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Association analysis of *IGF-I* gene expression with growth and reproductive traits in Jinghai yellow chickens

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ABSTRACT. The insulin-like growth factor, IGF-I, plays an important role in the development of growth and reproductive traits. Single-strand conformation polymorphism analysis was used to detect and analyze polymorphisms and expression profiles of the *IGF-I* gene and its association with growth and reproductive traits of Jinghai yellow chickens. A point mutation g.295T>C was detected in the *IGF-I* gene

with three genotypes CC, CT, and TT. The CT and TT genotypes were found to be significantly ($P < 0.05$) superior to the CC genotype in 8-, 10-, 12-, 14-, and 16-week-age weight of chickens (for growth traits) and in body weight at first egg-laying (for reproductive traits). Comparison of the expression level between males and females showed similarity in their expression curves, with females showing relatively higher *IGF-I* expression than males in all studied tissues. A similar *IGF-I* expression pattern was observed in the breast and leg muscles of both males and females, with the leg muscle showing relative higher *IGF-I* expression than the breast muscle. Our results indicate that, g.295T>C mutation in the *IGF-I* gene affects certain growth and reproductive traits, and it could be used to provide a theoretical basis as well as marker-assisted selection to upgrade the development of Jinghai yellow chickens in future.

Key words: Jinghai yellow chicken; Growth traits; Reproductive traits; Single nucleotide polymorphism

INTRODUCTION

The Jinghai yellow chicken was developed by traditional genetic breeding of the local yellow chickens in Nantong city, Jiangsu Province, China, for five generations (Zhang et al., 2015b). It is considered as a national cultivated meat breed characterized by its adaptability to poor quality feeds and environment (Zhao et al., 2012). According to previous studies, the Jinghai yellow chicken could be considered as a promising meat breed in terms of growth as many detected single nucleotide polymorphisms (SNPs) were associated with their growth and reproductive traits (Tang et al., 2014; Abdalhag et al., 2015; Zhang et al., 2015a; Wang et al., 2016). Insulin-like growth factors, IGF-I and IGF-II, are mitogenic polypeptides with a structure similar to insulin, and play an important role in the overall body and muscle growth in many species (Rotwein, 1991). The *IGF-I* gene is considered an essential player in many biological process by improving the metabolism and cell proliferation as well as doing its function by obligating to specific receptors (Zhou et al., 1995). The avian *IGF-I* affects the metabolic processes by increasing the amino acid and glucose uptake, thereby affecting the synthesis of DNA as well as proteins (McMurtry, 1998). The *IGF-I* gene is responsible for stimulating the differentiation, proliferation, and metabolism of a number of myogenic cell lines of many species (Duclos et al., 1999). *IGF-I* influences growth hormone by mediating growth-promoting effects in all mammals. The *IGF-I* gene is located on chromosome 1 (Darling and Brickell, 1996; Klein et al., 1996; Huo et al., 2006), and is significantly associated with skeletal traits, body composition, and growth traits (Amills et al., 2003; Zhou et al., 2005; Gouda and Essawy, 2010; Shah et al., 2012). The mRNA expression profile of the *IGF-I* gene could be useful for studying its association with body weight and feed conservation (Shah et al., 2012). In chickens, the molecular characterization of the *IGF-I* gene has provided a valuable evidence to understand its expression and regulation mechanisms (Shah et al., 2012). *IGF-I* gene expression can be detected in whole-chick embryos soon after fertilization through day 8 (de Pablo et al., 1990). Interestingly, *IGF-I* gene expression cannot be detected in the liver until after hatching (Kikuchi et al., 1991). Recently, many studies were conducted to detect mutations in the *IGF-I* gene, and to understand its association with growth and reproductive

traits, especially in large farm animals. In contrast, limited studies have been conducted in the chicken sector. In the present study, we studied the SNPs of the *IGF-I* gene in order to detect mutations, which might affect the growth and reproductive traits in Jinghai yellow chickens. Molecular biology techniques, including polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP), and real-time PCR (qPCR), were used in this study to analyze the polymorphisms, expression profiles, and expression regularity of the *IGF-I* gene in order to understand its association with growth and reproductive traits in Jinghai yellow chickens.

MATERIAL AND METHODS

Population and samples collection

A total of 336 blood samples were collected from the female Jinghai yellow chickens randomly selected from the same batch at the Jiangsu Jinghai Poultry Industry Group Co., Ltd., Jiangsu, China. All experimental chickens were hatched on the same day, and raised in floor pens with access to water and feed with commercial diets meeting the National Research Council nutrient requirements. The body weights of chickens were recorded on day 1 (BW0), and subsequently at weeks 2 (BW2), 4 (BW4), 6 (BW6), 8 (BW8), 10 (BW10), 12 (BW12), 14 (BW14), and 16 (BW16).

DNA extraction and genotyping

Genomic DNA was extracted from the blood samples using the phenol-chloroform extraction method, and dissolved in TE (pH 8.0) buffer: 10 mM TrisCl (pH 8.0); 10 mM EDTA (pH 8.0). The genomic DNA samples were quantified using a NanoDrop® Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and then stored at -20°C until further use.

Primer design and PCR amplification

Based on the chicken *IGF-I* gene sequence (GenBank accession No. NC_006088.4, Gene ID 418090), the Primer Premier 5® software (Premier Biosoft, Palo Alto, CA, USA) was used to design three pairs of primers to amplify parts of the exon regions of the *IGF-I* gene. For expression profile analysis, two pairs of primers were designed to perform qPCR (Table 1). All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The PCR was performed in a 20-μL reaction volume comprising 1.5 μL chicken genomic DNA (50 ng/μL), 10 μL 2X Taq Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China), 0.4 μL forward primer (10 μM); 0.4 μL reverse primer (10 μM), and 7.7 μL sterilized distilled water. The following amplification conditions were used: initial denaturation at 94°C for 7 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 10 min. The PCR products were verified by 10% non-denaturing polyacrylamide gel electrophoresis.

Table 1. Primer sequences for single-strand conformation polymorphism (PCR-SSCP) and qPCR of the *IGF-I* gene.

Primer's purpose	Primer name	Sequence (5'→3')	Annealing temperature (°C)	Product size (bp)	Location
Polymorphisms	P1 (IGF-I)	F: TGCACTTTTAAGAAGCAATGGA	59	246	Exon 1
		R: CAGCCATTTTCCAGATCACA			
	P2 (IGF-I)	F: CTTCCTTGGCAGGTGAAGAT	60	195	Exon 2
		R: GCAGTTGAATGAAAGGGTTGA			
	P3 (IGF-I)	F: ACAGGGTATGGATCCAGCAG	60	159	Exon 3
		R: CATATCAGTGTGGCGCTGAG			
Real-time PCR	P4 (IGF-I)	F: TGTACTGTGCTCCAATAAAGC	60	127	
		R: CTGTTTCCTGTGTTCCCTCTACTT			
Reference gene	P5 (β-actin)	F:CAGCCATCTTTCTTGGGTAT	60	165	
		R: CTGTGATCTCCTTCTGCATCC			

SSCP and sequencing

All amplified DNA samples were analyzed individually by SSCP as follows: 2 μL each amplification product was mixed with 7 μL denaturing buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 10 mM EDTA, pH 8.0, and 2% glycerol) and heated at 98°C for 10 min, followed by cooling on ice for 10 min. Subsequently, the denatured PCR products were subjected to 10% non-denaturing polyacrylamide gel (29:1) electrophoresis first at 200 V for 5 min, and then at 100 V for 10 to 12 h at 15°-25°C. The SSCP patterns on the polyacrylamide gels were visualized by silver staining according to the method of Bassam et al. (1991). For each genotype, four PCR product samples were selected to be sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Expression profile analysis of *IGF-I*

Total RNA was isolated from the heart, liver, spleen, lung, kidney, breast muscle, leg muscle, abdominal fat, hypothalamus, and small intestine of four male and female Jinghai yellow chickens on day 1 (W0), and subsequently at weeks 4 (W4), 8 (W8), 12 (W12), 16 (W16), and 20 (W20). The recovered RNAs were reverse-transcribed into cDNAs using the PrimeScript™ RT Master Mix kit (Takara Biotechnology Co., Ltd, Dalian, China). The qPCR was performed to determine the expression profiles of the *IGF-I* gene using SYBR® Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd, Dalian). The qPCR was performed in 20-μL reaction volume comprising 1 μL chicken cDNA, 10 μL SYBR® Premix, 0.4 μL 50X ROX Reference Dye II (Takara Biotechnology Co., Ltd, Dalian), 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), and 7.8 μL sterilized dH_2O. The following amplification conditions were used: holding stage (initial denaturation at 95°C for 30 s); cycling stage (40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 34 s); melting curve stage (95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s). Based on the expression profile results, the breast and leg muscles were chosen for studying the *IGF-I* expression pattern, and the heart sample was used as a reference. The total RNA was extracted, and qPCR was performed using the same reaction conditions as mentioned above. The *β-actin* gene was selected as a reference gene for expression analysis. The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method as described by (Livak and Schmittgen, 2001) to determine the relative changes in *IGF-I* gene expression.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0. The general linear model was used to analyze associations between the SNPs and nine growth and six reproductive traits according to the following equation:

$$Y_{ij} = \mu + G_i + e_j \quad \text{(Equation 1)}$$

where Y_{ij} is the phenotypic value of target traits, μ is the overall mean, G_i is the genotype effect of the *IGF-I* gene, and e_j is the random error.

The genotype and allele frequencies were calculated according to the following Equations 2 and 3, respectively:

$$Pi = \frac{Ni}{N} \quad \text{(Equation 2)}$$

where P_i is the genotype frequency, *Ni* is the number of different genotypes, and *N* is the total genotypes number.

$$Fi = \frac{2Nii + Nij}{2N} \quad \text{(Equation 3)}$$

where F_i is the allele frequency, *Nii* is the number of iith genotype, *Nij* is the number of ijth genotype, and *N* is the total genotypes number.

All SNPs were tested for Hardy-Weinberg equilibrium using the chi-square (χ^2) test:

$$X^2 = \sum_{i}^{n} \frac{(Oi - Ei)^2}{Ei} \quad \text{(Equation 4)}$$

where *Ei* is the expected value, *Oi* is the observed value, and *n* is the number of observations.

RESULTS

Polymorphisms of the *IGF-I* gene and genetic variation analysis

Products amplified by the primer pair P1 displayed polymorphisms as detected by PCR-SSCP. Three genotypes, CC, CT, and TT, were observed in Jinghai yellow chickens (Figure 1). The comparison between the sequences of three genotypes (sequenced by Sangon Biotech) indicated that the nucleotide mutation g.295T>C was located in exon 1 of the *IGF-I* gene in chromosome 1 at position number 55335498 (Figure 2). Sequence analysis by using the DNAMAN software package, version 5.2.2 (Lynnon BioSoft, USA) showed that the mutation g.295T>C resulted in an amino acid change from cysteine to arginine (Figure 3).

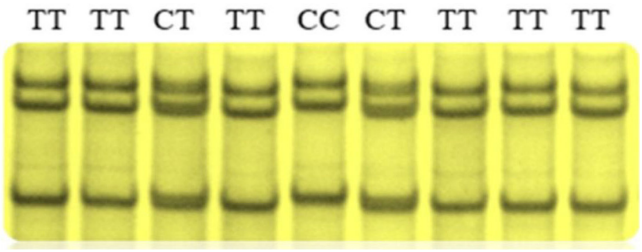

Figure 1. Polymerase chain reaction-based single-strand conformation polymorphism (PCR-SSCP) analysis of the mutation g.295T>C in the *IGF-I* gene.

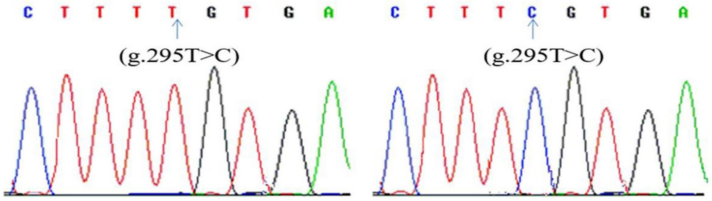

Figure 2. Sequencing of the point mutation g.295T>C in the exon 1 of the *IGF-I* gene.

Figure 3. Amino acid sequences of the wild-type and mutant (g.295T>C) *IGF-I* genes.

Allele and genotype frequencies of SNPs in the *IGF-I* gene

Genetic variation of the three genotypes based on the mutation g.295T>C was analyzed. The genotype frequencies of TT, CT, and CC, and allele frequencies of T, and C were calculated. The results showed that the genotype frequencies of the mutation g.295T>C were 0.423, 0.420, and 0.158 in CC, CT, and TT, respectively. The allele frequencies of C and T were 0.632 and 0.368, respectively. The chi-square test result showed that the polymorphism site fits the Hardy-Weinberg equilibrium ($P > 0.05$).

Association analysis with growth traits

Association between the mutation g.295T>C and growth traits of Jinghai yellow chickens was analyzed in three different genotypes CC, CT, and TT (Table 2). Significant

differences ($P < 0.05$) were observed in the BW8, BW10, BW12, BW14, and BW16 of the three genotypes, with the CT and TT genotypes being superior to the CC genotype in all these week-age weights. However, no significant differences were observed in the BW0, BW2, BW4, and BW6 of the three genotypes.

Table 2. Association of the mutation g.295T>C in exon 1 of the *IGF-I* gene with growth traits in Jinghai yellow chickens.

Traits	Genotype			SIG
	CC (53)	CT (141)	TT (142)	
First-day weight	33.40 ± 0.48	33.65 ± 0.29	33.17 ± 0.29	NS
2-week-age weight	117.62 ± 2.04	119.18 ± 1.25	117.18 ± 1.24	NS
4-week-age weight	244.43 ± 4.49	251.42 ± 2.76	248.13 ± 2.75	NS
6-week-age weight	454.43 ± 7.34	466.10 ± 4.50	458.38 ± 4.48	NS
8-week-age weight	576.89 ± 9.37^{b}	602.66 ± 5.74^{a}	607.15 ± 5.72^{a}	*
10-week-age weight	753.87 ± 11.19^{b}	780.39 ± 6.86^{a}	784.23 ± 6.83^{a}	*
12-week-age weight	931.51 ± 12.85^{b}	964.81 ± 7.88^{a}	966.02 ± 7.85^{a}	*
14-week-age weight	1077.45 ± 14.42^{b}	1119.55 ± 8.84^{a}	1125.08 ± 8.81^{a}	*
16-week-age weight	1266.30 ± 16.54^{b}	1309.96 ± 10.14^{a}	1310.49 ± 10.10^{a}	*

Means in the same row with different superscripts differ significantly; SIG = significance; *significant ($P < 0.05$); NS = not significant; the numbers between two brackets refer to genotype numbers.

Association analysis with reproductive traits

Association between the mutation g.295T>C and reproductive traits of Jinghai yellow chickens was analyzed in three different genotypes CC, CT, and TT (Table 3). Significant differences ($P < 0.05$) in the reproductive traits of the three genotypes were observed only in chicken weight at the first egg-laying, with the CT and TT genotypes being superior to the CC genotype. No significant differences were observed in the reproductive traits of the three genotypes, with respect to their age at the first egg-laying, weight of first egg, chicken weight at day 300, average egg-weight at day 300, and egg number at day 300.

Table 3. Association of the mutation g.295T>C in exon 1 of the *IGF-I* gene with reproductive traits in Jinghai yellow chickens.

Traits	Genotype			SIG
	CC (53)	CT (141)	TT (142)	
Age at first egg-laying	143.18 ± 1.67	146.56 ± 1.03	146.01 ± 1.02	NS
Chicken weight at the first egg-laying	1578.11 ± 24.90^{b}	1653.69 ± 15.26^{a}	1649.68 ± 15.21^{a}	*
Weight of the first egg	33.40 ± 0.93	33.30 ± 0.57	33.10 ± 0.57	NS
Chicken weight at day 300	2000 ± 40.40	2025.43 ± 24.77	2020.74 ± 24.68	NS
Average egg weight at day 300	50.82 ± 0.67	50.96 ± 0.42	50.88 ± 0.42	NS
Egg number at day 300	109.34 ± 0.97	109.67 ± 2.44	109.76 ± 2.43	NS

Means in the same row with different superscripts differ significantly; SIG = significance; *significant ($P < 0.05$); NS = not significant; the numbers between two brackets refer to genotype numbers.

Expression profile of the *IGF-I* gene in male and female Jinghai yellow chickens

To identify tissue distribution of the *IGF-I* gene, the qPCR was applied to 10 different tissues, including heart, liver, spleen, lung, kidney, breast muscle, leg muscle, abdominal fat, hypothalamus, and small intestine, of both male and female Jinghai yellow chickens. A relatively high expression of the *IGF-I* gene was detected in the liver, lung, and leg muscle

tissues of both male and female chickens as compared to other tissues. A relatively low level of *IGF-I* expression was detected in the kidney tissues of male chickens and the small intestine tissues of the female chickens. The female chickens were found to have higher *IGF-I* expression in the spleen, lung, kidney, abdominal fat, hypothalamus, and small intestine tissues, while the male chickens showed higher *IGF-I* expression only in the liver, breast muscle, and leg muscle tissues, considering the *IGF-I* expression level in heart as the reference (Figure 4).

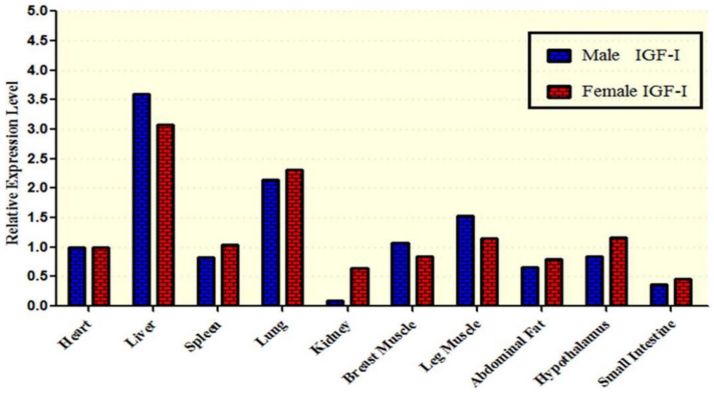

Figure 4. Comparison of the expression level of the *IGF-I* gene between male and female Jinghai yellow chickens.

Expression regularity of the *IGF-I* gene in male and female Jinghai yellow chicken

To study the expression regularity of the *IGF-I* gene in the breast and leg muscles, qPCR was applied to four male and four female Jinghai yellow chickens at W0, W4, W8, W12, W16, and W20 weeks of age. The expression pattern of the *IGF-I* gene in the breast and leg muscles was relatively similar in both male and female chickens. The qPCR results showed a relatively higher *IGF-I* expression in the leg muscle than in the breast muscle at all weeks of age both in male and female chickens. The *IGF-I* expression level at week 0 was taken as the reference (Figure 5).

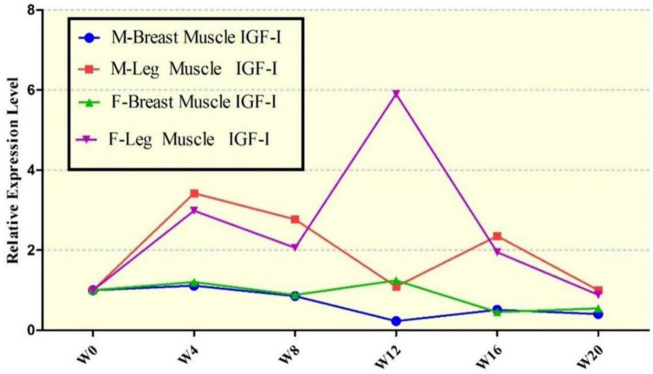

Figure 5. Expression level of the *IGF-I* gene in the breast and leg muscles of male and female Jinghai yellow chickens.

DISCUSSION

Previous studies have indicated that the growth is restricted by different genes, each of which has a little effect, while a fewer genes might be responsible for a relatively large ratio of genetic variation (Doosti et al., 2013). The researchers suggested that the genes, which are involved in the biology of traits, like genes coding for hormones and factors including *GH*, *GHR*, *STAT5*, *IGF-I*, and *IGF-II*, are considered as candidate genes for association studies (Siadkowska et al., 2006). A previous study showed no significant associations of the growth traits with the four SNPs for the *IGF-I* gene, when they studied their associations with growth and feeding traits in two strains of the Black Penedesenca chicken breed (Amills et al., 2003). However, they found significant associations between the *IGF-I* (SNP1) and average daily gain at day 107 and feed efficiency at 44, 73, and 107 days. Another study showed that a mutation of the *IGF-I* gene was associated with the egg and egg-shell weight in White Leghorn chickens (Nagaraja et al., 2000). Our findings indicated that, the mutation g.295T>C in the *IGF-I* gene was significantly associated with the growth and reproductive traits in Jinghai yellow chickens. This mutation in exon 1 of the *IGF-I* gene was significantly ($P < 0.05$) associated with the 8-, 10-, 12-, 14- and 16-week-age weights as well as chicken weight at the first egg-laying. This result is in agreement with the result reported by (Khadem et al., 2010) they found that the total number of eggs was significantly associated with the *IGF-I* gene, when they studied the SNPs of *IGF-I* and other genes in breeder hens of Mazandaran native fowls.

The results of the allele frequencies of the mutation g.295T>C in the *IGF-I* gene showed that this mutation was fit to the Hardy-Weinberg equilibrium, when tested by the chi-square test.

The expression of the *IGF-I* gene can be detected throughout the post-hatch growth in chickens, mostly in their livers (Rosselot et al., 1995; Tanaka et al., 1996). In order to determine the tissue distribution of the *IGF-I* gene, we applied qPCR to the 10 tissues, including heart, liver, spleen, lung, kidney, breast muscle, leg muscle, abdominal fat, hypothalamus, and small intestine, of both male and female Jinghai chickens. Our result showed that the expression level of the *IGF-I* gene was higher in the liver of both male and female chickens. This result is in agreement with the previous studies, which indicated that the liver is the major endocrine source of IGFs (Zahran and Aboul-Soud, 2007; Georgiev, 2010). Our result also showed that the expression pattern of the *IGF-I* gene was relatively difficult to assess among all studied ages, from W0 to W20, in the breast and leg muscles of both male and female Jinghai yellow chickens. A previous study strongly suggested a highly variable relationship between the circulating IGF-I and post-hatch growth in chickens (Vasilatos-Younken and Scanes, 1991). In contrast, another study showed that the circulating *IGF-I* gene increases with increasing age (McMurtry et al., 1997). Our result also showed that the expression level of the *IGF-I* gene was higher in the leg muscle than in the breast muscle at all week-ages in both male and female Jinghai yellow chickens. A previous study showed that *IGF-I* mRNA expression during hepatic development in rat and found that the fetal livers are characterized by relatively low levels of *IGF-I* mRNA as compared to the adult livers (Norstedt et al., 1988). Furthermore, in another study, the absolute amount of *IGF-I* mRNA in the liver was 8.90 ± 1.90 pg/mg in the Tilapia fish species (Caelers et al., 2004).

Our results indicate that the detected mutation g.295T>C in the *IGF-I* gene affects certain growth and reproductive traits in Jinghai yellow chickens. This mutation can be used as marker-assisted selection to upgrade and improve the progress of chicken genetics in future.

However, the study of more SNPs in the other exons of the *IGF-I* gene is highly recommended in order to delineate its further association with growth and reproductive traits.

CONCLUSION

The purpose of our study was to detect SNPs in the *IGF-I* gene and to analyze its association with the growth and reproductive traits in Jinghai yellow chickens. The association analysis indicated that the mutation g.295T>C in the *IGF-I* gene was significantly associated with the growth and reproductive traits of all three genotypes, with the CT and TT genotypes being superior to the CC genotype. The real-time qPCR results indicated that the expression level of the *IGF-I* gene was higher in the leg muscle than the breast muscle in all male and female Jinghai yellow chickens used in this study. Our results suggest that the mutation g.295T>C in the *IGF-I* gene could be used to upgrade Jinghai yellow chickens.

Conflicts of interest

The authors declare no conflict of interest.

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