

# Expression and effect of Calpain9 gene genetic polymorphism on slaughter indicators and intramuscular fat content in chickens

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**ABSTRACT** Calpain 9 (*CAPN9*) is expressed in the stomach and small intestine. *CAPN9* has regulatory roles in hypertension, heart disease, gastric mucosal defense, and kidney disease. The involvement of *CAPN9* has not been reported in the development of chickens. *CAPN9* mRNA was found in adipose and muscle tissue in this study. Two linkage single nucleotide polymorphisms (SNP; G7518A and C7542G) in intron 4 were screened from 160 birds of the D2 chicken line. The 2 mutation sites were associated with carcass weight, evisceration weight, abdominal fat weight (AFW), abdominal fat percentage (AFP), and breast muscle percentage (all  $P < 0.05$ ). Intramuscular fat (IMF) content was not significantly different in the 3 genotypes. But,

the AA(7518)/GG(7542) genotype had the highest IMF content, highest breast muscle weight, and lower AFW and AFP. Moreover, the mRNA level of *CAPN9* in abdominal fat tissue was significantly different ( $P < 0.05$  or  $P < 0.01$ ) between any 2 genotypes, consistent with AFW and AFP. In summary, the expression of *CAPN9* in adipose and breast muscle tissue is reported for the first time. *CAPN9* affected production performance of chickens. As a marker, the linkage G7518A and C7542G polymorphisms in intron 4 of *CAPN9* could affect the production traits by regulating mRNA expression. The findings concerning the marker enrich the theoretical foundation for molecular breeding of high-quality broilers.

**Key words:** Calpain 9 gene, expression, SNP, development, chicken

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## INTRODUCTION

The Calpain superfamily (*CAPN*) is a large group of calcium-activated cysteine proteases that are involved in a variety of functions, including cytoskeletal remodeling, cell signaling, and apoptosis (Shioda et al., 2006; Gafni et al., 2009; McClung et al., 2009). Among them,  $\mu$ -calpain will play a major role in meat quality changes of chicken breast muscle (Huang et al., 2014; Zhao et al., 2018). In addition, Calpain 10 plays an important role in regulating obesity and diabetes in mice (Cheverud et al., 2010).

Calpain 9 (*CAPN9*, also known as nCL-4) is a typical calcium protease that is expressed mainly in the stomach and small intestine (Lee et al., 1998). *CAPN 9*

associates with the common 28 kDa regulatory subunit calpain-4 (Lee et al., 1999). Most studies of *CAPN9* have focused on disease (Hata et al., 2010; Ookura et al., 2015), *CAPN9* and *CAPN8* have been functionally implicated in the gastrointestinal tract (Hata et al., 2010). But the involvement of *CAPN9* on animal production performance is unclear.

In this study, the expression and function of *CAPN9* gene were detected. It was found that *CAPN9* gene was expressed in adipose and breast muscle tissue of chicken. Further, the polymorphism analysis of *CAPN9* gene also was performed by direct PCR sequencing, and the linkage G7518A and C7542G polymorphisms in intron 4 of *CAPN9* were found, and associated with the body weight, muscle development, and fat deposition of chicken. The findings provide a marker and enrich the theoretical foundation for molecular breeding of high-quality broilers.

## MATERIALS AND METHODS

### *Animals and Ethics Statement*

All of the animal experiments were conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology

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(Beijing, China). Animal experiments were approved by the Animal Management Committee (in charge of animal welfare issues) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China). Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

D2 chickens ( $n = 160$ ), a yellow feather broiler with dwarf consanguinity, were obtained from Institute of Animal Science, Chinese Academy of Agricultural Science (Beijing, China). All birds were slaughtered at 90 d of age. Blood samples were collected from the wing vein for DNA extraction. For the detection of gene expression, samples of abdominal fat (**AF**) and pectoralis major muscle from 6 random birds were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for reverse transcription polymerase chain reaction (**RT-PCR**). For every effective SNP, 6 AF samples of every genotypes were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for the real-time quantitative reverse transcription polymerase chain reaction (**Q-PCR**). Other pectoralis major muscle samples were stored at  $-20^{\circ}\text{C}$  for intramuscular fat (**IMF**) assay.

### Slaughter Indicators and IMF Content

After birds were slaughtered, carcass weight (**CW**), eviscerated weight (**EW**), breast muscle weight (**BMW**), and abdominal fat weight (**AFW**) were determined, and abdominal fat percentage (**AFP**, %, mass of AF as a percentage of BW) and breast muscle percentage (**BMP**, %, mass of breast muscle as a percentage of BW) were calculated.

IMF content of pectoralis major muscle, trimmed of visible fat surrounding and lying around muscles, was measured by Soxhlet extraction (AOAC, 1990) and is presented as the weight percentage of dry muscle tissue.

### RNA Extraction

Total RNA was isolated from the frozen AF and pectoralis major muscle tissues using Trizol reagent according to the manufacturer's protocol (Takara Bio, Shiga, Japan). After removal of any genomic DNA using the DNA Removal Kit according to the manufacturer's protocol (Abnova, Wuhan, China), RNA was dissolved at  $1\ \mu\text{g}/\mu\text{L}$  and stored at  $-80^{\circ}\text{C}$  for use in RT-PCR and Q-PCR. The RNA integrity and purity were assessed by electrophoresis on 1.5% agarose gels and ultraviolet spectrophotometry.

### RT-PCR and Q-PCR

Total RNA ( $2.0\ \mu\text{g}$ ) from the frozen AF or pectoralis major muscle tissues was used to generate cDNA in a final volume of  $20\ \mu\text{L}$  using the GoScript<sup>TM</sup> reverse transcription system according to the manufacturer's instructions (Promega, Beijing, China). The specific

**Table 1.** The specific primers of *CAPN9* gene.

Primer	Sequence	Length (bp)
Q-1	5'-AGGGTCTGACACCACCTCC-3' 5'-GAGGTCTAGCAGAACAGGGC-3'	158
S-1	5'-CTAGGGAGGCTTGACAC-3' 5'-GGACAGGACCTCAAAAAGTA-3'	509
S-2	5'-GATCACCATGCAAGTGTACT-3' 5'-TCTTCACAGAACTGGCTAC-3'	537
S-3	5'-ACCAACCTAGCATGACACAG-3' 5'-GGGCAGCAGCACATAATACT-3'	416

Q-1: the primers used for Q-PCR;

S-1, -2, -3: the primers used for PCR of SNP.

primers of *CAPN 9* gene (Table 1) was designed with Primer Premier 5.0 software. Based on the sequence of *CAPN 9* gene (GenBank accession No. XM.419,585), the same specific primers (Q-1) were designed for RT-PCR and Q-PCR to detect the expression of *CAPN 9* gene.

RT-PCR was carried out in a  $20\text{-}\mu\text{L}$  reaction volume containing 100 ng cDNA template of AF or pectoralis major muscle,  $0.5\ \mu\text{L}$  of each primer (10 pM),  $10\ \mu\text{L}$  of  $2 \times$  Taq PCR MasterMix (Tiangen, Beijing, China) and the remaining volume being distilled deionized water ( $\text{ddH}_2\text{O}$ ). The amplification conditions were  $94^{\circ}\text{C}$  for 3 min (preliminary denaturation), followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $60^{\circ}\text{C}$  for 40 s, and extension at  $72^{\circ}\text{C}$  for 45 s; and a final elongation step at  $72^{\circ}\text{C}$  for 10 min.

Q-PCR was carried out in a  $20\text{-}\mu\text{L}$  reaction volume containing  $10\ \mu\text{L}$  of  $2 \times$  iQ<sup>TM</sup> SYBR Green Supermix,  $0.5\ \mu\text{L}$  (10 mM) of each primer, and 100 ng cDNA. Mixtures were incubated in an ABI 7500 Real-Time PCR Detection System (Applied Biosystems). A melting curve was constructed to verify that only a single PCR product was amplified. Samples were assayed in triplicate with standard deviations of CT values not exceeding 0.5 on a within-run basis.

### DNA Extraction and PCR Amplification

Genomic DNA was isolated from non-coagulated blood by the phenol-chloroform method (Sambrook and Russell, 2001) for PCR. The DNA integrity and purity were assessed by electrophoresis on 1.0% agarose gels and UV spectrophotometry.

According to the reference sequences (Chromosome 3: 40,615,856 to 40,650,876, Ensembl version 421,544.1), 60 pairs of primers were designed to amplify the sequences of *CAPN9* gene for screen the SNPs (Table 1, only listed 3 pairs primers, which had found the mutation in the according product). PCR was carried out in a  $20\text{-}\mu\text{L}$  reaction volume containing 50 ng genomic DNA template,  $0.5\ \mu\text{L}$  of each primer (10 pM),  $10\ \mu\text{L}$  of  $2 \times$  Taq PCR MasterMix (Tiangen, Beijing, China), and the remaining volume being distilled deionized water ( $\text{ddH}_2\text{O}$ ). The amplification conditions were  $94^{\circ}\text{C}$  for 3 min (preliminary denaturation), followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at

60°C for 40 s, and extension at 72°C for 45 s, and a final elongation step at 72°C for 10 min.

### SNPs Screen and Identification

For screening the SNPs of population, the pre-experiment was performed, and a genomic DNA pooling (100 ng/ $\mu$ L) with the equal amount DNA from every bird of 160 individuals was used for PCR as the template. Aliquots of the PCR products were analyzed by 1.5% agarose gel electrophoresis, and were then sequenced to identify the authenticity using an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Based on the results from an automated sequence shown in the form of a chromatogram, the sequence mutations would have been found, and the double peaks phenomenon in nucleotide base position was considered as the candidate SNP.

Based on these screened SNPs in pre-experiment, the fragments containing the SNPs were amplified again by the same PCR operation using the DNA from each bird. Every PCR product was directly sequenced to screen different genotypes. The sequence results with the chromatogram form were analyzed with the DNAMAN software (version 6.0, Lynnon Corporation, San Ramon, CA). As the evaluation criteria, the 2 homozygous types should have 1 peak for a single nucleotide base, and the heterozygous type has the double peaks for 2 different nucleotides. So the sequence mutations were identified between individuals, and the 3 different genotypes of every SNP were recorded.

### Statistical Analysis

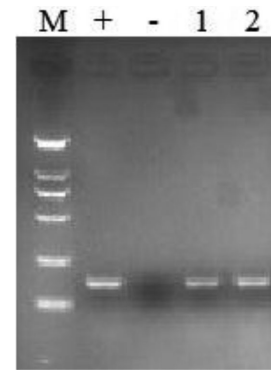
Data were analyzed using the general linear model procedure of statistical analysis systems software (SAS, Version 8.2; SAS Institute, Cary, NC). The general linear model  $Y_i = \mu + G_i + e$  was used to analyze the genotype effects of the *CAPN9* gene and the phenotype trait, where  $Y_i$ ,  $\mu$ ,  $G_i$ , and  $e$  are the measured value of trait phenotype, population mean, genotype effect, and residual error, respectively.

Differences in allelic or genotypic frequencies and between least squares means for the measured parameters in each of the genotypes were assessed. Correlations between several of the measured variables were analyzed using the Pearson's 2 test and, where appropriate, partial correlations were estimated. All experimental data are presented as mean  $\pm$  SEM. A  $P$ -value  $< 0.05$  was considered statistically significant.

## RESULTS

### Expression of Calpain 9 gene in Pectoralis Major Muscle and AF Tissues of Chicken

To determine if *CAPN9* was expressed in chicken muscle and adipose tissue, total RNA was isolated from



**Figure 1.** Expression of *CAPN9* mRNA in chicken abdominal adipose and breast muscle tissues. *CAPN9* mRNA expression was detected by RT-PCR. M: DL2000 Marker, +: positive control (stomach), -: negative control, 1: abdominal adipose tissue, 2: breast muscle tissue, respectively. The expected 158 bp product was verified by electrophoresis in abdominal adipose and breast muscle tissues.

**Table 2.** The related information of 8 mutation sites of *CAPN9* gene by 3 primers.

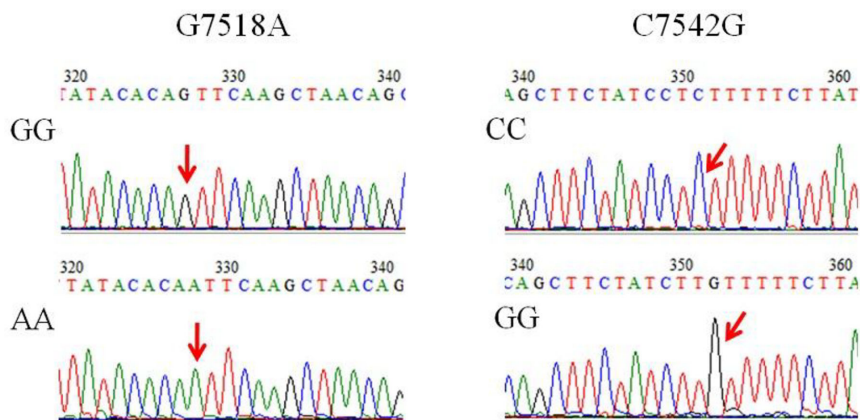
Mutation site	Genotype	Location	Primer
305	GG and AG	Exon 1	1
518	AA and AG	Intron 1	1
6689	TT, TC and CC	Intron 2	2
6769	CC, AC and AA	Intron 2	2
6820	TT, TG and GG	Exon 3	2
7518	GG, AG and AA	Entron 4	3
7540	CC, TC and TT	Entron 4	3
7542	CC, GC and GG	Entron 4	3

AF and pectoralis major muscle tissues, respectively. *CAPN9* mRNA expression was detected at the transcript level in AF and pectoralis major muscle tissues. After RT-PCR, the expected 158 bp product was verified by electrophoresis and confirmed by sequencing, and it was confirmed that *CAPN9* mRNA was present in adipose and breast muscle tissues (Figure 1).

### Screening of Calpain 9 gene SNPs

After qualified genomic DNA was obtained, the pooled DNA with equal amounts from each sample was used to amplify the *CAPN9* gene. Sequence analysis confirmed the correctness of the PCR products. Eight effective mutation sites were found in PCR products using 3 pair primers (S-1, S-2, S-3) (Table 1). Polymorphic loci were not found in other primers (not shown), so these 3 pair primers were chose for PCR amplification in the following experiment.

Among the 8 mutant sites (Table 2), 2 (G305A, A518G) were located in exon 1 and intron 1 for primer 1. They displayed only 2 genotypes (GG and AG, AA and AG). In addition, 3 sites were found in intron 2 (T6689C and C6769A) and exon 3 (T6820G) displayed 3 genotypes (TT, TC and CC; CC, AC and AA, TT, TG and GG) for primer 2. Similarly, 3 sites (G7518A, C7540T, and C7542G) were found in intron 4. These also displayed 3 genotypes (GG, AG and AA;



**Figure 2.** The genotyping of the linkage SNPs (G7518A and C7542G) in *CAPN9* gene. The mutation sites G7518A and C7542G were concatenated, and located in intron 4 of *CAPN9* gene. The homozygous genotypes were GG and AA (7518 site), CC and GG (7542 site), respectively.

CC, TC and TT; CC, GC and GG) for primer 3. Two sites (G7518A and C7542G) were identified as the linkage in intron 4. Their homozygous genotypes are provided in Figure 2.

### Effect of G7518A and C7542G Calpain SNPs on Carcass Traits

Analysis of genotypes and carcass traits by least square analysis revealed significantly different phenotypic values of CW, EW, AFW, AFP, and BMP traits (all  $P < 0.05$ ), but no significant difference on BMW ( $P > 0.05$ ) in birds with 2 linkage SNPs (G7518A and C7542G) among the 3 genotypes (Table 3). On the contrary, among the other SNPs, no significant differences were evident between genotypes and phenotypes (all  $P > 0.05$ ).

For the CW, EW, AFW, and AFP traits, the phenotype values in GG(7518)/CC(7542) homozygous genotype were significantly lower (all  $P < 0.05$ ) than AG(7518)/GC(7542) heterozygous genotype. There were no significant differences (both  $P > 0.05$ ) between GG(7518)/CC(7542) and AA(7518)/GG(7542), or AG(7518)/GC(7542) and AA(7518)/GG(7542) (Figure 3). However, for BMP, the phenotype values of AG(7518)/GC(7542) heterozygous genotype

were significantly lower ( $P < 0.05$ ) than of the AA(7518)/GG(7542) homozygous genotype. There were no significant differences (both  $P > 0.05$ ) between GG(7518)/CC(7542) and AG(7518)/GC(7542), or GG(7518)/CC(7542) and AA(7518)/GG(7542) (Figure 3).

### Effect of Calpain 9 SNPs on IMF Content

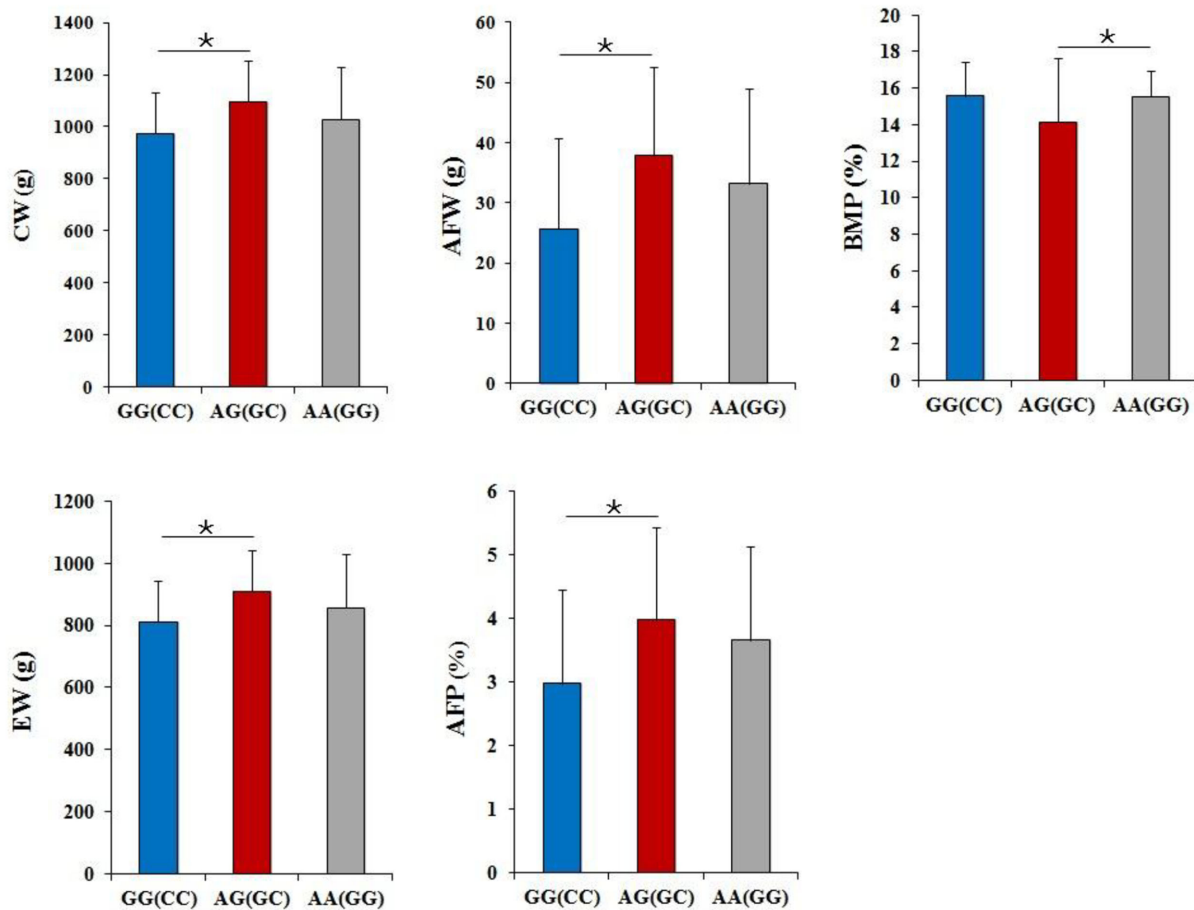
Least square analysis for each of the 8 mutant sites concerning the association between genotypes and IMF trait showed that the phenotypic values of IMF trait were not significantly different ( $P > 0.05$ ) among the 3 genotypes. However, for the 3 genotypes of SNPs G7518A and C7542G, the IMF content displayed a trend of gradual decrement of AA(7518)/GG(7542), AG(7518)/GC(7542), and GG(7518)/CC(7542) genotypes (Table 3). Similarly, BMW trait displayed the same trend with IMF content among 3 genotypes of SNPs G7518A and C7542G. In addition, the AA(7518)/GG(7542) genotype with the highest IMF content had lower AFW and AFP compared to the AG(7518)/GC(7542) genotype.

**Table 3.** Associations between genotypes with G7518A and C7542G and carcass traits and IMF content.

Locus	Genotype (n)	Genotype frequency (%)	Traits (M±S)						
			CW (g)	EW (g)	BMW (g)	AFW (g)	AFP (%)	BMP (%)	IMF (%)
7542	AA(64)	40.00	1028.44±201.65 <sup>a,b</sup>	856.38±173.33 <sup>a,b</sup>	133.35±30.13	33.13±15.75 <sup>a,b</sup>	3.66±1.48 <sup>a,b</sup>	15.54±1.40 <sup>b</sup>	2.68±1.15
	AG(68)	42.50	1096.41±157.41 <sup>b</sup>	910.19±131.22 <sup>b</sup>	128.89±35.46	37.77±14.88 <sup>b</sup>	3.98±1.46 <sup>b</sup>	14.16±3.51 <sup>a</sup>	2.56±1.00
	GG(28)	17.50	975.0±154.72 <sup>a</sup>	810.76±132.12 <sup>a</sup>	124.96±17.96	25.71±15.11 <sup>a</sup>	2.98±1.47 <sup>a</sup>	15.57±1.90 <sup>a,b</sup>	2.15±0.67
7518	GG(64)	40.00	1028.44±201.65 <sup>a,b</sup>	856.38±173.33 <sup>a,b</sup>	133.35±30.13	33.13±15.75 <sup>a,b</sup>	3.66±1.48 <sup>a,b</sup>	15.54±1.40 <sup>b</sup>	2.68±1.15
	GC(68)	42.50	1096.41±157.41 <sup>b</sup>	910.19±131.22 <sup>b</sup>	128.89±35.46	37.77±14.88 <sup>b</sup>	3.98±1.46 <sup>b</sup>	14.16±3.51 <sup>a</sup>	2.56±1.00
	CC(28)	17.50	975.0±154.72 <sup>a</sup>	810.76±132.12 <sup>a</sup>	124.96±17.96	25.71±15.11 <sup>a</sup>	2.98±1.47 <sup>a</sup>	15.57±1.90 <sup>a,b</sup>	2.15±0.67

AFP, abdominal fat percentage; AFW, abdominal fat weight; BMP, breast muscle percentage; BMW, breast muscle weight; CW, carcass weight; EW, eviscerated weight; IMF, intramuscular fat; different lowercase superscripts in each row indicate significant differences of phenotype.

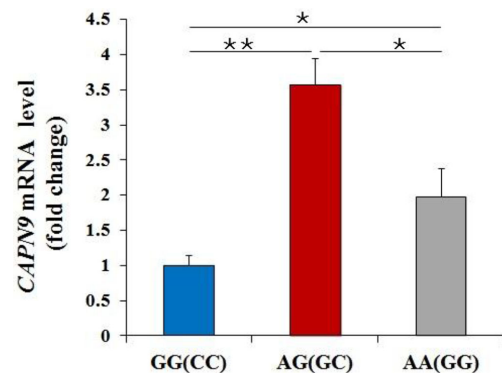




**Figure 3.** Association between of genotype of G7518A (C7542G) and slaughter indicator. By least square analysis, the phenotypic values of CW, EW, AFW, AFP, and BMP traits in different genotypes with G7518A/C7542G polymorphism were significant different (all  $P < 0.05$ ). Data are means  $\pm$  SEM,  $n = 28$ (GG/CC), 64(AA/GG), and 64 (GA/CG).

### Expression of Calpain 9 with Linkage SNPs (G7518A and C7542G) in AF Tissue of 3 Genotypes

Considering the important intron-mediated regulation of gene expression, the mRNA levels of *CAPN9* with linkage SNPs (G7518A and C7542G) in AF tissue of 3 genotypes were detected to explain the molecular regulation of SNP. As shown in Figure 4, the level of *CAPN9* mRNA in any 2 of 3 genotypes was significantly different ( $P < 0.05$  or  $P < 0.01$ ), and was significantly upregulated in the AG(7518)/GC(7542) genotype compared to the GG(7518)/CC(7542) genotype. The findings were consistent with the phenotypic values of AFW and AFP.



**Figure 4.** Expression level of *CAPN9* gene in AF tissue. The mRNA levels of *CAPN9* gene in AF tissue from different genotypes with G7518A and C7542G polymorphism were detected by Q-PCR. The level of *CAPN9* mRNA in any 2 of 3 genotypes had the significant difference ( $P < 0.05$  or  $P < 0.01$ ). Data are means  $\pm$  SEM,  $n = 6$ .

## DISCUSSION

The Calpain superfamily is a large group of calcium-activated cysteine proteases involved in a variety of functions, including cytoskeletal remodeling, cell signaling, apoptosis (Shioda et al., 2006; Gafni et al., 2009; McClung et al., 2009), and adipocyte differentiation (Patel and Lane, 1999; Yajima et al., 2006). *CAPN9*,

also known as nCL-4, a member of calpains (Lee et al., 1998), is expressed in the stomach and small intestine. In our current research, the expression of *CAPN9* gene in AF tissue and breast muscle tissue of chicken was detected, and the result shows for the first time that *CAPN9* is expressed in adipose and muscle tissue of chicken, and it was considered that

*CAPN9* might regulate the development of muscle or adipose tissue. Further, the polymorphism analysis of *CAPN9* by direct sequencing was performed to identify if *CAPN9* is functionally important in the development of muscle or adipose tissue of chicken.

DNA pooling was done to preliminarily screen the mutation site for *CAPN9* nucleotide sequences. After PCR amplification and sequencing, 8 mutation sites were found in different exons and introns by 3 pair primers. The corresponding nucleotide sequences with mutation sites in each bird in the detected population were sequenced to differentiate the genotypes. Least square analysis was done to explore the association between genotype and carcass traits. Two linkage SNPs (G7518A and C7542G) were found. The phenotype values of CW, EW, AFW, AFP, and BMP were significantly different among the 3 genotypes.

The phenotype values of CW and EW in GG(7518)/CC(7542) homozygous genotype were significantly lower than the AG(7518)/GC(7542) heterozygous genotype. Similarly, the phenotype values of AFW and AFP for the G7518A and C7542G linkage SNPs had the same result. Previous studies revealed the involvement of *CAPN9* in the regulation of gastric mucosal defense, hypertension, kidney disease, heart disease, and cancer (Markmann et al., 2005; Hata et al., 2010; Davis et al., 2014). However, the association of *CAPN9* with body weight, muscle development, and fat deposition has been unknown. The present findings support the view that *CAPN9* affects the development of body weight and fat deposition in chickens. The AG(7518)/GC(7542) heterozygote and GG(7518)/CC(7542) homozygote were the effective genotypes for body weight and fat deposition, respectively. However, different from other slaughter traits, the phenotype values of BMP for the G7518A and C7542G linkage SNPs in the GG(7518)/CC(7542) genotype were significantly lower than of the AG(7518)/GC(7542) genotype, although BMW was not significantly different. So, we believe that *CAPN9* could regulate the development muscle in chicken, but possibly in a different way than in adipose tissue.

IMF is deposited in muscle tissue. It is an important factor of meat quality (Farmer, 1999; Fernandez et al., 1999; Cui et al., 2017). The performance of high IMF and low AF is pursued in production of yellow-feathered broilers, and this goal was focal in this study. The AA(7518)/GG(7542) genotype had the highest IMF content, with the highest BMW and lower AFW and AFP. Although the phenotypic values of IMF were not significantly different among the 3 genotypes, the AA(7518)/GG(7542) genotype remains valid in production by long-term population selection.

Finally, the expression of *CAPN9* gene in AF tissue from 3 genotypes with linkage G7518A and C7542G polymorphisms was explored to uncover the expression regulation of *CAPN9* gene for this mutation site. An intron-mediated regulation on gene expression has

been described (Krizek, 2015; Visser et al., 2015; Cavalli et al., 2016). The mRNA levels of *CAPN9* in any 2 of 3 genotypes had a significant difference and consistency with the phenotypic value of AFW and AFP. So, it was confirmed that the G7518A and C7542G polymorphisms in intron 4 regulate *CAPN9* expression.

## CONCLUSION

In summary, we report for the first time that *CAPN9* is expressed in chicken adipose and breast muscle tissue, and affects production performance. Linkage G7518A and C7542G polymorphisms in intron 4 of *CAPN9* were found. Both affected body weight, muscle development, and fat deposition by regulating mRNA expression. The findings provide a marker and add to the knowledge of the molecular breeding of high-quality broilers. It is necessary to further clarify the molecular regulation mechanism of *CAPN9* on the development of chickens.

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## REFERENCES

- AOAC Official Methods of Analysis. 1990. Arlington, 15th edn. Association of Official Analytical Chemists, Virginia.
- Cavalli, M., G. Pan, H. Nord, and C. Wadelius. 2016. Looking beyond GWAS: allele-specific transcription factor binding drives the association of *GALNT2* to HDL-C plasma levels. *Lipids Health Dis.* 15:18.
- Cheverud, J. M., G. L. Fawcett, J. P. Jarvis, E. A. Norgard, M. Pavlicev, L. S. Pletscher, K. S. Polonsky, H. Ye, G. I. Bell, and C. F. Semenkovich. 2010. Calpain-10 is a component of the obesity-related quantitative trait locus Adip1. *J. Lipid Res.* 51:907–913.
- Cui, X. Y., R. R. Liu, H. X. Cui, G. P. Zhao, M. Q. Zheng, Q. H. Li, J. Liu, Z. Liu, and J. Wen. 2017. Effects of caponization and ovariectomy on objective indices related to meat quality in chickens. *Poult. Sci.* 96:770–777.
- Davis, J., S. G. Martin, P. M. Patel, A. R. Green, E. A. Rakha, I. O. Ellis, and S. J. Storr. 2014. Low calpain-9 is associated with adverse disease-specific survival following endocrine therapy in breast cancer. *BMC Cancer.* 14:995.
- Farmer, L. J. 1999. Poultry meat flavor [A]. Richardson Rl *et al.* Poultry meat science [C]. Pages 127–158. CAB International, New York.
- Fernandez, X., G. Monin, A. Talmant, J. Mourot, and B. Lebret. 1999. Influence of intramuscular fat content on the quality of pig meat-1. Composition of the lipid fraction and sensory characteristics of m.longissimus lumborum. *Meat Sci.* 53:59–65.
- Gafni, J., X. Cong, S. F. Chen, B. W. Gibson, and L. M. Ellerby. 2009. Calpain-1 cleaves and activates caspase-7. *J. Biol. Chem.* 284:25441–25449.
- Hata, S., M. Abe, H. Suzuki, F. Kitamura, N. Toyama-Sorimachi, K. Abe, K. Sakimura, and H. Sorimachi. 2010. Calpain 8/nCL-2 and calpain 9/nCL-4 constitute an active protease complex, G-calpain, involved in gastric mucosal defense. *PLoS Genet.* 6:e1001040.
- Huang, J. C., M. Huang, P. Wang, L. Zhao, X. L. Xu, G. H. Zhou, and J. X. Sun. 2014. Effects of physical restraint and electrical

- stunning on plasma corticosterone, postmortem metabolism, and quality of broiler breast muscle. *J. Anim. Sci.* 92:5749–5756.
- Krizek, B. A. 2015. Intronic sequences are required for AINTEGUMENTA-LIKE6 expression in Arabidopsis flowers. *BMC Res. Notes* 8:556.
- Lee, H. J., H. Sorimachi, S. Y. Jeong, S. Ishiura, and K. Suzuki. 1998. Molecular cloning and characterization of a novel tissue-specific calpain predominantly expressed in the digestive tract. *Biol. Chem.* 379:175–183.
- Lee, H. J., S. Tomioka, K. Kinbara, H. Masumoto, S. Y. Jeong, H. Sorimachi, S. Ishiura, and K. Suzuki. 1999. Characterization of a human digestive tract-specific calpain, nCL-4, expressed in the baculovirus system. *Arch. Biochem. Biophys.* 362:22–31.
- Markmann, A., S. Schäfer, W. Linz, M. Löhn, A. E. Busch, and P. Wohlfart. 2005. Down-regulation of calpain 9 is linked to hypertensive heart and kidney disease. *Cell Physiol. Biochem.* 15:109–116.
- McClung, J. M., A. R. Judge, E. E. Talbert, and S. K. Powers. 2009. Calpain-1 is required for hydrogen peroxide-induced myotube atrophy. *Am. J. Physiol. Cell Physiol.* 296:C363–C371.
- Ookura, T., E. Koyama, A. Hansen, J. H. Teeter, Y. Kawamura, and J. G. Brand. 2015. Phylogenetic analysis and taste cell expression of calpain 9 in catfish. *Nat. Sci.* 7:143–150.
- Patel, Y. M., and M. D. Lane. 1999. Role of calpain in adipocyte differentiation. *Proc. Natl. Acad. Sci. USA* 96:1279–1284.
- Sambrook, J., and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 1st edn. Cold Spring Harbor Laboratory Press, New York.
- Shioda, N., S. Moriguchi, Y. Shirasaki, and K. Fukunaga. 2006. Generation of constitutively active calcineurin by calpain contributes to delayed neuronal death following mouse brain ischemia. *J. Neurochem.* 98:310–320.
- Visser, M., R. J. Palstra, and M. Kayser. 2015. Allele-specific transcriptional regulation of IRF4 in melanocytes is mediated by chromatin looping of the intronic rs12203592 enhancer to the IRF4 promoter. *Hum. Mol. Genet.* 24:2649–2661.
- Yajima, Y., M. Sato, H. Sorimachi, M. Inomata, M. Maki, and S. Kawashima. 2006. Calpain system regulates the differentiation of adult primitive mesenchymal ST-13 adipocytes. *Endocrinology* 147:4811–4819.
- Zhao, L., T. Xing, J. Huang, Y. Qiao, Y. Chen, and M. Huang. 2018. Involvement of  $\mu/m$ -calpain in the proteolysis and meat quality changes during postmortem storage of chicken breast muscle. *Anim. Sci. J.* 89:423–431.