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Molecular Cloning and Characterization of a Novel Gene *STXBP6* from Chinese Banna Mini-Pig Inbred Line (BMI)

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Abstract: The complete CDS sequence of Banna Mini-pig Inbred Line (BMI) gene *STXBP6* was amplified using the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) based on the conserved sequence information of the rat, mouse or other mammals and known highly homologous swine ESTs. This novel gene was then deposited into NCBI database and located on Chromosome 7 of pig and assigned to accession number JF750402. Sequence analysis revealed that the BMI STXBP6 encodes a protein of 210 amino acids that has high homology with the Syntaxin Binding Protein 6 (STXBP6) of four other species rat (100%), mouse (99%), cattle (99%) and human (99%). The phylogenetic tree analysis revealed BMI STXBP6 has a closer genetic relationship with the rat STXBP6 than with those of mouse, cattle and human. Analysis by RT-PCR showed that BMI *STXBP6* gene was over-expressed in lung, moderately expressed in midbrain, ovary, diencephalon, spleen, nerve fiber, stomach, small intestine, large intestine, skin, muscle and fat, weakly expressed in kidney and heart and almost not expressed in lymph node, cerebrum, liver and pancreas. The experiment will establish a foundation for further insight into this swine gene.

Key words: Banna Mini-pig Inbred Line (BMI), pig, Syntaxin Binding Protein 6 (STXBP6), tissue expression, bioinformatics analysis, China

INTRODUCTION

Syntaxin Binding Protein 6 (STXBP6) also known as amisyn was isolated as a syntaxin binding protein enriched in brain (Scales *et al*., 2002) and is thought to act as an inhibitor of exocytosis (Constable *et al*., 2005). STXBP6 is also, expressed in the cement gland an amphibian-specific transient structure that is notable for being highly secretory.

STXBP6 is also, expressed in cranial ganglia before mid-tail bud stage. STXBP6, a 210 amino acid polypeptide has been identified to bind components of the SNARE complex and is known to be involved in regulating SNARE complex assembly and exocytosis through its C-terminal coiled-coil, a vesicle-SNARE-homology domain (Scales *et al*., 2002; John *et al*., 2005; Constable *et al*., 2005). Although, its characterization is still limited, amisyn has been proposed to form part of a vesicle-docking complex and is known to partially co-sediment with membranes (Scales *et al*., 2002).

Swine are generally considered to be the most ideal biomedical laboratory animals for their anatomical, physiological and metabolic characteristics are similar to human's. The inbred animals are good enough to be used as experimental animals, owing to their clear genetic background, high homozygosity, stable inheritance and so on. Inbred animals can also make less experimental errors using in biological research than noninbred ones (Wright, 1921; Harris, 1997). In 1980, the Banna Mini-pig Inbred line (BMI) was exploited by Yunnan Agricultural University based on the small-ear pigs at Xishuangbanna, Yunnan province, China. A pair of progenitors was a sow and her son with some degree inbreeding background. Then, the propagation was conducted by means of

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highly full sibling or parent-offspring inbreeding and each generation underwent the strict selection. As heterozygotic genes were separated and recombined in the process of inbreeding, BMI has already owned six families and eighteen substrains with different phenotypes and genotypes. Due to their consistent genetic background and minor interindividual differences, BMI is considered as an ideal model organism for biological studies (Yu *et al*., 2004; Zeng and Zeng, 2005).

Based on above described about the *STXBP6* gene, it is necessary to isolate this gene from pig for it is associated with energy metabolism, health and other important biological functions of animals. But until today the porcine STXBP6 has not been reported yet. The objective of this study was to isolate the full length coding sequence of BMI *STXBP6* gene according to the conserved sequence information of rat, mouse or other mammals and highly homologous swine ESTs sequence information, conduct sequence analysis and some necessary function analysis of established nucleotide sequence, finally examine the expression in a range of BMI tissues. These will provide a primary foundation for further research on this porcine gene.

MATERIALS AND METHODS

Samples collection, RNA extraction and first strand cDNA synthesis: Three matured female BMI were slaughtered for sampling. Fresh tissues (lymph node, midbrain, ovary, diencephalon, cerebrum, liver, kidney, spleen, heart, lung, nerve fiber, stomach, small intestine, large intestine, pancreas, skin, muscle and fat) were snap frozen in liquid nitrogen and stored at -80°C before use. Total RNA was extracted using the RNAiso Plus (TaKaRa, Dalian) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested with RNase-free DNase I (TaKaRa, Dalian). About 3 μg of RNA were reverse transcribed with oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase (Invitrogen, USA).

Isolation of the BMI *STXBP6* gene: The GenBank STXBP6 sequences for rat (NP_001178801), mouse (NP_653135) and their highly homologous pig ESTs sequences: EW180176, EW378526, EW550889 and FS692786 were used to design a primer pair to amplify the complete coding sequence of STXBP6 by using Primer Premier 5.0 Software. The primers for BMI *STXBP6* gene were: 5'-CGA CAA TGA GTG CCA AAT CT-3' and 5'-CTG CTG AAC AAA CTT CAA CTT C-3'. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed to isolate the BMI STXBP6 using the pooled cDNAs from different tissues. The 25 μL reaction system was: 2.0 μL cDNA (25 ng μL^{-1}), 2.0 μL 2.5 mM mixed dNTPs, 2.5 μL 10×Taq DNA polymerase buffer (Mg^{2+} plus), 0.5 μL 10 μM forward primer, 0.5 μL 10 μM reverse primer, 0.25 μL Taq DNA polymerase (5 U μL^{-1}, TaKaRa, Dalian) and 17.25 μL sterile water. The PCR program initially started with 94°C denaturation for 2 min followed by 35 cycles of 94°C/30 sec, 55°C/40 sec, 72°C/1.5 min then 72°C extension for 10 min, finally 4°C to terminate the reaction. After the PCR, the gene product was cloned into pMD18-T vector (TaKaRa, Dalian) and sequenced bidirectionally with the commercial fluorometric method. At least five independent clones were sequenced.

Bioinformatics analysis: Sequence analysis of BMI *STXBP6* gene was performed using software in NCBI (http://www.ncbi.nlm.nih.gov) and ExPaSy (http://www.expasy.org). The cDNA sequence was predicted using the GenScan Software (http://genes.mit.edu/GENSCAN.html). The Blastp Program and Conserved Domain Architecture Retrieval Tool were used to search for similar proteins and conserved domain, respectively (http://www.ncbi.nlm.nih.gov/Blast). The alignment of the nucleotide sequences and deduced amino acid sequences were computed using ClusterX and the phylogenetic tree was computed using the MEGA 4.0 Software with standard parameters. The theoretical isoelectric point (pI) and molecular weight (Mw) were predicted using the Compute pI/Mw Tool (http://us.expasy.org/tools/pi_tool.html). The putative signal peptide was predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The putative protein subcellular localization was predicted using PSort II (http://psort.hgc.jp/). Transmembrane topology prediction was performed using TMHMM-2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Secondary structures of deduced amino acid sequences were predicted with SOPMA (http://npsa-pbil.ibcp.fr/). Web-based microRNA (miRNA) predicting programs were used to locate conserved potential miRNA targets (http://www.mirbase.org/).

Semi-quantitative RT-PCR: To characterize the *STXBP6* gene further, RT-PCR was conducted to determine its expression in 18 BMI tissues. To eliminate the effect of cDNA concentration, we selected the housekeeping gene 18S rRNA (NR_002170) as a positive control. The control primers used were: 5'-GGA CAT CTA AGG GCA TCA CAG-3' and 5'-AAT TCC GAT AAC GAA CGA GAC T-3'. The BMI STXBP6 primers which were used to perform the semi-quantitative RT-PCR for tissue expression profile analysis were the same as the primers for isolation

RT-PCR above. The PCR reactions were optimized for a number of cycles to ensure product intensity within the linear phase of amplification.

RESULTS AND DISCUSSION

Cloning and identification of BMI STXBP6 cDNA: A STXBP6 fragment 690 bp of including the entire 633 bp coding region plus 5 bp of 5'-UTR and 52 bp of 3'-UTR was amplified (Fig. 1). This cDNA nucleotide sequence analysis using the BLAST Software at NCBI server revealed that BMI *STXBP6* gene was not homologous to any of the known porcine genes and it was then deposited into the GenBank database (Accession No.: JF750402). The sequence prediction was carried out using the GenScan Software and results showed that the 633 bp cDNA sequence represents a single gene which encoded 210 amino acids. The complete CDS and the encoded amino acids were shown in Fig. 2.

Physical and chemical characteristics of BMI STXBP6: The theoretical isoelectric point (pI) and Molecular weight (Mw) of STXBP6 were computed using the Compute pI/Mw Tool. The theoretical pI and the molecular weight of BMI STXBP6 are 9.30 and 23670.99, respectively. The result from SignalP revealed that there is has no putative signal peptide in BMI STXBP6 and indicated that it was probably a non-secretory protein (Petersen *et al*., 2011). The potential protein subcellular localization prediction by Reinhardt's method showed that BMI STXBP6 was probably located in the nuclear with up to 52.2% probability (Nakai and Horton, 1999). Using a hidden Markov Model algorithm, transmembrane topology prediction made by TMHMM program (Moller *et al*., 2001) showed that BMI STXBP6 was not a potential transmembrane protein.

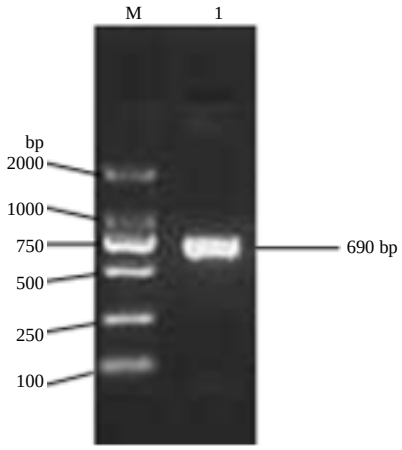

Fig. 1: RT-PCR result for BMI *STXBP6* gene; M: DL2000 DNA marker, 1: PCR product

Prediction and analysis of structures and conserved domains of BMI STXBP6: Proteins often contained several domains, each of which had their own evolutionary origins and functions. Examined using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) indicated that BMI STXBP6 contains one separated conserved domain synaptobrevin superfamily from 175- 207 amino acid residues (Fig. 3). The putative protein was also analyzed using prosite (http://expasy.org/prosite/). Four kind sites were found which were Protein kinase C phosphorylation sites (2-SaK-4, 29-TkK-31, 53-TnK-55, 117-SeK-119, 135-TdR-137); cAMP-and cGMP-dependent protein kinase phosphorylation sites (26-KRrT-29, 55-KKpT-58); N-myristoylation site (90-GIdpNR-95); Casein kinase II phosphorylation sites (115-TasE-118, 157-SaaD-160). The prediction of secondary structure by SOPMA indicates that the deduced BMI STXBP6 consists of alpha helices (105AA), extended strands (35AA), beta turn (11AA) and random coils (59AA) (Fig. 4).

Location of potential miRNA targets: MicroRNAs are noncoding single-stranded RNA molecules of 17-24 nucleotides that can regulate gene expression by binding to the coding region of target mRNAs (Bartel, 2004;

Fig. 2: The complete cDNA sequence and amino acid sequence of the protein encoded by STXBP6 (GenBank accession number: JF750402). ATG, start codon; TGA, stop codon; capital letters in pane, complete CDS and amino acid sequence; gray highlighted nucleotide sequence, primers

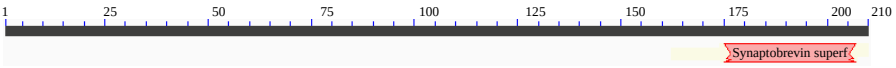

Fig. 3: The putative domains of the protein encoded by BMI STXBP6

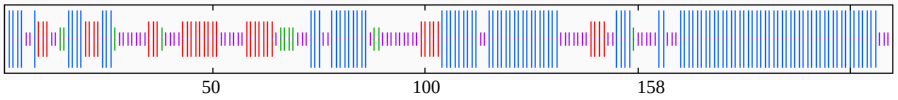

Fig. 4: The secondary structure of the BMI STXBP6 protein predicted by SOPMA. Helices, extended strands, beta turns and random coils are indicated, respectively with the longest, the second longest, the second shortest and the shortest vertical lines

Fig. 5: Alignment of the protein encoded by the BMI-pig STXBP6 and four other types of STXBP6 from rat (NP_001178801), mouse (NP_653135), cattle (DAA17536) and human (NP_054897)

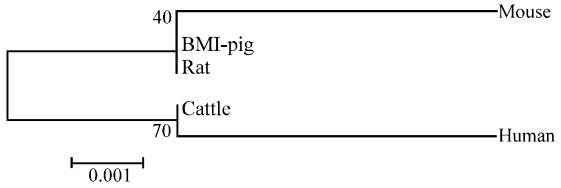

Fig. 6: Phylogenetic tree for STXBP6 protein from 5 species

Zeng *et al*., 2003). Researchers use web-based microRNA (miRNA) predicting programs to locate conserved potential miRNA targets: miRBase (http://www.mirbase.org/). The results showed *Sus scrofa* microRNA can't be found in the BMI STXBP6 CDS sequence.

Analysis of sequence identity and evolutionary relationships of BMI STXBP6: The deduced protein sequence of BMI STXBP6 was submitted to generate BLAST reciprocal best hits and similarity comparison revealed that BMI STXBP6 protein has high homology with the STXBP6 proteins of four other species rat (100%), mouse (99%), cattle (99%) and human (99%) (Fig. 5). To evaluate the evolutionary relationships of BMI STXBP6 with other species then researchers constructed a phylogenetic tree using DNAstar, Cluster, MEGA and DNAMAN softwares on the basis of the STXBP6 amino acid sequences. The phylogenetic tree analysis revealed that the BMI *STXBP6* gene has a closer genetic relationship with the rat *STXBP6* gene than with those of mouse, cattle and human (Fig. 6). Therefore, we can use rat as model organisms to study the pig *STXBP6* gene or use pig as a model organism to study the rat *STXBP6* gene.

mRNA tissue-specific expression profile: To check the relative expression levels of STXBP6 mRNA in various porcine tissues, semi-quantitative RT-PCR was performed in 18 BMI tissues mentioned earlier. The continuously expressed gene, 18S was used and served as an endogenous reference for determination of targeted mRNA profiles. Semi-quantification analysis was performed with Quantity One Gray Scanning Software. Result revealed that BMI *STXBP6* gene over-expressed in lung, moderately expressed in midbrain, ovary, diencephalon, spleen, nerve fiber, stomach, small intestine, large intestine, skin, muscle and fat, weakly expressed in kidney and heart and almost not expressed in lymph node, cerebrum, liver and pancreas (Fig. 7).

Comparative genomics determines the relationship of genome structure and function of different species. Researchers have learned a great deal about the function of human genes by examining their counterparts in simpler model organisms such as the mouse and some results

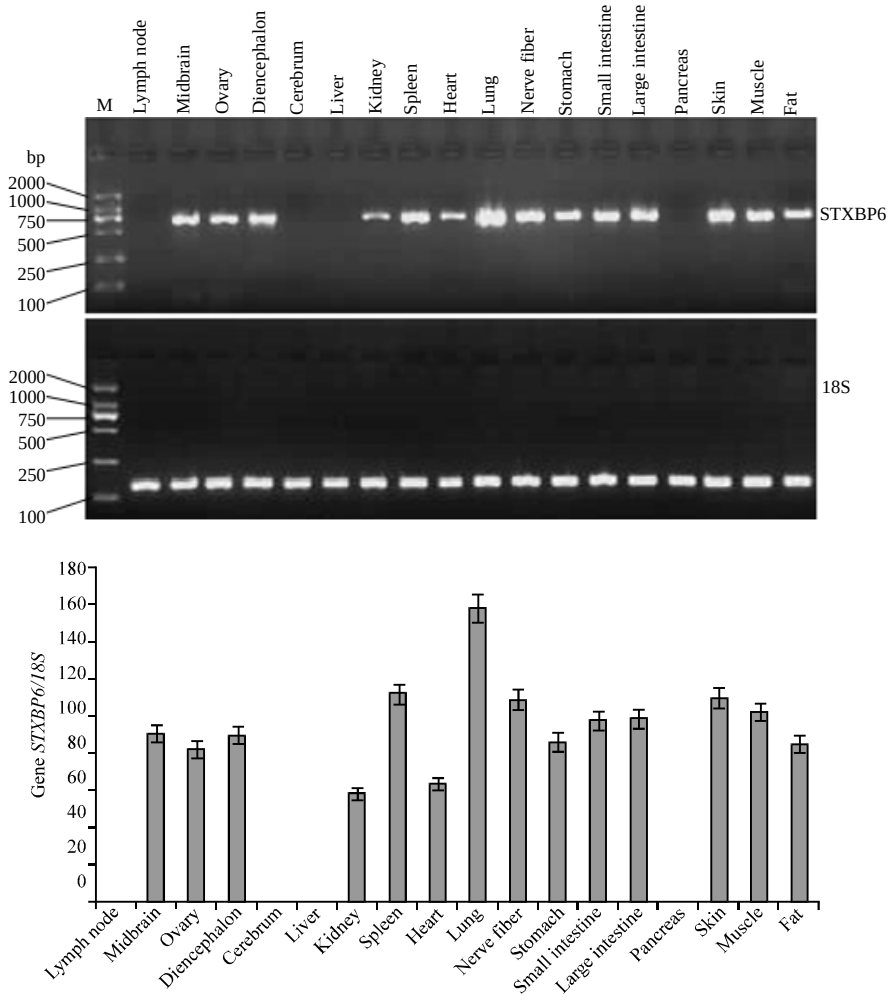

Fig. 7: Tissue transcription profile of BMI *STXBP6* gene. The 18S expression is the internal control

have revealed that virtually all (99%) of the protein-coding genes in humans align with homologues in mice and >80% are clear 1:1 orthologs (Hardison, 2003; Liu *et al*., 2008). This extensive conservation in protein-coding regions implied that the same protein-coding sequences may be expected in different mammals including pig. From the isolation of swine *STXBP6* gene, researchers can find that swine STXBP6 is highly homologous with STXBP6 of rat, mouse, cattle, human and other mammals. This further validated that comparative genomics method is one useful tool to isolate the unknown genes, especially the conserved coding region of genes for pig.

In this study, we not only cloned the CDS sequences of the BMI *STXBP6* gene but also conducted the sequence analysis and tissue expression profiles analysis. From the tissue expression profile analysis it can be seen that the gene was obviously differentially expressed in various tissues. As the researchers did not study functions at protein levels, there might be many possible reasons for differential expression of this porcine gene. The suitable explanation for this under the current conditions is that the biological activities associated with the functions of the gene were required in a different extent in different tissues at the same time.

CONCLUSION

In this study, the research firstly isolated BMI *STXBP6* gene and performed necessary functional analysis and tissue expression profile analysis. The cDNA clone, sequence information and function analyses of BMI *STXBP6* gene will be extremely important in elucidating the essential physiological function of STXBP6 protein using BMI and other miniature swines as experimental animal models in the future.

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