Linkage analysis of SNPs in *IGFBP-6* and its relation with the body sizes of pig

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ABSTRACT. Insulin-like growth factor binding protein-6 (IGFBP-6) is a member of the IGFBP family, which is known to be a key factor in regulating the effect of insulin-like growth factor-2 (IGF-2) on the animal growth and development. Gene sequences of 3'-untranslated regions (UTR) and exon 4 of *IGFBP-6* may influence the expression and proteolysis of IGFBP-6. In this study, 551 bp of the *IGFBP-6* (including 257 bp of intron 3, exon 4, and 170 bp of 3' UTR) were sequenced and compared in the Bama and Tibetan mini-pigs, the Landrace and Large White pigs, and the Northeast wild boars. Six single nucleotide polymorphisms (SNPs) were detected in the *IGFBP-6*, in which T593C, T636C, and T745C were in intron 3, A67G was in exon 4, and G37A was in 3' UTR. T636C, T745C, and A67G were in linkage and formed four kinds of haplotypes, with CCT being the dominant haplotype in the mini-pigs; however, the haplotype block was not formed in the Landrace pigs and Large White pigs or the Northeast wild boars. Based on the above results, we concluded that the SNPs and haplotype of the *IGFBP-6* may be related to the mini-size formation of the pig.

Key words: IGFBP-6; SNPs; Mini-pig; Haplotype; Body size

INTRODUCTION

Insulin-like growth factor binding protein-6 (IGFBP-6) is a member of the IGFBP family and functions through insulin-like growth factor (IGF)-dependent and IGF-independent systems (Clemmons, 2001; Firth and Baxter, 2002). IGFBPs help modulate IGF action in complex ways that involve inhibiting IGF action by preventing its binding to the IGF receptors and promoting IGF action, possibly by aiding in its delivery to the receptors and increasing the half-life of IGF (Firth and Baxter, 2002). IGFBP-6 is able to inhibit or promote the growth, development, cell adhesion, and other functions mediated by IGF-2 (Murphy, 1998) because of its high affinity to IGF-2. Studies on the osteoclasts, keratinocytes, muscle cells, and colonies of cancer cells and other cell lines have revealed that IGFBP-6 is involved in the growth, differentiation, and adhesion of cells, and formation of colonies (Ewton and Florini, 1995). Furthermore, it has been observed that down-regulation of IGFBP-6 leads to premature entry into cellular senescence. It has been reported that in contrast to other IGF binding proteins, IGFBP-6 is a negative regulator of cellular senescence in the human fibroblasts, because the overexpression of IGFBP-6 increases the cellular lifespan (Micutkova et al., 2011). Mice that overexpressed human IGFBP-6 suffered weight loss; a significant decrease in the reproductive capacity was observed in females and the development and metabolism of the brain were also influenced (Bienvenu et al., 2004, 2005). The transcription and expression of *IGFBP-6* are regulated by its 3'-untranslated regions (UTR) sequence (de Moor et al., 2005). The N-terminus sequence of exon 4 is relatively conserved, encodes a cysteine-rich motif, harbors multiple sites of interaction with proteases, and is involved in high-affinity binding between IGFBP-6 and IGF-2 (Clemmons, 2001; Duan, 2002). The quantitative trait loci (QTL) of pigs birth weight has been identified by the application of genetic markers. Miniature pigs have small body size and their physiology, and genetics are closest to those of humans; thus, they are an ideal animal model for biological and medical research. Tibetan and Bama mini-pigs are valuable germplasm resources of miniature pigs. However, the association between polymorphisms in the *IGFBP-6* gene and body size of pig is unknown. Studies on Chinese miniature pigs are still in their infancy and their growth mechanisms are unclear.

In the present study, Tibetan and Bama mini-pigs were used (along with Northeast wild boars, Large White and Landrace pigs used as controls) to screen and analyze the single nucleotide polymorphisms (SNPs) of IGFBP-6 gene in the exon 4, the 3' UTR, and partial sequences of intron 3 to investigate the association between the SNPs and body size of pigs. We hope this study will lay the foundation for further research on the mechanisms of dwarfism in miniature pigs.

MATERIAL AND METHODS

Animal resources

The Tibetan mini-pigs (42) were provided by Beijing Tongheshengtai Institute of Comparative Medicine while the Bama mini-pigs (42) were provided by the Yunfu Zhaoqing Pig Farm. The Northeast wild boars (40) were supplied by the Yezhulin Wild Boar Breeding Farm of Jiang Yuan County, Jilin Province. The Landrace pigs (50) and Large White pigs (54) were provided by the Pig Breeds Farm of Jilin University. The five pig breeds were grouped by body size into mini-pigs (<45 kg for adult; BamaXiang and Tibetan mini-pigs) and large-sized pigs (>200 kg for adult; Daibai, Northeast Wild, and Junmu No.1 White pigs). Number of males and females used was equal for all the breeds.

Reagents

LA *Taq*, DL2000 DNA marker, dNTPs, 6X loading buffer (TaKaRa Bio. Co., Ltd., Dalian, China), 2 X Power *Taq* PCR Master Mix (Bio Teck Biotechnology Co., Ltd., Beijing, China), DNA purification kit and animal tissue DNA extraction kit (Axygen BioScience, Inc., USA) were used in the experiments.

DNA extraction and determination of its purity and concentration

Genomic DNA was extracted from the liver of individual pigs of the 5 breeds according to the manufacturer instruction, and the purity and concentration of the genomic DNA were determined with a NanoDrop 2000 UV spectrophotometer (Thermo Fisher Scientific Inc., USA). DNA preparations in an appropriate amount were examined by gel electrophoresis on 1% agarose gel.

Primers and polymerase chain reaction (PCR) amplification

Primers were designed using Primer Premier 6.0 (Premier Biosoft International, Canada), according to the DNA sequence of the pig *IGFBP-6* in GenBank and synthesized by GENEWIZ, Inc., China. The extracted genomic DNA pool was used as a template to amplify the target gene fragments using PCR.

Partial sequence of *IGFBP-6* (NC_010447.4; 551 bp, from intron 3 to partial 3' UTR) was amplified by PCR of the genomic DNA pool of each pig breed (Bama and Tibetan mini-pigs, Landrace and Large White pigs, and Northeast wild boars) using the synthesized primers (forward primer: 5'-AGTCTCTAGTGATGCTGATGCT-3'; reverse primer: 5'-CCACGCCAACACCAACAAT-3'). The PCR of the partial sequences of *IGFBP-6* were performed in 25 µL reaction volumes containing 12.5 µL 2 X Power Taq PCR Master Mix, 0.5 µL upstream and downstream primers (10 µM), 2.0 µL template DNA (25 ng/µL), and 9.5 µL ultra-pure water. The PCR program used for the partial sequences *IGFBP-6* gene was as follows: 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 57°C for 20 s, 72°C for 30 s, 10 min extension at 72°C and final hold at 4°C. The PCR products (4.0 µL) were electrophoresed on 1% agarose gel to detect the amplifications.

SNP discovery and genotyping

The PCR products were purified according to the manufacturer instructions and submitted to GENEWIZ, Inc. (China) for DNA sequencing. The sequence alignment was performed on the sequencing data using DNASTAR Lasergene (DNAStar, Inc., USA) to screen for the inter-race mutations in the 5 pig breeds. The target peaks in the sequencing chromatographs from the five pig breeds were labeled and analyzed using Chormas (Technelysium Pty Ltd, Australia) to screen for the mutation sites. The SNPs were positioned, and a genotyping analysis was performed.

Statistical analysis

A chi-square test analysis was performed on each SNP locus using Graphpad prism 6.0 (Graphpad software, Inc., USA). A value of $P < 0.05$ was defined as statistically significant. Linkage analysis on the haplotype was conducted using HaploView 4.2 software (Daly Lab, USA) and the SNPs with a minor allele frequency (MAF), i.e., <1%, were excluded from the haplotype analysis.

RESULTS

PCR amplification of partial sequences of *IGFBP-6*

The gel electrophoresis of the PCR amplification of partial sequences of *IGFBP-6* is shown in Figure 1. Distinct bands of amplification products (551 bp) were observed.

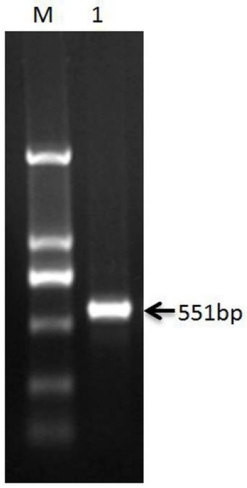

Figure 1. Gel electrophoresis of PCR products of the *IGFBP-6* partial sequences. *Lane M* = DNA marker; *lane 1* = *IGFBP-6* partial sequences.

Screening of SNPs on the partial *IGFBP-6* of pig

From the sequencing results of the PCR products by the pooled DNA templates, six SNPs were detected in the partial *IGFBP-6* of pig (Figure 2).

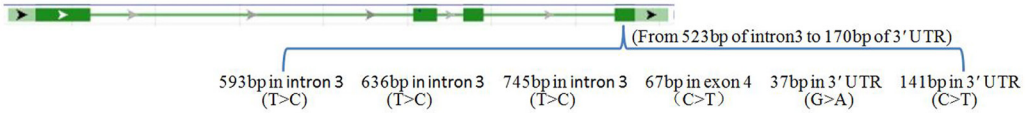

Figure 2. SNPs detected in the partial *IGFBP-6* of pig.

The SNPs, T593C, T636C, and T745C of intron 3, C67T of exon 4, G37A and C141T of the 3' UTR were detected in the partial *IGFBP-6*. In the exon 4, the mutation C67T was a nonsense mutation (codon change from GAC to GAU); therefore, it did not cause an amino acid sequence change in *IGFBP-6* (Figure 3). Among the SNPs, T593C, T636C, T745C, and C67T of exon 4 and C141T of 3' UTR have already been registered in the NCBI (accession No.: rs338341866, rs322857706, rs335307143, re80834317, and rs322436854).

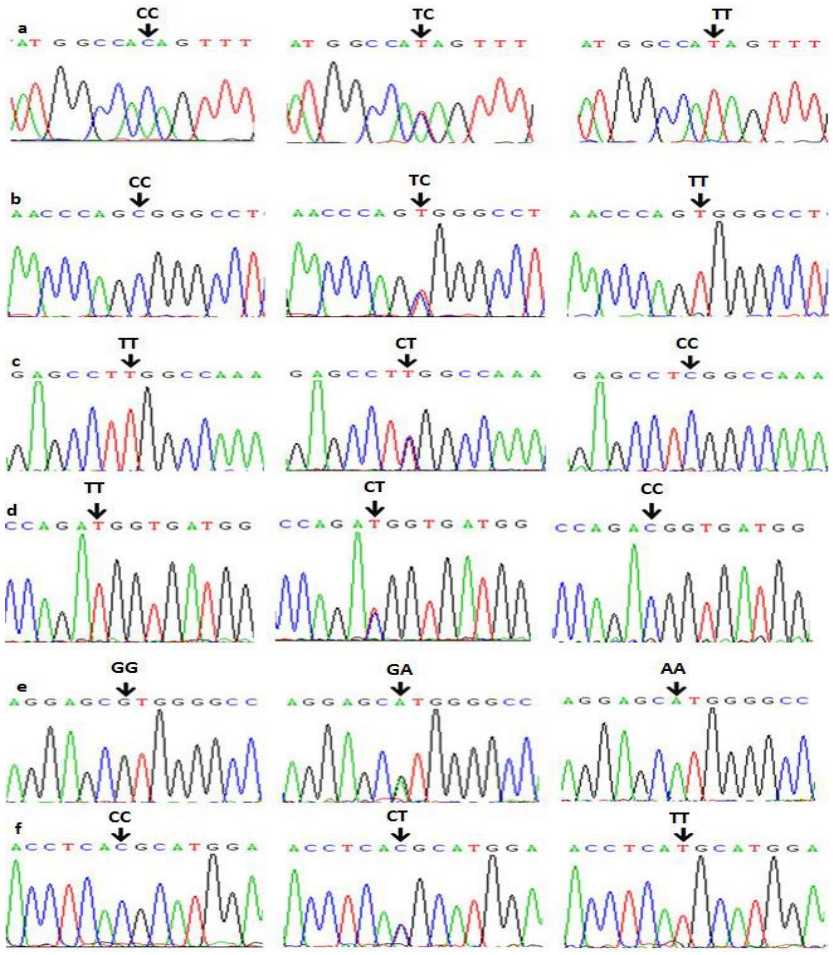

Figure 3. SNPs in the partial *IGFBP-6* of pig. **a.** T593C in intron 3; **b.** T636C in intron 3; **c.** T745C in intron 3; **d.** C67T in exon 4; **e.** G37A in the 3' UTR; and **f.** C141T in the 3' UTR.

SNP loci analysis

The analyses of the results for each locus of SNPs of *IGFBP-6* are shown in Table 1. T593C in intron 3 of the *IGFBP-6* gene revealed TT as the dominant genotype in Tibetan mini-pigs, TC and CC as the dominant genotypes in Bama mini-pigs, and TC as the dominant genotype in Northeast wild boars, Landrace pigs and Large White pigs. T636C in intron 3 of the *IGFBP-6* revealed CC as the dominant genotype in the Tibetan mini-pigs, Bama mini-pigs, Northeast wild boars, and Landrace and Large White pigs. At 745 bp of intron 3, the conversion of T to C occurred and TT was the dominant genotype in the Tibetan and Bama mini-pigs, Northeast wild boars, and Landrace and Large White pigs. T745C in intron 3 of the *IGFBP-6* showed that TT was the dominant genotype in the Bama and Tibetan mini-pigs, Northeast wild boars, and Junmu number 1 and Large White pigs. C67T in exon 4 of the *IGFBP-6* did not cause an amino acid sequence change, and TT was the dominant genotype in

the Bama and Tibetan mini-pigs, whereas CC was the dominant genotype in the Northeast wild boars and Large White pigs and CT was the dominant genotype in the Landrace pigs. The G37A mutation in the 3' UTR sequence of the *IGFBP-6* was only exhibited in the Tibetan mini-pigs, in which GG was the dominant genotype (0.87). No mutation was detected in other pigs at this locus, and they all exhibited the GG genotype. The C141T mutation in the 3' UTR sequence of the *IGFBP-6* did not occur in the Bama mini-pigs and Northeast wild boars, and they all exhibited the CC genotype. The dominant genotype was CC for the Tibetan mini-pigs, and the Landrace and Large White pigs.

Table 1. The frequencies and chi-square test of the genotypes of SNPs in *IGFBP-6* of pig.

Body size	Population	SNP location	Allele frequency		Genotype frequency			X^2, d.f.	P
		I 3 593 T>C	T	C	TT	TC	CC	46.27, 2	<0.0001
Mini	Bama		0.31	0.69	0.09	0.43	0.48		
	Tibetan		0.94	0.06	0.91	0.07	0.02		
Large	Boars		0.40	0.60	0.10	0.60	0.30		
	Landrace		0.64	0.36	0.30	0.68	0.02		
	White		0.40	0.60	0.02	0.76	0.22		
		I 3 636 T>C	T	C	TT	TC	CC	28.84, 2	<0.0001
Mini	Bama		0.29	0.71	0.10	0.38	0.52		
	Tibetan		0.13	0.87	0.02	0.21	0.77		
Large	Boars		0.05	0.95	0.02	0.05	0.93		
	Landrace		0.05	0.95	0.02	0.06	0.92		
	White		0.05	0.95	0.02	0.05	0.93		
		I 3 745 T>C	C	T	CC	CT	TT	28.95, 2	<0.0001
Mini	Bama		0.29	0.71	0.10	0.38	0.52		
	Tibetan		0.39	0.61	0.26	0.26	0.48		
Large	Boars		0.08	0.93	0.02	0.10	0.88		
	Landrace		0.14	0.86	0.02	0.24	0.74		
	White		0.10	0.90	0.02	0.16	0.82		
		E4 67 C>T	C	T	CC	CT	TT	68.58, 2	<0.0001
Mini	Bama		0.31	0.69	0.09	0.43	0.48		
	Tibetan		0.39	0.61	0.26	0.26	0.48		
Large	Boars		0.73	0.28	0.55	0.35	0.10		
	Landrace		0.67	0.33	0.36	0.62	0.02		
	White		0.87	0.13	0.76	0.22	0.02		
		3' UTR-37 G>A	G	A	GG	GA	AA	12.38, 2	0.003
Mini	Bama		1.00	0.00	1.00	0.00	0.00		
	Tibetan		0.90	0.10	0.84	0.14	0.02		
Large	Boars		1.00	0.00	1.00	0.00	0.00		
	Landrace		1.00	0.00	1.00	0.00	0.00		
	White		1.00	0.00	1.00	0.00	0.00		
		3' UTR-141 C>T	C	T	CC	CT	TT	0.7167, 2	0.5747
Mini	Bama		1.00	0.00	1.00	0.00	0.00		
	Tibetan		0.86	0.14	0.74	0.24	0.02		
Large	Boars		1.00	0.00	1.00	0.00	0.00		
	Landrace		0.82	0.18	0.70	0.24	0.06		
	White		0.95	0.05	0.93	0.05	0.02		

Large = large-sized pigs; Mini = mini-pigs; Bama = Bama mini-pigs; Tibetan = Tibetan mini-pigs; Boars = Northeast wild boars; Landrace = Landrace pigs; White = Large White pigs. I = intron; E = exon. Example: 'I 3 593 T>C' means a mutant of T to C was happened in the 593 bp of intron 3 (in the row of SNP location).

Linkage analysis of the SNP loci in the haplotypes

Results of the linkage analysis of the SNPs in the *IGFBP-6* are shown in Figure 4. In the five pig breeds, a strong linkage was observed between the SNPs at 593 and 636 nucleotide (nt) in the intron 3 (D' = 1.0, r^2 = 0.104), at 593 nt in the intron, 3 and at 141 nt in the 3' UTR (D' = 1.0, r^2 = 0.055), at 745 nt in the intron 3 and at 141 nt in the 3' UTR (D' = 1.0, r^2 = 0.244).

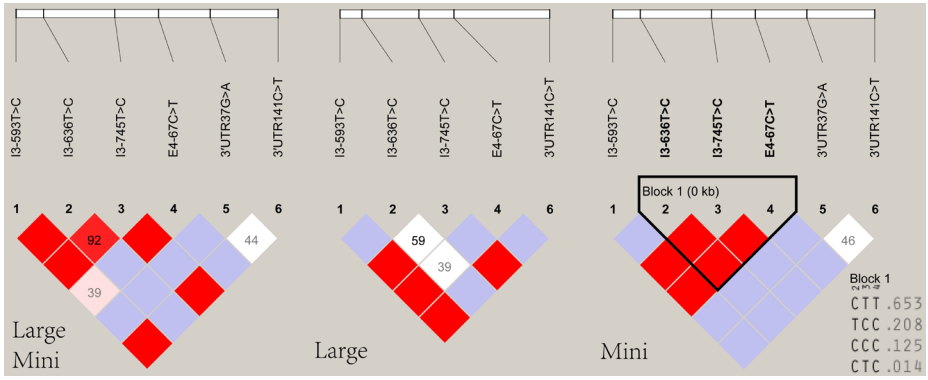

Figure 4. Linkage disequilibrium of the SNPs in the partial *IGFBP-6* of pig. Large: pig with large size; Mini: mini-pigs.

In the Large White and Landrace pigs and the Northeast wild boars, a strong linkage was observed between the SNPs at 593 nt and 745 nt in the intron 3 (D' = 1.0, r^2 = 0.129), at 593 nt in the intron 3 and at 67 nt in the exon 4 (D' = 1.0, r^2 = 0.269), at 593 nt in the intron 3 and at 141 nt in the 3' UTR (D' = 1.0, r^2 = 0.077), at 745 nt in the intron 3 and at 141 nt in the 3' UTR (D' = 1.0, r^2 = 0.611).

In Tibetan and Bama mini-pigs, a strong linkage was observed between the SNPs at 593 nt and 745 nt in the intron 3 (D' = 1.0, r^2 = 0.357), at 593 nt in the intron 3 and at 67 nt in the exon 4 (D' = 1.0, r^2 = 0.38), at 636 nt and 745 nt in the intron 3 (D' = 1.0, r^2 = 0.526), at 636 nt in the intron 3 and at 67 nt in the exon 4 (D' = 1.0, r^2 = 0.495), at 745 nt in the intron 3 and at 67 nt in the exon 4 (D' = 1.0, r^2 = 0.94). The SNPs at 636 nt and 745 nt in the intron 3 and 67 nt in the exon 4 formed a block, which exhibited four haplotypes: CTT (0.653), TCC (0.208), CCC (0.125), and CTC (0.014). The other SNPs showed weak linkages.

DISCUSSION

The IGFBPs consist of six mutually related peptides and exhibit high-affinity binding to IGF family proteins (Hwa et al., 1999). The IGFBPs and IGF receptors compete in the binding of IGF, and the outcome of this influences cell proliferation and animal growth and development. Although, the IGFBPs have same characteristics when they interact with IGF, the expression of each IGFBP is time- and tissue-specific, highly regulated, and thus, functions distinctively. The main biological function of IGFBP-6 is in binding with IGF-2, and it is also potentially involved in the regulations that are independent of the IGF system (Hwa et al., 1999).

The linkage analysis of 6 SNPs in the *IGFBP-6* demonstrated that in the Tibetan and Bama mini-pigs, the SNPs at 636 nt and 745 nt in the intron 3 and 67 nt in the exon 4 formed a haplotype block that exhibited four haplotypes, with CTT as the dominant haplotype. In the Large White and Landrace pigs and the Northeast wild boars, no such block was formed by the 3 SNPs, indicating that the these SNPs in the large-sized pigs were influenced by different selective pressure than those in the mini-pigs. In addition, the SNPs at 636 nt and 745 nt in the intron 3 and 67 nt in the exon 4 were correlated with dwarfism of the mini-pigs. Although the C67T mutation in the exon 4 did not introduce any changes in the amino acid sequence of *IGFBP-6*, the T allele at the locus was the dominant allele in the mini-pigs, whereas the C allele at the locus was dominant in the large-sized pigs, suggesting that the T allele at the locus may be related to dwarfism. Moreover, the SNP was previously identified and found to be significantly related to lumbar fat thickness and

water loss rate ($P < 0.05$) (Sini, 2006), suggesting that the haplotype formed by the 3 SNPs may influence the lumbar fat thickness and water loss rate of pigs, thus, affecting the pig body size. The haplotype can be used as a potential molecular marker that could be integrated into pig species improvement projects.

In the present study, the SNPs in the partial sequences of the *IGFBP-6* were investigated, and it was observed that C535A in large-sized pigs and T488C in mini-pigs did not comply with the law of HWE, which possibly was a result of the Landrace and Large White pigs having been introduced from abroad, whereas the Bama mini-pigs being a well-known inbred strain, and the Tibetan mini-pigs growing in a closed environment. In addition, the sample sizes in our study were small, which may have resulted in the failure to detect certain genotypes in the population; thus, the gene frequency of certain alleles failed to observe the law of HWE.

Based on the results of this study, it is suggested that the *IGFBP-6* may be one of the candidate genes that affects the pig body size. The specific mechanism of IGFBP-6 action requires further investigations. The present study provided molecular evidence of IGFBP-6, which influences the body size of mini-pigs and provides a good foundation for the investigation of the mechanisms of growth and development of mini-pigs. It also provides a theoretical basis for the development and application of mini-pig resources.

Conflicts of interest

The authors declare no conflict of interest.

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