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Growth hormone and receptor gene mutations in Chinese Banna miniature pig

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The Banna miniature pig (BNMP) is a representative miniature pig breed in China. Even though BNMP dwarfism is obvious, its underlying causative mutations remain unknown. In this study, the BNMP and Large White pig (LWP) serum growth hormone (GH) and insulin-like growth factor (IGF-1) levels were detected by ELISA and compared. BNMP serum IGF-1 levels were significantly lower than LWP levels ($P < 0.05$). The miniature condition may arise from mutations in the GH and GH receptor (GHR) genes. Therefore, GH and GHR cDNA from the BNMP were cloned into a pMD18-T vector by RT-PCR using the total RNA obtained from the BNMP's pituitary and liver tissues. Sequencing results indicated that the open reading frame of the BNMP GH gene is composed of a 26-residue signal peptide and a 191-residue mature peptide. The coding sequence of the BNMP GHR gene contained 639 amino acids, including a signal peptide that is 18 amino acids long. Two amino acid substitutions, A09V and R22Q, were found in the signal peptide of the GH gene. Additionally, the S104P mutation was found in the BNMP's mature GH protein. Four mutations in the cytoplasmic domain of GHR may influence the downstream signal transduction of GHR, which needs further experimental evidence.

Keywords: Banna miniature pig; growth hormone; growth hormone receptor; mutation

1. Introduction

Growth hormone (GH) has been shown to directly or indirectly affect numerous aspects of animal growth, reproduction, and lactation (Enright et al. 1993; Renville et al. 2002; Lucy 2008). GH is released from the adenohypophysis through a series of physiological stimuli such as GH-releasing hormone (GHRH), somatostatin, glucagon, insulin, IGF-1 and IGF-2 (Etherton and Bauman 1998). At the tissue level, the pleiotropic actions of GH result from its binding to specific GH receptors (GHRs) (Wells 1996), which results in transduction of an intracellular signal (Eleswarapu et al. 2008). This process initiates a cascade of intracellular metabolic events and culminates in the production of IGF-1 by the target tissues, notably the liver (Renville et al. 2002). Postnatal somatic growth is primarily regulated by GH, through IGF-1 activity, via stimulation of anabolic processes such as cell division, skeletal growth and protein synthesis.

The Banna miniature pig originated from the Xishuangbanna Prefecture, Yunnan Province, China, which belongs to the stock of Huanan-type pigs in Southern China. The Banna mini-pig inbred line has been maintained by inbreeding for approximately 30 years (Lian et al. 1993). BNMP F_{20} animals have been obtained through a current inbreeding method (inbreeding coefficient, 0.986). The BNMP has served as an ideal experimental model of human disease in the biomedical field due to its anatomical, physiological and metabolic similarities to human beings. Most recent studies on the mechanisms causing dwarfism have focused on the human Laron syndrome and on sex-linked dwarfism in chickens (Laron et al. 1966; Amselem et al. 1989; Burnside et al. 1991, 1992; Kelly et al. 1991; Huang et al. 1993; Laron and Klinger 1994). However, the growth-regulating mechanism of dwarfism in miniature pigs remains unclear. Our objective was to clone the cDNA of GH and GHR from BNMPs to determine whether GH or GHR mutations were apparent in these pigs.

2. Materials and methods

2.1. Animals

To detect the GH and IGF-1 levels in serum, the pool of the blood samples were collected via venipuncture from three male and three female F_{20} BNMPs from the conservation piggery at Yunnan Agricultural University and six male and six female LWPs from the conservation piggery at Jilin University.

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To identify BNMP GH and GHR polymorphisms, the pituitary and liver tissues were excised from two healthy 3-month-old male F_{20} BNMPs. Animal experiments were performed in accordance with the guidelines on animal care and use established by the Jilin University Animal Care and Use Committee.

2.2. Detection of serum GH and IGF-1 levels

Serum GH and IGF-1 levels were assessed in duplicate using an ELISA Kit (USCNLIFE, USA). Data are presented as the means ± SEM. Differences between data sets were evaluated using Student's *t*-test (SPSS16.0) (SPSS Inc., USA). A P value < 0.05 was considered to be a statistically significant difference.

2.3. RNA isolation and first strand cDNA synthesis

Total RNA was extracted from BNMP pituitary and liver samples using the SimplyP Total RNA Extraction Kit (BioFlux) according to the manufacturer's instructions. One microgram of total RNA from each sample was reverse-transcribed using PrimeScript Reverse Transcriptase with 50 μM of oligo dT primers according to the manufacturer's instructions (PrimeScript™ 1st strand cDNA Synthesis Kit, TaKaRa).

2.4. Amplification and cloning of BNMP GH and GHR genes

Primers for the full-length cDNA sequences of BNMP GH and GHR genes were designed and synthesized (Table 1, Sangon Biological Engineering Technology Company, Shanghai China) based on the cDNA sequences of porcine GH and GHR (GenBank accession numbers X53325 and X54429). Twenty-five microliter PCR amplification reactions were composed of standard 1 × PCR buffer, 1.0 U Ex Taq DNA polymerase (TaKaRa), 400 μM of each dNTP, 10 pmol of each primer and 1.5 μL of a cDNA template mixture. The PCR conditions used with the GH gene included a 3 min hot start at 95°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 1 min. For the GHR gene, PCR products were denatured at 95°C for 3 min, followed by 30 cycles of amplification at 94°C for 40 s, 55°C for 30 s, and 72°C for 2 min. Each amplification mixture was incubated for 10 additional minutes at 72°C. The amplified products were gel-purified using a Biospin Gel Extraction Kit (BioFlux, China) and sub-cloned into the pMD18-T vector (TaKaRa). Positive clones were confirmed by PCR and restriction enzyme digestion.

2.5. Sequencing and analysis of BNMP GH and GHR genes

After cloning, the plasmids that contained BNMP GH and GHR genes were sequenced in triplicate (Sangon, China). Sequences were submitted to Genbank and the identification was performed with the aid of www.ncbi.nlm.nih.gov/blast. Sequences were analyzed and compared using biology software (DNAStar, version 7.1 and DNAMAN, version 5.2).

3. Results

3.1. BNMP and LWP serum GH and IGF-1 levels

Serum GH and IGF-1 levels of 3-month-old BNMPs and LWPs were detected by ELISA. No significant differences were detected between BNMP and LWP serum GH levels (1.98 ± 0.05 ng/mL and 2.06 ± 0.03 ng/mL, $P > 0.05$). In contrast, BNMP serum IGF-1 levels were significantly lower than LWP levels (79.67 ± 18.20 ng/mL and 120.36 ± 15.00 ng/mL, $P < 0.05$).

3.2. Cloning and identification of BNMP GH and GHR genes

Agarose gel electrophoresis of BNMP GH and GHR RT-PCR products indicated a 724 bp and a 1937 bp fragment, respectively (Figure 1). The PCR products were gel-purified, cloned into a pMD18-T vector and screened. PCR, restriction enzyme digestion and sequencing confirmed the identity of the positive clones.

3.3. Sequence analysis of BNMP GH and GHR genes

Sequencing results were submitted to Genbank (accession numbers JF276446 and JF276447). The BNMP GH sequence illustrated that the open reading

Table 1. Primer details for PCR amplification of GH and GHR gene in BNMP.

Target genes	Primer	Primer sequence (5′-3′)	Primer location	Product length (bp)
GH	Forward	CGGCTGTGATGGCTGCAGG	2–20	724
	Reverse	CATTGGGGTGGCACTTTCCAG	705–725	
GHR	Forward	TAGAGGTCCTACAGGTATGGAT	3–24	1937
	Reverse	AAGGCTAAGGCATGATTTTGT	1919–1939	

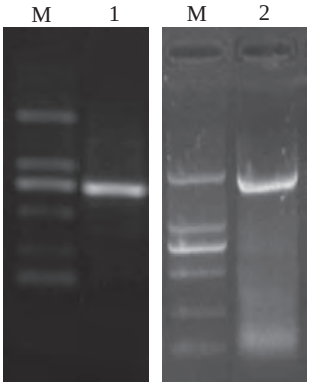

Figure 1. RT-PCR results of GH and GHR gene from BNMP. Lane M: 2000 bp ladder marker. 1: PCR products of GH gene from BNMP (724 bp). 2: PCR products of GHR gene from BNMP (1937 bp).

frame was composed of a 26-residue signal peptide and a 191-residue mature peptide. Compared with normal *Sus scrofa* GH cDNA (GenBank X53325), the BNMP GH sequence contained five mutations at nucleotides 26 (C/T), 65 (G/A), 114 (T/C), 252 (A/G) and 310 (T/C) (Figure 2). The predicted amino acid sequence of the BNMP GH indicated that the mutations located at 114 and 252 encoded synonymous mutations, whereas mutations at 26, 65 and 310 caused non-synonymous changes. The C26T, G65A and T310C mutations resulted in replacement of Ala with Val (A09V), Arg with Gln (R22Q) and Ser with Pro (S104P), respectively (Figure 3).

The coding sequence of the BNMP GHR gene contained 639 amino acids, including a signal peptide of 18 amino acids. Compared with normal *Sus*

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                  *         20         *         40         *         6
P-GH     : ATGGCTGCAGGCCCTCGGACCTCCGCGCTCCTGGCTTTCGCCCTGCTCTGCCTGCCCTG :  59
BNMP-GH  : ........................T..................................... :  59

                 0         *         80         *        100         *        1
P-GH     : GACTCGGGAGGTGGGCGCCTTCCCAGCCATGCCCTTGTCCAGCCTATTTGCCAATGCCG : 118
BNMP-GH  : .....A.....................................................C.... : 118

                20         *        140         *        160         *
P-GH     : TGCTCCGGGCCCAGCACCTGCACCAACTGGCTGCCGACACCTACAAGGAGTTTGAGCGC : 177
BNMP-GH  : ..................................................................... : 177

               180         *        200         *        220         *
P-GH     : GCCTACATCCCGGAGGGACAGAGGTACTCCATCCAGAACGCCCAGGCTGCCTTCTGCTT : 236
BNMP-GH  : ..................................................................... : 236

               240         *        260         *        280         *
P-GH     : CTCGGAGACCATCCCAGCCCCCACGGGCAAGGACGAGGCCCAGCAGAGATCGGACGTGG : 295
BNMP-GH  : ..............G.......................................... ..... : 295

               300         *        320         *        340         *
P-GH     : AGCTGCTGCGCTTCTCGCTGCTGCTCATCCAGTCGTGGCTCGGGCCCGTGCAGTTCCTC : 354
BNMP-GH  : ..............C.......................................... ..... : 354

               360         *        380         *        400         *
P-GH     : AGCAGGGTCTTCACCAACAGCCTGGTGTTTGGCACCTCAGACCGCGTCTACGAGAAGCT : 413
BNMP-GH  : ..................................................................... : 413

               420         *        440         *        460         *
P-GH     : GAAGGACCTGGAGGAGGGCATCCAGGCCCTGATGCGGGAGCTGGAGGATGGCAGCCCCC : 472
BNMP-GH  : ..................................................................... : 472

               480         *        500         *        520         *
P-GH     : GGGCAGGACAGATCCTCAAGCAAACCTACGACAAATTTGACACAAACTTGCGCAGTGAT : 531
BNMP-GH  : ..................................................................... : 531

               540         *        560         *        580         *
P-GH     : GACGCGCTGCTTAAGAACTACGGGCTGCTCTCCTGCTTCAAGAAGGACCTGCACAAGGC : 590
BNMP-GH  : ..................................................................... : 590

               600         *        620         *        640
P-GH     : TGAGACATACCTGCGGGTCATGAAGTGTCGCCGCTTCGTGGAGAGCAGCTGTGCCTTCT : 649
BNMP-GH  : ..................................................................... : 649

                *
P-GH     : AG : 651
BNMP-GH  : .. : 651
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Figure 2. Nucleic acid sequence alignment between BNMP's GH (bottom strand) and normal *Sus scrofa*'s GH (top strand).

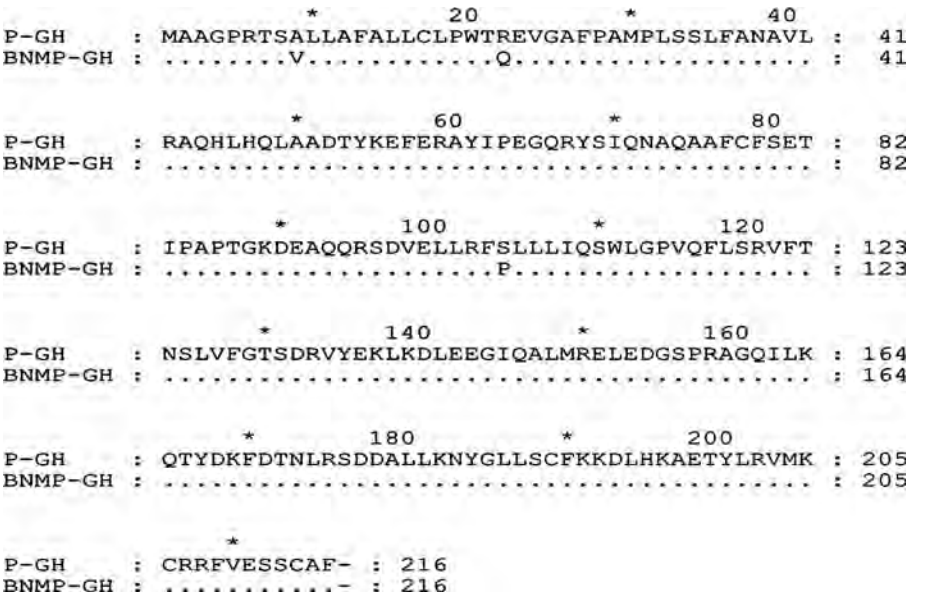

Figure 3. Amino acid sequence alignment between BNMP's GH (bottom strand) and normal *Sus scrofa*'s GH (top strand).

scrofa GHR cDNA (GenBank X54429), the BNMP GHR coding sequence contained nine mutations at nucleotides 564 (A/C), 759 (G/A), 1143 (G/T), 1225 (G/T), 1656 (T/C), 1666 (C/G), 1739 (C/G), 1827 (G/C) and 1914 (T/G). The 1143 (G/T), 1225 (G/T), 1666 (C/G) and 1739 (C/G) mutations resulted in replacement of Glu with Asp (E381D), Ala with Ser (A409S), Leu with Val (L556V) and Ala with Gly (A580G), respectively (Figure 4). All other mutations encoded synonymous mutations.

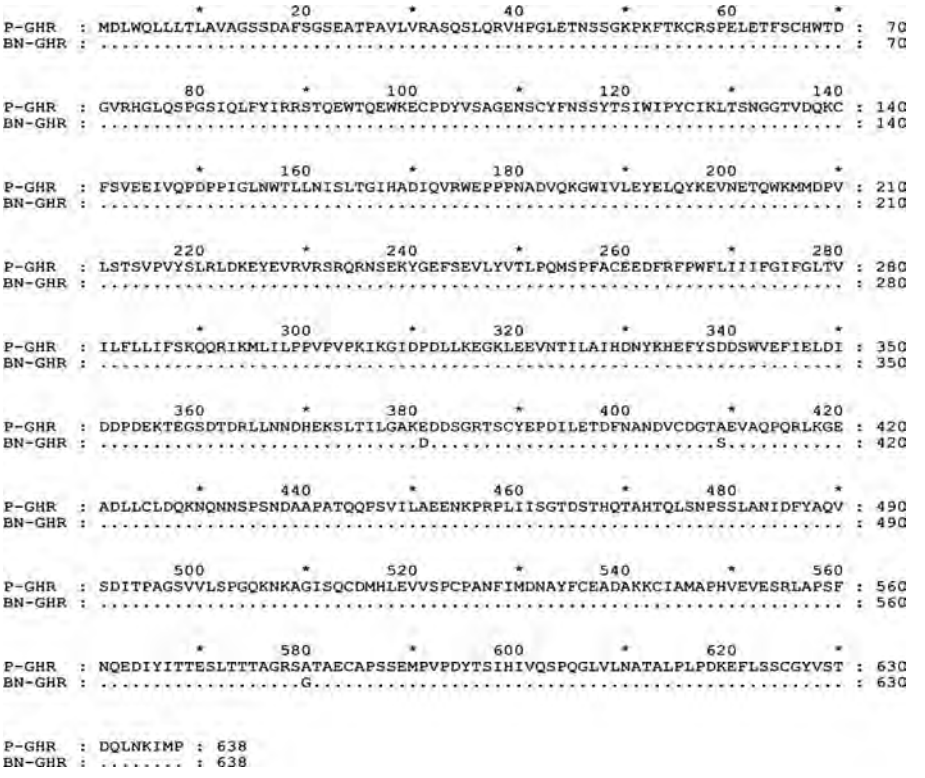

Figure 4. Amino acid sequence alignment between BNMP's GHR (bottom strand) and normal *Sus scrofa*'s GHR (top strand).

Discussion

GH deficiency associated with an abnormal growth hormone receptor is common across species. Deficiency in GHR signaling, due to gene mutations in humans, results in various degrees of dwarfism, with the most extreme cases resulting in Laron-type dwarfism (Laron et al. 1966; Amselem et al. 1989; Kelly et al. 1991; Laron and Klinger 1994). These disorders are controlled by defective alleles at major loci referring to hormones or hormone receptors. Causal point mutations and deletions have been detected in GH or GHR genes in chickens that inherit sex-linked recessive dwarfism (Burnside et al. 1991, 1992; Huang et al. 1993). Brahman miniature cattle have been shown to harbor a single nucleotide polymorphism that encodes an amino acid mutation involved in the binding of GH and GHR (Cormack et al. 2009); moreover, *Bos indicus* cattle have exhibited endocrine characteristics similar to those of human Laron-type dwarfism (Liu et al. 1999).

In this study, the full cDNA sequence of the BNMP GH gene was isolated from the pituitary tissue by RT-PCR. Sequencing results showed that the open reading frame of the BNMP GH gene is composed of a 26-residue signal peptide and a 191-residue mature peptide. Three point mutations, A09V, R22Q and S104P, were detected in the GH coding region. The A09V and R22Q substitutions encode the signal peptide. The BNMP GHR coding sequence is composed of 639 amino acids, including the 18-amino-acid signal peptide in which four point mutations have been detected. The four mutations, E381D, A409S, L556V and A580G, are located in the cytoplasmic domain of GHR.

Four mutations in the cytoplasmic domain of GHR may influence the downstream signal transduction of GHR, which needs further experimental evidence. We are currently further investigating the expression and biological activity of BNMP GH and GHR. The mutations in the BNMP GH and GHR genes may provide the foundation for further investigation of the dwarfism of Banna mini-pigs.

Acknowledgements

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