Molecular cloning, sequence characteristics, and tissue expression analysis of the SLC35D3 gene in lean, obese and mini-type pigs

Running title: characteristics of SLC35D3 gene in pigs

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Abbreviations: AA, amino acid; CDS, coding sequence; SLC35D3, solute carrier family 35, member D3; NCBI, National Center for Biotechnology Information; ORF, open reading frame; pl, isoelectric point; qPCR, real-time quantitative polymerase chain reaction; UTR, untranslated region; IMF, intramuscular fat; SAT, subcutaneous adipose tissue; PAT, perirenal adipose tissue; LM, longissimus dorsi muscle; DA, dopamine; DM, diabetes mellitus; AS, atherosclerosis; D1R, dopamine receptor D1; D2R, dopamine receptor D2; MetS, metabolic syndrome

Key words: SLC35D3, cDNA clone, sequence characteristics, tissues expression, adipose deposition

Abstract: Nowadays, with the development of people's life, obesity become one of the largest health problems today. Solute carrier family 35, member D3 (SLC35D3) protein has been reported to be involved in adipose deposition and metabolic control in mice. Because organ size in pig is comparable to that of the human, the pig is an ideal model that has been used to study diabetes mellitus, atherosclerosis, obesity, and other diseases. To better understand the structure and function of the SLC35D3 gene, the Meishan pig SLC35D3 gene was cloned and characterized, and its expression in different tissues was determined. The SLC35D3 cDNA consisted of a 1272 bp coding sequence that encoded a protein of 243 amino acids with a molecular mass of 44653.9 Da, and 966 bp 3′ untranslated regions. It is a pity that we have not got 5′ untranslated regions. Phylogenetic tree analysis revealed that porcine SLC35D3 had a closer genetic relationship and a shorter evolutionary distance with Vicugna; however, its evolutionary distance with that of the gorilla was longer than that of Vicugna. In addition, we quantified the SLC35D3 mRNA level by real-time polymerase chain reaction and detected expression in the liver, kidney, lung, heart, brain, LM, and spleen, as well as in the leaf lard, SAT, and PAT. In addition, we also tested the expression level of Meishan, Bama and Yorkshire in adipose tissue. The SLC35D3 mRNA level was highest in the leaf lard. These results serve as a foundation for further study on the porcine SLC35D3 gene.
Introduction

Chinese local pig breeds, such as the Meishan and the Bama pig, compare with western pig breeds, often possess valuable traits, such as disease resistance, good meat quality, high fertility, good maternal qualities, and the ability to adapt to harsh conditions, but slow growth rate[1]. European pig breeds, such as the Yorkshire, Landrace, and Duroc, are noted for their lean meat content and fast growth rate, but the litter size is poor, meat quality is not as good as local pig breeds[2]. Pig farming has application in animal husbandry, at the same time, the pig has been used as a model in many biological[3], agricultural, and biomedical studies[4-6]. Compared to other models of animals, the pig is similar to the human with regard to body size, physiological conditions, eating patterns, and fat deposition[7], so the pig is a good model in which obesity can be studied. The Guangxi Bama mini-pig is rich in unsaturated fatty acids, which prevent thrombosis formation and cardiovascular disease. Thus, the mini-pig holds promise for studies that involve liver microsomes[9](Liver microsomes is an important site of drug metabolism and biological transformation, which is essentially composed of endoplasmic reticulum fragments and RNA particles, if liver microsomes damage, drug metabolism will be disorders, prolong the time of drug action.), dermal regeneration[10], pharmacology, and toxicological studies[11-12], and vascular imaging[13]. It is also useful in the production of food for the elderly, because it fully meet the needs of human physiology and health[6-8]. Obesity is a complex disease influenced by genetic and environmental factors and their interactions, phenotypic difference is mainly determined by genetic differences[14-15]. Therefore, studying the genes involved in fat deposition is important in the breeding of pigs. The present study applied the qPCR and western blot to the identification of differentially expressed genes in two Chinese pig breeds (Meishan and Bama) and one European pig breed (Yorkshire).

In human, the SLC35D3 gene is a newly discovered gene associating with fat deposition. Liwei group found it close to human 6 chromosome long arm D6S1009 sites, and to be thought as a candidate gene for MetS, which is involved in metabolic control in the central nervous system by regulating dopamine signaling[16-19]. Recent research indicates that SLC35D3 is involved in the biogenesis of platelet dense granules, and the expression of SLC35D3 in the brain was limited to expressing D1R rather than D2R[20-22]. Other results suggest that SLC35D3 is a new regulator of tissue-specific autophagy and plays an important role in the increased autophagic activity required for the survival of subsets of DA neurons[23].

Moreover, reports on the expression and structure of the SLC35D3 gene are not available in swine[24]. Nowadays, the swine industry is facing several major problems, such as abundant fat deposits. The SLC35D3 maybe serve as a positional and functional candidate gene for understanding the mechanisms and therapeutic interventions of fat deposits in pig. In this study, we isolated the coding sequence of the pig SLC35D3 gene, analyzed its primary structure, and showed the tissue distribution of its expression. These results will establish a foundation for understanding the function of the pig SLC35D3 gene.

Materials and Methods
Animals and sample collection

Four-month-old pigs were purchased from the experimental farm of the Chinese Academy of
Agricultural Sciences (Tianjin, China). Yorkshire (lean), Meishan (obese) and Bama (mini and obese) pigs were obtained from Tianjin, China. Different tissues, including the liver, kidney, lung, heart, brain, spleen, and LM, as well as the leaf lard, SAT, and PAT were dissected from each pig, immediately frozen in liquid nitrogen, transported to the laboratory, and stored at -80°C until RNA extraction.

### RNA isolation and cDNA synthesis

Approximately 100 mg of each tissue was homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from the triturated sample using chloroform extraction, and the RNA was then dissolved in RNase-free water. The integrity of the RNA was detected by 1% agarose gel electrophoresis, and its concentration was determined by ultraviolet spectrophotometry. The first-strand cDNA was synthesized from 2 μg of purified total RNA using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Total RNA and the cDNA were stored at -80°C and -20°C, respectively.

### Cloning of an internal fragment of SLC35D

A pair of forward (F) and reverse (R) primers (Table 2) was designed with the Primer Premier 5 program using porcine SLC35D3 (XM_013986971.1) as the reference gene sequence. A cDNA fragment was amplified by RT-PCR using an oligo dT primer. The 25 μL reaction contained 1 μL of cDNA, 12.5 μL of 2×Es Taq Master Mix, 0.5 μL of each primer, and 10.5 μL of RNase-free water. The PCR program was performed at 94°C for 5 min, followed by 34 cycles at 94°C for 30 s, 57°C for 5 s, 72°C for 26 s, and 72°C for 10 min.

#### 5′/3′ RACE

The 5′/3′ RACE first-strand cDNA was synthesized using the SMARTer RACE 5′/3′ Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol.

#### 5′ RACE

5′ RACE was performed by nested PCR, and it employed the SLC35D3 specific primers GSP5 and NGSP5 (Table 2) and the universal primers UPM long and UPM short (Table 2). The 50 μL reaction contained 25 μL of 2×SeqAmp Buffer, 1 μL of SeqAmp DNA polymerase (TaKaRa), 5 μL of cDNA, 1 μL of GSP5, 5 μL of UPM long, and 15.5 μL of RNase-free water. The PCR program is divided into 4 stages, stage 1: 94°C, 5 min; stage 2: 94°C, 30 s, 72°C, 2 min, 5 cycles; stage 3: 94°C, 30 s, 70-60°C touchdown PCR, 30 s, 72°C, 2 min, every 5 cycles down 2°C, total 30 cycles; stage 4: 72°C, 10 min. The product was then diluted 50-fold with Tricine-EDTA buffer, NGSP5 and UPM short primers was used as primer, followed by the second PCR program, which was identical to the first PCR program.

#### 3′ RACE

The components of the 50 μL reaction were identical to those of 5′ RACE, except that the GSP3 primer was used (Table 2). Procedure is as follows: 94°C, 5 min; 94°C, 30 s, 72°C, 2 min, 5 cycles; 94°C, 30 s, 70°C, 30 s, 72°C, 2 min, 5 cycles; 94°C, 30 s, 68°C, 30 s, 72°C, 2 min, 25 cycles; then 72°C extension for 10 min, finally 4°C to terminate the reaction. All PCR products, including the internal fragment, 5′ RACE, and 3′ RACE, were detected by agarose gel electrophoresis, and the PCR products were recovered using an Agarose Gel DNA Purification Kit (Tiangen, Beijing, China). The products were cloned into the pEASY-T1 vector (Trans, Beijing, China) and sent to Sangon Biotech Co., Ltd. (Shanghai, China) for nucleotide sequencing.

### Bioinformatics sequence analysis
Sequence analysis of the Meishan pig SLC35D3 gene was performed using the BLAST program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF was predicted using the ORF Finder program at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool within the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the DNAMan program. The molecular weight and pI were calculated by the Compute pI/Mw program (http://us.expasy.org/tools/pi_tool.html). The signal peptide was predicted using the SignalP4.0 program (http://www.cbs.dtu.dk/services/SignalP/). The PSORT II program (http://psort.hgc.jp/) was used to predict protein sorting signals and the intracellular localization. The secondary structure of the deduced amino acid sequence was predicted by the SOPMA program (http://npsa-pbil.ibcp.fr/) [25]. The SWISS-MODEL program (http://www.expasy.org/swissmod/SWISSMODEL.html) was used to model the 3D protein structure [26-28].

**Phylogenetic analysis**

A phylogenetic tree was generated based on the SLC35D3 protein sequence by applying the neighbor-joining method in the MEGA4.0 program. Table 3 shows the GenBank Accession Numbers of the SLC35D3 sequence from different animals. The statistical significance of the different groups within the phylogenetic tree was evaluated using the bootstrap method.

**qPCR for tissue expression profile analysis**

The first strand cDNA was synthesized from 2 μg of total RNA that was extracted with TRIzol Reagent. Results were quantified using SDS1.4 software. The Q-F and Q-R primers (Table 2) were designed (Sangon Biotech Co., Ltd.) using the Primer Premier 5 program. The relative mRNA levels were normalized to that of β-actin. The 15 μL reaction volume contained 7.2 μL of 2×SYBR Premix Ex Taq (TaqKaRa), 0.3 μL of each primer, 1 μL of cDNA, 0.3 μL of Dye II, and sterile water to a volume 20 μL. The PCR program was performed at 95°C for 5 min, followed by 40 cycles at 95°C for 5 s and 60°C for 1 min. Thereafter, a dissociation program was carried out at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. All samples were amplified in triplicate, and the mean was used for further analysis. The stability of the target genes was evaluated by the 2^−ΔΔCt method.

**Western blot analysis**

Porcine adipose tissue was lysed in RIPA extraction buffer (Thermo Scientific, Waltham, MA, USA) and protease inhibitor (Roche, Beijing, China) mixture, the protease inhibitor supplemented with 1 mM Phenylmethanesulfonyl Fluoride. The protein concentration was determined with a BCA Protein Assay Kit (Sangon Biotech Co., Ltd.). Equal concentrations of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electro transferred to Polyvinylidene Fluoride membranes (Millipore, Bedford, MA, USA) for immunoblotting analysis. The membranes were blocked with 5% non-fat dry milk in Tris Buffered Saline, containing 0.1% Tween-20 (TBST) for 2 h at room temperature and then incubated with an anti-SLC35D3 (abcam, Cambridge, MA, USA, Cat. No. ab98334, 1:1000) or anti-GAPDH (abcam, Cambridge, MA, USA, Cat. No. ab85760, 1:2000) antibody overnight at 4°C. The membranes were washed three times with 10% TBST and then incubated with goat anti-rabbit IgG secondary antibody (abcam, Cambridge, MA, USA, Cat. No. ab72567, 1:5000). The proteins were detected using a ChemiDoc XRS Imaging System and analyzed with the Quantity One program (Bio-Rad, Hercules, CA, USA).
Results
cDNA cloning and sequence analysis of the SLC35D3 gene
This is the first study to report the nucleotide sequence of porcine SLC35D3. The 2238 bp SLC35D3 sequence was obtained by cloning and splicing using a cDNA from the liver of the Meishan pig as the template. It consisted of a 1272 bp CDS, and a 966 bp 3′ terminal UTR. A BLAST search of the NCBI's nucleotide sequence database revealed that the SLC35D3 fragment of Meishan pig origin was highly similar (99%) to the predicted SLC35D3 gene sequence of the pig (XM_013986971.1) (Fig. 1A). The Meishan pig SLC35D3 nucleotide and deduced amino acid sequences are shown in Figure 1B. In addition, the ORF Finder program identified the ORF between nucleotides 2–1273, encoding a protein of 423 amino acids (Fig. 1C)

Analysis of the amino acid sequence of SLC35D3
The deduced amino acid sequence had a molecular weight of 44653.9 Da and an pI of 6.94. SLC35D3 did not possess a signal peptide, as determined by the SignalP4.0 program (Fig. 2A, Table 1). In addition, the C-, S-, Y-, and D-scores were less than 0.5, indicating that SLC35D3 had no signal peptide and could not be secreted outside of cells. Prediction of its subcellular localization showed that 34.8% of the sequence may exist in the endoplasmic reticulum and 34.8% in plasma membrane, 13% may exist in the mitochondria, 4.3% may be in the vacuoles and 4.3% could be in the Golgi, and 4.3% could be in the nucleus, and 4.3% could be in the extracellular. The secondary structure of the protein was predicted to consist mainly of α-helices, β-folds, and random coils (Fig. 2B). TMHMM results indicated that the protein possessed eight transmembrane domains (Fig. 2C). The ProtScale program at ExPASy calculated the hydrophobicity profiles of porcine SLC35D3 (Fig. 2D). The ordinate represented the hydrophobic score of the protein; a high score was indicative of an overall high hydrophobicity, while a low score was indicative of an overall low hydrophobicity. The abscissa represented the position of the amino acids. As shown in Figure 2D, the first 300 amino acids of SLC35D3 were hydrophobic, while the remaining amino acids were hydrophilic. Furthermore, the amino acids near position 230 had the highest hydrophobicity. The amount of hydrophobic amino acids is larger than that of hydrophilic amino acids.

Predicted 3D structure of SLC35D3
The fully automatic feature on the SWISS-MODEL program was used to construct the 3D structure of SLC35D3 (amino acids 5–300) from the Meishan pig. Homology modeling revealed that this segment was not highly homologous to that of the human 5i20.1.A in the Protein Data Bank (PDB: D7A5Q8) (Fig. 3). The 3D structure of SLC35D3 from the Meishan pig may lay the foundation for studying the relationship between structure and function.

Characteristics of the deduced protein and phylogenetic analysis of SLC35D3
The deduced amino acid sequence of SLC35D3 from the Meishan pig was compared to that from nine other mammals using the MEGA4.0 program. The coding sequence and amino acid sequences accession number of the pig SLC35D3 gene are shown in Table 3. The phylogenetic tree was constructed from the deduced Meishan pig SLC35D3 and the SLC35D3 sequences from other mammals using the neighbor-joining method in the MEGA4.0 program(Fig. 1D). The results showed that SLC35D3 from the Meishan pig clustered with
SLC35D3 from other mammals; the highest homology was with Vicugna, and the lowest homology was with Gorilla.

Expression of SLC35D3 mRNA in different tissues
The SLC35D3 mRNA level was normalized against that of β-actin. qPCR was used to analyze the SLC35D3 mRNA level in different organs, including the liver, kidney, lung, heart, brain, spleen, dorsal muscle as well as the leaf lard, PAT and SAT from Meishan pigs. PAT, SAT, LM, leaf lard and brain from Bama pigs. PAT, SAT, LM, leaf lard from Yorkshire. The results showed that SLC35D3 expression was high in the lung, leaf lard, PAT, and SAT, but low in the brain (data not shown). The high expression of SLC35D3 in leaf lard, PAT, SAT suggested that SLC35D3 might have an important role in adipose deposition (Fig. 4F).

Expression of SLC35D3 in adipose tissues from different varieties of pigs
We further analyzed the SLC35D3 mRNA level in adipose tissues from Yorkshire, Meishan, and Bama pigs and found differences in expression across the three breeds. In SAT and PAT, the SLC35D3 mRNA level was highest in Yorkshire, followed by Bama and the Meishan pigs. In the leaf lard and LM, the SLC35D3 mRNA expression level was higher in Meishan and Yorkshire pigs than that in the Bama pig(Fig. 4).

Discussion
Obesity is caused by excessive intake of nutrients, the body's consumption of nutrients is certain, when excessive intake of nutrients, a imbalance between nutrients intake and consumption will be broken, excess nutrients will cause obesity. This is a complex physiological process that requires the participation of the nervous system[29-33]. In the past, it was believed that obesity was caused by abnormal endocrine and metabolic disorders. A recent study found that obesity is not only caused by abnormal metabolism, Li Wei research group found that the SLC35D3 protein was participate in fat deposition, meanwhile it is involved in the neural regulation process, and this is the first report about metabolic disorder caused by unusual central behavior not due to endocrine disorders. SLC35D3 localizes on mouse chromosome 10 and regulates platelet-dense granule content[20]. In the human, the SLC35D3 gene, which localizes on chromosome 6 close to the D6S1009, its mutation can cause obesity, it is a candidate gene for Mets[19]. However, there is no study on the function of SLC35D3 in the pig and its complete sequence remains unavailable. We report for the first time the cDNA sequence of SLC35D3 from the Meishan pig. It localizes on chromosome 1, possesses three exons, and contains a 1272 bp coding sequence (CDS) that encodes 423 amino acids, and a 966 bp 3’ terminal UTR. The deduced amino acid sequence of SLC35D3 was highly homologous (92–95%) with that of other mammalian species. The phylogenetic tree analysis revealed that the Meishan pig SLC35D3 amino acid sequence had a close genetic relationship with Vicugna. Porcine SLC35D3 did not possess a signal peptide, similar to SLC35D3 from other animals. It belongs to the intracellular signaling molecules, but contains obvious transmembrane domain. Our experimental results demonstrate that the sk35d3 gene mutation can lead to obesity and Mets.

From the NCBI, we know that SLC35D3 sequences of many species were just predicted sequences, and that most sequences are unavailable, including pig. The human SLC35D3 full-length cDNA sequence cloning is available[19]. According to the report, the human SLC35D3 protein is composed of 416 amino acid residues with a hydrophobic transmembrane region, a relatively stable structure, and a certain fluidity[25]. Our findings on
SLC35D3 in the Meishan pig agree with those in the human. IMF and fat deposition correlate closely with meat quality, and primary intramuscular preadipocytes are useful to study fat deposition mechanisms[33]. Previous studies have shown that ns mutant mice harboring a recessive mutation in the SLC35D3 gene show obesity and MetS[19]. However, it is still unclear whether SLC35D3 is differentially expressed in different pig breeds. Our results indicated that the mRNA level of SLC35D3 in the Meishan pig was similar in most of the tested tissues. However, it was more highly expressed in adipose tissues compared to other tissues, including heart, liver, and kidney. In the mouse, SLC35D3 is primarily expressed in the liver and kidney compared with other organs, but not in adipose tissues[20]. Based on our results, we conclude that SLC35D3 may have an important function in adipose tissue.

The different expression patterns in the different fat tissues of lean, obese and mini-type pigs indicate that SLC35D3 gene may play an important role in porcine fat accumulation and metabolism. Gardan et al. (2006) evaluated the fat deposition in different parts of 80–210 day old pigs, the sequence of deposition was: the muscle<SA< PAT. Adipose deposition in the growth stage showed a rising trend, but the rate of deposition in the muscle is higher than that in SAT and PAT[34-35]. The expression level of SLC35D3 gene in the leaf lard and LM was low in the Bama miniature pig compared with Yorkshire and Meishan pigs. The Yorkshire pig is a foreign breed, while Meishan and Bama pigs are local breeds. The Yorkshire leaf lard content was significantly higher than that in Bama and Meishan pigs, which may be because individual reasons resulting in less fat deposition. Meanwhile, the Bama pig muscle myoglobin content is high and stable, IMF content is high than that of Landrace, Landrace and Yorkshire are foreign pig breeds, so it consistent with the expression level of SLC35D3 in LM. The Meishan pig is defined as an obese pig, and its fat content is higher than that of Yorkshire and Bama pigs and its backfat thicker than the Bama and Yorkshire. The Bama pig is a mini-type pig, and its backfat thickness is higher than that of the Yorkshire pig, which is widely distributed across the world because of its fast growth-rate and lean meat content[36].

The fat percentage and the IMF content of local breed pigs were significantly greater than that of the Yorkshire pig.

In conclusion, the SLC35D3 gene from the Meishan pig was cloned. It consisted of a 1272 bp CDS that encoded 243 amino acids with a molecular mass of 44653.9 Da, and 966 bp 3′ UTR. In addition, the phylogenetic tree analysis revealed that the pig SLC35D3 had a closer genetic relationship and evolutionary distance with Vicugna; however, its evolutionary distance with that of the gorilla was longer than that of Vicugna. Furthermore, the qPCR results showed that SLC35D3 was highly expressed in adipose tissues, indicating that this gene may be involved in fat deposition. The results of this study provide a good basis for further study on the function and regulation of SLC35D3.

Disclosure of Potential Conflicts of Interest
The authors have no conflict of interest to disclose.

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21. Meng, R. et al. SLC35D3 delivery from megakaryocyte early endosomes is required for platelet dense granule biogenesis and is differentially defective in Hermansky-Pudlak syndrome models. Blood. 2012; 120:
Figure 1. (A) BLAST search of NCBI's nucleotide sequence database showing a distribution of BLAST hits on the queried sequence (all BLAST hits are not shown). (B) Nucleotide and predicted amino acid sequences of SLC35D3 in the Meishan pig. The start codon (ATG) is highlighted in red, and the stop codon (TGA) is highlighted in red and denoted with an asterisk. (C) ORF Finder. (D) Phylogenetic tree and alignment of SLC35D3 amino acid sequences from the Meishan pig and other species. The GenBank Accession Numbers of the SLC35D3 sequences are listed in Table 3. The tree was constructed using the neighbor joining method in the MEGA 4.0 program.

Figure 2. (A) Prediction of the signal peptide of SLC35D3 (C-score: Cleavage site score, S-score: Signal peptide score, Y-score: combined cleavage site score). (B) Predicted secondary structure of the porcine SLC35D3 amino acid sequence. The secondary structure prediction method was used. The blue lines represent α-helices, the red lines represent extended strands, and the purple lines represent random coils. (C) Protein transmembrane domains of porcine SLC35D3 by TMHMM tool. (D) Hydrophobicity profile of porcine SLC35D3 analyzed by the ProtScale program.
**Figure 3.** Relative mRNA and protein levels of *SLC35D3* in adipose tissue of the pig analyzed by qPCR (A–D) and immunoblotting (E). β-actin (A–D) and GAPDH (E) served as the internal references in qPCR and immunoblotting experiments, respectively (LL represents leaf lard). (F) Relative mRNA level of *SLC35D3* in different tissues of the pig analyzed by qPCR. β-actin served as the internal reference. This experiment was repeated three times. Data are shown as means ± S.E.M.

**Figure 4.** Three dimensional model prediction of porcine SLC35D3 by the SWISS-MODEL.

**Table 1.** Prediction of a signal peptide sequence in porcine SLC35D3.

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### Table 2. Primer sequences and their use in this study

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### Table 3. Homology of SLC35D3 nucleotide and amino acid sequences between Sus scrofa and other species.

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