

A novel pig gene, *CPNE1*, differentially expressed in the muscle tissues from Wujin pigs and Large White pigs*

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The mRNA differential display technique was performed to investigate gene expression differences in the *longissimus dorsi* muscle from Wujin and Large White pigs. A fragment of one differentially expressed gene was isolated and sequenced. A complete cDNA sequence of the gene was obtained using the rapid amplification of cDNA ends (RACE) method. The open reading frame of this gene encodes a protein of 537 amino acids, which is homologous with the Copine I (*CPNE1*) of eight species: greater horseshoe bat (95%), mouse (92%), rat (93%), cattle (95%), sheep (95%), rabbit (94%), human (95%) and dog (94%). This newly identified gene was respectively defined as the swine *CPNE1* gene and had been assigned GeneID: 100233174. The phylogenetic analysis revealed that the swine *CPNE1* gene has a closer genetic relationship with the *CPNE1* gene of a dog. The tissue expression analysis indicated that the swine *CPNE1* gene has a broad tissue distribution. The experiment described is the first to establish the primary foundation for further research on the swine *CPNE1* gene.

KEY WORDS: CPN1 / differential display / muscle / mRNA / pig

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The mRNA differential display first described by Liang and Pardee [1992], remains an efficient tool for comparative profiling of gene expression under different experimental conditions. It has statistically been shown that 80-120 primer combinations would be sufficient to cover all the transcript populations in the cell [Liang *et al.* 1993]. Such assay has the following advantages: (1) it is based on well established methods, (2) more than two samples can be compared simultaneously, and (3) only a small amount of starting material is needed [Yamazaki and Saito 2002].

Pigs of Chinese indigenous breeds such as Wujin, Meishan, Erhualian and Tongcheng often have superabundant fat and too low lean meat content, while European breeds, such as Large White, Landrace and Duroc, have lower fat and higher content of lean of the carcass. Therefore, Chinese indigenous pigs are always named fat-type, while exotic pigs the lean-type [Pan *et al.* 2004]. Given that phenotypic variances are mainly determined by the genetic differences, the identification of differentially expressed genes between Chinese indigenous and European pig breeds can give considerable promises for breeding.

Our present study was carried out with the mRNA differential display technique to identify the differentially expressed genes in the muscle tissues from Chinese indigenous breed, Wujin, and European Large White pig. The results are provided of the identification of previously unrecognised porcine gene, *CPNE1*, which is differentially expressed in Wujin *versus* Large White skeletal muscle tissues

Material and methods

Sample collection

The *longissimus dorsi* (LD) muscle samples were collected from five males and five females of Large White and five males and five females of Wujin pigs all aged 120 days for mRNA differential display and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analyses. The tissues including spleen, ovary, heart, small intestine, liver, lung, kidney, muscle and backfat were collected from one adult Wujin x Large White F1 crossbred pig for the later tissue expression profile analysis. The samples were immediately frozen in liquid nitrogen and stored at -80 °C. The total RNA was extracted from tissues using the total RNA extraction kit (GIBCO, Grand Island, NY, USA) following the manufacturer's recommendations. Before the first-strand complementary DNA (cDNA) synthesis, DNase I treatment of the total RNA was done.

Differential display

The differential display PCR amplification of each reverse transcription product was carried out with ten arbitrary and nine oligo (dT) primers as described by Liu *et al.* [2004]. The PCR products were then separated on the 8% non-denaturing polyacrylamide gel and displayed using the silver stain following Pan *et al.* [2003] and Liu *et al.* [2004].

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described by Liu [2010], Liu and Xiong [2009] and Yan *et al.* [2010]. To avoid the influence of cDNA concentration on semi-quantitative RT-PCR, we repeated PCR amplifications using 100, 200, 300, 400 and 500 ng cDNA as template. We selected the housekeeping gene *GAPDH* as the internal control. The control gene primers used were: 5'-ACCACAGTCCATGCCATCAC-3' (forward primer 1) and 5'-TCCACCACCCTGTTGCTGT-3' (reverse primer 1). The 420-bp PCR product was verified by sequencing. The following primers were used to perform the RT-PCR for identification and tissue expression profile analysis of the swine *CPNE1* gene: 5'-TGTACTTGCAGAAGTACC-3' (forward primer 2) and 5'-GAAAGTGCAAGCACTGAGG-3' (reverse primer 2). The PCR product was 216-bp in length (verified by sequencing). The 25 µl reaction system contains 2 µl cDNA (100-500 ng), 5 pmoles each oligonucleotide primer (forward primer 1 and 2, reverse primer 1 and 2), 2.5 µl 2 mmol/l mixed dNTPs, 2.5 µl 10×Taq DNA polymerase buffer, 2.5 µl of 25 mmol/l MgCl₂ and 2 units of Taq DNA polymerase. The PCR programme initially started with a 94°C denaturation for 4 min, followed by 25 cycles of 94°C / 50 s, 59°C / 50 s, 72°C / 50 s, then 72°C extension for 10 min, and finally 4°C to terminate the reaction.

PCR products were analysed in the linear range of amplification by agarose gel electrophoresis and intensity of bands was estimated using Glyco BandScan Software (PROZYME®, San Leandro, CA, USA). The ratio of *CPNE1* to *GAPDH* was calculated using EXCEL programme. Significance of differences between the ratios of *CPNE1* to *GAPDH* was analysed with the least square method (GLM procedure, SAS version 8.0).

Rapid amplification of cDNA ends (5'- and 3'-RACE)

5'- and 3'-RACE were performed according to the instructions of BD SMART™ RACE cDNA Amplification Kit (BD Science, USA). The Gene-Specific Primers (GSPs) were: 5'-RACE GSP: 5'-CTTTATTGAATGAGGGGCTCCTGGG-3' and 3'-RACE GSP: 5'-AAGGTTGGGCTCCATTCAAGCCA-3'.

RACE touchdown PCRs were carried out with 5 cycles of 94°C / 30 s and 72°C / 3 min, followed by 5 cycles of 94°C / 30 s, 70°C / 30 s and 72°C / 3 min, finally with 30 cycles of 94°C / 30 s, 67°C / 30 s, 72°C / 3 min to terminate reaction. The RACE PCR products were then cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced bidirectionally with the commercial fluorometric method (SHENGGONG, Shanghai, China). At least five independent clones were sequenced for each PCR product.

Sequence analysis

The cDNA sequence prediction was conducted using GenScan software (<http://genes.mit.edu/GENSCAN.html>). Protein sequence prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the

National Centre for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov/BLAST>) and ClustalW software (<http://www.ebi.ac.uk/clustalw>).

Results and discussion

mRNA differential display

From the mRNA differential display, one band, nominated as the band 135, later identified as a fragment of the *CPNE1* gene, was predominantly expressed in the LD muscle of Large White, while barely visible in the band pattern of the LD of Wujin pigs (Photo 1).

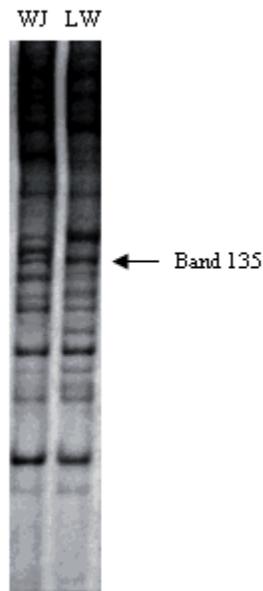


Photo 1. Exemplary representative band pattern on mRNA differential display analysis showing the up-regulated band 135 (about 300 bp) in Wujin (WJ) and Large White (LW) longissimus dorsi (LD) muscles.

Semi-quantitative RT-PCR

The differentially expressed band was recovered from gel and used as the template for the re-amplification, which was performed with the corresponding oligo(dT) and arbitrary primers used in the mRNA differential display assay. The resulting PCR product was 309 bp being consistent with that of the differential display shown on Photo 1. The purified PCR product was then cloned into the pMD18-T vector and

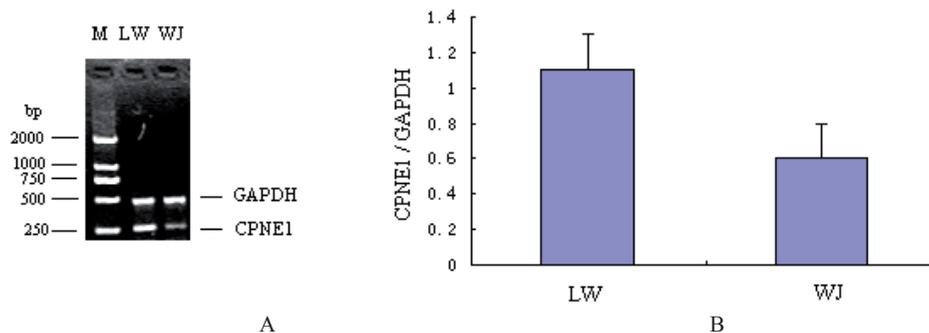


Photo 2. *CPNE1* mRNA expression levels relative to *GAPDH* in Large White (LW) and Wujin (WJ) LD muscle samples (RT-PCR, n=5). A – representative, B – calculated.

the recombinant plasmid was sequenced. Semi-quantitative RT-PCR was conducted and the results (Photo 2) indicated that the band 135 (*CPNE1*) was predominantly expressed in the LD muscle of Large White pigs.

5'- and 3'-RACE and sequence analysis

Through 5'-RACE, one PCR product of 1882 bp was obtained. The 3'-RACE product was 266 bp. These products were then cloned to T-vector and sequenced. The alignment of these 238-bp overlapping sequences yielded a 1910-bp cDNA sequence (Fig. 1). The nucleotide analysis, using the BLAST software at NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>), revealed that a 1910-bp cDNA sequence was not homologous to any of the known porcine genes, and it was then deposited into the GenBank database (accession number: FJ436394). The sequence prediction was carried out using the GenScan software. An open reading frame (ORF) encoding 537 amino acids was found in the 1910-bp cDNA sequence. A probability of exon was 0.810, while poly-A signal ranged from 1876-bp to 1881-bp (consensus: AATAAA). The complete cDNA sequence of this gene and the encoded amino acids are shown in Figure 1.

Further BLAST analysis revealed that the protein sequence was characterized by a high homology with that of the Copine I (*CPNE1*) of greater horseshoe bat (accession number: ACC68958; 95%), mouse (accession number: NP_733467; 92%), rat (accession number: EDL85869; 93%), cattle (accession number: NP_001070408; 95%), sheep (accession number: NP_001155362; 95%), rabbit (accession number: ACJ76628; 94%), human (accession number: NP_690902; 95%), and dog (accession number: XP_534404; 94%).

Based upon own investigations we assume that the gene in question can be defined as the porcine *CPNE1*. Based on the results of the alignment of eight known *CPNE1* proteins, a phylogenetic tree was constructed, using the ClustalW software (Fig. 2).

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CGGGAAGCCGTTCTGGCTGGGGCTGTCGACCGCGGGGGCGGAGGCACTCGCGCGGGGGTAACTCGGG
TCTGGGTTTCGGGTGCCGCGCAGTTCTCCCGATGGGCCCACTGCCTGACCTTGGTTTCAGCTGTCCATT
M A H C V T L V Q L S I
TCATGTGACCACCTCACTGACAAGGACATCGGCTCCAAGTCTGACCCACTCTGTGTCTTTTACAG
S C D H L T D K D I G S K S D P L C V L L Q
GATGTAGGAGGGGGCACCTGGGCTGAGCTTGGCCGTACAGAGCGAGTACGGAACCTGCTCGAGCCCT
D V G G G T W A E L G R T E R V R N C S S P
GAGTCTCAAGACTCTGCAGCTTGTGAGTACCCTTTGAAACGGTTCAAAAACCTCCGATTTGGTATC
E F S K T L Q L E Y H F E T V Q K L R F G I
TATGACATGACAATAAGACGCCTGAGCTGGGGGATGATGACTTCCTAGGAGGGGCTGAGTGTCC
Y D I D N K T P E L G D D D F L G G A E C S
CTAGGACAGATTGTATCCAGCAGATACTAACTTACCCTTGTGTTGAAGCTGGAAAACCTGCT
L G Q I V S S Q I L T L P L M L K P G K P A
GGGCGAGGACCATCAGGCTCAGCTCAGGAGTTGAAGGATAGTCAGTAGTGACCATGGAGGTG
G R G T I T V S A Q E L K D S R V V T M E V
GAGGCCAGAACTAGATAAGAAGGACTTCTGGGGAAATCAGATCCATTCTTGGAGTTCTTCCCG
E A R N L D K K D F L G K S D P F L E F F R
CAGGTGATGGGAAATGGCACCTGGCATACAGATCAGAGGTCAAGAACAACCTGAACCTACT
Q G D G K T W H L A Y R S E V I K N N L N P T
TGGAAGCGCTTCTGTTCTTTTCCAGATTCTGTGGGGGAGACCCAGTACACCCATCCAGGTG
W K R F S V P F Q H F C G G D P S T P I Q V
CGATGTTCCGATTATGACAGCGATGGTTCACATGATCTCATTGGTCCCTTCCACTACCTTGGCC
R C S D Y D S D G S H D L I G P F H T T L A
CAACTGCAGGCAGTCCCGGCTGAGTTTGAATGCATCCACCTGAGAAAACAGCAAAAAGAAAAG
Q L Q A V P A E F E C I H P E K Q Q K K K S
TACAAGAACTCTGGAATAGTCCGTGTCAAGATTTGTCAGGTAGAAACAGAGTATTCATTCTCGGAC
Y K N S G I V R V K I C Q V E T E Y S F L D
TATGTGATGGGAGGCTGTCAAATCAACTTCACTGTGGGTGTGGACTCACAGGCTCCAATGGAGAT
Y V M G G C Q I N F T V G V D F T G S N G D
CCTTCTCACCTGATTCTGCACTACCTGAGCCCAACAGGGGTCAATGAGTACCTGACAGCACTG
P S S P D S L H Y L S P T G V N E Y L T A L
TGGAGTGTGGGACGCTGGTTCAGGACTATGACTCGGACAAGCTGTCCAGCATTGGATTGGG
W S V G S V V Q D Y D S D K L F P A F G F G
GCTCAGGTACCCCTGACTGGCAGGTCTCCCATGAATTTGCCTTGAACCTCAACCCAGTAACCCC
A Q V P P D W Q V S H E F A L N F N P S N P
TACTGTGCAGGCATCCAGGCACTTGGATGCCTACCGCCAGGCCCTACCCCAAGTTTCGCCTCTAT
Y C A G I Q G I V D A Y R Q A L P Q V R L Y
GGTCCCAACCTTGCACCCATCATCAACATGTGGCCAGGTTTGCAGCCAGGCTGCTCATCAG
G P T N F A P I I N H V A R F A A Q A A H Q
AAGAGTGCCTCGAATACTTCGTGCTGTGCTGCTAACTGATGGTGTGTGACAGATGTGGAGGCC
K S A S Q Y F V L L L L T D G A V T D V E A
ACACGTGAGGCTGTGGTTCGAGCCTCCTACCTGCCATGTCAAGTATCATCTGGGTGTGGTGGT
T R E A V V R A S Y L P M S V I I V G V G G
GCTGACTCGAGGCCATGGAGCAACTGGACGCTGATGGCGGACCCCTGCATACAGCTCCGGGGAG
A D F E A M E Q L D A D G G P L H T R S G E
GCAGCTGCTGTGACATAGTGAATTTGTGCCCTACCGCCGCTTTCAGAATGCCCTCGAGAGGCA
A A A R D I V Q F V P Y R R F Q N A P R E A
TTGGCGCAGACTGTACTTGCAGAAGTACCCACGCAACTGGTTTCTACTTCAAGGCCCAAGGTTGG
L A Q T V L A E V P T Q L V S Y F K A Q G W
GCTCCATTCAAGCCACTCCACCCCAACCAAGGGCCCTGCACAGGCCCTCAGGCTTAGATTCCCTT
A P F K P L P P P T K G P A Q A P Q A *
GGCCTAGATTCCCTAGGACTGTGGCCAGTCCCTCAATCCTATGTTCCCAAGGTCACCCCAACCTGAACA
CACATTCCTCAGTGCTTGCATTTCTGTTTGTACTTTTATACTTCTCTCCATCCCACTGGCTTGC
CCAGGAGCCCTCATTCAATAAAGATCAATGAAGAACAACAAAAA

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Fig. 1. The cDNA and amino acid sequence of the porcine gene containing band 135 (GenBank accession number: FJ436394). ATG - start codon; TAG - stop codon (* stop codon).

The porcine *CPNE1* gene has a closer genetic relationship to the greater horseshoe bat *CPNE1* gene than to mouse, rat, cattle, sheep, rabbit, human and dog genes.

The RT-PCR profiling of tissue expression of the porcine *CPNE1* gene was carried out using tissue samples collected from an adult Wujin × Large White crossbred pig.

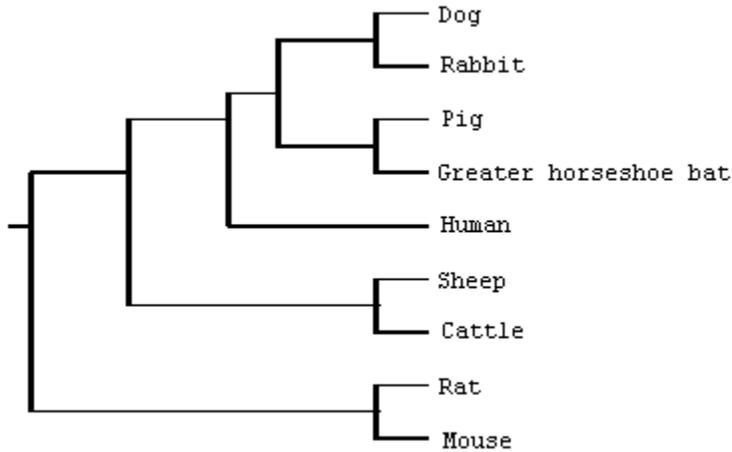


Fig. 2. The phylogenetic tree for the *CPNE1* gene

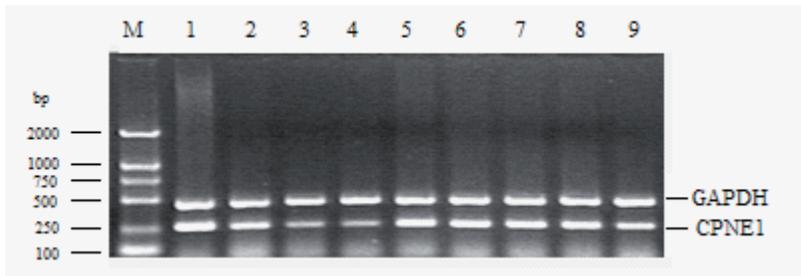


Photo 3. RT-PCR profiling of the porcine *CPNE1* gene expression. M – DL2000 marker; 1 – ovary; 2 – spleen; 3 – backfat; 4 – small intestine; 5 – skeletal muscle; 6 – heart; 7 – lung; 8 – kidney; 9 – liver.

As shown in Photo 3 the gene in question is predominantly expressed in ovary, spleen, muscle, heart, lung, kidney and liver, and moderately in backfat and small intestine (Photo 3).

CPNE1 is a member of calcium-dependent membrane-binding proteins which may regulate molecular events at the interface of the cell membrane and cytoplasm. The *CPNE1* encodes a calcium-dependent protein that also contains two N-terminal type II C2 domains and an integrin A domain-like sequence in the C-terminus. However, the encoded protein does not contain a predicted signal sequence or transmembrane domains. This protein has a broad tissue distribution and it may function in membrane trafficking [Tomsig *et al.* 2004, Zhang *et al.* 2008, Yang *et al.* 2008]. To this date, the *CPNE1* gene was indentified and characterized in mouse, rat, cattle, sheep, rabbit, human, dog and other animal species, but not in pig. The present results show that the *CPNE1* gene is differentially expressed in the LD muscle being a more abundant in

Large White than in Wujin pigs. The latter is a fat-type breed, depositing much more body fat than does the lean type Large White. On the other hand, Large White is a typical lean-type pig breed, presenting the opposite phenotype to that found in the Wujin breed. The two pig breeds used in this study, differ in lean meat content of carcass. It is, therefore, interesting that the expression of the porcine *CPNE1* gene in the LD muscle shows the trend of a higher expression in Large White as compared to Wujin. A major question is the extent to which such predominant expression could be developmentally or metabolically significant in terms of acquiring of any phenotypic change in favour of a higher lean-type rate. Clearly, this merits further study.

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