Single-nucleotide polymorphism analysis of GH, GHR, and IGF-1 genes in minipigs

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Abstract

Tibetan (TB) and Bama (BM) miniature pigs are two popular pig breeds that are used as experimental animals in China due to their small body size. Here, we analyzed single-nucleotide polymorphisms (SNPs) in gene fragments that are closely related to growth traits [growth hormone (GH), growth hormone receptor (GHR), and insulin-like growth factor (IGF-1)] in these pig breeds and a large white (LW) control pig breed. On the basis of the analysis of 100 BMs, 108 TBs, and 50 LWs, the polymorphic distribution levels of GH, GHR, and IGF-1 were significantly different among these three pig breeds. According to correlation analyses between SNPs and five growth traits – body weight (BW), body length (BL), withers height (WH), chest circumference (CC), and abdomen circumference (AC) – three SNP loci in BMs and four SNP loci in TBs significantly affected growth traits. Three SNP sites in BMs and four SNP sites in TBs significantly affected growth traits. SNPs located in the GH gene fragment significantly affected BL and CC at locus 12 and BL at locus 45 in BMs, and also BW, WH, CC, and AC at locus 45 and WH and CC at locus 93 in TBs. One SNP at locus 85 in the BM GHR gene fragment significantly affected all growth traits. All indices were significantly reduced with a mixture of alleles at locus 85. These results provide more information regarding the genetic background of these minipig species and indicate useful selection markers for pig breeding programs.

Key words: minipigs; SNP; GH; GHR; IGF-1

Introduction

Dwarfism is caused by a genetic disease that results in a disproportionately short stature. Although the mechanisms of human Laro-type dwarfism, sex-linked dwarfism in chickens, and other dwarfism conditions have been well studied (1), the underlying cause of dwarfism in miniature pigs remains elusive. Growth hormone (GH), growth hormone receptor (GHR), and insulin-like growth factor-1 (IGF-1) are candidate genes for evaluating pig growth traits (2). GH is a polypeptide produced by the adenohypophysis, and is a main factor that promotes somatic growth in vertebrates (3). GH is also involved in morphology, physiology, metabolism, immunology, reproduction, and behavior (4), and it promotes skeletal system growth and facilitates amino acid incorporation during protein synthesis (5). GHR is a member of the type I cytokine receptor family and is a transmembrane receptor for growth hormone, which is related to drip loss (6), body weight (BW) (7,8), and marbling score (9). GHR is expressed in many tissues and mediates the effect of GH. IGF-1 has complex biological functions and plays important roles in cell differentiation and proliferation, animal growth, and metabolism. IGF-1 function is particularly important for embryonic development (10). The porcine IGF-1 gene is also important for regulating body growth, development, and metabolism. Due to the wide and diverse geographic features of China, many minipig breeds are naturally distributed around the country. Over the last two decades, some of these minipig breeds have been adopted and used as laboratory animals, including Tibetan minipigs (TBs), Guizhou xiang minipigs, Guangxi Bama minipigs (BM), Guizhou xiang minipigs, Guangxi Bama minipigs (BM), Wuzhishan minipigs, and Banna minipigs (11). The Laboratory Animal Center of Southern Medical University (China) first imported TBs from Tibet to Guangzhou for laboratory animal research in 2004. The acclimatization and experimental animalization of these minipigs have been completed (12). TB is a unique breed that lives in high altitude environments (13). These pigs, which grow slowly and have thin skin, high meat cutability, and extra-fine muscle fibers, exhibit strong adaptability and resistance to harsh environments (14).
Minipig breeding has been partially hindered by the paucity of studies regarding functional genes associated with growth and development (15). BMs and TBs are valuable, rare Chinese species that play a unique role in studying new pig breeds. We previously performed single-nucleotide polymorphism (SNP) analysis and molecular genetics research on MSTMN, ESR, H-FABP, SLA, DQA, and PPARr-2 genes. In the present study, we analyzed GH, GHR, and IGF-1 SNPs by performing DNA sequencing in BMs and TBs to further explore the effects of these genes on growth traits. Large white pigs (LWs) from the United Kingdom, also known as Yorkshire pigs, were used as the control.

### Material and Methods

#### Animals

Animal experiments were performed according to the Guidelines on Animal Care and Use established by the Southern Medical University Animal Care and Use Committee. A total of 100 blood samples from 3- to 8-month-old BMs and 108 blood samples from 3- to 8-month-old TBs were collected at the Laboratory Animal Center, Southern Medical University, Guangzhou. Fifty ear samples from LWs were randomly collected at the Dalingshan Food Company, Dongguan, China. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). The animal use protocol was approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Genomic DNA was extracted using Tiangen genomic DNA extraction kits (Tiangen Biotech, China). The purity and concentration of the DNA samples were determined using agarose gel electrophoresis and ultraviolet spectrophotometry. The samples were diluted to 100 ng/μL and stored at –20°C.

**GH, GHR, and IGF-1 gene fragment amplification**

Primers for GH (Gene ID: 347618782), GHR (Gene ID: 347618778), and IGF-1 (Gene ID: 347618789) gene fragments were designed using the Primer 5.0 software (PREMIER Biosoft, Canada). Polymerase chain reaction (PCR) was carried out in a 50-μL volume containing 50 ng template DNA, 2 μL primers (10 μM each), 25 μL of 2 x MasterMix (0.05 units/μL Taq DNA polymerase, 4 mM MgCl2, 4 mM dNTPs), and double-distilled (dd)H2O (Aidlab Biotechnologies Co., Ltd., China). PCR reactions were performed under the following conditions: a 3-min hot start at 95°C, 35 cycles of denaturation at 94°C for 30 s, annealing (GH at 60°C, GHR at 58°C, IGF-1 at 55°C) for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. PCR products were loaded onto a 1% agarose gel and visualized using a gel imaging system (Bio-Rad, USA).

**DNA sequencing**

The PCR products from pig samples were sequenced by the Invitrogen Trading Company (China).

### Data analysis

Polymorphism results were analyzed using DNA STAR Lasergene v7.1, Chromas, Variant Reporter, Popgene32, polymorphism information content (PIC) Calc 0.6, SPSS13.0 (ANOVA, chi-square test), and other biological software by searching SNP loci sites and identifying genotypes.

### Results

**SNP loci of GH, GHR, and IGF-1 gene fragments in three pig breeds**

A 221-bp fragment of the GH gene, a 254-bp fragment of the GHR gene, and a 486-bp fragment of the IGF-1 gene were amplified by PCR. GH, GHR, and IGF-1 gene fragments from the three pig breeds were of the expected sizes.

GH gene fragments revealed five SNP loci (A12G, T45C, G84A, G93A, and C133T) in BMs and TBs, but three SNP loci (A12G, T45C, and G93A) in LWs. Only one SNP locus (A85C) was found in the GHR gene fragment of BMs, and no loci were found in the other two pig breeds. In IGF-1 gene fragments, three SNP loci (A100T, A285G, T300C) were observed in BMs and TBs, whereas two SNP loci (A285G and T300C) were found in LWs. On the basis of the number of SNP loci in these three gene fragments among the three pig breeds, the most diversity was observed in the BM breed, and the least diversity was observed in LWs. The differences were also closely related to their geographical distribution and breeding methods. In general, BMs came from the Guangxi Provinces of China, and they were bred from an individual family with very small numbers and without any selection. TBs have a relatively smaller geographical distribution than BMs. In contrast, LWs are bred on large commercial pig farms with specific selection. The LW samples collected for this experiment came from a limited number of farmers.

**GH, GHR, and IGF-1 gene fragments and their genotype frequencies**

We statistically analyzed the genotype frequencies of GH, GHR, and IGF-1 gene fragments (Tables 1-3, K independent samples test), and the PICs are shown in Tables 1-3. The following PICs were identified at each locus of the GH gene fragments from BMs, TBs, and LWs, respectively: 0.372, 0.360, and 0.310 at A12G; 0.373, 0.375, and 0.340 at T45C; 0.122, 0.256, and 0 at G84A; 0.029, 0.211, and 0.374 at G93A; and 0.143, 0.369, and 0 at C133T. The G84A and C133T SNP loci were not found in the LW breed. In the GH gene fragment GG was only observed in TBs at locus 84. The dominant allele was G, and no AA was present at locus 93 in BMs. Ninety-four percent GA was found in LWs at locus 93. The
percentages of T at locus 133 were 42.6% in TBs and 0% in LWs. The genotype distribution among the three groups was significantly different (P<0.05, chi-square test) for four of the five SNP sites.

For the GHR gene, the SNP at A85C was found only in BMs. Both TBs and LWs contain the AA allele in this locus. The genotype distribution among the three groups was significantly different (P<0.05, chi-square test).

For the IGF-1 gene fragment, three SNPs were observed (A100T, A285G, and T300C) in the two minipig breeds, but two SNPs (A285G and T300C) were observed in LWs. AA and TT were present at 98% at locus 285 and locus 300 in LWs, respectively. The percentages of A were 70.5% in BMs and 29.6% in TBs at locus 100, whereas the percentages of A were 24.5% in BMs and 68.1% in TBs at locus 285. The genotype distribution among the three groups was significantly different (P<0.05, chi-square test).

Among the three gene fragments, the average observed and expected heterozygosities of the GH gene in the TB populations were higher than those of BMs. However, it was lower for the IGF-1 gene, and no SNP sites were observed in the GHR gene of TBs (Table 4).

### Relationship between growth traits and GH, GHR, and IGF-1 gene polymorphisms

SNPs in the GH gene fragment significantly affected body length (BL) and chest circumference at locus 12 and BL at locus 45 in BMs; BW, withers height (WH), CC, and abdomen circumference (AC) at locus 45; and WH and CC at locus 93 in TBs. One SNP was observed at locus 85 in BMs. The GHR gene fragment significantly affected all of the growth trait characteristics. All indices were significantly reduced with a mixture of alleles in this locus. These results indicate that this locus has the potential to be used as a breeding selection marker for BMs. SNPs

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### Table 1. Genotype and allele frequency of GH gene in three pig breeds.

| Breed | Number | Locus | Genotype frequency | Allele frequency | PIC |
|-------|--------|-------|--------------------|------------------|-----|
|       |        |       | AA                | AG              | GG  |
| BM    | 100    | 12    | 0.3500 (35)       | 0.4100 (41)     | 0.2400 (24) |
|       |        |       |                   | 0.555           | 0.445 |
| TB    | 108    |       | 0.1111 (12)       | 0.5370 (58)     | 0.3519 (38) |
|       |        |       |                   | 0.380           | 0.620 |
| LW    | 50     |       | 0.7000 (35)       | 0.0800 (4)      | 0.2200 (11) |
|       |        |       |                   | 0.740           | 0.260 |
|       |        |       |                   | 0.372           |       |
|       |        |       | TT                | TC              | CC  |
| BM    | 100    | 45    | 0.2500 (25)       | 0.4100 (41)     | 0.3400 (34) |
|       |        |       |                   | 0.455           | 0.545 |
| TB    | 108    |       | 0.2685 (29)       | 0.4629 (50)     | 0.2685 (29) |
|       |        |       |                   | 0.500           | 0.500 |
| LW    | 50     |       | 0.2800 (14)       | 0.0800 (4)      | 0.6400 (32) |
|       |        |       |                   | 0.320           | 0.680 |
|       |        |       |                   | 0.340           |       |
|       |        |       | GG                | GA              | AA  |
| BM    | 100    | 84    | 0.9700 (97)       | 0.0300 (3)      | 0.0300 (3) |
|       |        |       |                   | 0.985           | 0.015 |
| TB    | 108    |       | 0.7593 (82)       | 0.2037 (22)     | 0.0370 (4) |
|       |        |       |                   | 0.861           | 0.139 |
| LW    | 50     |       | 0.0000 (0)        | 0.9400 (47)     | 0.0600 (3) |
|       |        |       |                   | 0.470           | 0.530 |
|       |        |       |                   | 0.374           |       |
|       |        |       | GG                | GA              | AA  |
| BM    | 100    | 133   | 0.8600 (86)       | 0.1100 (11)     | 0.0300 (3) |
|       |        |       |                   | 0.085           | 0.915 |
| TB    | 108    |       | 0.2222 (24)       | 0.4074 (44)     | 0.3704 (40) |
|       |        |       |                   | 0.426           | 0.574 |
| LW    | 50     |       | 1.0000 (50)       | 0.0000 (0)      | 0.0000 (0) |
|       |        |       |                   | 0.000           | 1     |
|       |        |       |                   | 0     | 0     |

BM: Bama miniature pigs; TB: Tibetan miniature pigs; LW: large white pigs; PIC: polymorphism information content.

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### Table 2. Genotype and allele frequency of GHR gene in three pig breeds.

| Breed | Number | Locus | Genotype frequency | Allele frequency | PIC |
|-------|--------|-------|--------------------|------------------|-----|
|       |        |       | AA                | AC              | CC  |
| BM    | 100    | 85    | 0.7300 (73)       | 0.0700 (7)      | 0.2000 (20) |
|       |        |       |                   | 0.765           | 0.235 |
| TB    | 108    |       | 1.0000 (108)      | 0 (0)           | 0 (0) |
|       |        |       |                   | 1               | 0    |
| LW    | 50     |       | 1.0000 (50)       | 0 (0)           | 0 (0) |
|       |        |       |                   | 1               | 0    |

BM: Bama miniature pigs; TB: Tibetan miniature pigs; LW: large white pigs; PIC: polymorphism information content.

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in the IGF-1 gene fragment did not affect growth trait characteristics in BMs. However, the SNPs at loci 285 and 300 significantly affected BW, BL, and CC in TBs. These three indices were significantly reduced with the mixture of alleles at these two loci. These two SNPs may be used as breeding selection markers for TBs.

**Discussion**

The GH gene fragment displayed the most diversity among the three gene fragments and pig breeds as demonstrated by the number of SNP loci and PIC values. Only one SNP locus was found in the GHR gene fragment of BMs. Although the IGF-1 gene fragment was selected to evaluate the genetic diversity among pig breeds, our results suggest that GH is the best candidate for this purpose.

Polymorphism levels were different among the SNP sites. The average PIC is an ideal index to assess gene fragment polymorphisms. A PIC value >0.5 indicates a highly polymorphic region, 0.25< PIC<0.5 indicates a moderately polymorphic site, and a PIC value <0.25 signifies a low polymorphic site (16). In the GH gene fragment, moderately polymorphic sites were observed at A12G and T45C in BMs; A12G, T45C, G84A, and C133T in TBs, and A12G, T45C, and G93A in LWs. The only moderately polymorphic site found in BMs was at A85C in the GHR gene fragment. The three SNP sites in the IGF-1 gene fragment were moderately polymorphic for BMs and TBs but showed low polymorphism for LWs. However, only low polymorphism was observed in TBs in two previous studies (17-19) using the same IGF-1 gene fragment. The difference in these studies may be due to the different sample sizes. LW body size is much bigger than that of TBs and BMs (20), and only three moderately polymorphic sites were found in LWs. These results indicate that the values of each SNP site vary among the three pig breeds.

The different SNP sites might differentially affect pig growth traits (21-25). Among the nine SNP sites in these

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**Table 3. Genotype and allele frequency of IGF-1 gene in three pig breeds.**

| Breed | Number | Locus | Genotype frequency | Allele frequency | PIC  |
|-------|--------|-------|--------------------|------------------|------|
|       |        |       | AA                 | AT               | TT   |
| BM    | 100    | 100   | 0.4600 (46)        | 0.4900 (49)      | 0.0500 (5) |
| TB    | 108    |       | 0.0463 (5)         | 0.5000 (54)      | 0.4537 (49) |
| LW    | 50     |       | 0.00 (0)           | 0.00 (0)         | 1.0000 (50) |
|       |        |       | A                  | T                |      |
| BM    | 100    | 285   | 0 (0)              | 0.4900 (49)      | 0.5100 (51) |
| TB    | 108    |       | 0.3796 (41)        | 0.6019 (65)      | 0.0185 (2) |
| LW    | 50     |       | 0.9800 (49)        | 0.0200 (1)       | 0 (0) |
|       |        |       | A                  | G                |      |
| BM    | 100    | 300   | 0.5500 (55)        | 0.4500 (45)      | 0 (0) |
| TB    | 108    |       | 0.4537 (49)        | 0.5185 (56)      | 0.0278 (3) |
| LW    | 50     |       | 0.9800 (49)        | 0.0200 (1)       | 0 (0) |

BM: Bama miniature pigs; TB: Tibetan miniature pigs; LW: large white pigs; PIC: polymorphism information content.

**Table 4. Genetic diversity of GH, GHR, IGF-1 genes in 3 pig breeds.**

| Gene | Population | Number | A        | Ae       | H<sub>o</sub> | H<sub>e</sub> |
|------|------------|--------|----------|----------|-------------|-------------|
| GH   | BM         | 100    | 2.000    | 1.4622   | 0.2138      | 0.2656      |
|      | TB         | 108    | 2.000    | 1.7183   | 0.3704      | 0.4038      |
|      | LW         | 50     | 1.800    | 1.0135   | 0.9867      | 0.0133      |
| GHR  | BM         | 100    | 2.000    | 1.4818   | 0.7760      | 0.2240      |
|      | TB         | 108    | 1.000    | 1.000    | 0.000       | 0.000       |
|      | LW         | 50     | 1.000    | 1.000    | 0.000       | 0.000       |
| IGF-1| BM         | 100    | 2.000    | 1.6575   | 0.5455      | 0.4156      |
|      | TB         | 108    | 2.000    | 1.6978   | 0.5156      | 0.4142      |
|      | LW         | 50     | 1.6667   | 1.0135   | 0.9867      | 0.0133      |

A: average number of alleles; Ae: effective allele number; H<sub>o</sub>, H<sub>e</sub>: observed and expected heterozygosity, respectively; BM: Bama miniature pigs; TB: Tibetan miniature pigs; LW: large white pigs.
three gene fragments, three SNP sites in BMs and four SNP sites in TBs significantly affected growth traits. SNPs in the GH gene fragment significantly affected BL and CC at locus 12 and BL at locus 45 in BMs, whereas they affected BW, WH, CC, and AC at locus 45 and WH and CC at locus 93 in TBs. The SNP at locus 85 in the GHR gene fragment in BMs significantly affected all of the examined growth traits. All indices were reduced significantly with the mixture of alleles at this locus. These results indicate that this locus has the potential to be used as a breeding selection marker in BMs. SNPs in the IGF-1 gene fragment did not affect growth characteristics in BMs. However, the SNPs at loci 285 and 300 significantly affected BW, BL, and CC in TBs. These three indices were significantly reduced with the mixture of alleles at these two loci. Therefore, these two SNPs may be used as breeding selection markers for TBs.

Genetic heterozygosity indicates the proportion of the group with site heterozygotes at some loci. The average heterozygosity of loci reflects the level of variation in the genetic structure, and variability is directly correlated with heterozygosity and the ability to adapt to the environment (20). Among the three gene fragments, the average observed and expected heterozygosities of the TB population was higher than those of BMs for the GH gene, lower for the IGF-1 gene, and no SNP sites were observed in the GHR gene of TBs.

In summary, the GH gene fragment represents the best candidate for SNP analysis among these three gene fragments, and some SNP sites are closely related to growth traits.

Acknowledgments

This research was supported by the Guangdong Natural Science Foundation (#S2013010014720); China Postdoctoral Science Foundation (#2014M50439); the National High Technology Research and Development Program of China (863 Program: #2011AA100304); Science and Technology Planning Project (#2010U1E00811) of Guangzhou City, Guangdong Province, China; Conditions for scientific research project (#2010B060500001, #2011B060300014) of Guangdong Province, China; Key Program for International S&T Cooperation Projects of China (#2011DFA33290); Torch Program (#SQ2012GGHE000003A08), and Development Foundation of Special High-tech Zone (#SQ2011SF11CO1543).

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