Cloning and Identification of Porcine SMPX Differentially Expressed in F1 Crossbreds and Their Parents

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Abstract

In order to investigate porcine heterosis on the molecular basis, Large White (L), a European purebred, and Meishan (M), a Chinese indigenous purebred, were hybridized directly and reciprocally to produce F1 hybrids, Large White × Meishan (LM) and Meishan × Large White (ML) pigs. Using mRNA differential display, we found an expression sequence tag (EST) differentially expressed in F1 hybrids and their parents, designated as EST55, which was homologous to human and murine skeletal muscle protein (SMPX), and the full-length cDNA of porcine SMPX was cloned by the rapid amplification of cDNA end (RACE) method. Translation of the mRNA transcript revealed an open reading frame (ORF) of 86 amino acid residues encoding a nuclear location signal peptide, two overlapping casein kinase II phosphorylation sites and one N-glycosylation site with theoretical molecular weight of 9.3 kDa. Alignment analysis revealed that the deduced protein sequence shared 94%, 83% and 78% homology with that of its human, mouse and rat counterparts, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that it was expressed predominantly in skeletal and heart muscles, whereas at a moderate level in backfat, spleen, stomach and uterus tissues. Two single nucleotide polymorphism (SNPs), located in 5'- and 3'-untranslated region (UTR), respectively, were identified by PCR and sequencing. Phylogenetic tree and the secondary structure prediction were also performed. The possible relationship between porcine SMPX and heterosis was discussed.

Key words skeletal muscle protein; pig; mRNA differential display; expression pattern

Heterosis, which is defined as superiority of hybrids to their parents in areas such as growth and reproduction, is a significant phenomenon in the field of life science. Heterosis has made significant contributions to world food needs [1]. Three classical hypotheses, dominance, over-dominance and epistasis, may be the genetic basis of heterosis, but our understanding for heterosis is far from adequate [2]. It is known that the hybrid’s genome is derived from the parents, so the hybrids do not create new genes. This suggests that gene expression probably varies under a new genetic background [3].

The mRNA differential display technique, first described by Liang and Pardee [4], is a fast and efficient method for investigating the difference of gene expression in different types. This technique possesses advantages over other similar techniques as follows: more than two samples can be compared simultaneously, and only a small amount of total RNA is required. There were significant correlations between patterns of differential gene expression and heterosis of some economic traits in plants [3,5], also in animals [6], by using the mRNA differential display technique.

In the present study, we identified a differentially expressed gene, skeletal muscle protein gene (SMPX) encoding small muscle protein, in backfat between F1 crossbred pigs, Meishan × Large White (ML) and Large White × Meishan (LM), and their parents, Meishan (M) and Large White (L).
Materials and Methods

Animals

F1 hybrids, ML and LM pigs and their parents, M and L pigs used for mRNA differential display were derived from Jingpin Pig Station of Huazhong Agricultural University.

cDNA synthesis and differential display of mRNA

Total RNAs were isolated using Trizol reagent (Invitrogen, Grand Island, USA) from the backfat of six F1 hybrids, ML and LM pigs, respectively, and six purebreds, M and L pigs, respectively, and mixed to construct RNA pools. In cases where the samples were contaminated with genomic DNA, DNase I (Promega, Madison, USA) was used. Total RNAs were isolated using Trizol reagent (Invitrogen, Grand Island, USA) from the backfat of six pigs. The house-keeping gene GAPDH was used as an internal control (the forward primer is 5'-ACCACAGTCCAT-GCCATCAC-3' and the reverse primer is 5'-TCCACACC-CTGTTGCTGTA-3'). The PCR amplification was performed as follows: initial denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s; and an additional extension step for 10 min at 72 °C.

RT-PCR analysis of porcine SMPX expression

For spatial expression analysis, total RNAs were also isolated from various tissues including backfat, Longissimus dorsi, heart, liver, spleen, lung, kidney and small intestine of M pigs. The house-keeping gene GAPDH was used as an external control (the forward primer is 5'-ACCACAGTCCAT-GCCATCAC-3' and the reverse primer is 5'-TCCACACC-CTGTTGCTGTA-3'). The PCR amplification was performed as follows: initial denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s; and an additional extension step for 10 min at 72 °C.

Rapid amplification of cDNA ends-PCR

The differentially expressed cDNA fragment (EST55) was homologous to human SMPX with 85% identity. In order to obtain the full-length cDNA of porcine SMPX, 5' and 3' rapid amplification of cDNA ends (RACE) were used according to BD SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, USA). The gene-specific primers were: 5'-CTCGCGGTCAACCTATCGGAGATCAG-3' for 5' RACE and 5'-CTGGAGGTTGTTGTTTTTGAGGAGGA-3' for 3' RACE.

Sequence analysis software

Sequence similarity analysis in GenBank was carried out using the Blast 2.1 search tool. The amino acid sequence of porcine IDH3β gene deduced by New Genescan Web Server at Massachusetts Institute of Technology (MIT) was analyzed by online ExPASy Molecular Biology Server (http://www.expasy.org/tools). Secondary structure prediction and phylogenetic tree were produced employing the Lasergene software from DNASTar (DNASTar Inc., USA). Transcription factors prediction in 5'-untranslated region (UTR) was performed by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). PSORT Version II (http://psort.hgc.jp/) was used to predict protein sorting signals. Prediction of potential biologically significant sites was performed by PROSITE (http://www.expasy.org/prosite). In addition, destabilizing PEST motif was predicted by PESTfind (https://emb1.bcc.univie.ac.at/content/view/21/45).

Results

Identification of EST55 as differentially expressed gene between F1 hybrids and their parents

A band, designated as EST55 and occurring only in L
pigs, was isolated from the mRNA differential display gel (Fig. 1) and reamplified. Semiquantitative RT-PCR analysis found that EST55 presented high-level expression in backfat of L pigs (Fig. 2), whereas, very low abundance in that of M, ML and LM pigs. This was approximately consistent with the result of mRNA differential display.

**Cloning of the porcine SMPX cDNA**

5’ and 3’ flanking sequences of porcine SMPX were amplified by 5’ and 3’ RACE-PCR (Fig. 3), respectively, and sequenced. Therefore, an 863-bp full-length porcine SMPX cDNA was obtained. The sequence data have been submitted to the GenBank database under accession number of DQ845243.

**Nucleotide sequence analysis**

Porcine SMPX mRNA contains an open reading frame (ORF) of 261 nucleotides, encoding a protein of 86 amino acids (Fig. 4). The ORF begins the longest reading frame and one in-frame stop codon (TAA in Fig. 4) is presented at position 105–107 in the 5’-UTR. It is often the first methionine that initiates translation [8]. Therefore, we infer that the ATG codon at position 124–126 is the true start site of translation. In addition, a polyadenylation signal, AATAAA, is found at position 788–793. Six destabilizing motifs, ATTTTA or ATTTT, which probably accelerate mRNA degradation, are found in 3’-UTR (Fig. 4).

Sequence alignments between M and L pigs show two putative base mutations, C111T and G525A, in 5’-UTR.

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and 3'-UTR region, respectively. Three trans-factors, heat shock factor (HSF), myoblast determining factor (MyoD) and runt-factor AML-1 (AML-1a) were found based on the prediction using TFSEARCH (Fig. 4). However, the AML-1a could not bind the cis-sequence when T was substituted by C at position 111 in 5'-UTR.

Analysis of the porcine skeletal muscle protein sequence

Translation of the porcine SMPX mRNA transcript revealed an ORF of 86 amino acid residues with theoretical molecular weight of 9.3 kDa and isoelectric point of 9.2. Prediction of protein sorting signals (PSORT) identified two overlapping Pat 7 nuclear localization motifs (PPRKKEC and PRKKECT) (Fig. 4). Two overlapping casein kinase II (CKII) phosphorylation sites (TSDE and SDEE) and one N-glycosylation site (NLSE) were found by PROSITE prediction (Fig. 4). SMPX protein sequence was also analyzed using PESTfind, which identified a destabilizing PEST motif (ECTPETEEA VLPTSDEE) (Fig. 4). Similarity comparison for the SMPX amino acid residues of five species was carried out (Fig. 5). The porcine SMPX amino acid sequence shares 94% identity with that of human, and 83% and 78% identities with that of mouse and rat, respectively. Based on the available sequence of vertebrate SMPX proteins, a phylogenetic tree, constructed (Fig. 6) using the Jotun Hein method [9], revealed that two clusters were formed between mammalian, including porcine SMPX, and other vertebrate. Therefore, we can further confirm that the gene isolated in our experiment is porcine SMPX gene.

Hydropathy plots of porcine SMPX, based on both Garnier-Robson and Chou-Fasman secondary structural predictions, are shown in Fig. 7. Alpha helical, beta sheet and turn structure are ordered throughout the protein sequence with several hydrophobic domains.

Expression profile of porcine SMPX mRNA

SMPX mRNA was expressed predominantly in Longissimus dorsi and heart, whereas in backfat, spleen, stomach and uterus tissues at a lower level (Fig. 8). In human, SMPX was constitutively expressed in skeletal
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Discussion

Skeletal muscle protein plays a role in skeletal muscle hypertrophy in response to stretch, increased load, and exercise [10]. It may also participate in the regulation of cytoskeletal dynamics through the Rac1-p38 pathway [11]. Until now, it has not been reported about the porcine SMPX.

In the present study, we isolated its cDNA, which contained a 261-bp ORF encoding 86 amino acids, using the mRNA differential display technique.

The porcine putative protein predicted to contain two overlapping putative CKII phosphorylation sites (PPRKEC and PKKECT), which are conserved with the CKII consensus sequence, so it can be regarded as a genuine candidate site for phosphorylation. One N-glycosylation site was also found by prediction. Protein and cardiac muscles [10].

Fig. 6  Phylogenetic tree of the skeletal muscle protein (SMPX)

Fig. 7  The secondary structure prediction by Garnier-Robson and Chou-Fasman methods

Fig. 8  mRNA expression of porcine IDH3β in heart, liver, spleen, lung, kidney, stomach, small intestine, ovary, uterus, longissimus dorsi and backfat

GAPDH, glyceraldehydes-3-phosphate dehydrogenase gene; SMPX, skeletal muscle protein. M, marker; 1, heart; 2, liver; 3, spleen; 4, lung; 5, kidney; 6, stomach; 7, small intestine; 8, ovary; 9, uterus; 10, Longissimus dorsi; 11, backfat.

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N-glycosylation is a metabolic process that has been highly conserved in evolution, functioning by modifying appropriate asparagine residues of proteins with oligosaccharide structures, thus influencing their properties and bioactivities [12].

Six ATTTA and ATTTT motifs within the 3'-UTR were found, as well as a PEST sequence in protein sequence 9 (Fig. 4). ATTTA and ATTTT motifs were first noted by Shaw and Kamen [13], who identified the highly conserved motif in several short-lived lymphokines associated with instability. PEST sequence indicates instability within skeletal muscle [14]. These suggest that SMPX mRNA has a short half-life.

The mRNA expression is predominant in porcine heart and skeletal muscles, which is consistent with the results in human and murine [10,15]. This indicates that the SMPX gene probably functions preferentially in muscles. However, the porcine SMPX is expressed in backfat detected in the present study, so it is implied that porcine SMPX functions in other ways. The mutation of C111T, resulted in the absence of AMI-1a binding to 5'-UTR of SMPX based on prediction, may be one of factors that induce the change of the transcriptional level between F1 hybrids’ and their parents’ backfat, and increased expression of CKII was involved in the regulation of MyoD and MRF4 gene and CKII acts indirectly on myogenic regulatory factors (MRFs) via regulation by other proteins [16]. So the different expression levels between F1 hybrids and their parents may induce the change of trait performance in F1 hybrids versus their parents, and accordingly, produce heterosis. It’s not enough that we understand the molecular basis of heterosis from a single gene. So we tried to isolate the amount of genes differentially displayed between hybrids and parents [17–19] in our laboratory.

We isolated and identified porcine SMPX the first time. But this is a preliminary work, and future efforts should be focused on the role of this protein in muscle and in backfat tissue, and its relation to heterosis.

References

9 Hein J. A new method that simultaneously aligns and reconstructs ancestral sequences for any number of homologous sequences, when the phylogeny is given. Mol Biol Evol 1989, 6: 649–668

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