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Associations between GHR and IGF-1 Gene Polymorphisms, and Reproductive Traits in Wenchang Chickens*

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Abstract: Alleles of physiological candidate genes for reproductive traits, insulin-like growth factor-1 (IGF-1), and growth hormone receptor (GHR) were assessed to determine their associations with total egg production (NE), average days of continual egg-laying (ADCE), and number of double yolk eggs (DYE) in Wenchang chickens (Chinese indigenous breed). PCR-RFLP was used for genotype identification. The frequency of restriction enzyme C1/C2 alleles in the population was 0.53 (C1) and 0.47 (C2) for IGF-1. For GHR-intron 2 it was 0.06 and 0.94 for (A1) and (A2), respectively, while for GHR-intron 5 it was 0.20 (B1) and 0.80 (B2). Four significant associations were found ($P < 0.05$): between IGF-1 polymorphism, and NE (300 d), NE (400 d), and ADCE, and between GHR polymorphism and DYE. Two significant effects were observed: for IGF-1 and NE (300 d), and for GHR intron 2 and DYE. The current research supports the effects of GHR and IGF-1 genes on the reproductive traits of chickens.

Key Words: Chicken, IGF-1, GHR, reproduction, single nucleotide polymorphism (SNP)

Introduction

The Wenchang chicken is a special indigenous breed in China. They have a small body and a dual purpose for meat and egg production. The qualities of the chicken meat and eggs suit Chinese consumers' tastes. It is, therefore, necessary to study the Wenchang chicken by the molecule marker method in an effort to efficiently improve its reproductive traits, which will help to meet the market demand for increased production.

Most traits of economic importance in farm animals show continuous variation; however, their underlying genetic natures are very complex. Molecular marker-assisted selection is efficient and leads to improvements in production performance. A candidate gene approach is a powerful method for understanding the direct genetic basis involved in the expression of quantitative differences between individuals (1,2).

Reproduction is a comprehensive reflection of the development of various parts of a chicken body and its final expression is the result of interaction among genetic, nutritional, and environmental factors. The components

that constitute the growth hormone (GH) axis have a major influence on a diverse array of biological processes, ranging from growth and differentiation to reproduction (3-5). The growth hormone receptor (GHR), insulin-like growth factor-1 (GH-IGF-1) system controls the number of follicles in animals that are recruited to the rapid growth phase (6,7). It is also known that the GH-IGF-1 system has been modified as a result of selection for enhanced growth rate (8,9). In chickens divergently selected for high or low growth rates, there were significantly higher IGF-1 mRNA levels in the high growth rate line than in the low growth rate line (10). There are obvious physiological connections between body weight homeostasis and the reproductive axis in both sexes. The rate of sexual maturation is much more closely associated with body growth than with chronological age (11).

Some understanding of the genetic architecture of quantitative traits may be gained by systematically analyzing genetic markers in major metabolic pathways. In addition to this, the major endocrine pathway mediated by the hypothalamus, pituitary gland, liver, other tissues that produce GH and IGF-1 have been identified,

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indicating that these hormones, together with their receptors and binding proteins, provide a complex regulatory network that coordinates a multitude of traits (12). Several studies have shown that the GH-IGF-1 system affects reproductive traits. Thus, the IGF-1 gene was chosen as a candidate gene that might be associated with egg-laying performance or double yolk egg (DYE) production (13). In addition, researchers have demonstrated that the identified markers in the IGF-1 and GH-receptor genes, which are still segregating in many non-inbred strains of White Leghorn chickens, are associated with changes in body weight (14) and egg production (2,15).

The objective of the present study was to identify polymorphisms of GHR and IGF-1 genes by developing PCR-RFLP methods to detect those DNA polymorphisms in Wenchang chickens (Chinese indigenous breed). In particular, we searched for a genotypic interaction between the 2 genes, and analyzed the effects of genotype on the relationship between these polymorphisms and reproductive traits of Wenchang chickens.

Materials and Methods

Experimental Chickens and Traits

The study included 120 purebred Wenchang chickens from Hainan province that were bred in the poultry quality testing center of the Chinese Ministry of Agriculture. Data on egg production, including total egg production, continual egg-laying, and number of DYE, were collected daily using trap nests to identify individual birds. Data for each hen were collected during an 8-month experimental period, which began at 25 weeks of age. All birds were raised in the same conditions, and were fed a commercial corn-soybean-based diet that met all NRC requirements (16) and fresh water ad libitum. DNA and trait data were obtained from 117 birds.

Establishment of a PCR Assay

Blood was sampled from plumage veins and collected in test tubes containing an anticoagulant solution. Genomic DNA was isolated and eluted into 350 μ l of Tris-EDTA (TE) buffer. A 718-base pair (bp) fragment of the GHR gene intron 2 (GHR-intron 2) was amplified by polymerase chain reaction (PCR) using forward (5'-GGCTCTCCATGGGTATTAGGA-3') and reverse (5'-GCTGGTGAACCA ATCTCGGTT -3') primers (14). The

applied cycles were denaturation at 94 °C for 5 min, followed by 35 cycles. Each cycle was 45 s at 94 °C, 45 s at 59 °C, and 60 s at 72 °C, followed by synthesis at 72 °C for 10 min. Primers used to detect the GHR gene intron 5 (GHR-intron 5) were 5'-ACGAAAAGTGTTCAGTGTGA-3' (forward) and 5'-TTTATCCCGTGTCTCT TGACA-3' (reverse). The PCR reaction conditions were the same as for GHR-intron 2, except that the annealing temperature was 56 °C (17).

A 621-base pair (bp) fragment of the IGF-1 gene was amplified by PCR using forward (5'-GACTATACAGA AAGAACCAC-3') and reverse (5'-TATCACTCAAGTG GCTCAAGT-3') primers (2). The applied cycles were denaturation at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C, 45 s at 60 °C, 60 s at 72 °C, and final synthesis at 72 °C for 10 min.

Statistical Analysis

Data for 300-day egg production (NE 300 d), 400-day egg production (NE 400 d), ADCE, and the number of DYE were obtained from farm records. Statistical calculations were performed using SPSS procedures. Frequencies of distribution of alleles within the lines were compared with the chi-square test. The effects of IGF-1 and GHR genotypes on egg production were analyzed using the GLM procedure (SAS 8.0). The following model was used: $Y_{ijk} = \mu + G_i + I_k + B_{ik} + E_{ijk}$, where Y_{ijk} is the trait analyzed in 2 lines, μ is the overall mean, G_i is the fixed effect of the GHR marker genotypes (including intron 2 and intron 5), I_k is the fixed effect of the IGF-1 marker genotypes, B_{ik} is the interaction between the 2 genotypes, and E_{ijk} is random error.

As the interaction term was not significant for any of the traits analyzed, the model was subsequently reduced to $Y_{ijk} = \mu + G_i + I_k + E_{ijk}$.

Screening for Restriction Enzyme-Detectable Single Nucleotide Polymorphisms

PCR of DNA from each bird was performed according to the conditions described above. For GHR-intron 2, the PCR product was digested overnight using 15 U of Hind III enzyme at 37 °C. The digestion products were separated by horizontal electrophoresis (50 volts, 60 min) in 2% agarose gels in 1 \times TBE and 1.0 μ M of ethidium bromide. For GHR-intron 5, 10 U of NspI was used to digest at 37 °C overnight and the digested products were electrophoresed for 1 h at 80 V on 2.5% agarose gel. For the IGF-1 gene, 10 U of PstI was used

to digest at 37 °C overnight and the digested products were electrophoresed for 1 h at 100 V on 3.5% agarose gel. Individual PCR-RFLP fragment sizes of each gene were determined by visualizing the banding pattern under ultraviolet light (Table 1).

Results

Sequence Variation and PCR-RFLP Analysis

For GHR, the 718-bp product from the intron 2 was sequenced for each Wenchang chicken. The following DNA restriction fragments were obtained for GHR-Hind III polymorphism: 428 bp/290 bp for the A₁A₁ genotype and 258 bp/170 bp/290 bp for the A₂A₂ genotype (Figure 1). Accordingly, we revealed an additional Hind III site located 250 bp upstream of the polymorphic Hind III site. For GHR-intron 5, a 740-bp fragment was amplified and 2 single nucleotide polymorphisms (SNPs) were discovered that were linked, both containing cytosine-thymidine transversions (Figure 2). The 2 genes' genotypes differed from the expected Hardy-Weinberg equilibrium (Table 2).

For IGF-1, a 621-bp fragment of 5'-UTR (5'-untranslated region) was obtained. The restriction enzyme PstI-digested PCR products had fragments of 257 and 364 bp for the C₂C₂ genotype, 257, 364, and 621 bp for the C₁C₂ genotype, and 621 bp (no digestion) for the C₁C₁ genotype (Figure 3). The observed distribution of genotypes was not different than the distribution expected under the assumption of Hardy-Weinberg equilibrium (Table 2).

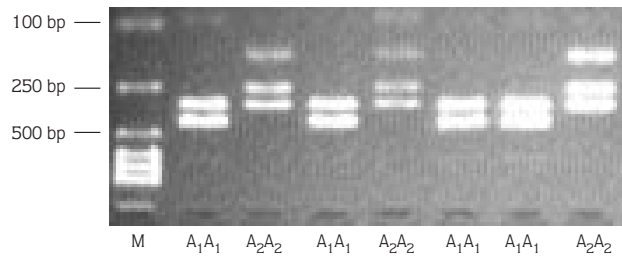


Figure 1. PCR-RFLP pattern for GHR-intron 2 with *Hind III* digestion.

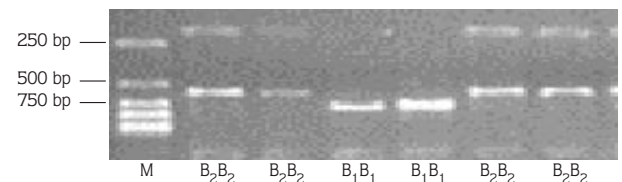


Figure 2. PCR-RFLP pattern for GHR-intron 5 with *NspI* digestion.

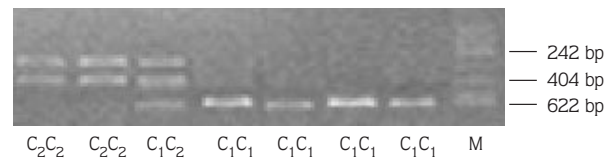


Figure 3. PCR-RFLP pattern for 5'UTR of IGF-1 with *PstI* digestion.

Associations between Genotypes and Traits

Associations between genotypes and egg production traits were initially analyzed using a linear model that included terms for the GHR genotype, the IGF-1 genotype, and the interaction between the 2 genotypes; however, the interaction term was not significant ($P > 0.05$) and was therefore removed from the model.

Table 1. Gene polymorphic loci and sources.

Gene	Location	Position	Diagnostic Enzyme	Type of polymorphism
GHR	Intron 2	294 and 541	<i>Hind III</i>	A/G transversion
GHR	Intron 5	571	<i>NspI</i>	C/T transversion
IGF-1	5'-UTR	364	<i>PstI</i>	C/T transversion

Table 2. Frequency of GHR and IGF-1 genotypes and alleles.

Genes	Genotype Frequency			Allele Frequency		χ^2
GHR-intron 2	0.06 (A ₁ A ₁)	0.94 (A ₂ A ₂)		0.06 (A ₁)	0.94 (A ₂)	89.97
GHR-intron 5	0.20(B ₁ B ₁)	0.80(B ₂ B ₂)		0.20 (B ₁)	0.80 (B ₂)	45.07
IGF-1	0.32(C ₁ C ₁)	0.41(C ₁ C ₂)	0.27(C ₂ C ₂)	0.53 (C ₁)	0.47 (C ₂)	3.64

There were no associations between the GHR gene, and the NE and ADCE traits; however, we were able to detect associations between the DYE trait and the GHR-intron 2 marker in single analyses. A significant association between GHR-intron 2 polymorphism and the number of DYE was found ($P < 0.05$) (Table 3), as well as an additive effect of GHR-intron 2 on the DYE trait ($P < 0.05$).

There were no associations between the IGF-1 gene and the DYE trait; however, significant ($P < 0.05$) associations were found between IGF-1 polymorphism, and NE 300 d, NE 400 d, and ADCE (Table 4). An additive effect of IGF-1 on NE 300 d was also observed ($P < 0.05$).

Discussion

Reproduction is a composite of complex developments that are influenced by genetic, nutritional, and environmental factors. Although association studies cannot determine if the GHR and IGF-1 gene allele markers are responsible for the variation in a particular trait or whether the variation is due to a closely linked

locus, we think that 2 genes would influence the traits in chickens.

Indications that IGFs may be involved in avian reproductive performance come from previous in vivo studies that used injections of GH, gonadotropins, and even IGFs. Hocking et al. (13) and Bruggeman et al. (18) found higher levels of IGF-1 in the systemic blood of food-restricted broiler breeder hens during rearing than in those that were fed ad libitum. The injection of IGF-1 in sex-linked dwarf chickens that lack GH receptors resulted in increased reproductive performance (19). The number of follicles in laying hens increased after GH or gonadotropin injection (20). The latter studies suggest that IGF is a local mediator of GH or gonadotropin action in the ovary. Thus, changes in the GH/IGF axis may be associated with poor reproductive performance.

Recent in vitro studies using cell cultures showed that IGF-1 and IGF-2 have major roles to play in avian ovarian function. Both IGFs regulate follicular growth and differentiation. This is consistent with the localization of the IGFs and their receptors in ovarian cells.

In the present study the associations detected by analyzing a single generation of Wenchang hens suggest

Table 3. Correlation analysis between GHR genotypes and egg-laying traits.

Traits	GHR-intron 2		Additive	GHR-intron 5		
	A ₁ A ₁	A ₂ A ₂		B ₁ B ₁	B ₂ B ₂	Additive
NE 300 d	84.14	84.46	-0.006	83.23	85.22	-0.067
NE 400 d	129.43	130.87	-0.016	129.55	131.42	-0.035
ADCE	2.72	3.01	-0.054	3.04	3.00	0.011
DYE	1.00 ^a	0.30 ^b	0.208*	0.27	0.32	-0.027

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).
* $P < 0.05$.

Table 4. Correlation analysis between IGF-1 genotypes and egg-laying traits.

Traits	IGF-1 Genotype			Additive	Dominant
	C ₁ C ₁	C ₁ C ₂	C ₂ C ₂		
NE 300 ^d	82.61 ^a	82.94 ^a	89.03 ^b	0.199*	0.106
NE 400 ^d	127.82 ^a	128.56 ^{ab}	137.84 ^b	0.177	0.089
ADCE	2.96 ^a	2.78 ^a	3.38 ^b	0.114	0.143
DYE	0.34	0.29	0.42	0.034	0.053

^{a,b}Means within a row without a common superscript differ significantly ($P < 0.05$).
* $P < 0.05$.

that the GHR gene plays a role in controlling the DYE trait and the IGF-1 gene affects NE and ADCE. Some previous studies have identified markers in the GH and GHR genes, which continue to segregate in many non-inbred strains of White Leghorn chicken, and have shown that they are associated with changes in body weight (14) and the rate of egg production (15). Nagaraja et al. (2) found that the IGF-1 genotype significantly influences egg weight and specific gravity. Differences exist between the results of previous studies and our study, which may be due to the

fact that SNP identified different alleles in these unrelated populations.

In conclusion, the current study found strong evidence of significant and simultaneous beneficial effects of GHR-SNP and IGF-1-SNP associated with chicken reproductive traits. Whether or not the behavior of GHR and IGF-1 variants represents a paradigm for other genes remains to be determined. Furthermore, the same genetic variants may have different effects in different genetic backgrounds.

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