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Full-length cDNA, Expression Pattern and Association Analysis of the Porcine *FHL3* Gene

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ABSTRACT : Four-and-a-half LIM-only protein 3 (FHL3) is a member of the LIM protein superfamily and can participate in mediating protein-protein interaction by binding one another through their LIM domains. In this study, the 5'- and 3'- cDNA ends were characterized by RACE (Rapid Amplification of the cDNA Ends) methodology in combination with *in silico* cloning based on the partial cDNA sequence obtained. Bioinformatics analysis showed *FHL3* protein contained four LIM domains and four LIM zinc-binding domains. *In silico* mapping assigned this gene to the gene cluster *MTF1-INPP5B-SF3A3-FHL3-CGI-94* on pig chromosome 6 where several QTL affecting intramuscular fat and eye muscle area had previously been identified. Transcription of the *FHL3* gene was detected in spleen, liver, kidney, small intestine, skeletal muscle, fat and stomach, with the greatest expression in skeletal muscle. The A/G polymorphism in exon II was significantly associated with birth weight, average daily gain before weaning, drip loss rate, water holding capacity and intramuscular fat in a Landrace-derived pig population. Together, the present study provided the useful information for further studies to determine the roles of *FHL3* gene in the regulation of skeletal muscle cell growth and differentiation in pigs. (**Key Words :** Pig, *FHL3*, Meat Quality, *In silico* Mapping)

INTRODUCTION

The successful application of marker assisted selection in the animal population will depend on the identification of major genes or tightly linked markers. The candidate gene approach allows the identification of single nucleotide polymorphisms in genes likely to cause variation in a trait based on physiological, immunological, or endocrine evidence, such as adiponectin gene as a candidate gene for fat deposition traits in pigs (Dai et al., 2006; Shin et al., 2007). LIM domain proteins are defined as proteins having a double zinc finger motif with a consensus amino acid sequence CX2CX16-23HX2(C/H) X2CX2CX16-23CX2(C/H/D) (Morgan and Madgwick, 1999). Four and a half LIM domain (FHL) proteins belong to the LIM protein superfamily, which are considered to be important regulators in cell growth, cell fate determination, cell differentiation and remodelling of the cell cytoskeleton (Li et al., 2001). Four-and-a-half LIM-only protein 3 (FHL3) is one of the members of FHL proteins that contain four types of LIM-only protein. The FHL3 protein is expressed predominantly in skeletal muscle and can participate in mediating protein-protein interaction by binding one another through their LIM domains (Coghill et al., 2003; Fimia et al., 2000; Mils et al., 2003; Turner et al., 2003). Therefore, the *FHL3* gene might be an ideal candidate gene for the production traits.

In pigs, we have cloned partial cDNA and genomic sequence, and identified an A→G missense mutation in exon II segregating only in Landrace pigs (Zuo et al., 2004). To further understand the molecular structure and comprehensively evaluate this SNP as a genetic marker for screening breeding pigs, we determined the complete genomic structure of the full-length cDNA sequence and examined the effect of the A/G substitution on the growth and meat quality traits. Additionally, the chromosomal location and expression pattern of the *FHL3* were also investigated in pigs.

MATERIALS AND METHODS

Cloning and sequence analysis of full-length cDNA

SMART (Switching Mechanism At 5' end of the RNA

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Transcript) RACE (Rapid Amplification of the cDNA Ends) polymerase chain reaction (PCR) was used to amplify the 5' end of the *FHL3* gene. The double-strand cDNA library was constructed from 1 μg of total RNA from skeletal muscle with the use of the SMART RACE cDNA Amplification kit (BD Biosciences). To obtain the 5'-UTR of *FHL3* gene, 2 μl of the 5'-RACE cDNA library was used as the template for PCR. The *FHL3* gene specific primer was 5'-GCC GTT GTC CGT CTG AAT GTA-3', designed based on the obtained porcine *FHL3* sequence (AY277587), against the SMART II oligonucleotide (5'-AAC GCA GAG TAC GCG GG-3'). PCR was performed in 25 μl reactions mix containing: 1×PCR buffer, 1.5 mM $MgCl_2$, 150 μM of each dNTP, 0.25 μmol of each PCR primer, 2 units Taq DNA polymerase (Biostar International, Toronto, ON, Canada), and 2 μl cDNA. PCR was carried out in a GeneAmp PCR system 9600 (Perkin-Elmer Company, USA) thermocycler as follows: 95°C initial denaturation for 4 min, 35 cycles of 95°C denaturation for 40 s, 62°C annealing for 40 s, and 72°C extension for 3 min. A final extension was performed at 72°C for 10 min.

To obtain the 3' end of *FHL3* cDNA, expressed sequence tags (ESTs) database mining was performed with BLASTN using the obtained sequence information of pig *FHL3* gene (AY277587). One partially overlapping porcine ESTs with polyA highly identical to the *FHL3* sequence (CK462267) was selected. From this EST sequence, primer pair FH1 (Forward: 5'-ACT AAG GCT CCT CTT CCA GAC CAC-3'; Reverse: 5'-CGT TAT TTC GTC ATA CTC CTT TTT TT-3') was designed using Primer 5.0 software. PCR reaction components were the same as above described. PCR was run with the following cycling parameters: 95°C initial denaturation for 4 min, 35 cycles of 95°C denaturation for 40 s, 62°C annealing for 40 s, and 72°C extension for 3 min. A final extension was performed at 72°C for 10 min.

The PCR products were loaded on 1.0% (W/V) agarose gel, and selected bands were purified using a gel extraction kit (Sangon, Shanghai, China). The purified PCR products were ligated into the pGEM-T vector (Promega, USA) and transformed into DH5α competent cells. Bacteria were grown in LB-ampicillin agar. Cloned PCR products were sequenced by Shanghai Sangon Biotechnology Company. The nucleotide sequence of each clone will be compared with DNA, EST, and protein sequences from various databases by means of the basic local alignment search tool (BLAST). The open reading frame finder (http://www.ncbi.nlm.nih/gov/gorf/gorf.html) was used to deduce the amino acid sequences. The deduced amino acid sequence was analyzed at the ExPASy Molecular Biology Server (http://au.expasy.org/).

Isolation of RNA and tissue distribution

The tissue distribution of *FHL3* mRNA was determined by RT-PCR. Total RNAs were extracted from spleen, lung, liver, kidney, small intestine, skeletal muscle, heart, fat and stomach with Trizol reagent (Invitrogen, USA). One microgram of treated total RNA was used to synthesize the first-strand cDNA using Superscript reverse transcriptase and oligo (dT) primer (Promega, USA). PCR was carried out as described above using gene-specific primer pair FH2 (Forward: 5'-GCC ACC ATG AGC GAG ACC TT-3' and Reverse: 5'-GGC ACG CAG TAG TGA GCA CC-3'). This produced a 476-bp product. The glyceraldehydes-3-phosphate dehydrogenase (*G3PDH*) gene was used as endogenous reference gene. The primers (Forward: 5'-ACC ACA GTC CAT GCC ATC AC-3'; Reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3') were designed based on the known sequence. PCR reaction components were the same as above described. Amplification procedures were 94°C for 4 min; followed by 30 cycles of 94°C for 50 s, 64°C for 50 s, and 72°C for 50 s; and a final extension step at 72°C for 10 min. 5 μl of the PCR products were analysed by electrophoresis on a 1.0% agarose gel. To validate the results, the RT-PCR was repeated.

Traits measurement and association studies

One hundred and five pigs including 42 Landrace pigs, 28 Yorkshire×Landrace crossbred and 35 Landrace×Yorkshire crossbred pigs, were used as experimental materials to perform the PCR-RFLP and association analysis. The finishing animals were slaughtered and measured according to the method of Xiong and Deng (1999). The growth traits analyzed were birth weight, average daily gain before weaning and average daily gain from weaning to testing periods. Meat quality traits included pH of *m. Longissimus Dorsi*, pH of *m. Biceps Femoris*, pH of *m. Semipinalis Capitis*, drip loss rate, water holding capacity, meat color of *m. Longissimus Dorsi*, meat color of *m. Biceps Femoris*, intramuscular fat, meat moisture. All the animals were genotyped for A/G transversion in the exon II by PCR-*Pst*I-RFLP (Zuo et al., 2004). The association between genotypes and traits was performed with the GLM procedure of SAS version 8.0 software package (SAS Institute, Cary, NC.). Both additive and dominance effects were estimated using REG procedure of SAS version 8.0, where the additive effect was denoted as -1, 0 and 1 for *AA*, *AB* and *BB*, respectively, and the dominance effect was denoted as 1, -1 and 1 for *AA*, *AB* and *BB*, respectively (Liu et al., 1998). The model used to analyze the data was assumed to be:

$$Y_{ijk} = \mu + S_i + B_j + G_k + b_{ijk}V_{ijk} + e_{ijk}$$

Where, Y_{ijk} is the observation of the trait; μ is the least square mean; S_i is the effect of i^{th} sex (i = 1 for male or 2 for

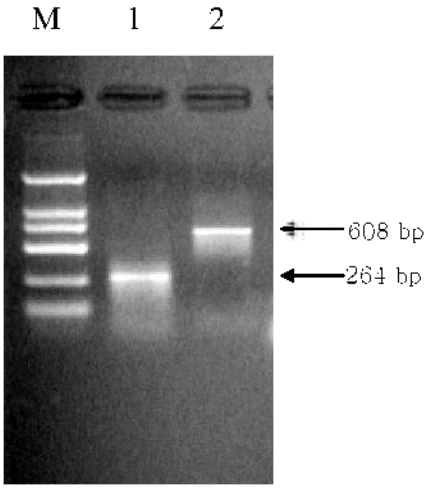

Figure 1. RT-PCR amplification results of 5'-RACE and primer pair FH1. Lane M, DNA molecular marker DL2000; Lane 1, amplified fragment of 5'-RACE, 264 bp; Lane 2, amplified fragment of 3' PCR products, 608 bp.

female). B_j is the effect of j^{th} breed (j = 1 for Landrace, or 2 for Yorkshire×Landrace, or 3 for Landrace×Yorkshire); G_k is the effect of k^{th} genotype (k = *AA*, *AB* and *BB*). b_{yk} is the regression coefficient of the covariate. X_{ijl} is the age at slaughter used as a covariate for meat quality traits. e_{ijk} is the random residual.

RESULTS AND DISCUSSION

Cloning and sequencing of PCR products

Amplified cDNA products were loaded on 1.0% agarose gel, and clear amplified bands of primer of 5' SMART - RACE and primer FH1 were obtained (Figure 1). RT-PCR products were cloned into vector pGEM-T and sequenced. Sequencing results showed that the sizes of the 5' and 3' PCR products were 264 bp and 608 bp, respectively.

Sequence analysis and *in silico* mapping

The sequence of the above PCR products and the known sequence (AY277587) were assembled into one 1,491-bp cDNA and deposited into the Genbank database under the accession number DQ472001. The full-length cDNA contained a 843-bp open reading frame flanked by a 172-bp 5' - untranslated region (UTR) and a 469-bp 3'-UTR. The potential polyadenylation signal "AATAAA", was found at nt 11 upstream of the poly (A) tract. The coding sequence encoded 280 amino acids (Figure 2). Four LIM domains, termed LIM domains I-IV, starting from the N-terminus to C-terminus and four LIM zinc-binding domains were identified in this protein of 280 amino acids. In porcine FHL3, it was inferred that domain I extended from N-38 to S-98, domain II from S-99 to P-160, domain III from R-161 to A-218 and domain IV from R-219 till the C-terminal of

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CCCACACAGTCCGTGTGCAGCCGGCAGCCTAGCCGGTAGCTCGCTCGGCCCCGCTCGGCCT
GGCCCGCAGGTAGGAGCCGCCGAGGGACGCGGCCCCGGCTGCCCGGCCCTCCCTGCTCGA
GCGCCGCGCCTCGCTCCCGCCGCCCGCTTCGAGGGCTCCATCACAGCCACCATGAGCGAG
                                                  M  S  E
ACCTTTGACTGTGCAAAATGCAGCGAGTCCCTGTACGGGCGCAAATACATTCAGACGGAC
 T  F  D  C  A  K  C  S  E  S  L  Y  G  R  K  Y  I  Q  T  D
AACGGCCCCTACTGTGTGCCCTGCTATGACAGCACCTTTGCCAACACGTGCGCTGAGTGC
 N  G  P  Y  C  V  P  C  Y  D  S  T  F  A  N  T  C  A  E  C
CAGCAGCTTATCGGGCATGACTCAAGGGAGCTGTTTTACGAGGATCGCCACTTCCACGAG
 Q  Q  L  I  G  H  D  S  R  E  L  F  Y  E  D  R  H  F  H  E
GGCTGTTTCCGCTGCTGCCGCTGCCAGCGCTCCCTCGCCGACGAGCCCTTCACCTGCCAG
 G  C  F  R  C  C  R  C  Q  R  S  L  A  D  E  P  F  T  C  Q
GATAGCGAGCTGCTCTGTAACGACTGCTACTGCAGCGCCTTCTCCTCTCAGTGCTCCGCC
 D  S  E  L  L  C  N  D  C  Y  C  S  A  F  S  S  Q  C  S  A
                ▼
TGCAGAGAGACCGTCATGCCTGGGTCCCGGAAGCTGGAGTACGGAGGCCAGACGTGGCAT
 C  R  E  T  V  M  P  G  S  R  K  L  E  Y  G  G  Q  T  W  H
GAGCACTGCTTCCTGTGCAGCGGCTGTGAGCAGCCGCTGGGCTCCTGTTCCTTTGTGCCC
 E  H  C  F  L  C  S  G  C  E  Q  P  L  G  S  C  S  F  V  P
GACAAGGGTGCTCACTACTGCGTGCCCTGCTATGAGAACAAGTTCGCGCCTCGCTGCGCC
 D  K  G  A  H  Y  C  V  P  C  Y  E  N  K  F  A  P  R  C  A
CGCTGCAGCAAGACGCTGACACAGGGTGGCGTGACGTACCGTGACCAACCCTGGCATCGA
 R  C  S  K  T  L  T  Q  G  G  V  T  Y  R  D  Q  P  W  H  R
GAATGCCTGGTCTGCACAGGGTGCCAGACGCCCCTGGCAGGGCAGCAGTTCACCTCCGG
 E  C  L  V  C  T  G  C  Q  T  P  L  A  G  Q  Q  F  T  S  R
GACGATGATCCTTATTGTGTGGCCTGTTTTGGAGAACTCTTTGCACCCAAGTGCAGCAGC
 D  D  D  P  Y  C  V  A  C  F  G  E  L  F  A  P  K  C  S  S
TGCAAGCGCCCCATCACAGGACTCGGCGGAGGCAAGTATGTGTCCTTTGAAGACCGCCAC
 C  K  R  P  I  T  G  L  G  G  G  K  Y  V  S  F  E  D  R  H
TGGCACCACAGCTGCTTCTCCTGTGCCGCTGCTCCACCTCCCTGGTGGGTCAAGGCTTC
 W  H  H  S  C  F  S  C  A  R  C  S  T  S  L  V  G  Q  G  F
GTGCCGGATGGAGACCAAGTGCTGTGCCAGGGTTGCAGCCAGGCAGGGCCCTGAGCCAGG
 V  P  D  G  D  Q  V  L  C  Q  G  C  S  Q  A  G  P  *
GCTCCTGGGCCCAGGCCCTCCCATACCAGGGCTCCAGGACTAAGGCTCCTCTTCCAGACC
ACCTCTGGGTCCCAGCCCCGCCCCAAATGGGGTTCCCCCTAGGCTCCAGGATTCAGCCTC
CCCACTCCAACATCCCCAATCTGGTACTCCAGGCTTAATGGAGCCTCCATCGTTTAAATC
CCCCCCAAACCAGGACCCCAGGACTCAGTCTATATGGGTGACTGAAAAGTCCTCAGAAGT
GGACCCTCCCCACCCTCATCCCACCTTTCTCCACAGGGATGGGGCTTTCTGGGCAACAGA
TGAGAGGTTCAGTGCTTAGGGGGGGTCCTAGGGTACAGTTTTGCCCAGCCCGTGTTGTCA
AGTGTTTTCTCGTTTCAGTTCAGCTCTGTTTTGCCCAGAGATGGGCAGAGGGGTGGGATT
GGCTCACCCCACCATTCAGATTCTGCAATAAAGCAGTATGAGGAAAAAAA
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Figure 2. Full-length nucleotide and predicted amino acid sequences of the porcine *FHL3* gene (DQ472001). The start (ATG) codon is boxed and the stop (TGA) codon is indicated with an asterisk. The polyadenylation signal (AATAAA) is underlined. The nucleotide letters in boldface type indicate the splice donor and acceptor site. Four LIM domains are indicated in shadow and four LIM zinc-binding domains underlined and shadowed. The missense mutation G/A is indicated by "▼".

porcine FHL3 (Figure 2).

The full-length cDNA sequence was compared with the pig nucleotide database by BLASTN. One DNA sequence from clone PigE-92E11 on pig chromosome 6 (accession number: CR956649) significantly matched with *FHL3* sequence. From this contig, the complete exon-intron structure of the pig *FHL3* gene was determined and the full-length cDNA region was organized in six exons (Figure 2), which updated our previous studies (Zuo et al., 2004). By BLASTN similarity search of this contig against the human genome sequence, five functional genes (*MTF1*, *INPP5B*, *SF3A3*, *FHL3*, *CGI-94*) were found and the gene order was *MTF1-INPP5B-SF3A3-FHL3-CGI-94*, which was highly conserved with that of human genome. As the human 1p34

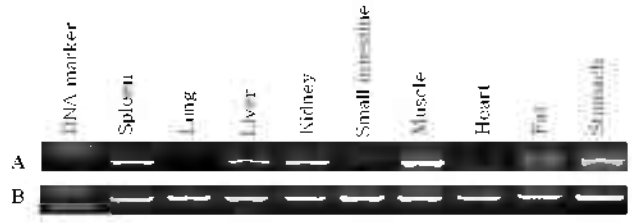

Figure 3. Reverse transcription (RT) PCR tissue expression analysis of porcine *FHL3* in spleen, lung, liver, kidney, small intestine, muscle, heart, fat and stomach. A shows RT-PCR amplification results of porcine *FHL3* gene, 461 bp; B shows the control PCR products with *G3PDH* specific primer, 480 bp; DNA marker: DNA molecular marker DL2000 (TaKaRa, Dalian, China).

is a syntenic region of the porcine chromosome 6 by bidirectional chromosome painting (Goureau et al., 1996), our results improve the pig-human comparative map for human chromosome 1 and pig chromosome 6.

Tissue distribution of the *FHL3* gene

The tissue distribution and transcript size of pig *FHL3* was determined by RT-PCR (Figure 3). The RT-PCR results showed the porcine *FHL3* was highly expressed in skeletal muscle and relatively low expressed in spleen, liver, kidney, small intestine, fat and stomach, with no detectable expression in heart and lung. Using an *FHL3* cDNA probe to hybridize with poly (A) RNA of various human tissues, Lee et al. (1998) detected a very strong signal in skeletal muscle. Fimia et al. (2000) determined that mouse *FHL3* was expressed predominantly in skeletal muscle, with low but detectable levels in ovary, spleen, and adrenal gland. From the above results, porcine *FHL3* gene had a highly expression level in the skeletal muscle tissue, consistent with reports of the expression of its homolog in human and mouse. The high abundance of pig FHL3 in skeletal muscle gave more evidence of this gene as candidate for traits of interest in pigs.

Association studies with growth and meat quality traits

The results of tests for *FHL3* genotypes and growth and meat quality traits were given in Table 1. Statistically significant associations with birth weight ($p<0.05$), average daily gain before weaning ($p<0.05$), drip loss rate ($p<0.05$), water holding capacity ($p<0.05$), and intramuscular fat ($p<0.05$) were found. This locus seemed to be significantly additive in action for birth weight, average daily gain before weaning, and intramuscular fat, and allele *A* was associated with increase of birth weight and average daily gain before weaning, but decrease of intramuscular fat. In our previous studies, it was found that this locus had significant effects on some carcass traits such as loin eye width and loin eye area, and allele *A* was associated with increase of the trait (Zuo et al., 2004). Considering all these results, we can draw the conclusion that allele *A* presented positive and desirable effects on growth and carcass traits, but negative and undesirable effects on meat quality traits.

The FHL3 proteins could participate in mediating protein-protein interaction by binding one another through their LIM domains and the second LIM domain-LIM2 of FHL3 was identified as the principal LIM domain for their interaction (Li et al., 2001; Mils et al., 2003). Recent studies revealed that FHL3 could bind MyoD and negatively regulate myotube formation (Cottle et al., 2007). As the Arg→Gly substitution which we have been found was just located in the LIM2 domain (Figure 2), the results presented here probably resulted from the functional change of FHL3 protein, and hereby further argued for *FHL3* gene as a candidate gene for the production traits related to skeletal muscle. Additionally, the *FHL3* gene was very close linked to the *MTF1* gene on chromosome 6. Interestingly, several quantitative trait loci (QTL) affecting intramuscular fat, eye muscle area and backfat thickness had been mapped in an Iberian×Landrace F_2 pig population (Olivo et al., 2000; 2002), and the position of QTL was just between maker *S0228* and *Sw1881* which were very close to the *MTF1* gene on pig chromosome 6 (http://cmap.medvet.angis.org.au/cmap/index.php). On the basis of association results, the position in QTL region and the important role of FHL3 in the regulation of cell growth and cell differentiation, this missense SNP could be considered a good candidate site for the observed QTL effects in the Landrace-derived populations. Thus, it will be of interest to continue the functional study of the *FHL3* gene.

Table 1. Statistical analysis of *Pst*I-RFLP genotypes with growth and meat quality traits

Traits	*FHL3* genotype (μ±SE)			Genetic effects (μ±SE)	
	AA (22)	*AB* (60)	*BB* (23)	Additive	Dominance
Birth weight (kg)	1.610±0.046 ^{a}	1.580±0.03 ^{ab}	1.470±0.052 ^{b}	-0.070±0.033*	-0.020±0.026
Average daily gain before weaning (kg/d)	0.265±0.019 ^{a}	0.261±0.012 ^{a}	0.207±0.021 ^{b}	-0.029±0.013*	-0.013±0.011
Drip loss rate (%)	6.586±0.381 ^{a}	7.605±0.239 ^{b}	7.585±0.430 ^{ab}	0.499±0.276	-0.260±0.211
Water holding capacity (%)	91.222±0.503 ^{a}	89.854±0.316 ^{b}	89.924±0.568 ^{ab}	-0.650±0.364	0.359±0.278
Intramuscular fat (%)	1.686±0.063 ^{a}	1.806±0.039 ^{ab}	1.894±0.071 ^{b}	0.104±0.045*	-0.008±0.035

Mean values with different letters are significantly different: small letter, $p<0.05$. Negative values of the additive effects denote a decrease of the trait due to the allele *B*. * $p<0.05$.

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