Research Article

Association of a PIT1 gene polymorphism with growth hormone mRNA levels in pig pituitary glands

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Abstract

Fourty-six non-castrated, halothane-free, male Landrace pigs were genotyped by PCR-RFLP for the Rsa I polymorphism in the PIT1 gene and classified into AA and AB genotypes. Total RNA was extracted from the pituitaries and the relative quantities of growth hormone (GH) mRNA were determined by semi-quantitative RT-PCR. Pigs with the AB genotype had higher levels of GH mRNA than those with the AA genotype (p = 0.034; Kruskal-Wallis test). This result suggests that the Rsa I polymorphism may be involved in Pit-1 protein expression or function, which in turn may influence GH transcription and expression. Thus, the Rsa I PIT1 gene polymorphism in this pig line may be used as a molecular marker to identify higher GH expression and possibly select for carcass and performance traits affected by GH.

Key words: PIT1 gene, growth hormone mRNA, semi-quantitative RT-PCR, pig.

Introduction

Pit-1 is a pituitary transcription factor for GH, prolactin and TSHβ genes (Yu *et al.*, 1995; Cogan and Phillips, 1998). Yu *et al.* (1995) studied the association between PIT1 polymorphisms and carcass and performance traits in pigs and concluded that this gene may be a candidate for a quantitative trait locus (QTL) in these animals. Supporting evidence for this has been provided by this same group, who identified a chromosomal region close to PIT1 with evidence of QTL (Yu *et al.*, 1999). Stancekova *et al.* (1999) and Brunsch *et al.* (2002) also studied associations of PIT1 polymorphisms with carcass and performance traits, and also suggested that PIT1 might be a candidate gene for a QTL in pigs.

According to Parks *et al.* (1993), the Pit-1 protein is located in somatotroph, lactotroph and tireotroph nuclei, and alterations in GH secretion in humans may be caused by PIT1 gene mutations. Farmer *et al.* (1993) reported that pituitary GH secretion undergoes dramatic changes with age. In agreement with this, GH mRNA levels decrease with age, and this may be associated with the onset of puberty (Te Pas *et al.*, 2001).

Matteri and Carroll (1997) studied male piglets (1, 7, 14, 21, 28, 35 and 42 days old) and found that the levels of PIT1 and GH mRNAs were significantly correlated, and that the quantity of GHRH receptor mRNA correlated with GH mRNA levels. Sun *et al.* (2002) obtained similar results for the age-dependent expression of PIT1. However, the polymorphisms detected in the PIT1 gene have not yet been shown to affect Pit-1 function or activity in pigs (Tuggle and Trenkle, 1996).

Several techniques for quantifying gene expression have been used, particularly dot blot and northern blot analyses (Taniyama *et al.*, 1999), and semi-quantitative RT-PCR (Uchide *et al.*, 1999).

The purpose of this study was to investigate the association between the Rsa I polymorphism of the PIT1 gene and the relative levels of GH mRNA in pig pituitaries.

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Material and Methods

Biological material

To determine the levels of GH gene expression, 46 pituitaries were collected from Landrace pigs at Granja Rezende S/A, Brazil. The pigs were classified into AA and AB genotypes (n = 23 each), based on the Rsa I polymorphism of the PIT1 gene. The frequency of the BB genotype in this population was < 1%, so this genotype was not included in the study on GH gene expression.

All of the pigs were non-castrated males and were halothane-free, as confirmed by PCR-RFLP of the HAL gene (data not shown).

The animals were slaughtered during the months of May through July in accordance with Brazilian legislation, during which time the seasonal temperature was relatively constant (average of 22 °C). The pigs were fasted for 24 h before slaughtering. One hour after slaughtering, the pituitaries were removed and immediately frozen in liquid nitrogen for transportation to the Molecular Genetics Laboratory of the Universidade Federal de Uberlândia (UFU), where they were stored at -80 °C until RNA extraction.

To search for an alternative splicing site, pituitaries of each of the three genotypes were used (n = 2 each).

DNA extraction

For Rsa I polymorphism genotyping by PCR-RFLP, genomic DNA was obtained from blood samples by adding 1 mL of lysis buffer 20 mM Tris-HCl, pH 7.5, 5 mM ethylenediaminetetracetic acid (EDTA), 640 mM sucrose, 10 mM $MgCl_2$, 4% Triton X-100 to 500 µL of white blood cells kept on ice for 10 min. The lysed cells were precipitated (white pellet) by centrifugation for 1 min at 7200 g and 4 °C, followed by two or more washes with lysis buffer diluted 1:1 in water. The resulting pellet was digested with 10 µL of proteinase K (10 mg/mL) and 200 µL of proteinase K buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1% sarkosyl) by incubation overnight at 50 °C. After this, 500 µL of 8 M guanidine isothiocyanate/0.49 M ammonium acetate was added to the digested material, followed by shaking for 1 h at room temperature to dissolve the pellet. DNA was precipitated with 800 µL of 100% isopropanol by centrifugation at 7200 g for 10 min, and the supernatant was discarded. The pellet was washed once with 60% isopropanol and centrifuged at 7200 g for 2 min. The DNA was dried and re-dissolved in 0.2 mL of TE (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA), followed by incubation at 65 °C for 1 h to ensure complete suspension.

Genotyping at the PIT1 locus

Genotyping at the PIT1 gene was done using a pair of primers designed by Yu *et al.* (1994), with the following sequences: 5' AGTGTAGCCAGAGCATCT 3' (forward) and 5' ACCACATCTGCACACTCA 3' (reverse), which amplifies an amplicon of 1745 bp, corresponding to the region from intron 4 to the 3' untranslated region (3' UTR). The PCR reactions were carried out in a PTC-100 MJ Research thermocycler. After preheating at 95 °C for 4 min, amplification was done using 30 cycles at 95 °C for 1 min, 61 °C for 1 min, and 72 °C for 3 min. The final incubation was done at 72 °C for 5 min. For the PCR assays, 2.5 U of Taq DNA polymerase (Cenbiot Enzimas, Biotechnology Center - UFRGS, Porto Alegre, Brazil), 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM each of both primers, and 200 ng of genomic DNA in a final volume of 25 µL were used. After amplification, 15 µL of the PCR amplicon were digested with 12 U of Rsa I restriction enzyme for 8 h at 37 °C; genotyping was performed in 2% agarose gels stained with ethidium bromide (10 mg/mL), and photographed under UV illumination.

RNA extraction

Total RNA was extracted from pituitaries using Trizol reagent (Gibco BRL) according to the manufacturer's protocol, with a Trizol/sample weight ratio of 1.5 mL of Trizol per pituitary. All samples were quantified by spectrophotometry and diluted to 50 ng/µL in diethylpyrocarbonate (DEPC)-treated water.

Semi-quantitative RT-PCR

Total RNA from each pig was analyzed by semi-quantitative RT-PCR, using Ready-To-Go™ RT-PCR beads (Amersham Biosciences) for the GH and β-actin genes. The GH gene primer pair was designed at the UFU Molecular Genetics Laboratory, using the Primer Designer program - version 2.0. The primers were based on the GH gene sequence (Vize and Wells, 1987). The β-actin gene primer pair was used to normalize the samples (Matteri and Caroll, 1997). The primers sequences were: GH - 5' CGATCGCAGAGGTACTCCATCCAGAA 3' (forward) and 5' CGATCGTATGTCTCAGCCTTGTGCAG 3' (reverse), which amplified a 418 bp product, and β-actin - 5' GCTGTATTCCCCTCCATCGT 3' (forward) and 5' ACGGTTGGCCTTAGGGTTCA 3' (reverse), which amplified a 264 bp product.

Reverse transcription (RT) and PCR amplification reactions were subsequently performed, using the following conditions: 42 °C for 30 min, 95 °C for 5 min, 25 cycles at 95 °C for 30 s, 58°C for 40 s, and 72°C for 1 min. The final incubation was done at 72°C for 10 min. Each RT-PCR mixture included 25 ng of total RNA and 0.08 µM of each primer in a final volume of 50 µL. Both genes were amplified simultaneously in the same tube.

The exponential phase of the RT-PCR was determined by using the same reaction conditions as described above for 20, 22, 25, 28, 30, and 35 cycles (data not shown). After amplification, 10 µL of RT-PCR products were run in

2% agarose gels, stained with ethidium bromide (10 mg/mL), and photographed under UV illumination.

Densitometric readings

All samples were run in the same agarose gel, and the levels of expression were quantified by densitometry using ImageMaster[TM] VDS Software version 2.0 (Amersham Biosciences). The relative quantities of GH mRNA were determined for each pig by the IOD_{GH}/IOD_{β}-actin ratio, where IOD is the integrated optical density.

Statistical analysis

The differences in age and weight between AA and AB pigs was analyzed by using variance analyses, and the association between the PIT1 genotypes and the relative quantities of GH mRNA was analyzed by using the Kruskal-Wallis test, run in the Jandel SigmaStat program, version 2.0, 1995. The results are expressed as means ± S.E.M. p values < 0.05 indicated significance.

Search for an alternative splicing site

To search for an alternative splicing site within the region from exon 4 to 3' UTR of the PIT1 cDNA, RT-PCR was carried out with 200 ng of total RNA from each of the three Rsa I PIT1 genotypes (n = 2 each), using Ready-to-Go[TM] RT-PCR beads (Amersham Biosciences).

The primers used were 5' ACCTACAGCTCAGC TTCA 3' (forward) and 5' ACCACATCTGCACACTCA 3' (reverse). The forward primer was designed at the UFU Molecular Genetics Laboratory. The reverse primer was described by Yu *et al*. (1994). A concentration of 0.2 µM of each primer was used in a final volume of 50 µL. The RT and PCR amplification reactions were performed using the following conditions: 42 °C for 30 min, 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min. The final incubation was done at 72 °C for 10 min.

After amplification, 15 µL of the RT-PCR amplicons were run on 1.8% agarose gels, then stained with ethidium bromide (10 mg/mL), and photographed under UV illumination.

The smallest RT-PCR amplicon was cut from the agarose gel, macerated, filtered through a glass-wool plug by centrifugation at 6000 g, and collected in a 1.5 mL microtube. Purification was done by adding 2 µL of 7.5 M ammonium acetate and 2 µL of glycogen (10 mg/mL) to 8 µL of filtered solution, which was mixed and incubated with an equal volume of 100% ethanol for 3 h at -20 °C. The DNA solution was centrifuged at 19000 g for 5 min, and the pellet was subsequently washed with 70% ethanol. The purified amplicon was cloned by using the TOPO TA cloning kit (Invitrogen Life Technologies), and the selected fragment was sequenced using a dideoxy fluorescence terminator system (ABI 377).

Control reactions

Negative controls for all semi-quantitative RT-PCR were obtained by using total RNA without reverse transcription, in order to check for DNA contamination. The total RNA from all samples used to search for an alternative splicing site was treated with RNase-free DNase I. Negative controls for all RT-PCR assays were run without DNA/RNA template.

Results

Genotyping at the PIT1 locus

The pigs were separated according to their PIT1 genotype, with an equal number (n = 23) per group. Figure 1 shows the genotype patterns. Allele A contained one 710 bp fragment, whereas allele B had two fragments (388 and 322 bp). Monomorphic fragments of 774, 153 and 108 bp were also present.

RNA extraction, semi-quantitative RT-PCR and statistical analysis

RNA extractions were of similar quality (Figure 2). Semi-quantitative RT-PCR produced a 418 bp fragment for the GH and a 264 bp fragment for the β-actin gene. The co-amplified amplicons are shown in Figure 3.

The statistical analysis showed no differences between AA and AB pigs regarding age and weight (p > 0.05); the AA pigs were 141.56 ± 1.01 days old and weighed 92.60 ± 1.36 kg, while the AB pigs were
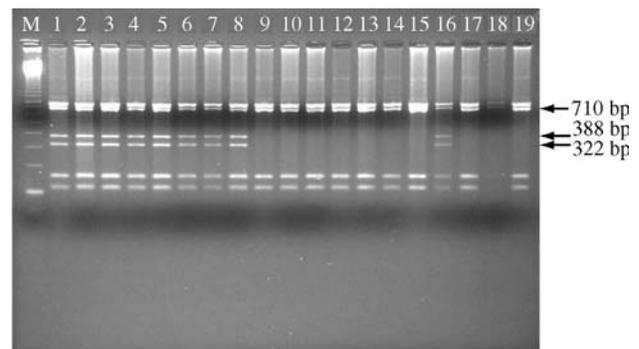

Figure 1 - RsaI polymorphisms in the PIT1 gene detected by PCR-RFLP. The PCR products were run on 2% agarose gels. M - 100 bp ladder of molecular weight markers (Gibco/BRL); lanes 1 - 8 and 16 show the AB genotype; lanes 9 - 15 and 17 - 19 show the AA genotype. Allele A contained a 710 bp fragment whereas allele B had two fragments, 388 and 322 bp, that are indicated by arrows. Monomorphic fragments of 774, 153 and 108 bp were also present.
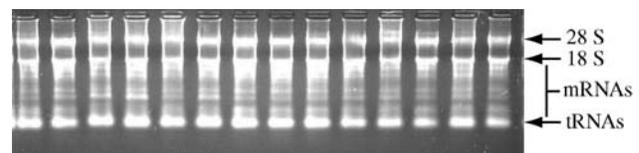

Figure 2 - The quality of the total RNA extracted from the pituitaries of 14 pigs. The samples were run on 1.2% agarose gels.
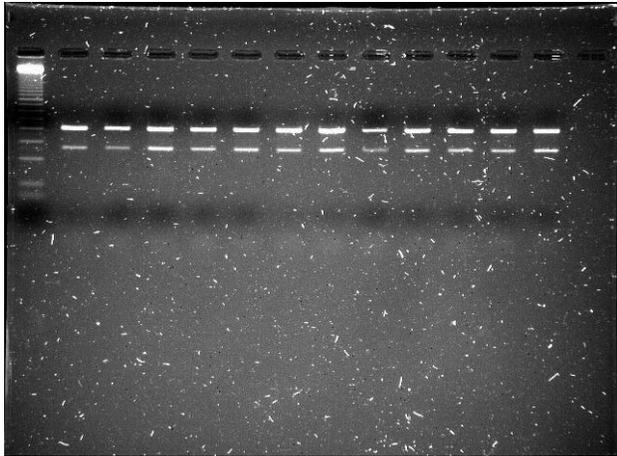
Figure 3 - Semi-quantitative RT-PCR for the GH gene. First lane - 100 bp ladder of molecular weight markers. The 12 lanes represent co-amplified samples of GH cDNA (418 bp) and the β-actin cDNA (264 bp). Lanes 1-6 show the AB genotype; lanes 7-12 show the AA genotype. The samples were run on 2% agarose gels.

142.65 ± 0.93 days old and weighed 89.23 ± 2.00 kg. Besides that, the relative abundance of GH mRNA was higher in AB pigs than in AA pigs: 2.37 ± 0.15 in AA pigs, and 3.52 ± 0.40 in AB pigs (p = 0.034).

Alternative splicing site

The RT-PCR amplifications from exon 4 to 3'-UTR of PIT1 cDNA generated two stronger bands, 650 bp and 496 bp, including the primer sequences (Figure 4). The 650 bp fragment had the expected size based on the PIT1 cDNA sequence, whereas the 496 bp fragment could be an alternative splicing fragment. After sequencing, the smallest fragment was deposited at GenBank (accession number AY309500) and used to search for homology in the GenBank database using BLAST software (www.ncbi. nlm.nih.gov/blast). No homology was found for the PIT1 gene as expected, but it showed high homology with *Homo*
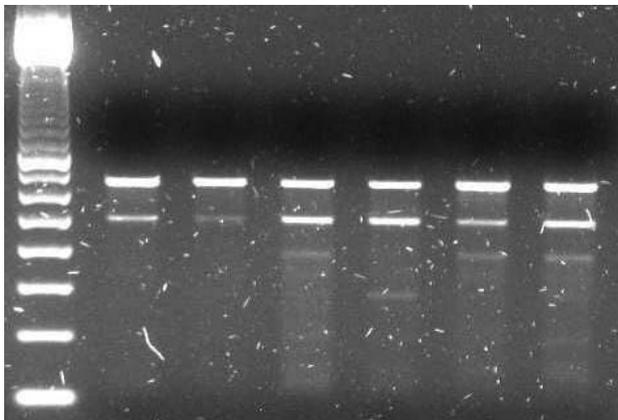
sapiens and *Mus musculus* A kinase (PRKA) anchor protein 13 isoform genes. These primers are specific for the PIT 1 gene sequence of the pig, but they probably bound unspecifically and sufficiently to generate the smallest fragment.

Control reactions

Figure 5 shows the control reactions for DNA contamination in the total RNA samples.

Discussion

For semi-quantitative RT-PCR 25 cycles were used, since at this number the reaction was in its exponential phase, an important and necessary condition for the quantification of gene expression. The two primer pairs were co-amplified in the same reaction tube, thus avoiding different amplification conditions and reducing the possibility of mistakes. An internal control (β-actin) was used to normalize the quality and quantity of the RNA samples, as discussed by Siebert and Larrick (1992). This method for quantifying gene expression is fast and efficient and is an important tool in gene expression studies.

Tatsumi and Amino (1999) showed that the Pit-1 protein was involved in modulating the expression of GH, TSH, prolactin and PIT1 genes. Korytko *et al*. (1996) mentioned that little is known about GHRH receptor regulation, although Pit-1 may modulate its expression.

Te Pas *et al*. (2001) studied the growth rate and the reduction in back fat thickness in Large White pigs, as well as changes in the mRNA levels of GH and PIT1 genes, and concluded that for each generation of selection the GH and PIT1 mRNA levels increased by 50%. Nevertheless, there was considerable variation in the mRNA levels of both genes among animals within each generation.

The availability of a DNA marker related to the variations in GH and PIT1 mRNA levels could be useful for identifying and selecting animals with favorable genotypes.

As shown here, Rsa I genotypes in the PIT1 gene presented an association with GH mRNA levels, suggesting


Figure 4 - PIT1 cDNA amplicons. First lane - 100 bp ladder of molecular weight markers. Lanes 1 and 2 - genotype AB; lanes 3 and 4 - genotype BB; lanes 5 and 6 - genotype AA. The samples were run on 2% agarose gels.
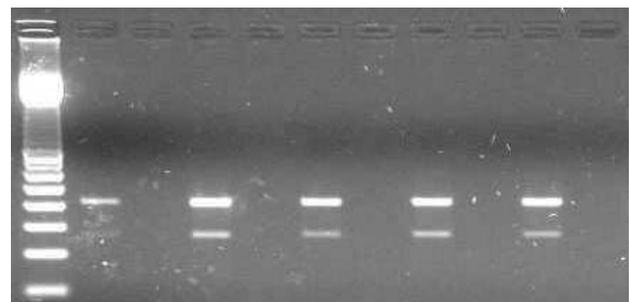
Figure 5 - The control of DNA contamination using total RNA. First lane - 100 bp ladder of molecular weight markers. Lanes 1, 3, 5, 7, and 9 are amplicons of GH cDNA (418 bp) and the β-actin cDNA (264 bp) obtained using untreated total RNA with RNase-free DNase I as template. Lanes 2, 4, 6, 8, and 10 are equal reactions with untreated total RNA without reverse transcription. The samples were run on 2% agarose gels.

that this polymorphism may be a useful molecular marker for production traits that are controlled by GH.

This conclusion is reinforced by the findings of Sun *et al*. (2002), who observed an important association of an Msp I PIT1 polymorphism with the plasma levels of GH and PRL, which suggested an association between this genotype and gene expression in the pituitary. Together, these results show the important physiological effect of the PIT1 gene on other genes in this gland.

Yu *et al*. (1999) detected a strong association between PIT1 polymorphism and microsatellites that flank this gene and the phenotypic traits of young animals. The expression of PIT1 is age-dependent, with higher expression in young animals. Matteri and Carroll (1997) also observed age-dependent changes in the expression of various other genes.

As shown here, an association between PIT1 gene polymorphism and GH mRNA expression was detected close to puberty. Although the importance of PIT1 in early life has been shown previously, we also found an association between this gene polymorphism and the level of GH gene expression in adult animals.

The genes involved in the pathway of GH action have great potential as molecular markers, with a direct application for animal production, because of their physiological effects on bone and muscle growth, fat metabolism and other important effects on animal development. Most of the studies in this area have used humans, mice and rats as models, with few studies of economically important animals. Studies of candidate genes based on direct physiological effects are simple and straightforward, as compared to the use of anonymous DNA markers, which requires long, labor-intensive protocols.

The results shown here suggest that the Rsa I polymorphism could be related with levels of PIT1 gene expression. This polymorphism could be involved in the production of alternative transcripts, thereby altering the protein and its function. Since the Pit-1 protein is involved in GH expression, a difference in GH mRNA levels would be expected with different PIT1 genotypes.

The presence of two amplicons in the RT-PCR amplification of the PIT1 region from exon 4 to the end of the cDNA, containing the Rsa I mutation in intron 5, raised the possibility of alternative gene splicing. The 650 bp fragment (Figure 4) had the expected size based on the known gene sequence, whereas sequencing of the smallest 496 bp fragment (accession number AY309500) showed that it was not an alternative transcript, since BLAST analysis indicated high homology with *Homo sapiens* and *Mus musculus* A kinase (PRKA) anchor protein 13 isoform genes. It is the first time that this sequence is described in pigs.

The possible interference of this mutation with other alternative splicings, already described (Tuggle and Trenkle, 1996), remains to be determined. Furthermore, the quantification of PIT1 mRNA may also associate this mutation with PIT1 self-expression.

The Rsa I PIT1 polymorphism may be related to GH mRNA levels through self-regulation of the PIT1 gene and/or interference with the production of alternative gene transcripts. Either of these possibilities would result in altered PIT1 gene expression and consequently in GH gene expression. Additional studies to analyze the PIT1 gene expression will be important for a better understanding.

We can conclude that this PIT1 polymorphism may be a useful molecular marker for selecting higher GH mRNA levels as well as phenotypic traits related to GH action in this population.

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