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Full Length Research Paper

Effects of calpastain (CAST) polymorphisms on carcass and meat quality traits in Mongcai pigs

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Calpastain (CAST) activity plays a major role in muscle growth and proteolytic changes post-mortem and the CAST gene has been considered as a candidate gene for carcass and pork quality characteristics. The aim of this study was to analyze the association of two polymorphisms namely CAST_*Hinf*I (allele A and B) and CAST_*Msp*I (allele C and D) with carcass and meat quality traits in Mongcai, a Vietnamese indigenous pig breed. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype the animals at these loci. Results indicate that the CAST_*Hinf*I single nucleotide polymorphism (SNP) had a low frequency of allele A as compared to allele B, while the C and D allele distribution was almost the same for the CAST_*Msp*I SNP. In the association analysis, significant effects on dressing percentage of carcass were detected. The CAST_*Hinf*I locus was associated with the pH_{24}, while the CAST_*Msp*I position was in association with $pH_{45\ min}$, drip $loss_{48}$ and redness color. Additional analysis showed a variation in muscle fiber type composition with higher proportion of IIx fiber in pigs with AB genotype ($P < 0.05$). Three constructed haplotypes namely AB/CD, AB/DD and BB/CC also had significant effects on carcass, type IIa and IIb fiber percentages.

Key words: Association, carcass, pork quality, Vietnamese local pig.

INTRODUCTION

Calpastatin (CAST) is a specific inhibitor of calpain, a Ca^{2+} activated protease family and is considered to be responsible for the initiation of myofibrillar protein degradation in living muscle (Goll et al., 1992). Genes coding for calpastatin and calpain are therefore suggested as candidate genes for growth and meat quality of skeletal muscle. In this perspective, Kurył et al. (2003) characterized the polymorphisms of CAST using three restriction enzymes (*Hinf*I, *Msp*I and *Rsa*I) in several pig breeds. The results showed that pigs with different genotypes had various back fat and weight and that there was an association between eye muscle area and the CAST genotype. This was further supported by Krzęcio et al. (2005), who demonstrated the effect of CAST_*Msp*I on drip loss and nutritional value of the meat, that is, protein content. In the Jinhua x Pietrain F2 population, the three genotypes at these loci were proven to associate with loin area and $pH_{45\ min}$ value as well as the percentage of intramuscular connective tissue (Wu et al., 2007). Moreover, the association of CAST_*Hinf*I locus with pork quality traits including pH, drip loss and meat color was reported in a cross of commercial Pietrain-based breed. Although, there has been extensive research on the effect of this gene on carcass and meat quality in different pig breeds, there is still limited information with regards to the Vietnamese native pigs, especially the Mongcai (MC) breed. This study aimed to

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Abbreviations: CAST, Calpastain; **$pH_{45\ min}$,** pH at 45 min post-mortem; **pH_{24}**, pH at 24 h post-mortem; **Drip $loss_{24}$**, Drip loss 24 h post-mortem; **Drip $loss_{48}$**, Drip loss 48 h post-mortem; **SNP**, single nucleotide polymorphism.

examine the distribution of genotypes from the CAST_*Msp*I and CAST_*Hinf*I loci and investigated the association of these polymorphisms with carcass and meat quality in MC pigs.

MATERIALS AND METHODS

Animals and sampling

This study was carried out on castrated male MC pigs that were reared at a state farm in Quangninh province, Vietnam. All animals were fed under similar conditions and they were slaughtered at 197 ± 17 days of age with the slaughter weight of 28.5 ± 6.0 kg. The carcasses were assessed following the standard commercial procedures. Hot carcass weight was recorded and dressing percentage was defined as the ratio of carcass weight to live weight. In addition, *Longissimus dorsi* (LD) muscle samples (from the seventh thoracic vertebra to the last lumbar) were collected and divided into three parts: a small sample was kept in RNAlater (Qiagen) reagent for RNA isolation, a second sample was stored at -20°C for DNA extraction and the remaining samples were vacuum-packed.

Meat quality trait measurements

Meat color classified as lightness (*L**), redness (*a**) and yellowness (*b**), were determined on a fresh cut surface 24 h post-mortem by using a Minolta Chromameter (CR310, Minolta, Japan). Muscle pH was measured at 45 min ($pH_{45\ min}$) and 24 h (pH_{24}) post-mortem with a Delta-320 pH meter (Mettler Toledo, USA). Drip loss was calculated as the weight loss of a meat sample (40 ± 5 g) placed in a bag at 4°C for 24 (drip $loss_{24}$) and 48 h (drip $loss_{48}$) (Rehfeldt et al., 2008).

Muscle fiber composition

Total RNA was isolated from individual LD samples of MC pigs using TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Subsequently, cDNA was synthesized following the instruction for the High Capacity RNA to cDNA Kit (Applied Biosystems). A real-time RT-PCR approach was applied using five primer pairs from an earlier study (Wimmers et al., 2008) and the expression level of each fiber was determined base on the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). From this expression level, type IIb was assumed as 1, and the relative ratios of type I, IIa or IIx to IIb were calculated as the corresponding expression of type I, IIa and IIx fibers. The muscle fiber percentage was then calculated from these proportions for each muscle type (Li et al., 2009). In total, there were 30 MC pigs used for muscle fiber typing.

Genotyping

Total DNA was extracted using phenol-chloroform extraction. A working solution of DNA was prepared by diluting DNA in 1x TA buffer to the concentration of 50 ng/µl. The primer sequence and PCR conditions to amplify the product of the CAST gene (GenBank Accession No. EU137105.1) were applied following the method detailed by Ernst et al. (1998). For genotyping, genetic variants were identified by the PCR-RFLP method using two restriction enzymes namely *Hinf*I and *Msp*I (MBI, Fermentas). In each PCR-RFLP reaction, a mixture containing 1 unit of enzyme, 1 µl of 10 x restriction buffer and 1 µg of PCR product was incubated at 37°C for 15 min to ensure complete digestion. The digested products were checked by electrophoresis on a 2% agarose gel at 80 V for 1 h. From the two detected SNPs, a construction of haplotype using Merlin software (Abecasis et al., 2002) was performed and these haplotypes together with two SNPs were analyzed in the association study.

Statistical analysis

Analysis of variance was performed by using a general linear model in Minitab (version 13.20) to investigate the effect of genotypes on carcass and meat quality traits. Factors found to affect the traits such as genotype, boar and mother were used in the model and body weight at slaughter was added as a covariate. A *P*-value less than 0.05 was used as significant threshold using the Tukey's option in the least squares procedure. Data were presented as least square means ± standard error.

RESULTS

The primer pair amplified a 1421-bp fragment of the CAST gene. The product was digested with *Hinf*I and *Msp*I restriction enzymes and PCR-RFLP patterns are shown in Figures 1 and 2, respectively. Genotype and allele frequencies for the two SNPs are listed in Table 1. The CAST_*Hinf*I SNP showed a higher frequency of allele B than allele A and as a result, very few AA animals were observed in the population. At the CAST_*Msp*I locus, the frequency of CC and DD genotypes was slightly different.

Table 2 presents the effects of CAST_*Hinf*I, CAST_*Msp*I and the constructed haplotypes on carcass and meat quality characteristics of LD muscle. The dressing percentage was significantly ($P < 0.05$) affected by the CAST_*Hinf*I SNP with a lower value in BB animals. With meat traits, the $pH_{45\ min}$ showed similar values but pH_{24} appeared to be higher in BB pigs ($P < 0.05$). None of the significant effects of genotype were found for other meat quality parameters. In addition, hot carcass and the dressing percentage of MC pigs were associated ($P < 0.05$) with genotypes at CAST_*Msp*I locus and the highest values were observed in CD and DD animals. This SNP significantly affected $pH_{45\ min}$. drip $loss_{48}$ ($P < 0.05$) and meat redness color ($P < 0.01$). Similarly, the haplotypes showed significant effects on carcass and redness color of the meat.

The association of two SNPs and haplotypes with muscle fiber content is shown in Table 3. The percentages of all fibers were almost the same among genotypes at both loci with the exception of the intermediate (type IIx) fiber proportion that was higher ($P < 0.05$) in pigs of AB genotype. Furthermore, the CAST haplotypes were associated with type IIa and IIb fibers ($P < 0.05$) with the highest proportion in BB/CC and AB/DD haplotypes, respectively.

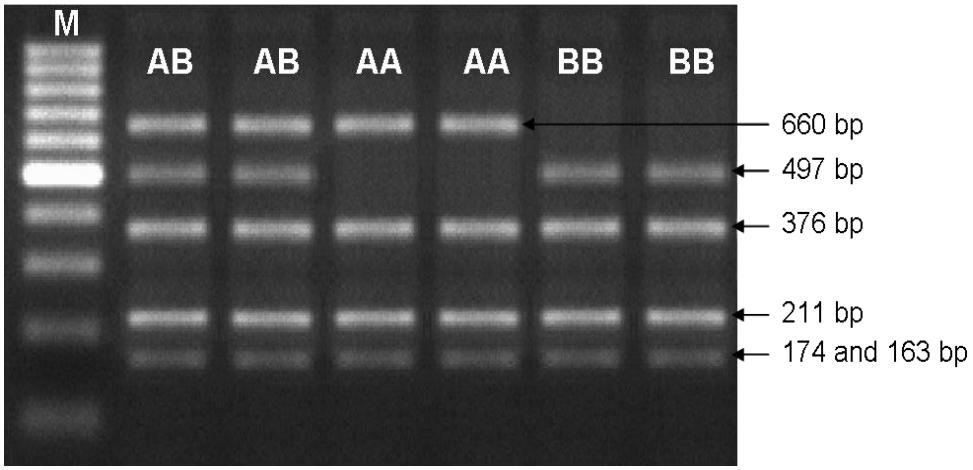

Figure 1. Agarose gel electrophoresis of CAST gene after *Hinf*I digestion. AA genotype shows five fragments (660, 376, 211, 174 and 163 bp), AB genotype shows six fragments (660, 497, 376, 211, 174 and 163 bp) and BB genotype shows five fragments (497, 376, 211, 174 and 163 bp). The separation of 174 and 163 bp bands cannot be seen on the gel. M: 100 bp DNA ladder, Fermentas.

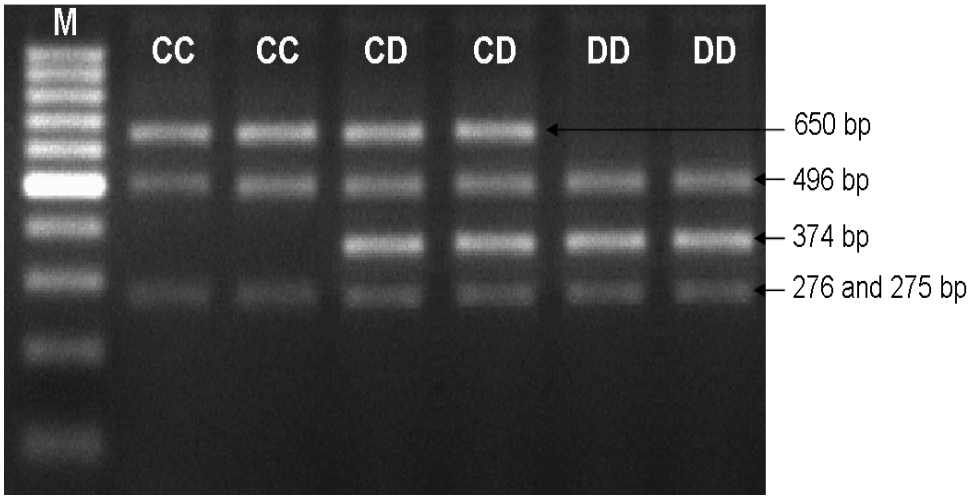

Figure 2. Agarose gel electrophoresis of CAST gene after *Msp*I digestion. CC genotype shows four fragments (650, 496, 276 and 275 bp), CD genotype shows five fragments (650, 496, 374, 276 and 275 bp) and DD genotype shows four fragments (496, 374, 276 and 275 bp). The separation of 276 and 275 bp bands cannot be seen on the gel. M: 100 bp DNA ladder, Fermentas.

Table 1. Genotype and allele frequencies of two SNPs in MC population.

SNPs	Genotype frequency			Allele frequency	
CAST_*Hinf*I (n = 101)	AA	AB	BB	A	B
	0.04	0.70	0.26	0.39	0.61
CAST_*Msp*I (n = 109)	CC	CD	DD	C	D
	0.25	0.57	0.18	0.53	0.47

Table 2. Association of the CAST SNPs and haplotypes with carcass and meat quality traits.

Trait	CAST_*Hinf*I genotypes				CAST_*Msp*I genotypes				CAST haplotypes			
	AA (n = 4)	AB (n = 71)	BB (n = 26)	P	CC (n = 27)	CD (n = 62)	DD (n = 20)	P	AB/CD (n = 40)	AB/DD (n = 14)	BB/CC (n = 16)	P
Carcass												
Hot carcass (%)	79.4±2.5	79.2±0.6	78.3±0.9	0.510	77.5±1.0^{b}	78.8±0.6^{ab}	81.5±1.2^{a}	0.022	78.3±0.8^{b}	82.1±1.3^{a}	78.0±1.1^{b}	0.015
Dressing (%)	68.4±1.8^{ab}	68.5±0.4^{a}	66.5±0.7^{b}	0.033	66.9±0.9^{b}	69.1±0.5^{a}	70.7±1.2^{a}	0.011	67.7±0.6^{b}	70.6±1.0^{a}	66.5±0.9^{b}	0.015
Meat quality traits												
$pH_{45\ min}$	6.83±0.11	6.62±0.03	6.66±0.04	0.155	6.71±0.04^{a}	6.60±0.02^{b}	6.74±0.05^{a}	0.025	6.58±0.04	6.71±0.06	6.63±0.06	0.190
pH_{24}	6.22±0.09^{ab}	6.11±0.02^{b}	6.20±0.04^{a}	0.045	6.20±0.04	6.13±0.03	6.16±0.04	0.331	6.10±0.03	6.15±0.06	6.20±0.05	0.211
Drip $loss_{24}$ (%)	2.10±0.39	1.82±0.09	1.83±0.14	0.783	1.97±0.18	1.92±0.12	1.61±0.21	0.365	1.87±0.12	1.32±0.20	1.85±0.18	0.062
Drip $loss_{48}$ (%)	2.49±0.53	2.66±0.12	2.82±0.21	0.732	2.83±0.20^{a}	2.75±0.13^{a}	2.09±0.23^{b}	0.032	2.89±0.18	2.25±0.29	2.82±0.26	0.163
*L** (Lightness)	50.5±0.8	49.4±0.2	49.2±0.3	0.307	49.3±0.3	49.2±0.2	49.7±0.4	0.499	49.2±0.3	49.6±0.5	49.2±0.4	0.728
*a** (Redness)	5.6±1.5	5.9±0.4	5.6±0.6	0.684	4.9±0.5^{b}	5.4±0.3^{b}	8.0±0.6^{a}	0.001	5.4±0.5^{b}	8.8±0.8^{a}	4.1±0.7^{b}	0.001
*b** (Yellowness)	8.6±0.7	8.6±0.2	9.1±0.3	0.356	9.1±0.3	8.5±0.2	8.4±0.3	0.119	8.3±0.2	8.5±0.4	8.9±0.3	0.243

Table 3. Association of the CAST SNPs and haplotypes with muscle fiber type composition.

Muscle fiber type	CAST_*Hinf*I genotypes			CAST_*Msp*I genotypes				CAST haplotypes			
	AB (n = 5)	BB (n = 25)	P	CC (n = 5)	CD (n = 17)	DD (n = 8)	P	AB/CD (n = 17)	AB/DD (n = 9)	BB/CC (n = 4)	P
Type I	21.6±2.2	24.5±2.9	0.160	24.3±3.2	21.1±2.3	23.0±2.7	0.294	20.2±1.3	24.3±1.7	22.9±2.4	0.168
Type IIa	26.4±2.4	29.5±3.3	0.213	29.9±2.6	28.1±2.6	24.1±3.0	0.135	27.4±1.1^{ab}	24.7±1.3^{b}	32.0±1.8^{a}	0.024
Type IIx	40.1±2.3	34.8±3.1	0.023	35.1±3.5	39.6±2.5	39.8±2.9	0.238	41.2±1.5	37.8±1.7	35.2±2.4	0.055
Type IIb	11.9±1.8	11.2±2.4	0.645	10.7±2.6	11.2±2.0	13.1±2.2	0.347	11.2±0.6^{ab}	13.2±0.7^{a}	9.9±1.0^{b}	0.044

DISCUSSION

In MC pigs, there are three possible genotypes at both loci, which are similar to other breeds, such as Yorkshire and Large White (Ernst et al., 1998), Stamboek and Złotnicka Spotted pigs (Kurył et al., 2003). In comparison with other indigenous pigs, for example, the Meishan breed, only one genotype (BB and DD for *Hinf*I and *Msp*I, respectively) was found in the population and hence this breed was considered to be conservative for the loci examined (Wang et al., 2007).

In the present work, all tested animals were free of $RYR1^{T}$ allele. Therefore, it excluded the effect of gene interaction on carcass and meat quality traits analyzed. In the association analysis, both loci were significantly associated with the dressing percentage of carcass. The results are in agreement with the study of Koćwin-Podsiadła et al. (2004) that used these SNPs for pig selection and found the increased ham in those bearing BB or improved loin muscle weight in animals of CC genotype for CAST_*Hinf*I and CAST_*Msp*I, respectively. In our work, a higher value in CC animals for dressing percentage and carcass, two factors showing a positive correlation with ham

weight, was detected. However, this relationship was not confirmed in data presented by Judyma (2010) and no association was found between the CAST_*Hinf*I locus with carcass traits. Also, in Stamboek pigs, the CAST_*Hinf*I did not show a relationship with carcass traits, whereas the CAST_*Msp*I polymorphism had an effect on loin eye area (Kurył et al., 2003).

Judyma (2010) pointed out the significant effect of CAST_*Hinf*I on pH_{24} with higher value in AB pigs; however in this report, the homozygous animals (BB) had higher pH_{24}. Previously, Kapelański et al. (2004) also demonstrated an effect of this locus on LD muscle at 45 min post-mortem following a trend that $pH_{45\ min}$ measured in rare AA animal group was usually higher than that of the others. Such an association was not confirmed in our study due to limited number of animals tested but it appeared that this SNP, corresponding with that reported by Đurkin et al. (2009), had no influence on $pH_{45\ min}$.

Of the investigated traits related to sensory meat quality, the significant influence of CAST was found on drip loss, of which allele D did show a trend of decreasing drip $loss_{48}$. In a research on Italian Duroc x (LxLW) pigs, Gandolfi et al. (2011) also indicated the relationship of this locus with drip loss with the greater drip loss reported in CD and DD animals. The relationship between specific genotype with drip loss could be explained by the activity of calpain upon the effect of pH based on the hypothesis that higher drip loss of meat might be a result of fast pH drop leading to early activation of calpain (Gandolfi et al., 2011). Consequently, heterozygous pigs at the CAST_*Msp*I locus (CD) had the lowest $pH_{45\ min}$ and highest drip loss percentage, while the homozygous DD animals had the lowest drip loss meat, which could be more favorable for meat selection.

For meat color trait, Pietrain-based pigs carrying AB genotype displayed a less intensity of redness (*a**) and yellowness (*b**) color as compared to BB meat (Rybarczyk et al., 2010). These results were confirmed by the present data that the SNP was highly associated with meat color of MC pigs with redder meat in DD animals. Nevertheless, it should be mentioned here that there was a weak link between meat color, particularly redness value and the expression of muscle fiber in MC pigs. It can be explained by the fact that depending on each breed, there is a negative, positive or no correlation between these two factors in LW, Duroc and traditional breeds (Berkshire, Tamworth), respectively (Chang et al., 2003). With regards to the effects of CAST gene on muscle fiber types, limited number of comparable reports are available for the two SNPs. However, Klosowska et al. (2005) concluded on significant associations between CAST_*Rsa*I, another locus in intron 6 of the gene with diameters of fibers and the proportion of fast-twitch fibers in a bundle. Additionally, in F2 Jinhua x Pietrain crossbred pigs, different genotype patterns of CAST polymorphisms (*Msp*I, *Hinf*I and *Rsa*I) are statistically related to loin muscle area (Wu et al., 2007), of which BB/DD/FF pigs provided higher muscle area than those of AB/CD/EF individuals. This difference is probably from the discrepancy between the IIb fiber content of the two animal groups as mentioned by Wimmers et al. (2008). In the present study, pigs of AB/DD haplotype showed a higher proportion of IIb fiber than those from other two haplotypes. Moreover, individuals being DD homozygous at the CAST_*Msp*I locus tended to have increase in the percentage of IIb fiber; thus, it may be inferred that the D allele could be profitable in producing meat with increasing eye muscle area in MC pigs. This finding confirmed the report of Kurył et al. (2003) that genotype DD was the most advantagous for improved eye-muscle area in Stamboek fatteners.

In summary, two analyzed SNPs of the CAST gene are associated with some traits of interest such as dressing percentage, pH, drip loss and redness color. The effect of haplotype on muscle fiber type composition is interesting but this should be analyzed in a larger number of animals or in another indigenous pig breed for confirmation. The results provide further evidence on the use of this gene for improving carcass and meat quality characteristics in MC pigs.

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