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Associations of Diplotypes of *Vldlr* Gene with Egg Production Traits in Laiwu Black Chickens

Yan Zhou[1,2], Hai Xia Han[1,2], Qiu Xia Lei[1,2], Jin Bo Gao[1,2], Wei Liu[1,2], Fu Wei Li[1,2], Jie Liu[1,2] and Ding Guo Cao[1,2]*

[1]*Institute of Poultry Science, Academy of Agricultural Sciences of Shandong Province, Jinan, Shandong, China*
[2]*Poultry Breeding Engineering Technology Center of Shandong Province, Jinan, Shandong, China*

Yan Zhou and Hai Xia Han contributed equally to this study.

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Authors' Contribution
YZ, DGC and HXH conceived and designed the experiments. YZ, HXH, QXL, JBG, WL, FWL and JL performed the experiments. YZ, HXH, QXL and JBG analyzed the data. YZ and HXH wrote the paper.

Key words
Gallus gallus, *VLDLR* gene, Diplotype, Egg production traits

ABSTRACT

Very low density lipoprotein receptor (VLDLR) is of vital importance for egg production in mediating the synthesis of yolk protein precursors. To better understand the effects of VLDLR on reproduction in chickens, the haplotypes and diplotypes based on three genetic mutations (NC_006127.2:g.8467G>A, NC_006127.2:g.12321G>A and NC_006127.2:g.13876A>G) were constructed, and the associations of diplotypes with reproduction traits were assessed, their effects on gene expression were evaluated also. As a result, three haplotypes H1 (G-G-A), H2 (G-G-G) and H3 (A-A-G) were obtained, H1 was the main haplotype with a frequency of 91.75%. The correlation analysis showed that diplotypes (H1H1, H2H2 and H3H3) were significantly associated with egg production at age of 40W (E40) (P=0.0342) and egg production at age of 43W (E43) (P=0.0184). The egg production at age of 38W (E38), E40 and E43 of H2H2 chickens were all higher than those of the H1H1 and H3H3 chickens. Compared with H1H1 and H3H3 chickens, the highest mRNA levels of VLDLR were found in ovary, 6 mm - 8 mm follicles and 4 mm follicles from H2H2 chickens, and significant difference compared with those of H3H3 chickens (P<0.01). These findings suggest that VLDLR could be considered a candidate gene for egg production in chickens.

INTRODUCTION

Very low density lipoprotein receptor (VLDLR) is a member of the low density lipoprotein receptor (LDLR) gene superfamily (Bujo *et al*., 1994). As a multifunctional receptor, VLDLR has many biological functions, including the regulation of lipid metabolism (Beisiegel, 1995; Tacken *et al*., 2001; Takahashi *et al*., 2003), cell proliferation and differentiation (Wada *et al*., 2000; Kosaka *et al*., 2001), and Reelin and Wnt signaling pathways (Sharaf *et al*., 2013; Khialeeva and Carpenter, 2017; Chen *et al*., 2016); VLDLR is also related to type 2 diabetic (Yuan *et al*., 2011; Pardina *et al*., 2016).

In poultry, VLDLR was found to be a key event in the control of oocyte growth (Stifani *et al*., 1990; Barber *et al*., 1991). During the rapid final stage of growth, chicken oocytes take up massive amounts of plasma components and convert them to yolk (Shen *et al*., 1993). More than 30% of the yolk weight is composed of lipids imported in the form of serum-borne lipoproteins, mainly including yolk lipoprotein precursors, vitellogenin (VTG) and very low density lipoprotein (VLDL) (Bujo *et al*., 1997). VLDLR is the key receptor that mediates VTG and VLDL endocytosis into growing chicken oocytes. Based on this, multiple studies have assessed its relationship with egg production in oviparous species. Nimpf *et al*. (1989) found that VLDLR absence from oocytes is responsible for the R/O (Restricted Ovulator) phenotype. This was further confirmed by Bujo *et al*. (1995). The latter authors described a single nucleotide substitution (G→C), which results in Cys-682→Ser replacement. Hens harboring this mutation are sterile and display severe hyperlipidemia. Subsequently, several VLDLR mutations were shown to have close associations with egg quality and production traits in duck (Wang *et al*., 2011), quail (Wu *et al*., 2015) and chicken (Cao *et al*., 2012). Interestingly, changes of ovary VLDLR mRNA expression are correlated with clutch size, laying interval, and egg mass in zebra finch (Han *et al*., 2009).

Despite a wealth of data regarding VLDLR, its biological effects on chicken reproduction remain largely unknown. In this study, we aimed to examine the sequence

* Corresponding author: jqsyzs@163.com
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variants of VLDLR in Lai Wu Black chicken and assess their genetic effects on reproduction traits.

MATERIALS AND METHODS

Animals and data collection

Four hundred LaiWu Black chickens selected randomly from the Local Breed Genetic Resources Bank of Shandong Province were used for the detection of VLDLR SNPs and association analyses. Four reproduction traits, including age at first egg (AFE), egg production at age of 38W (E38), egg production at age of 40W (E40), and egg production at age of 43W (E43), were measured according to The Poultry Production Performance Terms and Measurement Statistics Method (NY/T823-2004). All animal procedures were carried out in accordance with the Directory Proposals on the Ethical Treatment of Experimental Animals, established by the Ministry of Science and Technology (Beijing, China).

Detection of SNPs, genotyping, and diplotype construction

Venous blood samples were collected from all four hundred LaiWu Black chickens by venipuncture, then the genomic DNA was isolated with TIANamp Blood DNA Kit (DP318, Tiangen, Beijing, China) and stored at -20°C. According to the CDS sequence of VLDLR (GenBank Accession no. NC_006127.2, GI: 118136399), nineteen sets of PCR primers were designed to detect VLDLR SNPs, but only three genetic mutations were found and genotyped by PCR-RFLP with specific primers (Table I). PCR-RFLP reactions were performed in a 15 µL system containing 0.5 µL of restriction enzymes (10U/µL) *(HinfI, Eco47I or MvaI, N*ew England Biolabs, Inc., USA), 1 µL of 10× NEBuffer, 8 µL of PCR product, and 5.5 µL ddH_2O. The PCR products were digested at 37°C for 4 h; the digestion products were separated by 2.5% agarose gel electrophoresis for 30 min at 120V. After genotyping, two samples per SNP genotype were sequenced by Jinan Li Ge Technology Co., Ltd (Jinan, China) to confirm the variation. Haplotypes and diplotypes were constructed based on the SNPs identified in the 400 chicken, using the PHASE 2.0 software.

Tissues and real-time quantitative PCR

To characterize the mRNA expression of chicken VLDLR gene of individuals with different diplotypes, five LaiWu Black chickens for each diplotype were euthanized, and the 4 mm diameter follicles, 6 mm-8 mm diameter follicles and ovary (O) were sampled rapidly. All the tissues were frozen and stored in liquid nitrogen.

Total RNA was isolated from tissue samples using Ultrapure RNA Kit (CW0581, CWbio. Co. Ltd., Beijing, China) according to the manufacturer's instructions, and assessed by the A260/A280nm ratio, with the expected values falling between 1.8 and 2.0 (Eppendorf, Hamburg, Germany). 1µg of total RNA was used for cDNA synthesis with HiFi-MMLV cDNA Kit (CW0744, CWbio Co. Ltd., Beijing, China). The chicken β-actin gene was used as an internal control for qRT-PCR. The primers used in this study are listed in Table II. qRT-PCR was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The cycle threshold (CT) value of the control gene was used to normalize target gene signals in each sample. Specimens were assessed in triplicate. The $2^{-\triangle\triangle CT}$ method was used to derive the relative amounts of target gene transcripts based on the control gene.

Statistical analysis

The Hardy-Weinberg equilibrium was evaluated by $\chi 2$ test. The associations of diplotypes with reproduction traits were determined by the general linear model (GLM) procedures of SAS8.12 (SAS Inst. Inc., Cary NC, USA) with the following statistical model: $Y = \mu + G_i + e$, Where Y is the observed value of reproduction traits, µ is the population mean, G is the fixed effects of the diplotype, and e is random error. Multiple comparisons were performed with least squares means. One-way ANOVA was used to assess gene expression differences among tissues with various diplotypes. Data are least squares means ± standard error of the means; $P < 0.05$ was considered statistically significant.

RESULTS

Genotype and allele frequencies, and associations

There were three genetic mutations, with a total of six genotypes detected. By sequencing, nucleotide changes at various loci were further confirmed. Based on GenBank Accession No. NC_006127.2, a G→A synonym mutation at nucleotide 8467 (NC_006127.2: g.8467G>A) was located in exon 6; a G→A mutation at position 12321 (NC_006127.2: g.12321G>A) was found in intron 15, and an A→G mutation at position 13876 (NC_006127.2: g.13876 A >G) in intron 17.

According to genotype and allele frequency analysis (Table III), the GG genotype had advantage over others, with G being the dominant allele in NC_006127.2: g.8467G>A. In NC_006127.2: g.12321G>A, G was the dominant allele since the GG genotype (0.975) occurred much more frequently than the AA genotype (0.025). In NC_006127.2: g.13876A>G, AA genotype frequency was higher than that of the GG genotype, and the allele A was dominant. The genotype distribution in the experimental chicken for all three SNPs did not fit the Hardy-Weinberg equilibrium with a *P*-value lower than 0.05.

Table I. Parameters of primers for SNP identification and genotyping of *VLDLR*.

Primer	Sequences (5′–3′)	Length of products/bp	Annealing temp (°C)	Usage
NC_006127.2:g.8467G>A	F: GGCTCAGGTGAATGTATC	474	50.3	Genotyping with *Hinf*I
	R: CAGTCTCCGTGATGGTTA			
NC_006127.2:g.12321G>A	F: ATTGGGAATCAGGATACTAAAC	640	50.5	Genotyping with *Eco47*I
	R: CCTACTCATTTCAGGCTCT			
NC_006127.2:g.13876A>G	F: GGCTGTTCTTCCTATCTG	441	50	Genotyping with *Mva*I
	R: GGTCCCTTCTGATTGC			

Table II. Parameters of primers for RT-qPCR.

Gene	Primer sequence(5'-3')	Annealing temp (°C)	Product size(bp)
VLDLR	F: CCGTTTGTATTGGCTTGATTC R: CACCATAGACTGCCTCGTTC	60	173
β-actin	F: CCATCTATGAAGGCTACGC R:CTCGGCTGTGGTGGTGAA	60	124

Table III. Genotype and allele frequencies at NC_006127.2: g.8467G>A, NC_006127.2: g.12321G>A and NC_006127.2: g.13876A>G.

SNPs	Location	Genotype frequency		Allele frequency		P-value [a]
NC_006127.2: g.8467G>A	Exon6	GG	AA	G	A	5.51E-89
		0.973	0.027	0.973	0.027	
NC_006127.2: g.12321G>A	Intron15	GG	AA	G	A	5.51E-89
		0.975	0.025	0.975	0.025	
NC_006127.2: g.13876A>G	Intron 17	AA	GG	A	G	5.51E-89
		0.915	0.085	0.915	0.085	

[a] *P-value* is the probability in χ2-test for the Hardy-Weinberg equilibrium

Table IV. Haplotypes and diplotypes inferred based on the 3 single nucleotide polymorphisms.

Haplotype	NC_006127.2: g.8467G>A	NC_006127.2: g.123217G>A	NC_006127.2: g.13876A>G	Frequency (%)	Diplotype	Frequency (%)
H1	G	G	A	91.750	H1H1	91.750
H2	G	G	G	5.750	H2H2	5.750
H3	A	A	G	2.500	H3H3	2.500

Construction of haplotypes and associations

PHASE 2.0 data are shown in Table IV. Based on the three SNPs, three haplotypes were obtained. G-G-A was the main haplotype with a frequency of 91.75%. In addition, three diplotypes were obtained at frequencies higher than 2%; H1H1 was the main diplotype, accounting for 91.75% of all cases.

Table V showed that significant associations of diplotypes with E40 (P=0.0342) and E43 (P=0.0184) were found. H3H3 chickens were superior to H1H1 and H2H2 chickens in AFE, but their egg production from 38 to 43 weeks was poorest among all chickens. For egg production, H2H2 chickens showed an absolute advantage, with E38, E40 and E43 higher than those of the other chickens.

Comparison of VLDLR mRNA expression in tissues with different diplotyps

The mRNA expression of VLDLR in ovary, 4 mm and 6 mm-8 mm diameter follicles were analyzed and compared among H1H1, H2H2 and H3H3 chickens. The results in Figure 1 showed that, in the same tissue, mRNA levels in different diplotypes chickens were ordered as: H2H2 > H1H1> H3H3; the highest mRNA levels were found in H2H2 chickens, and significant difference

compared with those of H3H3 chickens (P<0.01).

Table V. Associations of diplotypes of the chicken *VLDLR* gene with reproductive traits.

Traits	P value	H1H1(367)	H2H2(23)	H3H3(10)
AFE	0.8404	139.69±0.47	139.08±1.88	138.20±2.86
E38	0.0552	94.16±0.88	102.36±3.50	90.00±5.48
E40	0.0342	104.15±0.96^{ab}	112.81±3.85^{a}	95.88±6.032^{b}
E43	0.0184	117.33±1.11^{ab}	127.04±4.431^{a}	104.66±6.932^{b}

The least square means within a row lacking a common lowercase letters differ significantly (P<0.05).

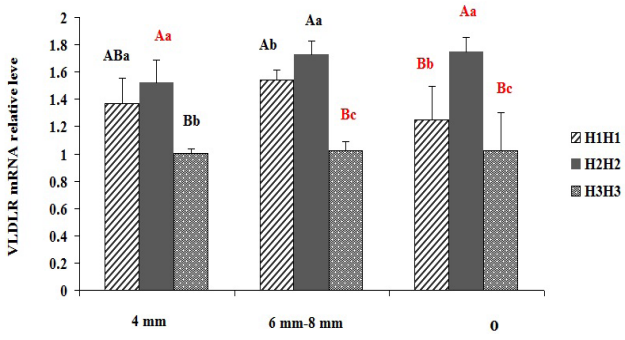

Fig. 1. Relative expression levels of *VLDLR* mRNA of chickens with different diplotypes in ovary (O), 4 mm and 6 mm-8 mm follicles; Comparisons of mRNA expression of VLDLR gene in 4 mm, 6 mm - 8 mm diameter follices, and ovary among chickens with H1H1, H2H2 and H3H3 diplotypes. X-axis represents different tissues: ovary (O), 4 mm and 6-8 mm diameter follicles. Y-axis is the VLDLR mRNA relative level. In the same tissue, the highest mRNA level were all found in H2H2 chickens, and significant difference compared with those of H3H3 chickens (P<0.01). Menas ± SE were calculated for each tissue. Bars with different superscript letters (a, b or A, B) mean significantly different (P<0.05 or P<0.01).

DISCUSSION

Previous studies have demonstrated that VLDLR is essential for vitellogenesis and influences subsequent egg production in chicken. Its point mutation at position 2177bp of the chicken VLDLR cDNA, termed restricted ovulator, characterized by leghorn female sterility (Nimpf *et al*., 1989; Takahashi *et al*., 2004). To further explore relevant VLDLR mutations affecting chicken reproduction, sequencing and PCR-RFLP were used to screen all eighteen VLDLR exons to examine their variants in four hundred LaiWu Black chicken. Interestingly, three genetic mutations (NC_006127.2: g.8467G>A, NC_006127.2: g.12321G>A and NC_006127.2: g.13876A>G) were detected. NC_006127.2: g.8467G>A was a synonymous mutation at positon 8467 in exon 6; the other two mutations were located in introns. The R/O genotype was not found in this experimental population, indicating that the harmful mutation might be deleted during the breeding process.

Zhan *et al*. (2009) found five SNPs in introns 2, 7 and 9 by PCR-SSCP, PCR-RF-SSCP and sequencing in 390 chickens; intron 2 polymorphisms (T3967G) were significantly associated with age at first egg and egg shell thickness (P<0.01). In most eukaryotic genes, the coding sequence is interrupted by introns, which are eventually removed by a precise splicing mechanism in the nucleus during mRNA maturation (Sharp, 1987). Although introns do not directly encode proteins, more and more studies have revealed that they might play a role in chromatin structure and its associations with gene function (Svaren and Chalkley, 1990; Sattar *et al*., 2019), e.g. regulation of gene expression both transcriptionally and post- transcriptionally (Breitbart *et al*., 1987; Jonsson *et al*., 1992). In addition, mutations in introns have close relationships with human diseases (Yang, 2016; Kim *et al*., 2016; Karakus *et al*., 2013) and important economic traits in animals (Maitra *et al*., 2014; Yang *et al*., 2017; Zhang *et al*., 2017). The currently known VLDLR mutations mainly occur in intronic regions (Sharp, 1987; Zhan *et al*., 2009). In this study, two of the three SNPs were also located in introns, correlation analysis showed that diplotypes were significantly associated with E40 and E43, and the association between these parameters increased with chicken age. E38, E40 and E43 in H2H2 chickens were all higher than those of the remaining chickens. It is worth noting that E43 of H2H2 chickens had 9.71 and 22.38 eggs more than those of H1H1 and H3H3 chickens, respectively. Previous studies showed that the expression of VLDLR mRNA were related with reproduction traits e.g. egg mass in zebra finch (Han *et al*., 2009). VLDLR is also found highly expressed in the ovary, oviduct, pituitary gland in duck (Wang *et al*., 2011). To further assess the effects of VLDLR on reproduction, we further compared the mRNA expression of VLDLR gene in ovary, 6 mm-8 mm diameter and 4 mm diameter follicles among different diplotypes. In all the three tissues, the highest expression level of VLDLR gene were found in H2H2 chickens, showing significant difference compared with those of H3H3 chickens. The data were consistent with the results of association analysis on egg production. Therefore, H2H2 may be considered an advantageous diplotype for the reproduction trait.

CONCLUSIONS

In summary, three VLDLR mutations were detected and characterized in LaiWu Black chickens. The diplotypes were significantly associated with egg production traits.

The E38, E40 and E43 of H2H2 chickens were all higher than those of the H1H1 and H3H3 chickens, and the mRNA expressions of VLDLR in H2H2 chickens were highest in ovary, 6 mm-8 mm and 4 mm follicles than those in H1H1 and H3H3 chickens. H2H2 might be considered a potential favorable molecular marker for egg production in chicken. These findings provide further insights into the molecular mechanism by which VLDLR regulates egg production.

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Statement of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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