Expression and genomic imprinting of the porcine Rasgrf1 gene

Yue-Yun Ding a,1, Li-Yuan Liu b,1, Jie Zhou b,*, Xiao-Dong Zhang a, Long Huang a, Shu-Jing Zhang b, Zong-Jun Yin a,⁎

a Anhui Provincial Laboratory for Local Livestock and Poultry Genetic Resource Conservation and Bio-Breeding, Department of Animal Science, College of Animal Science and Technology, Anhui Agricultural University, Hefei City, Anhui 230036, PR China
b Department of Veterinary, College of Animal Science and Technology, Anhui Agricultural University, Hefei City, Anhui 230036, PR China

Abstract

Imprinted genes play important roles in mammalian growth, development and behavior. The Rasgrf1 (Ras protein-specific guanine nucleotide exchange factor 1) gene has been identified as an imprinted gene in mouse and rat. In the present study, we detected its sequence, imprinting status and expression pattern in the domestic pigs. 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 G/A transition, was identified in Rasgrf1 exon 14, and then, the reciprocal Berkshire × Wannan black F1 hybrid model and the RT-PCR-RFLP method were used to detect the imprinting status of porcine Rasgrf1 gene at the developmental stage of 1-day-old. The expression profile results indicated that the porcine Rasgrf1 mRNA was highly expressed in brain, pituitary and pancreas, followed by kidney, stomach, lung, testis, small intestine, ovary, spleen and liver, and at low levels of expression in longissimus dorsi, heart, and backfat. The expression levels of Rasgrf1 gene in brain, pituitary and pancreas tissues were significantly different between the two reciprocal F1 hybrids. Imprinting analysis showed that porcine Rasgrf1 gene was maternally expressed in the liver, small intestine, paternally expressed in the lung, but bi-allelically expressed in brain, heart, spleen, kidney, stomach, pancreas, backfat, testis, ovary, longissimus dorsi and pituitary tissues.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

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). In domestic livestock species, imprinted genes have been shown to contribute to economically important production traits (Qiao et al., 2012; Zhang et al., 2007). Recently, extensive comprehension of imprinted genes is economically important production traits (Qiao et al., 2012; Zhang et al., 2007). Recently, 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, there are only a small number of imprinted genes identified in livestock (Lefebvre, 2012). Comparing to the extensive researches of 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 guanine-nucleotide exchange factor and activates the small g-proteins Ras and Rac in a calcium dependent manner, forms part of the MAPK signaling pathway (Talebian et al., 2013). The rat Rasgrf1 gene is located on chromosome 8, shows paternal-specific monoallelic expression in neonatal brain (Pearsall et al., 1999). The mouse Rasgrf1 gene is located on chromosome 9 (Puente et al., 2002), is predominantly expressed from the paternal allele in neonatal brain, liver, heart, and stomach, with expression becoming bi-allelic during weaning and later into adulthood, whereas other tissues (e.g., lung, ovary and testis) show bi-allelic expression at all times (Charalambous et al., 2007; Dockery et al., 2009; Plass et al., 1996). It is the first imprinted gene demonstrated to regulate postnatal rather than prenatal growth (Tiet et al., 1998). Imprinting status of Rasgrf1 gene has been well elucidated in mouse and rat. However, no research on the imprinting status of Rasgrf1 gene has been carried out in swine.

Studies of genome-wide scanning for QTL (quantitative trait loci) in pigs revealed that many QTLs are maternally or paternally imprinted, which significantly affect growth, backfat thickness, carcass composition and reproduction (de Koning et al., 2000; Duthie et al., 2011; Rohrer et al., 2006). For example, on Sus scrofa chromosome 7, a maternally expressed QTL at 56 cM (confidence interval, 41–72 cM) was identified with strong effect on muscle depth between the third and fourth rib (de Koning et al., 2000), a QTL at 77 cM (confidence interval, 57–99 cM) showed a suggestive association with longissimus moisture.
content when paternally inherited (Rohrer et al., 2006). The porcine Rasgrf1 gene is located on SSC7 at 62.51–62.66 cM (http://www.ncbi.nlm.nih.gov/projects/mapview/), is located inside of the two QTLs (at 56 cM and 77 cM, respectively) confidence interval, this led to the speculation that Rasgrf1 is likely to be associated with pork quality and body composition traits in pigs. Therefore, identification and characterization of more imprinted genes such as Rasgrf1 gene to improve porcine production traits are very important, not only for studying the imprinting mechanism and function of imprinted genes in livestock, but also for the comparative genomic analysis of genomic imprinting among different species.

In this study, we focused on the identification of the imprinting status of Rasgrf1 in neonatal porcine tissues, utilizing the method of RT-PCR-RFLP (reverse transcription-polymerase chain reaction-restricted fragment length polymorphism). In addition, tissue expression patterns of porcine Rasgrf1 gene of 16 piglets of the reciprocal Berkshire × Wannan black F1 hybrids were investigated.

2. Materials 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.

2.1. Tissue samples and DNA preparation

Ear tissues from an adult Berkshire pig and an adult Wannan black pig were obtained to search for single nucleotide polymorphisms (SNPs). Genomic DNA was isolated from the white blood cells of 16 1-day-old F1 hybrid pigs (8 piglets of a Berkshire boar × Wannan black sow matings and 8 of a Wannan black boar × Berkshire sow mating) and their parents according to the standard phenol-chloroform method. Tissues samples (brain, heart, liver, spleen, lung, kidney, stomach, pancreas, backfat, testis, ovary, small intestine, longissimus dorsi, pituitary) from 16 heterozygous pigs (based on SNP) of the 16 1-day-old F1 hybrid piglets were collected for imprinting analysis, from the 16F1, hybrid piglets were collected for analysis of Rasgrf1 gene expression pattern. The backfat was collected from the subcutaneous adipose at the 3/4rd last ribs and 3/4rd last lumbar vertebra.

2.2. RNA isolation and cDNA synthesis

Total RNA from all tissues collected 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 of total RNA treated with Dase I (TaKaRa, Tokyo, Japan) according to the instructions of the manufacturer. Reverse transcription was performed in a 20-μl volume containing 5 μM oligo (dt) 16 primer, 1 × M-MLV first-strand buffer, 40 U of M-MLV reverse transcription (RT), 1 mM of each dNTP, and 8 U of RNase inhibitor (TaKaRa, Tokyo, Japan) at 42 °C for 60 min.

2.3. PCR of DNA and cDNA

Highly conserved ESTs were obtained through standard BLAST analysis according to partial sequences of the human (NCBI Gene ID: 5923), mouse (NCBI Gene ID: 19417) and S. scrofa (NCBI Gene ID: 100157221) Rasgrf1 genes. Gene-specific primers for Rasgrf1 gene were designed using the highly conserved ESTs (Table 1).

Polymerase chain reactions were performed in a 25 μl volume containing 25 ng of porcine cDNA or 50 ng of DNA, 1 × PCR buffer, 0.2 μM of each primer, 150 μM of each dNTP, 1.5 mM MgCl2, and 1 U of Taq DNA polymerase (TaKaRa, Tokyo, Japan), on a Bio-rad MyCycler PCR system (Bio-Rad, California, USA). The PCR conditions were as follows: 94 °C for 4 min, 32 cycles of 94 °C for 45 s, annealing for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Table 1

<table>
<thead>
<tr>
<th>Method primer sequences (5′–3′)</th>
<th>Annealing (°C)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rasgrf1 F-GGT CCT TGC GGA TGT CG 58.6 cDNA/</td>
<td>62.4</td>
<td>DNA/</td>
</tr>
<tr>
<td>Rasgrf1 R-CAC GAG GAT GAC TGT GAA GGG 51.6 cDNA/</td>
<td>228 bp</td>
<td>DNA/</td>
</tr>
<tr>
<td>Rasgrf1 F-GGT CCT TGC GGA TGT CG 58.6 cDNA/</td>
<td>64.0</td>
<td>DNA/</td>
</tr>
<tr>
<td>Rasgrf1 R-CAC GAG GAT GAC TGT GAA GGG 51.6 cDNA/</td>
<td>193 bp</td>
<td>DNA/</td>
</tr>
</tbody>
</table>

2.4. 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 amplified by the primer pairs (exon 14F/exon 14R, exon 1F/exon 1R) in the Berkshire and Wannan black pigs were compared using DNASTAR software (Madison Wisconsin, USA).

2.5. RFLP of PCR and RT-PCR products

Ten microliters of PCR or RT-PCR products (contain 0.1–1 μg DNA) amplified by 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 Vpalk1AI (Thermo, Massachusetts, USA) and 1 μl attached 10 × Vpalk1AI Basal Buffer. Digested products were separated through 3% agarose gels and stained with ethidium bromide.

2.6. Imprinting analysis

RT-PCR-RFLP method was used to detect the imprinting status of porcine Rasgrf1 gene. We chose polymorphic locus g.84709G→A and restriction enzyme Vpalk1AI for imprinting analysis. The first step was screening for heterozygous piglets on polymorphic locus g.84709G→A. PCR was carried out on neonatal piglet's and parental DNA using primer pair exon 14F/exon 14R, RT-PCR was performed on heterozygous piglet's RNA using primer pair exon 14F/exon 14R′, which were followed by Vpalk1AI RFLP. By comparing the digestion patterns of piglet's DNA/RNA and parental DNA, we could define the imprinting status of heterozygous piglets.

2.7. Quantitative real-time PCR analysis of Rasgrf1 gene expression pattern

The expression level of Rasgrf1 were detected by Go Taq® qPCR assay using Rotor-gene 6000 real-time PCR thermal cycle instrument (Corbett, Australia). PCRs were performed using gene specific primers pairs (Rasgrf1F/Rasgrf1R, β-actinF/β-actinR, Table 1). The reactions contained 1 × Go Taq® qPCR Master Mix (Promega, USA), 1 μl diluted cDNA template and each primer at 200 nM in a 25 μl reaction volume. The specificity of PCR products was confirmed by melting curve analysis. Gene expression levels were quantified relatively to the expression of the β-actin using Rotor-Gene Q Series Software (Corbett, Australia) by employing an optimized comparative Ct (ΔΔCt) value method. The expression level was calculated as 2−ΔΔCt to compare the relative expression. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. The differences in extent of gene expression at the same tissue between the two reciprocal F1 hybrids of Berkshire and Wannan black were compared by SPSS19.0 statistics software (SPSS Inc., Chicago, USA) using the Student’s t-test, the differences in extent of gene expression at tissues tested within each one of the two reciprocal F1 hybrids were compared by SPSS19.0 statistics software.
using one-way ANOVA and LSD method test. A p value < 0.05 was considered significant.

3. Results

3.1. Sequence analysis of domestic pigs Rasgrf1 gene

In the present study, we obtained 228 bp and 193 bp partial sequences of the domestic pig Rasgrf1 gene. The two partial sequences were located in exon 14 and exon 1 of Rasgrf1, respectively. Sequence analysis showed that the exon 14 of domestic pigs Rasgrf1 gene was high in GC content (59.29%), the 228 bp sequence shared 93.4% and 83.9% nucleotide similarities with the human and mouse orthologs; the exon 1 of domestic pigs Rasgrf1 gene was high in GC content (67.60%), the 193 bp sequence shared 91.2% and 79.3% nucleotide similarities with the human and mouse orthologs.

3.2. SNP detection and screening for heterozygous piglets

Comparison of the sequences from Berkshire and Wannan black breeds revealed one SNP in exon 14 of the porcine Rasgrf1 gene: the G-to-A SNP in exon 14 (NCBI Gene ID: 100157221 g.84709G>A). The SNP (G/A(4709)) in the coding region was a synonymous mutation and could be detected with the restriction enzyme Vpak11AI. 16 piglets of the two reciprocal Berkshire × Wannan black hybrids were used to identify heterozygous individuals at the SNP site. Amplification of genomic DNA with the primer pair exon14F/exon14R was conducted. PCR products were digested by Vpak11AI, allele g.84709G was 116, 75 and 38 bp, and allele g.84709A was 191 and 38 bp (Fig. 1). The results of this PCR-RFLP test indicated that three Berkshire boar × Wannan black sow F1 hybrid piglets were heterozygous at the site.

3.3. Allelic expression of the porcine Rasgrf1 gene

Vpak11AI-RFLP analysis of the RT-PCR products showed that the 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, backfat, tests, ovary, longissimus dorsi and pituitary tissues. The g.84709G allele was monoallelically expressed in the lung tissue, the g.84709A allele was monoallelically expressed in the liver and small intestine tissues of three heterozygous Berkshire boar × Wannan black sow F1 hybrid piglets, and both alleles originated from their Berkshire fathers and Wannan black mothers respectively (Fig. 2a). The g.84709A allele was monoallelically expressed in the liver tissue, the g.84709G allele was monoallelically expressed in the liver and small intestine tissues of three heterozygous Wannan black boar × Berkshire sow F1 hybrid piglets, and the both alleles originated from their Wannan black fathers and Berkshire mothers respectively (Fig. 2b).

3.4. Tissue expression patterns of porcine Rasgrf1 gene

Real-time RT-PCR was performed and revealed the mRNA expression of porcine Rasgrf1 gene in 14 different tissues. Preliminary indications could be obtained about the differences of Rasgrf1 gene expression level among tissues. As shown in Fig. 3, the porcine Rasgrf1 gene in 8 piglets of a Berkshire boar × Wannan black sow mating and in 8 piglets of a Wannan black boar × Berkshire sow mating had a similar tissue expression pattern, displayed a significantly high expression in brain, pituitary, pancreas, followed by kidney, stomach, lung, testis, small intestine, ovary, spleen, liver, and at low levels of expression in longissimus dorsi, heart, and backfat. Interestingly, we also observed that the expressions of Rasgrf1 in Berkshire boar × Wannan black sow F1 hybrid piglets were greater than that in Wannan black boar × Berkshire sow F1 hybrid piglets at the brain, pituitary and pancreas tissues.

4. Discussion

It has been reported that the rat Rasgrf1 gene, located on chromosome 8, shows paternal-specific monoallelic expression in neonatal brain (Pearsall et al., 1999). While, mouse Rasgrf1, located on chromosome 9, is imprinted and exclusively expressed from the paternal allele in the neonatal brain, heart, stomach and liver (Dockery et al., 2009; Plass et al., 1996). This imprinting in special tissues in mouse is relaxed during development, the transition from strongly and predominantly paternal allele expression to biallelic expression can be seen over time, and expression becomes biallelic around the time of weaning (Nadia et al., 2009). Interestingly, compared to the imprinting expression of Rasgrf1 gene in mouse and rat, we observed an imprinting variance of Rasgrf1 gene in 1-day-old domestic pigs. In our study, porcine Rasgrf1 was shown to have paternal-specific expression in the lung, maternal-specific expression in the liver and small intestine, whereas other tissues (e.g., testis, ovary) showed biallelic expression.

Imprinting mechanisms of Rasgrf1 gene has been well elucidated in mouse. The mouse Rasgrf1 gene was found associated with a region that is a differentially methylated domain (DMD) and shows paternal-specific methylation, is conserved in some rodent species and therefore plays a role as an important cis-acting imprinting control region (Holmes et al., 2006; Pearsall et al., 1999; Yoon et al., 2005). This DMD located 30 kbp upstream of the Rasgrf1 promoter, is responsible for controlling the imprint status of the gene in mice. Immediately 3’ of the DMD is a repeat unit consisting of 40 copies of a 41-bp tandem repeat element which is responsible for establishing methylation of the DMD (Holmes et al., 2006). This DMD serves as a CCCTC-binding factor (CTCF) binding site on the unmethylated maternal allele. The unmethylated maternal allele binds to CTCF resulting in blocked RasGrf1 expression. In contrast, CTCF binding is prevented by DMD-mediated methylation of the paternal allele, thus allowing expression of RasGrf1 (Yoon et al., 2005).

But in swine, it is the first time to investigate the imprinting status of the Rasgrf1 gene. By first obtaining the partial exon 14 sequence, we found that, typical to imprinting genes (Amarger et al., 2002; Zhang et al., 2004), the porcine Rasgrf1 gene was highly GC rich in the exon 14 region. Furthermore, the identification of an expressed SNP (g.84709G>A) in Rasgrf1 gene also allowed us to study its imprinting status by following the expression of the parental alleles in heterozygous pigs. We found that the Rasgrf1 gene was maternally expressed in the liver and small intestine, paternally expressed in the lung, but biallelically expressed in brain, heart, spleen, kidney, stomach, pancreas, backfat, testis, ovary, longissimus dorsi and pituitary. These observations are not consistent with those imprinting reports about mouse and rat Rasgrf1 gene (Dockery et al., 2009; Nadia et al., 2009; Pearsall et al., 1999; Plass et al., 1996). The differential imprinting status of the Rasgrf1 gene among rat, mouse and swine may be caused by the different species or the different tissues examined.
Studies of the murine Rasgrf1 ortholog showed that its expression was high in the central nervous system, low levels of expression had been identified in other tissues (Fernandez-Medarde et al., 2009; Plass et al., 1996; Punita et al., 2012). Studies of human Rasgrf1 ortholog showed that full length p140 Rasgrf1 protein was heavily expressed in the human brain but significant levels of expression were also detected in other tissues such as the lung and pancreas (Font de Mora et al., 2003; Guerrero et al., 1996; Tung et al., 1997). The tissue expression patterns of genes can somehow imply their corresponding functions. As its expression pattern suggested and as predicted by the conflict hypothesis, mouse Rasgrf1 played a role in learning and memory (Giese et al., 2001). Analysis of various knockout mouse strains had uncovered a specific functional contribution of RasGrf1 in processes of photoreception, pancreatic β-cell function and glucose homeostasis (Fernandez-Medarde and Santos, 2011). Interestingly, the differential Rasgrf1 gene expression levels in the mice models resulted in concordant differential expression of IGF1 (the insulin-like growth factor 1 gene) and molecular components of the GH/IGF1 (growth hormone/insulin-like growth factor 1) axis, which are essential for normal growth and development (Magee et al., 2010). In our study, we found that porcine Rasgrf1 was highly expressed in brain, pituitary and pancreas tissues, followed by kidney, stomach, lung, testis, small intestine, ovary, spleen and liver, and at low levels of expression in longissimus dorsi, heart, and backfat. The high expression of Rasgrf1 in brain, pituitary and pancreas tissues were in agreement with previous researches. We also observed that the expression levels of Rasgrf1 gene in brain, pituitary and pancreas tissues were significantly different between the two reciprocal F1 hybrids of Berkshire and Wannan black. One possible explanation is that traits such as production and growing performance are differentially selected in different pig breeds. Because Rasgrf1 is a growth-regulating gene, the difference in Rasgrf1 expression levels in the two reciprocal F1 hybrids is one of the underlying mechanisms of such growth properties. The data presented in this study suggest that the porcine Rasgrf1 ortholog may similarly play an important physiological role in the growth, development, maintenance and performance of pigs, possibly through involvement with the GH/IGF1 axis.

One of the limitations of this study is that no imprinting data at other porcine developmental stages, especially around weaning, was available to confirm the imprint status of Rasgrf1. To identify the conservation of genomic imprinting at different developmental phases and in different species, the imprint status of Rasgrf1 around the time of weaning in pigs needs to be investigated in future studies. Another limitation of this study is that no data about imprinting mechanisms of Rasgrf1 gene was available. Compared to the expressed Rasgrf1 gene in mouse, imprinting variation of Rasgrf1 in swine is

---

**Fig. 2.** RT-PCR-RFLP/PCR-RFLP image of the allele-specific expression of domestic pigs Rasgrf1 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 respectively (lanes 16, 17, and 18). Digestions with Vpak11Al revealed that the RasGrf1 gene is biallelically expressed in most of the tissues tested. The Rasgrf1 gene of Berkshire boar × Wannan black sow (a) and Wannan black boar × 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 is marker GM303 (the bands are 500, 400, 300, 200, 100 and 75 bp from top to bottom respectively).

**Fig. 3.** The tissue distribution of Rasgrf1 mRNA assessed by quantitative real-time PCR. The values shown in this figure are the averages of three independent experiments. Data are expressed as mean ± SEM (n = 8). Asterisks indicate significant differences in extent of gene expression at the same tissue between the two reciprocal F1 hybrids of Berkshire and Wannan black (P < 0.05). Different letters indicate significant differences in extent of gene expression at tissues tested within each one of the two reciprocal F1 hybrids (P < 0.05).
reminiscent of a series of questions about whether the porcine Rasgrf1 gene is regulated by the same imprinting mechanism as the mouse Rasgrf1 locus, whether the domestic porcine Rasgrf1 gene contains the repeat sequences and DMDs, whether these DMDs are paternally or maternally methylated. The imprinting mechanism work was not within the scope of the current study but could be pursued in the future. Moreover, the functional significance of its imprinting in swine, if any, is still unknown. Further studies will refine this knowledge and, we predict, will shed light on imprinting biological functions, mechanisms and evolution of the porcine Rasgrf1 gene.

In conclusion, this is the first study on the imprinting status of Rasgrf1 gene in domestic pigs other than mouse and rat. Our results showed that there is a general variation of imprinting for Rasgrf1 gene in species that have been studied so far, suggesting that the Rasgrf1 gene shows both species- and tissue-specific variation in imprint expression. Differences in tissue expression patterns of Rasgrf1 gene observed in the two reciprocal F1 hybrids of Berkshire and Wannan black provide a basis for future studies of the porcine Rasgrf1 gene function.

Conflicts of interest

The authors of this paper have no conflicts of interest. We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled "Expression and genomic imprinting of the porcine Rasgrf1 gene".

Acknowledgments

This research was jointly supported by grants from the 2012 Expert Yard on Agricultural Science-Technology of Anhui Province (Grant No. 20), the Transformation of Agriculture Scientific and Technological Achievements Foundation of China (Grant No. 2011GR2C300017), the National Natural Science Foundation of China (Grant Nos. 31171200; 30771581) and the Natural Science Foundation of Anhui Province (Grant No. 1208085MC44).

References