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Molecular characterization of porcine *NECD*, *SNRPN* and *UBE3A* genes and imprinting status in the skeletal muscle of neonate pigs

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Abstract Imprinted genes are expressed monoallelically depending on their parental origin, and play important roles in embryo survival and postnatal growth regulation. In this study, we characterized the porcine *NECD* (necdin), *SNRPN* (small nuclear ribonucleoprotein polypeptide N) and *UBE3A* (UBE3A ubiquitin protein ligase E3A) genes, analyzed their expression in nine tissues including liver, lung, small intestine, skeletal muscle, heart, kidney, spleen, inguinal lymph nodes and fat, and also examined their imprinting status in the skeletal muscle of neonate pigs. Results indicated that these three genes were highly homologous between pigs and cattle, being 95.02 % in nucleotide and 99.17 % in amino acid with the cattle *SNRPN* gene, and 96.46 % in nucleotide and 98.63 % in amino acid with the cattle *UBE3A* gene, respectively. The three genes were expressed in all the tissues investigated. Three single nucleotide polymorphisms (SNPs) in the coding region of these genes, i.e. g.263G>C, g.402T>C and g.340A>G for porcine *NECD*, *SNRPN* and *UBE3A* genes, respectively, were revealed; and imprinting analysis with which indicated that, in the skeletal muscle of neonate pigs, both *NECD* and *SNRPN* were maternally imprinted, while *UBE3A* was not imprinted.

Keywords Pig · Imprinting · *NECD* · *SNRPN* · *UBE3A* · Gene

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Introduction

Genomic imprinting in mammals is an epigenetic gene-marking phenomenon, by which only paternally or maternally inherited genes but not both are expressed, and also is usually described as monoallelic expression. Imprinting has a great impact on normal mammalian development, fetal growth, metabolism and adult behaviors [1]. The human necdin gene (*NECD*, also called *NDN*) is maternally imprinted and located in the Prader-Willi syndrome (PWS) chromosomal region [2]; it mediates skeletal muscle regeneration by promoting myoblast survival and differentiation [3] and also plays an important role in regulating cytoskeleton polarization during development [4]. Small nuclear ribonucleoprotein polypeptide N gene (*SNRPN*) is located within the PWS and Angelman syndrome (AS) region containing multiple imprinted genes that are coordinately regulated by a bipartite imprinting center (IC) [5]. Abnormal imprinting of *SNRPN* gene was found in day-40 bovine fetuses generated by in vitro culture and somatic cell nuclear transfer (SCNT) [6]. Human *SNURF* (*SNRPN* upstream reading frame)-*SNRPN* transcript extends from *SNURF* to *UBE3A* (UBE3A ubiquitin protein ligase E3A) which is antisense to *UBE3Aas*, spans 460 kb and 148 exons [7, 8] and is exclusively paternally expressed. *UBE3A* is involved in targeting cellular proteins for degradation, and in human, a defect in the maternal copy of *UBE3A* produces AS. Human *UBE3A* is imprinted in the brain, fibroblast, lymphoblast and neural-precursor cells [9, 10] and mice with maternal deficiency for *UBE3A* resembles human AS with motor dysfunction, inducible seizures, and a context-dependent learning deficit [11].

Most records concerning genomic imprinting were found in human and mouse, less imprinted genes were identified in pigs (http://igc.otago.ac.nz/home.html). Recently, the

imprinting of *NNAT* and *DLX5* [12], *DIRAS3* [13], *MAGEL2* [14], *NAP1L5* and *PEG3* [15] were reported in pigs. Bischoff et al. [16] characterized the conserved and nonconserved imprinted genes in pigs. However, whether *NECD*, *SNRPN* and *UBE3A* are imprinted in pigs remains unknown. In this study, we first obtained the cDNA sequence of *SNRPN* and *UBE3A* genes [*NECD* sequence is downloaded from the National Center for Biotechnology Information (NCBI)], then analyzed their expression pattern in adult pig tissues and examined their imprinting status in the skeletal muscle of neonate pigs.

Materials and methods

Tissue collection

Tissues from skeletal muscle *longissimus dorsi* were collected from 10 neonate pigs of Landrace boars × Laiwu sows cross and nine of the reciprocal cross which were reared under the same conditions. The two pig breeds of Landrace and Laiwu (an indigenous pig breed in China) differ in many economic traits such as growth rate, litter size and meat quality, and the pedigree established between crosses of these two breeds are expected to be heterozygous at many sites, therefore are appropriate for imprinting analysis. Tissues from liver, lung, small intestine, skeletal muscle, heart, kidney, spleen, inguinal lymph nodes and fat were gathered from 15 adult Landrace × Laiwu crossbred pigs, which were also reared under the same conditions. All neonate and adult pigs were slaughtered according to the standard of Animal Management of Shandong Agricultural University. After slaughter, the samples were washed briefly with phosphate-buffered saline (PBS) and snap frozen in liquid nitrogen.

Nucleic acid isolation and cDNA synthesis

Genomic DNA was isolated with DNA isolation Kit (TIANGEN, Beijing, PRC) according to the manufacturer's instructions and stored at −20 °C. Total RNA was isolated from the above mentioned porcine tissues with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at −80 °C. The quality and concentration of nucleic acids were checked by running electrophoresis with 1 % agarose gel and quantified with BioPhotometer plus UV spectrophotometer (Eppendorf AG, Hamburg, Germany). First-stranded cDNA was synthesized through a two-step procedure in a nuclease free reaction tube: first mix 1 μg of DNaseI(Fermentas, Glen Burnie, USA) treated total RNA, 2 μl of 10 pmol/μl oligo$(dT)_{15}$ primer and 13 μl nuclease-free H_2O, incubate at 65 °C for 10 min and place the tube immediately on ice; then add 0.5 μl of RNase inhibitor (20 U/μl, Fermentas, Glen Burnie, USA), 2 μl of dNTPs (10 mM, TaKaRa, Dalian, PRC), 4 μl of Transcriptor RT reaction buffer and 0.5 μl of Transcriptor Reverse Transcriptase (20 U/μl, Roche, Penzberg, Germany), mix and incubate at 55 °C for 30 min, 85 °C for 5 min. The resultant cDNA was stored at −20 °C.

Reverse transcription (RT)-PCR amplification, cDNA cloning and mRNA expression

The cDNA of porcine *SNRPN* and *UBE3A* genes were obtained by RT-PCR, sequenced and assembled with DNAMAN software (Version 5.2.2, Lynnon Biosoft, Canada). First, we used human *SNRPN* (GenBank: NM_022807) and *UBE3A* cDNA sequences (GenBank: NM_130838) as seeds to search 'EST-others' database with standard BLAST (http://www.ncbi.nlm.nih.gov/blast/) and obtained porcine ESTs. Then, we assembled the corresponding porcine genes with DNAMAN software (Version 5.2.2, Lynnon Biosoft, Canada) that are used for designing primers (Table 1). RT-PCR was performed in 20 μl volume containing 4 μl 5× PrimeSTAR™ Buffer (Mg^{2+} plus), 1.6 μl of dNTPs (10 mM, TaKaRa, Dalian, PRC), 0.5 μl of each forward and reverse primers (10 μM), 100 ng of cDNA and 0.2 μl of PrimeSTAR™ HS DNA polymerase (2.5 U/μl, TaKaRa, Dalian, PRC). The PCR amplification was performed with normal PCR or Touchdown PCR procedure (marked with an asterisk in Table 1) at the annealing temperature shown in Table 1. The blunt-end RT-PCR amplicons with PrimeSTAR™ HS DNA polymerase were purified and ligated into pJET1.2/blunt cloning vector (Fermentas, Glen Burnie, USA), then transformed into the competent *Escherichia coli* DH5α cells. For each RT-PCR amplicon, at least four positive clones were sequenced with ABI 3730 sequencer (Applied Biosystems, CA, USA).

The mRNA expression of porcine *NECD* gene was analyzed with primers cND263F and cND263R which were designed according to the sequences of porcine *NECD* gene (GenBank: NM_001123144) in a 20 μl volume containing 2 μl of 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 1.6 μl of dNTPs (10 mM, TaKaRa, Dalian, PRC), 0.6 μl of 50 mM $MgCl_2$, 0.5 μl of each forward and reverse primers (10 μM), 100 ng of cDNA and 0.15 μl of Platinum® Taq DNA polymerase (5 U/μl, Invitrogen, Carlsbad, CA, USA). The thermal profile was as follows: 94 °C 5 min, 94 °C 30 s, 58 °C 30 s, 72 °C 20 s, for 34cycles; and 72 °C 5 min. For mRNA expression analysis of porcine *SNRPN* and *UBE3A* genes, primer pairs SNR3F/SNR4R and cUB2F/cUB3R for RT-PCR were designed according to sequences obtained above and performed using the same condition as above except that different annealing temperatures (Table 1) were

Table 1 Primer sequence, annealing temperature and fragment size of *NECD*, *NRPN*, *UBE3A* and *GAPDH* genes in pigs

Gene	Primers	Sequence (5′–3′)	Annealing temperature (°C)	Size (bp)	Usage
NECD	NDF	ACTTGCTCCTTGTCCAGTG	60	1,983	SNP screen
	NDR	CTCATTCCCAACCCATACTAC			
	cND263F	CTGAGCGAAGAAGGGAGA	56*	221	Expression and imprinting
	cND263R	CTTGTAACTGCCGATGACA			
SNRPN	SNR1F	TGGAGATGCCTGAGGTTA	62	408	cDNA cloning
	SNR2R	AAGGGAAGGCTACAGATAAAGG			
	SNR3F	ACCTTTATCTGTAGCCTTCCC	61	360	SNP screen, imprinting and cDNA cloning
	SNR3R	GAGCAAT(C/T)CCAGTATCTTTGGG			
	SNR4F	CCCAAAGATACTGGRATTGC	57	255	Expression
	SNR4R	GCCTGGTGGATACTGAGTTG			
	SNR3F	ACCTTTATCTGTAGCCTTCCC	58	593	Expression
	SNR4R	GCCTGGTGGATACTGAGTTG			
	SNR5F	CAACTCAGTATCCACCAGGC	61	293	cDNA cloning
	SNR5R	CTCCACACATTTCACAAGACAC			
UBE3A	cUB1F	TCCTCTGAAGTTTGGCGA	60	262	cDNA cloning
	cUB1R	CCTCATTTCCACAGCCTT			
	cUB2F	AAGGCTGTGGAAATGAGG	59	534	SNP screen, imprinting, cDNA cloning and expression
	cUB3R	GCCATCACTTATCCTTGAGG			
	cUB4F	CTCAAGGATAAGTGATGGCTC	58	264	cDNA cloning
	cUB4R	GGGCTGTGGAGATTTCTATTC			
	cUB5F	GAATAGAAATCTCCACAGCCC	58*	425	cDNA cloning
	cUB6R	CACCAAGTTCAGTTTCCAGTG			
	cUB7F	GACCCACTGGAAACTGAACT	56*	686	cDNA cloning
	cUB8R	GAGTCTCCCAAGTCACGAA			
	cUB9F	CAGTTCACTCTGATTGGCAT	56*	408	cDNA cloning
	cUB10R	TAAGGGAGATTCATTGGTCACC			
	cUB11F	GAAGGAAGTGTGGAAGATGATA	56*	779	cDNA cloning
	cUB11R	CAGACATAGGTGACTACTGTGGT			
GAPDH	GAPDHF	ACCACAGTCCATGCCATCAC	58	452	Expression
	GAPDHR	TCCACCACCCTGTTGCTGTA			

* Touchdown PCR procedure with a range of 70 °C to annealing temperature as shown

used. The housekeeping gene *GAPDH* was used as internal control for mRNA expression analysis with primers and annealing temperature listed in Table 1.

Homology analysis

The cDNA sequences of porcine *NECD*, *SNRPN* and *UBE3A* genes were translated into amino acids sequences with DNAMAN software (Version 5.2.2, Lynnon Biosoft, Canada), then, with the three genes' amino acid sequences of other species retrieved from the GenBank and Ensembl database, the phylogenetic trees were constructed with MEGA 4.0 software [17]. The neighbor joining method was used in bootstrap, and the replication was 500. The 'Complete Deletion' for the gaps/miss data and amino 'Poisson Correction' for the substitution model were chosen. The numbers of each branch (Fig. 1) indicates percentage of reality in the process of bootstrap.

Single nucleotide polymorphism identification and imprinting analysis

In order to distinguish paternal and maternal alleles of each gene, at least one single nucleotide polymorphism (SNP) located in the transcript is required. The SNPs in the transcript of porcine *NECD* and *SNRPN* genes were identified by sequencing and alignment of PCR amplicons from porcine genomic DNA of neonate pigs with primer pairs NDF/NDR and SNR3F/SNR3R, respectively. The SNP in the coding region of *UBE3A* was identified through

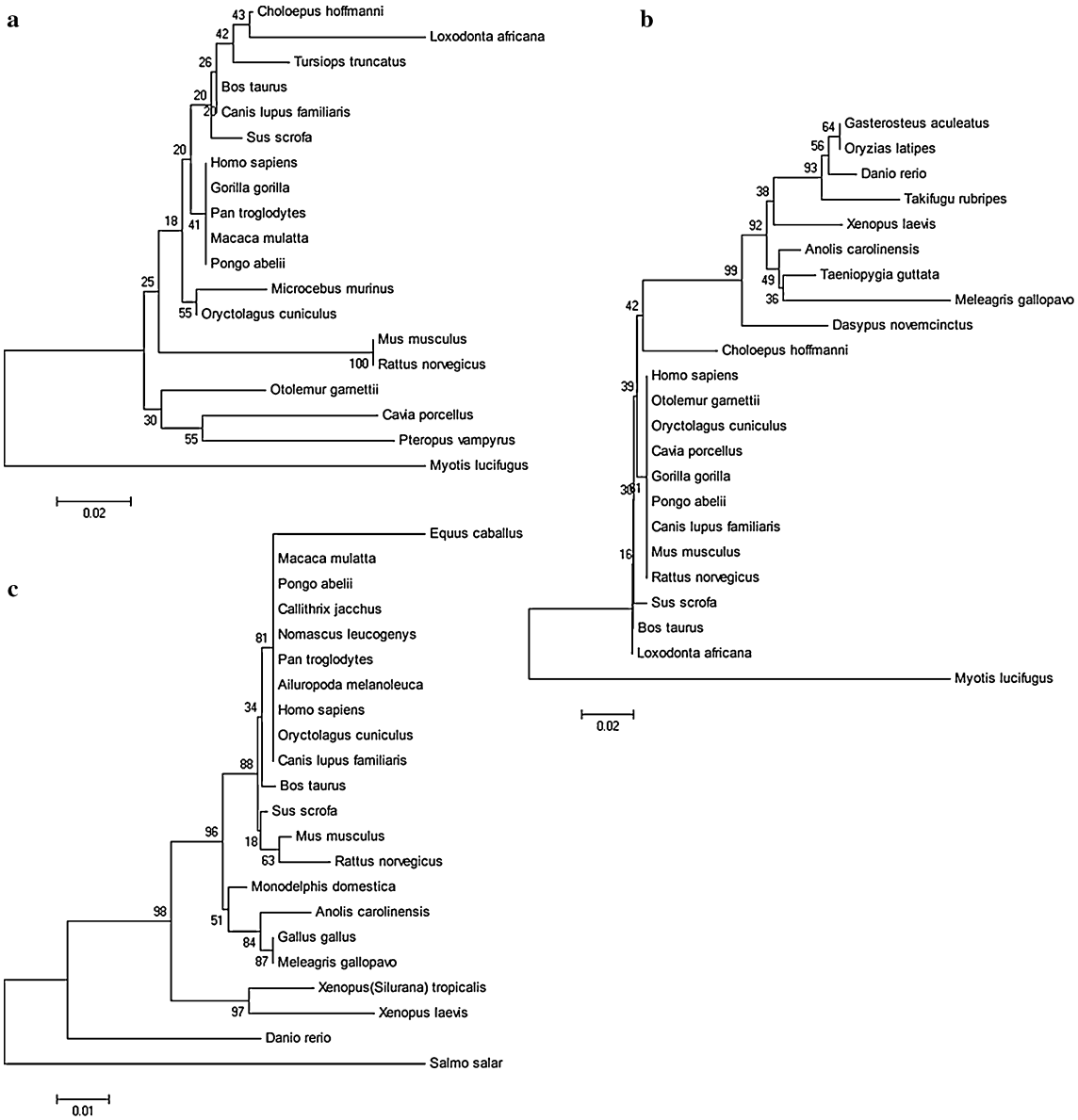

Fig. 1 Cladogram tree of the NECD (**a**), SNRPN (**b**) and UBE3A (**c**) amino acid sequences of several species retrieved from the GenBank or Ensembl database

alignment of porcine *UBE3A* cDNA sequences and confirmed by sequencing the fragment amplified from porcine genomic DNA with primer pair cUB2F/cUB3R (Table 1). PCR amplification, sequencing and alignment were performed as above, the primer sequence and annealing temperature used for amplification were shown in Table 1.

Imprinting assay was performed by deciding which allele was expressed in the heterozygous offspring. The genotype at each SNP for genomic and cDNA of porcine *NECD*, *SNRPN* and *UBE3A* genes was determined by PCR/RT-PCR amplification and direct sequenced using the BigDye v3.1 kit (Applied Biosystem). Imprinting assay was performed by comparing the genotype of the heterozygous offspring, its expressed transcript in the skeletal muscle *longissimus dorsi* of neonate pigs and the genotype of the dam to determine whether the maternally inherited allele is expressed in the offspring. Both pedigrees of Landrace × Laiwu and Laiwu × Landrace were used for determining imprinting status of each gene.

Results

cDNA cloning and homology

The cDNA sequence of porcine *SNRPN* and *UBE3A* genes was obtained by RT-PCR from skeletal muscle tissues. The cDNA sequence of 1304 bp including the complete coding

region (CDS, 723 bp) of porcine *SNRPN* gene was obtained, which shared 95.02 % in nucleotide and 99.17 % in amino acid with the cattle *SNRPN* gene, respectively; for porcine *UBE3A* gene, the cDNA sequence of 2,740 bp including full-length of CDS (2,628 bp) was obtained, which shared 96.46 % in nucleotide and 98.63 % in amino acid with the cattle *UBE3A* gene, respectively. The cDNA sequence of porcine *SNRPN* and *UBE3A* genes was deposited in GenBank with accession numbers JQ316195 and JQ316194, respectively. Phylogenetic trees (Fig. 1) indicated the *NECD*, *SNRPN* and *UBE3A* genes were highly conserved among mammalian species, such as pig, cattle, mouse, orangutan, monkey and human; while evolutionarily distal from fish, reptiles and amphibians.

mRNA expression in porcine tissues

The mRNA expression in nine porcine tissues of liver, lung, small intestine, skeletal muscle, heart, kidney, spleen, inguinal lymph nodes and fat of adult pigs was analyzed by RT-PCR with *GAPDH* as housekeeping gene. The amplified fragment size was 221, 593 and 533 bp for porcine *NECD*, *SNRPN* and *UBE3A* genes, respectively (Fig. 2). The results showed that porcine *NECD*, *SNRPN* and *UBE3A* genes were expressed in all of the nine tissues investigated. The expression levels were not different among tissues for *NECD* and *UBE3A* genes; however, for *SNRPN* gene, the expression in liver, skeletal muscle and fat was slightly lower compared with those expressed in other tissues.

SNPs in the transcript

With primer pair NDF/NDR, a fragment of 1983 bp was amplified and sequenced from porcine genomic DNA, alignment of sequences amplified from 19 neonate pigs revealed one SNP named as g.263G>C, existing in the coding region of porcine *NECD* gene. With primer pair SNR3F/SNR3R, a fragment of 360 bp was amplified and sequenced from porcine genomic DNA, alignment of sequences amplified from 19 neonate pigs revealed that one SNP named as g.402T>C in the coding region of porcine *SNRPN* gene exists. A SNP (g.340A>G) in the coding region of *UBE3A* was identified through alignment of porcine *UBE3A* cDNA sequence and confirmed by PCR from porcine genomic DNA with primer pair cUB2F/cUB3R (Fig. 3). The g.263 G>C and g.402 T>C mutations do not change the encoded amino acids, while the g.340A>G mutation of porcine *UBE3A* gene results in amino acid substitution of lysine with glutamic acid.

Imprinting in the skeletal muscle of neonate pigs

Imprinting assay in the skeletal muscle *longissimus dorsi* of neonate pigs was carried out in both Landrace × Laiwu and Laiwu × Landrace pedigrees. For *NECD* gene, five Landrace × Laiwu and three Laiwu × Landrace neonate pigs were used for imprinting analysis; for *SNRPN* gene, three Landrace × Laiwu and seven Laiwu × Landrace neonate pigs; and for *UBE3A* gene, two Landrace × Laiwu and four Laiwu × Landrace neonate pigs, were used. The results indicated that the maternally inherited allele of both *NECD* and *SNRPN* genes was not expressed in the skeletal muscle *longissimus dorsi* of neonate pigs, suggesting that these two genes were maternally imprinted in skeletal muscle. For *UBE3A* gene, however, both alleles were detected in the offspring, which suggest that *UBE3A* was not imprinted in the skeletal muscle of neonate pigs (Fig. 3).

Discussion

More and more studies reveal the importance of genomic imprinting in mammalian development and growth. Although the imprinted genes reported in pigs were growing in recent years, the imprinted genes identified are still very less compared with those in human and mice.

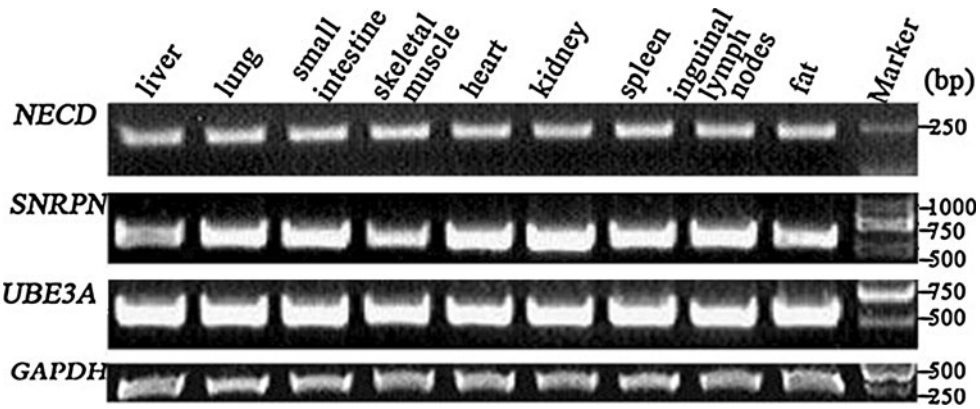

Fig. 2 The mRNA expression of *NECD*, *SNRPN* and *UBE3A* genes in adult pigs. Porcine *GAPDH* was used as housekeeping gene

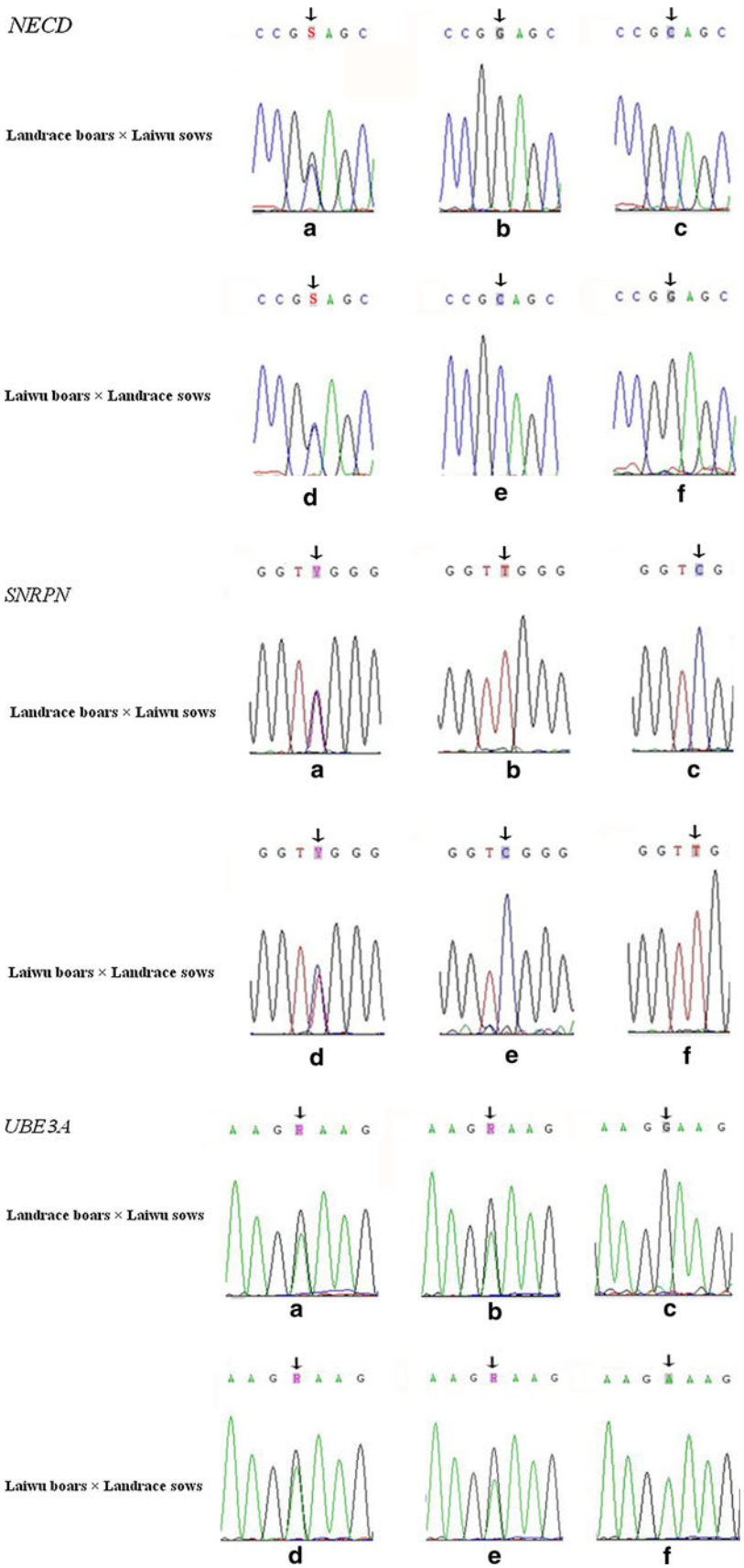

Fig. 3 Single nucleotide polymorphism (SNP) detection and imprinting analysis of *NECD*, *SNRPN* and *UBE3A* genes in the skeletal muscle of neonate pigs. The arrows point to SNP sites. **a**, **d** Sequence at SNP sites of genomic DNA from the hybrid pigs, showing heterozygosity at the polymorphic sites of the three genes. **b**, **e** cDNA sequence at SNP sites of the three genes expressed in the skeletal muscle of the heterozygous pigs. **c**, **f** Sequence at SNP sites of genomic DNA from the skeletal muscle of the mother. For both *NECD* and *SNRPN* genes, the maternal alleles are not expressed in the heterozygous pigs, so they are maternally imprinted; whereas for the *UBE3A* gene, both alleles are expressed

Some imprinted genes like *IGF2* play important roles in controlling skeletal muscle growth and lean mass in pigs [18], therefore, identifying more imprinted genes will be helpful for explaining the parental-specific-effect in affecting economic traits such as meat mass and meat quality. The *NECD*, *SNRPN* and *UBE3A* genes were located in an imprinted region affecting human PWS and AS, and *NECD* mediates skeletal muscle regeneration by promoting myoblast survival and differentiation [3]. In this study, the homology, expression and imprinting status of these three genes were investigated in pigs.

Phylogenetic trees constructed based on amino acid sequence indicated that *NECD*, *SNRPN* and *UBE3A* genes were highly conserved in mammals, and the highest homology was found between pigs and cattle, which is consistent with the species evolutionary relationship between them, suggesting that *NECD*, *SNRPN* and *UBE3A* genes were essential for development. With *GAPDH* as housekeeping gene, we further characterized the mRNA expression of these genes in nine tissues including liver, lung, small intestine, skeletal muscle, heart, kidney, spleen, inguinal lymph nodes and fat of adult pigs, and found that they were expressed in all the tissues investigated (Fig. 2). Previous studies have shown that the *SNRPN* gene was abundantly expressed in rodent brain and heart and relatively lower in lung, liver, spleen, kidney, skeletal muscle and gonads [19], which is consistent with the results of the present study revealing that, in adult pigs, its expression in liver and skeletal muscle was slightly lower compared with other tissues. A previous study on 180-day-old Wujing pig showed that only in muscle and kidney the *NECD* was expressed, and was not detected in small intestine, large intestine, liver, muscle, backfat, lung, spleen and kidney [20]. We found that, in adult Landrace × Laiwu crossbred pigs, *NECD* gene was expressed in all of the nine tissues including liver, lung, small intestine, skeletal muscle, heart, kidney, spleen, inguinal lymph nodes and fat. The difference is likely due to that pigs with different age, breed, or genotypes were used.

Previous studies on the imprinting status of *NECD* and *SNRPN* genes that were carried out by quantitative allelic pyrosequencing after comparing biparental (BP) and parthenogenetic (PRT) fetal tissues showed that both genes were predominantly paternally expressed in brain, fibroblast, liver, and placenta of day 30 porcine fetuses [16]. In this study, the polymorphism-based approach was used to detect the imprinting status of *NECD*, *SNRPN* and *UBE3A* genes in reciprocal pedigrees constructed with Landrace and Laiwu pigs. With the three SNPs identified in the coding region, the maternal alleles of *NECD* and *SNRPN* were found not to be expressed in the skeletal muscle of neonate pigs. These results indicated that the *NECD* and *SNRPN* genes are maternally imprinted in both porcine fetuses and the skeletal muscle of neonate pigs. As for other mammals, the human and mouse *NECD* genes are maternally imprinted [2], and *SNRPN* is also maternally imprinted in mouse [21] and human [22], suggesting that the imprinting status of *NECD* and *SNRPN* was conserved among mammals. In cattle, the methylation profile of CpG island of *SNRPN* gene are comparable with mouse and human [23], and artificial reproductive techniques, such as prolonged in vitro culture and SCNT, may lead to abnormal reprogramming of imprinting of *SNRPN* gene by altering methylation levels at this locus [23, 24]. These results imply that the imprinting status of *SNRPN* is prone to environmental changes such as nutrition. In human and rodents neurons, the paternal allele of *UBE3A* is intact but epigenetically silenced, while in most tissues *UBE3A* is biallelically expressed [25, 26].We also found that *UBE3A* gene was not imprinted in the skeletal muscle of neonate pigs, consistent with the results obtained in human and rodents.

In conclusion, porcine *NECD*, *SNRPN*, and *UBE3A* genes were highly conserved among mammals and were expressed in most tissues. Both *SNRPN* and *NECD* genes are maternally imprinted genes while *UBE3A* is not imprinted in the skeletal muscle of neonate pigs. The role of these three genes in porcine skeletal muscle development requires further investigations.

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