EVIDENCE FOR NEW ALLELES IN CALPASTATIN GENE ASSOCIATED WITH MEAT QUALITY TRAITS IN PIGS

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INTRODUCTION

Calpastatin (*CAST*) is a specific inhibitor of calpains, a Ca^{2+}-activated protease family, considered to be the primary cause of initiation of myofibrillar protein degradation in living muscle (Goll *et al*. 1992). Calpains seem to play an important role in postmortem tenderization of skeletal muscle due to the degradation of key myofibrillar and associated proteins (Koohmaraie, 1992). Using a Berkshire x Yorkshire (BxY) pig family (Malek *et al*. 2001), a suggestive QTL for average Instron force was revealed for chromosome 2. Based on its function and location, we considered the *CAST* gene to be a good candidate for the QTL.

MATERIAL AND METHODS

Linkage mapping. Mapping of the *CAST* gene to the BxY family linkage map (Malek *et al*. 2001) was performed using a CAST *Msp*I substitution (Ernst *et al*. 1998), and was accomplished using CRI-MAP (Green *et al*. 1990).

PCR, RT-PCR and polymorphism discovery. Based on *CAST* pig cDNA sequence available in GenBank (M20160), we designed primers to amplify the entire coding region of type III *CAST* skeletal muscle isoform. The PCR was performed using Promega *Taq* DNA polymerase and standard PCR protocols. The reverse transcription of total RNA was performed by random hexanucleotide priming and Superscript II (Gibco BRL) according to the manufacturer's protocol. The amplicons were sequenced using dye terminators (PE Applied Biosystems) on an ABI 377 automated sequencer. We used Sequencher software (Gene Codes) to assemble the sequences and to identify polymorphisms.

Genotyping and PCR-RFLP analysis. The region flanking each newly discovered missense mutation (*Apa*LI, *Hpy*188I and *Pvu*II) was PCR amplified and after that digested with *Apa*LI, *Hpy*188I and *Pvu*II. The non-cutting allele was designated allele 1. The *Apa*LI substitution was not used in these association studies as it is in almost complete linkage disequilibrium (LD) with *Hpy*188I and does not add information after *Hpy*188I genotypes are included.

Phenotypic trait measurement. Meat quality measures for the BxY family were made on the *Longissimus dorsi* using typical industry techniques and included several sensory traits and average Instron force. *Longissimus dorsi* % drip loss data were determined at a packing plant on samples from two commercial pig lines: Duroc Synthetic and Synthetic.

Statistical Analysis. Associations between the *CAST Hpy*188I and *CAST Pvu*II substitutions and meat quality traits in the BxY F_2 family were tested using the general linear models

procedure (SAS) with a model that included dam as random, and slaughter date, sex and marker genotype as fixed effects. Least squares (LS) means for all genotypes were obtained for the *CAST* substitutions. In the BxY family and in the commercial lines, the combined effects of the two substitutions were estimated as haplotype substitution effects. Contrasts between haplotypes in the BxY animals were estimated from a mixed model (SAS) including dam (random), and slaughter date, sex and one variable for each haplotype as fixed. For the commercial lines, sex was not included (the trait was measured in females only), and sire (random) replaced dam in the model. The haplotype substitution effects are presented as deviations from the effect of haplotype 3.

Phosphorylation sites prediction. Potential phosphorylation sites for c-AMP dependent protein kinase (PKA) were identified using NetPhos2.0 prediction server (Blom *et al*. 1999).

RESULTS AND DISCUSSION

Linkage mapping and marker development. Using the BxY intercross family a suggestive QTL for average Instron force was detected on SSC2 (Malek *et al*. 2001) and located at 72 cM (F = 3.97). We mapped *CAST* at 73.1 cM on the BxY map. By sequencing the entire coding region of the *CAST* gene in BxY F_3 individuals with extreme values for meat quality, we identified three new missense mutations: *CAST Apa*LI (Ser – Asn) located in domain L, *CAST Hpy*188I (Arg – Lys) in domain 1 and *CAST Pvu*II (Arg – Ser) in domain 4. In the BxY family *CAST Apa*LI is in complete LD with *Hpy*188I. Both *CAST Hpy*188I and *Pvu*II are located in subdomain C of their respective domains. This subdomain potentiates CAST inhibitory activity (Takano and Maki 1999). Single mutations in any of the subdomains (A, B and C) affect CAST activity (Ma *et al*. 1994). The role of the domain L was found recently to be involved in reactivation of the L-type Ca^{2+} channel activity (Hao *et al*. 2000).

Association study. An association analysis on the BxY F_2 animals, revealed significant effects for both polymorphisms tested on average Instron force and in some traits associated with it, like firmness and juiciness (data shown only for *CAST Hpy*188I - Table 1). The *CAST Hpy*188I -11 genotype is favorable in terms of meat quality being associated with lower firmness, chew score and Instron force and higher tenderness and juiciness.

Table 1. Association results between genotypes of *CAST Hpy*188I and meat quality traits in BxY F_2 animals[A, B].

Traits	Genotype			P
	11	12	22	
Firmness	3.21 e,c	3.44 f	3.43 d	0.001
Juiciness	6.23 a	6.05	5.76 b	0.05
Tenderness	8.01 a	7.74 b	7.75	0.11
Chew score	2.32	2.51	2.54	0.11
Avg. Instron Force (kg)	4.39 a	4.45 a	4.63 b	0.05

[A] n=136 (11), 228-233 (12) and 129-130 (22).
[B] Significant differences: a-b $p<.05$; c-d $p<.005$; e-f $p<.0005$.

Differences between genotype LS means were statistically significant except for chew score. Haplotype analysis was performed in order to dissect the possible effect of each polymorphism. In the BxY family just three haplotypes were observed (Table 2). The analysis showed important differences between the effects of haplotypes 1 and 3 for juiciness ($p < .01$), average Instron force ($p < .01$) and chew score ($p < .05$). The differences between haplotype 3 and haplotype 1 are at both polymorphic sites. Some significant differences were revealed for firmness between the effects of haplotype 1 and 2 ($p < .01$) and also between 2 and 3 ($p < .05$). This analysis showed haplotype 3 as unfavorable, being associated with lower juiciness and tenderness, higher chew score and average Instron force.

Table 2. Haplotype substitution effects for meat quality traits in BxY F_2 animals[A].

Trait	Haplotype[B] effect		
	1	2	3
Juiciness	0.22 [c]	0.06	0 [d]
Tenderness	0.14	0.10	0
Chew score	-0.12 [a]	-0.02	0 [b]
Avg. Instron force (kg)	-0.14 [c]	-0.21	0 [d]
Firmness	-0.06 [c]	0.18 [d,a]	0 [b]

[A] haplotype 1: *Hpy*188I –1 and *Pvu*II – 1 (frequency = 0.50); haplotype 2: *Hpy*188I –2 and *Pvu*II – 1 (0.07); haplotype 3: *Hpy*188I –2 and *Pvu*II – 2 (0.43); n=448 – 482.
[B] Significant differences: a-b $p<.05$; c-d $p<.01$

In order to evaluate the potential roles of *CAST Hpy*188I and *Pvu*II, their effects for % drip loss were estimated in two commercial lines. This trait is correlated with average Instron force and in general with tenderness measures. The same haplotypes revealed in the BxY family were detected in both lines (Table 3). Significant differences were discovered between the effects of haplotype 1 and 3 in the Duroc Synthetic ($p < .01$) and in an across line analysis ($p < .001$). Significant differences were also revealed between haplotype 2 and 3 in the Duroc Synthetic and in the combined analysis ($p < .05$). As expected from the BxY study, haplotype 3 is again unfavorable and is associated with higher % drip loss. Moderate differences between the estimated effects of haplotype 2 and 3 (different only at *CAST Pvu*II site) could be associated with an effect of *CAST Pvu*II.

Table 3. Haplotype substitution effects for % drip loss in two commercial pig lines[A].

Line	n	Haplotype* frequency			Haplotype effect		
		1	2	3	1	2	3
Duroc Synthetic (DS)	154	0.61	0.19	0.20	-0.55 [c]	-0.46 [a]	0 [d,b]
Synthetic (S)	93	0.62	0.28	0.10	-0.47	-0.24	0
DS+S	297	0.61	0.22	0.17	-0.58 [e]	-0.40 [a]	0 [f,b]

[A] Significant differences: a-b $p<.05$; c-d $p<.01$; e-f $p<.001$.

Phosphorylation sites prediction. PKA phosphorylates CAST, which influences its aggregation, changing its intracellular location (Averna *et al*. 2001). An increase in Ca^{2+} level

activates the proteolytic system but also CAST, as a result of disaggregation through the action of a phosphoprotein phosphatase. Using this mechanism, calpains could avoid CAST inhibition in the first steps of Ca^{2+} activation process (Averna *et al*. 2001). Using NetPhos 2.0 we predicted six potential phosphorylation sites in CAST. *CAST Apa*LI and *Pvu*II affect the consensus sequence of two of them. Haplotype 3 has a *CAST Pvu*II – 2 allele that encodes a peptide that will potentially not be phosphorylated by PKA. We can speculate that this variant will be always ready to inhibit calpains even in the earliest moments of Ca^{2+} activation. Further study will be needed to establish if PKA phosphorylates CAST *PvuII* and/or CAST *Apa*LI sites. In the latter case it will be interesting to determine the CAST phosporylation effect on the L-type Ca^{2+} channel activation.

CONCLUSIONS

The newly discovered *CAST* genetic markers have significant effect on tenderness and related pork quality traits. It remains to be further demonstrated if the revealed effects are caused by these substitutions alone, or due to linkage disequilibrium. A combined use of the polymorphisms discovered could have an important potential to improve overall meat quality and hence the economic value for pork supply chain and quality products for consumers.

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