Pakistan J. Zool., vol. 51(3), pp 855-863, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.3.855.863

Association of *ChREBP* Expression and Polymorphism with Serum Biochemical Indices in Cherry Valley Duck

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ABSTRACT

ChREBP (carbohydrate-responsive element-binding protein) plays a crucial role as a central regulator of lipid synthesis and glycolysis in animal liver. In this study, the relative quantitative real-time PCR analysis indicated that the duck ChREBP mRNA is widely expressed in all examined tissues. ChREBP mRNA level was the highest in abdominal fat and the lowest in gizzard. The g.247075G>A silent mutation in exon 10 was first identified by direct sequencing approach, and resulted in three genotypes of AA, GA and GG. Association analysis demonstrated that the liver ChREBP mRNA expression level was significantly positive or negative effect on serum total protein (TP), albumin (Alb), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) ($P<0.05$ or $P<0.01$). The g.247075G>A mutation was significantly association with these measured serum biochemical indexes except for LDL-C. Significant additive effects were detected for all detected serum biochemical indexes except for LDL-C between allele A and G, and significant dominance effects for TP. These findings suggested that ChREBP might be a crucial candidate genes for the selection of duck lipid content.

Article Information
Received 18 May 2018
Revised 23 July 2018
Accepted 01 August 2018
Available online 15 March 2019

Authors' Contribution
YYZ is project leader, and conceived and designed the study. HLL carried out the experimental operation of the study and wrote the article. YYQ carried out the experimental operation of the study. LW analyzed the data of the study.

Key words
Duck, *ChREBP* gene, Gene expression, Single nucleotide polymorphism (SNP), Serum biochemical indices.

INTRODUCTION

ChREBP, carbohydrate-responsive element-binding protein also known as MLX-interacting protein-like (MLXIPL), derives from the protein's interaction with carbohydrate response element sequences of DNA. It is a basic helix 1oop helix/leucine zipper (bHLH/LZ) protein of the Myc/Max/Mad superfamily, widely expressed on multiple metabolic tissues in mammals, especially high expressed in liver, small intestine, skeletal muscle, adipose tissue, brain, spleen and kidney. ChREBP as a determinant transcriptional factor, forms a heterodimeric complex and binds and activates, in a glucose-dependent manner, regulated lipid synthesis in liver at the transcriptional level by binding to conservative sequences carbohydrate response element (ChoRE) in promoter of glycolysis and triglyceride synthesis genes, including fatty acid synthase (FAS), sterol regulatory element–binding protein 1 (SREBP1), acetyl CoA carboxylase (ACC) and L-type pyruvate kinase (L-PK) (Ma *et al*., 2006; Liu *et al*., 2018). ChREBP is translocated to the nucleus and binds to DNA after dephosphorylation of a p-Ser and a p-Thr residue by PP2A, which itself is activated by Xylulose-5-phosphate (Xu5p). Hepatic ChREBP is critically responsive to fructose intake and that fructose-mediated activation of ChREBP may link to hypertriglyceridemia, hepatic steatosis, and both hepatic and peripheral insulin resistance. Using tissue-specific ChREBP deletion method has shown that hepatic ChREBP is not required for fructose tolerance. Intestine-specific ChREBP-KO mice recapitulate the fructose-mediated toxicity observed in global ChREBP-KO mice and intestinal ChREBP is essential for fructose tolerance. However, liver-specific ChREBP-KO mice tolerate HFrD without evidence of significant liver inflammation. ChREBP gene is deleted in Williams-Beuren syndrome which a multisystem developmental disorder caused by the deletion of contiguous genes at chromosome 7q11.23. Hepatic ChREBP is protective in regards to hepatic insulin sensitivity and whole body glucose homeostasis. Hepatic ChREBP action can influence other peripheral tissues and is likely essential in coordinating the body's response to different feeding states (Jois *et al*., 2017). Based on the role of ChREBP in regulation of glycolysis and lipid metabolism. *ChREBP* gene may affect the economic traits of animals. However, few studies have been reported on expression and genetic variation of the *ChREBP* gene in birds. The properties and functions of *ChREBP* gene are still unknown in poultry. Therefore, in this study, we

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0030-9923/2019/0003-0855 $ 9.00/0

sought to examine the association of the mRNA expression levels and polymorphisms of ChREBP with duck serum biochemical indices which may suggest an insight into the genetic mechanism of economic traits and be helpful for improving production performance in duck breeding.

MATERIALS AND METHODS

Animal, sample preparation and data collections

A introduced duck variety of Cherry Valley ducks (321 females) were hatched on the same day. All of individuals were raised in a semi-open house and fed commercial corn–soybean diets based on the NRC requirements and subjected to same management environment in the experimental farm of Guizhou University, Guiyang, Guizhou, P.R. China. Blood samples (1.5mL) were obtained from the wing vein of each individual at 70 days of age, and then the serum was isolated in centrifuge tube. After blood was collected, all of ducks were euthanized that the specific implementation plan was taken all experimental ducks placed in an operating room filled with a mixture of 90% argon and 10% nitrogen, the ducks were quickly bled and dissected after they were unconscious. Sixteen kinds of tissues samples were collected from liver, heart, spleen, kidney, lung, cerebellum, cerebrum, proventriculus, gizzard, intestine, large intestine, abdominal fat, subcutaneous fat, pituitary, hypothalamus, and breast muscle, and frozen in liquid nitrogen until being used.

Serum biochemical indexes, including triglyceride (TG), total cholesterol (TCHO), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), albumin (ALB), globulin (GLB), and total protein (TP) were measured by Multiskan Spectrum (ThermoFisher, USA).

Primer sequences

According to the sequence of duck *β-Actin* gene (GenBank Accession No: EF667345) and *ChREBP* gene (NW_004676726.1). Eleven pair primers were designed to detect the mRNA expression level and polymorphisms of *ChREBP* gene. The sequences information of the primers was shown in Table I.

Table I.- The information of primer sequences.

Primers name	Primer sequence (5'→3')	Position/Product size (bp)	Tm (°C)
C1-F/R	F: GCAGACCACTAAGGGCTTCC	g.229706-230105/exon1/400	60
	R: ACTGGGGGATCAGTGAGACG		
C2-F/R	F: TCCCTTCAGCCTCTCTTTTG	g.237698-237891/exon2/194	58
	R: ACATGGCATTACCCAGCACT		
C3-F/R	F: GCACCCAGCCCTAGAGTGT	g.237920-238138/exon3/209	56
	R: CAACCTGCTCCTGGCTACAG		
C4-F/R	F: CCACCAGCTCCTTTTCTCC	g.239529-239775/exon6/247	60
	R: ATGGCTTGGGGCACCTAC		
C5-F/R	F: GTGAGGTTTGGTCCCTGATG	g.246886-247179/exon10/294	60
	R: AGTTCCCAGTCACCCCACTC		
C6-F/R	F: TCAGCCTGTCTTCCCTAAGC	g. 247822-248062/exon11/241	56
	R: GTGGGGGTGAATTTGGTTG		
C7-F/R	F: GGGTCAGCCCATATCCAGT	g.248079-248301/exon12/223	58
	R: TCTGCTCTGTCCTGCACACT		
C8-F/R	F: CAAGACCTCATGCCTTTTGC	g.248457-248679/exon13/223	58
	R: CCCAACACAGGTAGCAGCTC		
C9-F/R	F: AGCCTCCTGACTGCAGACTC	g.248967-249152/exon14/186	60
	R: CACCAGCCCCAAAGGATAC		
β-Actin-F/R	F: GGGTTCAGGGGAGCCTCTGT	107	60
	R: AACTGGGATGACATGGAGAAGA		
ChREBP-F/R	F: CAGCACTTCGACTTGGACAC	133	60
	R: AGTCTGGCTGGATCATGTCG		

DNA isolation, RNA isolation and cDNA synthesis

Genomic DNA of 321 birds were extracted from the blood samples using QIAamp DSP DNA Blood Mini Kit (Shanghai Labpal Co. Ltd., China). The total RNA of sixteen kinds of tissues samples from 6 ducks and the liver total RNA of all individual were isolated by Trizol Reagent (RNA Extraction Kit, Invitrogen, USA), respectively. The concentration and quality of total RNA and genomic DNA were estimated by spectrophotometer Nano-Drop 2000 (USA) and agarose-gel electrophoresis. For expression analysis, total RNA was treated by DNAse I (Invitrogen, USA). Each treatment contained 20-50 μg total RNA, 5 μL 10 ×RNase I buffer, 20 U RNase inhibitor, 2 μL(10 U) DNase I (RNase-free), RNase-free dH_2O up to 50 μL, and then 37°C for 20 min, subsequently detected and adjusted concentration to 100 ng/μL. All of total RNA were reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time) (Dalian TaKaRa Co. Ltd., China) for real-time quantitative RT-PCR (qRT-PCR) analysis.

Real-time qRT-PCR analysis

A housekeeping gene, duck*β-Actin*, was used in parallel for each run as an internal control. Two sets of primers (Table I), β-Actin-F/R and ChREBP-F/R, were used to detect the ChREBP mRNA levels in the sixteen different tissues. For duck *ChREBP* mRNA lever, the relative quantitative real-time polymerase chain reaction (qRT-PCR) was performed with an ABI 7500 (ABI, USA) using SYBR® Premix Ex Tap™ II (Takara Dalian, Japan). Each reaction mixture contained 10 μL SYBR® Premix *Ex Tap™ II*, 1 μL of each primer (10 μM), 0.5 μL of ROX Reference Dye II, 1 μL of cDNA and 6.5 μL of RNase-free water in a final volume of 20 μL. The experimental run protocol was a denaturation step at 95°C for 1 min, 40 cycles of amplification (95°C for 15s, 60°C for 34s), and final denaturation at 95°C for 15s, at 60°C for 60s, at 95°C for 15s, and at 60°C for 15s. To check the specificity of the PCR reaction, melting curves were acquired and analyzed by continuous fluorescence acquisition using SDS1.4 software.

Quantitative values were obtained from the threshold cycle (Ct) at which a significant increase in the magnitude of the signal generated by the PCR reaction was detected. The relative amount of duck ChREBP mRNA in various tissues was calculated by the formula $2^{-\Delta\Delta Ct}$. Statistical analyses of the relative expression of duck ChREBP mRNA in distinct tissues were processed with the least square method using SPSS 17.0 software. The expression levels in different tissues were presented as least square mean ± standard error.

PCR amplification, direct sequencing and SNP detection

Nine pairs of primers (C1-F/R-C9-F/R, as shown in Table I) were used to detect polymorphism of the ChREBP gene. The PCR was performed in a final volume of 25μL that consisted of 10μL 2×Taq Master Mix (pfu) (Shanghai Labpal Co. Ltd., China), 12μL free water, 1μL of each primer and 1μL genomic DNA. PCR profile was used: initial denaturation for 10 min at 95°C; 35 cycles of 94°C for 45 s, annealing for 30 s at the temperature shown in Table I, extension for 35 s at 72°C; final extension for 10 min at 72°C, preserved at 4°C. The amplified fragments were characterized by 1.5% agarose gel electrophoresis. The gels were visualized by GoldenView. All of the PCR products were purified and sequenced by Shanghai Labpal Co. Ltd., China. Sequence was edited and screened single nucleotide polymorphisms (SNPs) by software MEGA 6.0.

Statistical analysis

Allele frequency, genotype frequency, chi-squar (χ^2), polymorphism information content (PIC), effective number of alleles (Ne) and heterozygosities (h) were calculated by PopGen32 V1.31. Correlation coefficients were calculated between gene expression and serum biochemical indexes by bivariate Correlation. SPSS 16.0 (General linear model procedures) was used to analyze relationships between genotypes and serum biochemical indexes according to the following model: Y =μ+ G + e, Y-dependent variable (analyzed traits), μ-overall mean, G-fixed effects of genotype or mRNA expression level, and e-random error. Multiple comparisons were performed with the least squares means, were given as means ± standard error. Values were regarded to be statistically significant with $P < 0.01$ or $P < 0.05$. The additive effect and dominance effect were estimated according to Falconer and Mackay (1996) and Zhu and Jiang (2014).

RESULTS AND DISCUSSION

ChREBP gene expression profile

To detect the tissue distribution of duck ChREBP mRNA, qRT-PCR was carried out with cDNA from sixteen duck tissues. The result was shown in Figure 1, the ChREBP mRNA was observed in all examined tissues from the six 70-day-old female ducks. The relative expression of duck ChREBP mRNA in sixteen tissues were identified as follows: abdominal fat > pituitary > subcutaneous fat > lung > small intestine > large intestine > hypothalamus > heart > kidney > breast muscles > cerebrum > cerebellum > liver > proventriculus > spleen > gizzard. Salamanca *et al*. (2015) reported that the metabolic pathways of lipid deposition and elimination are regulated tightly by the nutritionally regulated transcription factors Foxo1, Foxa2, ChREBP and SREBP-1c. The subcellular distribution of ChREBP between the nucleus and cytosol is controlled

by different metabolites including xylulose 5-phosphate, glucose 6-phosphate, fructose 2,6-biphosphate and ketone bodies (Nakagawa *et al*., 2013). In mammals, ChREBP was expressed in key metabolic tissues including liver, adipose tissue, small intestine, kidney, and skeletal muscle, brain and spleen (Iizuka *et al*., 2004). In lean and obese patients, ChREBP increased in the liver from obese compared to lean subjects, whereas the expression decreased in both adipose tissues. The expression levels of ChREBP mRNA and protein were dramatically increased during the differentiation of human omental and subcutaneous preadipocytes (Hurtado del Pozo *et al*., 2011). In chicken, ChREBP mRNA level from high to low: liver > duodenum > kidney > pancreas > brain > spleen > abdominal fat > hypothalamus > heart > breast muscles. ChREBP demonstrated the most tissue-specific expression pattern with the highest mRNA levels detected in liver and duodenum and the lowest in heart and skeletal muscle (Proszkowiec-Weglarz *et al*., 2008). However, in duck, ChREBP mRNA level was the highest in abdominal fat and the lowest in gizzard, what's more, it's mRNA level in the pituitary was second only to abdominal fat in this study. Numerous studies had indicated that the growth hormone secreted by the pituitary gland can significantly affect the lipid metabolism of animals (Angelin and Rudling, 1994; Oscarsson *et al*., 1999). From the experimental results, we speculated that whether ChREBP had an inevitable link with growth hormone to jointly achieve the regulation of fat metabolism, which will be the content we will further study in the future. As a whole, the result indicated that the ChREBP mRNA expression level was similar between chicken and duck, and it has significant difference to mammals and human, and suggested the germplasm difference between birds and mammals. Our research results indicated that the level of duck ChREBP mRNA may play a role in adipogenesis.

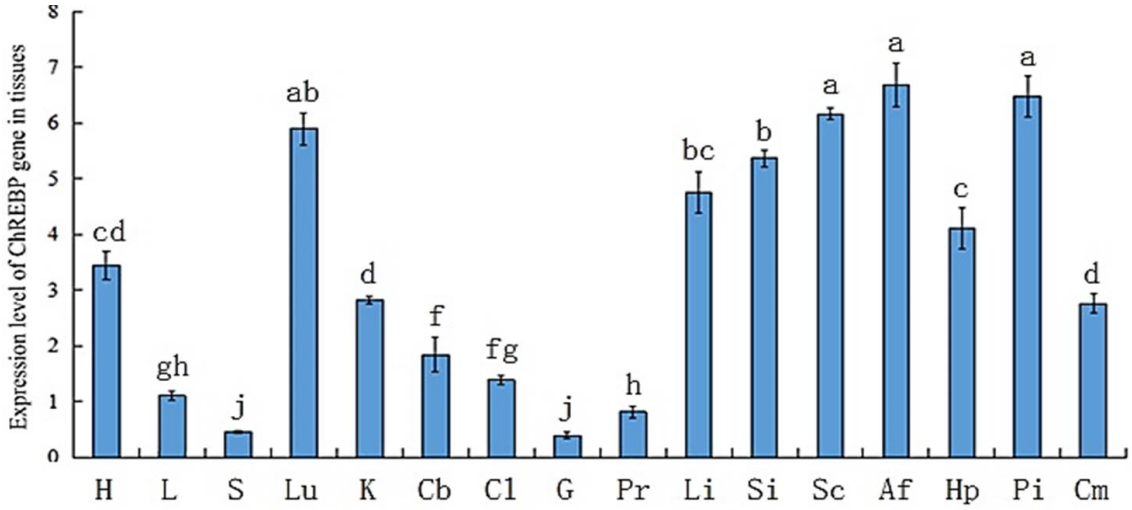

Fig. 1. Expression profiles of duck ChREBP mRNA in different tissues. Samples are identified as follows: H, heart; L, liver; S, spleen; Lu, lung; K, kidney; Cb, cerebrum; Cl, cerebellum; G, gizzard; Pr, proventriculus; Li, large intestine; Si, small intestine; Sc, subcutaneous; Af, abdominal fat; Hp, hypothalamus; Pi, pituitary and Cm, chest muscle.

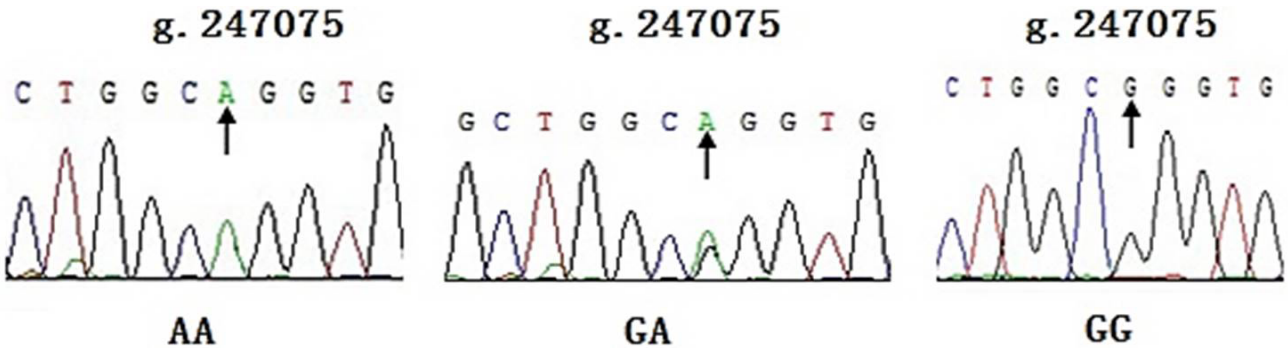

Fig. 2. Sequencing map with different genotypes in primer C5-F/R.

Polymorphism identification, allele and genotype distribution

All of the target fragments were successfully amplified using nine pairs primers (C1-F/R-C9-F/R, as shown in Table I) for the ChREBP gene, which could be analysed by direct sequencing. Only g.247075G>A silent mutation was found in exon 10 by sequence alignment (Fig. 2). Properties of the g.247075G>A mutation are listed in Table II. The dominant genotype and allele were GG and G, with frequencies of 0.4486 and 0.5982, respectively. Heterozygosity (h) and polymorphism information content (PIC) were 0.4807 and 0.3652, respectively. Botstein *et al*. (1980) reported that a PIC values from 0.25 to 0.5 is considered to be moderately informative. Under this formulation, the g.247075G>A locus was a moderate degree of polymorphism in Cherry Valley ducks. Chi-square test indicated that the g.247075G>A locus deviated from Hardy-Weinberg equilibrium (P<0.01), in which genotypes frequencies was likely to been distorted by migration, mutation, long-term artificial selection or other reasons. Furthermore, only a single SNP was detected in this species, suggesting that the ChREBP gene sequence might have been highly conserved through evolution.

Correlation between ChREBP gene expression and serum biochemical indices

ChREBP is a key lipogenic transcription factors responsible for the induction of gene encoding enzymes involved in fatty acid and triacylglyceride synthesis in the liver (Filhoulaud *et al*., 2013). In our study, correlation analysis between ChREBP mRNA expression level in liver of Cherry Valley ducks (321 females) and serum biochemical indices were shown in Table III. Significant positive or negative correlations were found between liver ChREBP mRNA expression level and serum total protein (TP), albumin (Alb), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) (P<0.05 or P<0.01), respectively. Furthermore, ChREBP expression was not significantly correlated with serum globulin. Our research results indicated that the level of duck ChREBP mRNA may play a role in adipogenesis. Hong *et al*. (2014) reported that the accumulation of intra-renal free fatty acids and triglycerides increased through activation ChREBP and sterol regulatory element-binding protein-1 (SREBP1). Proctor *et al*. (2006) found significant increases in renal cholesterol, triglyceride content and fatty acid related to increase expression of SREBP1 and ChREBP, decrease expression of ATP-binding cassette transporter-1, liver X receptor-alpha (LXRα), and LXR-beta (LXRβ), which results in decreased cholesterol efflux. Glucose uptake and GLUT4 expression are important to induce ChREBP expression and synthesis of FAs from glucose and to regulate the release of fatty acids and other lipids as well as endocrine hormones and cytokines, which enhances lipogenesis and the synthesis of these fatty acid esters of hydroxy fatty acids (Smith and Kahn, 2016). Linden *et al*. (2018) showed that ChREBP and SREBP-1c are both required for coordinated induction of glycolytic and lipogenic mRNAs in mice. Glucose enhanced total ChREBP expression and triglyceride synthesis but was associated with improved hepatic insulin signaling (Softic *et al*., 2017). RetSat depletion may cause at least some of the observed metabolic alterations by interfering with hepatic ChREBP in HS/HFD-fed (high-fat/high-sucrose diet) mice, and further affected liver steatosis and serum TG (Heidenreich *et al*., 2017). In summary, our results were similar to that a lot of results indicated that the expression level of ChREBP was significantly related to lipid metabolism, and speed up both TG and TC by the liver to cells in vivo of transhipment, and caused the fatty deposits (Poupeau and Postic, 2011; Du *et al*., 2017; Sae-Lee *et al*., 2016).

Table II.- Characteristics of the g.247075G>A locus of ChREBP gene in Cherry Valley ducks.

SNP	Genotype frequencies			Alleles frequencies		h	PIC	χ2
	AA	GA	GG	A	G			
g.247075G>A	0.2523	0.2991	0.4486	0.4018	0.5982	0.4807	0.3652	23.03**

H, heterozygosity; PIC, polymorphism information content; χ^2-test, Hardy-Weinberg equilibrium, χ2 0.01(2) = 9.21, χ2 0.05(2) = 5.99. *, P<0.05; **, P<0.01.

Table III.- Association of ChREBP expression with serum biochemical indices in Cherry Valley ducks.

mRNA level	TP (g/L)	ALB (g/L)	GLB (g/L)	TG (mM)	TC (mM)	HDL-C (mM)	LDL-C (mM)
1.23±0.22	33.79±0.94	15.10±0.35	21.14±0.76	0.84±0.03	5.44±0.12	3.33±0.11	2.01±0.05
	0.385**	0.401**	-0.091	0.322**	0.235*	0.245*	-0.277*

TP, total protein; ALB, albumin; GLB, globulin; TG, triglyceride; TC, total cholesterol; HDL-C, highdensity lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *Correlation is signifcant at the 0.05 level, **Correlation is signifcant at the 0.01 level.

Table IV.- Least-square means and estimated effects of g.247075G>A locus of ChREBP gene on serum biochemical indices in Cherry Valley ducks.

Indexes	Genotypes			Additive effect	P value	Dominant effect	P value
	AA(81)	GA(96)	GG(144)				
TP (g/L)	42.15±1.19a	32.40±1.28b	30.02±0.85b	6.07±0.86	0.00**	3.68±2.12	0.02*
ALB (g/L)	17.48±0.37a	14.47±0.55b	14.19±0.40b	1.64±0.35	0.00**	1.36±0.46	0.25
GLB(g/L)	26.70±1.10a	21.13±1.22b	18.01±0.91b	4.34±0.76	0.01*	1.22±1.01	0.09
TG (mmol/L)	0.93±0.05a	0.82±0.05b	0.81±0.04b	0.06±0.04	0.01*	0.05±0.07	0.42
TC (mmol/L)	6.23±0.15a	5.21±0.17b	5.15±0.09b	0.54±0.13	0.00**	0.48±0.45	0.34
HDL-C (mmol/L)	4.14±0.08a	3.01±0.16b	3.08±0.07b	0.53±0.12	0.00**	0.60±0.68	0.18
LDL-C (mmol/L)	2.11±0.08	2.01±0.11	1.96±0.06	0.07±0.05	0.29	0.02±0.14	0.73

For abbreviations, see Table III. Data are least square means ± standard errors; Values with different superscript letters (a, b) are significantly different (Duncan's Multiple Range Test) in genotypes AA, GA, and GG. *, level of 0.05; **, level of 0.01.

Association of g.247075G>A mutation with serum biochemical indices

ChREBP genes involved in glucose, carbohydrate and lipid metabolism, and influence accumulation of hepatic triglycerides. Overexpression of a constitutively active form of ChREBP in the liver enhanced insulin sensitivity, but also increased steatosis. Regulation of ChREBP appears to be very complex and responds to environmental conditions, dietary or hormonal changes being clearly tissue-specific. ChREBP isoforms may have different role of the pathophysiological and physiological functions (Jois *et al*., 2017). In our study, the g.247075G>A locus of ChREBP gene associated with Cherry Valley duck serum biochemical indices were showed in Table IV. The results of correlation analysis indicated that the g.247075G>A locus was significantly associated with the measured serum biochemical indices except for LDL-C. With the exception of LDL-C, genotype AA was significant higher than genotypes GA and GG, and then there had significant additive effects between allele G and A at P<0.05 or 0.01. However, only TP had significant dominant effect between allele G and A at P<0.05.

Comparatively, Chambers *et al*. (2011) showed that the g.72664314T/C (rs17145750) synonymous mutation of ChREBP associate with hypertriglyceridemia and liver function abnormalities, and suggested that it may participate in the progression from simple steatosis to NASH by genome-wide association study (GWAS). Herman *et al*. (2012) demonstrated that ChREBP can be transcribed from an alternative promoter and alternative exon 1 that skips the canonical exon 1a and can be translated from a start codon in exon 4. Kooner *et al*. (2008) found association of a G771C nonsynonymous mutation (rs3812316, Gln241His) in ChREBP gene with plasma triglyceride levels in Indian Asians. Kathiresan *et al*. (2008) and Willer *et al*. (2008) indicated that the T/C (rs17145738) mutation at 7q11 near TBL2 and MLXIPL had the strongest effect on blood triglyceride concentrations. Gao *et al*. (2016) found 2 SNPs (rs1051921, rs17145750) had not identified any significant association between individual SNP or haplotype and levels of ChREBP DNA methylation, and ChREBP DNA methylation was associated with TC, TG and LDL-C. The allele G of rs3812316 was significantly lower in coronary artery disease (CAD) group for age, sex, BMI, SBP and DBP. Haplotypes GGC, CGT, CCC and CGC were constructed based on rs3812316, rs7798357 and rs1051921. Haplotype CGC was significant higher in CAD group. Haplotypes GGC, CGT and CCC were significant lower in CAD group. The SNPs of rs7798357, rs17145750 and rs1051921 were no significant differences between cases and controls (Guo *et al*., 2011). Serum biochemical indexes are necessary for the maintenance of normal homeostasis in vivo and are used to evaluate livestock performance, such as laying and meat value. Thus, knowledge on serum biochemical parameters in poultry will facilitate the diagnosis of existing diseases and optimize the value of birds. Whether the AA genotype and A allele of the g.247075G>A mutation can contribute to the improved the content of lipids, and it may be used in marker assisted selection to improve duck lipid metabolism. However, serum biochemical indexes are complex quantitative traits involving multiple genes, loci, and interactions of various factors. Our research results still requires further physiological and biochemical evidence under the same test conditions.

ACKNOWLEDGMENTS

The funding for this work was supported by the National Natural Science Foundation of China (31760663)

and science and technology project of Guizhou province (QKHPTRC(2017)5788).

Statement of conflict of interest

Authors have declared no conflict of interest.

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