Analyses of Chicken Tenderness Traits Based on Transcriptome Sequencing

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Abstract The calpain system is ubiquitous in cells, mainly comprising calpains and calpain inhibitors, and is a widespread calcium-dependent cysteine protease in organisms that is involved in many cellular processes such as muscle degradation *in vivo* and affects the tenderness of meat after animal slaughter. The study found 128 DEGs that probably regulated tenderness traits were selected from 16 significantly enriched GO terms by transcriptome sequencing analysis, and found that the developmental changes in the expression levels of the *CAPN*1 gene in the pectoral and leg muscles were significantly positively correlated ($P < 0.05$) with the cumulative growth values of live weight and comb weight. The developmental changes in the expression levels of the *CAST* gene in the pectoral and leg muscles were not significantly correlated with the cumulative growth values of live weight and comb weight. Our results helped demonstrate the potential molecular mechanisms of tenderness in chickens and provide valuable information for chicken breeding.

Key words Chicken; Tenderness traits; Transcriptome sequencing

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The calpain system is ubiquitous in cells, mainly comprising calpains and calpain inhibitors. Calpain (Calpain, CAPN) is a widespread calcium-dependent cysteine protease in organisms that is involved in many cellular processes such as muscle degradation *in vivo* and affects the tenderness of meat after animal slaughter. Calpastatin (Calpastatin, CAST) is an endogenous, Ca^{2+}-activated calpain inhibitor that can inhibit the degradation of muscle proteins and inhibit the activity of calpains after slaughter, reducing protein hydrolysis[1-2]. Studies have shown that the genes of the calpain system are involved in the renewal of proteins during muscle growth and are closely related to the growth and tenderness of animal muscles[3-4].

Currently, the research on the calpain system in mammals mainly focuses on the correlation between calpain activity and the tenderness of postmortem muscle and the degradation amount of muscle protein. MicroRNAs (miRNAs) contain 20 - 22 nucleotides and are small non-coding RNAs responsible for post-translational regulation in plants and animals[5-6]. Several researchers have reported the association of miRNAs indicated in this study with chicken growth. The seed region of miR-1657 was found to be correlated with chicken growth and tenderness traits[7]. Transcriptome sequencing can provide comprehensive information about the chicken genome, including the structure and expression patterns of genes. Studying the expression of genes in different tissues and their association with tenderness traits helps to gain a deeper understanding of the molecular mechanisms that affect tenderness. In this study, global miRNA sequencing was used to investigate the differential expression of miRNA and its potential regulatory mechanisms related to tenderness and growth rates between the MC, ZJ and DH chicken.

Materials and Methods

Sample collection

MC, ZJ and DH chickens were obtained from the Sichuan Dahen Animal breeding company. All tissues were collected from 10-week-old chickens, which were then snap-frozen in liquid nitrogen and stored at -80 ℃. All experiments on chickens were carried out based on the guidelines of EU legislations on the ethical use and care of laboratory animals.

RNA isolation, Small RNA sequencing quality control and expression analysis

Total RNA was isolated from breast muscle using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.) per the manufacturer's instructions. The quantity and purity of RNA were determined using NanoDrop ND-1000 spectrophotometer at 260/280 nm (Nano Drop Technologies, Wilmington, Delaware). RNA integrity was measured via agarose gel electrophoresis. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). The differentially expressed miRNAs were identified by counts screening using the DESeq2[8] R package, with a threshold of Log2FC >1 or <-1 and P value <0.05. The miRNA target prediction was performed through the combination of Miranda (http://www.microrna.org/microrna/) with a score $\geqslant 150$ and energy <-20 and RNAhybrid[9] with energy <-25. The results were obtained by the intersection of the two prediction tools. The heat map of differential expression was generated by Heml 1.0.

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Bioinformatics analysis

Gene oncology analysis (http://geneontology.org) was performed to analyze the main function of the target genes based on Fisher's exact test[10-12]. Pathway analysis was done to find out the significant pathway of miRNA targets based on the KEGG database (http://www.genome.jp/Kegg/).

Quantitative real-time PCR

Total RNA was extracted from tissues using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Two micrograms of RNA was used for cDNA synthesis through reverse transcription using PrimeScript RT Master Mix (Takara). Real-time PCR was performed in triplicate in a 96-well plate using 1 μl of cDNA and SYBR Green PCR mix (Bio-Rad) on a Real-Time PCR System (Thermo Fisher). The primer sequences of these miRNAs are listed. Expression of beta-actin was used to normalize gene expression. By convention, changes in expression were determined using $2^{-\Delta\Delta CT}$ method.

Results and Analysis

Analysis of DEGs between lines

To compare genetic difference among these three lines, the significantly expressed genes between lines MC and ZJ, lines MC and DH, and lines ZJ and DH under different development stages were mined separately. Initially, we compared the gene expression profile among lines MC, ZJ and DH, and found that 1953 DEGs were screened between ten-week-old line DH and line B, and 932 genes were more highly expressed in line ZJ than in line DH.

Gene-act-network and candidate genes for tenderness trait

After GO analysis and pathway analysis, 128 DEGs that probably regulated tenderness trait were selected from 16 significantly enriched GO terms (including terms related to muscle cell differentiation, muscle structure development, growth) and 13 significantly enriched pathways. To further explore the interactions between these DEGs, the gene-act-network was established based on the relationships between these DEGs in terms of expression and interaction. Several DEGs played a core role in the PPI network, including calcium-dependent cysteine protease, calpastatin, insulin like growth factor 2, apoptosis regulator, indicating that these genes may play key roles in regulating tenderness trait of chicken.

Correlation analysis of relative expression levels of *CAPN*1 gene with traits

The developmental changes in the expression levels of *CAPN*1 gene in pectoral muscle, leg muscle, heart, and liver tissues were respectively correlated with the cumulative growth values of the above traits by Pearson correlation analysis. As shown in Table 1, the developmental changes in the expression levels of *CAPN*1 gene in both pectoral muscle and leg muscle were significantly positively correlated with the cumulative growth values of live weight and comb weight ($P<0.05$); and the developmental changes in the expression levels of *CAPN*1 gene in heart and liver tissues had no significant correlation with the three traits under investigation ($P>0.05$).

Table 1 Results of correlation analysis between the relative quantities of *CAPN*1 gene and traits

Trait		Relative quantity of *CAPN*1 gene			
		Breast muscle	Liver	Heart	Leg muscle
Live weight	R	0.726	0.543	0.457	0.711
	P	0.023	0.095	0.124	0.022
Intramuscular fat content	R	0.390	0.195	0.330	0.272
	P	0.232	0.543	0.242	0.361
Comb weight	R	0.641	0.430	0.417	0.743
	P	0.042	0.147	0.212	0.019

R = Pearson Correlation Coefficients; P = Probability.

Correlation analysis between the relative expression of *CAST* gene and traits

The developmental changes of *CAST* gene expression in pectoral muscle, leg muscle, heart and liver tissues were respectively correlated with the cumulative growth values of the above traits by Pearson correlation analysis. As shown in Table 2, there was no significant correlation between the developmental changes of *CAST* gene expression in pectoral muscle, leg muscle, heart and liver tissues and the three traits under investigation ($P>0.05$).

Table 2 Results of correlation analysis between the relative quantities of *CAST* gene and traits

Trait		Relative quantity of *CAST* gene			
		Breast muscle	Heart	Liver	Leg muscle
Live weight	R	-0.423	-0.572	-0.331	0.423
	P	0.171	0.056	0.283	0.172
Intramuscular fat content	R	-0.185	0.05	-0.07	0.272
	P	0.541	0.871	0.754	0.369
Comb weight	R	-0.446	-0.612	-0.451	0.342
	P	0.162	0.057	0.163	0.271

R = Pearson Correlation Coefficients, P = Probability.

Conclusions

Chicken muscle growth is important traits to evaluate the production of poultry meat. miRNAs play a vital role in the growth and development[13-14].

The calpain system controls the degradation of myofibrillar proteins. Myofibers are the basic units that make up muscles, and the state of myofibers will directly affect the quality of chicken meat. In this study, we found 128 DEGs that probably regulated tenderness trait were selected from 16 significantly enriched GO terms and 13 significantly enriched pathways.

The expression changes of the *CAPN*1 and *CAST* genes in the pectoral and leg muscle tissues mainly manifest in the degradation of muscle proteins and the growth and development changes of myofibers. It is known that the development of the comb can directly reflect the sexual maturity of chickens. The early-maturing MC chicken has a small body weight. Therefore, comb weight can also reflect the growth and development of chickens to a certain extent. Meanwhile, this study found that the developmental changes in the expression levels of the *CAPN*1 gene in the pectoral and leg muscles were significantly positively correlated ($P < 0.05$) with the cumulative growth values of live weight and comb weight. The developmental changes in the expression levels of the *CAST* gene in the pectoral and leg muscles were not significantly correlated with the cumulative growth values of live weight and comb weight.

In summary, it is hypothesized that the expression level of the *CAPN*1 gene in muscle tissues may be closely related to the growth and development of myofibers and plays an important regulatory role in the metabolism of myofibrillar proteins. The research on *CAST* genes as candidate genes for meat quality has just begun. In general, the specific regulatory mechanism of the calpain system is not very clear and requires further in-depth research.

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