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EVALUATION OF THE *IGFs* (*IGF1* AND *IGF2*) GENES AS CANDIDATES FOR GROWTH, BODY MEASUREMENT, CARCASS, AND REPRODUCTION TRAITS IN BEIJING YOU AND SILKIE CHICKENS

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Insulin-like growth factors are crucial in cellular growth, differentiation, and reproduction by mediating many of the actions of growth hormone in chickens. To determine whether insulin-like growth factors genes (*IGFs*) are associated with important economic traits in chicken or not, we herein analyzed the association between two single nucleotide polymorphisms (SNPs) within* IGF1 *and* IGF2 *and twenty-seven growth, body measurement, carcass, and reproduction traits in two Chinese native breeds, i.e., Beijing You and Silkies. With marker-trait association analysis, we found that SNP IGF1-PstI, within the 5' flanking region of* IGF1, *was significantly associated with body weight at 8 (BW8), 10 (BW10), and 13 (BW13) wk of age; and shank length (SL13) and shank circumference (SC13) at 13 wk of age in Silkie population ($P < 0.05$). The SNP IGF2-MspI within the exon2 of* IGF2 *showed a significant association with body weight (BW17) and carcass weight (CW17) at 17 wk of age in Beijing You population ($P < 0.05$). Our findings implied that the SNPs within* IGF1 *and* IGF2 *genes could be in linkage disequilibrium with the actual causative mutations that affect growth and carcass traits.

Keywords: Chicken; Economic traits; *IGF1*; *IGF2*; Single nucleotide polymorphism

Insulin-like growth factors 1 and 2 (*IGF1* and *IGF2*) are mitogenic polypeptides with structural similarity to insulin[1,2] and are related to insulin with multifunctional metabolic and anabolic properties.[3] Both *IGF1* and *IGF2* play major roles in cellular growth by mediating many of the actions of growth hormone and can affect a wide range of biological processes, ranging from growth and differentiation to

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reproduction in poultry.[4] As a result, the chicken *IGFs* are considered to be the most important candidate genes that can influence chicken performance traits including growth, body measurement, carcass, and reproduction. Extensive studies have concluded that *IGF1* was associated with body weight,[5–9] carcass traits,[8] and reproduction traits[10] in chicken. Also, a C/G mutation in the exon2 of *IGF2* was reported to be significantly associated with growth and carcass traits in chicken.[11]

In addition, previous studies have revealed several chromosomal regions that are likely to harbor QTLs affecting growth, carcass, and reproductive traits in chicken. Both *IGF1* and *IGF2* are located within the linkage regions where some QTL have been detected by other investigators.[12,13] The *IGF1* has been mapped to chromosomal 1p1.4–1.3 with approximately 166.0 cM, and is composed of 4 exons and 3 introns.[14,15] *IGF2* is known to be located at 14.8 Mb on chromosome 5, approximately 48.0 cM, and has 3 exons and 2 introns[16,17]. Sewalem et al.[12] found a QTL affecting BW at 6 wk at 160 cM (confidence interval 114 to 180 cM) on chromosome 1 in a broiler-layer F_2 population. McElroy et al.[13] reported one QTL for weight of abdominal fat pad at 168 cM (confidence interval 162 to 259 cM) on chromosome 1, and two QTLs of WM% (weight of the white meat percentage live weight) and FNTH (weight of the half of the carcass) at 48 cM on chromosome 5 in a F_2 population generated form two commercial lines.

As aforementioned, *IGF1* and *IGF2* represent two important positional candidates for chicken performance traits. Therefore the objective of the present study was to investigate the association of *IGF1* and *IGF2* with growth, carcass, and reproduction traits in Beijing You and Silkies, and whether they could be genetic markers in marker-assisted selection (MAS) of important economical traits.

MATERIALS AND METHODS

Animals and Phenotypic Data

A total of 1239 Beijing You Chicken, including 632 sires and 606 dams, and 1529 Silkies, consisting of 452 sires and 1077 dams, were used in this study. Twenty-nine male and 290 female parents were included in each line. All birds were fed in cages under the same conditions at the National Centre for Poultry Performance Testing. Both the two lines were native Chinese chicken breeds with specific performance characteristics. Beijing You chicken is a yellow-feathered and slow-growing meat-type quality breed, and quite valuable for the delicious meat and good egg quality. Silkie has fluffy plumage, dark blue flesh and bones, and possesses high medical value.

Phenotypic values for five growth traits and three body measurement traits were measured in each individual. Body weight was recorded at 8 (BW8), 10 (BW10), 13 (BW13), 17 (BW17), and 40 (BW40) weeks old, as well as at the first egg of age (BWAFE). Body measurement traits at 13 wk of age include shank length (SL13), shank circumference (SC13), and keel length (KL13). In addition, sixty males and sixty females were randomly selected from both Beijing You Chicken and Silkies, slaughtered and measured for carcass traits at 17 wk of age, including slaughter weight (SW17), carcass weight (CW17), eviscerated weight with giblet (EWG17), eviscerated weight (EW17), wing weight (WW17), leg weight (LW17),

leg muscle yield (LMY17), breast muscle yield (BMY17), abdominal fat weight (AFW17), and skinfold thickness (SFT17). For reproduction traits, the number of eggs (EN) was recorded daily for all dams from the AFE (age at first egg) until 40 wk of age and calculated every month until 40 wk, while egg weight (EW) was measured individually at 40 wk of age.

Genomic DNA Extraction

Approximately 1-ml blood samples were collected from the wing vein of each chicken, and stored at −20°C with ACD anticoagulant. Genomic DNA was isolated and purified using TIANamp Genomic DNA kit (TianGen, Beijing, China) according to the instructions of manufacturer with some modifications, and kept at −20°C.

PCR Amplifications and Genotyping

Restriction fragment length polymorphisms (PCR-RFLPs) were used to detect the SNP within the 5' flanking region of *IGF1*. By using Oligo 6.0, primers were designed according to the genomic sequence of chicken *IGF1* (GenBank accession no. EF198877). The forward and reverse primer sequences were 5′-TCATTTAAGATCCAGCCTCCA-3′ and 5′-AATATGAGCAGCACGGTTGA-3′ with an expected PCR product size of 470 bp, encompassing a distal 5' flanking region. The PCR reaction was carried out in a final volume of 25 μl containing 50 ng of template DNA, 2.5 μl of buffer, 2.0 μl of dNTP, 1.5 μl of Mg^{2+}, 0.6 μM of each primer, and 1.0 U of Taq DNA polymerase (Takara, Dalian, China). The profile of amplification was: 94°C for 7 min; 35 cycles with 95°C for 1 min, 56°C for 50 s, 72°C for 50 s; finally the extension was performed at 72°C for 7 min. The PCR products were digested at 37°C overnight with 10 U of *Pst*I (New England Biolabs, Beverly, MA, USA), and then electrophoresed on a 2% agarose gel with ethidium bromide.

Primer-introduced restriction analysis (PCR-PIRA) was performed to detect the SNP within the exon2 of *IGF2*. Based on the chicken *IGF2* sequence (GenBank accession no. AP003796), primers were designed through online application (http://cedar.genetics.soton.ac.uk/public_html/primer2.html) for amplification of the exon2 of *IGF2*, one of which contains a single-base mismatch close to its 3' end for introducing a *Msp*I (CCGG) recognition site into the PCR product.[18] The primer sequences were 5′-GTCAAGTCAGAGCGTGACCTCCC-3′ and 5′-CATAGAGCACCCAGCAGTCCTCC-3′ with an expected PCR product of 151 bp. The PCR reaction condition was the same as that for *IGF1* with the exception of annealing temperature of 64°C. The PCR products were digested at 37°C overnight with 10 U of *Msp*I (New England Biolabs, Beverly, MA, USA). Restriction digests were separated on a 5% agarose gel electrophoresis with ethidium bromide.

Sequencing

The PCR products from two homozygotes were isolated and purified with BioGene Geneclean III (Carlsbad, CA, USA) and then sequenced by an ABI 377 DNA sequencer.

Statistical Analysis

The association between each SNP and phenotypic value of each trait was analyzed using GLM procedure of SAS 8.02 (SAS Institute, 1989), respectively. Differences among genotypes were tested by using Scheffe multiple-range test for all traits investigated. The linear models are given as:

$$y = \mu + S + L + D(L) + G + e \quad (1)$$

$$y = \mu + S + L + D(L) + G + \beta \times \mathrm{CW17} + e \quad (2)$$

and

$$y = \mu + L + D(L) + G + \beta \times AFE + e \quad (3)$$

where Y is the phenotypic value of each trait; μ is overall mean; S is a fixed effect of sex; L is a fixed effect of paternal family; D (L) is a fixed effect of dam nested in the corresponding paternal family; G is a fixed effect corresponding to the genotype of IGF1-PstI or IGF2-MspI (AA, BB, or AB); CW17 acts as a co-variable for carcass traits; AFE denotes as a co-variable for egg weight (EW); β is a regression coefficient; e is the random residual. The additive and dominance effects were estimated according to the expressions $a = (AA - BB)/2$ and $d = AB - (AA + BB)/2$, respectively, where AA, AB, and BB are the genotypic values.

Hardy-Weinberg equilibrium was tested at each SNP locus using GENEPOP software.[19] The FREQ procedure of SAS 8.02 software (SAS Institute, 1989) was used to perform chi-square tests aimed to identify the existence of significant differences between breeds with regard to genotypic frequencies of IGF1-PstI and IGF2-MspI.

RESULT

Identification of Polymorphisms

With PCR-RFLP analysis, three kinds of genotypes, named AA (470 bp), AB (470 bp + 335 bp + 115 bp), and BB (335 bp + 115 bp), were observed at the SNP (IGF1-PstI) within the 5′ flanking region of *IGF1* gene in Silkies. However, only

Table 1 Genotypic and allele frequencies of IGF1-PstI and IGF2-MspI

	Beijing You		Silkies	
Genotype	Genotypic frequency	Allele frequency	Genotypic frequency	Allele frequency
IGF1-PstI				
AA	1.00	A = 1.00	0.18	A = 0.45
AB	0.00	B = 0.00	0.53	B = 0.55
BB	0.00		0.29	
IGF2-MspI				
CC	0.69	C = 0.84	0.78	C = 0.89
CD	0.29	D = 0.16	0.21	D = 0.11
DD	0.02		0.01	

AA genotype was found in Beijing You chickens. By comparing the sequences of two types of homozygotes, we found that IGF1-PstI showed a C/T substitution at position 279 locating 7 kb upstream *IGF1* promoter, which is the same as detected in other chicken populations.[6,10]

Table 2 P-values of associations between IGF1-PstI and IGF2-MspI and performance traits

	Beijing You		Silkies		
Traits[a]	IGF2-MspI	N[b]	IGF1-PstI	IGF2-MspI	N
Body weight					
BW8(g)	0.83	1239	0.04*	0.35	1529
BW10(g)	0.87	1239	0.01**	0.45	1529
BW13(g)	0.60	1239	0.04*	0.44	1529
BW17(g)	0.03*	1239	0.13	0.50	1529
BW40(g)	0.95	1239	0.74	0.63	1529
BWAFE(g)	0.58	413	0.08	0.38	820
Body measurement traits					
SL13(cm)	0.35	1239	0.01**	0.38	1529
SC13(cm)	0.98	1239	0.05*	0.59	1529
KL13(cm)	0.69	1239	0.20	0.90	1529
Carcass traits					
SW(g)	0.37	120	0.34	0.84	120
CW(g)	0.02*	120	0.78	0.87	120
EWG(g)	0.48	120	0.70	0.63	120
EW(g)	0.49	120	0.80	0.72	120
WW(g)	0.65	120	0.15	0.33	120
LW(g)	0.89	120	0.48	0.62	120
LMY(g)	0.85	120	0.56	0.67	120
BMY(g)	0.98	120	0.79	0.91	120
AFW(g)	0.75	120	0.90	0.35	120
SFT(mm)	0.42	120	0.75	0.90	120
Egg traits					
AFE(d)	0.75	413	0.15	0.18	820
Egg weight					
EWAFE(g)	0.99	413	0.62	0.33	820
EW40(g)	0.99	267	0.49	0.54	472
Number of eggs					
EN24	0.66	413	0.89	0.88	820
EN28	0.36	413	0.92	0.95	820
EN32	0.94	413	0.70	0.68	820
EN36	1.00	413	0.68	0.62	820
EN40	0.96	413	0.20	0.50	820

[a]BW8, BW10, BW13, BW17, BW40, BWAFE = body weight at 8, 10, 13, 17, 40 wk, respectively; BWAFE = body weight at the age of first egg; SL13, SC13, and KL13 = shank length, shank circumference and keel length at 13 wk respectively; SW = slaughter weight; CW = carcass weight; EWG = eviscerate weight with giblet; EW = eviscerated weight; WW = wing weight; LW = leg weight; LMY = leg muscle yield; BMY = breast muscle yield; AFW = abdominal fat weight; SFT = skinfold thickness; AFE = age at first egg; EWAFE = egg weight at first egg; EW40 = egg weight at first egg; EN24, EN28, EN32, EN34, EN36, EN40 = egg numbers from AFE to 24, 28, 32, 36, 40 wk, respectively.

[b]N means the number of animals for which the phenotypes were collected.

*$P < 0.05$; **$P < 0.01$.

Table 3 Comparison between different genotypic values, additive, and dominance effects of IGF1-PstI and IGF2-MspI on performance traits

	Silkies (IGF1-PstI)					Beijing You (IGF2-MspI)				
Traits[1]	AA	AB	BB	Additive effect	Dominance effect	CC	CD	DD	Additive effect	Dominance effect
BW8(g)	$594.57^{AB} \pm 5.60^{b}$	$603.77^{B3} \pm 4.06$	$584.51^{A} \pm 6.74$	5.05 ± 4.36	$14.23 \pm 5.91^{**}$	617.00 ± 3.52	620.65 ± 5.99	605.58 ± 40.87	5.72 ± 20.57	9.35 ± 21.11
BW10(g)	$780.02^{AB} \pm 7.08$	$795.69^{B} \pm 5.14$	$767.35^{A} \pm 8.56$	6.33 ± 5.52	$21.10 \pm 7.50^{**}$	791.11 ± 4.08	793.87 ± 6.95	773.40 ± 47.07	8.85 ± 23.69	11.61 ± 24.33
BW13(g)	$1012.45^{ab} \pm 8.68$	$1029.51^{b} \pm 6.53$	$1000.24^{a} \pm 10.79$	6.10 ± 6.94	$23.17 \pm 9.44^{**}$	990.68 ± 5.96	990.29 ± 10.23	921.64 ± 68.33	34.52 ± 34.40	34.14 ± 35.34
SL13(cm)	$8.57^{AB} \pm 0.05$	$8.65^{B} \pm 0.04$	$8.45^{A} \pm 0.06$	0.06 ± 0.04	$0.13 \pm 0.05^{**}$	8.50 ± 0.03	8.58 ± 0.05	8.72 ± 0.33	-0.01 ± 0.09	-0.02 ± 0.09
BW17(g)	1354.53 ± 12.57	1379.99 ± 9.19	1351.74 ± 14.95	13.42 ± 22.79	16.55 ± 31.65	$1317.86^{B} \pm 8.42$	$1296.86^{b} \pm 13.86$	$1096.69^{A} \pm 88.52$	$110.59 \pm 44.50^{**}$	$89.58 \pm 45.56^{*}$
CW17(g)	1172.79 ± 33.44	1175.91 ± 29.49	1145.94 ± 42.55	1.40 ± 9.70	26.85 ± 13.22	$1129.36^{B} \pm 34.68$	$1083.42^{B} \pm 55.79$	$753.40^{A} \pm 129.27$	$187.98 \pm 62.55^{**}$	$142.04 \pm 67.62^{*}$

[1]BW8, BW10, BW13, BW17 = body weight at 8, 10, 13, 17 wk respectively; SL13 = shank length at 13 wk; CW17 = carcass weight at 17 wk.

[2]Least square mean values (±SE).

[3]Different letters denoting significant difference between groups: $^{a,b}P < 0.05$, $^{A,B}P < 0.01$, $^{**}P < 0.01$, $^{*}P < 0.05$.

As for *IGF2*, three kinds of genotypes were found in both Beijing You and Silkies populations. The CC genotype showed a single band of 151 bp, while DD genotype, revealed a fragments of 129 bp and a short fragment of 22 bp, but the 22-bp band was not visualized on the 5% gel. Sequence analysis identified a substitution from C to G at the position 285 within exon2 of *IGF2* gene.

Allele and Genotypic Frequency

The allele and genotypic frequencies of IGF1-PstI and IGF2-MspI in Beijing You and Silkies are summarized in Table 1. At the IGF1-PstI site, the frequencies of alleles A and B were 45% and 55% in Silkies; however, all individuals of Beijing You detected in the present research expressed AA genotype. Chi-square test showed that the genotypes of IGF1-PstI for Silkies violated Hardy-Weinberg equilibrium ($P < 0.05$). At the IGF2-MspI site, the frequencies of alleles C and D were 84% and 16% in Beijing You, while were 89% and 11% in Silkies, respectively. The distribution of genotypes of IGF2-MspI in Silkies population is in Hardy-Weinberg equilibrium ($P > 0.05$). However, this locus exhibited a large number of birds with CD genotypes than that was expected from the Hardy-Weinberg equilibrium principle in Beijing You population ($P < 0.05$). Chi-square tests indicated that genotypic frequencies of IGF1-PstI and IGF2-MspI between Beijing You and Silkies differed significantly ($P < 0.05$).

Association of *IGF1* and *IGF2* Genes with Performance Traits

With marker-trait association analysis, we found that the associations with growth, carcass, and reproduction traits were not simultaneously found in two breeds. In Silkies, IGF1-PstI was significantly associated with BW8 ($P < 0.05$), BW10 ($P < 0.01$), BW13 ($P < 0.05$), SL13 ($P < 0.05$) and SC13 ($P < 0.01$) (Table 2). Multiple comparisons showed that AB genotype had highest values for BW8 ($P < 0.01$), BW10 ($P < 0.01$), BW13 ($P < 0.05$), and SL13 ($P < 0.01$), while BB homozygote had lowest values for these traits. However, individuals with AA and AB genotypes had higher SC13 than those with BB genotype ($P < 0.05$). Dominant and additive effects of IGF1-PstI genotypes were also estimated on BW8, BW10 BW13, SL13, and SC13. Significant dominant effects on BW8, BW10 BW13, SL13 ($P < 0.01$) and a significant additive effect on SC13 ($P < 0.05$) were found. Allele A was associated with increase in the SC13 value (see Table 3).

Additionally, IGF2-MspI was significantly associated with 17-wk body weight and carcass weight in Beijing You ($P < 0.05$, Table 2). Chickens with genotypes CC and CD showed significantly greater 17-wk body weight and carcass weight compared to those with DD genotype ($P < 0.01$). Also, we found there were significant additive and dominant effects on BW17 and CW17, and allele C was associated with higher value of BW17 and CW17 (see Table 3). However, there was no significant association observed between the genotypes of IGF2-MspI and any trait in Silkies ($P > 0.05$).

Discussion

In the present study, we found that the frequency of allele A on IGF1-PstI locus was higher in Beijing You (1.00) than that in Silkies (0.45). Furthermore, it

was different from the allele A frequency in White Leghorn (0.83) and Korean Native Ogol Chicken (0.43).[6,10] For IGF2-MspI, allele C was found in most chickens, which is the same as previous study.[11] The different breeding aims and selection intensity might be the main causes for such differences across chicken populations. It is interesting that the polymorphism analyzed in *IGF1* was associated with almost all body weight traits in Silkies while with no association with any reproduction traits in this study. In previous study,[10] association analysis revealed a significant influence of *IGF1* genotype on reproduction traits. In the case of growth weight at 8, 10, and 13 wk of age, the allele A was dominant and animals carrying this allele were significantly higher than those carrying allele B, this finding herein is consistent with previous research.[6,7] However Nagaraja et al.[10] and Amills et al.[5] revealed there are no significant associations between IGF1-PstI and growth traits. Such difference may arise from the different chicken populations and sample sizes.

Another SNP (IGF2-MspI), within the exon2 of *IGF2*, was associated with BW17 and CW17, but not associated with any reproduction traits in Beijing You, while chickens with different genotypes had no significant differences in all recorded traits in Silkies. In Beijing You, chickens expressing CC and CD genotype had higher BW17 and CW17 than DD ones. It can be deduced that allele C may be related to higher BW17 and CW17 and allele D may lead to lower BW17 and CW17. These results were not consistent with the association analysis reported by Wang et al.[11] since they found significant associations between this SNP and BW and carcass traits in a chicken population developed by Silkies reciprocally crossing to Broilers. In addition, we found that IGF2-MspI was only significantly associated with BW17, but not with BW8, BW10, and BW13. Growth is a very complicated trait. For Beijing You chicken, the period from birth to 13 weeks is defined as the early growth period while that between 13 and 17 weeks is a fast-growing period. There may be different genes controlling the chicken growth in different periods. This may be the reason for the difference results at different times. Also, because the CW DD genotypic group consists of only 2 or 3 birds, the putative significant difference is likely simply due to sampling and small group size.

The positional candidate gene approach considers a candidate after the establishment of its proximity to a QTL identified in the genome scan study.[20] It has been successfully used to find the QTL responsible for genetic variation in domestic animals.[21] Many QTLs have been detected in the regions including *IGF1* and *IGF2*.[12,13] In previous studies, both *IGF1* and *IGF2* have been considered to be positional candidate genes of growth and reproduction traits, because they are members of the growth hormone (GH) axis which affects growth, differentiation, reproduction, immune responsiveness, and aging.[22] In the current study, we found that the C/T mutation at base 279 in the 5′ region of *IGF1* and C/G mutation at base 285 in the exon2 of *IGF2* were significantly associated with chicken growth and carcass traits, which is consistent with previous reports.[5–9] Though the observed significant association of IGF1-PstI and IGF2-MspI with some performance traits in the present study, it is more likely that IGF1-PstI and IGF2-MspI may not be the causative mutations, but in linkage disequilibrium with the actual causative mutations that affect growth and carcass traits in our resource population because such association was mildly significant. Also, such associations did not achieve experimental significance. This may be due to a small sample size. To provide readers with reliable

information, we have listed the original P value in Table 2. Further investigation is needed in future endeavors, to consider expanding the type of experimental samples and species in the next step to further explore whether the significance level is up to the experimental level in a larger group.

In summary, these aforementioned results indicated that two SNPs within *IGFs* were associated with growth, body measurement, and carcass traits and could be a potential genetic marker in chicken breeding systems.

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