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Molecular Cloning, Tissue Distribution and Expression of the Porcine Cationic Amino Acid Transporter CAT3

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Abstract: The Cationic Amino acid Transporter CAT3 (*HGMW*-approved gene symbol SLC7A3; solute carrier family 7, member 3) plays a crucial role in amino acid nutrition. In this study, researchers cloned and sequenced porcine CAT3 and examined its expression in the porcine small intestine. A 2276 bp porcine CAT3 cDNA fragment was obtained that includes a 127 bp 5'UTR, 1860 bp Open Reading Frame (ORF) and 289 bp 3'UTR. The predicted porcine CAT3 has 619 amino acids with a molecular weight of 66.88 kDa and is 92.4, 94.5, 89.7 and 89.8% identical in amino acid sequence to human, cattle, mouse and rat CAT3, respectively. Real-time RT-PCR analysis showed that porcine CAT3 transcripts are expressed across a panel of tissues of Landrace pigs at the age of day 7 with the highest expression in the brain and heart and moderate expression in the liver, kidney, muscle, intestinal tract and lung. The CAT3 mRNA abundance was significantly higher at day 1 than at other ages in the duodenum and colon ($p<0.05$) however, it was highest at day 30 in the jejunum ($p<0.05$) and ileum. There was no significant difference in the CAT3 mRNA abundance in either the duodenum or the jejunum between Landrace and Lantang pigs ($p>0.05$). The CAT3 mRNA abundance in the ileum of Lantang pigs was significantly higher than that in the ileum of Landrace pigs at day 30 ($p<0.05$) however, the colon of Landrace pigs demonstrated significantly higher CAT3 mRNA than that of Lantang pigs at day 1 ($p<0.05$).

Key words: Cationic amino acid transporter, CAT3, SLC7A3, ontogenetic regulation, pigs

INTRODUCTION

Animal cellular membranes possess many amino acid transport carrier systems (Christensen, 1990). According to their Na^+ dependence, amino acid transporters are divided into Na^+-dependent and Na^+-independent transporters (Closs *et al*., 2006). Cationic amino acids mainly consist of four systems: $B^{0,+}$, y^+L, $b^{0,+}$ and y^+. System y^+ is the most typical and widespread Na^+-independent cationic amino acid transporter system. Additionally, this system relies on the chemical potential coupled with the cytoplasmic membrane for substrate transport and aggregation (Rojas and Deves, 1997). The CAT family which belongs to system y^+, plays key roles in the uptake of cationic amino acids into cells (Closs, 2002) and has an affinity for cationic amino acids such as lysine and arginine. However, in the presence of sodium ions, the CAT family displays a low affinity for neutral micromolecular amino acids (Wang *et al*., 1991). CAT-1, CAT-2 and CAT-3 show a high amino acid sequence homology (approximately 60%) among each other (Verrey *et al*., 2004). Porcine CAT-1 (Cui *et al*., 2005) and CAT-2 (Zou *et al*., 2009) have been cloned successfully; however, the complete sequence of the *CAT-3* gene has not been obtained. Most of the studies conducted previously on CAT3 focused on human and mouse CAT3. In contrast to the detailed information available on the structure and function of human and mouse CAT3, a dearth of information exists regarding porcine CAT3. The Lantang pig is a Chinese native breed with excellent meat quality and reproductive capacity. The Landrace pig, originating from Denmark is a famous pork breed with outstanding growing performance. In the present study, researchers cloned the *CAT-3* gene, investigated the tissue specificity and developmental regulation of CAT-3 mRNA and compared the difference between them to improve the understanding of the relationships among CAT-3 expression, age and cationic amino acid enteric absorption.

MATERIALS AND METHODS

Animals and diets: Five Landrace boars at day 7 (Body Weight (BW) = 2.57±0.02 kg) from the same litter were used for the cloning of CAT3 cDNA and analysis of CAT3 mRNA expression in different tissues. Under the

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similar genetic background, 20 purebred Lantang boars (BW = 0.45±0.03 kg) and 20 purebred Landrace boars (BW = 1.25±0.04 kg) at day 1 from five sow, 4 boars each sow were selected to determine regular characters of CAT3 ontogeny expression in small intestine from days 1-30, respectively. Piglets were fed creep feeds (Table 1) starting at day 7-35 and weaned at the age of day 28. Throughout the experiment from days 1-30, all of piglets were provided with food and water *ad libitum*. The treatment of the animals strictly followed the necessary procedures and was approved by the Animal Care Committee of South China Agricultural University.

Tissue sample collection: At the ages of days 1, 7, 26 and 30, five Lantang boars and five Landrace boars with the Body Weight (BW) closing to average BW of group were euthanized with an overdose injection of 10% sodium pentobarbital before sampling. The entire small intestine was then removed, dissected free from mesenteric attachments and placed on a smooth, cold surface. The duodenum, jejunum, ileum and colon were separated. The isolated intestinal segments were immediately opened lengthwise following the mesentery line, flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF (pH 7.4)), divided into 15 cm segments and deposited in marked tubes. Each tube, containing approximately 15 g of tissue was tightly capped and stored at -80°C until further analysis. Moreover, the brain, lung, liver, kidney, muscle, heart and intestine (a mixed sample of duodenum, jejunum, ileum and colon) tissue samples were also gained from five pigs at the age of day 7 and used to detect the expression and tissue distribution of CAT3.

RNA extraction and cDNA synthesis: Total RNA was isolated from 100 mg of intestinal tissue samples using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen) according to the manufacturer's instructions.

The RNA quality was checked by 1% agarose gel electrophoresis and stained with 10 μg mL^{-1} ethidium bromide. The RNA had an OD_{260}: OD_{280} ratio between 1.8 and 2.0. Synthesis of the first strand cDNA was performed using oligo (dt) 20 and Superscript II reverse transcriptase (Invitrogen).

cDNA cloning strategy: All the primers except for those provided by the Clontech RACE kit are shown in Table 1. Based on two ESTs of porcine SLC7A3 (GenBank Accession No. BG609729.1 and EW667542.1) from the NCBI, porcine *SLC7A3* gene-specific primer pairs C1 and C2 were synthesized (Table 2). Two overlapping fragments for the porcine *CAT3* gene were amplified. The PCR was performed in 25 μL reaction mixtures containing 1.25 U of ExTaq polymerase (Takara, Osaka, Japan), 5 μL of 5×buffer supplied by the manufacturer and 200 μM of dNTPs. The PCR conditions were 3 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at the Tm and 1 min at 72°C and a final extension of 5 min at 72°C in a Mastercycler gradient (Eppendorf Limited, Hamburg, Germany). The amplified fragments were cloned into the pGEM-T Easy plasmid vector (Promega, Tokyo, Japan) and then sequenced by Biosune Co., Ltd. (Shanghai, China).

The 3' RACE was performed according to the manufacturer's instructions (BD Biosciences Clontech). Briefly, the first strand cDNA was generated from 1 μg of

Table 1: Formulation of diets and nutrient content

	7-30 days	
Ingredients	Landrace	Lantang
Corn (%)	57.00	59.00
Fish meal, white (%)	4.00	3.00
Soybean meal (%)	-	16.00
Whey (%)	5.00	4.00
Lactose (%)	3.00	3.00
Inflated soybean (%)	11.00	7.00
Replace milk (%)	8.00	4.00
Soybean protein (%)	8.00	-
Premix (%)	4.00	4.00
Total (%)	100.00	100.00
Nutrient levels		
DE (MJ kg^{-1})	14.23	14.00
CP (%)	19.00	18.50
Ca (%)	0.72	0.72
AP (%)	0.40	0.40
Lys (%)	1.40	1.30
Met+Cys (%)	0.88	0.80

The pigs were supplied the following per kilogram of completed diet: vitamin A: 20000 IU; vitamin D3: 2000 IU; vitamin E: 60 IU; vitamin K: 2 mg; thiamin: 2 mg; riboflavin: 10 mg; calpan: 20 mg; niacin: 50 mg; pyridoxine: 5 mg; vitamin B12: 40 μg; folacin: 1.5 mg; biotin: 0.15 mg; vitamin C: 200 mg; choline chloride: 600 mg; Mn: 75 mg; Zn: 120 mg; Fe: 140 mg; Cu: 8 mg; I: 0.4 mg; Se: 0.30 mg

Table 2: Detailed information on primers

Primers	Primer sequences (5'-3')	Size (bp)	Annealing temperature Tm (°C)
C1	F: TGAACTCGGCATCCCCAACC	376	65
	R: GCACAGCCCAGCCAACATAGAAG		
C2	F: GCCAAAGATAAAGCAGGACC	1279	54
	R: AGCAGTGAGGAAAAACATAGAC		
3' RACE	GSP: CAGGAGATGAGGAACGAGGAAGGTGAAGT	786	70-65
	NGSP: GAGAAGCTGACCCTACAGGGACTATTTTG		
C3:ORF	F: GAACCCTATCCGAGACTCTCTG	1941	54
	R: GTGATGATGTCAAACTGAATGG		
C4	F: CTGGAGGATTTGTGCCATTT	200	59
	R: GAGCGTGAGTGCCGAAGA		
β-actin	F: TGCGGGACATCAAGGAGAA	133	59
	R: TCGTTGCCGATGGTGATG		

total RNA using 3' RACE CDS primer A (3'CDS). For 3' RACE, an amplification reaction was performed first using touch down PCR for 40 cycles (5 cycles of 94°C for 5 min, 94°C for 30 sec, 70°C for 30 sec and 72°C for 2 min; 5 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 2 min and 30 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 2 min) using GSP2 (Table 1) and the reverse primer UPM (Clontech). A second (nested) PCR was performed under similar conditions using the nested primer NGSP2 (Table 1) and the reverse primer NUP (Clontech). The amplified fragments were cloned into the pGEM-T Easy plasmid vector (Promega, Tokyo, Japan) and then sequenced by Biosune Co., Ltd. (Shanghai, China).

Three obtained overlapping sequences were assembled to obtain the full-length cDNA sequence of the porcine *CAT3* gene. Based on the newly obtained sequence for the full-length cDNA, the pair of PCR primers C3 (Table 1) was designed to amplify the sequence covering the ORF (open reading frame) of porcine SLC7A3.

Sequence and structural analysis: Nucleotide and amino acid sequence alignment were analyzed using the DNAMAN 5.2.2 Software package. Homology searches were performed using BLAST at the National Center for Biotechnological Information (NCBI).

Detection of tissue distribution and ontogenetic regulation of porcine SLC7A7 by Real-time RT-PCR analysis: β-actin was used as an internal control in quantitative real-time RT-PCR with the SYBR Green dye to quantify the relative mRNA levels of the porcine *CAT3* gene.

Real-time RT-PCR was performed using the one-step SYBR Green PCR Mix (Takara, Dalian, China) containing $MgCl_2$, dNTPs and Hotstar Taq polymerase. The 2 μL of cDNA template was added to make a total volume of 25 μL containing 12.5 μL of SYBR Green mix, 0.25 μL of RT mix and 1 μM each of forward and reverse primers of C4 for samples or β-actin for control.

Real-time PCR was performed at 94°C for 3 min followed by 40 cycles of 30 sec at 94°C, 30 sec at 59°C and 40 sec at 72°C in an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The identities of the PCR products were confirmed by sequencing analysis (Biosune Co., Ltd. Shanghai, China). Varying lengths of oligonucleotides produced dissociation peaks at different melting temperatures. Following 40 cycles, the PCR products were consequently analyzed using the heat dissociation protocol to confirm that one single PCR product was detected by the SYBR green dye. Each data point was repeated five times. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which the increase in signal associated with an exponential growth of PCR product starts to be detected. The relative mRNA levels in each sample were normalized to its content. The relative expression levels of the porcine *CAT3* gene were indicated by $2^{-\Delta CT}$ (Livak and Schmittgen, 2001) for which $\Delta Ct = Ct_{target\ gene}-Ct_{\beta\text{-}actin}$.

Statistical analysis: Developmental data of mRNA abundance were subjected to analysis of variance of mRNA abundance among days 1, 7, 26 and 30 using the LSD test by SPSS 11.0. The data are presented as the means±SE. Treatment effects were deemed significant at $p<0.05$.

RESULTS AND DISCUSSION

Cloning of the porcine CAT3 cDNA sequence: The obtained cDNA of the porcine *CAT3* gene amplified by the C1 (0.376 kb), C2 (1.279 kb) and 3' RACE (0.786 kb) primer pairs was 2276 bp in length, comprising a 127 bp 5'UTR, 1860 bp ORF and 289 bp 3'UTR. The cDNA sequence was deposited in the NCBI database with an accession number of EU780707 and it encodes porcine CAT3, a 619 amino acid peptide with an estimated molecular weight of approximately 66.88 kDa (Fig. 1).

A typical conserved AA-permease superfamily domain was predicted in the sequence. Hydrophobicity prediction indicated 14 putative membrane-spanning domains within porcine CAT3. Analysis of the amino acid sequence by ScanProsite (De Castro *et al*., 2006) revealed several consensus sites for post-translational modification. The consensus site for protein kinase C phosphorylation was located at positions 599-601 of the amino acid sequence. The consensus site for cAMP and cGMP-dependent protein kinase phosphorylation was located at positions 15-18. The consensus site for tyrosine kinase phosphorylation was located at positions 218-224 and the consensus site for N-glycosylation was located at positions 232-235. Additionally, there are 15 N-myristoylation sites and 8 casein kinase II phosphorylation sites.

A Blast search showed that porcine CAT3 is 92.4, 94.5, 89.7 and 89.8% identical in amino acid sequence to human (GenBank Accession No. NM_001048164), cattle (GenBank Accession No. NM_001078019), mouse (GenBank Accession No. NM_007515) and rat (GenBank Accession No. NM_017217) CAT3, respectively (Fig. 2).

Tissue-specific expression by real-time PCR analysis: The tissue distribution of CAT3 mRNA in Landrace pigs

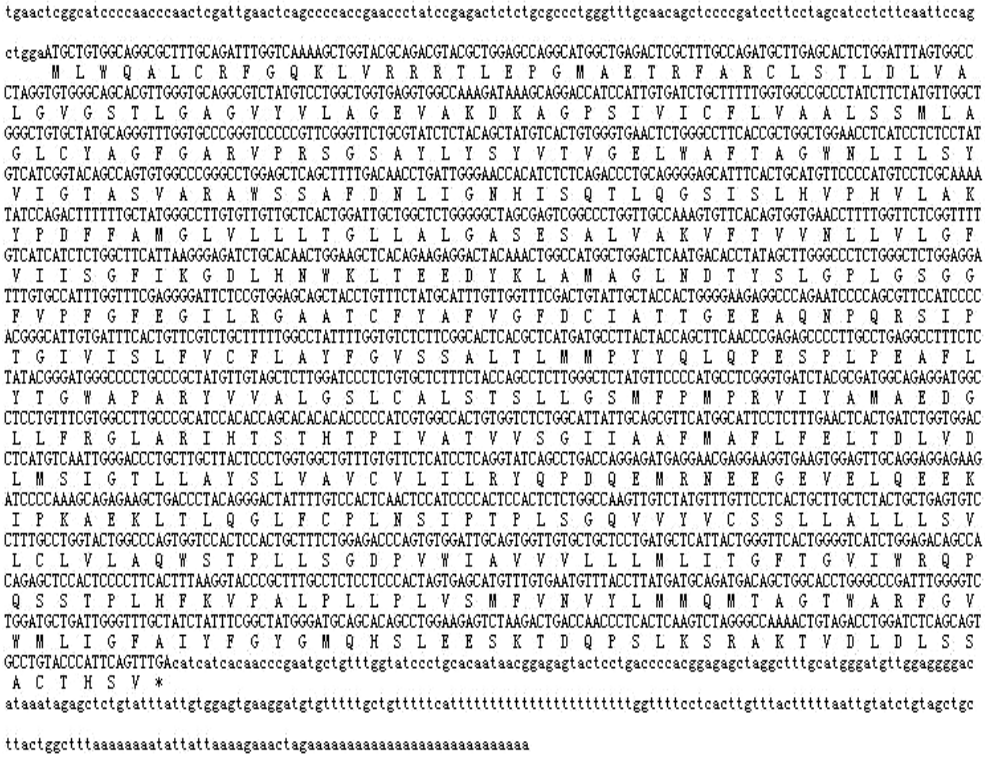

Fig. 1: cDNA and deduced amino acid sequences of the porcine *CAT3* gene. Nucleotides that are in lowercase letters indicate the 5'UTR and 3'UTR whereas '*' refers to the stop codon

at day 7 is presented in Fig. 3. The RT-PCR results indicated that porcine CAT3 transcripts were expressed in the heart, liver, kidney, muscle, intestine, brain and lung. The brain had the highest CAT3 mRNA abundance whereas the liver had the lowest ($p<0.05$) (Fig. 3).

Ontogenetic regulation of CAT3 mRNA expression: Developmental changes in CAT3 mRNA expression along the small intestine are presented in Fig. 4. The highest level of CAT3 mRNA in the duodenum was observed in Lantang pigs at day 1 ($p<0.05$). The CAT3 mRNA abundances in Landrace pigs at day 30 were significantly higher than those at other ages in the jejunum ($p<0.05$) whereas the CAT3 mRNA abundances in Lantang pigs were the same as those in the ileum. The CAT3 mRNA abundances in Landrace pigs at day 1 were significantly higher than those at day 7 in the colon. However, there was no significant difference in the CAT3 mRNA abundance in either the duodenum or the jejunum between Landrace and Lantang pigs ($p>0.05$). For the ileum, the CAT3 mRNA abundance in Lantang pigs at day 30 was significantly higher than that of Landrace pigs ($p<0.05$); however for the colon, the CAT3 mRNA abundance in Landrace pigs at day 1 was significantly higher than that in Lantang pigs ($p<0.05$) (Fig. 4).

Feed proteins are absorbed in the small intestine in the form of peptides and free Amino Acids (AAs) (Johnson, 1997). Three types of cationic amino acids (lysine, arginine and histidine) among 20 types of amino acids are essential amino acids in the swine. Moreover, lysine is the first limiting amino acid in the porcine cationic ration that can have a great influence on porcine performance and meat quality. The proportions of these three amino acids are fairly high in the porcine ideal protein model. Cationic amino acid absorption is related to porcine performance.

The study of tissue specificity and developmental regulation of cationic amino acid transport mRNA expression is conducive to a better understanding of the intestinal absorption mechanism of amino acids and growth of animals. Sequence analysis indicated that the CAT3 cDNA sequence was 2276 bp (ORF 1860 bp) which encoded 619 amino acids. This sequence predicts a conserved AA-permease superfamily domain that is related to amino acid transport. These results are in agreement with the study conducted by Closs *et al.* (2004) that showed the *CAT-3* gene possessing the functions of transport and absorption of arginine and lysine (Verri *et al*., 2005). Other functions of CAT3 involve the transport of special enzymes (Huang *et al*., 2004;

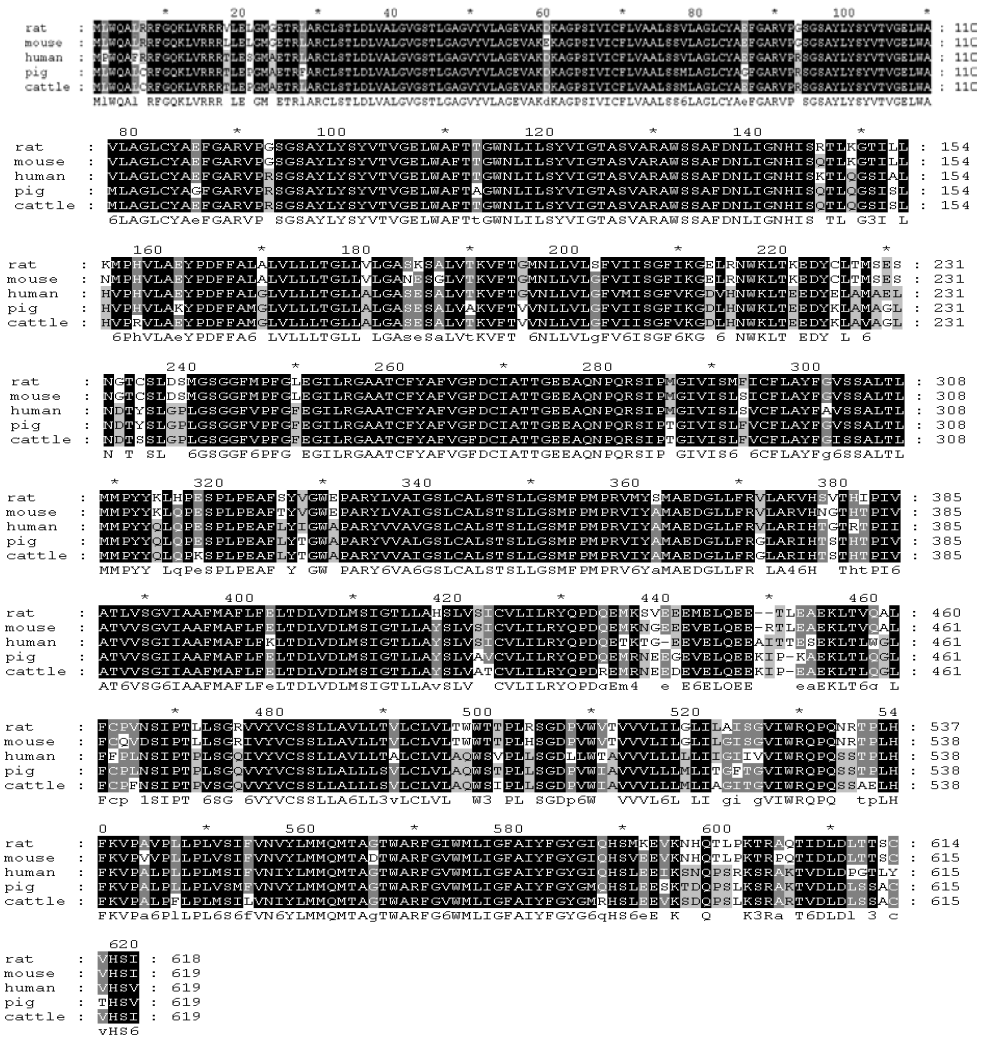

Fig. 2: Comparison of CAT-3 amino acid sequences among pig, cattle, human, rat and mouse. Porcine CAT3 is 92.4, 94.5, 89.7 and 89.8% identical in amino acid sequence to human (GenBank Accession No. NM_001048164), cattle (GenBank Accession No. NM_001078019), mouse (GenBank Accession No. NM_007515) and rat (GenBank Accession No. NM_017217) CAT3, respectively

Bae *et al*., 2005) and increasing CAT3-mediated cationic amino acid transport which functionally compensates in CAT1 knockout cell lines (Nicholson *et al*., 1998).

Research on tissue-specific expression of transporter CAT3 mRNA in pigs has not been reported thus far. Hosokawa *et al*. (1999) found CAT-3 in central neurons such as the ectocinerea of the cerebrum and cerebellum. Vekony *et al*. (2001) showed that in humans, most *CAT-3* gene expression occurred in the brain; however, CAT-3 was also expressed in other tissues such as the thoracic gland and peripheral tissues. Closs *et al*. (2004) found that the *CAT-3* gene was expressed in the thoracic gland, uterus, testicle, lacteal gland, brain, ovaries and stomach. In the present study, the *CAT-3* gene was expressed in the heart, liver, kidney, muscle, intestinal tract, brain and lung and the highest expression level occurred in the brain. The tissue distribution indicates the primary function of this gene. Similarly with previous results, the cause of the high expression level was presumptively that this gene possesses certain biological functions in the nervous system besides the role of amino acid transport.

The mRNA expression of intestinal cationic amino acid transporter is a process that is regulated by multi-factors (mainly developmental stage, humoral factors, nutrition and physiological status). Until now, few studies have been concerned with ontogenetic regulation. The current study aimed to explore the significant

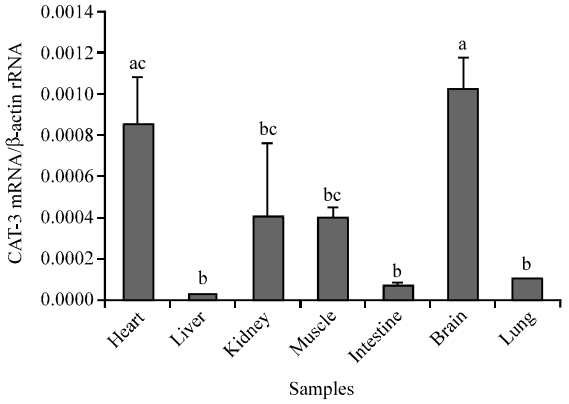

Fig. 3: Tissue distribution of porcine CAT3 in the brain, lung, liver, kidney, muscle, intestine and heart (n = 5). All samples were normalized using β-actin expression as an internal control in each real-time PCR. The relative levels of CAT3 mRNA were analyzed by the 2(-Delta Ct) Method. The data are presented as the means±SE (n = 5) in arbitrary units

variations of CAT3 expression abundance at different stages and proved the reliability of CAT3 expression abundance and that ontogenetic regulation results in expression of the porcine intestinal *NHE2* gene (Feng *et al*., 2009), $b^{0,+}$*AT* gene (Zhi *et al*., 2008), *GLUT2* gene (Huang *et al*., 2008) and *CAT2* gene (Zou *et al*., 2009). In the present study, researchers also found similar ontogenetic regulation changes in the CAT3 mRNA expression between Landrace and Lantang pigs in the duodenum, jejunum, ileum and colon. In pigs from days 1-30, CAT3 mRNA expression in the duodenum was depressed initially and then elevated; CAT3 mRNA expression in the jejunum and ileum was initially at a low level and then reached its highest level at day 30; CAT3 mRNA expression in the colon showed no significant difference except at day 1 ($p<0.05$). These results indicated similar ontogenetic regulation of CAT3 mRNA between the two pig breeds at different intestine sections; however, a difference in expression abundance was noted between the two pig breeds at the same stage.

Humphrey *et al*. (2004) stated that the adaptation reaction of animals to changes in feed ingredients and nutrition serves to alter the types and quantity of the nutritional transporter and the chemical composition in the diet can influence gene expression directly or indirectly.

Similarly, the present study showed that at 30 day after weaning (28 days), in the ileum, the CAT3 mRNA expression abundance in Lantang pigs displayed a significant difference from that at 26 day ($p<0.05$) with no

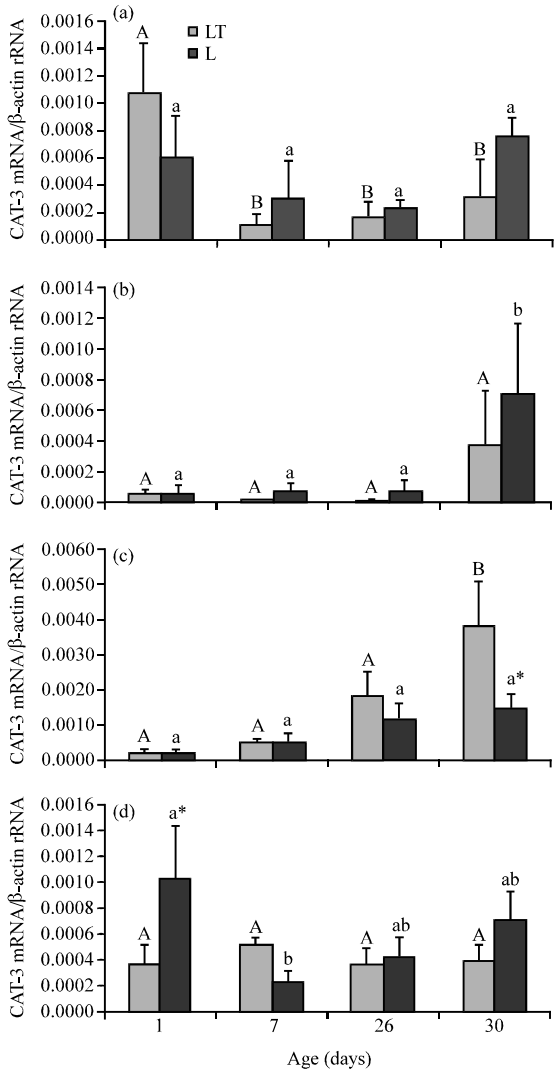

Fig. 4: Relative mRNA expression of porcine CAT3 in the duodenum, jejunum, ileum and colon of the pig during postnatal development; a) duodenum, b) jejunum, c) ileum and d) colon. All samples were normalized using β-actin expression as an internal control in each real-time PCR. Relative levels of CAT3 mRNA were analyzed by the 2(-Delta Ct) Method. Bars without common letters differ significantly ($p<0.05$). The data are presented as the means±SE (n = 5) in arbitrary units

significant difference regarding other developmental stages indicating that the weaning stress response of Lantang pigs was more sensitive than that of Landrace pigs. The differences of both species and diet might regulate the intestinal amino acid transporter expression in the developmental stage.

CONCLUSION

Researchers have cloned the cationic amino acid transporter CAT3 from the pig. This cationic amino acid transporter revealed significant homology with human, cattle and murine CAT3. The mRNA of CAT3 was not only developmentally expressed but also distributed segment-specifically along the small intestine of pigs at both early and growing stages of life, a finding that may be related to the luminal substrate concentration, amino acid requirement and hormonal status. This finding was also similar to that reported by Feng *et al.* (2008) regarding two heterodimeric amino acid transporter mRNAs.

Further studies are needed to elucidate which cationic amino acid is transported by CAT3 as well as the function of CAT3 in porcine nutrition and physiology. Further research is also necessary concerning developmental changes of CAT3 at the protein level in the whole small intestine to comprehensively understand ontogenetic regulation.

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NOMENCLATURE

LT = Lantang pigs
Ld = Landrace pigs
CP = Crude Protein
DE = Digestible Energy
AP = Available Protein

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