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 composition 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 NcoI 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 F2 offspring showed that the NcoI 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 CO2 diffusion and diverse processes involving H+ and HCO3 transport (Edwards et al., 1992). Pig muscle carbonic anhydrase III has been found to be a 30 kDa protein displaying three activities (CO2 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

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Polymorphism of the porcine CA3 gene.

MATERIALS AND METHODS

Experimental animals

One hundred and forty F2 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'-GTCCAGTGCCCACGAAAG-3’ and Reverse: 5’-GCCAGAGCGGATTTGA-3’) and primer pair 2 (Forward: 5’-CAGACACCAAT-3’ and Reverse: 5’-GGAATAGGACAAAGAAA-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 MgCl2, 200 µM of each dNTP, 0.4 µmol of each
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- NcoI-RFLP

According to the BLAST results of intron 1 in Yorkshire, landrace and Meishan. Primer pair 3 (Forward: 5'- GCTA CGCGGACCAAAATG-3’ and Reverse: 5'-GGCAACCC AAGGCTACA-3’) were used to detect for PCR- NcoI-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 NcoI (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:

\[ Y_{ijk} = \mu + G_i + S_j + F_k + b_{ik}X_{ijk} + e_{ijk} \]

Where, \( Y_{ijk} \) is the observation of the trait; \( \mu \) is the least square mean; \( G_i \) is the effect of ith genotype (\( i = AA, AB, BB \)); \( S_j \) is the effect of jth sex (\( j = 1 \) for male or 2 for female); \( F_k \) is the effect of family; \( b_{ik} \) 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

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.

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

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

| Breed        | Number of pigs | Genotype frequencies (%) | Allele frequencies (%) |
|--------------|----------------|--------------------------|------------------------|
|              |                | 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(12)     | 25.0(3)      | 66.7(8)      | 20.8        | 79.2        |

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

| Traits                                | CA3 genotype | 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±0.641a| 58.225±0.448b| 58.243±0.652ab| 0.901±0.459*| -0.441±0.320|
| Backfat thickness at shoulder (cm)    | 3.361±0.109Aa| 2.904±0.076Bb| 3.023±0.111ABb| -0.169±0.078b| 0.144±0.055*|
| Backfat thickness at thorax-waist (cm)| 1.923±0.088Aa| 1.683±0.062Bb| 1.853±0.090ABb| -0.035±0.063| 0.103±0.045*|
| 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  | 2.084±0.060  | 2.238±0.087  | -0.086±0.061| 0.121±0.043**|
| Backfat thickness at 6-7th thorax (cm)| 2.714±0.096  | 2.342±0.068  | 2.373±0.098  | -0.170±0.069| 0.101±0.048*|
| Loin eye height (cm)                  | 8.844±0.125Aa| 9.096±0.088ABb| 9.385±0.128Bb| 0.270±0.089**| 0.009±0.063 |
| Lion eye area (cm²)                   | 28.973±0.786Aa| 29.765±0.557Bb| 31.695±0.814Bb| 1.316±0.565*| 0.284±0.398 |

* Digits in the bracket are the number of pigs.

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 NcoI 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 NcoI 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 offspring (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 and significant conclusion can be made on other carcass traits.
(+0.542 cm) and LEA (+2.723 cm²) 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). SJ160 (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 NcoI PCR-RFLP genotype and carcass traits.

**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.
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