Identification of *Ava*I Polymorphisms in the Third Intron of *GH* Gene and Their Associations with Abdominal Fat in Chickens

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ABSTRACT Growth hormone (GH) plays a diverse role in animals together with other hormones of somatotropic axis. In the current research, chicken *GH* (c*GH*) as a candidate gene affecting carcass traits was investigated in the chickens from 2 local chicken breeds [Mountainous Black-Bone (Wugu) and Caoke chicken] in the Sichuan province, 1 pure line of a quality chicken (Sanhuang chicken) from the Guangdong province, and commercial crossbreds. The RFLP method was used to identify polymorphisms of the c*GH* gene. Three restriction enzyme polymorphic sites were detected in the c*GH* gene. Sequence alignment from GenBank revealed 2 mutations in the third intron of the c*GH* gene, which were identified by the *Ava*I enzyme. Two novel *Ava*I polymorphic sites were genotypied in 240 chickens from the above-mentioned chicken populations. One *Eco*RV polymorphic site, the previously reported polymorphism, was also detected in these populations. Significant differences in allelic and genotypic frequencies among all the chicken populations were observed. In *Ava*I polymorphic sites, allele *A2* and *B1* had higher frequencies than allele *A1* and *B2*, respectively. In *Eco*RV polymorphic sites, the frequency of allele *N2* was higher than that of allele *N1*. Associations of polymorphisms of the c*GH* gene with carcass traits were analyzed by using a GLM procedure. Significant associations were found between *Ava*I genotypes or combined genotypes and abdominal fat weight and abdominal fat percentage ($P \leq 0.05$). The allele *A2* and *B1* had a beneficial effect on increasing the live BW, breast muscle weight, and breast muscle percentage while decreasing the abdominal fat weight, abdominal fat percentage, and s.c. fat thickness. No significant associations were observed between *Eco*RV genotypes and carcass traits. In conclusion, the c*GH* gene may be a potential marker affecting the abdominal fat trait of chickens.

Key words: chicken, growth hormone gene, polymorphism, carcass trait, abdominal fat

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INTRODUCTION

The molecular markers, either linked to QTL influencing economically important traits or directly having an effect, are unaffected by environmental conditions. Therefore, they could enhance the speed and effectiveness of progress in animal breeding. Once an association between a DNA polymorphism and an important trait is found, the DNA marker could be used in the molecular MAS.

The approach of using a candidate gene, selected because of known relationships between physiology and production traits, is a useful method to investigate associations of gene polymorphisms directly, with certain traits of interest in farm animals (Rothschild and Soller, 1997). Growth hormone (**GH**), a polypeptide hormone, is very important in animals for its broad range of activities. It plays important roles in promoting-growth, protein and muscle accretion, and fat catabolism together with other hormones of the somatotropic axis (Hou and Cheng, 1984; Etherton and Bauman, 1998). Studies in animals have shown that treatment with GH in vivo and in vitro, increases average daily weight gain, feed conversion efficiency, and milk production and reduces fat deposition (Hoj et al., 1993; Vasilatos-Younken, 1995; Klindt et al., 1996). Therefore, *GH* may be a potential candidate gene for MAS schemes.

In farm animals, many polymorphisms have been identified in the *GH* gene of pigs (Kirkpatrick and Huff, 1991; Franco et al., 2005), bovine (Lucy et al., 1993; Grochowska et al., 2001), and goat (Malveiro et al., 2001). Compared with other animals, the intron regions of the chicken *GH* (***cGH***) gene is highly polymorphic, and the studies using RFLP showed that these polymorphisms are associated with abdominal fat, egg production, resistance to Marek's disease or avian leucosis, and meat yield traits (Fotouhi et al., 1993; Kuhnlein et al., 1997; Yan et al., 2003). The

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objectives of the current study were to detect polymorphisms in the third intron of the c*GH* gene using the RFLP method and to analyze the associations of these polymorphisms with carcass traits in the Chinese local populations of chickens.

MATERIALS AND METHODS

Resource Populations

The chicken populations were the Mountainous Black-Bone chicken (n = 120), Caoke chicken (**CK**; n = 60), Sanhuang chicken (**SH**; n = 60), and a commercial cross-bred (**CC**) chicken (n = 30). The Mountainous Black-Bone and CK chickens are indigenous breeds in the Sichuan province, having spotty feathers and black or yellow skin. These chickens have favorable meat quality but grow slowly. The SH chicken is an indigenous breed in the Guangdong province. It was named by its yellow plumage, skin, and shank and has a high quality of meat. The CC was designed by crossing SH cocks with CK hens. All birds were hatched on the same day, housed on the deep-litter bedding, and moved to the growing pens at the age of 7 wk. Birds had access to feed (commercial corn-soybean diets meeting NRC requirements) and water ad libitum. Before slaughter, blood was collected, and the genomic DNA was isolated by phenolic extraction and was used to genotype the *GH* gene.

Phenotyping for Carcass Traits

At the age of 90 d, BW was measured on live birds after 12 h with no access to feed. After slaughter at the same day of age, the carcass traits were measured, including carcass weight (**CW**), eviscerated weight, semieviscerated weight, breast muscle weight (**BMW**), leg muscle weight, abdominal fat weight (**AW**), and s.c. fat thickness (**SFT**). The CW was measured on the chilled carcass after removal of the feathers. Semieviscerated weight was measured on the carcass after removal of the trachea, esophagus, gastrointestinal tract, spleen, pancreas, and gonad. The eviscerated weight was measured on the semieviscerated weight after removal of the head, claws, heart, liver, gizzard, glandular stomach, and abdominal fat. The ratios of these traits to CW were calculated as eviscerated percentage, semieviscerated percentage, breast muscle percentage (**BMP**), leg muscle percentage, and abdominal fat percentage (**AP**). Subcutaneous fat thickness was measured at the caudal spondyle including the skin and fat width with a vernier caliper after processing.

Amplification and Population Genotyping

Primer pairs 5′-GTC CGT GCT CTT CTC TTA TC-3′ (forward) and 5′-GCC AGG CTT CCA TCA GTA T-3′ (reverse) were used (Yan et al., 2003) to amplify the fragment (664 bp) of the third intron of the c*GH* gene. The PCR was performed in a final volume of 15 μL containing 0.3 μ*M* each primer, 0.5 U of *Taq* polymerase with its buffer, 0.2 m*M* each deoxynucleotide triphosphate, 1.5 m*M* $MgCl_2$, and 50 ng of DNA. Genomic DNA was denatured for 4 min at 94°C, and the PCR was run at 94°C for 30 s for 35 cycles, 62°C for 45 s for annealing, 72°C for 50 s for extension, and 72°C for 7 min as final elongation.

The polymorphic sites could be detected by the alignment of DNA sequences deposited in the GenBank (AF289468, D10484, and AY461843), and the *Ava*I restriction enzyme sites in the sequence were predicted by DNASTAR (DNASTAR Inc., Madison, WI). The amplified fragment was digested with restriction enzymes *Ava*I and *Eco*RV, respectively. In a total volume of 15 μL of reaction buffer containing 7 μL of PCR product and 8 U of enzyme after maintaining at 37°C overnight, restriction patterns with the *Ava*I enzyme were visualized by electrophoresis of the digestion product through 8% acrylamide gel. The digests with the *Eco*RV enzyme were electrophoresed through 2% agarose gel, and gels were both visualized on Gel Doc EQ170-8060 (Bio-Rad Laboratories Inc., Hercules, CA) and photographed.

Statistical Analysis

Data were analyzed with the GLM procedures of SAS (SAS Institute Inc., Cary, NC). The genetic effects were analyzed by a GLM procedure in the SAS package, and the following model was used: $Y = \mu + B + S + G + (S \times G) + e$, where Y = the traits measured; μ = the population mean; e = the random error; B = the fixed effect of breed; S = the fixed effect of sex; G = the fixed effect associated with the genotype; and S × G = the interaction between the breed and sex. The S × G interaction was excluded from the model if its effect was $P > 0.05$ for a given trait. The values were presented as least square means ± SEM. The significant differences of least square means were tested with Duncan's multiple range tests ($P \leq 0.05$).

The data of some carcass traits were not normally distributed. The BW, CW, leg muscle weight, AW, and SFT were analyzed as the linear model, with parameters estimated on the square root scale. The eviscerated percentage, semieviscerated percentage, BP, and AP traits were shifted and rescaled to give approximate normality and equality of variance.

RESULTS

Genotypic and Allelic Frequencies of cGH Gene With the AvaI Enzyme

Chickens (240) from 4 breeds or strains were examined for the c*GH* gene polymorphisms in the current study. Two new polymorphic sites were identified in the 664-bp fragment with the *Ava*I enzyme. The polymorphisms were at 240 bp (A locus) and 347 bp (B locus). In locus A, allele *A1*, with the polymorphic restriction site, was cut into 240- and 424-bp fragments, and allele *A2* was characterized by a 664-bp fragment. In locus B, allele *B1*, with the polymorphic restriction site, was cut into 317-

Table 1. The genotypic and allelic frequencies at *Ava*I and *Eco*RV polymorphic sites of the chicken growth hormone genes[1]

		Genotype			Allele		Genotype			Allele		Genotype			Allele	
Strain	n	*A1A1*	*A1A2*	*A2A2*	*A1*	*A2*	*B1B1*	*B1B2*	*B2B2*	*B1*	*B2*	*N1N1*	*N1N2*	*N2N2*	*N1*	*N2*
CK	60	26.67	28.33	45.00	40.83	59.17	45.00	28.33	26.67	59.17	40.83	0.00	30.00	70.00	15.00	85.00
CC	30	10.00	33.33	56.67	26.67	73.33	56.67	33.33	10.00	73.33	26.67	0.00	16.67	83.33	8.34	91.66
BB	90	21.11	36.67	42.22	39.45	60.55	44.45	36.67	18.89	62.78	37.22	1.33	26.67	70.00	16.67	83.34
SH	60	28.34	30.00	41.66	43.34	56.66	41.66	30.00	28.34	56.66	43.34	1.67	25.00	73.34	14.17	85.84

[1]CK = Caoke chicken; CC = commercial crossbred; BB = Mountainous Black-Bone chicken; and SH = Sanhuang chicken. Locus A and B were the polymorphic sites identified by the *Ava*I enzyme and *N* represent the *Eco*RV polymorphic site, respectively.

and 347-bp fragments, and allele *B2* was characterized by a 664-bp fragment.

The genotypic and allelic frequencies of *Ava*I polymorphic sites in the different chicken populations are shown in Table 1. In locus A, allele *A2* was identified as the dominant allele among all populations due to the highest allele frequency (average = 62.4%). The frequency of *A1A1* homozygous genotype was the lowest among all populations, whereas the *A2A2* genotype had the highest frequency. In locus B, allele *B1* was the dominant allele among all chicken populations because of the highest allele frequency (average = 63.0%). The frequency of *B1B1* genotype was the highest, and *B2B2* genotype had the lowest frequency among all populations.

Genotypic and Allelic Frequencies of cGH Gene With the EcoRV Enzyme

The amplified product was digested with *Eco*RV restriction enzyme, and 1 polymorphic site was found. The fragment sizes of 431 and 233 bp were designated as the *N1* allele, whereas the allele *N2* showed only 1 fragment of 664 bp.

The genotypic and allelic frequencies of *Eco*RV polymorphism in 4 chicken populations are shown in Table 1. The frequency of genotype *N1N1* was very low, even 0 in the CK and CC populations. The frequency of genotype *N2N2* was the highest, and *N2* was the dominant allele among all populations.

Associations Among AvaI Genotypes and Growth Traits

The results of the GLM analysis of associations between the *GH* RFLP polymorphisms and carcass traits in the local chicken populations are summarized in Table 2. In locus A, AW and AP were significantly associated with *GH* genotypes ($P = 0.0219$ and $P = 0.013$). The AW of *A1A1* chickens was notably higher than that of *A2A2* ($P < 0.05$). There were no differences among other genotypes ($P > 0.05$). The *A1A1* chickens had higher AP than *A1A2* and *A2A2* chickens by 0.12 and 0.45%, respectively ($P < 0.05$), and *A1A2* chickens had 1.64% AP, which was higher than that of the *A2A2* chickens ($P < 0.05$). No significant differences were detected for other carcass traits. The allele *A2* had a favorably positive effect on the BW, CW, BMW, and BMP, and it also beneficially decreased the AW, AP, and SFT of chickens. In locus B, differences among *Ava*I genotypes were also significant for trait AW ($P = 0.0283$) and AP ($P = 0.0080$). The *B1B1* chickens had significantly lower AW than *B2B2* chickens ($P < 0.05$), but there was no difference when compared with *B1B2* ($P > 0.05$). The AW of the *B2B2* chickens was not different from that of the *B1B2* chickens ($P > 0.05$). The AP of *B1B1* chickens was 2.53%, which was lower than that of *B2B2* by 0.48% ($P < 0.01$) and lower than that of *B1B2* by 0.33% ($P < 0.05$). There was no significant difference between *B1B2* and *B2B2* chickens ($P > 0.05$). No differences were observed for other carcass traits. The *B1* allele had a favorably positive effect on BW, BMW, and BMP and also beneficially decreased the AW, AP, and SFT of chickens.

Association Between AvaI Combined Genotypes and Carcass Traits

Based on the 2 polymorphic sites, 5 combined genotypes were constructed as *M1* (*A1A1-B1B1*), *M2* (*A2A2-B1B1*), *M3* (*A1A1-B2B2*), *M4* (*A1A2-B1B1* and *A1A1-B1B2*), and *M5* (*A1A2-B1B2*). The frequency of the *M4* combined genotype was very low (only 4 chickens), so it was deleted from further analysis. There were significant associations of combined genotypes with AW and AP ($P = 0.0487$ and $P = 0.0334$). The AW and AP of the *M2* chickens were lower than that of *M3* by 7.64 g ($P = 0.0108$) and 0.47% ($P = 0.0104$), respectively (Table 2). No associations were observed between combined genotypes and other carcass traits.

Association Between EcoRV Genotypes and Growth Traits

The associations of *Eco*RV genotypes with carcass traits in chickens were analyzed. No significant associations between genotypes and carcass traits were observed, although the results indicated that allele *N1* had an additive effect on all the carcass traits.

DISCUSSION

Because essential GH is for growth and metabolism of the chicken, the c*GH* gene, which directly controls the synthesis of chicken GH, has received a lot of attention for a long time. The c*GH* gene contains 4 exons and 5 introns, and the intron sequences of c*GH* gene are longer

Table 2. Effect of chicken growth hormone genotypes identified with *Ava*I enzyme on the carcass traits (least square mean and SE)

	Genotype or combined genotype								
Trait[1]	*A1A1* (55)[2]	*A1A2* (78)	*A2A2* (107)	*B1B1* (115)	*B1B2* (73)	*B2B2* (52)	*M1* (13)	*M2* (107)	*M3* (52)	*M5* (64)
AW (g)	48.81 ± 2.61^{a}	45.76 ± 2.16^{ab}	41.46 ± 1.83^{b}	41.73 ± 1.78^{a}	45.39 ± 2.23^{ab}	49.28 ± 2.66^{b}	46.89 ± 5.45^{ab}	41.80 ± 1.85^{b}	49.26 ± 2.68^{a}	44.89 ± 2.43^{ab}
%AP	2.98 ± 0.15^{a}	2.86 ± 0.12^{b}	2.53 ± 0.10^{c}	2.53 ± 0.10^{a}	2.86 ± 0.13^{b}	3.01 ± 0.15^{b}	2.90 ± 0.31^{ab}	2.54 ± 0.11^{a}	3.01 ± 0.15^{b}	2.82 ± 0.14^{ab}

$^{a-c}$Means within a row with no common superscripts differ significantly ($P \leq 0.05$).
[1]AW = abdominal fat weight; %AP = AW to carcass weight.
[2]The numbers in the brackets are the number of chicken of the respective genotypes.

than exons compared with mammals (Stephen et al., 2001). Previous reports confirm that the exons and 5′ regulatory region of c*GH* gene are conservative, and mutations predominantly occur in the intron sequences (Li and Zhang, 1996; Zhang and Li, 1998; Stephen et al., 2001), so the expression of *cGH* may be regulated by the introns or 3′ untranslation region. Polymorphisms of c*GH* are associated with abdominal fat pad content (Fotouhi et al., 1993). Association analysis also shows that *Eco*RV genotypes in the third intron are related to AF trait (Yan et al., 2003). The results indicated the possibility that these sequences are involved in the regulation of cGH production. However, it is unclear whether the introns indeed regulate the c*GH* expression and how the introns exert their roles during the transcription or translation.

Abdominal fat has been recognized as by-product in the processing, and numerous studies have been carried out to detect the genes whose expression or mutations are related to AF. To investigate the possible function of the mutations, the associations of c*GH* genotypes with carcass traits were analyzed. The *Ava*I genotypes were significantly associated with AW and AP. The mutations in the third intron in the current study did not change amino acids in the protein sequence but possibly affected the efficiency of transcription or translation and interfered with the quantity of GH secretion. The distribution of energy and metabolism is controlled by hormone factors, so expression profiles of these genes could affect the metabolism of animals.

The *Eco*RV polymorphic site was detected by Yan et al. (2003). Studies of this polymorphism in the F_2 offspring derived from the populations of crossing Mingxing chickens with Silkies found a significant association of the genotypes with AP. However, the present results did not show significant associations between this polymorphism and carcass traits. The effects of this mutation have varied in different studies, which could be caused by the different populations studied, different statistical models used, and numbers of animal genotyped. Therefore, further study is still needed to identify the effects of *Eco*RV genotypes.

In the current study, the new *Ava*I polymorphisms were found in the third intron. Associations between c*GH* genotypes and abdominal fat were identified. The associations with polymorphisms do not necessarily mean that selection based upon these polymorphisms will have a direct effect on AW and AP. Resource chickens in the current study had lower abdominal fat, s.c. fat, and more i.m. fat in comparison with fast-growing chickens. Therefore, the results from the association analysis implied that 3 polymorphic sites could be used as genetic markers notably affecting the abdominal fat deposition. Considering that allele *A2* and *B1* had a beneficial effect on decreasing abdominal fat, it would be possible to make selection schemes favoring the 2 alleles for decreasing the abdominal fat in chickens; this hypothesis must be tested in selection experiments. To make the selection schemes applicable, it would be necessary to further analyze the effects of

c*GH* polymorphisms by using populations from different genetic backgrounds and increasing the size of samples.

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