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Original article

NNAT and *DIRAS3* genes are paternally expressed in pigs

Huan-Chen CHENG[a], Feng-Wei Zhang[a], Chang-Yan DENG[a]*, Cao-De JIANG[b], Yuan-Zhu XIONG[a], Feng-E LI[a], Ming-Gang LEI[a]

[a] Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education and Key Laboratory of Swine Genetics and Breeding of Ministry of Agriculture, Huazhong Agriculture University, Wuhan, P. R. China
[b] Department of Bio-engineering, College of Animal Sciences, Southwest University, Chongqing, 400716, P. R. China

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Abstract – Although expression and epigenetic differences of imprinted genes have been extensively characterised in man and the mouse, little is known on livestock species. In this study, the polymorphism-based approach was used to detect the imprinting status of *NNAT* and *DIRAS3* genes in five heterozygous pigs (based on SNP) of Large White and Meishan F_1 hybrids. The results show that both genes were paternally expressed in all the tested tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary and pituitary). In addition, the *NNAT* gene had two transcripts in all tested tissues, which is consistent with its counterpart in man and cattle.

pig / *NNAT* / *DIRAS3* / imprinting / paternally expressed

1. INTRODUCTION

Genomic imprinting is a parent-of-origin-dependent epigenetic mechanism, in which a subset of autosomal genes is expressed from only one allele [15]. Imprinted genes have important roles in the regulation of foetal growth, development, function of the placenta and postnatal behaviour, in mammals in particular [14]. At present, more than 120 imprinted genes have been identified in man and mice, but only ten imprinted genes have been identified in sheep, seven in cattle and three in pigs (http://igc.otago.ac.nz/home.html). Therefore, it is of interest to identify other imprinted genes in pigs in order to analyse the conservation of genomic imprinting among different species.

* Corresponding author: zfw2004790921521@126.com

NNAT located on chromosomes 20q11.2-q12 in man and 2H1 in mice is preferentially expressed from the paternal allele [6, 17]. The protein encoded by *NNAT* is a proteolipid that may be involved in the regulation of ion channels during brain development [5]. Chu and Tsai [3] have found that *NNAT* plays an important role in insulin secretion. In man and cattle, *NNAT* has two alternatively spliced transcripts (α and β), either with or without exon 2 [8,21]. *DIRAS3* is a maternally imprinted tumour suppressor gene and its expression decreases dramatically in ovarian cancers [9, 19]. Yu *et al*. [20] have reported that transgenic expression of the human *DIRAS3* gene in the mouse produces a small stature and that the gene inhibits growth.

In this study, we obtained the complete coding region of porcine *NNAT* and *DIRAS3* genes. The imprinted status of porcine *NNAT* and *DIRAS3* genes was detected using SNP present in the exons of the two genes in five heterozygous pigs (based on SNP). We demonstrate that *NNAT* also has two transcripts in all tested tissues.

2. MATERIALS AND METHODS

2.1. Experimental animals and DNA isolation

All animals in this study were from the Experimental pig station of Huazhong Agricultural University. Ten two-month F_1 hybrid pigs from Large White boars × Meishan sows and ten from Meishan boars × Large White sows were used to search for individuals heterozygous for the two genes. Genomic DNA from the twenty F_1 hybrids and their mothers were isolated according to the standard phenol-chloroform method.

2.2. RNA isolation and cDNA synthesis

Total RNA from twelve tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary and pituitary) of five heterozygous pigs of the twenty F_1 hybrids were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions. First strand cDNA was synthesised from 2 μg total RNA treated with DNAse I (TaKaRa, Tokyo, Japan) in a 20 μL reaction volume containing 5 μM oligo$(dT)_{18}$ primer, 1 X M-MLV first-strand buffer, 40 U M-MLV reverse transcriptase, 1 mM each dNTP and 8 U RNase inhibitor (Promega, Madison, WI, US) at 42 °C for 60 min.

Table I. Primer sequences and products amplified from the porcine *NNAT*, *DIRAS3* and *GAPDH* genes.

Gene	Primer	Sequence	Annealing temperature	Size (bp) DNA	Size (bp) cDNA	Acc no and SNP position
NNAT	NN1F	ACCCACCACCCTTGGAAC	58 °C	1758	595 and 514	DQ666422 no SNP
	NN1R	GGCTTGATTGGCGCTGTC				
NNAT	NN2F	CAGCACCGACAATGACGA	60 °C	645	645	DQ666422 2135 bp
	NN2R	AATCTAGCCGGGGAGACA				
DIRAS3	DIF	CCCCATCAATACCCAACG	55 °C	1227	1227	DQ666421 66bp
	DIR	TCTCTGCTCCCACCCTCA				
DIRAS3	DIIF	CCCCATCAATACCCAACG	56 °C	508	508	DQ666421 no SNP
	DIIR	CCTTCTCACTCACCTCCC				
GAPDH	HouseF	ACCACAGTCCATGCCATCAC	55 °C	780	480	
	HouseR	TCCACCACCCTGTTGCTGTA				

2.3. PCR of DNA and cDNA

The human *NNAT* cDNA sequence (GenBank: NM_005386) and *DIRAS3* cDNA sequence (GenBank: NM_004675) were used to identify porcine expressed sequence tags (EST) through standard BLAST (http://www.ncbi.nlm.nih.gov/blast/) searches in the 'EST-others' database. Pig EST sharing more than 85% sequence identity with the human cDNA sequences were assembled into EST-contigs. The exon-intron structures of porcine *NNAT* and *DIRAS3* genes were estimated according to the structure of the two genes in man. All primers were designed from the consensus sequences of EST-contigs (Tab. I). The PCR conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 45 s, annealing at optimal temperature (Tab. I), 72 °C for 1 min and a final extension at 72 °C for 7 min. Primer pair HouseF/HouseR, which amplified the fragment spanning intron 8 of the *GAPDH* gene was applied to exclude the possibility of DNA contamination during all RT-PCR reactions (Fig. 1A).

2.4. Sequencing and SNP detection

PCR products amplified by primer pairs NN1F/NN1R, NN2F/NN2R and DIF/DIR were purified with the Wizard prep PCR purification system (Promega) and sequenced commercially. The site was considered as a heterozygous mutation if double peaks appeared at similar heights in the sequence bands.

3. RESULTS

3.1. Sequence analysis and SNP discovery

Primer pairs NN1F/NN1R and NN2F/NN2R amplified a total of 2234 bp containing the complete open reading fragment (ORF) of *NNAT*. Primer pair DIF/DIR amplified a 1227 bp fragment covering the 687 bp complete coding region of *DIRAS3*. Both of the DNA sequences were deposited in the GenBank database. According to the sequence bands, one SNP (C/T) was found in the *NNAT* gene and one SNP (G/T) was found in the *DIRAS3* gene. Three F_1 hybrid pigs of Large White boars $\times$ Meishan sows and two of Meishan boars $\times$ Large White sows were heterozygous for both genes. The accession number and the position of the SNP are listed in Table I.

3.2. Imprinting analysis and transcript identification of the *NNAT* gene

RT-PCR of primer pair NN1F/NN1R indicated that the *NNAT* gene has two transcripts in all tissues examined (heart, liver, spleen, lung, kidney, stomach, small intestine skeletal muscle, fat, uterus, ovary and pituitary) (Fig. 1B). Sequencing of the two RT-PCR products showed that the two transcripts differ in exon 2. Transcript α has 81 amino acids and transcript β has 54 amino acids. Primer pair NN2F/NN2R amplified DNA from the heterozygous pigs and their mothers and cDNA from various tissues of the heterozygous pigs. Sequencing of the PCR and RT-PCR products revealed that the *NNAT* gene is paternally expressed in all the tissues tested. The three heterozygous pig hybrids of Large White boars $\times$ Meishan sows expressed the T allele but their mothers showed the C allele in genomic DNA, and the two heterozygous pig hybrids of Meishan boars $\times$ Large White sows expressed the C allele but their mothers showed the T allele in genomic DNA (Fig. 2). The five heterozygous pigs had the same imprinted status.

3.3. Imprinting analysis of the *DIRAS3* gene

RT-PCR of the same tissues as above from the five pigs with primer pair DIIF/DIIR indicates that the *DIRAS3* gene was expressed in all tissues examined (Fig. 1C). The primer pair also amplified DNA from the heterozygous pigs and their mothers. Sequencing of the PCR and RT-PCR products showed that the *DIRAS3* gene is also paternally expressed in all tissues tested. The three heterozygous pigs of Large White boars × Meishan sow hybrids expressed the T allele but their mothers showed the G allele in genomic DNA, and the two heterozygous pigs of Meishan boars × Large White sows expressed the G allele but their mothers showed the T allele in genomic DNA (Fig. 2). There was no difference in the imprinted status of the gene in all tissues from the five heterozygous pigs.

4. DISCUSSION

At present, most imprinted genes have been identified and studied in man and mice and few reports exist on imprinting in livestock species. Therefore, identifying more imprinted genes in livestock species should be useful for the study of the conservation and function of imprinted genes. It has been reported that in the adult mouse, *NNAT* is expressed in the brain, pituitary gland, lungs, adrenal glands, uterus, skeletal muscles, ovaries, and pancreas [2,7,12,16]. Our study, as compared with these studies, shows that in the pig, *NNAT* is expressed in the pituitary gland, lungs, uterus, skeletal muscles and ovaries, but also in the heart, liver, spleen, kidney, stomach, small intestine and fat. These observations indicate that *NNAT* is expressed in a wide range of tissues. Xu *et al*. [18] showed that the *DIRAS3* gene acts as a negative regulator in mouse growth and development. Luo *et al*. [10] reported that an appropriate methylation status of the CpG islands in the promoter region may play a role in the down-regulation of *DIRAS3* gene expression. Therefore, our study should be useful to study whether the appropriate methylation status of the CpG islands affects porcine growth and development.

NNAT and *DIRAS3* genes are both preferentially expressed paternal alleles in the human and mouse foetus [6, 17]. In the present study, the imprinting analysis of the two genes in five heterozygous pigs shows that the two genes are both paternally expressed in all tested tissues. The results confirm the conservation of genomic imprinting among different species, although there are some studies reporting species-specific and tissue-specific imprinting [4, 11].

Aikawa *et al*. [1] and Zaitoun *et al*. [21] have reported that the *NNAT* gene has two transcripts in the pig and bovine foetus, and that the two transcripts

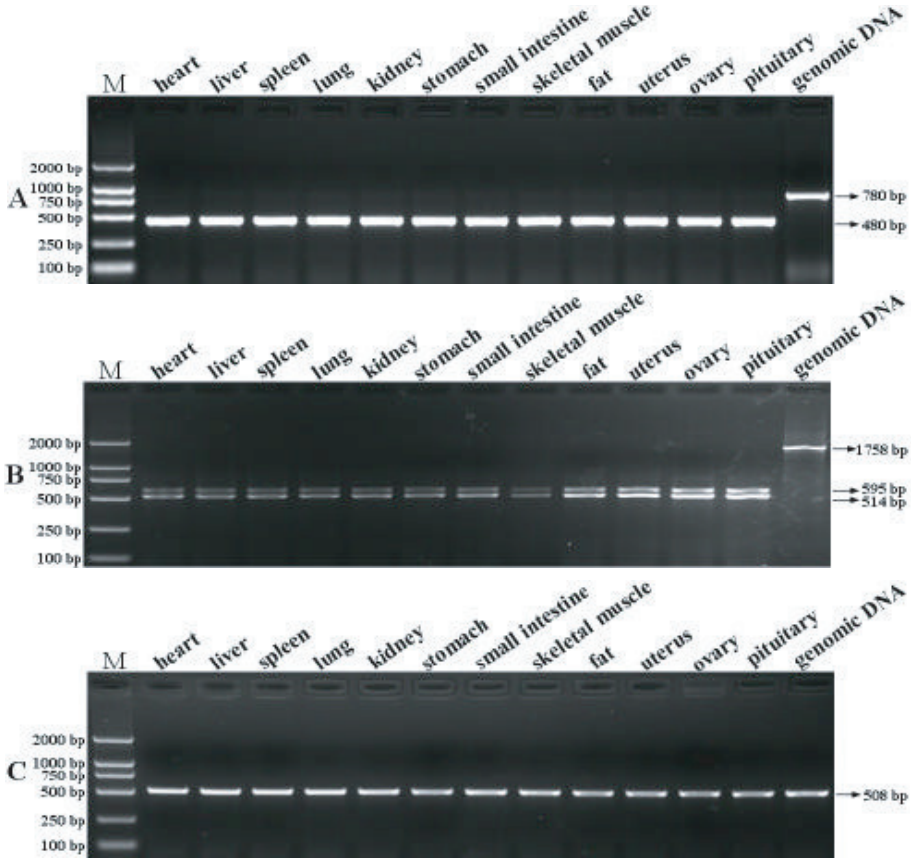

Figure 1. Expression patterns of porcine *NNAT* and *DIRAS3* genes in 12 tissues analysed by RT-PCR. A is the amplification with primer pair HouseF/HouseR of the *GAPDH* gene to exclude the DNA contamination. B is the expression patterns amplified with primer pair NN1F/NN1R of the *NNAT* gene, which shows the two transcripts of the gene. C is the expression patterns amplified with primer pair DI1F/DI1R of the *DIRAS3* gene.

are paternally expressed in cattle. Our results were consistent with these reports since they indicate that in all tissues tested from the five two-month old pigs, the *NNAT* gene also has two transcripts and both express paternal alleles. Alternative splicing has recently emerged as a major mechanism of generating protein diversity in higher eukaryotes [13]. The alternative splicing of the *NNAT* gene may be useful to study the function of the *NNAT* protein.

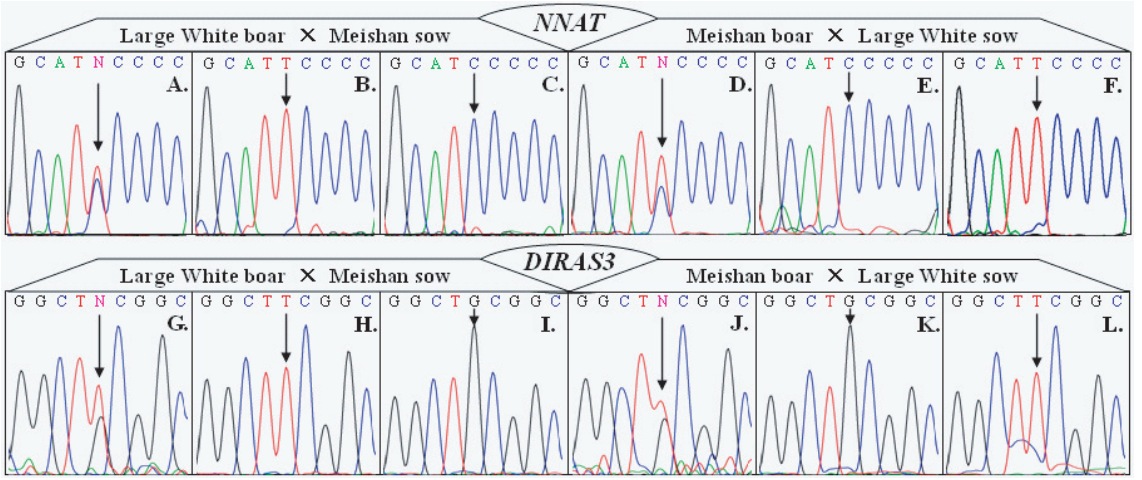

Figure 2. Imprinting analysis of porcine *NNAT* (A, B, C, D, E and F) and *DIRAS3* (G, H, I, J, K and L) genes revealed by sequencing. The arrows point to SNP sites. A and D, sequence analysis of genomic DNA from the hybrid pigs shows heterozygosity (C/T) at position 2135 of the *NNAT* gene. B and E, sequence analysis of cDNA from skeletal muscle shows monoallelic expression of alleles T and C at position 2135, respectively. C and F, sequence analysis of maternal genomic DNA shows alleles C and T at position 2135, respectively. G and J, sequence analysis of genomic DNA from the hybrid pigs shows heterozygosity (G/T) at position 66 of *DIRAS3* gene. H and K, sequence analysis of cDNA from skeletal muscle shows monoallelic expression of alleles T and G at position 66, respectively. I and L, sequence analysis of maternal genomic DNA shows alleles G and T at position 66, respectively. The maternal alleles are not expressed in the heterozygous pigs, so porcine *NNAT* and *DIRAS3* genes are both maternally imprinted and preferentially express the paternal alleles.

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