of 12 weeks	±1.15	±0.70	±0.46		±0.69	±0.56	±0.68		±0.48^{b}	±0.63^{a}	±0.93^{ab}	
Weight	79.02	86.42	88.94	*	84.94	89.60	86.14	-	83.70	92.04	88.73	**
of 30 weeks	±3.29^{a}	±2.00^{ab}	±1.31^{b}		±1.97	±1.60	±1.95		±1.42^{b}	±1.84^{a}	±2.74^{ab}	
Carcass	67.43	71.49	70.55	-	70.56	71.48	69.22	-	68.05	73.72	71.43	**
weight	±2.48	±1.51	±0.99		±1.46	±1.18	±1.44		±1.05^{b}	±1.37^{a}	±2.04^{ab}	
Body fat	8.32	9.97	10.01	-	9.24	10.15	9.93	-	9.21	11.04	9.55	**
	±0.90	±0.55	±0.36		±0.54	±0.43	±0.53		±0.38^{b}	±0.50^{a}	±0.75^{ab}	
Backfat	18.82	21.36	21.85	-	20.46	21.89	21.66	-	19.96	23.58	21.87	**
thickness	±1.51	±0.92	±0.60		±0.90	±0.73	±0.89		±0.65^{b}	±0.84^{a}	±1.26^{ab}	
Crude fat	2.12	1.69	2.61	*	2.24	2.41	2.20	-	2.05	2.46	2.78	-
	±0.51^{ab}	±0.31^{b}	±0.20^{a}		±0.31	±0.25	±0.30		±0.22	±0.29	±0.44	
pH 24 h	5.63	5.58	5.64	-	5.60	5.66	5.58	*	5.60	5.65	5.62	-
	±0.04	±0.02	±0.01		±0.02^{ab}	±0.02^{a}	±0.02^{b}		±0.02	±0.02	±0.04	
WHC	59.42	61.19	61.36	-	59.96	62.49	60.36	**	60.59	62.34	60.27	**
	±1.02	±0.62	±0.41		±0.59^{b}	±0.48^{a}	±0.58^{b}		±0.44^{b}	±0.58^{a}	±0.86^{b}	
Drip loss	3.56	4.35	3.21	**	3.82	3.58	3.27	-	3.48	3.58	3.42	-
	±0.46^{ab}	±0.28^{a}	±0.18^{b}		±0.28	±0.22	±0.27		±0.20	±0.26	±0.38	
Cooking	33.43	33.02	33.28	-	32.90	32.50	34.48	**	33.51	32.59	32.85	-
loss	±0.82	±0.50	±0.33		±0.48^{b}	±0.39^{b}	±0.47^{a}		±0.35	±0.46	±0.68	

[1] A and B alleles = 790 and 500 bp for *Hinf* I, 760 and 370 bp for *Msp* I, and 360 and 250 bp for *Rsa* I, repectively.

[2] Frequency is a percentage of each genotype.

[a, b] Means in the same row with a common superscript do not differ (* $p<0.05$, ** $p<0.01$).

WHC = Water holding capacity.

Table 2. Results of QTL mapping associated with growth traits on chromosome 2

SSC	Trait	Locus (cM)	F-value	LOD	Threshold		Additive		Dominance	
					5%	1%	Estimate	S.E.	Estimate	S.E.
2	Weight at 3 weeks	106	12.81**	1.97	8.30	10.62	1.64	0.88	1.63	0.99
	Weight at 30 weeks	82	32.84**	2.88	6.48	9.21	-45.13	6.24	29.20	6.54

** $p<0.01$ for F-statistic threshold.

markers SW395, SW776 and SW14, which also showed significant linkage to CAST (lod = 7.90, 9.72 and 6.02, respectively). This indicates that the porcine CAST gene is placed in the region between SW395 and SW1695.

QTL mapping analysis

Single position significance thresholds (5% and 1%) were derived by 1,000 replicates of the permutation test. In the QTL mapping, a significant association was detected at 82 cM between SW395 and CAST-*Rsa* I for body weight at 30 weeks of age and body weight at 3 weeks of age (Figure 2, Table 2). This point is meaningful when the results of the QTL mapping is compared with the association study for the CAST-*Rsa* I on a body weight at 30 weeks of age (Table 1). These results suggest that CAST gene might be an influential candidate gene underlining the QTL on chromosome 2 for growth traits in pigs.

For the body weight at 30 weeks of age, the additive effect suggested that the Korean native pig alleles were inferior to the Landrace pig alleles in this study. On the other hand, the additive effect for the body weight at 3 weeks of age suggested that the Korean native pig alleles were superior to the landrace pig alleles (Table 2). The threshold differences between the traits were considered to be due to the differences in the phenotypic distributions and to random sampling (Lee et al., 2003).

The putative QTL for growth rate (birth to 35 kg and weaning to 35 kg) was identified based on a nominal significance near the SW395 marker on chromosome 2 in swine (Paszek et al., 1999). These QTL regions for growth traits on chromosome 2 were in accordance with our results detected at 82 cM between SW395 and CAST-*Rsa* I for body weight at 30 weeks of age. This suggests that the CAST gene might be a powerful candidate gene for growth traits on chromosome 2.

In conclusion, the QTL mapping of porcine CAST will assist the evaluation of this gene as a candidate gene for growth and pork quality. Also, we suggest that the polymorphisms and the QTL for growth traits identified in this study need to be further evaluated for potential relationships of CAST functions in growth and metabolic processes.

Association study

A PCR fragment of approximately 1.6 kb was obtained and confirmed to be the same as a fragment of the CAST gene previously reported (Ernst et al., 1998) by direct sequencing. For PCR-RFLP, sizes of the polymorphic fragments were separated by 790 and 500 bp for *Hinf* I, 760 and 370 bp for *Msp* I, and 360 and 250 bp for *Rsa* I.

As is shown in Table 1, CAST- *Hinf* I was significantly associated with birth weight ($p<0.05$), body weight at 30 weeks of ages ($p<0.05$), crude fat ($p<0.05$) and drip loss ($p<0.01$). Highly significant associations ($p<0.01$), were detected at CAST-*Rsa* I for body weight at 12 and 30 weeks of ages, carcass weight, body fat, and backfat thickness. These polymorphisms affected mainly growth traits by dominant effects.

In our previous study (Choy et al., 2002), body fat and crude fat (intramuscular fat in the longissimus muscle) had higher variations (CV = 120%) than the other carcass characteristics. This suggests that the great difference in fat deposition represents appreciable variations in growth patterns and the degree of maturity at slaughter age of the pigs.

The percentage of drip loss for the longissimus muscle had been related with the percentage of crude fat (intramuscular fat content) (Moeller et al., 2003). Choi et al. (2002) reported that the high percentage of intramuscular fat in the longissimus muscle prevents a loss of wetness (low drip loss) and improves the texture of meat in Hanwoo cattle.

The development of muscle may be related with meat quality, especially tenderness. Kristensen et al. (2002) reported that an increase in the vivo protein turnover may be a result of regulated levels of CAST that affects the meat quality. Thus, genetic variations at the porcine CAST gene locus could be used as markers to evaluate muscle development-related growth and meat quality traits.

ACKNOWLEDGEMENTS

We thank the USDA-supported U.S. Pig Genome Coordination Project for the distribution of the primers (Coordinator; Professor Max F. Rothschild).

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