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Characterization of porcine *ENO3*: genomic and cDNA structure, polymorphism and expression

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Abstract – In this study, a full-length cDNA of the porcine *ENO3* gene encoding a 434 amino acid protein was isolated. It contains 12 exons over approximately 5.4 kb. Differential splicing in the 5'-untranslated sequence generates two forms of mRNA that differ from each other in the presence or absence of a 142-nucleotide fragment. Expression analysis showed that transcript 1 of *ENO3* is highly expressed in liver and lung, while transcript 2 is highly expressed in skeletal muscle and heart. We provide the first evidence that in skeletal muscle expression of *ENO3* is different between Yorkshire and Meishan pig breeds. Furthermore, real-time polymerase chain reaction revealed that, in Yorkshire pigs, skeletal muscle expression of transcript 1 is identical at postnatal day-1 and at other stages while that of transcript 2 is higher. Moreover, expression of transcript 1 is lower in skeletal muscle and all other tissue samples than that of transcript 2, with the exception of liver and kidney. Statistical analysis showed the existence of a polymorphism in the *ENO3* gene between Chinese indigenous and introduced commercial western pig breeds and that it is associated with fat percentage, average backfat thickness, meat marbling and intramuscular fat in two different populations.

pig / *ENO3* / polymorphism

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1. INTRODUCTION

Enolase is a glycolytic enzyme (2-phospho-D-glycerate hydrolyase) that catalyses the interconversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. In higher vertebrates, the active enolase enzyme is a dimer composed of three different subunits, alpha, beta and gamma, encoded by separate genes. The expression of these genes is regulated in a tissue-specific and development-specific manner: *ENO1* (alpha enolase) is expressed in many tissues while *ENO2* (gamma enolase) is expressed only in cells of the nervous system and *ENO3* (beta enolase) is localized to muscle tissue [2]. A switch from alpha enolase to beta enolase occurs in muscle tissue during development in rodents [14]. During ontogenesis, striated muscle differentiation is accompanied by an increase in beta-enolase expression and by a decrease in the expression of the alpha isoform [6,11]. It has been shown that *ENO3* is expressed in proliferating adult myoblasts as well as in differentiated myotubes and that it is one of the earliest markers of myogenic differentiation in man [6]. In adult human muscle, over 90% of the enolase activity is accounted for by the beta-enolase subunit encoded by the *ENO3* gene. Mutations in this gene can be associated with metabolic myopathies that may result from a decreased stability of the enzyme [4].

Feo *et al.* [5] have mapped *ENO3* to human chromosome 17pter-p11 by analysis of rodent-human somatic cell hybrids and transfectant cell lines carrying different portions of human chromosome 17. In pig, the *ENO3* gene has been assigned to pig chromosome SSC12 [10]. However, data on the characterization of the porcine *ENO3* gene are still scarce. In the present study, we describe the cDNA cloning and the expression analysis of two transcripts of the porcine *ENO3* gene. In addition, the genomic structure, polymorphism and association analysis of this gene were studied.

2. MATERIALS AND METHODS

2.1. Isolation of porcine *ENO3* gene

Initially, pig ESTs were identified using the cDNA sequence of human *ENO3* (NM_053013) and mouse *Eno3* (NM_007933) by running a BLASTN search against the GenBank 'EST-others' databases. These ESTs were retrieved and then assembled into one contig. From this contig and the human sequence, primers (EN-1F, EN-1R) and (EN-2F, EN-2R) were designed using Primer 5.0 software (<http://www.premierbiosoft.com>). These primers yielded two overlapping polymerase chain reaction (PCR) products. The purified PCR products were cloned into the pGEM-T vector (TaKaRa, Dalian, China) and sequenced using standard M13 primers.

The cDNA sequence of pig *ENO3* gene was compared with the human and mouse orthologous mRNAs and their genomic sequences to predict the genomic organization of the pig gene, which was confirmed by PCR amplification and sequencing. Four primer pairs were designed to amplify the genomic fragments including all introns (Tab. I). Three genomic DNA mixture pools from three pig breeds (Yorkshire, Landrace and Meishan) were used. DNA sequences were compiled using the DNA star software (Madison, WI, USA). Sequence data of *ENO3* in different pig breeds were compared by BLAST (<http://www.ncbi.nlm.nih.gov>).

2.2. RACE-PCR and identification of transcripts

To amplify the 5' end of the *ENO3* gene, cDNA was generated by the SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) using as template total RNA isolated from the *longissimus dorsi* of Yorkshire pig and the 5'- rapid amplification of cDNA ends (RACE) primer complementary to exon 2 and the SMART II oligonucleotide primer (SMART 5' in Tab. I).

Possible alternative splice donor sites in the 5' end were predicted by comparing the genomic DNA sequence of the pig *ENO3* gene with its cDNA sequence and by using information available in man and mouse on the two beta-enolase mRNA forms. Based on the pig genomic sequence, the primer pair (EN-3F, EN-3R) was designed to amplify the other transcript from the cDNA of Yorkshire pigs' *longissimus dorsi*. These primers bind to the 5' end of intron 1 and the exon 4-exon 5 region, respectively. The RNA secondary structures in 5'-untranslated regions (UTR) of the two transcripts of the *ENO3* gene were predicted using RNA secondary structure prediction software (http://www.genebee.msu.su/services/rna2_reduced.html).

2.3. Analysis of *ENO3* expression levels

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the *longissimus dorsi* of Yorkshire pigs at postnatal day-1, day-60 and day-120 and Meishan pigs at postnatal day-60, respectively. We used three animals of each breed and at each stage, which were reared in same conditions and slaughtered at the same slaughterhouse on the same day. RNA samples were also isolated from other tissues of Yorkshire breed at day-120 *i.e.* fat, heart, liver, spleen, kidney, lung, stomach, small intestine and uterus. Reverse transcriptase PCR was carried out using M-MLV Reverse Transcriptase (Promega, USA).

For amplification, two transcript primer pairs (trans-1F, trans-1R and trans-2F, trans-2R) were designed using cDNA sequences as templates and Primer Express

Table I. Primers used in this study.

Primer name	Sequence (5'-3')	Binding region	Annealing temperature (°C)	Size (bp)
5'-RACE	GCAGGTCCACCTCCACTGT	Exon 2	59	155
SMART 5'	GCAGTGGTATCAACGCAGAGTACGC			
EN-1F	AGCTGCCACCTCTACTCC	Exon 1	55	218
EN-1R	CCAGGTAGCGAGATTGTC	Exon 3		
EN-2F	CTTCCACGGGTATCTATGA	Exon 3	55	1269
EN-2R	GTTGGCACCAGTGTTTATT	Exon 12		
EN-3F	CTGATGACTCTTCCAGCCTC	Intron 1	58	295
EN-3R	ACACTTAGTTTCTTTTCCAGCA	Exon 4 ~ exon 5		
trans-1F	GTTGGAGCAAGCAGGACG	142 bp insert sequence	60	201
trans-1R	TGCCCAGGTAGCGAGATT	Exon 3		
trans-2F	GCGAAGACATCCCAGCCAT	Exon 1 ~ exon 2	60	194
trans-2R	GCCCAGGTAGCGAGATTG	Exon 3		
GAPDH-F	GAAGGTCGGAGTGAACGGAT	Exon 2	60	251
GAPDH-R	CTCATTTGATGTTGGCGGG	Exon 4		
Genomic 1-2F	AGCTGCCACCTCTACTCC	Exon 1	55	1191
Genomic 1-2R	GTCCCGTCCTCTTTCCTA	Intron 3		
Genomic 3-6F	CCGATTCCGAGCTGCTGT	Exon 3	62	2438
Genomic 3-6R	CATTGGTGGCGTCCTTCC	Exon 7		
Genomic 7-8F	ATCAAGGGCAAATACGGG	Exon 7	54	820
Genomic 7-8R	GAGGTCCAGGTCTTCCAGT	Exon 9		
Genomic 8-11F	GGCATCTGAGTTCTACCGC	Exon 8	55	1702
Genomic 8-11R	GGCACCAGTGTTTATTTTCG	Exon 12		
SNP-F	AGATTCTGCTTCGTCCCA	Intron 9	55	687
SNP-R	GGCTTCCACCTTCTCACT	Exon 12		

software (Tab. I). Quantitative PCRs were performed in 25 μL volumes containing 12.5 μL of $2 \times$ SYBR Green Real-time PCR Master Mix (TOYOBO, Japan), 1 μL cDNA and 0.3 μM (final concentration) of each primer. PCR was run in the Applied Biosystems 7500 Real-Time PCR System. Fluorescent signals were continually monitored at the end of each PCR cycle consisting of 1 min at 95 °C for the initial denaturing step, 15 s at 95 °C, 15 s at 60 °C and 45 s at 72 °C for a total of 40 cycles. The relative expression levels of the gene were analysed using the Comparative Ct method, in which glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as an internal control, to correct for the differences in the mRNA quantities. For amplification of *GAPDH*, a specific primer pair (*GAPDH*-F and *GAPDH*-R, see Tab. I) was used producing a 251 bp fragment under the same PCR conditions as above. *t*-test was performed to conclude on the significance of the observed differences.

2.4. Detection of polymorphisms and association analysis

Based on the BLAST results of intron 9 sequences in Yorkshire, Landrace and Meishan, primer pair (SNP-F, SNP-R) was used to detect PCR-*Stu*I- restriction fragment length polymorphism (RFLP). PCR was performed in 20 μL reaction mix containing: 25 ng of genomic DNA pool, 150 μM dNTP, 0.25 μmol of each PCR primer, 1 U Taq DNA polymerase supplied by the manufacturer. PCRs were run as follows: an initial step at 94 °C for 4 min, 35 cycles at 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s and a final extension step at 72 °C for 10 min. For the PCR-RFLP assays, 7.5 μL of PCR products was digested with 5 U *Stu*I (TaKaRa) in 1 X digestion buffer with 1 X BSA added in a total volume of 10 μL . Following the digestion at 37 °C for 4 h, the products were separated by electrophoresis on a 1.5% agarose gel in 1 X TAE buffer and stained with 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide.

Allele frequencies were determined in eight different populations. The *Stu*I PCR-RFLP was genotyped in 132 pigs (34 Yorkshire pigs, 37 Landrace pigs, 35 Landrace $\times$ Yorkshire and 26 Yorkshire $\times$ Landrace pigs) [18] and 140 F_2 pigs of a Yorkshire $\times$ Meishan reference family [17]. The association between genotype and production traits was performed with the least square method (GLM procedure, SAS[®] version 8.0). The model used to analyse the data was assumed to be:

$$Y_{ijk} = \mu + G_i + S_j + B_k + b_{ijk}X_{ijk} + e_{ijk} \quad [18]$$

$$Y_{ijk} = \mu + G_i + S_j + F_k + b_{ijk}X_{ijk} + e_{ijk} \quad [16]$$

where Y_{ijk} is the observation of the trait; μ is the least square mean; G_i is the effect of i th genotype; S_j is the effect of j th sex ($j = 1$ for male or 2 for female);

B_k is the effect of k th breed; F_k is the effect of k th family; b_{ijk} is the regression coefficient of the slaughter weight and e_{ijk} is the random residual.

3. RESULTS AND DISCUSSION

3.1. Two mRNA forms for the porcine *ENO3* gene

Primer pairs (EN1F, EN1R) and (EN2F, EN2R) amplified two fragments 218 bp and 1269 bp long, respectively, which resulted in a consensus sequence of 1426 bp corresponding to pig *ENO3* coding sequence (GenBank Accession No. DQ355513). Using RACE-PCR, we obtained a 155 bp fragment containing all of exon 1 and part of exon 2. The sequence comparison of this 155 bp fragment with the 1426 bp *ENO3* coding sequence produced a full-length consensus sequence of 1437 bp (GenBank Accession No. EU418440), which shares the highest sequence identity with variant 2 of human *ENO3* transcript that lacks exon 1 as compared to variant 1. Aligning pig *ENO3* cDNA and genomic sequences with the human *ENO3* sequence revealed a possible splice donor site in the 3' end of exon 1. Primer pair (EN-3F, EN-3R) amplifies a 393 bp cDNA fragment containing 140 bp of intron 1, exon 2, exon 3, exon 4 and part of exon 5. Thus, two forms of pig *ENO3* mRNA exist that differ by the presence or absence of a 142-nucleotide fragment in the 3' end of exon 1. Based on the sequence of the human *ENO3* [7], porcine *ENO3* transcript 1 containing the 142-nucleotide insert is the long form of *ENO3* mRNA *i.e.* 1579 bp (GenBank Accession No. EU418439) while transcript 2 corresponds to the short form of *ENO3* mRNA *i.e.* 1437 bp (GenBank Accession No. EU418440). Aligning this pig sequence with the human *ENO3* and mouse *Eno3* cDNA sequences revealed a 54 bp or 196 bp of 5'-untranslated sequence, 1305 bp of coding sequence and 78 bp of 3'-untranslated sequence. The sequence "AATAAA" indicating a putative polyadenylation signal was found in the 78 bp fragment of the 3' end sequence. The coding region of the porcine *ENO3* as determined by alignments shares 90 and 85% identity with human and mouse cDNAs, respectively. The deduced protein contains 434 amino acids showing 97% sequence similarity with the human and mouse proteins.

Computer-generated predictions of the most stable leader secondary structure of the two splice forms are illustrated in Figure 1. Introduction of the 142-nucleotide fragment (transcript 1) produced a markedly altered folding pattern, leading to the formation of a very stable stem-loop structure with a predicted free energy = $-42.7 \text{ kcal}\cdot\text{mol}^{-1}$. In contrast, the potential structure of the short transcript 2 had a predicted free energy = $-4.8 \text{ kcal}\cdot\text{mol}^{-1}$, representing a significantly less stable secondary structure.

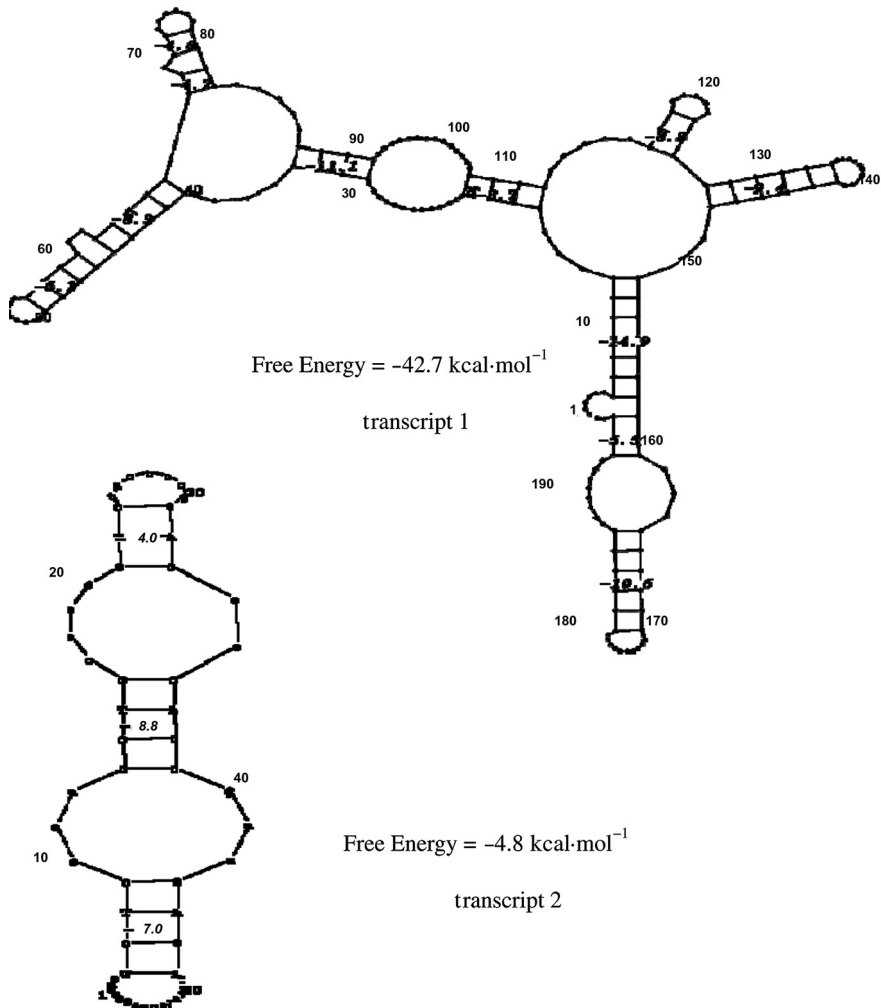


Figure 1. Predicted secondary structure of the 5'-UTR of *ENO3* mRNA. The software predicted the most stable stem-loop structure in the 5'-UTR of both transcripts with the smallest free energy method. Overall free energy of formation values is $-42.7 \text{ kcal}\cdot\text{mol}^{-1}$ and $-4.8 \text{ kcal}\cdot\text{mol}^{-1}$ for the long (transcript 1) and the short (transcript 2) splice forms, respectively.

3.2. Differential expression of the two transcripts

Spatial expression analysis of *ENO3* showed that the expression level of transcript 1 in muscle, fat, heart, small intestine, stomach and uterus is extremely weak and slightly higher in liver, lung and kidney (Fig. 2A). The level of transcript 1 mRNA is undetectable in spleen (Fig. 2A). Real-time PCR revealed that

the expression level of transcript 2 was high in skeletal muscle and heart, with medium levels in fat, lung and stomach, with low levels in liver, spleen, kidney, small intestine and uterus (Fig. 2B). Compared to that of transcript 2, the expression level of transcript 1 is very low in all tissues, with the exception of liver and kidney (Fig. 2B).

Expression of *ENO3* transcript 1 mRNA showed no variation during the three stages of skeletal muscle development in Yorkshire pigs, while expression of transcript 2 was highest in skeletal muscle at postnatal day-1 and lower at day-60 but was upregulated with age (Fig. 3). Compared with that of transcript 2, the expression level of transcript 1 is very low in skeletal muscle (Fig. 3). An expression pattern was also analysed in the skeletal muscle of both Western Yorkshire and Chinese Meishan pigs at day-60. The two transcripts were differentially expressed in the skeletal muscle between Yorkshire and Meishan (Fig. 4). Moreover, the transcript level of pig *ENO3* transcript 1 was very low in all the samples of skeletal muscle. The expression level of transcript 2 was higher in the skeletal muscle of Yorkshire than that of Meishan but the expression of transcript 1 showed no difference between both breeds (Fig. 4).

3.3. Genomic structure of the *ENO3* gene

The resulting overlapping PCR products were assembled into a single contig revealing the complete sequence of pig *ENO3* gene (GenBank Accession No. DQ676935). A 5376 bp genomic DNA sequence covering the entire coding region of the porcine *ENO3* gene was amplified using four gene-specific primer pairs (Tab. I) and compared with the cDNA sequence to clarify the exon/intron organization. The porcine *ENO3* gene is composed of 12 exons and 11 introns (Fig. 5) with the first intron interrupting the 5'-untranslated sequence, just 2 bp upstream of the initiation methionine codon. All splice donor and acceptor sites conform to the typical 5' GT-AG 3' rule, with the exception of the donor site of intron 4, which is GC instead of GT. Sizes of *ENO3* exons vary between 52 and 223 bp and sizes of the introns vary between 82 and 1155 bp in size (Tab. II). This sequence will be useful to search for SNPs and for association analyses.

3.4. Detection of the *ENO3* polymorphism and association analysis

By comparing the sequence from these different pig breeds, we detected several SNPs and a T deletion mutation (g.404delT) at position 404 in intron 9 that spans a *StuI* restriction site. Primer pair 4 amplifies a 687 bp fragment containing part of intron 9, exon 10, intron 10, exon 11, intron 11 and part of exon 12

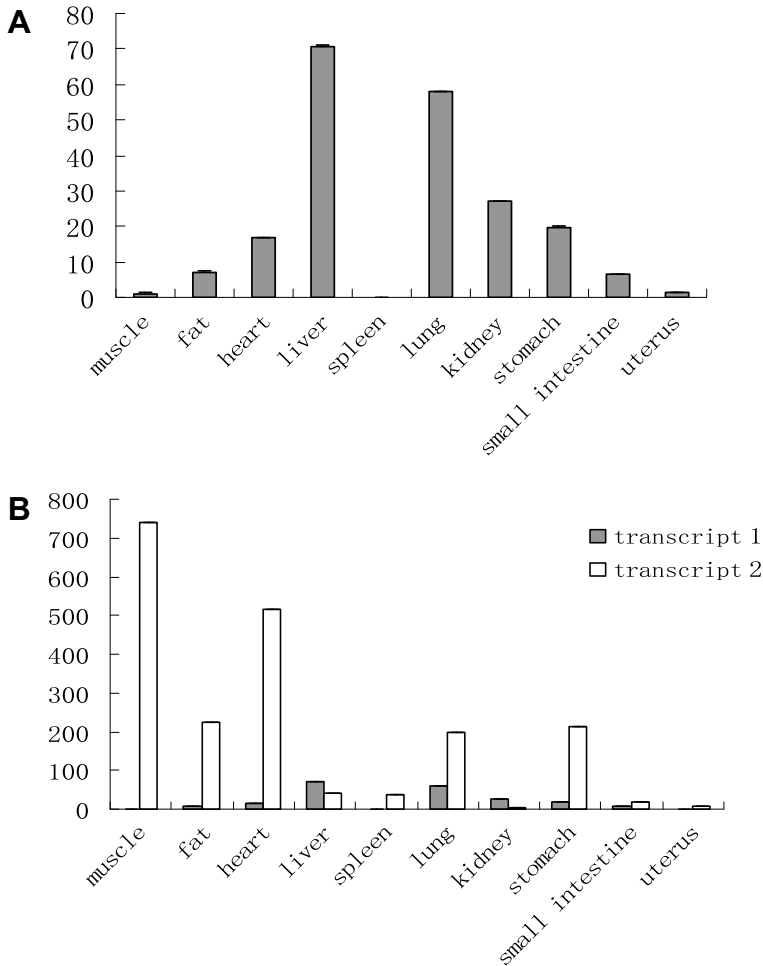


Figure 2. Spatial expression profiles of the two porcine *ENO3* transcripts. (A) Expression of *ENO3* transcript 1 in different tissues. (B) Relative expression levels of both transcripts. Real-time PCR analysed the expression of *ENO3* in different tissues of an adult Yorkshire pig. The relative expression levels of *ENO3* mRNA were analysed using the Comparative Ct method, with *GAPDH* as the reference gene in each sample. The difference in the levels of expression was significant ($P < 0.05$) by *t*-test analysis.

and was scanned for polymorphism. The 687 bp (allele *A*) PCR product was digested into two fragments of 169 bp and 518 bp, respectively, the latter corresponding to allele *B*. Allele frequencies for the *ENO3* *StuI* PCR-RFLP were studied in a sample of 244 unrelated pigs, belonging to eight different populations (Tab. III). The allele distribution revealed that in Chinese indigenous

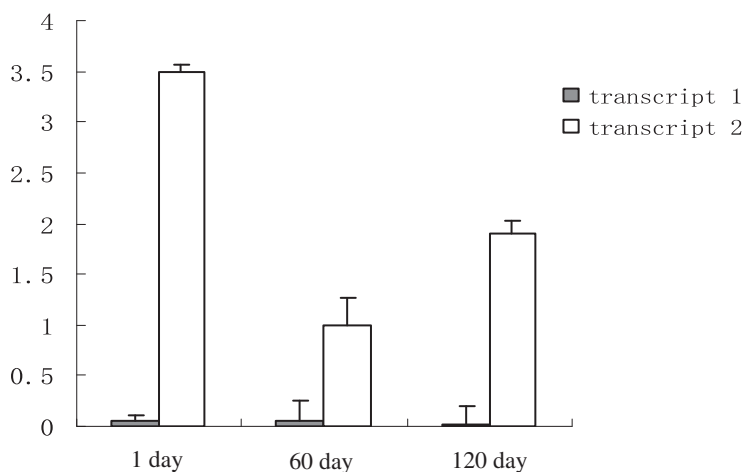


Figure 3. Temporal expression profiles of the two porcine *ENO3* transcripts. Relative levels of *ENO3* mRNA were calculated using the Comparative Ct method with *GAPDH* as the reference gene in each sample. Bars represent the mean $\pm$ SE ($n = 3$). 1 day, 60 day and 120 day indicate three stages of skeletal muscle development in Yorkshire pigs, postnatal day-1, day-60 and day-120, respectively. For transcript 1, the differences in expression level for the three stages examined were not significant ($P > 0.05$).

breeds, with the exception of the Bamei pigs, allele *A* was more frequent than in western pig breeds *i.e.* genotype *BB* was prevalent in Landrace and Duroc but not in Yorkshire pigs in which allele *A* was prevalent.

The results of the association analysis between *ENO3* genotypes and product traits in two different populations are given in Table IV. Statistically significant associations with fat percentage, average backfat thickness, meat marbling and intramuscular fat were found in 140 F₂ Yorkshire $\times$ Meishan pigs. In the other population of 132 pigs composed of 34 Yorkshire pigs, 37 Landrace pigs, 35 Landrace $\times$ Yorkshire and 26 Yorkshire $\times$ Landrace pigs, significant associations with fat percentage, meat marbling and intramuscular fat were also found. No significant conclusion could be drawn for other carcass traits and meat quality traits. Pigs with the *AA* genotype tended to have more desirable characteristics. Allele *A* was associated with an increase in meat marbling, intramuscular fat and decrease of fat percentage in the two populations.

4. DISCUSSION

In the present study, we report the isolation of pig *ENO3* gene and its structural features. The deduced polypeptide shows a high degree of homology with

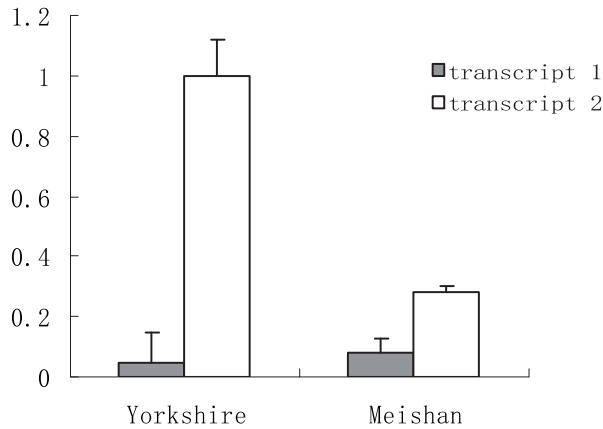


Figure 4. Differential expression analysis of the two porcine *ENO3* transcripts in different pig breeds. Real-time PCR analysis of both transcripts in the skeletal muscle of Yorkshire and Meishan pigs at day-60. Relative levels of *ENO3* mRNA were calculated using the Comparative Ct method with *GAPDH* as the reference gene in each sample. Bars represent the mean $\pm$ SE ($n = 3$). The differences in the expression levels of transcript 2 between both pig breeds and between both transcripts in each breed were significant ($P < 0.05$) by *t*-test analysis.



Figure 5. Schematic diagram of the genomic organization of pig *ENO3* gene. Boxes correspond to the 12 exons and horizontal lines correspond to the 11 introns. The hatched box in the untranslated exon 1 represents the 142-nucleotide insert present in the long form of *ENO3* mRNA (transcript 1).

that of other species. As in man and rat [13,15], porcine *ENO3* gene spans a region of about 6 kb and contains 12 exons. All intron/exon splice donor and acceptor sites conform to the human *ENO3* gene. In man, two mRNA forms exist that differ from one another by the presence or absence of a 42 bp fragment due to differential splicing in the leader sequence [7]. This mechanism of alternative splicing, generating two mRNA forms, is also conserved in mouse [12], in which a 110 bp sequence is inserted in the 5'-UTR of the *Eno3* gene

Table II. Genomic structure of the porcine *ENO3* gene.

Exon	Sequence (5'–3')	Intron	Sequence
I (52/194 bp)	CATCCCAG gt tctga/GGACGGAG gt tactc	1 (612/470 bp)	cctcttgccttgc ag CC[ATG]GCC
II (87 bp)	GGCCAAGG gt aact	2 (270 bp)	ttctcccacccc ag GCCGATTC
III (96 bp)	GGGCAAAG gt gaga	3 (168 bp)	cttcggcctctet ag GGGTCCTG
IV (59 bp)	TGGAAAAG gc aagt	4 (185 bp)	aacctgcccttcc ag AAACTAAG
V (70 bp)	AAATAAGT gt gagt	5 (397 bp)	acttgcttctcc ag CCAAGTTT
VI (134 bp)	CCGTCCCT gt gagt	6 (1155 bp)	gtctcccttctc ag GCCTTCAA
VII (223 bp)	CAATGAAG gt tcagt	7 (91 bp)	cttgccccctccc ag CCCTGGAG
VIII (198 bp)	CTATCCTG gt tgagc	8 (397 bp)	gccccctcccacc ag TGGTCTCC
IX (202 bp)	ATCCAGGC gt tgagt	9 (426 bp)	gtctctcccccc ag CTGCAAAC
X (109 bp)	CGGGACAG gt acgc	10 (159 bp)	ctttccctctc ag ATCAAGAC
XI (59 bp)	CTCATGAG gt acag	11 (82 bp)	gactttgctttcc ag GATTGAGG
XII (148 bp)	CCAAG[TGA]GAAGGTGGAAGCCCCTGGAGCCCGCAGGGCAGATCTGGGCCTTCAAGCCCCTTCCCCCGA <u>AATAAA</u> CACTGGTGCCAAC		

Uppercase letters correspond to exon sequences and lowercase letters to intron sequences. Exon and intron sizes are indicated in brackets. Putative 3'-mRNA processing signals are underlined. The initiation codon ATG and stop codon TGA are inserted in a box.

Table III. Genotype and allele frequencies of pig *ENO3* gene in eight pig breeds.

Breeds	Number	Genotype			Allele frequencies	
		<i>AA</i>	<i>AB</i>	<i>BB</i>	<i>A</i>	<i>B</i>
Yorkshire	38	20	15	3	0.724	0.276
Landrace	39	3	22	14	0.359	0.641
Duroc	22	0	3	19	0.068	0.932
Meishan	42	25	15	2	0.774	0.226
Huainan	15	7	8	0	0.733	0.267
Qingping	45	26	19	0	0.789	0.211
Erhualian	31	28	3	0	0.952	0.048
Bamei	12	3	6	3	0.500	0.500

(GenBank Accession No. X70182). As in man and mouse, two beta-enolase transcripts with distinct sequences in their 5'-UTR are present in pig muscle with a 142 bp insert in the long form of pig beta enolase mRNA.

As an early marker of human myogenesis [6], *ENO3* is responsible for over 90% of the enolase activity in adult human muscle and plays an important role in muscle diseases [4]. In our study, we used the real-time PCR technique to examine the tissue expression of both *ENO3* transcripts and expression changes of these mRNAs during three important stages of skeletal muscle development in different pig breeds. This technique is useful to identify some functions of *ENO3* during muscle development. A previous analysis of the relative expression of both spliced forms in developing and adult muscle did not show any stage-specific or tissue-specific pattern [12]. Our findings demonstrate that the expression of pig *ENO3* transcript 2 is high in adult skeletal muscle and heart. The two spliced forms are expressed during the three postnatal stages of skeletal muscle development in Yorkshire pigs and the highest levels are observed in skeletal muscle at postnatal 1 day. In mouse, [1] have shown that the level of beta enolase is increased in newborn and adult muscle, and [8] and [9] have reported that it is also increased during foetal muscle development and during terminal differentiation of myoblasts, respectively. Hence, it is possible that beta enolase plays important roles during skeletal muscle development, including muscle differentiation. In addition, transcript 2 is relatively more abundant in skeletal muscle tissue and has a higher expression level in Western Yorkshire than in Chinese Meishan pigs. Thus, we propose that the *ENO3* transcript 2 might play an important and different role in different breeds in terms of transcript level during muscle development.

As shown, the alternative splice site of the different transcripts is in the 3' end of exon 1. However, only one product is obtained after amplification with primer

Table IV. Association analysis of the *ENO3* polymorphism.

Population	Genotype	Number	Fat (%)	Average backfat thickness (cm)	Marbling of <i>m. longissimus dorsi</i>	Marbling of <i>m. biceps femoris</i>	Intramuscular fat (%)
132 pigs = 34 Yorkshire	<i>AA</i>	29	11.555 ± 0.438 ^{ab}	1.761 ± 0.134	2.853 ± 0.060 ^a	3.778 ± 0.078 ^a	1.968 ± 0.055 ^A
37 Landrace	<i>AB</i>	59	11.379 ± 0.315 ^a	1.646 ± 0.046	2.714 ± 0.042 ^{ab}	3.609 ± 0.054 ^{ab}	1.840 ± 0.038 ^A
35 Landrace × Yorkshire	<i>BB</i>	44	12.388 ± 0.357 ^b	1.754 ± 0.440	2.686 ± 0.047 ^b	3.554 ± 0.061 ^b	1.680 ± 0.043 ^B
26 Yorkshire × Landrace							
140 F ₂ Yorkshire ×	<i>AA</i>	63	18.173 ± 0.566 ^a	2.172 ± 0.062 ^a	3.291 ± 0.024 ^a	4.214 ± 0.022 ^A	2.643 ± 0.075 ^A
Meishan offspring	<i>AB</i>	59	19.701 ± 0.630 ^{ab}	2.213 ± 0.069 ^{ab}	3.231 ± 0.027 ^{ab}	4.096 ± 0.024 ^B	2.226 ± 0.083 ^B
	<i>BB</i>	18	20.985 ± 1.218 ^b	2.459 ± 0.133 ^b	3.146 ± 0.052 ^b	4.064 ± 0.046 ^B	1.917 ± 0.161 ^B

Different letters indicate significantly different least square mean values, small letters (a/b) indicate $P < 0.05$ and capital letters (A/B) indicate $P < 0.01$.

pair (EN1F, EN1R) and 5'-RACE primers and it contains the 3' end of exon 1 and shares similar structural features with those of human *ENO3* transcript variant 2. It is possible that the short form of mRNA has a high expression and the long form of mRNA possesses a specific structure leading to low expression. Furthermore, real-time PCR experiments revealed that the expression level of pig *ENO3* transcript 1 is very low in skeletal muscle and all other tissue samples. Alternative splicing within the 5'-UTR is usually linked to alternative promoter usage. However, multiple beta-enolase mRNA forms arise from the same promoter, suggesting the possibility that regulated splicing may further control gene expression [7]. Variation in mRNA stability may result in different transcript levels [3]. Predictions of secondary structures indicate that the two transcripts of pig *ENO3* gene could form different secondary structures. A more stable secondary structure may exist in the 5' end of transcript 1 than in transcript 2 resulting in a low expression of transcript 1. In addition, it has been shown in man that transcripts containing the long and short leader sequences showed differences in the translational rate, suggesting a role for the *ENO3* 5'-UTR in the regulation of translation [12]. Thus, the 5'-untranslated sequences of pig *ENO3* transcripts may regulate expression of both transcripts.

In this study, we have detected a new SNP (g.404delT) and carried out association studies. Allele frequencies are clearly different in Chinese indigenous breeds and in introduced commercial western breeds. Results of the association analysis with product traits in two different populations are nearly consistent. The polymorphism may be associated with variation in several carcass traits and meat quality traits of interest for pig breeding. Thus, we suggest that *ENO3* might affect not only skeletal muscle growth during muscle development but also meat flavour and carcass quality. However, the number of animals analysed here is limited, which may affect the observed results. Further investigations in other pig populations are required to confirm the association study. In summary, our study shows that the porcine *ENO3* gene may be an important candidate gene for meat quality and carcass traits in animal breeding and could be involved in muscle developmental mechanisms. These considerations are important for applications in animal breeding and provide a foundation for future research on the *ENO3* gene.

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