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Characterization of the expression profile of *calpain-3* (*CAPN3*) gene in chicken

Zeng-Rong Zhang · Qing Zhu · Yong-Gang Yao · Xiao-Song Jiang · Hua-Rui Du · Yi-Ping Liu

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Abstract Calpain-3 is a skeletal muscle-specific protease and participates in the regulation of myogenesis. In this study, we quantified the expression of *calpain-3* (*CAPN3*) mRNA in a Chinese local chicken breed (Sichuan Mountainous Black-boned chicken [MB]), to discern the tissue and ontogenic expression pattern. Meanwhile, we compared the *CAPN3* mRNA expression pattern in MB chicken at 10 weeks with a commercial meat type chicken line (S01) of the same age to identify the unique expression pattern under different genetic background. A real time quantitative PCR (qRT-PCR) assay was developed for an accurate measurement of its expression in various tissues from chickens at different ages (0, 2, 4, 6, 8, 10, and 12 weeks). Expression of the *CAPN3* mRNA was detected in the selected tissues, regardless of age. The breast muscle and leg muscle tissues had a significantly higher expression than the other tissues from the same individual ($P < 0.01$). Overall, the *CAPN3* mRNA level exhibited a "rise-decline" developmental change in detected tissues except for brain. The S01 chicken had a higher expression of the *CAPN3* mRNA in detected tissues than the MB chicken at 10 weeks. The present expression data of chicken *CAPN3* gene may provide some information to shed light on the tissue and ontogenic expression pattern during chicken development.

Keywords Chicken · *CAPN3* · qRT-PCR · Ontogenic expression · Tissue distribution

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Z.-R. Zhang · Q. Zhu · Y.-P. Liu (✉)
College of Animal Science and Technology, Sichuan Agricultural University, 46# Xinkang Road, Ya'an, Sichuan 625014, China
e-mail: liuyp578@yahoo.com

Y.-G. Yao
Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan 650223, China

Z.-R. Zhang · X.-S. Jiang
Sichuan Animal Science Academy, Chengdu, Sichuan 610066, China

H.-R. Du
Sichuan Dahen Poultry Breeding Company, Chengdu, Sichuan 610066, China

Introduction

The calpain system contains two well-characterized calcium-dependent neutral proteinases (calpain-1 and calpain-2) and their specific inhibitor (calpastatin) [1]. Several lines of evidence indicate that calpain-1, but not calpain-2, plays an important role in postmortem meat tenderization [2–4]. Of the other calpain family members, the skeletal muscle-specific calpain 3/p94 is of interest to meat scientists because it binds specifically to titin at the N_2 line, a site where proteolytic susceptibility in the early postmortem period has been closely linked to the tenderization process, and at the M line, where p94 may have a regulatory role in myofibrillogenesis [5, 6]. Although the *calpatin-3* (*CAPN3)* gene was initially suggested to be expressed only in skeletal muscle [7, 8], later studies have shown that this gene also expressed in other tissues [9].

The role of calpain-3 in postmortem meat tenderness and muscle proteolysis is controversial. In a pioneer study by Parr et al. [10], neither the abundance nor the rate of

autolysis of calpain-3 has been found to be associated with variability in porcine longissimus muscle tenderness. However, subsequent studies by Ilian et al. [11–14] observed significant correlations between meat tenderization and the expression and autolysis rate of the calpain-3. It was suggested that calpain-3 may regulate the activity of calpain-1 and calpain-2 by degrading calpastatin [15, 16]. The calpain-3 was present in all porcine fibers, but at a significantly lower level in slow type I fibers compared to fast type IIA/IIB fibers; the *CAPN3* mRNA expression in pig longissimus dorsi muscle was significantly decreased in different periods [17–19]. In cultured C2C12 cells (mouse myoblast cell line), calpain-3 regulated myogenesis via its action on the myogenic regulator factor [20].

Presently, few studies have been performed in chicken to evaluate the expression of the *CAPN3* gene in skeletal muscle tissue [21]. In a recent study, we identified two single nucleotide polymorphisms (SNPs) in chicken *CAPN3* gene in our established commercial pure lines of the meat-type quality chicken and four native Chinese chicken breeds, and discerned an association between the haplotypes of the *CAPN3* gene polymorphisms and some carcass traits [22]. However, it is necessary to verify the role of calpain-3 in controlling meat quality and carcass traits for the sake of future studies. In this study, we aimed to (1) develop a convenient approach to quantify the abundance of the *CAPN3* transcripts in chicken tissues, and (2) to determine tissue distribution and ontogenic expression of this gene, particularly in muscle tissues.

Materials and methods

Animals

Thirty-six chickens (half male and half female) at different ages from two breeds, Sichuan Mountainous Black-boned chicken (MB) and a commercial chicken (S01) from Sichuan Province were used in this study. Sichuan Mountainous Black-boned chicken (MB) is a native breed in Sichuan province, with spotty feathers, black or yellow skin, small body weight, low growth rate, and high meat tenderness [23]. The pure line S01 was developed by Sichuan Dahen Poultry Breeding Company using local breeds from Sichuan and Guangdong Provinces of China, and has yellow partridge plumage with blue shanks and white skin, relative high growth rate, and large body weight. All populations were raised under the same condition and were randomly selected. Tissue samples (including heart, liver, breast muscle, leg muscle, and brain) from MB chickens were collected at 0, 2, 4, 6, 8 and 12 weeks, respectively; at each time point, four chickens were slaughtered. We also collected tissue samples from six S01 chickens and six MB chickens at 10 weeks. Tissue samples were snap-frozen in liquid nitrogen and then stored at −80°C for total RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was isolated from heart, liver, brain, breast muscle, and leg muscle tissues using the TRIzol reagent (Invitrogen). The quality of RNA was determined by the $A_{260/280}$ absorbance ratio (1.61.8) and the integrity of the 18S and 28S rRNA bands on 1% formaldehyde agarose gel. Isolated RNAs were treated with 8 μl DNase (Fermentas) for 20 min at 37°C and stored at −80°C. The cDNA was synthesized using the ImProm-II Reverse Transcription System (TaKaRa) according to the manufacture's instructions. The reaction was performed in a volume of 10 μl containing 5× PrimerScript Buffer, 10 mM of each dNTPs, 40 U/μl RNase Inhibitor, 2.5 μM oligo-dT Primer. The reverse transcription was maintained at 30°C for 10 min, 45°C for 25 min, 99°C for 5 min, and ended with incubation at 4°C for 5 min, then stored at −20°C.

Real-time quantitative PCR (qRT-PCR) assay for the *CAPN3* gene

According to the chicken *CAPN3* mRNA sequence in GenBank (accession number NM_001004405.1), a pair of primers was designed by using Oligo 6.0 (Table 1).

The expression levels of chicken *CAPN3* gene were detected using the SYBR Green I assay on an IQ5 real-time PCR thermal cycle instrument (version 3.0a, Bio-Rad, German) and were normalized to the *beta-actin* gene expression (*ACTB*; GenBank Accession No. NM_205518.1). Relative transcript quantification was performed using standard curves generated for the *ACTB* and *CAPN3* genes based on an eight-fold serial dilution of the pooled cDNA product prepared from a subset of the gastrocnemius samples. The cycling conditions consisted of an initial single cycle of 95°C for 3 min, followed by 35 cycles of 15 s at 94°C and 1 min at 60°C. Reactions were performed in a volume of 25 μl, which included a 2.0 μl cDNA template, 1.0 μl of each specific primer (Table 1), 12.5 μl SYBR Green PCR Master Mix (TaKaRa, Japan), 1.0 μl calibration liquid (Bio-Rad), 7.5 μl PCR-grade water, and 1.0 μl cDNA. All real-time quantitative PCR amplifications were performed in triplicate for each sample and were analyzed using the $2^{-\Delta\Delta Ct}$ method described previously [24].

Statistical analysis

Expression data were described as Mean ± SD and were analyzed using the SAS 8.0 for Windows Software (SAS Institute Inc., Cary, NC). The expression levels of the

Table 1 Primers of real-time PCR assay for chicken *ACTB* and *CAPN3* genes

Gene	Primer sequences (5′ → 3′)	Location	Tm (°C)	Product length (bp)
beta-actin	F: GCCAACAGAGAGAAGATGACAC	408–429	60	140
	R: GTAACACCATCACCAGAGTCCA	526–547		
CAPN3	F: AGCACTACCACTGAAGGTACAGG	289–311	60	177
	R: GAGGGAAGTCTCATTAGGTGGA	444–465		

Note: The locations of primers for *beta-actin* and *CAPN3* genes were scored relative to their respective GenBank entries (*CAPN3* gene, NM_001004405.1; *beta-actin* gene, NM_205518.1)

Tm annealing temperature

CAPN3 gene between the tissue and age-related samples of the same breed and those samples from the two breeds with the same age were analyzed by the one-way ANOVA and student's *t*-test, respectively. A P value < 0.05 was considered statistically significant.

Results

Validation of the RT-qPCR assay for the *CAPN3* gene

Relative mRNA quantification was performed using standard curves generated for the *ACTB* and *CAPN3* genes based on a serial dilution (10^{-1}–10^{-8}) of cDNA. The crossing point, where the fluorescence curve of the sample turned sharply upward, indicating exponential amplification, was automatically determined by the real-time PCR software. The ranges of the Ct values for the *CAPN3* and *ACTB* genes were 9.62-32.14 and 9.35-33.16, respectively. Plotting the obtained Ct values relative to the relative concentrations of *CAPN3* and *ACTB* resulted in a linear correlation ($Y = -3.446X–0.445$ and $Y = -3.534X–0.962$, respectively) with square regression coefficient of 0.992 and 0.997, respectively, suggesting that quantification of the target cDNA was possible.

Tissue distribution of the *CAPN3* mRNA

qRT-PCR analysis showed that the *CAPN3* gene was expressed in all five MB chicken tissues. The *CAPN3* gene transcript had the highest expression level in breast muscle and leg muscle, followed by heart and liver, whereas the brain tissue had the lowest expression level. The difference of the *CAPN3* mRNA expression levels between muscle tissues (breast muscle and leg muscle) and other tissue from the same chicken was statistically significant ($P < 0.01$) (Table S1).

Ontogenic expression of the *CAPN3* mRNA

We analyzed the developmental changes of the *CAPN3* mRNA expression for each tissue in MB chickens of different ages. To compare the overall expression pattern in different tissues, every tissue expression level of the *CAPN3* mRNA at 12 weeks was arbitrarily set to 1. As shown in Fig. 1 and Table S2, the *CAPN3* mRNA in breast muscle had the highest expression at 4 weeks and the lowest expression at birth, the difference was significant ($P < 0.05$). Among liver tissue, the heart and brain tissues had the highest expression of the *CAPN3* gene at 2 weeks and the lowest expression at 6 weeks ($P < 0.05$). The leg muscle had the highest expression of the *CAPN3* mRNA at 8 weeks. Overall, the *CAPN3* mRNA levels exhibited a "rise-decline" developmental change in all tissues except for brain.

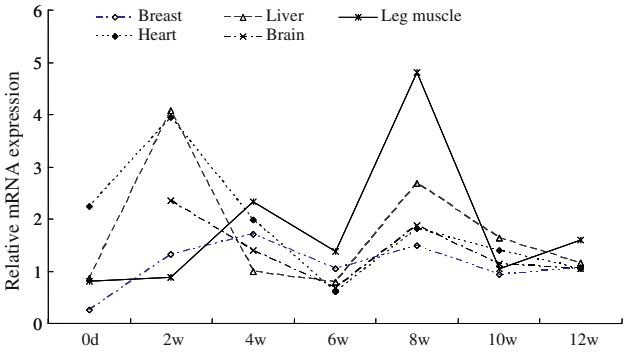

Fig. 1 Ontogenic expression of the *CAPN3* mRNA in tissues collected from chickens at different ages. The values were normalized to the endogenous *ACTB* expression in the same tissue; the expression level of the *CAPN3* gene in different tissues at 12 weeks was arbitrarily set to 1.0

CAPN3 gene expression in MB and S01 chickens at 10 weeks

To characterize whether the expression of the *CAPN3* mRNA showed differences between breeds, we analyzed the expression levels of this gene in two chicken breeds. Figure 2 presented the expression pattern of the *CAPN3* mRNA in the MB and S01 chickens at 10 weeks. The meat-type S01 chicken had a higher expression of the *CAPN3* gene than that of MB chicken in detected tissues

except for brain, and the difference was statistically significant ($P < 0.05$).

Discussion

In this study, we quantified the tissue distribution and ontogenic expression of *CAPN3* mRNA in two Chinese chicken breeds, and found the *CAPN3* mRNA was expressed in all five different tissues with a dominant expression in muscle tissues. This pattern was in agreement with the pattern observed by others [9–11]. We speculate that the *CAPN3* gene plays an active role in muscle development. However, the exact mechanism for the increasing expression of the *CAPN3* remains elusive, especially as a protease, it is likely to have an important in protein breakdown or protein turnover, and it was seemingly associated with the increasing of muscle deposition in chicken.

It is well known that the breast muscle of chicken is made of white and fast oxidative glycolytic fibers, whereas the leg muscle has red and slow oxidative aerobic fibers. The red oxidative fibers have a faster protein degradation rate than the white glycolytic fibers [25]. Several studies have showed that growth stage from 2 weeks to 8 weeks is an important period for muscle fiber development [26, 27]. In this study, we quantified the ontogenic expression of the *CAPN3* mRNA in the five tissues collected at different growth points, and observed a significant change in the *CAPN3* gene expression during the development. The relative expression level of the *CAPN3* mRNA in breast muscle was dominant between 2 weeks and 8 weeks. The overall pattern of the *CAPN3* expression in breast muscle was remarkably different from that in leg muscle; this may be consistent with the notion that CAPN3 is involved in degradation of the myofibrillar proteins [28]. We also found that the relative expression level of the *CAPN3* mRNA in the S01 chicken was higher than that in the MB chicken at 10 weeks. Although we did not analyze the *CAPN3* mRNA expression in S01 chicken at all seven developmental stages like we have done for the MB chicken, we thought that the comparative analysis of the *CAPN3* mRNA in the two chicken breeds at 10 weeks may provide some insight into the uniquely expression pattern of *CAPN3* mRNA in chickens of different genetic backgrounds. Our observed expression pattern of *CAPN3* mRNA was in agreement with the marked morphological differences between the two chicken breeds, despite the fact that we have no data showing specifically, in a mechanistic way, how an increase in *CAPN3* expression leads to a change in the number of myofibrillar proteins. Investigation of all developmental stages, and of calpain-3 and its muscle protein substrates in these two chicken breeds may further explain the observed variation in meat tenderness.

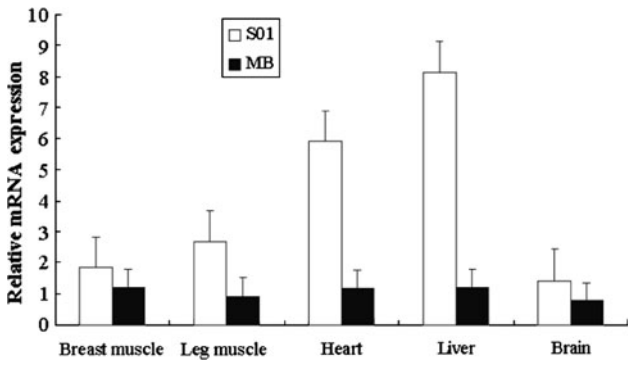

Fig. 2 The relative mRNA expression of the *CAPN3* gene in tissues collected from chickens belonging to the meat-type S01 line and Sichuan Mountainous Black-boned chicken (MB) breed at 10 weeks

In conclusion, we have developed a real-time PCR method to detect tissue distribution and ontogenic expression of the *CAPN3* mRNA in chicken. We found that *CAPN3* expression may be related to muscle fiber development. These results, together with our recent association study of the *CAPN3* gene polymorphisms with chicken carcass traits [22], suggest that the *CAPN3* gene plays an important role in the regulation of muscle deposition in chicken.

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On-line Supplementary Data

Table S1 Tissue distribution of the *CAPN3* mRNA at the same growth point

Growth point	Breast muscle	Heart	Liver	Brain	Leg muscle
0 week	301.34^{A}±45.13	9.23^{B}±0.84	1.30^{B}±0.09	\	467.61^{A}±65.25
2 weeks	356.06^{A}±36.59	3.18^{C}±0.97	1.18^{C}±0.93	0.59^{C}±0.08	99.67^{B}±6.59
4 weeks	1794.76^{A}±89.31	6.14^{C}±0.99	1.11^{C}±0.64	1.36^{C}±0.78	1013.09^{B}±76.03
6 weeks	1563.31^{A}±213.97	2.62^{C}±0.99	1.26^{C}±0.95	0.94^{C}±0.65	842.98^{B}±112.42
8 weeks	555.68^{A}±16.44	2.00^{B}±0.87	1.08^{B}±0.42	0.65^{B}±0.21	747.59^{A}±16.92
10 weeks	647.39^{A}±19.58	2.81^{B}±0.97	1.21^{C}±0.48	0.72^{C}±0.32	298.47^{A}±9.85
12 weeks	926.92^{A}±25.14	3.25^{B}±0.98	1.24^{B}±0.78	0.94^{B}±0.41	744.14^{A}±21.73

Note: Values in a line without a common uppercase mean that the relative quantities of the *CAPN3* gene differ significantly ($P < 0.01$). Expression data were based on Sichuan Mountainous Black-boned chicken (MB) and were normalized to the endogenous *ACTB* expression in the same tissue.

Table S2 Ontogenic expression of the chicken *CAPN3* mRNA

Week	Breast muscle	Heart	Liver	Brain	Leg muscle
0w	0.26^{b}±0.04	2.24^{a}±0.55	0.86^{b}±0.08	\	0.80^{b}±0.20
2w	1.31^{a}±0.39	3.94^{a}±0.68	4.07^{a}±0.62	2.34^{a}±0.23	0.88^{b}±0.21
4w	1.71^{a}±0.40	1.98^{a}±0.28	0.98^{b}±0.32	1.39^{b}±0.13	2.32^{a}±1.22
6w	1.05^{a}±0.93	0.60^{b}±0.14	0.79^{b}±0.07	0.68^{b}±0.07	1.36^{b}±0.42
8w	1.48^{a}±0.23	1.81^{a}±0.27	2.68^{a}±1.64	1.86^{a}±0.26	4.81^{a}±0.88
10w	0.94^{a}±0.07	1.38^{b}±0.45	1.63^{b}±0.13	1.12^{b}±0.13	1.04^{b}±0.56
12w	1.06^{a}±0.19	1.05^{b}±0.56	1.14^{b}±0.51	1.04^{b}±0.21	1.60^{b}±0.72

Note: Values in a column without a common lowercase mean that the relative quantities of the *CAPN3* gene in a tissue at different growth points differ significantly ($P < 0.05$). Expression data were based on Sichuan Mountainous Black-boned chicken (MB) and were normalized to the endogenous *ACTB* expression in the same tissue. The expression level of the *CAPN3* gene in different tissues at 12 weeks was arbitrarily set to 1.0.