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Cloning of *TPO* gene and associations of polymorphisms with chicken growth and carcass traits

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Abstract Thyroid peroxidase (TPO), which located on the apical membrane surface of thyrocytes, is the key enzyme involved in thyroid hormone synthesis, mainly catalyses the iodination of tyrosine residues and the coupling of iodotyrosines on thyroglobulin to form thyroxine and triiodothyronine. The objectives of this study were to identify genetic polymorphisms of the chicken *TPO* gene and to analyze potential association between single nucleotide polymorphisms (SNPs) and growth and carcass traits in chicken. Partial sequences of *TPO* gene were cloned firstly. The nucleotide sequence was found to have 72 % identity with that of humans. The chicken TPO amino acid sequence was 71 %. Through polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing methods, three novel mutations of the chicken *TPO* gene were detected in the F_2 resource population from Gushi chickens and Anka broilers. The association analysis indicated that all of the three SNPs showed association with chicken growth at different periods. The g.29996C>T polymorphisms was significantly associated with body weight, breast bone length, pectoral angle at 12 weeks, claw weight and leg muscle weight ($P < 0.05$). In addition, individuals with the TT genotype had higher value for almost all the traits than CC and CT genotype. Meanwhile for CLW, the additive effects were significant ($P < 0.05$). Hence, we suggest that genotype TT can be regarded as a potential molecular marker for later growth and carcass traits in chicken.

Keywords Thyroid peroxidase gene · Clone · SNPs · Chicken · Growth traits · Carcass traits · Association analysis

Introduction

Thyroid hormones are produced by the thyroid gland, which in birds consists of two separate lobes located on either side of the trachea [1]. Thyroid peroxidase or thyroperoxidase is a glycosylated membrane bound hemoprotein localized on the apical membrane surface of follicular cells [2]. Thyroid peroxidase (TPO) plays an essential role in the process of thyroid hormone synthesis by catalyzing the iodination of tyrosine residues and the coupling of iodotyrosnes on thyroglobulin to form thyroxine (T4) and triiodothyronine (T3). Thyroid hormones play important and diverse roles in animal growth. Treating chicken embryos with thyroid hormones between day 18.5 and 19 of incubation can advances hatching time [3]. Children may have cretinism for lake of thyroid hormone when he was young.

Full-length human *TPO* mRNA transcribed from the gene consists of about 3048 bases and encodes 933 amino acids. The human *TPO* gene was mapped to chromosome 2p25 and spans over 150 kb, containing 17 exons. The expression of TPO *gene* is controlled by thyroid specific transcription factors such as TTF-1, TTF-2, and Pax-8, play

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a critical role in the determination and maintenance of cellular phenotype [4, 5].

Mutations in the *TPO* gene in humans are known to be associated with thyroid-related disease. For instance, mutations of *TPO* gene cause human congenital hypothyroidism [6], thyroid dyshormonogenesis [7], iodide organification defect [8, 9] and congenital goiter [10, 11] in human beings and animals. Mutations in the *TPO* gene described up to now have been classified as frameshift mutations, nonsense mutations, missense mutations, and gene deletion, mutations affecting splicing [7, 12–15].

Growth is a composite of complex developments that result from genetic, nutritional, and environmental factors. Growth traits are important traits in chicken and they are controlled by multiple genes [16]. Significant QTL affecting body weight of chicken was mapped on 258 cM of chromosome 3 where the chicken *TPO* gene located nearby [17]. Therefore, in this study we cloned the partical cDNA of chicken *TPO* gene and detected the single nucleotide polymorphisms (SNPs), aiming to investigate the associations of SNPs with growth and carcass traits of chicken. The results could provide new information and evidence regarding the usefulness of these markers for gene-assisted selection in chicken breeding programs.

Materials and methods

Resource populations and measurement of phenotypes

An F_2 resource population as described by Han et al. [18] was used in this study. The population was generated from Gushi (G) chickens representing a slow-growing Chinese native breed and Anka (A) broilers representing a fast-growing broiler. The F_2 population consisted of four cross-bred families (A-roosters mated with G-hens) and two reciprocal families (G-roosters mated with A-hens). To build the F_2 population, nine F_1 females were selected from each of six families; the 54 F_1 females were mated by six F_1 males from six families. The complete resource population included 42 grandparents, 60 F_1 parents, and 849 F_2 hens. Over two hatches that occurred at 2-week intervals, the resource population was established. All F_2 birds had free access to feed and water. All chickens were managed in cages, according to the national standard.

Thirty-one growth traits at different weeks were measured in the F_2 population, which included body weight (BW, calculated every 2 weeks from birth to slaughter) and body size index calculated every 4 weeks containing shank length (SL), shank girth (SG), chest depth (CD), breast bone length (BBL), pectoral angle (PA), pelvis breadth (PB), chest width (CWI) and body slanting length (BSL).

The F_2 chickens were slaughtered at the age of 84 days and their carcass traits were measured, including carcass weight (CW), semi-evisceration weight (SEW), breast muscle weight (BMW), leg muscle weight (LMW) and claw weight (CLW). The SEW is the CW excluding the trachea, esophagus, crop, intestine, spleen, pancreas, gallbladder and reproductive organs.

cDNA cloning of chicken *TPO* gene

Total RNA was extracted from 20-week-old male chicken thyroid glands using the TRIZOL (TAKALA Cat. No. D9108) and reverse-transcribed into cDNA using a First-Strand cDNA Synthesis Kit (Fermentas; Cat. No. K1621) following the manufacturer's instructions.

A pair of primers (Forward primer: TCT GGG ATG GAT TGC TTG Reverse primer: GTT TCC AGT CTT GGT AAG TCG C) based on GenBank sequences (XM_001235672) was used to clone a fragment of the coding sequence. 1 μl of the resulting cDNA was amplified in a mix (20 μl) containing 1 μl of each primer, 10 μl Taq Mix (Cwbiotech, Beijing, China), 7 μl ddH_2O. After denaturation at 94 °C for 5 min, 35 cycles of 45 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C were conducted, followed by a final 10-min elongation step at 72°C. The resulting amplicon were purified using the Gel Extraction Kit (Tiangen Bio Co.) and sequenced by Taihegene Biotechnology (Beijing) Co., Ltd.

DNA samples, PCR amplification and genotyping

Genomic DNA samples were extracted from whole blood of F_2 individuals by the phenol–chloroform method. DNA quality was assessed by running samples on 1 % agarose gels and DNA concentration was measured with an UV spectrophotometer. Seventy-two DNA samples from the F_2 individuals (each 2 μl) were used to construct DNA pool which was sequenced by Taihegene Biotechnology (Beijing) Co., Ltd. to find SNPs of the *TPO* gene.

Primers used to amplify chicken *TPO* gene were designed according to the two published gene sequences (GenBank accession no: NW-0014716171.1 and XM001235672.1) (Supplementary Table 1). PCR was performed in a 10 μl of reaction volume, containing 50 ng genomic DNA, 0.5 μl of each primer, 5 μl Taq Mix (Cwbiotech, Beijing, China). The cycling conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55–64 °C depending on the primer for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Aliquots of 10 μl PCR products of the *TPO* gene were digested with 3 U endonucleases for 6 h at 37 °C following the supplier's directions. Restriction enzymes used are summarized in Supplementary Table 1. The digested products were detected by electrophoresis in 3.0 % agarose gel, stained with ethidium bromide. The

different genotypes of the three SNPs were confirmed by sequencing the PCR products.

Statistical analysis

Genotypic frequencies and allelic frequencies and were directly calculated. Population genetic indexes, such as gene heterozygosity (He) [19], gene homozygosity (Ho), effective allele numbers (Ne) [20] and polymorphism information content (PIC) [21] were calculated according to Nei's methods, respectively. The linkage disequilibrium (LD) was estimated in the population using SHEsis software (http://analysis.bio-x.cn) [22, 23] based on the phase information, which was used as an accelerated expectation maximization algorithm similar to the partition–ligation method and the default algorithm was used.

The associations between single SNP marker genotypes of the *TPO* gene and traits were analyzed by the mixed liner models procedure of SPSS 17.0. Mixed linear Model I was used to investigate the associations between genotypes and growth traits. Mixed linear Model II was applied to carcass traits, with carcass weight as a covariate to investigate its effect on carcass traits. The effect of genotypes of the polymorphisms on target traits was investigated by least-squares analysis. Significance was determined to be $P < 0.05$ and a Bonferroni test was used to control for multiple comparisons.

$$\text{ModelI(forgrowthtraits)}:$$
$$Y = \mu + G + S + H + G \times S + G \times H + F + D + e$$
$$\text{ModelII(forcarcasstraits)}:$$
$$Y = \mu + G + S + H + G \times S + G \times H + F + D + b(W - \overline{W}) + e$$

Y is the observation on the traits; μ is the overall population mean; G is the fixed effect of genotype; S is the fixed effect of sex; H is the fixed effect of hatch; G × S is the fixed effect of genotype-sex interaction; G × H is the fixed effect of genotype-hatch interaction; F is the random effect of family; D is the random effect of sire and dam; b is the regression coefficient for CW; W is the individual CW; $\bar{W}$ is the average CW; e is the the random error.

The additive and dominance effects were estimated for the traits identified to have significant SNP associations using REG procedure of SAS 8.0, where the additive effect was estimated as 0, 1 and −1 for the heterozygote, the favorable homozygote and the unfavorable homozygote, respectively; and the dominance effect was represented as 1 for the homozygotes and −1 for the heterozygote.

Results

Cloning of the chicken *TPO* gene

An 968 bp cDNA sequence was obtained (Fig. 1), with a open reading frame (ORF) of 960 bp which encodes 319 amino acids. Comparison to predicted mRNA of chicken, there is a G481A mutation in the coding sequences, lead Ala to Thr.

The nucleotide sequence was 72 % identity to that of humans (NM_000547.5), 69 % to rats (NM_009417.2) and mice (NM_019353.1), 68 % to bovine (XM_002683944.1) and 70 % with xenopus (XM_002936302.1). Amino acid sequence identities were 71, 72, 70, 65 and 64 %, respectively.

Polymorphisms of the chicken *TPO* gene

Three SNPs of c.111G>A, c.430G>A and g.29996C>T of the *TPO* gene were found by DNA pool sequencing in exon 2, exon 4 and intron 13, respectively. The DNA sequences

```
1    TCTGGGATGG ATTGCTTGCC CTTCTACCGC TCATCTCCTG CATGTGGCAC TGGTGATCAC
61   AGTATTCTCT TTGGAAATCT ATCAGCCTTA AATCCAAGAC AACAGATTAA TGGTTTAACC
121  TCCTTCATTG ATGCATCTAC TGTCTATGGC AGCACTTCCA CTGTTGAAAA CAAGCTGAGG
181  AATTTAACAA GTGAAGAAGG CCTTCTCAGA GTCAACAGTA AACATAATGA TAATGGTCAG
241  GAATACCTGC CTTTTACAGA CCGGGTTCCA TCACCTTGTG CACAGGACTC AAATGCAAGT
301  GAAGATGAAA GGATTGAATG CTTTATGGCT GGGGACAGCC GATCGAGTGA GGTCACATCA
361  TTGACAGCCA TGCACACACT GTGGCTGAGA GAACACAACC GCCTGGCCCG GGCCCTCAAA
421  GCCATCAACA GCCACTGGAG TGCTGAGACT GTCTACCAAG AAGCAAGGAA AATTGTTGGA
481  ACTCTCCATC AGATCATTAC TTTAAGAGAT TATATTCCCA AAATCATTGG CCCAGATGCT
541  TTTAATCAGT ATATTGGCCT TTATAAAGGT TATGATCCCA CAGTGAATCC TACAGTTTCT
601  AACGTATTTG CAACAGCTGC TTTTCGTTTC GGTCATGCAA CAATCCAACC GATAGTAAGG
661  CGATTGAATG CACAGTATTT AGATGATCCA GAACTCCCAA ATCTTCATTT GCATGAAGTT
721  TTCTTTAGTC CTTGGAGGCT TATTAAAGAA GGAGGCTTGG ATCCATTGAT AAGGGGTCTT
781  CTTGCACATC CAGCAAAACT GCAGGTGCAA GGTCAACTGA TGAATGAAGA GTTAACAGAT
841  AAGCTGTTTG TGTTGTCCAA TAATGGTTCA CTTGATTTAG CATCGCTGAA TTTACAGCGT
901  GGCCGTGATC ATGGACTCCC AGGTTATAAT GACTGGCGAG AATTCTGCGA CTTACCAAGA
961  CTGGAAAC
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Fig. 1 Clone sequence of the chicken *TPO* gene

were submitted to the GenBank database (GenBank accession no. JN601422, JN601423, JN601424, JN601425, JN60 1426 and JN601427).

The c.430G>A mutation identified a missense SNP (p. D144N), namely, Asp (GAT) > Asn (AAT) at position 144. The g.29996C>T was in a non-coding region, whereas the c.111G>A in exon 2 mutation identified a silent SNP, p. A37A, namely, Ala (GCG) > Ala (GCA) at position 37 of the TPO protein.

Linkage disequilibrium analysis

Genotypic and allelic frequencies of the three SNPs in the F_2 resource population were shown in Table 1. On the basis of Nei's methods, the population genetic indexes, including He, Ho, Ne and PIC were calculated (Supplementary Table 2).

The results of linkage disequilibrium tests between c.111 G>A and c.430G>A was $r^2 = 0.013$ and 0.056 between c.111G>A and g.29996C>T, and for c.430G>A and g.29996C>T, it was $r^2 = 0.009$ (Fig. 2). In large samples, $r^2 = 1$ indicates complete LD, that is, no evidence for recombination between the SNP pairs —such SNPs are good surrogates for each other; $r^2 = 0$ indicates no LD. "Strong" LD was defined as having pairwise $r^2 > 0.33$. Among the three polymorphisms, strong linkage disequilibrium was not observed suggesting that this region cannot be inherited as a unit.

Association of polymorphisms with growth and carcass traits

A total of 31 growth traits were used for analysis of association in this study. Results showed that the c.111G>A mutation polymorphism was significantly associated with BW at 2 weeks, SG at 12 weeks, CD at 12 weeks, BBL at 8, 12 weeks, BMW and LMW ($P < 0.05$), and highly significantly associated with BBW, PA at 12 weeks ($P < 0.01$), and G rather than A was advantageous for chicken growth (Table 2). For CD at 12 weeks and PA at 12 weeks, the additive effects were significant ($P < 0.05$; Table 5).

Table 1 Allele frequency of TPO SNP in the F_2 population

Mutation	Genotype			Allele	
c.111G>A	GG	GA	AA	G	A
	0.6130	0.2196	0.1673	0.7229	0.2771
c.430G>A	GG	GA	AA	G	A
	0.4380	0.4276	0.1343	0.6518	0.3482
g.29996C>T	CC	CT	TT	C	T
	0.7178	0.2320	0.0503	0.8338	0.1623

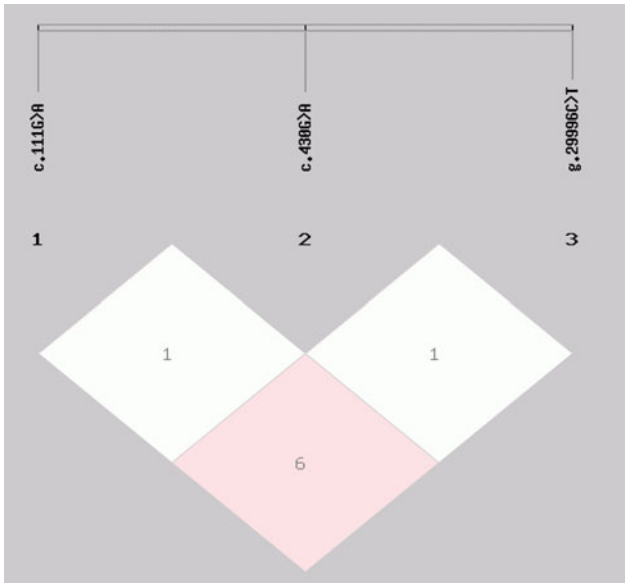

Fig. 2 r^2 value of pairwise linkage disequillibrium analysis in the three loci of *TPO* gene

The c.430G>A polymorphism was significantly associated only with SL at 8 weeks, PB at 4 weeks ($P < 0.05$) and highly significantly associated with and CWI at 8 weeks, BSL at 8 weeks ($P < 0.01$), and A allele was advantageous for chicken growth (Table 3). For CWI at 8 weeks, the additive effects were significant ($P < 0.05$; Table 5).

The g.29996C>T polymorphism was significantly associated with BW at 12 weeks, BBL at 12 weeks, PA at 12 weeks, CLW and LMW ($P < 0.05$). T allele was advantageous for chicken growth (Table 4). Meanwhile for CLW, the additive effects were significant ($P < 0.05$; Table 5).

Discussion

Significant QTL affecting body weight of chicken was mapped on 258 cM of chromosome 3 where the chicken *TPO* gene located nearby [17]. Meanwhile Wang et al. [26] found that the SNP of A/G 642 in the fourteenth exon of *TPO* gene was significantly associated with ham weight in porcine.

For growth traits, the c.111G>A mutation was significantly associated with SG at 12 weeks, CD at 12 weeks, BBL at 8, 12 weeks, PA at 12 weeks. The c.430G>A polymorphism was significantly associated with PB at 4 weeks, SL at 8 weeks, CWI at 8 weeks and BSL at 8 weeks and the g.29996C>T polymorphism was significantly associated with BBL at 12 weeks and PA at 12 weeks. Thyroid hormones, which were known to affect a wide variety of metabolic processes, are necessary for growth and development

Table 2 Associations of c.111G>A genotypes of *TPO* gene with chicken growth and carcass traits

Traits	GG	AG	AA	*P* value
BBW (g)	30.826 ± 0.623[a]	30.037 ± 0.644[b]	30.423 ± 0.655[a,b]	0.006
BW2 (g)	122.453 ± 5.026	118.400 ± 5.159	118.135 ± 5.226	0.022
SG12 (cm)	3.859 ± 0. 024[a]	3.854 ± 0.028[a,b]	3.787 ± 0.030[b]	0.016
CD12 (cm)	7.820 ± 0.065[b]	8.027 ± 0.080[a]	7.877 ± 0.086[a,b]	0.011
BBL8 (cm)	8.929 ± 0.123[a]	8.906 ± 0.130[a,b]	8.725 ± 0.134[b]	0.015
BBL12 (cm)	11.032 ± 0.109[a]	11.006 ± 0.117[a,b]	10.853 ± 0.121[b]	0.044
PA12 (°C)	78.768 ± 0.439[b]	78.712 ± 0.507[b]	80.028 ± 0.541[a]	0.009
BMW (g)	70.632 ± 4.003[a]	70.899 ± 4.106[a]	66.206 ± 4.169[b]	0.011
LMW (g)	99.776 ± 5.437[a]	98.921 ± 5.535[a,b]	94.801 ± 5.591[b]	0.022

BBW birth body weight, *BW2* body weight at 2 weeks, *SG12* shank girth at 12 weeks, *CD12* chest depth at 12 weeks, *BBL8,12* breast bone length at 8 and 12 weeks, *PA12* pectoral angle at 12 weeks, *BMW* breast muscle weight, *LMW* leg muscle weight

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$)

Table 3 Associations of c.430G>A genotypes of *TPO* gene with chicken growth and carcass traits

Trait	GG	AG	AA	*P* value
PB4 (cm)	5.091 ± 0. 046[b]	5.193 ± 0.047[a]	5.211 ± 0.061[a,b]	0.023
SL8 (cm)	7.823 ± 0.069[b]	7.982 ± 0.069[a]	7.919 ± 0.089[a,b]	0.025
CWI8 (cm)	5.725 ± 0.121	5.621 ± 0.121	5.498 ± 0.129	0.003
BSL8 (cm)	16.070 ± 0.092[b]	16.319 ± 0.090[a]	16.468 ± 0.130[a]	0.008

PB4 pelvis breadth at 4 weeks, *SL8* shank length at 8 weeks, *CWI8* chest depth at 8 weeks, *BSL8* body slanting length at 8 weeks

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$)

Table 4 Associations of g.29996C>T genotypes of *TPO* gene with chicken growth and carcass traits

Trait	CC	CT	TT	*P* value
BW 12 (g)	1347.655 ± 48.451[a,b]	1338.398 ± 50.259[b]	1427.340 ± 57.890[a]	0.036
BBL12 (cm)	10.945 ± 0.115[b]	11.102 ± 0.125[a]	11.120 ± 0.162[a,b]	0.039
PA12 (°C)	79.205 ± 0.390[a]	78.209 ± 0.486[b]	79.247 ± 0.798[a,b]	0.035
LMW (g)	98.331 ± 5.496[b]	98.783 ± 5.623[b]	106.295 ± 6.203[a]	0.035
CLW (g)	58.487 ± 2.235[a,b]	57.941 ± 2.343[b]	62.705 ± 2.520[a]	0.032

BW12 body weight at 12 weeks, *BBL12* breast bone length at 12 weeks, *PA12* pectoral angle at 12 weeks, *LMW* leg muscle weight, *CLW* claw weight

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$)

especially to nervous and skeletal in humans. On one hand, thyroid hormones regulate the growth hormone (GH) gene by rapidly increasing its transcription rate [24]. On the other hand, thyroid hormone stimulates the production of growth factors, particularly EGF and NGF [25].

For carcass traits, the c.111G>A polymorphism was significantly associated with LMW and BMW. The g.29996C>T polymorphism was significantly associated with LMW and CLW. Wang et al. [26] found that the SNP of A/G 642 in the fourteenth exon of *TPO* gene was significantly associated with ham weight in porcine. In addition T3 and T4 were proved at increasing rear leg growth [24]. Thyroid hormone has its complex metabolic effects involved in the general mechanisms of body growth, for example thyroid hormones increased protein degradation in skeletal muscle by regulating proteolytic and other lysosomal enzyme activities in tissues [27]. *TPO* plays an essential role in the process of thyroid hormone synthesis so we assumed that the *TPO* mutations may change the expression of thyroid hormones, which affect the growth of skeletal and muscle.

It was further indicated that polymorphisms of the *TPO* gene affected chicken growth at different periods. The c.430G>A

Table 5 Estimated additive and dominance effects for the traits identified to have significant SNP associations

	Traits	Additive effect	*P* value	Dominance effect	*P* value
c.111G>A	BBW (g)	0.219 ± 0.134	0.101	0.385 ± 0.121	0.002
	BW2 (g)	0.867 ± 0.937	0.355	0.776 ± 0.847	0.360
	SG12 (cm)	0.013 ± 0.012	0.283	−0.024 ± 0.011	0.033
	CD12 (cm)	0.060 ± 0.030	0.048	−0.021 ± 0.027	0.445
	BBL8 (cm)	0.061 ± 0.034	0.073	−0.044 ± 0.030	0.144
	BBL12 (cm)	0.034 ± 0.034	0.325	−0.042 ± 0.031	0.178
	PA12 (°C)	−0.552 ± 0.207	0.008	0.253 ± 0.187	0.178
	BMW (g)	0.394 ± 0.760	0.650	−1.819 ± 0.686	0.008
	LMW (g)	0.597 ± 0.861	0.488	−1.240 ± 0.779	0.112
c.430G>A	PB4 (cm)	0.044 ± 0. 027	0.106	−0.026 ± 0.018	0.141
	SL8 (cm)	0.019 ± 0.040	0.624	−0.059 ± 0.026	0.024
	CWI8 (cm)	0.111 ± 0.033	0.001	−0.015 ± 0.021	0.470
	BSL8 (cm)	0.109 ± 0.067	0.105	−0.014 ± 0.044	0.743
g.29996C>T	BW 12 (g)	16.682 ± 16.625	0.316	24.602 ± 10.825	0.023
	BBL12 (cm)	0.038 ± 0.058	0.516	−0.030 ± 0.038	0.427
	PA12 (°C)	−0.150 ± 0.357	0.675	0.601 ± 0.233	0.010
	LMW (g)	1.229 ± 1.455	0.398	1.869 ± 0.954	0.051
	CLW (g)	1.972 ± 0.846	0.020	1.314 ± 0.556	0.018

polymorphism was associated with SL at 8 weeks, CWI at 8 weeks and BSL at 8 weeks but was associated with none of chicken carcass traits. This was surprising because some carcass traits were correlated with growth traits to some extent. So the c.430G>A polymorphism may effects on chicken growth only in middle stage. The missense mutation may potentially change the structure of the TPO protein then affects the growth of chicken. Otherwise, c.111G>A polymorphism seemed to have higher effects on chicken growth in later stage, as it was associated with SG, CD, BBL and PA at 12 weeks, as well as some carcass weight for example BMW and LMW. The g.29996C>T polymorphisms seemed to have higher effects on chicken growth in later stage, too. It was significantly associated with BW, BBL, PA at 12 weeks, claw weight and leg muscle weight ($P < 0.05$). Individuals with the TT genotype had higher value for almost all the traits than CC and CT genotype. Meanwhile for CLW, the additive effects were significant. Hence, we suggest that genotype TT can be regarded as a potential molecular marker for growth and carcass traits in chicken. These results are preliminary; however, further investigation is essential to confirm these findings.

In conclusion, our present results suggest that the *TPO* gene can play an important role in the control of chicken growth. It provided important basis for future studies on the molecular regulation of the *TPO* gene in chicken and will potentially lead to a better understanding of the regulation function of thyroid gland during the growth and development in chicken. On the other hand, it appears clear that further studies to test the *TPO* gene in different populations are required.

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