ORIGINAL ARTICLE

Differential expression of six genes and correlation with fatness traits in a unique broiler population

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Abstract Previous results from genome wide association studies (GWASs) in chickens divergently selected for abdominal fat content of Northeast Agricultural University (NEAUHLF) showed that many single nucleotide polymorphism (SNP) variants were associated with abdominal fat content. Of them, six top significant SNPs at the genome level were located within *SRD5A3*, *SGCZ*, *DLC1*, *GBE1*, *GALNT9* and *DNAJB6* genes. Here, expression levels of these six candidate genes were investigated in abdominal fat and liver tissue between fat and lean broilers from the 14th generation population of NEAUHLF. The results showed that expression levels of *SRD5A3*, *SGCZ* and *DNAJB6* in the abdominal fat and *SRD5A3*, *DLC1*, *GALNT9*, *DNAJB6* and *GBE1* in the liver tissue differed significantly between the fat and lean birds, and were correlated with abdominal fat traits. The findings will provide important references for further function investigation of the six candidate genes involved in abdominal fat deposition in chickens.

1. Introduction

Because of the strong association with a number of diseases, including insulin resistance, type 2 diabetes mellitus, atherosclerosis and ischemic heart disease, obesity produces adverse health consequences in humans (Spiegelman and Flier, 2001; Hotamisligil, 2006; Shoelson et al., 2006). A similar problem exists in chickens. Excessive accumulation of fat in chicken abdomens does not only reduce carcass yield and feed efficiency, but is also a less desirable product for consumers. Therefore, mechanisms of obesity occurrence, genes regulating fat deposition and the development of adipose tissue are issues

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identified either by traditional research methods or high-throughput techniques (Wang et al., 2006; Gesta et al., 2007).

Notwithstanding increased knowledge of obesity, the genes influencing fatness remain incompletely detected. As one of the major tools, genome wide association studies (GWAS) have resulted in a dramatic increase in the identification of susceptibility variants associated with obesity in humans and domestic animals (Scherag et al., 2010; Day and Loos, 2011; Hu et al., 2013).

In recent years, many variants and genes associated with obesity in chickens have been successfully identified using GWAS. Abasht et al. revealed cryptic alleles as an important factor in heterosis for fatness in a chicken F_2 population (Abasht and Lamont, 2007). Liu and Sun identified some candidate genes associated with abdominal fat traits in an F_2 resource population derived from a cross between a Chinese local breed and a commercial rapid-growing broiler line (Liu et al., 2013; Sun et al., 2013).

Previously, many variants associated with abdominal fat traits have been identified using GWAS in our laboratory (unpublished data). Of them, six top significant single nucleotide polymorphisms at the genome level were located within *SRD5A3* (Steroid 5α-reductase 3), *SGCZ* (Sarcoglycan, zeta), *DLC1* (Deleted in liver cancer 1), *GBE1* (Glucan (1,4-alpha-), branching enzyme 1), *GALNT9* (N-acetylgalactosaminyltrans ferase 9) and *DNAJB6* (DNAJ homology subfamily B member 6), suggesting that these genes play important roles in fat deposition in chickens. Here, we investigate whether these six genes are differentially expressed in fat and liver tissues between fat and lean broilers and the relationship between their expression levels and abdominal fat content, which would help in our understanding of the roles these genes play in chicken adipose tissues.

2. Materials and methods

2.1. Experimental animals

The broilers used in this study were derived from the Northeast Agricultural University (NEAU) broiler lines divergently selected for abdominal fat content (NEAUHLF). The NEAUHLF line has been selected since 1996 and the selection procedure and raising conditions have been described in detail previously (Wang et al., 2007; Guo et al., 2011). For each line, a total of 10 male and 6 female birds from the 14th generation population were used. Birds were slaughtered at 7 weeks of age, the average abdominal fat weight (AFW) with standard error and average abdominal fat percent (AFP) with standard

error of the lean line were 12.53 ± 1.17 g and 0.59% ± 0.05%, respectively, however, for the fat line, they were 54.09 ± 1.93 g and 3.29% ± 0.13%. There were significant differences in both AFW and AFP between the two lines. Samples were collected from abdominal fat and liver tissues, then weighed and immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. RNA extraction and cDNA synthesis

Total abdominal and liver RNA was isolated using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in DEPC-treated water and the purity and integrity were estimated by an ultraviolet/visible spectrophotometer (Pharmacia, USA) at a 260/280 nm absorbance ratio (range 1.8–2.0 indicates a pure RNA sample) and agarose gel electrophoresis. Total RNA was reverse transcribed to cDNA in a reaction volume of 20 μL containing 1 μg total RNA, 0.5 μL of 50 pmol/L Oligo d(T)18 Primers and finally supplemented with nuclease-free water to a volume of 5 μL for the first step. This mixture was heated at 70 °C for 5 min and incubated on ice-water for 5 min. Then 5× Reverse transcription Buffer 4 μL, MgCl$_2$ (25 mM) 2.5 μL, dNTP Mixture 1 μL, RNase Inhibitor (Promega Biotech Co. Ltd) 0.5 μL, Improm-II Reverse Transcriptase (Promega, Madison, WI, USA) 1 μL and nuclease-free dH$_2$O were added to a final volume of 20 μL. The RT mixture was incubated at 25 °C for 5 min, then 42 °C for 60 min and finally inactivated by heating at 70 °C for 15 min. The cDNA was directly for use in quantitative real-time PCR.

2.3. Quantitative analysis of mRNA expression

Special primers for amplifications of these genes were designed spanning at least one intron to avoid genomic DNA contamination using Primer Premier 5.0 software according to Ensembl. All primers were synthesized by Invitrogen Biotechnology (Shanghai) Co., Ltd. (Table 1).

SYBR Green real-time PCR amplifications were conducted using an AB Applied Biosystems 7500 Real Time PCR System (Life Technologies, USA). The stably expressed gene, GAPDH, served as the endogenous reference for determination of targeted mRNA profiles (Bustin, 2002). Quantitative PCR amplifications were performed in a final volume of 10 μL reaction mixture under the optimum reaction conditions including 5 μL SYBR® Permix Ex Taq™ II (TaKaRa, Japan), 0.2 μL ROX Reference Dye II (TaKaRa, Japan), 0.2 μL

Table 1 Primer sequences used in this study.

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)	Production size (bp)	Anneal temp (°C)	GenBank No.
SRD5A3	TGGACTTGGCTATTACGTTGCTG	CATCGCAACGCCTATGATGTG	122	60	ID: 422750
SGCZ	GCTCTGCGTCTGTCCCAATG	AGCTCCACAAGCAGATGTTGCTA	92	60	ID: 422739
DLC1	ATGAGAGTTCAACAGACAG	TAAAAGCATAATGGCAG	194	60	ID: 422740
GBE1	ATTTGTGGATGGTGGACT	CATACCCTTTACCCTCAA	132	60	ID: 427964
GALNT9	AGATTGGCTTGCTTGAC	TGTAGGGTTTCTTTGTGC	153	60	ID: 416796
DNAJB6	AGCCTTTGCTGAGGAGT	CTTGCTGCCTTCTTTGTAT	211	60	ID: 420448
GAPDH	AGAACATCATCCCAGCGT	AGCCTTCACTACCCTCTTG	184	60	ID: 374193

forward primer, 0.2 μL reverse primer (10 μmol/L) of target gene or housing genes, 3.4 μL water and 1 μL template cDNA. Amplification conditions were performed starting with 30 s template denaturation step at 94 °C, followed by 40 PCR cycles of 5 s at 95 °C, 34 s at 60 °C, where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle for 15 s at 95 °C, followed by 60 °C for 1 min and increased to 95 °C with acquired fluorescence.

All samples were amplified in triplicate as technical replicates and specific amplification was confirmed by single peak observation on dissociation curves. The means of Ct values were obtained for further calculations.

2.4. Statistical analysis

The expression levels of the six genes were measured using real-time PCR. The $2^{-\Delta Ct}$ (ΔCt = Ct of the target gene – Ct of the housekeeping gene) method was used to analyze the relative quantitative data. Values were expressed as mean ± standard error of the mean. Data of expression were subjected to square root and arcsine transformation to normality distribution.

Model-based tests were carried out to evaluate the different gene expression levels on abdominal fat and liver between the two lines using $Y = \mu + Line + Sex + Line \times Sex + e$, by the GLM procedure of JMP4.0 (SAS, Chicago, IL, USA), which fitted with Line and Sex as fixed effects, Line × Sex as interaction of Line and Sex, where Y is the dependent variable for different gene expression levels of birds, μ is the overall population mean, and e is the residual random error. The Pearson coefficient of correlation between expression levels and abdominal fat traits was estimated. $P < 0.05$ was taken to indicate significant differences or significant correlation.
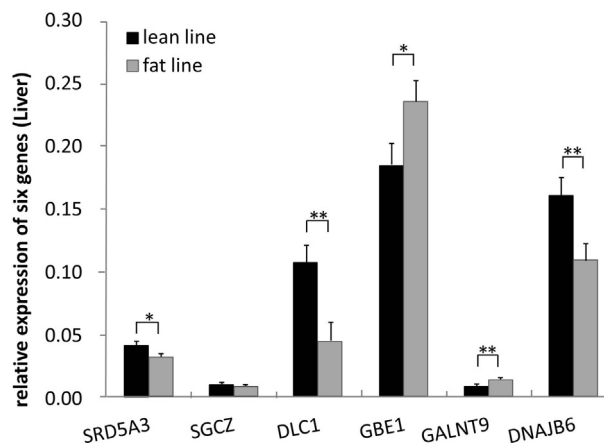
3. Results

3.1. The expression of six genes in abdominal fat tissues

As shown in Fig. 1, all six genes were expressed in abdominal fat tissues. *SRD5A3* and *SGCZ* were differentially expressed between the fat and lean birds ($P < 0.01$), and the expression
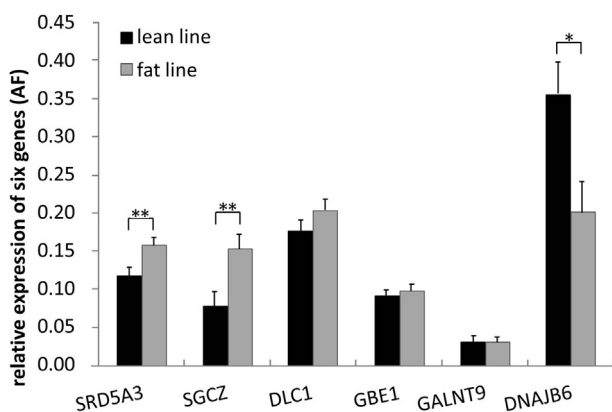
Figure 1 Comparison of expression level of six genes in abdominal fat tissue between two lines.

Figure 2 Comparison of expression level of six genes in liver tissue between two lines.

levels were higher in fat birds. In contrast, *DNAJB6* expression level in fat birds was significantly lower than that of lean birds ($P < 0.05$). For the other three genes, *DLC1*, *GBE1* and *GALNT9*, no significant differences in the expression levels were observed in abdominal fat tissues between the two lines.

3.2. The expression of six genes in liver tissues

As shown in Fig. 2, all six genes were expressed in liver tissues of fat and lean birds. *SRD5A3* expression levels in lean birds were significantly higher than that of fat birds ($P < 0.05$). Both *DLC1* and *DNAJB6* expression levels in lean birds were significantly higher than that of fat birds ($P < 0.01$). In contrast, *GBE1* expression levels in lean birds were significantly ($P < 0.05$) lower than that of fat birds, and *GALNT9* expression levels in lean birds were significantly ($P < 0.01$) lower than that of fat birds.

3.3. The correlation analyses between these six gene expression levels and AFW and AFP

The results of a correlation analysis between these six gene expression levels and AFW are given in Table 2. *SRD5A3* and *SGCZ* expression levels in abdominal fat tissues were significantly positively correlated with AFW and AFP ($P < 0.05$ or $P < 0.01$). *DNAJB6* expression levels in abdominal fat tissues were significantly negatively correlated with AFW and AFP ($P < 0.01$). *SRD5A3*, *DLC1* and *DNAJB6* expression levels in liver tissues were negatively and significantly correlated with AFW and AFP ($P < 0.01$). *GBE1* and *GALNT9* expression levels in liver tissue were significantly positively correlated with AFW and AFP ($P < 0.05$ or $P < 0.01$).

4. Discussion

Adipose and hepatic tissues play important roles in the growth and development of animals. The former not only maintains energy balance, but also functions as a crucial endocrine organ; the latter, especially in avian species, is a major site for lipid metabolism. Here, for the first time, *SRD5A3*, *SGCZ*, *DLC1*, *GBE1*, *GALNT9* and *DNAJB6* genes were investigated at the transcriptional level in abdominal fat and liver tissues of

Table 2 Correlation coefficients between mRNA expression levels of six genes in abdominal fat and liver tissues and abdominal fat traits.

	SRD5A3		SGCZ		DLC1		GBE1		GALNT9		DNAJB6	
	AF	Liver	AF	Liver	AF	Liver	AF	Liver	AF	Liver	AF	Liver
AFW	0.35[*]	−0.53[**]	0.47[**]	−0.21	0.13	−0.47[**]	0.04	0.35[*]	−0.10	0.47[**]	−0.47[**]	−0.54[**]
AFP	0.39[*]	−0.49[**]	0.55[**]	−0.18	0.24	−0.45[**]	0.14	0.38[*]	−0.04	0.48[**]	−0.46[**]	−0.51[**]

[*] Indicates $P < 0.05$.
[**] Indicates $P < 0.01$.

chicken. Significant differences in expression levels of these genes between fat and lean birds were observed for both tissues, coupled with a significant correlation of their expression levels with AFW and AFP, which suggested that these genes could be associated with abdominal fat deposition in chickens.

SRD5A3 was expressed in abdominal fat and liver tissues of chickens, and *SRD5A3* expression levels in abdominal fat tissues were significantly positively correlated with AFW and AFP. It has been reported that *SRD5A3* plays a crucial role in N-linked protein glycosylation, which was a process that involves many steps including the assembly of a lipid carrier for the oligosaccharide, the flip-flopping of this lipid between leaflets of the endoplasmic reticulum membrane, and multiple cycles of phosphorylation and dephosphorylation of lipids in yeast and mammals (Stiles and Russell, 2010; Cantagrel et al., 2010). In addition, *SRD5A3* participates in the process of steroid hormone biosynthesis, and fatty acids had been identified as the substrates of this steroid 5α-reductase family member (Moon and Horton, 2003). Based on the above evidence, it is speculated that *SRD5A3* might be essential for fat deposition.

DNAJB6 was expressed in both adipose and liver tissues, and the expression levels in both tissues differed between the two lines, and were negatively correlated with AFW and AFP. *DNAJB6,* named MRJ [Mammalian relative of DnaJ (HSP40)], is a member of the HSP40 family, subfamily B, owning two spliced variants, MRJ (S) (smaller isoform) and MRJ (L) (long isoform). It was found that MRJ (L) up-regulates expression of *DKK1* (Mitra et al., 2010), a Wnt inhibitor, which regulates aspects of placental lipid deposition through the Wnt signaling pathway (Strakovsky and Pan, 2012). Sustained activation of the Wnt signaling pathway prevents adipogenic differentiation (Nakamura et al., 2013). Accordingly, we infer that *DNAJB6* is critical for regulating abdominal fat accumulation, potentially by impacting the transcription of *DKK1* involved in lipid metabolism.

SGCZ was differentially expressed between the fat and lean birds, and the expression levels in abdominal fat tissues were significantly correlated with AFW and AFP. *SGCZ*, a well-known gene whose protein product belongs to the sarcoglycan protein family, had been identified as a basilic factor in the pathogenesis of muscular dystrophy and is expressed mainly in vascular smooth muscle (Hack et al., 2000; Wheeler et al., 2002; Aurino et al., 2008). Vascular smooth muscle cells are the essential factor of activity and configuration of blood vessels that could be changed in cardiovascular diseases (Cannon, 2013), and obesity could induce a series of cardiovascular diseases (Lppoliti et al., 2013). Additionally, the report suggested that muscle could be seen as an important mediator for fat

deposition (Brockmann et al., 2009). Therefore, we could deduce that *SGCZ* participates in the process of fat deposition.

Our results also showed that *DLC1* was expressed in the adipose and liver tissues, which was in line with the results from human studies and proved by Durkin et al. (2002). The expression level of *DLC1* in the liver was significantly different ($P < 0.01$) between the two lines. Coincidentally, it was negatively and significantly correlated with AFW. As a potential tumor suppressor gene in the liver (Xue et al., 2008), suppressive function of *DLC1* relies on *DLC1*'s RhoGAP activity activated by lipid interaction and the START domain (Erlmann et al., 2009), which is typically found in lipid transfer proteins and forms a hydrophobic pocket to accommodate a single lipid molecule (Alpy and Tomasetto, 2005). When liver cancer occurs, normal lipid metabolism may be significantly influenced (Jiang et al., 2007). Therefore, it could be concluded that *DLC1* indirectly influences fat metabolism *in vivo*.

GBE1 and *GALNT9* genes were expressed in both abdominal fat and liver tissues, and the mRNA expression levels in the liver were positively significantly correlated with AFW and AFP. It was reported that a mutation on *GBE1* was found causing glycogen storage disease type IV, an autosomal recessive disorder of the glycogen synthesis (Andersen, 1956). *GALNT9* participates in the process of biosynthesis of O polyose and has been identified as being responsible for mitochondrial myopathy and glycobiology (Casas et al., 2004; Van der Zwaag et al., 2009). It is well known that in the liver, glycogen is converted to fat, and delivered to other tissues to be used or stored in the form of lipoprotein. Thus, these two genes could influence the synthesis of fat acid through the regulation of glycometabolism.

To sum up, our findings will provide important references for further function investigation of the six candidate genes involved in abdominal fat deposition in chickens.

Conflict of interest

The authors declare that there is no conflict of interest.

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