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# Differential expression of six chicken genes associated with fatness traits in a divergently selected broiler population

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ABSTRACT

A genome-wide association study has shown a number of chicken (*Gallus gallus*) single nucleotide polymorphism (SNP) markers to be significantly associated with abdominal fat content in Northeast Agricultural University (NEAU) broiler lines selected divergently for abdominal fat content (NEAUHLF). The six significant SNPs are located in the kinase insert domain receptor (*KDR*), tumor suppressor candidate 3 (*TUSC3*), phosphoribosyl pyrophosphate amidotransferase (*PPAT*), exocyst complex component 1 (*EXOC1*), v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (*MYBL2*) and *KIAA1211* (undefined) genes. In this study, the expression levels of these genes were investigated in both abdominal fat and liver tissues using 32 14th generation chickens from the NEAUHLF. The levels of expression of *KDR* in abdominal fat and *KDR* and *TUSC3* in liver differed significantly between the two lines. The expression level of *KDR* in the abdominal fat was significantly correlated with the abdominal fat weight (AFW) and abdominal fat percentage (AFP). The expression levels of *KDR*, *TUSC3* and *PPAT* in liver were significantly correlated with AFW and AFP, indicating that the six genes, especially *KDR* and *TUSC3*, could be associated with fat traits in domestic chickens. This study could provide insight into the mechanisms underlying the formation of abdominal fat in chickens.



## 1. Introduction

Chickens selected for rapid growth have an increased risk of physiological disorders such as obesity [1]. The excessive deposition of abdominal fat can lead to diseases such as ascites, leg malformation and sudden death syndrome in broiler chickens [2,3]. Breeding chickens with less abdominal fat has become a goal of the poultry industry.

Genetic improvement of meat quality and carcass traits through traditional selection strategies is difficult because these traits have low or moderate heritability and, in general, can only be measured post slaughter [4,5]. Abdominal fat is an important factor in meat quality and carcass traits in chickens. Efficient selection for improved meat quality and carcass traits through marker-assisted selection (MAS) or genomic selection using high-throughput genomic techniques is achievable. Genome-wide association studies (GWASs) are commonly used for the identification of genes responsible for complex traits in farm animals, which greatly facilitates MAS or genomic selection. GWASs have been used to identify major genomic loci associated with important economic traits in chickens [6].

In this study we have used a GWAS to identify a number of single nucleotide polymorphisms (SNPs) associated significantly with abdominal fat weight (AFW) and abdominal fat percentage (AFP) in chickens. Six significant SNPs located in the *KDR*, *TUSC3*, *PPAT*, *EXOC1*, *MYBL2* and *KIAA1211* genes were chosen to investigate whether these six genes affect the accumulation of abdominal fat via analysis of differential expression in abdominal fat and liver and by analysis of the correlation between the level of gene expression and AFW and AFP values.

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## 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 using the AFP (abdominal fat weight/body weight) and the plasma very low-density lipoprotein (VLDL) concentration as selection criteria. The entire $G_0$ generation of NEAUHLF came from the same Arbor Acres broiler grandsire line, which was then divided into two lines according to VLDL concentration at seven weeks of age. From $G_1$ to $G_{14}$, birds from each line were raised in two hatches. Plasma concentrations of VLDL were measured in all male birds at seven weeks of age and the AFP of the male birds in the first hatch was measured after slaughter at seven weeks of age. Sibling birds from the families with AFPs lower or higher than the average value of the population were selected as candidates for breeding. The plasma concentration VLDL and the body weight (BW) of male birds in the second hatch and egg production of female birds in both hatches were taken into consideration. The selection procedure and rearing conditions have been described [7]. The AFW and AFP differed significantly between the two lines from the fourth generation onwards, with the AFP of the fat line being nearly 4.45-fold greater than that of the lean line at 49 days old. BW at seven weeks of age was not significantly different between the two lines, indicating that selection for AFP was very efficient in the subsequent generations (Fig. 1). All birds were housed under identical environmental conditions with free access to food and water. They were fed a commercial soybean-based diet that met all of the NRC requirements. The birds received a starter diet of 3000 kcal ME/kg and 210 g/kg CP until they reached three weeks of age. They were fed a grower diet of 3100 kcal ME/kg and 190 g/kg CP from three to seven weeks of age [8].

Sixteen fat (ten male and six female) and 16 lean (ten male and six female) age-matched birds of 14th generation ($G_{14}$) were used in this study. The birds were slaughtered when they were seven weeks old. There were significant differences in both the AFW and AFP between the two lines. The average (±standard error) AFW and AFP values were 12.53 (±1.17) g and 0.59 (±0.05)%, respectively, for the lean line, and 54.09 (±1.93) g and 3.29 (±0.13)%, respectively, for the fat line. The average (±standard deviation) plasma concentration VLDL value of the birds of the $G_{14}$ was 0.18 (±0.06) $mmol \cdot L^{-1}$ for the lean line, and 0.32 (±0.14) $mmol \cdot L^{-1}$ for the fat line, respectively.

Abdominal fat and liver samples were collected and immediately frozen in liquid nitrogen and stored at −80 °C [9].

### 2.2. RNA extraction and cDNA preparation

Total RNA was extracted from the abdominal fat and liver samples using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions then dissolved in DEPC-treated water. The concentration, purity and integrity were assessed using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany) to measure the 260 nm/280 nm absorbance ratio (range 1.8–2.0 indicates pure RNA) and electrophoresis in 1% (w/v) agarose gel was used to verify the integrity.

Total RNA was reverse transcribed to cDNA in a reaction volume of 20 μL containing 1 μg of total RNA, 0.5 μL of 50 pmol/L oligo$(dT)_{18}$ primers and 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 then incubated in ice-water for 5 min. Subsequently, 4 μL of 5× reverse transcription buffer, 2.5 μL of 25 mM $MgCl_2$, 1 μL of dNTP mixture, 0.5 μL of RNase inhibitor (Promega Biotech Co. Ltd, Beijing, China), 1 μL of Improm-II Reverse Transcriptase (Promega, Madison, WI, USA) and nuclease-free distilled water were added to make a final volume of 20 μL. The mixture was incubated at 25 °C for 5 min, 42 °C for 60 min and inactivated by heating at 70 °C for 15 min. The cDNA was subsequently used in real-time quantitative PCR (qPCR) [9].

### 2.3. Quantitative analysis of mRNA expression of six chicken genes

The expression levels of six genes were measured using real-time PCR. Primers for the amplification of the six target genes and two internal controls were designed spanning one intron to avoid genomic DNA contamination. Premier 5.0 software was used to design the oligonucleotide primers set for the eight genes (Table 1).

SYBR Green real-time PCR amplifications were performed using an Applied Biosystems® 7500 Real Time PCR System (Life Technologies, Gaithersburg, MD, USA). The internal controls *GAPDH* and β-*actin* served as endogenous references. qPCR amplifications were performed in a reaction volume of 10 μL consisting of 5 μL of SYBR® Permix ExTaq™ (Perfect Real Time, Dalian, China), 0.2 μL of ROX™ Reference Dye II (50×), 0.4 μL of 10 μM primer for target or internal control and supplemented with 3.4 μL of water and 1 μL of cDNA.

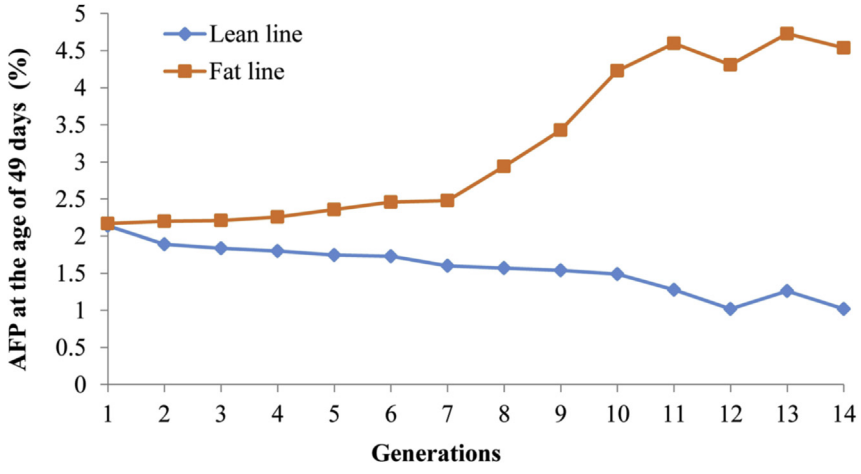


**Fig. 1.** Phenotypic changes after 14 generations of divergent selection for high and low abdominal fat content.

**Table 1**
Primer sequences used during real-time PCR to measure the expression levels of six target genes associated with abdominal fat deposition in chickens.

| Gene symbol | Forward primer (5′-3′) | Reverse primer (5′-3′) | Production length (bp) | Anneal temp (°C) | GeneBank no. |
|---|---|---|---|---|---|
| *PPAT* | ATGAACGAAACTCCAACTT | CCACCCATCCTTCTGTC | 163 | 60 | NM_001004401.1 |
| *TUSC3* | ATTGCTCTGGCTCTTCTG | GGTCCACGGATGTGATTC | 194 | 60 | XM_420692.4 |
| *KDR* | AGCATCACGAGCAGCCAGAG | GGCCACCTGGAAGCTATAACAGA | 150 | 60 | NM_001004368.1 |
| *EXOC1* | CGTTGTATTGAACCTGAG | TTTCCAAGTGTTGTGCTG | 152 | 60 | XM_004936022.1 |
| *MYBL2* | CTTCCTTGACTCCTG | TCTTGTCCCTGTGC | 188 | 60 | NM_205318.1 |
| *KIAA1211* | CCTGCCCGTGTTTGTCTT | GTGGCTCGGCTTCAGTTT | 136 | 60 | XR_210592.1 |
| *GAPDH* | AGAACATACATCCCAGCGT | AGCCTTCACTACCCTCTTG | 184 | 60 | NM_204305.1 |
| *β-actin* | TCTTGGGTATGGAGTCCTG | TAGAAGCATTTGCGGTGG | 331 | 60 | NM_205518.1 |

Amplification started with a template denaturation step at 94 °C for 30 s followed by 40 PCR cycles at 95 °C for 5 s and 60 °C for 34 s, at which point fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 °C for 15 s followed by 60 °C for 1 min and ramped to 95 °C with acquired fluorescence [9].

### 2.4. Statistical analysis

The $2^{-\Delta Ct}$ ($\Delta C_t = C_t$ of the target gene $- C_t$ of the internal control) method was used to analyze the relative quantitative data. Values were expressed as mean ± standard error of the mean (S.E.M.). Expression levels were subjected to square root and arcsine transformation to yield a normalized distribution.

Model-based tests were used to evaluate the expression levels of the different genes in the abdominal fat and liver of the two lines with the fitted model as follows:

$$Y = \mu + Line + Sex + Line \times Sex + e$$

With $Y$ being the dependent variable for gene expression levels, $\mu$ the overall population mean and $e$ the residual random error. The GLM procedure of JMP4.0 (SAS, Chicago, IL, USA) was used, with *Line* and *Sex* as fixed effects and *Line* × *Sex* as the interaction between *Line* and *Sex*. The Pearson coefficient of correlation between expression levels and abdominal fat traits was estimated. The level of statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Differential expression of the mRNA of six genes in abdominal fat and liver of two lines of chicken

As shown in Figs. 2 and 3, all six genes were expressed in the abdominal fat and liver of the lean and fat lines. The same pattern of expression was observed when the internal controls *GAPDH* and β-*actin* were used as endogenous references. The results indicated that the level of mRNA expression of *KDR* was significantly ($P < 0.01$) higher in the abdominal fat and liver of the lean line than of the fat line. Furthermore, the expression level of *TUSC3* in the liver of birds from the fat line was significantly ($P < 0.01$) higher than in those from the lean line. However, mRNA expression by the other four genes was not significantly different between the two lines in the two tissues.

### 3.2. Correlation of mRNA expression of six genes with AFW and AFP

Correlation coefficients between the expression of mRNA by the six genes and the AFW and AFP are given in Table 2. The level of expression of *KDR* was significantly ($P < 0.01$) negatively correlated with the AFW and AFP in both tissues. The level of expression of *TUSC3* in liver was significantly ($P < 0.05$) positively correlated with the AFP and AFW. The level of expression of *PPAT* in liver was significantly ($P < 0.05$) negatively correlated with the AFW and AFP. There was no significant correlation between the AFW or AFP and the expression of the other three genes in either the abdominal fat or liver.

## 4. Discussion

The qRT-PCR method is widely used when housekeeping genes act as the internal control in the calculation of accurate data normalization. In this study, it was necessary to use two reference genes to evaluate the accuracy and credibility [10–13]. *GAPDH* and *β-actin* were used as the two endogenous reference genes and the same pattern of expression was observed, which enhanced the reliability of the results.

The differential expression of *KDR* mRNA between the two lines, coupled with the correlation analysis of the expression levels of *KDR* with the AFW and AFP, indicated that *KDR* could be involved in lipid metabolism or inhibit the production of lipids. *KDR*, a type III receptor tyrosine kinase, is the indispensable mediator of several physiological and pathological effects of VEGF (vascular endothelial growth factor) on vascular endothelial cell development [14]. The VEGF family is associated with vasculogenesis and hematopoiesis [15]. Earlier studies showed inhibition of angiogenesis reduced adipose tissue mass and ameliorated obesity [16–19]. Studies of the development of adipose tissue showed angiogenesis precedes adipogenesis in embryos [20]. The results of recent research indicated obesity is related to lower skeletal muscle capillarization and the appearance of new blood vessels is coupled to adipocyte differentiation, which indicated angiogenesis is essential for adipogenesis and VEGF is a key mediator of that process [21,22]. Although it is not clear how angiogenesis and adipogenesis interact and what role blood vessels have in adipogenesis in obesity, there are close relationships between blood vessel formation and adipogenesis. In addition, a recent report indicated genetic variants in the *KDR* transcriptional regulatory region affected promoter activity and intramuscular fat deposition in Erhualian pigs [23]. Thus, it was concluded that *KDR* could be associated with fat traits in chickens.

The level of expression of *TUSC3* in liver differed significantly ($P < 0.01$) between the two lines of chicken and correlated significantly ($P < 0.05$) with the AFP and AFW. The *TUSC3* gene is related to human cancer [24–26] and was initially described as a tumor suppressor candidate [27]. This gene is highly expressed in human adipose tissue [28], indicating it is likely associated with fat deposition in humans. To date, there has been no report of a relationship between *TUSC3* and fat traits in other animals. In chickens, lipogenesis occurs mainly in the liver [29] and *TUSC3* was found to play an important role in the liver in the present study. It therefore appears likely that this gene is related to lipogenesis in chicken liver.

The level of expression of *PPAT* in liver was higher than that of the other genes and was correlated significantly ($P < 0.05$) with the

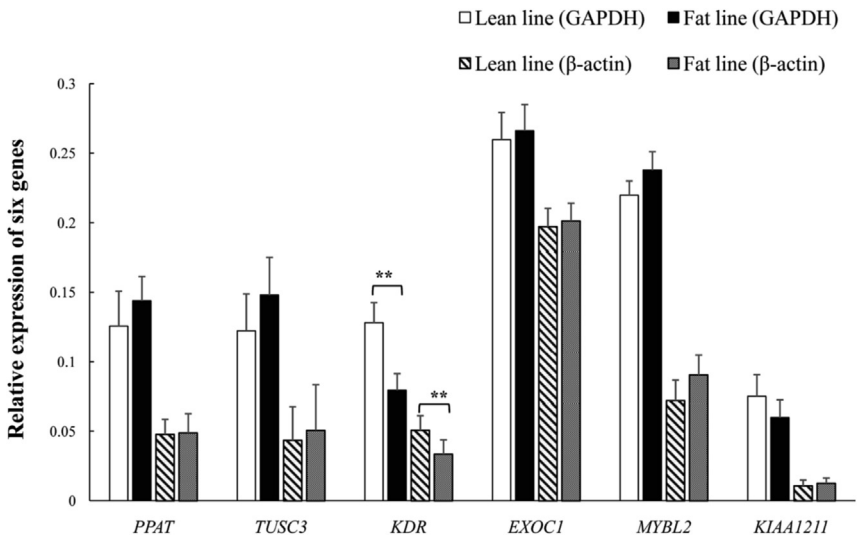


**Fig. 2.** Relative expression of six genes in abdominal fat tissue. **denote the significant differences of expression levels ($P < 0.01$). *GAPDH* and *β-actin* are the internal controls.

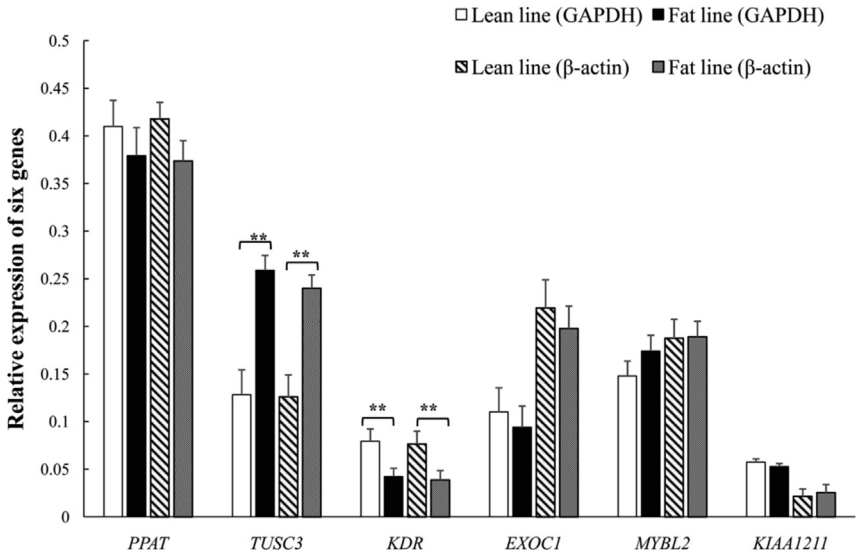


**Fig. 3.** Relative expression of six genes in liver tissue. **denote the significant differences of expression levels ($P < 0.01$). *GAPDH* and *β-actin* are the internal controls.

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

| | *KDR* | | *TUSC3* | | *PPAT* | | *EXOC1* | | *MYBL2* | | *KIAA1211* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AF | Liver | AF | Liver | AF | Liver | AF | Liver | AF | Liver | AF | Liver |
| AFW | −0.59** | −0.59** | 0.13 | 0.42* | 0.20 | −0.41* | 0.07 | −0.30 | 0.22 | −0.09 | −0.13 | 0.19 |
| AFP | −0.56** | −0.56** | 0.20 | 0.50* | 0.29 | −0.38* | 0.15 | −0.24 | 0.31 | −0.05 | −0.13 | 0.28 |

* Indicates $P < 0.05$; ** indicates $P < 0.01$.

AFW and AFP. This gene may therefore be related to lipogenesis in chicken liver. A previous study concluded that this gene regulated the key penultimate step in the essential coenzyme A (CoA) biosynthetic pathway [30]. The fatty acids needed for the deposition of animal body fat are derived mostly from de novo fatty acid synthesis, and acetyl-CoA and malonic acid single acyl CoA are catalyzed by fatty acid synthase to produce triglycerides [31,32]. CoA is important in fat synthesis and the gene likely participates in lipogenesis.

There was no difference in the expression of *MYBL2*, *KIAA1211* or *EXOC1* in the adipose tissue or liver between the two lines and they were not correlated with the AFW or AFP. However, *MYBL2* was identified in a GWAS responsible for human obesity [33]. There is no report in the literature of any relationship between *KIAA1211* or *EXOC1* and the formation of adipose tissue in any species but these two genes were found to be expressed in the adipose tissue and liver of chickens. The expression levels found for these genes in this study as well as GWASs suggest that *MYBL2*, *KIAA1211* and *EXOC1*

may be associated with fat deposition in chickens. However this requires confirmation.

In summary, the findings of this study could provide valuable information for further research on the six candidate genes involved in abdominal fat deposition in chickens.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgments

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