cDNA Cloning and Polymorphism of the Porcine Carbonic Anhydrase III (*CA3*) Gene

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ABSTRACT : Carbonic anhydrase III (*CA3*) is a member of a multigene family that encode carbonic anhydrase isozymes. In this study, a complete coding sequence of the pig *CA3* gene which encodes a 260 amino-acid protein was determined. The amino acid comparison showed high sequence similarities with previously identified human (86.5%) *CA3* gene and mouse (91.5%) *Car3* gene. The partial genomic DNA sequences were also investigated. The length of intron 1 was 727 bp. Comparative sequencing of three pig breeds revealed that there was a T→C substitution at position 363 within intron 1. The substitution was situated within a *Nco*I recognition site and was developed as a PCR-restriction fragment length polymorphism (RFLP) marker for further use in population variation investigations and association analysis. Two alleles (*A* and *B*) were identified, and 617 bp fragments were observed for the *AA* genotype and 236 bp and 381 bp fragments for the *BB* genotype. The polymorphism of *CA3* was detected in 8 pig breeds. Allele B was predominant in the Western pig breeds. In addition, association studies of the *CA3* polymorphism with carcass traits in 140 Yorkshire×Meishan F_2 offspring showed that the *Nco*I PCR- RFLP genotype may be associated with variation in several carcass traits of interest for pig breeding. Allele *B* was associated with increases in lean meat percentage, loin eye height and loin eye area. Statistically significant association with backfat thickness was also found; pigs with the *AB* genotype had much less backfat thickness than *AA* or *BB* genotypes. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 3 : 324-328)***

Key Words : Carbonic Anhydrase III, Pig, PCR-RFLP, Polymorphism

INTRODUCTION

Carbonic anhydrases form a large family of genes encoding zinc metalloenzymes of great physiologic importance. They participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid (Dodgson et al., 1991). Carbonic anhydrase 3 (*CA3*) is an abundant muscle protein characteristic of adult type 1, slow-twitch, fibres. The protein plays an important role in facilitated CO_2 diffusion and diverse processes involving H^+ and $^-HCO_3$ transport (Edwards et al., 1992). Pig muscle carbonic anhydrase III has been found to be a 30 kDa protein displaying three activities (CO_2 hydratase, acetate esterase, p-nitrophenyl phosphatase) (Pullan et al., 1985).

The *CA3* gene was first isolated from human (Lloyd et al., 1985) and the *Car3* gene was later isolated from mouse (Tweedie et al., 1989). Studies showed that the expression of the *CA3* gene is strictly tissue-specific and at high levels in skeletal muscle and much lower levels in cardiac and smooth muscle (Lloyd et al., 1986). However, relatively little is known concerning the porcine *CA3* gene. In the present study, we describe the cDNA cloning and Polymorphism of the porcine *CA3* gene.

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MATERIALS AND METHODS

Experimental animals

One hundred and forty F_2 pigs of a Yorkshire×Meishan reference family were used in this study (Bo Zuo et al., 2003a). All the animals had unlimited access to food and water. The finishing animals were slaughtered and carcass traits were recorded according to the method of Xiong and Deng (1999). For each trait, one hundred and forty phenotypic records were available.

Isolation of the cDNA of porcine CA3 gene

A number of pig ESTs were initially identified using the cDNA sequence of human *CA3* (NM_005181) and mouse *Car3* (NM_007606) by running a BLASTN search against the GenBank 'EST-others' databases. These ESTs were retrieved and then assembled into one contig. From this contig, primer pair 1 (Forward: 5'-GTCCAGTGCCC ACGAAGA-3' and Reverse: 5'-GGCAGAGCCAGGGTCA TA-3') and primer pair 2 (Forward: 5'- CCAAGGG AGACAACCAAT-3' and Reverse: 5'-GGAAT AAGGA GCACCAAAA-3') were designed using Primer 5.0 software (http://www.premierbiosoft.com). These primers yielded two overlapping PCR products. PCR was performed in 25 μl reactions mix containing: 1×PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μmol of each

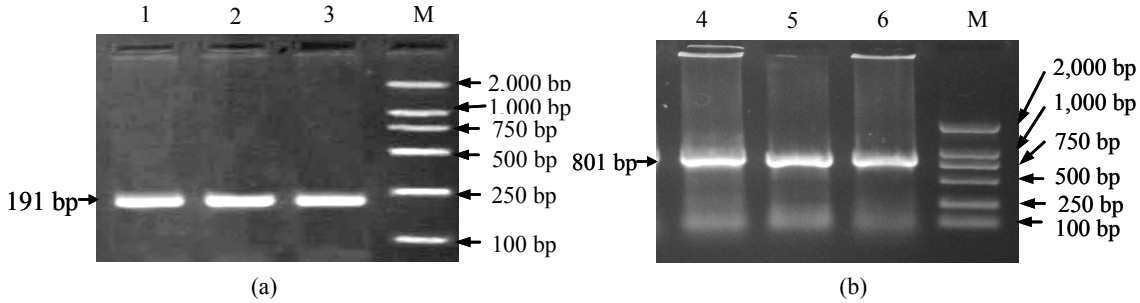

Figure 1. The cDNA isolated products of the porcine *CA3* gene. (a): PCR product with primer pair 1,191 bp; (b): PCR product with primer pair 2,801 bp. M: DL2000 DNA marker. 1, 2, 3, 4, 5, 6: each lane.

PCR primer, 3 U Taq DNA polymerase (Biostar International, Toronto, Canada), 2 μl cDNA derived from Musculus longissimus dorsi muscle. PCR was run in the GeneAmp PCR system 9600 (Perkin-Elmer Co., Norwalk, CT, USA) thermocycler as follows: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 50 s, 59°C or 57°C for 50 s, 72°C for 1 min, and a final extension time of 10 min at 72°C. The purified PCR products were cloned into the pGEM-T vector (TaKaRa, Dalian, China) and was sequenced using standard M13 primers.

Genomic DNA amplification of intron 1

The cDNA sequence of the pig *CA3* gene was compared with the human and mouse orthologue mRNA and their genomic sequence in order to predict the genomic organization of the pig gene which was confirmed by PCR amplification and sequencing. The intron 1 primers were the same as primer pair 1 above. Three genomic DNA mixture pools from three pig breeds (Yorkshire, landrace and Meishan) were used. PCR was performed in 25 μl reactions mix containing: 200 ng of genomic DNA pool, 200 μM dNTP, 0.4 μmol of each PCR primer, 2 U Taq DNA polymerase in the reaction buffer supplied by the manufacturer. Run PCR as follows: 94°C for 4 min, 35 cycles of 94°C for 50 s, 59°C for 50 s, 72°C for 1 min and a final extension step at 72°C for 10 min. The sequencing results of different pig breeds were compared by using BLAST (http://www.ncbi.nlm.nih.gov).

Detection of PCR- *Nco*I-RFLP

According to the BLAST results of intron 1 in Yorkshire, landrace and Meishan. Primer pair 3 (Forward: 5'- GCTA CGCCGACCACAATG-3' and Reverse: 5'-GGCAACCC AAGGCTCACA-3') were used to detect for PCR- *Nco*I-RFLP. PCR was performed in 20 μl reactions mix containing: 25 ng of genomic DNA pool, 150 μM dNTP, 0.25 μmol of each PCR primer, 1 U Taq DNA polymerase in the reaction buffer supplied by the manufacturer. Run PCR as follows: 94°C for 4 min, 35 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 50 s and a final extension step at 72°C for 10 min. For the PCR-RFLP assays, 7.5 μl of PCR products were digested with 5 U *Nco*I (TaKaRa) in 1×digestion buffer with 1×BSA added in a total volume of 10 μl. Following digestion for 4 h at 37°C, digested products were separated by electrophoresis on a 1.5% agarosegel in 1×TAE buffer and stained with 0.5 μg/ml ethidium bromide.

Statistical analysis

The association between genotype and carcass traits was performed with the least square method (GLM procedure, SAS version 8.0). According to the method of Liu (1998), both additive and dominance effects were also estimated using REG procedure of SAS version 8.0, where the additive effect was denoted as -1, 0 and 1 for *AA*, *AB* and *BB*, respectively, and the dominance effect represented as 1, -1 and 1 for *AA*, *AB* and *BB*, respectively. The model used to analyze the data was assumed to be:

$$Y_{ijk} = \mu + G_i + S_j + F_k + b_{ijk}X_{ijk} + e_{ijk}$$

Where, Y_{ijk} is the observation of the trait; μ is the least square mean; G_i is the effect of ith genotype (i = *AA*, *AB*, *BB*); S_j is the effect of j^{th} sex (j = 1 for male or 2 for female); F_k is the effect of family; b_{ijk} is the regression coefficient of the slaughter weight and e_{ijk} is the random residual.

RESULTS

Complete coding sequence of porcine *CA3* gene

A 191-bp fragment and a 801-bp fragment were amplified by primer pair 1 and primer pair 2, respectively (Figure 1). They produced a consensus sequence of 891 bp for pig *CA3* (Genbank accession number AY789514). Aligning this pig sequence with the human *CA3* and mouse *Car3* cDNA sequence revealed 23 bp of 5'-untranslated sequence, 783 bp of coding sequence and 85 bp of 3'-untranslated sequence. The coding region of the porcine

Table 1. The genotype frequencies and allele frequencies of pig *CA3* gene in 8 pig breeds

Breed	Number of pigs	Genotype frequencies (%)			Allelefrequencies (%)	
		AA	*AB*	*BB*	*A*	*B*
Yorkshire	36	0	11.1(4)*	88.9(32)	5.6	94.4
Landrace	36	0	0	100(36)	0	100
Duroc	25	0	20.0(5)	80.0(20)	10.0	90.0
Meishan	44	100(44)	0	0	100	0
Tongcheng	20	0	75.0(15)	25.0(5)	37.5	62.5
Qingping	40	0	100(40)	0	50.0	50.0
Erhualian	22	54.5(12)	45.5(10)	0	77.3	22.7
Bamei	12	8.33(1)	25.0(3)	66.7(8)	20.8	79.2

* Digits in the bracket are the number of pigs.

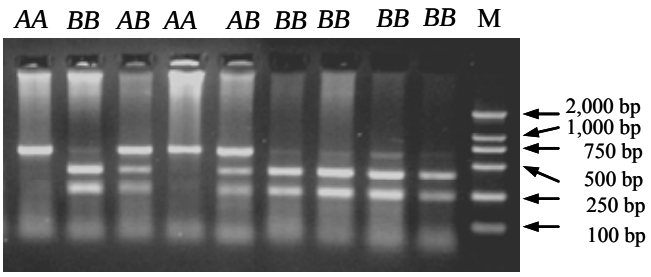

Figure 2. PCR-RFLP at the *Nco*1 locus. The genotypes are indicated at the top of each lane. M, DL 2,000 DNA marker.

CA3 as determined by alignments shares 87% identity with human and mouse cDNAs. The deduced protein contains 260 amino acids showing 86.5% and 91.5% sequence similarity with the proteins from human and mouse, respectively.

Isolation of intron 1 and restriction site analysis

The intron 1 primers amplify a 918-bp fragment containing a part of exon 1, complete intron 1 and a part of exon 2. The results of PCR sequencing showed that the length of intron 1 was 727 bp (Figure 3). Sequence analysis revealed that the T363/C substitution within intron 1 of the *CA3* gene can be detected as a *Nco*I PCR-RFLP. The primer pair 3 amplify a 617-bp fragment containing part of exon 1 and part of intron 1 which was scanned for polymorphism. The 617-bp (*A* allele) PCR product was digested into two fragments of 236 and 381-bp fragment (*B* allele) (Figure 2).

Frequencies of allele and genotype of different pig breeds

Allele frequencies for the *CA3 Nco*I PCR-RFLP were studied in a sample of 235 unrelated pigs, belonging to eight different populations (Table 1). The genotype *BB* was the predominant genotype and allele *B* was predominant in Western pig breeds, such as, the *B* allele frequencies in Yorkshire pigs, Landrace pigs and Duroc pigs were 94.4%, 100% and 90%, respectively. There existed three genotypes (*AA*, *AB* and *BB*) in Chinese indigenous pig populations and no predominant allele was observed. Allele *A* was fixed in Meishan pigs.

Analysis of phenotype value about carcass traits

The analysis results for *CA3* genotypes and carcass traits in F2 offsping (Yorkshire×Meishan) were given in Table 2. At the locus, the number of animals genotyped *AA*, *AB* and *BB* was 35, 71 and 34, respectively. Statistically significant associations with Lean meat percentage(LMP), Backfat thickness at shoulder (BFT1), Backfat thickness at thorax-waist (BFT2), Backfat thickness at 6-7th thorax (BFT4), Average backfat thickness (ABF), Loin eye height (LEH) and Lion eye area (LEA) were found, but no significant conclusion can be made on other carcass traits. Pigs with the *BB* genotype had more LMP (+1.802%), LEH

Table 2. Association between carbonic anhydrase III (*CA3)* genotype and carcass traits

Traits	*CA3* genotype（μ±SE）			Effect (μ±SE）	
	AA	*AB*	*BB*	Additive	Dominance
Dressing percentage (%)	70.828±0.771	70.225±0.547	71.604±0.791	0.387±0.554	0.495±0.389
Lean meat percentage (%)	$56.442±0.641^{a}$	$58.225±0.448^{b}$	$58.243±0.652^{ab}$	$0.901±0.459^{*}$	-0.441±0.320
Backfat thickness at shoulder (cm)	$3.361±0.109^{Aa}$	$2.904±0.076^{Bb}$	$3.023±0.111^{ABb}$	$-0.169±0.078^{*}$	$0.144±0.055^{*}$
Backfat thickness at thorax-waist (cm)	$1.923±0.088^{a}$	$1.683±0.062^{b}$	$1.853±0.090^{ab}$	-0.035±0.063	$0.103±0.044^{*}$
Backfat thickness at buttock (cm)	1.609±0.091	1.394±0.064	1.594±0.092	-0.007±0.065	0.104±0.045
Average backfat thickness (cm)	$2.411±0.085^{A}$	$2.084±0.060^{B}$	$2.238±0.087^{AB}$	-0.086±0.061	$0.121±0.043^{**}$
Backfat thickness at 6-7th thorax (cm)	$2.714±0.096^{Aa}$	$2.342±0.068^{Bb}$	$2.373±0.098^{ABb}$	$-0.170±0.069^{*}$	$0.101±0.048^{*}$
Loin eye height (cm)	$8.844±0.125^{A}$	$9.096±0.088^{AB}$	$9.385±0.128^{B}$	$0.270±0.089^{**}$	0.009±0.063
Lion eye area (cm^2)	$28.973±0.786^{a}$	$29.765±0.557^{ab}$	$31.695±0.814^{b}$	$1.316±0.565^{*}$	0.284±0.398

Least square mean values with different letters are significantly different: small letter: $p<0.05$.
Capital letter: $p<0.01$; * $p<0.05$, ** $p<0.01$. Additive effect = (*BB*-*AA*)/2; Dominance effect = *AB*-(*AA*+*BB*)/2.

Figure 3. Intron 1 sequence of the porcine *CA3* gene. Nucleotides at the end of exon 1 and beginning of exon 2 are indicated in bold,the GT-AG consensus sequences are indicated in the box. The length of the isolated intron 1 is 727 bp.

(+0.542 cm) and LEA (+2.723 cm^2) than pigs with *AA* genotype and in these traits value this locus was significantly additive in action and allele *B* was associated with increases. Pigs with the *AB* genotype had much less BFT1, BFT2, BFT4 and ABF than pigs with *AA* or *BB* genotype. Effect of dominance was significant at backfat thickness. Heterozygous pigs tended to have more desirable characteristics.

DISCUSSION

As we know, gene sequence is an entry point to study the gene expression and function. In our study, we isolated the cDNA and partial genomic DNA sequences of porcine *CA3* gene. Our results revealed that the porcine *CA3* gene shares the high sequence identity with its mammalian counterparts at both the nucleotide level and the amino acid level, which suggested the significance and conservatism of their biological functions during evolution.

It is clearly that seeking the single nucleotide polymorphism (SNP) of the important functional region of the candidate gene and taking the association analysis with the economic traits is the very useful tool to study the gene function (Wang et al., 2004). In this study, we detected a new SNP in intron 1 and did the association studies. The genotype *BB* was the predominant genotype and allele *B* was predominant in Western pig breeds. But, allele *A* was fixed in Meishan pigs. In 140 F2 pigs of a Yorkshire×Meishan reference family, association analysis indicated allele *B* (from Yorkshire pig) was associated with increasing LMP, LEH and LEA. Heterozygous pigs had little backfat thickness and tended to have more desirable characteristics. This may be the phenomenon of heterosis. In addition, pigs with *AA* genotype had much less LMP, LEH and LEA and more backfat thickness than pigs with *AB* or *BB* genotype. So it was unfavourable for selecting pigs with the *AA* genotype in pig production.

The SSC4 encompasses several quantitative trait Loci that are important in pig breeding for economic benefit (Walling et al. 2000). The porcine *CA3* gene was assigned to SSC4q11-q12 (Fujishima-Kanaya et al. 2004). S*J*160 (associated with *CA3* gene) was located 46.9 cM on SSC4 linkage map (Fujishima-Kanaya et al., 2003). In the same 140 F2 individuals, chromosome-wise evidence for QTL affecting backfat thickness (BFT1, ABF and BFT4) was found around 53 cM, between marker SW835 and SW752 (Zuo et al., 2003b). The significant effect on backfat thickness was observed expect for BFT3 in our present study. According to the results obtained, the pig *CA3* gene is located close the QTL affecting backfat thickness and may be responsible for these QTL. Then we may use this site as an molecular marker that can be applied to the Marker Assistant Selection (MAS) in pig breeding. However, the number of individuals analyzed is limited and other closely linked genes on SSC4 might affect the observed results. Further investigation is required among more populations of pigs to confirm the association between the *Nco*I PCR-RFLP genotype and carcass traits.

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