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Original Article

Analysis on Differentially Expressed Genes of Muscle Tissues in Rugao Chicken at the Ages of 2 and 12 Weeks by Microarray

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Wei Zhou[1] Yan Liu[2] Guo Hong Chen[1]*

Abstract

Microarray containing 13319 probes was used to construct gene expression profiles in order to screen differentially expressed genes of muscle tissues in Rugao chicken and investigate the molecular mechanism related with muscle tissue traits at 2 and 12 weeks old of chicken. Two hundred and eight differentially expressed genes, of which 108 were known genes, 94 up-regulated, 114 down-regulated were involved in growth, molecular mechanism, fat metabolism, cell proliferation, transcription and splicing factor, protein synthesis and degradation. Meanwhile, some genes of these differentially expressed genes that had no annotation in GenBank were screened out, they were presumed to be unknown new genes. The differential expressions of 4 genes were confirmed by real-time quantitative PCR and the results indicated that the expression changes of these genes were generally consistent with the results of GeneChip, a technology that could be used in breeding and selection of Chinese native chicken breeds.

Keywords: chicken, GeneChip, muscle tissues, gene expression profile, real-time quantitative PCR

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บทคัดย่อ

การวิเคราะห์การแสดงออกของยีนในกล้ามเนื้อของไก่สายพันธุ์ Rugao ในช่วงอายุ 2 และ 12 สัปดาห์ โดยเทคนิคไมโครอะเรย์

De Qin Luan[1] Guo Bin Chang[1] Ting Zhen[1] Zheng Yang Huang[1] Wei Zhou[1] Yan Liu[2] Guo Hong Chen[1]*

เทคนิคไมโครอะเรย์ซึ่งประกอบด้วย 13319 probes ถูกนำมาใช้ในสร้างรูปแบบ เพื่อศึกษาการแสดงของยีนในระยะการพัฒนาในกล้ามเนื้อของไก่สายพันธุ์ Rugao และศึกษากลไกระดับอณูชีวโมเลกุลที่เกี่ยวข้องกับกล้ามเนื้อในไก่อายุ 2 และ 12 สัปดาห์. ทำการศึกษาการแสดงออกของยีนจำนวน 208 ชนิด ซึ่งประกอบด้วย 108 known genes 94 up-regulated และ 114 down-regulated ซึ่งยีนดังกล่าวมีบทบาทในการเจริญ กลไกระดับอณูชีวโมเลกุล เมตาบอลิสมของไขมัน การงอกขยายของเซลล์ transcription and splicing factor การสังเคราะห์และการสลายโปรตีน ในขณะเดียวกันยีนบางชนิดยังไม่มีรายงาน การแสดงออกของยีนที่แตกต่าง 4 ชนิดถูกทดสอบโดยวิธี real-time quantitative PCR และผลการแสดงออกแสดงในรูปแบบของ GeneChip ซึ่งเป็นเทคโนโลยีที่ใหม่สำหรับการคัดเลือกสายพันธุ์ ของไกพันธุ์พื้นเมืองในประเทศจีนได้

คำสำคัญ: ไก่ GeneChip กล้ามเนื้อ รูปแบบการแสดงออกของยีน real-time quantitative PCR

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Introduction

Chicken is an important model organism that bridges the evolutionary gap between mammals and other vertebrates (Hillier et al., 2004). Recently, studying the function of growth genes in muscle tissues was a hotspot and a difficulty (Oudin et al., 1998). As we all know, the growth of muscle tissues directly affects the quality of chicken. So one of the issues which should be solved as soon as possible for chicken production is how to make production that is good in quality and unique in flavor. Exploring the molecular mechanism of related genes of the growth for muscle tissues in synthesis and decomposition, transshipment and sedimentary not only is an effective way but also shows the physiological mechanism. Therefore, in order to broaden the knowledge of science and production of chicken, studying the growth of muscle tissues in molecular level is important.

With the development of chicken production modernization and the fast improving of living standard, the chicken muscle growth is now the issue of common concern by researchers. The study of muscle fiber had been emphasized by earlier researchers. In fact, Alberle (1979) researched on the chicken skeletal muscle fiber, and Ono (1993) found the basic discipline of earlier broiler growth in different muscle fiber. Later, researchers applied molecular technology to the growth of chicken muscle, some scholars began to focus on the effect of IMP (inosine-5'-monophosphate) and fatty acids content of chicken muscle on meat quality (Chen et al., 2009), Zhang et al. (2009) used PCR-SSCP (Polymerase Chain Reaction-Single Strand Conformation Polymorphism) and DNA sequencing to detect SNPs in three genes, the results showed that GPAT (glutamine-PRPP aminotransferase), AIRC (aminoimidazole ribonucleotide carboxylase) and PURH (ATIC : IMP cyclohydrolase) genes had significant effect on the muscle IMP content in Baier chickens (p<0. 05), they were candidate loci or linked to major genes that affected muscle IMP content. Tu et al. (2009) used RT-PCR technique to study gene expression in fatty acid-binding protein (H-FABP) gene in Luyuan chicken and Recessive white chicken at 12 weeks old, finding that H-FABP mRNA expression level had significantly negative correlation with IMF (intramuscular fat) content. The H-FABP mRNA expression level in IMF content in recessive white was significantly higher than that of Luyan which growed slowly. However, until now there has been few report involving the molecular mechanisms of growth and development of chickens, especially the study of local chicken breeds, and there are considerable important factors which affect the muscle growth (William et al., 1991). Actually, the advances in meat growth research was restricted by basic discipline on the growth of muscle tissues which was not completely researched and the complicated living activity of the growth of muscle tissues and molecular metabolism of which made future research involve poly-gene, multi-way and multi-step. Nowadays, the approaches to candidate gene and

whole genome scanning are used as the main investigative means, but have not been able to solve the problem yet because of the limitation. Therefore, this study aimed for a high throughput approach for screening differentially expressed genes to examine the effects and mechanism of regulation genes on the growth and development and molecular metabolism of muscle. The emergence and application of GeneChip have paved the way to achieve this goal. GeneChip is a technique used to detect specific gene expression by fixing intensive oligonucleotide or gene fragment on slides, silicon or nylon membrane (Morgan et al., 2001). The technology is mainly used in the expression of different genes, search for new genes, molecular diagnosis of disease, gene mutation and polymorphism analysis and drug development in order to analyze and judge the specificity of these comprehensive gene expressions to large-scale (Yutaka et al., 2008).

In this study, the microarray of gene expression profiles were used to screen differently expressed genes of muscle tissues of Rugao chicken at 2 and 12 weeks old in order to analyze the differences of expression profiles and study the possible connection and discipline among them in molecular biology, and then classify the different related-gene by GO to provide a theoretical basis for further research.

Materials and Methods

Chips: Every chip (Agilent Company, 15K gene chip, America) had 43604 points in which every 3 point represents 3 repeats of one gene. It had 13319 probe sequences of chicken, excluding negative controls and housekeeping genes.

Experimental animals: Full sib family of Rugao chicken (China native breeds, Jiangsu province) were from Institute of Poultry, China Academy of Agricultural Sciences. The birds were slaughtered at 2 and 12 weeks old, breast muscle and leg muscle were isolated, immediately frozen in liquid nitrogen and stored at -70°C. The eight birds used in the present study were divided into 2 groups: group A consisted of 2 males and 2 females aged 2 weeks old and group B consisted of 2 males and 2 females aged 12 weeks old, each muscle tissue for one chip.

RNA isolation and total RNA purification: Pieces of the muscles were crushed under liquid nitrogen. 50-100 mg tissues of each bird were used to isolate total RNA using TRIzol reagent (Agilent, America), according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 ml of TRIzol reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenization, insoluble material was removed from the homogenate by centrifugation at 12,000xg for 10 min at 4°C. Then total RNA of intestine by Ribonucleic Acid Analysis, 2100 (Agilent, America), was purified by QIAGEN RNeasy Kit (QIAGEN Company, Germany), according to the introduction of Qiagen (QIAGEN Company, Germany).

Preparation of DNA probe and fluorescein mark: cRNA samples were purified and marked with kit instructed by QIAGEN Company, which were synthesized from mRNA by two reversed transcriptions, then the concentration and the purity of the samples were analyzed at OD 260 nm and OD 280 nm with ultraviolet spectrophotometer by Cy3-marked, the content of cRNA was adjusted, the molecular concentration of fluorescence was analyzed, the content of Cy3 at 552 nm was tested and the incorporation efficiency was calculated.

Table 1 Primer pairs used to analyze gene expression by quantitative RT-PCR, and size of product

Gene	Forward primer[a]	Reverse primer[a]	Size
ENO1	TCAAGATGTCCATTCTGAAGATCCA	CCAAGTAGCGTGTCTTGTCATTGTC	180 bp
CCNB2	CCAGGTCCACTCAAGGTTCCA	GCTGTTACACCCACCAACTGAAG	123 bp
PSMA7	ATCCGGTCACCGTGGAGTACA	AGGTCTGCGACGTTTGCTTTG	80 bp
POLR1B	ACATGGTCTCCGACAAGTTCCAG	ATGAGCCAGCAACGCATCAC	134 bp
GAPDF	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	152 bp

a: Sequences of oligonucleotides are indicated from 5' to 3' end

cRNA fragmentation and microarray hybridization: The mixed fragmentation liquor was prepared with Cy3 cRNA 875 ng, 10x blocking agent 11 µl, 25x fragmentation buffer 2.2 µl, nuclease-free water x µl, then incubated in 60°C water for fragmentation for 30 min, which was added 55 µl 2x GEx hybridization buffer, and 100 µl of which was taken on the chip, they were rolling hybridized at 65°C, at 10 rpm, for 17 hours.

Washing and scanning: The chip was dislodged into solution 1(GE washing buffer 1, Agilent, America) for washing for 1 min, and then put into solution 2 (GE washing buffer 2, Agilent, America) for washing for 1 min at 37°C, which was scanned in Agilent scanner with a resolution of 5 µm, and the scanner automatically scanned the chip to 100% and 10% PMT (poly-3-methyl-thiophene) for each, respectively.

Microarray data normalization and Analysis: The chip data were received from Agilent scanner. Raw data sets were normalized to total fluorescence, which represented the total amount of cRNA hybridized to a microarray using the quantile method and analyzed by Genespring software and the SAS system (Shanghai Biotechnology Corporation, China). The functional annotation and different functional classifications were divided by Gene Ontology (GO: http://www.geneontology.org/).

Real-time quantitative RT-PCR: Oligonucleotide

primers were designed to amplify a fragment containing sequences from two adjacent exons in order to avoid contamination with genomic DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered to be a stably expressed housekeeping gene and was used as an internal reference gene. The primer pairs were used to analyze gene expression and the size of product are shown in Table 1.

Four genes were selected from all differently expressed genes for further investigation into their expression. Real-time quantitative RT-PCR was used to evaluate. The reaction was performed in a volume of 20 µl and carried out in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) (Applied Biosystems) programmed to conduct one cycle at 95°C for 30 sec and 40 cycles at 95°C for 15 sec and 60°C for 34 sec. The dissociation curves were analyzed for each PCR reaction to detect and eliminate possible primer-dimer artifacts. Results (fold changes) were expressed as 2-ΔΔCt with ΔΔCt = (Ct,Target-Ct, GAPDH) sample X - (Ct,Target –Ct, GAPDH) sample 1, where Ct,Target and Ct, GAPDH in the first brackets were the Ct for target gene and for GAPDH in a sample (named X), and Ct,Target and Ct, GAPDH in the second brackets were the Ct for target gene and for GAPDH in a sample 1, expressed as the standard, T-test *p*-values were determined for each gene (Bourneuf et al., 2006).

Table 2 BW, BMP and LMP of chickens used in the study

Groups	Body weight	Percentage of breast muscle	Percentage of leg muscle
2 wks	168.21±25.44**	2.61±0.12**	2.33±0.16**
12 wks	2808.71±58.55**	12.31±2.80**	16.64±1.93**

** Stands for the level of significant difference of the same line (p<0.01)

Results

Characterization of the two chicken groups: It is clear that the BW of 12 week old birds was significantly higher than that of 2 week-old birds. The percentage of breast muscle and percentage of leg muscle are different. The BMP and LMP of 12 week old and 2 week old birds were 12.31% and 16.64%, and 2.61% and 2.33%, respectively (Table 2). The birds used in this study differed significantly not only in body weight but also in percentage of breast muscle and percentage of leg muscle (Fig 1) and were chosen on that basis. BMP and LMP of 12 week old birds were six times those of 2 week old birds (BW: body weight; BMW: breast muscle weight; BMP: Percentage of breast muscle; LMW: leg muscle weight; LMP: Percentage of leg muscle).

The chip scanning results: From the results obtained by the chip in the scan, the light and dark of fluorescent colors represented the signal strength of gene dot blot. Samples were marked by using green fluorescent (cy3) dye in Agilent microarray (single marked). If the scanning results were green, the lighter color meant the stronger signal and vice versa. If they were black, it meant that the signal was very weak or there was no signal. If they were white, it meant that the signal was saturated. The background was homogenized after scanned. The scanned images results (partial enlargement) are shown in Fig 2.

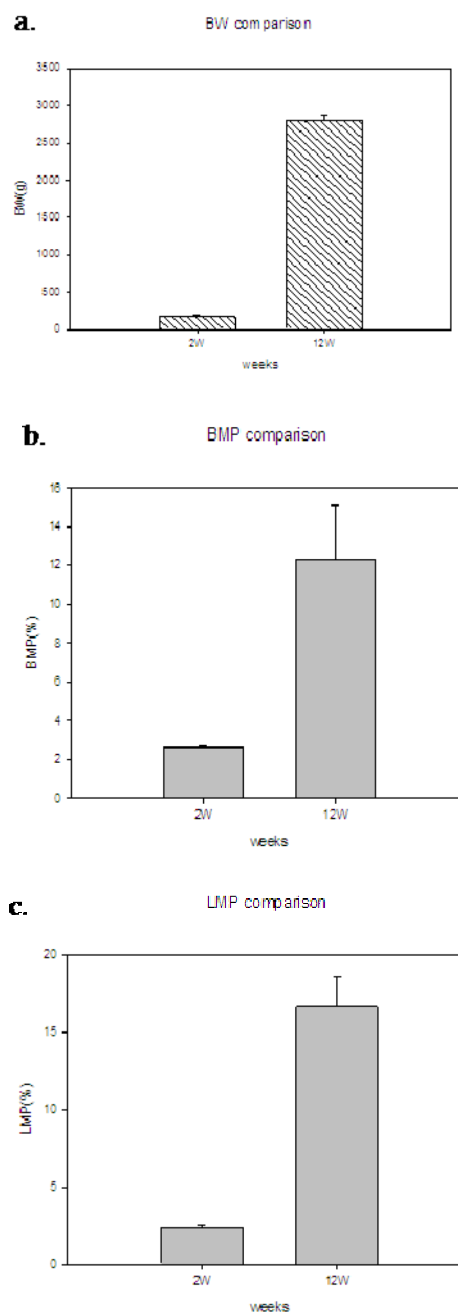

Figure 1 Comparison of BW, BMP and LMP in chickens used in the study. The BMP was calculated as BMP (%) = BMW (g)/BW (g). The LMP was calculated as LMP (%) = LMW (g)/BW (g). **p<0.01, significant difference between two groups.

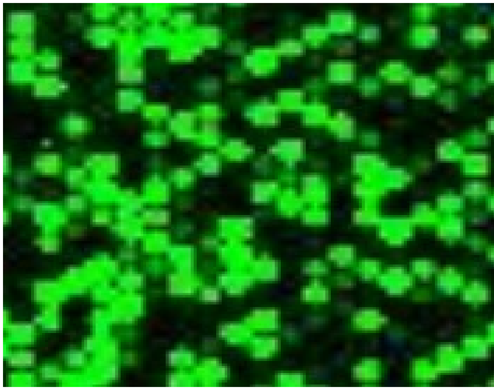

Figure 2 Microarray results (partial enlargement)

Microarray data analysis: 208 differentially expressed genes, of which 108 are known genes, 94 up-regulated, 114 down-regulated were involved in growth, molecular mechanism, fat metabolism, cell proliferation, transcription and splicing factor, protein synthesis and degradation. Meanwhile, some genes among these differentially expressed genes that had no annotation in GenBank were screened out and were presumed to be unknown new genes (Table 3), in which there were 59 genes that were associated with growth, 34 with metabolic process, 54 with molecular processes, 3 with bio-adhesion process, 5 with anatomical structure and 12 with development process and so on. The distributions of related differentially expressed genes of the growth were shown in Fig 3.

Cluster dendrogram of differential expressed gene: The results of the samples and related differentially expressed genes in this study classified and clustered by Heatmap function of SAS system are shown in Fig 4, in which abscissa was a sample cluster, namely, breast and leg muscle of 2 and 12 weeks old , Two experimental groups had 4 for each, whereas vertical axis represented the differentially expressed gene cluster. The details of the results of the 208 differentially expressed genes were divided into two major categories, which were shown in Fig 4a, the right side of which were the corresponding probe numbers of differentially expressed genes and different colors represented the expression strength of genes in tissues, the color bar was shown in Fig 4b. The concrete expression of a gene in different samples could be seen by using TreeView software, and the gene expression could also be analyzed according to the classification of samples. For example, differentially expressed gene that corresponded to probe No A_87_P026998 was calcium binding gene (NID2).

Validation of gene expression data by quantitative realtime PCR: To validate the microarray results, we performed quantitative real-time PCR for: Gallus gallus enolase 1, (alpha) (ENO1), Mrna (RCJMB04_24e12), Gallus gallus cyclin B2 (CCNB2), mRNA(CCNB2), Gallus gallus proteasome (prosome, macropain) subunit, alpha type, 7 (PSMA7), Gallus gallus similar to RNA polymerase I polypeptide B (POLR1B) (Table 1). All the real-time RT-PCR fold differences were in complete correspondence with the microarray data. Table 4 compares the microarray and real-time RT-PCR results.

Discussion

The growth and development of animal body were the results of the interaction of genetic factors and environment (Susanne et al., 1995), a very complicated process that includes gene expression and environmental conditions ensuring the gene expression. As the body growth and development is closely related to molecular metabolism and cell proliferation, so far studies have mainly focused on these three processes, and the research strategy which is often selected is the use of molecular biology methods to study the differences between different periods of the same species (Douglas et al., 2007).

Two groups of samples were analyzed in the experiment. There were significant differences in growth and development and molecular metabolism of Rugao chicken muscle tissue samples between 2 and 12 weeks old, which indicated that they were good animal models for studying the growth and development and molecular metabolism of chicken.

From the analysis of experimental results, ENO1 (alpha) and CCNB2 were both involved in the growth and development of chicken muscle. The analysis of northern blot indicated that alpha-enolase mRNA could be expressed in a wide range of chicken tissues (Tanaka et al., 1995). Alpha-enolase and beta-enolase are important genes in the growth and development of chicken muscle, whose cDNAs are cloned and analyzed to reveal that alpha- but not beta-enolase had a Src-dependent phosphorylation site, the gene expression of alpha-like enolase switched from alpha to beta-enolase occurred just after hatching in developing chicken muscle, which gene expression of α-enolase began to decline (Tanaka et al., 1995). Our microarray results were consistent with previous research findings. Cyclin proteins form complexes with members of the p34cdc2 kinase family and they were essential components of the cell cycle regulatory machinery (He et al., 1995). They were thought to determine the timing of activation, the subcellular distribution, and/or the substrate specificity of cdc2-related kinases, but their precise mode of action remained to be elucidated (Gallant et al., 1992). Gallant and Nigg (1992) also described the subcellular distribution of cyclin B2 (CCNB2) in chick embryo fibroblasts and in DU249 hepatoma cells. By indirect immunofluorescence microscopy they showed that cyclin B2 (CCNB2) was cytoplasmic during interphase of the cell cycle, but underwent an abrupt translocation to the cell nucleus at the onset of mitotic prophase. Moreover, they examined the phenotypic consequences of expressing wild-type and mutated versions of avian cyclin B2 (CCNB2) in HeLa cells and found that expression of cyclin B2 (CCNB2) carrying a mutation at arginine 32 (to serine) caused HeLa cells to arrest in a pseudomitotic state.

Table 3 Part of genes differentially expressed (*p*<0.001) between group A and group B

Catalog	GenBank ID	Definition	FoldChange	regulation
Growth	D37900	Gallus gallus enolase 1, (alpha) (ENO1), Mrna(RCJMB04_24e12)	0.45	Down
	X62531	Gallus gallus cyclin B2 (CCNB2), mRNA(CCNB2)	0.27	Down
Cellular metabolism	NM_204613	Gallus gallus proteasome (prosome, macropain) subunit, alpha type, 7 (PSMA7)	2.16	Up
	XM_415007	Gallus gallus similar to RNA polymerase I polypeptide B (POLR1B)	2.21	Up
Transcription and splicing factor	CR354335	Gallus gallus GA binding protein transcription factor(GABPA)	2.31	Up
	CR733249	Gallus gallus similar to SRF-related protein(RCJMB04_5e9)	2.10	Up
Biosynthetic process	Y11870	Gallus gallus bone morphogenetic protein receptor, type II(BMPR2)	0.33	Down
	AF525027	Gallus gallus STT3, subunit of the oligosaccharyltransferase complex, homolog A(STT3A)	0.45	Down
Cell proliferation	NM_205086	Gallus gallus ferritin, heavy polypeptide 1 (FTH1)	1.35	Up
	NM_001024578	Gallus gallus tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15)	4.67	Up
Intracellular part	NM_204182	Gallus gallus creatine kinase, mitochondrial 1A (CKMT1A), nuclear gene encoding mitochondrial protein	0.27	Down
	NM_204645	Gallus gallus centromere protein F, 350/400ka (mitosin) (CENPF)	0.16	Down
Protein binding	NM_204318	Gallus gallus gamma-aminobutyric acid (GABA) A receptor, alpha 1 (GABRA1)	0.45	Down
	NM_001001858	Gallus gallus stathmin 1 (STMN1)	0.24	Down
Others	BX933266	Gallus gallus finished cDNA, clone ChEST985d19（AMPD3）	7.53	Up
	XM_421005	Gallus gallus similar to sulfonylurea receptor, transcript variant 2（ABCC8）	0.35	Down
	BX950547	Gallus gallus finished cDNA, clone ChEST565l13（USP20）	2.58	Up
	NM_204969	Gallus gallus phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (PLA2G7)	3.02	Up

That growth and development had close relationship with molecular metabolism had been a recognized fact (Xiao et al., 2010). PSMA7 (alpha subunit of proteosome) and POLR1B (RNA polymerase) are important genes in the molecular metabolism of chicken muscle in screened genes, the gene expression of which in 12 weeks old of Rugao chicken muscle was significantly higher than that of 2 weeks old. Hutson et al. (1997) used subtractive hybridization to identify differentially expressed messages of proteasomal subunit after neural crest ablation in chick embryos, finding that a alpha subunit of a Drosophila proteosome was differentially expressed in embryos lacking neural crest. An increase in GPROS-28 (a 1 kb clone, homologous to PROS-28, a 28 kD alpha subunit of a Drosophila proteosome) expression in the head and pharyngeal arches of stages 12-21 chick embryos without cardiac neural crest accompanied generalized low-level expression of GPROS-28 throughout experimental and normal embryos, which was important to the gene expression of proteosomal subunit in chicken embryos, whereas RNA polymerase POLR1B was one of the major components of protein binding mechanism (Fion et al., 2007).

Cell proliferation and differentiation are important phenomenon in muscle growth and development process (Claudia et al., 2008). In the early stage of muscle cell proliferation and differentiation, the expression of ferritin heavy peptide factor (FTH1), tumor necrosis factor (TNFSF15) and other related genes show a rising trend by process in which irregular-shaped cells transform into a round shape, bone morphogenetic protein (BMPR2) and other related proteins show a clearly descendent trend, and down up to 40%. Adhesion regulator (ADRM1), the oligosaccharide transferase enzyme synthesis factor (STT3A) and other related proteins are involved in biosynthetic process, the gene expression in Rugao chicken muscle decreases, indicating that the activities related with the biosynthetic process in Rugao chicken muscle decrease over the time. In addition, GA-binding protein factor (GABPA) and phospholipase A2 (PLA2G7) are involved in the transcription process, and some related protein metabolism genes, the expression in broilers tends to increase, suggesting that the protein synthesis is very active in chicken, the mechanism needed to be further elucidated.

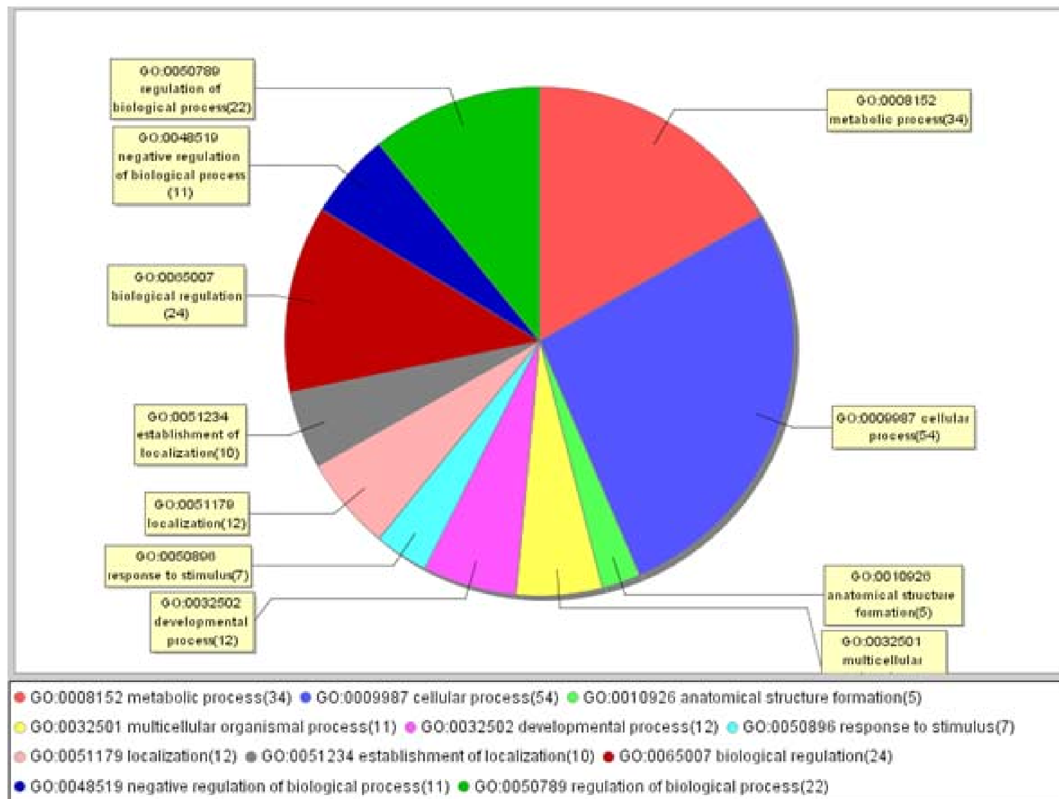

Figure 3 Pie chart of differential genes in biological processes

The genes, some sequences of which had no annotation in GenBank, were blasted with the chicken genome database, the results showed that the information of sequence location could be known, but there was not enough information for the corresponding genes, which were also hypothetical genes in the human or the mouse, so they were speculated to be unknown new genes in the chicken genome.

In this study, the selected genes were divided into different functional classifications by Gene Ontology (GO). GO constructs three relatively independent ontology. Among them, the biological processes, molecular function and cellular components are all attributes of genes and gene products (Wacław, 2008). The 208 genes screened by the Agilent microarray were classified in the study, For example, the 59 different genes were screened in the biological process of all, which were divided into different categories according to their functions. Information could be subtle to the function of each gene and its participation in the relevant biological path. GO functional classification reduces the tedious validation of gene function by biological methods, and can divide known genes into different functional classifications. GO can also speculate that the 'unknown' genes, some of which sequences had no annotation in GenBank, may be involved in the same biological process. New genes can be discovered, but it has to rely on biological methods to verify.

The expression of different samples of a particular gene and the classification of samples according to gene expression can be studied in Heatmap cluster diagram by the use of Java TreeView software. For example, the expression of calcium binding gene (NID2) in group A was significantly higher than that of group B, which suggested that the gene in the growth and development process of chicken muscle showed descendent trend, prompting that calcium ion binding activity was relatively weaker in broilers over time, and resulting in the loss of calcium ions, the biological process of which needed to be further validated by biological methods.

Table 4 Comparision of microarray and real-time RT-PCR analyses

Gene	Microarray	Real-time RT-PCR	
	Fold change	Fold change	T-test (*p*-value) [a]
ENO1	0.45	0.53	0.027*
CCNB2	0.27	0.268	0.0073**
PSMA7	2.16	2.31	0.031*
POLR1B	2.21	2.19	0.0011**

*a: *p<0.05, **p<0.01*

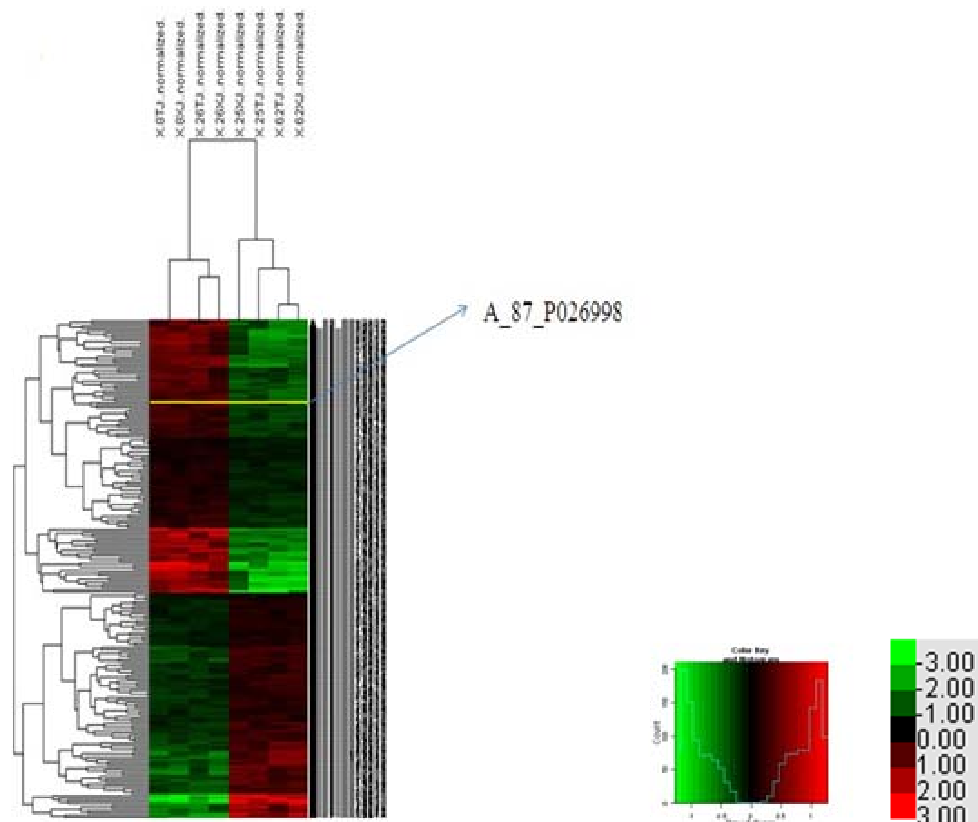

Figure 4a Differentially expressed genes cluster dendrogram of group A and group B b: Colorbar scale

Conclusion

In conclusion, the chicken muscle tissue gene expression profile was investigated comprehensively in the present study, and specific genes that were differentially expressed at 2 and 12 weeks old Rugao chiken were identified with the aid of Genechip. Further analysis indicating the pyruvate dehydrogenase complex and the protein tyrosine phosphatase pathway may play key roles in our research on local chicken meat quality. The research on the level of protein and miRNA of differentially expressed genes need to be further verified in the future.

Acknowledgements

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