



Detection of a novel single nucleotide polymorphism and imprinted status analysis of the Ras protein-specific guanine nucleotide-releasing factor 1 gene in domestic pigs

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ABSTRACT. The aim of this study was to determine the imprinting status of the Ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) gene in domestic pigs. In this study, a 228-bp partial sequence located in exon 14 and a 193-bp partial sequence located in exon 1 of the *Rasgrf1* gene in domestic pigs were obtained. A novel single nucleotide polymorphism, a G/A transition, was identified in *Rasgrf1* exon 14, and then the reciprocal Berkshire x Wannan black F1 hybrid model and the reverse transcription-polymerase chain reaction-restriction fragment length polymorphism method were used to detect the imprinting status of the porcine *Rasgrf1* gene at the 1-day-old developmental stage.

Imprinting analysis showed that, compared to the imprinted expression of the *Rasgrf1* gene in mouse and rat, a variable imprinting status was observed in domestic pigs. In principle, the porcine *Rasgrf1* gene was maternally expressed in the liver and small intestine, paternally expressed in the lung, and biallelically expressed in brain, heart, spleen, kidney, stomach, pancreas, fat, testis, ovary, longissimus dorsi, and pituitary tissues. In conclusion, our results indicated that the *Rasgrf1* gene shows both species- and tissue-specific variation in imprinted expression.

Key words: Domestic pigs; Single nucleotide polymorphism; *Rasgrf1*; Imprinting analysis

INTRODUCTION

Genomic imprinting is an unusual epigenetic phenomenon in which the allele inherited from one parent is epigenetically silenced in the offspring and escapes Mendel's laws of heredity (Mannens and Alders, 1999). In mammals in particular, imprinted genes have an important function in the regulation of fetal growth, development, function of the placenta, and postnatal behavior (Isles and Holland, 2005; Zhang et al., 2007). Recently, an extensive comprehension of imprinted genes is becoming more and more important to animal breeders. However, most imprinted genes have been identified in humans and mice, and there are only a small number of imprinted genes identified in livestock (Lefebvre, 2012; Qiao et al., 2012). While there are extensive researches on imprinting in humans and mice, there is still a dearth of knowledge about imprinting in swine.

The Ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) is a 140-kD protein that acts as a guanine-nucleotide exchange factor and activates the small G-proteins Ras and Rac in a calcium-dependent manner and forms part of the MAPK signaling pathway (Talebian et al., 2013). The mouse *Rasgrf1* gene is located on chromosome 9 (De la Puente et al., 2002) and is predominantly expressed from the paternal allele in neonatal mouse brain, liver, heart, and stomach, with expression becoming biallelic during weaning and later into adulthood, whereas other tissues (e.g., lung, ovary, and testis) showed biallelic expression at all times (Plass et al., 1996; Charalambous et al., 2007; Dockery et al., 2009). The rat *RasGrf1* gene is located on chromosome 8 and shows paternal-specific monoallelic expression in the neonatal brain (Pearsall et al., 1999). It was the first imprinted gene that was demonstrated to regulate postnatal rather than prenatal growth (Itier et al., 1998). Analysis of various knockout mouse strains uncovered a specific functional contribution of *Rasgrf1* in the processes of photoreception, control of postnatal growth, body size, and pancreatic β -cell function and glucose homeostasis (Fernández-Medarde and Santos, 2011). However, no research on the imprinting status of the *Rasgrf1* gene has been carried out in swine (<http://www.geneimprint.com>; <http://igc.otago.ac.nz>).

In this study, we identified the imprinting status of *Rasgrf1* in neonatal porcine tissues, utilizing the polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method. To achieve our purpose, we subcloned the partial sequence of the domestic pig *Rasgrf1* gene, identified a single nucleotide polymorphism (SNP) in *Rasgrf1* exon 14, and analyzed its imprinting status in 14 organ/tissues of 1-day-old piglets. These results may be useful in marker-assisted and genomic selection breeding schemes.

MATERIAL AND METHODS

All procedures involving animals were approved by the Animal Care and Use Committee of Anhui Agricultural University. All animals used in this study were derived from “the pig experimental station” of Anhui Agricultural University.

Tissue samples and DNA preparation

Ear tissues from an adult Berkshire pig and an adult Wannan black pig were obtained to search for SNPs. Genomic DNA was isolated from the white blood cells of sixteen 1-day-old F1 hybrid pigs (8 piglets of a Berkshire boar x Wannan black sow mating and 8 of a Wannan black boar x Berkshire sow mating) and their parents according to the standard phenol-chloroform method. Tissue samples (brain, heart, liver, spleen, lung, kidney, stomach, pancreas, fat, testis, ovary, small intestine, longissimus dorsi, and pituitary) from 7 heterozygous pigs (based on SNPs) of the 16 F1 hybrid pigs were collected for imprinting analysis.

RNA isolation and cDNA synthesis

Total RNA from all collected tissues was isolated with Trizol reagent (TaKaRa, Tokyo, Japan) according to the instructions of the manufacturer. First-strand complementary DNA (cDNA) was synthesized from 2 µg total RNA treated with DNase I (TaKaRa) in a 20-µL reaction volume containing 5 µM oligo(dT)₁₆ primer, 1X M-MLV first-strand buffer, 40 U M-MLV reverse transcriptase, 1 mM of each dNTP, and 8 U RNase inhibitor (TaKaRa) at 42°C for 60 min.

PCR of DNA and cDNA

Highly conserved expressed sequence tags (ESTs) were obtained through standard Basic Local Alignment Search Tool analysis according to partial sequences of the human [National Center for Biotechnology Information (NCBI) Gene ID: 5923], mouse (NCBI Gene ID: 19417), and *Sus scrofa* (NCBI Gene ID: 100157221) *Rasgrfl* genes. Gene-specific primers for the *Rasgrfl* gene were designed using the highly conserved ESTs (Table 1).

Table 1. Primers used in this study.

Method	Primer sequence (5'-3')	Annealing (°C)	Product/size
PCR	Exon 14 F: CCGACGCCTCCTTGATTGT	62.4	DNA/228 bp
	Exon 14 R: TGGGCTTCCTGTAGATGGTGAT		
	Exon 1 F: GCGTGAGAAGGAGCGAGCCA	64.0	
RT-PCR	Exon 1 R: TTGCGTGCCAGCAGTCCC	63.0	cDNA/332 bp
	Exon 14 F': CCGACGCCTCCTTGATTGT		
	Exon 14 R': GTGGCTCGTGGGACTTG		

PCRs were performed in a 25-µL volume containing 25 ng porcine cDNA or 50 ng DNA, 1X PCR buffer, 0.2 µM of each primer, 150 µM of each dNTP, 1.5 mM MgCl₂, and 1 U Taq DNA polymerase (TaKaRa) using a Bio-Rad MyCycler PCR system (Bio-Rad, USA).

The PCR conditions were as follows: 94°C for 4 min; 32 cycles of 94°C for 45 s, annealing for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Sequencing and SNP detection

PCR products were purified with the Wizard Prep PCR purification system (Promega, USA), cloned with the pMD18-T easy vector (TaKaRa), and sequenced commercially (Shanghai Sangon, China). To search for SNPs, sequences that were amplified by the primer pairs exon 14F/exon 14R and exon 1F/exon 1R in the Berkshire and Wannan black pigs were compared using the DNASTAR software (USA).

RFLP of PCR and reverse transcription (RT)-PCR products

Ten microliters of PCR or RT-PCR products (containing 0.1-1 µg DNA) amplified by the primer pair exon 14F/exon 14R or exon 14F'/exon 14R' was incubated at 37°C for 9 h with 3 U of the restriction enzyme *Vpa*k11AI (Thermo, USA) and 1 µL attached 10X *Vpa*k11AI Basal Buffer. Digested products were separated on 3% agarose gels and stained with ethidium bromide.

Imprinting analysis

The RT-PCR/RFLP method was used to detect the imprinting status of the porcine *Rasgrfl* gene. We chose the polymorphic locus g.84709 G>A and the restriction enzyme *Vpa*k11AI for imprinting analysis. The first step was screening for heterozygous piglets at the polymorphic locus g.84709 G>A. PCR was carried out on neonatal piglet and parental DNA using the primer pair exon 14F/exon 14R, and RT-PCR was performed on heterozygous piglet RNA using the primer pair exon 14F'/exon 14R', which were followed by *Vpa*k11AI RFLP. By comparing the digestion patterns of the piglet's DNA/RNA and parental DNA, we could define the imprinting status of heterozygous piglets.

RESULTS

Subcloning of the domestic pig *Rasgrfl* gene

In this study, we obtained 228- and 193-bp partial sequences of the domestic pig *Rasgrfl* gene. The two partial sequences are located in exon 14 and exon 1 of *Rasgrfl*, respectively. Sequence analysis showed that exon 14 of the domestic pig *Rasgrfl* gene had a high GC content (59.29%), and the 228-bp sequence shared 93.4 and 83.9% nucleotide similarity with the human and mouse orthologs, respectively. Exon 1 of the domestic pig *Rasgrfl* gene had a high GC content (67.60%), and the 193-bp sequence shared 91.2 and 79.3% nucleotide similarity with the human and mouse orthologs, respectively.

SNP detection and screening for heterozygous piglets

Comparison of the sequences from the Berkshire and Wannan black breeds revealed

one SNP in exon 14 of the porcine *Rasgrfl* gene: the G-to-A SNP in exon 14 (NCBI Gene ID: 100157221 g.84709 G>A). The SNP (G/A⁸⁴⁷⁰⁹) in the coding region was a synonymous mutation and could be detected with the restriction enzyme *Vpak11AI*. Sixteen F1 piglets of the Berkshire x Wannan black cross were used to identify heterozygous individuals at the SNP site. Amplification of genomic DNA with the primer pair exon 14F/exon 14R was conducted. PCR products were digested by *Vpak11AI*. Allele g.84709G produced fragments that were 116, 75, and 38 bp, and allele g.84709A produced fragments that were 191 and 38 bp (Figure 1). Results of this PCR/RFLP test indicated that three Berkshire boar x Wannan black sow F1 hybrid piglets and four Wannan black boar x Berkshire sow F1 hybrid piglets were heterozygous at the site.

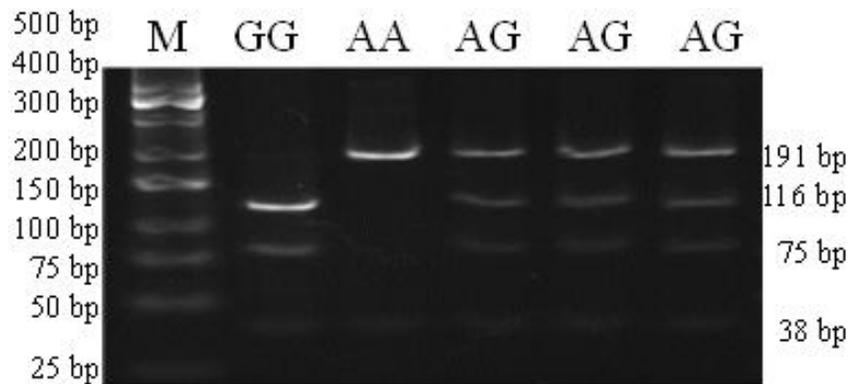


Figure 1. PCR-RFLP of the porcine *Rasgrfl* gene. Lane M = marker GM303 (the bands are 500, 400, 300, 200, 100, and 75 bp from top to bottom, respectively; Sangon, Shanghai, China). Lanes GG, AA, and AG = each of the different g.84709 G>A genotypes.

Allelic expression of the porcine *Rasgrfl* gene

RT-PCR of the brain, heart, liver, spleen, lung, kidney, stomach, pancreas, fat, testis, ovary, small intestine, longissimus dorsi, and pituitary from the seven heterozygous piglets with the primer pair exon 14F'/exon 14R' showed that the porcine *Rasgrfl* gene was expressed in all of the tissues tested. *Vpak11AI* RFLP analysis of the RT-PCR products showed that the *Rasgrfl* gene was maternally expressed in the liver and small intestine, paternally expressed in the lung, and biallelically expressed in the brain, heart, spleen, kidney, stomach, pancreas, fat, testis, ovary, longissimus dorsi, and pituitary tissues. The g.84709G allele was monoallelically expressed in the lung tissue, and the g.84709A allele was monoallelically expressed in the liver and small intestine tissues of three heterozygous Berkshire boar x Wannan black sow F1 hybrid piglets; the alleles originated from their Berkshire fathers and Wannan black mothers, respectively (Figure 2a). The g.84709A allele was monoallelically expressed in the lung tissue, and the g.84709G allele was monoallelically expressed in the liver and small intestine tissues of four heterozygous Wannan black boar x Berkshire sow F1 hybrid piglets; the alleles originated from their Wannan black fathers and Berkshire mothers, respectively (Figure 2b).

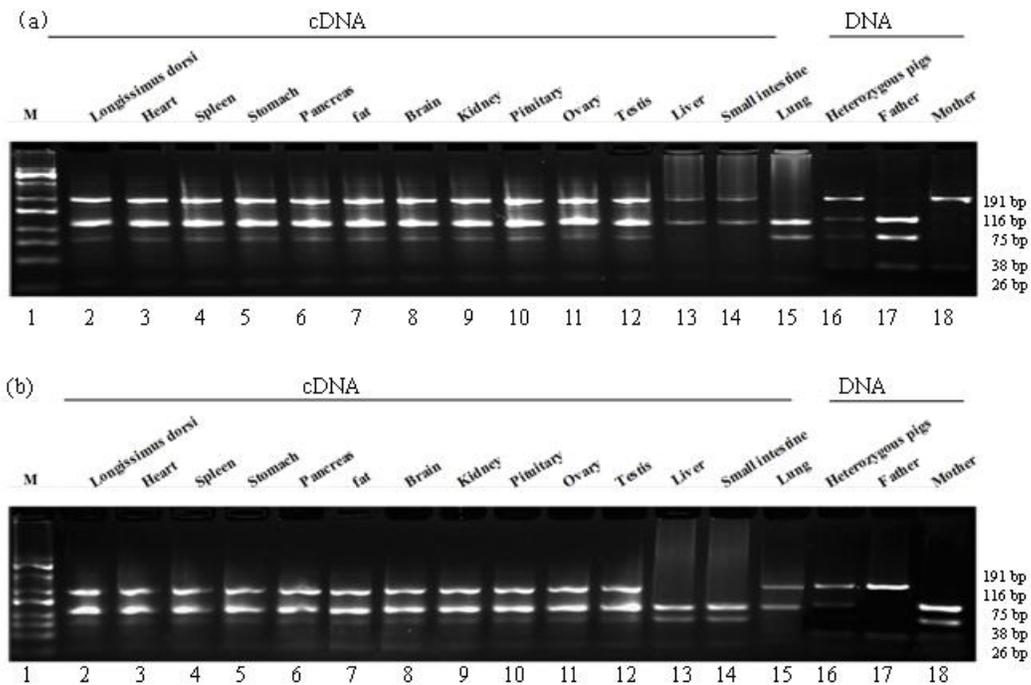


Figure 2. RT-PCR-RFLP/PCR-RFLP image of the allele-specific expression of the domestic pig *Rasgrfl* gene. The complementary DNA (cDNA) samples are from 14 different tissues of a G-A heterozygous piglet (lanes 2 to 15), and genomic DNA samples are from the piglet, its father, and its mother (lanes 16, 17, and 18, respectively). Digestions with *Vpa*K11AI revealed that the *Rasgrfl* gene is biallelically expressed in most of the tissues tested. The *Rasgrfl* gene of Berkshire boar x Wannan black sow (a) and Wannan black boar x Berkshire sow (b) hybrid piglets are maternally expressed in the liver and small intestine (lanes 13 and 14), paternally expressed in the lung (lane 15). Lane 1 = marker GM303 (the bands are 500, 400, 300, 200, 100, and 75 bp from top to bottom, respectively).

DISCUSSION

In this study, a Berkshire x Wannan black F1 hybrid model and the RT-PCR/RFLP method were used to detect the imprinting status of the porcine *Rasgrfl* gene. Berkshire pigs are Western breeds, whereas Wannan black pigs are of Asiatic origin. These 2 breeds are genetically very different; therefore, it is easy to search for SNPs between Berkshire and Wannan black pigs, including SNPs in exons (Qiao et al., 2010). The frequency of heterozygous pigs, which was a prerequisite for imprinting analysis, was high in F1 hybrid pigs (Bischoff et al., 2009). Furthermore, we used the reciprocal F1 hybrids of Berkshire x Wannan black crosses to analyze the imprinting status of the porcine *Rasgrfl* gene. This model avoids the effect of gene expression in a single pig breed and makes the results reliable. The RT-PCR/RFLP method is rapid and convenient to detect the imprinting status compared with sequencing (Cheng et al., 2007). Therefore, the combination of the method and the model is convenient and believable for imprinting analysis.

It has been reported that the rat *Rasgrfl* gene, located on chromosome 8, shows paternal-specific monoallelic expression in the neonatal brain (Pearsall et al., 1999). The mouse *Rasgrfl*, located on chromosome 9, is imprinted and exclusively expressed from the paternal allele in the

neonatal brain, heart, stomach, and liver (Plass et al., 1996; Dockery et al., 2009). The imprinting of the mouse *Rasgrfl* gene in a few tissues is relaxed during development, the transition from strongly and predominantly paternal allelic expression to biallelic expression can be seen over time, and expression becomes biallelic around the time of weaning (Drake et al., 2009). Interestingly, compared to the imprinted expression of the *Rasgrfl* gene in mouse and rat, we observed a variance in the imprinting of the *Rasgrfl* gene in domestic pigs. In our study, porcine *Rasgrfl* was shown to have paternal-specific expression in the lung, maternal-specific expression in the liver and small intestine, and biallelic expression in other tissues (e.g., testis and ovary).

The imprinting status of the *Rasgrfl* gene has been elucidated in mouse and rat. However, this is the first investigation of the imprinting status of the *Rasgrfl* gene in swine. By first obtaining the partial exon 14 sequence, we found that, typical of imprinted genes (Amarger et al., 2002; Zhang et al., 2004), exon 14 of the porcine *Rasgrfl* gene was highly GC-rich. Furthermore, a *de novo* polymorphism located in exon 14 of the porcine *Rasgrfl* gene was discovered and was presented for the first time here (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=100157221). The identification of an expressed SNP in the *Rasgrfl* gene also allowed us to study its imprinting status by following the expression of the parental alleles in heterozygous pigs. We found that the *Rasgrfl* gene was maternally expressed in the liver and small intestine, paternally expressed in the lung, and biallelically expressed in the brain, heart, spleen, kidney, stomach, pancreas, fat, testis, ovary, longissimus dorsi, and pituitary. These observations are not consistent with those from imprinting reports about the mouse and rat *Rasgrfl* gene (Plass et al., 1996; Dockery et al., 2009; Drake et al., 2009). The differential imprinting status of the *Rasgrfl* gene among rat, mouse and pig may be caused by the different species or the different tissues that were examined.

One of the limitations of this study is that no imprinting expression data at other porcine developmental stages, especially around weaning, was available to confirm the imprinting status of *Rasgrfl*. To identify the conservation of genomic imprinting at different developmental phases and in different species, the imprinting status of *Rasgrfl* around the time of weaning in pigs needs to be investigated in future studies.

In conclusion, this is the first study on the imprinting status of the *Rasgrfl* gene in domestic pigs. Our results showed that there is a general variation in the imprinting of the *Rasgrfl* gene in species that have been studied so far, suggesting that the *Rasgrfl* gene shows both species- and tissue-specific variation in imprinted expression.

Conflicts of interest

The authors declare no conflict of interest.

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