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Research paper

Effect of a single nucleotide polymorphism in the growth hormone secretagogue receptor (*GHSR*) gene on growth rate in pigs

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ABSTRACT

The growth hormone secretagogue receptor (*GHSR*) gene controls growth hormone (GH) release by inducing a strong stimulatory effect on the endogenous ligand, ghrelin. In this study, we examined the possible role of *GHSR* in the growth traits of four pig breeds, namely Tibetan pigs (n = 45), Diannan small-eared pigs (n = 40), Yorkshire pigs (n = 45), and New Huai pigs (n = 122). Single nucleotide polymorphisms (SNPs) in these pigs were identified by polymerase chain reaction (PCR) sequencing and genotyping was performed using PCR-restriction fragment length polymorphisms (PCR-RFLPs). A SNP (C/A) named C-1595A (the "C" allele), which is located 1595 bp upstream of the initiation codon of the *GHSR* gene, was found at a higher frequency in the fast-growing Yorkshire pigs than in the slow-growing Tibetan and Diannan small-eared pigs. In preliminary assays, the C-1595A genotype was found to be associated with growth traits in New Huai pigs. Quantitative real-time PCR and western blotting assays were used to measure the levels of GHSR1a, a functionally active form of the GHSR protein, in the tissues of the growth axis. The estimated levels of mRNA and protein in pituitary and liver tissues were significantly higher in Yorkshire pigs than in Diannan small-eared or Tibetan pigs ($P < 0.05$). The results indicated that *GHSR* had a positive influence on the growth rate of pigs and suggested that the C-1595A SNP could be of value as a molecular marker for improving the production performance of pig breeds.

1. Introduction

The growth hormone secretagogue receptor (*GHSR*) gene, which was first isolated from rats and humans (Kojima et al., 1999), comprises two exons and one intron in mammals and chicken. The pig *GHSR* is located on chromosome 13 (range 110,981,465–111,006,149 on Reference Sscrofa11.1). Alternative splicing of the transcribed *GHSR* RNA generates two protein isoforms, GHSR1a and GHSR1b. The functionally active form of GHSR, called GHSR1a, is a seven-transmembrane domain receptor that is expressed in multiple tissues, including the pituitary gland, kidney, thymus, pancreas, myocardium, and adipose (Abizaid et al., 2006; Dixit et al., 2007; Venables et al., 2011). In addition, the expression of ghrelin and GHSR1a has been detected in reproductive tissues, including the uterus (Unsal and Sönmez, 2013) and ovary (Gupta et al., 2015). The GHSR protein binds to ghrelin to play a critical role in the central and peripheral regulation of growth hormone secretagogues, appetite stimulation, food intake, energy homeostasis, lipogenesis, suppression of brown fat thermogenesis, and improvement of cardiovascular functions (Schellekens et al., 2010; Yin et al., 2014; Müller et al., 2015; Yuan et al., 2016).

Naturally occurring mutations of *GHSR* are associated with growth disorders, metabolic syndromes, and obesity in humans (Baessler et al., 2005; Liu et al., 2007; Mager et al., 2008; Inoue et al., 2011). *GHSR* has been identified as a potential candidate gene in quantitative trait locus and half-sib regression analyses of growth traits in Japanese black cattle (Malau-Aduli et al., 2005). In chicken, four single nucleotide polymorphisms (SNPs) in *GHSR* are associated with growth and carcass traits (Fang et al., 2010); other SNPs are significantly associated with fat deposition and muscle fiber traits (Lei et al., 2007). An association between a ghrelin gene polymorphism and chicken growth has also been established (Li et al., 2006; Fang et al., 2007). In cattle, two *GHSR* gene mutations have been shown to have a significant effect on body weight in Nanyang cattle (Zhang et al., 2009).

In light of the information available from various species, we

Abbreviations: DNA, deoxyribonucleic acid; DSP, Diannan small-eared pig; GHSR, growth hormone secretagogue receptor; *GHSR1a*, growth hormone secretagogue receptor 1a; LD, *longissimus dorsi* muscle; mRNA, messenger RNA; NHP, New Huai line of pig; PBST, phosphate buffer saline with 0.1% Tween 20; PCR, polymerase chain reaction; PCR-RFLP, PCR-restriction fragment length polymorphism; RNA, ribonucleic acid; SNPs, single nucleotide polymorphisms; TP, Tibetan pigs; YY, Yorkshire pigs

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suggest that *GHSR* might be a candidate gene for growth traits in pigs. Therefore, the aim of this study was to investigate differences in *GHSR1a* levels among different pig strains and to identify SNPs that could be used to analyze the association of *GHSR* with growth traits in pigs. The identification of such SNPs could provide molecular markers for use in marker-assisted selection of pigs.

2. Materials and methods

2.1. Experimental materials

For DNA extraction, ear tissue samples were collected from Tibetan pigs (TP, $n = 45$) in Linzhi of Tibet, Diannan small-eared pigs (DSP, $n = 40$) in Xishuang Banna of Yunnan (China), Yorkshire pigs (YY, $n = 45$) and New Huai pigs (NHP, $n = 122$) in Hefei of Anhui (China). RNA and protein analyses were performed on liver and pituitary gland tissues, and on *longissimus dorsi* (LD) muscle of DSP, TP, and YY individuals that were raised under the same conditions in Beijing. Ten individuals of each strain were humanely slaughtered at 6 months of age and the collected tissues were immediately frozen in liquid nitrogen and stored at − 80 °C.

All procedures were carried out in strict accordance with the protocols approved by the Animal Welfare Committee of China Agricultural University (Permit Number: XK622).

2.2. DNA, RNA, and protein extraction and cDNA preparation

Genomic DNAs were isolated from ear tissue samples using the method previously described by Sambrook and Fussell (2001). The isolated DNAs were dissolved in Tris-EDTA buffer and stored at − 20 °C.

Total RNAs were extracted from the tissues using TRIzol Reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were checked using a Nanodrop 2000 Biophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and their integrity was verified by electrophoresis. RNA samples (2 μg) were reverse transcribed to cDNA in a 20-μL reaction volume using M-MLV Reverse Transcriptase (Beijing HT-biotech Co., Ltd., Beijing, China).

Total protein was isolated from the liver and LD muscle using the SDS Lysis Buffer (P0013B, Beyotime Ltd., Haimen, China). Protein content was measured using an Enhanced BCA Protein Assay Kit (P0010, Beyotime Ltd.).

2.3. SNP screening and genotyping

Five pairs of primers for pig *GHSR* (NC_010455) were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA); these primers amplify coding regions (exons 1 and 2) and 5′-flanking sequences of the gene (Table 1). Polymerase chain reaction (PCR) products amplified from 10 samples of each group were pooled and sequenced to identify SNPs using the software programs Chromas Pro v.1.33 (Technelysium Pty Ltd., Helensvale, Australia) and DNAMAN 6.0 (Lynnon, Pointe-Claire, QC, Canada). The genotypes of the SNPs found in the pooled sequences were determined using individual PCR and sequencing.

Screening for PCR-restriction fragment length polymorphisms (PCR-RFLPs) was used to identify SNPs. One of the SNPs, named C-1595A (see Section 3.1), was used to screen the *GHSR* gene of different pig strains. The primers for *GHSR*C-1595A were: forward, 5′-AAA CGC ACT AAT AAA TGC TTC A-3′ and reverse, 5′-CTT CCT GCC CTC ACC TTT T-3′. The restriction enzyme was AccII (New England Biolabs, Ipswich, MA, USA), and the amplicon size was 176 bp. Individuals with 26- and 150-bp fragments are described as having a CC genotype; individuals with 150- and 176-bp fragments have an AC genotype; and individuals with a 176-bp fragment have an AA genotype.

2.4. Association of GHSR genotype with growth traits

The body weights of the 122 NHP individuals were monitored to determine the time required to reach 30 and 90 kg in Anhui Kexin Farm. According to the agricultural industry criteria of China (NY/T 822-2004), the number of days to reach a body weight of 30 kg was adjusted using the following equation:

$$\text{Adjusted days} = \text{actual age} + [(30 - \text{actual weight}) \times 1.55.$$

The number of days to reach a body weight of 90 kg was adjusted using the following equation:

$$\text{Adjusted days} = \text{actual age} - [(\text{actual weight} - 90) \times (\text{actual age}/\text{actual weight}) \times 1.82.$$

The association of the *GHSR* C-1595A genotype with growth traits was statistically analyzed using the PROC GLM procedure in SAS 9.1 (SAS Institute, Inc., Cary, NC, USA).

2.5. Semi-quantitative RT-PCR

Primers for the *GHSR1a* mRNA sequence (NM_214180) were designed using Primer Premier 5.0 software: 5′-CAG GGA CCA GAA CCA CAA-3′ and 5′-AAG AGG ACA AAG GAC ACG AG-3′. We selected the *β-actin* gene (DQ845171) as the internal standard (primers: 5′-TCT GGC ACC ACA CCT TCT A-3′ and 5′-AAG GTC TCG AAC ATG ATC TG-3′). A semi-quantitative RT-PCR was carried out using a thermal cycler (Eppendorf, German) in a 20-μL reaction volume containing 150–750 ng cDNA. The PCR cycling protocol included an initial denaturation at 95 °C for 5 min, 28 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 10 min.

2.6. Quantitative PCR

The quantitative gene expression measurements were performed in triplicate in a 20-μL reaction volume containing 1.0 μL cDNA, 0.5 μL of

Table 1
Description of five primer pairs used for SNP identification in the pig *GHSR* gene.

Primer	Primer sequences (5′ to 3′)	Amplicon region	Amplicon size (bp)	Annealing temperature
5′-FR1	F: AGCCGCTTAATACCAGTCTG R: TAGAACCTATCATCCGTCGTG	− 1643/ − 775 bp	869	58 °C
5′-FR2	F: TAACACGACGGATGATAGGT R: TCCACTAGCGAGTCGTTTT	− 778/87 bp	892	55 °C
GHSR-P1	F: CTCCACCCTCTCGGTCCTA R: GGAAGCAGATGGCGAAGTAG	Exon 1	491	59 °C
GHSR-P2	F: TACTTCGCCATCTGCTTCCC R: ACAAAAGACGGACACGCACA	Exon 1	574	61 °C
GHSR-P3	F: CACAAAATCACTCAGAATATGG R: CTGCTGTGCTATGGCTTC	Exon 2	612	58 °C