



Gene Expression of Heart and Adipocyte Fatty Acid-binding Protein in Chickens by FQ-RT-PCR*

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ABSTRACT : This study was to detect the expression of heart fatty acid-binding protein (H-FABP) and adipocyte fatty acid-binding protein (A-FABP) gene mRNA in different tissues of Rugao and Luyuan chickens at 56 d and 120 d by real-time fluorescence quantitative reverse transcription polymerase-chain reaction (FQ-RT-PCR). The primers were designed according to the sequences of H-FABP, A-FABP and GAPDH genes in *Gallus gallus*, which were used as target genes and internal reference gene, respectively. The levels of H-FABP and A-FABP gene expression were detected by SYBR Green I FQ-RT-PCR. The relative H-FABP and A-FABP gene mRNA expression level was calculated with $2^{-\Delta C_t}$. Melting curve analysis showed a single peak of three genes. Intramuscular fat (IMF) content in breast muscle and leg muscle of the two chicken breeds at 120 d was higher than at 56 d. IMF content in breast muscle and leg muscle at 56 d and 120 d in Luyuan was significantly higher than in Rugao, however, abdominal fat of Luyuan was significantly lower than that of Rugao. The relative H-FABP gene mRNA expression level in cardiac muscle was the highest in both chicken breeds. The relative H-FABP and A-FABP gene expression of different tissues in Luyuan was higher than in Rugao. H-FABP gene mRNA expression had a negative effect on IMF of leg and breast muscles, and was significantly negatively correlated with IMF content. The relative A-FABP gene mRNA level in abdominal fat was higher than in liver. The A-FABP gene mRNA was not expressed in leg, breast and cardiac muscles. A-FABP gene mRNA expression level was significantly positively correlated with abdominal fat and had a significant effect on abdominal fat but not IMF content. (**Key Words :** Gene Expression, Fluorescent Quantitative Reverse PCR, A-FABP Gene, H-FABP Gene, Chicken)

INTRODUCTION

Intramuscular fat (IMF) content is a major determinant of the eating quality of chicken and has moderate heritability (Hovenier et al., 1993; Fisher et al., 2001; Nade et al., 2003; Okumura et al., 2007), but genetic correlations with other production traits are unfavorable (Hovenier et al., 1992). However, the need for carcass measurement has made it difficult to improve through selective breeding (Yuan et al., 2007). Marker or gene-assisted selection is a promising strategy for improvement of such carcass traits (Meuwissen and Goddard, 1996). Many researchers have focused on the correlation between H-FABP, A-FABP and IMF content. Ye reported that variations in the first exon

and the first intron from the H-FABP gene were correlated with IMF content (Ye et al., 2003). Genetic variation on the 1805 site of the third exon in the A-FABP gene had an effect on amino acid sequence: Pro→Ser (Tu et al., 2004; Chen et al., 2006). These findings are consistent with the function and tissue-specific expression of these FABP (Veerkamp and Van Moerkerk, 1993; Veerkamp and Maatman, 1995). Functionally, both A-FABP and H-FABP are members of the FABP family that comprises a group of small cytosolic proteins which are involved in intracellular fatty acid transportation, cell growth and differentiation, cellular signaling, gene transcription, and protection of enzymes from the toxic effects of free fatty acids (Wang et al., 2007) (Gerbens et al., 1998, 1999, 2001). The essential role of FABP is in long-chain fatty acid uptake and metabolic homeostasis which has been demonstrated for H-FABP by gene disruption experiments (Schaap et al., 1999). Furthermore, A-FABP is exclusively expressed in adipocytes (Xu et al., 2007; Vogel-Hertzel and Bernlohr, 2000), whereas H-FABP is expressed in various tissues but predominantly in cardiac and skeletal muscle cells (Gerbens

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et al., 1999). In order to control IMF, genetic variation in these FABP genes ultimately has to affect the expression level or functionality of the respective mRNA transcripts.

The sensitivity and specificity of fluorescence quantitative Real-time RT-PCR (FQ-RT-PCR) assays have made it widely used to estimate the expression level of genes of interest in DNA quantitative detection (Scheda et al., 1995; Murphy et al., 2001; Zhang et al., 2001; Yin et al., 2005; Agüero et al., 2007). Since little work has been done on A-FABP and H-FABP gene expression in chickens, the objective of this research was to detect the relative expression of A-FABP and H-FABP genes in different tissues of chickens using FQ-RT-PCR, and further analyze the correlation between transcription level of A-FABP and H-FABP genes and IMF content. This should provide further understanding of the genetic mechanism of IMF and indicate new approaches to improve meat quality of birds in the future.

MATERIALS AND METHODS

Experimental materials and procedures

Experimental design : The study was conducted on 60 Rugao and 60 Luyuan chickens (provided by National Chicken Genetic Resources in China). The birds were housed in the same test station and fed with a complete broiler diet in all the breeding periods.

60 Rugao and 60 Luyuan chickens were randomly selected for slaughtering at 56 d and 120 d with a 1:1 sex ratio. The average body weight of Rugao and Luyuan was about 594 g and 1,510 g, respectively, at 56 d and 580 g and 1,500 g, respectively, at 120 d. Twenty-four hours after slaughter, a slice of the breast and leg muscle was isolated from the right carcass carefully to avoid fat depots for assessment of IMF content. The IMF content was determined using Soxhlet petroleum-ether extraction and expressed as a percentage of the wet weight of muscle tissue. Abdominal fat ratio indicated the ratio of abdominal fat weight to body weight. 200-300 mg of leg muscle, breast muscle, cardiac muscle, abdominal fat and liver were sampled and stored at -70°C for extraction of total RNA.

RNA isolation and sequences synthesis : Total RNA was extracted from each tissue using the Trizol method according to the manufacturer's instructions (Invitrogen,

CA). All RNA extractions were performed in 2 days in batches of 20 samples each. The RNA concentration was quantified spectrophotometrically at 260 nm with A260/A280 ratios between 1.7 and 1.9, indicating pure and clean RNA isolates. The RNA integrity was checked by agarose gel (2%) electrophoresis to ensure that RNA was intact.

The primers (Table 1) were designed according to the sequences of the H-FABP (NM_204290), A-FABP (NM_001030889) and GAPDH (NM_204305) genes on Gen Bank, with Beacon Designer 3.0 (Beacon Designer 3.0 from Premier Biosoft International, CA). Oligo6.0 and primer5.0 were also used, which provided the parameters analysis. Difference in tissues of H-FABP and A-FABP gene expression was analyzed with the internal parameter of the house-keeping gene GAPDH. Primers were synthesized by TaKaRa, and the concrete parameters are shown in Table 1.

The first strand of cDNA was performed according to the recommended method of the Promega reverse transcription Kit (AMLV). The reaction volume was 20 µl, including 1 µl 100 µM oligo (dT)₁₈ primer, 4 µl 5×reverse transcriptase buffer (Promega, WI), 2 µl 10 mM dNTPs mixture, 1 µl rRNasin and 1 µl AMLV reverse transcriptase, 2 µl RNA template (500 ng), and 9 µl water. The reaction conditions for reverse transcription were as follows: annealing reaction for 30 min at 42°C, inactivated reverse transcriptase for 5 min at 99°C and cooling for 5 min at 4°C.

The regular PCR reaction system was 25 µl, containing 1×reaction buffer, 1 unit of Taq DNA polymerase, 2.0 mmol/L of Mg²⁺, 300 pmol of each dNTP, 2.5 pmol forward and reverse primers, and 100 ng of cDNA template. The following PCR conditions were used: 300 s at 95°C, 35 cycling of 50 s at 94°C, 50 s at annealing temperature of 55-60°C and 50 s at extension temperature of 72°C, and final extension step 300 s at 72°C in a Perkin-Elmer thermocycler 9600 (Tu et al., 2006).

PCR products were isolated by 1% agarose gel electrophoresis and target fragments were also cut under an ultraviolet lamp then purified with DNA purification kit. The recovered DNA fragments were linked with pMD18-T Simple Vector and then transformed into DH5α strain. Positive clones were selected and identified by PCR, then sent to be sequenced by TaKaRa (Dalian).

Table 1. Primer sequence, product size and loci of PCR

Gene	Primers (5'-3')	Product (bp)	Loci	Annealing temperature (°C)
H-FABP	F: CCTTACCCACCACCATCAT	199	110-129	55
	R: GTCTCCTTGGGATCCCACTT		289-309	
A-FABP	F: TGTGGGGTTTGCTA	189	107-123	60
	R: TAGGGAAATGACATTCAAAGT		275-296	
GAPDH	F: ATGGCATCCAAGGAGTGA	141	1,042-1,060	60
	R: GGGAGACAGAAGGGAACAG		1,173 -1,182	

FQ-PCR : Fluorescence Quantitative PCR (FQ-PCR) was used to analyze the difference in tissues of H-FABP and A-FABP gene expression after validation of three gene sequences. The resulting cDNA solution was diluted 10, 50, 100, 500 and 1,000 fold before further use. 100 fold was the best concentration in FQ-PCR. The primer concentration and T_m were optimized. FQ-PCRs were carried out in 20 μ l, including 10 μ l 2 \times SYBRGreenI Master mix (Brilliant SYBR Green QPCR master mix), 1 μ l forward and reverse primers (100 nM), 3 μ l cDNA, and 6 μ l water. The PCR conditions were as follows: 95°C 10 min, 95°C 30 s, 55-60°C 1 min, 35 cycles, 72°C 5 min in Stratagene's Mx3000P. Three repetitions in each sample were detected.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0. All values are shown as means \pm SE. Statistical significance in the parameter values among the different groups was tested by ANOVA procedure, the treatment means were separated by Duncan's multiple range test, and accepted if $p < 0.05$. The raw FQ-PCR values (Ct) had to be processed by comparative Ct (Δ Ct) method to obtain gene expression levels. The fold change of expression of the genes normalized against GAPDH gene mRNA levels from different tissues were calculated with $2^{-\Delta\Delta Ct}$ (Houng et al., 2000; Shaw et al., 2007) where $\Delta\Delta Ct = Ct (A-FABP \text{ or } H-FABP) - Ct (GAPDH)$.

RESULTS AND DISCUSSION

Cloning for H-FABP and A-FABP gene

Three nucleotide sequences were obtained and compared; the sequences were completely in accordance with the nucleotide sequences of H-FABP, A-FABP and

GAPDH genes on Genbank utilizing DNA Star analysis.

Melting curve analysis

Melting (Dissociation) curves of a typical PCR run where only a specific product was amplified are shown in Figure 1 and 2. Dissociation temperature of H-FABP, A-FABP and GAPDH gene was 83.9°C, 78.2°C and 85.5°C, respectively. The arrowheads represent the temperature where fluorescent data were collected in order to avoid interference from non-specific amplifications. In this study, melting curves of the three genes had a single melt-curve peak which showed better DNA quantitative detection with FQ-RT-PCR.

Relative quantification of H-FABP and A-FABP gene

The IMF content and abdominal fat ratio determined in Rugao and Luyuan at 56 d and 120 d are shown in Table 2. IMF content in breast muscle and leg muscle and abdominal fat of the two chicken breeds at 56 d were significantly lower than at 120 d. IMF content in Luyuan was significantly higher than in Rugao; however, abdominal fat of Luyuan was significantly lower than that of Rugao at the two growing ages.

Relative variation of H-FABP and A-FABP genes of different tissues in two chicken breeds is compared in Figure 3 and 4. The relative H-FABP gene mRNA level in cardiac muscle was significantly higher than in other tissues. There were significant differences between leg and breast muscles in mRNA level ($p < 0.01$). The relative variation of H-FABP and A-FABP genes of different tissues in Luyuan was higher than in Rugao. The relative H-FABP mRNA level in the two chicken breeds was 0.548-1.490 fold, which showed that it was down-regulated in these tissues, and it increased along with growing stage. The correlations

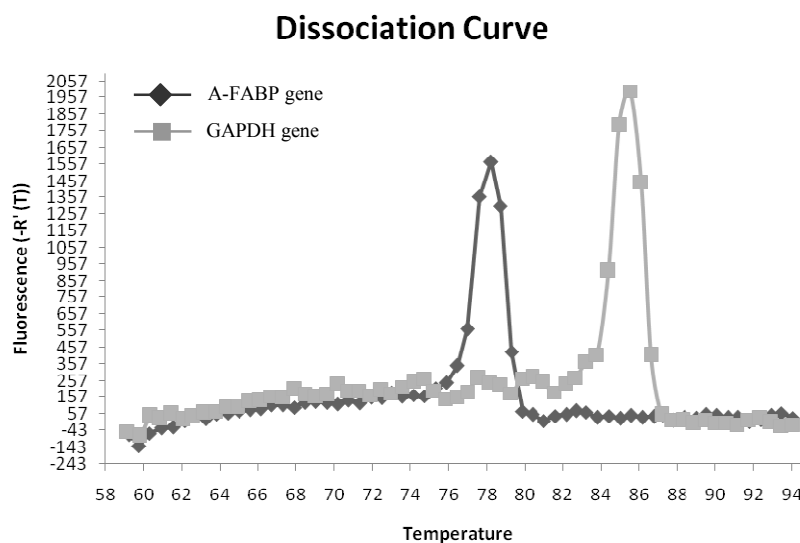


Figure 1. Dissociation curve of A-FABP and GAPDH gene.

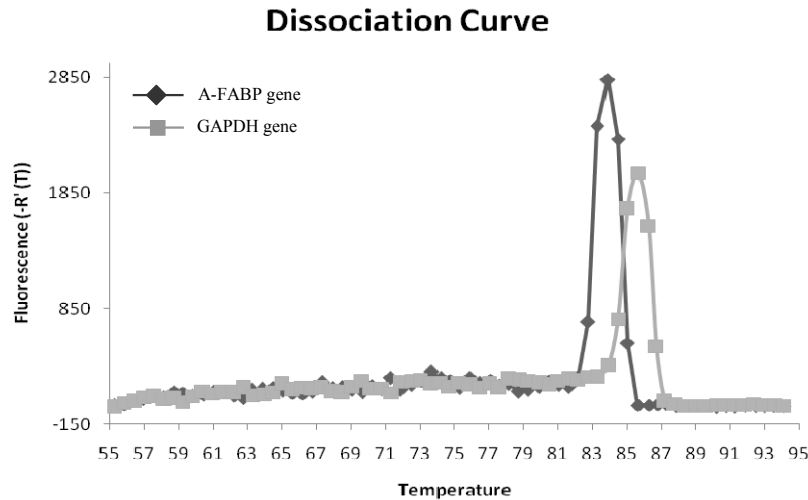


Figure 2. Dissociation curve of H-FABP and GAPDH gene.

between H-FABP and A-FABP gene mRNA expression and IMF content and abdominal fat are shown in Table 3. The correlation coefficients between H-FABP gene mRNA and cardiac muscle, breast muscle and leg muscle were -0.749, -0.668 and -0.723 respectively. H-FABP gene mRNA expression level was significantly negative correlated with IMF content. Moreover, H-FABP gene mRNA was mainly expressed in cardiac, leg and breast muscles, which was consistent with the results of Zschiesche and Gerbens (Zschiesche et al., 1995; Gerbens et al., 1999).

The relative A-FABP gene mRNA level in abdominal fat was higher than in liver ($p < 0.01$). A-FABP gene mRNA was not expressed in leg, breast and cardiac muscles in the two breeds. The relative gene mRNA level of A-FABP was 9.799-20.84 fold, which showed that A-FABP gene mRNA expression had a positive effect on lipocytes and liver. The correlation coefficients between A-FABP gene mRNA and abdominal fat was 0.858. Moreover, A-FABP gene mRNA was mainly expressed in fat tissue (Fisher et al., 2001), which is further confirmed in this study. Ye and Xu reported that A-FABP was the candidate gene of IMF (Ye et al., 2003; Xu et al., 2007); however, in this study, we showed that A-FABP was a candidate gene for abdominal fat but not IMF. It is possible that A-FABP induces differential effects on gene expression in the different fat depots due to the

regulation of gene transcription by fatty acids. A-FABP is expressed only in differentiated adipocytes, and the primary function is to carry fatty acids away from intracellular lipid droplets after triglyceride hydrolysis, and this was also validated in this study.

Previous studies demonstrated associations between DNA polymorphisms in the A-FABP, H-FABP genes and IMF content in chickens and pigs (Gerbens et al., 1999, 2001; Chen et al., 2006; Uemoto et al., 2007; Cho et al., 2009). However, these analyses did not exclude other closely linked genes from being responsible for these effects on IMF content. Obviously, to exert an effect, a particular mutation needs to alter the function (quality) or the abundance (quantity) of the respective gene products (i.e., mRNA molecules). This paper explicitly investigated A-FABP and H-FABP gene mRNA quantity and not the quality of the respective mRNA molecules. The relative H-FABP and A-FABP mRNA quantitative levels in different tissues of the two chicken breeds were better reflected using FQ-RT-PCR in this study. The number of chicken breeds and ages of the breeds will be enlarged and validated on protein level in future research work. The genetic mechanism of IMF and abdominal fat depots will be further understood and new approaches to inherit improvement in meat quality of birds will be obtained in the future.

Table 2. IMF content (%) in breast and leg muscle and abdominal fat ratio of Rugao and Luyuan chickens

	Rugao at 56 d	Rugao at 120 d	Luyuan at 56 d	Luyuan at 120 d
IMF content in leg muscle	0.602±0.1011 ^a (n = 60)	0.911±0.105 ^b (n = 60)	0.723±0.087 ^g (n = 60)	1.217±0.114 ^h (n = 60)
IMF content in breast muscle	0.483±0.056 ^c (n = 60)	0.712±0.021 ^d (n = 60)	0.593±0.067 ⁱ (n = 60)	1.085±0.075 ^j (n = 60)
Adominal fat ratio	1.414±0.012 ^e (n = 60)	2.028±0.012 ^f (n = 60)	1.238±0.149 ^k (n = 60)	1.934±0.164 ^m (n = 60)

The different lowercase in row and in line indicate that it is different significantly ($p < 0.05$).

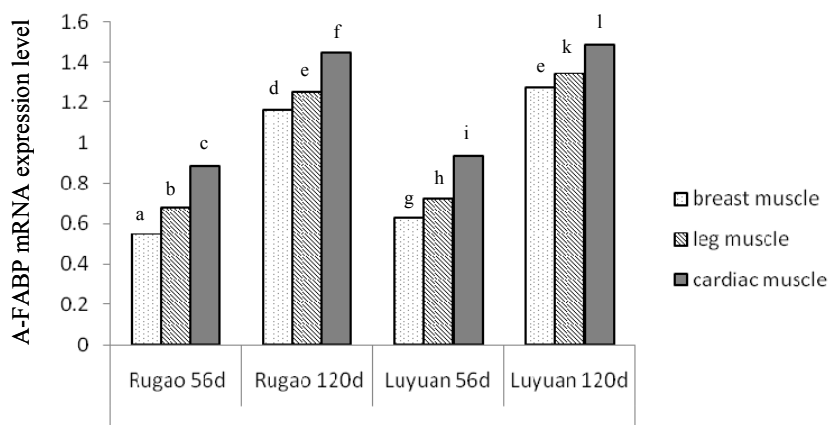


Figure 3. Relative variation of H-FABP gene mRNA expression in different tissues of Rugao and Luyuan chickens at 56 d and 120 d. The different lowercase indicate significant difference (p<0.01).

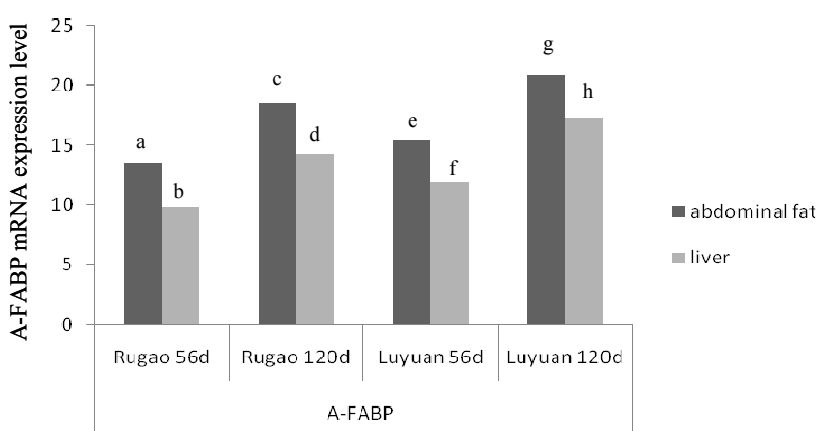


Figure 4. Relative variation of A-FABP gene mRNA expression in different tissues of Rugao and Luyuan chickens at 56 d and 120 d. The different lowercase indicate significant difference (p<0.01).

CONCLUSION

Statistical analyses in this study showed that H-FABP gene mRNA expression had a negative effect on IMF content. Moreover, the relative H-FABP gene mRNA level in cardiac muscle was very high. H-FABP gene mRNA expression level was significantly negatively correlated with IMF content. A-FABP gene mRNA expression level was significantly positively correlated with abdominal fat and had a significantly positive effect on abdominal fat ratio but not on IMF content.

Table 3. Correlations between H-FABP and A-FABP gene mRNA expression and IMF content and abdominal fat in Rugao and Luyuan chickens

	H-FABP gene mRNA	A-FABP gene mRNA
Cardiac muscle	-0.749 (p<0.01)	-
Breast muscle	-0.668 (p<0.05)	-
Leg muscle	-0.723 (p<0.01)	-
Abdominal fat	-	0.858 (p<0.01)

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