Journal of Animal and Veterinary Advances 11 (19): 3662-3667, 2012
ISSN: 1680-5593

Associations of Very Low Density Lipoprotein Receptor (*VLDLR*) Gene Polymorphisms with Reproductive Traits in a Chinese Indigenous Chicken Breed

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Abstract: Chicken Very Low Density Lipoprotein Receptor (*VLDLR*) is a physiological candidate gene for reproductive traits. The objective of the current research was to investigate the association of VLDLR Single Nucleotide Polymorphisms (SNPs) and the reproductive traits in a Chinese Indigenous chicken breed (Wenshang Luhua chicken). A total of 528 individuals were genotyped with PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). As a result, an A→G mutation on exon 6 (A12321G) and an A→G mutation on intron 17 (A13876G) were identified. In locus 12321, genotypes have significantly effect on Egg Weight at 300 days (EW) and the chickens harboring genotype A2A2 had significantly higher EW ($p<0.05$) than that of A1A1 chickens. Also the Egg Weight at First Egg (EWFE) and Egg Production at 300 days (EP) values of A2A2 chickens were higher than A1A1 chickens and A2A2 chickens had lower Living Weight at First Egg (LWFE), Living Weight at 300 days (LW) and Age at First Egg (AFE) than A1A1 chickens. In locus 13876, genotypes had significantly effect on EW ($p<0.05$). The EW values of B2B2 chickens were significantly higher than B1B1 chickens ($p<0.05$). For LWFE, LW and EP, B2B2 chickens were superior to B1B1 chickens but no significant difference between them ($p>0.05$). Four diplotypes were constructed on the two SNPs. Significantly dominant effects of diplotypes H1H1 were observed for traits EW whereas H4H4 had a negative effect on it. Also for EWFE, LWFE, LW and EP, the H1H1 chickens were superior to H4H4 chickens it maybe tell that H1H1 is an advantaged diplotype for chicken reproductive traits.

Key words: Chicken, *VLDLR* gene, PCR-RFLP, reproductive traits, locus, China

INTRODUCTION

The Very Low Density Lipoprotein Receptor (VLDLR), a member of the LDL receptor gene superfamily is an important multifunctional receptor and mediates the deposition of the Very Low Density Lipoprotein (VLDL) and Vitellogenin (VTG) which were the yolk mass components of chicken oocytes (Bujo *et al*., 1994; Nykjaer and Willnow, 2002).

In 1992, Takahashi and colleagues cloned a cDNA from rabbit heart that encoded a protein strikingly similar in sequence and structure to the LDLR. Expression of this cDNA in cultured cells produced a protein that bound rabbit VLDL but not LDL with high affinity. And later it was cloned in chicken, human, mouse, cattle, goose and duck. Its structure in these different species was elucidated in great detail (Bujo *et al*., 1994; Sakai *et al*., 1994; Oka *et al*., 1994; Magrane *et al*., 1998; Wang *et al*., 2011). *VLDLR* gene encodes a protein with six functional domains: a signal sequence followed by an amino-terminal ligand-binding domain constituted by multiple cysteine-rich repeats; an Epidermal Growth Factor (EGF) precursor homologous domain; an O-linked sugar domain; a transmembrane domain and a cytoplasmic domain with a FDNPVY sequence (Wyne *et al*., 1996; Wang *et al*., 2011). The chicken *VLDLR* gene is located on the avian sex chromosome Z, in agreement with the sex linkage of a single-gene defect in animals that fail to reproduce because of the lack of expression of functional VLDL/VTGR (Bujo *et al*., 1994). It encodes an 863 amino acid protein and contains 18 exons and 17 introns in its genomic DNA sequence. Studies established that *VLDLR* gene mediates the endocytosis of VTG and VLDL into growing chicken oocytes and plays a key role in

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control of the development of oocytes and yolk lipoprotein deposition (Barber *et al*., 1991; Shen *et al*., 1993; Hirayama *et al*., 2003; Takahashi *et al*., 2003).

Bujo *et al*. (1994) detected a point mutation (G/C) at position 2177 bp of the chicken VLDLR cDNA (mutation named Restricted Ovulation or RO) and showed that the mutant had a reduced egg production (Nimpf *et al*., 1989). Liu *et al*. (2000) cloned a chick 423 bp fragment using primers designed according to sequence published by orthogonal design. Zhan *et al*. (2009) found five SNPs in three introns (T3967G in intron 2, C8099T and A8296G in intron 7, A8839T and G9084A in intron 9) by methods of PCR-SSCP, PCR-RF-SSCP and sequencing. Results showed that *VLDLR* gene has important effect on the egg performance and the egg qualities in chicken. Apart from chicken, the regulation and dynamics of VTG/VLDL receptors in relation to vitellogenesis and follicle development also have been characterized in Drosophila (Schonbaum *et al*., 2000), trout (Rodriguez *et al*., 1996) white perch (Hirayama *et al*., 2003), Zebra finch (Han *et al*., 2009).

All the earlier studies has proved the important role of *VLDLR* gene for deposition of the yolk mass components of chicken oocytes but the associations between the SNPs of *VLDLR* gene and the chicken reproductive traits were researched scarcely. Marker-assisted selection has become an important approach towards improving production traits in animal breeding. So, in the present study, researchers investigated the association between VLDR SNPs with reproductive traits in an indigenous chicken breed in China to identify useful single nucleotide polymorphisms for genetic selection of chicken reproductive traits.

MATERIALS AND METHODS

Animals: An indigenous breed of Wenshang Luhua chicken (WL) in Shandong province of China was used for the current study. The WL chickens were covered with barred feather and the egg production at 500 days was ranging from 200-230. A total of 528 WL hens for this study were randomly selected form the Poultry Institute of Shandong Academy of Agricultural Science. All birds were hatched on the same day and transferred to single-gen cages at the age of 16 weeks. All birds were kept in the same laying house to minimize environmental effects. Blood samples were collected from all 528 individuals. The genomic DNA was isolated by the standard phenol/chloroform method.

Phenotypic measurements: The Egg Weight at First Egg (EWFE), Living Weight at First Egg (LWFE), Age at First Egg (AFE), Living Weight at 300 days (LW), Egg Weight at 300 days (EW) and Egg Production at 300 days (EP) were measured for each bird. Living weight was measured after 12 h with no access to feed. Data for EW were obtained from 528 hens on 3 days consecutively when hens were 300 days of age. The average for the 3 days was used as the value for each hen. All the experiments were complied with the requirements of the directory proposals on the Ethical Treatment of Experimental Animals of China.

Genotyping for *VLDLR* gene polymorphisms: Sixteen pairs of primers were designed for genotyping the polymorphisms in the *VLDLR* gene according to the *Gallus gallus* VLDLR sequence (GenBank Accession No: NC-006127.2) but only two pairs of primers were polymorphic (Table 1). Primer set 1 was used to amplify the fragment (640 bp) of the exon 6 of *VLDLR* gene, the primer set 2 was used to amplify the intron 17 of the *VLDLR* gene. The PCR reaction was performed in a final volume of 10 μL containing 0.8 μL of genomic DNA (2.5 ng μL^{-1}), 0.3 μL of each primer (10 pmol μL^{-1}), 3.6 μL ddH_2O, 5 μL of 2×MasterMix (Tiangen, Beijing, China). The following PCR cycle condition was used: an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 20 sec, 50.5°C (Primer set 1) or 50°C (Primer set 2) for 20 sec and 72°C for 40 sec and a final elongation at 72°C for 6 min.

The fragment amplified by primer set 1and 2 were digested with restriction enzyme XbaI and MvaI respectively, in a total volume of 15 μL reaction buffer containing 8 μL PCR product and 5U of enzyme at 37°C overnight. The digests with the restriction enzyme were detected through 1.5% agarose gel electrophoresis, genotypes were recorded according to the band patterns. PCR products that had polymorphism as revealed by PCR-RFLP were further amplified, purified and sequenced by a commercial sequencing company (Invitrogen, Shanghai, China).

Statistical analysis: Associations of single nucleotide polymorphisms or diplotypes with reproductive traits were analyzed using the GLM procedures of SAS (SAS Inst. Inc., Cary NC). The genetic effects were analyzed by mixed procedure according to the following model:

Table 1: Forward (F) and Reverse (R) primers for amplification of the chicken *VLDLR* gene

Primer set	Forward primer (5'-3')	Annealing temprature (°C)	Product length (bp)
1	F: ATTGGGAATCAGGATACTAAAC R: CCTACTCATTTCAGGCTCT	50.5	640
2	F: GGCTGTTCTTCCTATCTG R: GGTCCCTTCTGATTGC	50.0	441

$$Y = \mu + G + X + e$$

Where:

Y = The dependent variable
μ = The population mean
G = Fixed effects of genotype or diplotype
X = Reproductive traits
e = Random error

Multiple comparisons were performed with the least squares means. Type III sum of squares was used in each F-test. Values were considered significant at $p<0.05$ and presented as least square means±standard error means.

Haplotype reconstruction: Haplotypes were constructed based on the SNPs identified in all 528 experimental birds using the PHASE 2.0 Program. The function of this program is to reconstruct haplotypes from the population data. The genetic statuses of the subjects were expressed as the combination of two haplotypes (diplotype configuration). Genetic effects of the diplotypes were performed with the mixed model.

RESULTS AND DISCUSSION

The electrophoretic profiles of RFLP analysis of the fragment obtained from primer set 1 and 2 are shown in Fig. 1. Total of four genotypes were found and the heterozygous genotypes were not detected for both the two SNPs in the experimental population.

The homozygous genotype for each locus was further confirmed by sequencing. For locus 12321, the A→G mutation at position 12321 nt (relative to GenBank Accession No: NC-006127.2) was located on the exon 6 and this SNP didn't cause amino acid change. For locus 13876, the A→G mutation at position 13876 nt was located on the intron 17. The A12321G of *VLDLR* gene was resulted in two different gel profiles (A1A1 and A2A2) (Fig. 1a). The two genotypes (B1B1 and B2B2) of A13876G could be well recognized by two different gel profiles (Fig. 1b).

The effects of SNPs of the *VLDLR* gene on 6 reproductive traits were estimated and the results were shown in Table 2. In locus 12321, genotypes have significantly effect on egg weight at 300 days ($p<0.05$) but no association was observed for the other 5 traits. In chickens but no significant difference for these five traits between them ($p>0.05$). In locus 13876, genotypes had significantly effect on EW ($p<0.05$). The EW values of B2B2 chickens were significantly higher than B1B1 chickens ($p<0.05$). For LWFE, LW and WP, B2B2 chickens were superior to B1B1 chickens but no significant difference between them ($p>0.05$).

All haplotypes that were reconstructed from the 2 SNPs identified in all 528 experimental birds were shown in Table 3. Four haplotypes were identified. Haplotype-GGGG accounted for 78.41% of the observations. Four particular, the chickens harboring genotype A2A2 had significantly higher EW ($p<0.05$) than that of A1A1 chickens. Also the EWFE and EP values of A2A2 chickens were higher than A1A1 chickens and A2A2 chickens had lower LWFE, LW and AFE than A1A1 diplotypes were obtained from these 4 haplotypes (Table 4). The mixed model analysis indicated

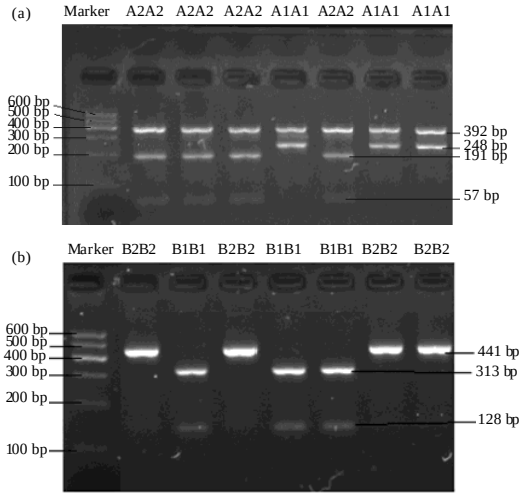

Fig. 1: The genotypes of the A12321G and A13876G single nucleotide polymorphisms in the chicken *VLDLR* gene; a) Genotypes of A12321G; b) Genotypes of A13876G

Table 2: The GLM analysis of association between the chicken reproductive traits and *VLDLR* gene SNPs

	Locus 12321		Locus 13876	
Traits	A1A1 (109)	A2A2 (419)	B1B1 (111)	B2B2 (417)
Egg weight at first egg (g)	29.86±0.360	29.99±0.18	29.97±0.360	29.96±0.18
Living weight at first egg (g)	1437.92±14.26	1433.11±7.27	1435.22±14.13	1433.80±7.29
Age at first egg (g)	161.09±1.050	161.06±0.53	160.97±1.040	161.10±0.54
Living weight at 300 days (g)	1496.42±18.47	1493.37±9.46	1495.47±18.29	1493.61±9.49
Egg weight at 300 days (g)	45.86±0.350^{b}	46.79±0.17^{a}	45.90±0.350^{b}	46.78±0.17^{a}
Egg production at 300 days	97.86±1.870	99.94±0.95	97.94±1.850	99.93±0.95

The least square means within a row lacking a common lowercase superscript differ significantly ($p<0.05$). The numbers in the brackets are the chicken individuals of respective genotypes

that there was significant association between diplotypes and reproductive traits (Table 5). Significantly dominant effects of diplotypes H1H1 were observed for traits EW whereas H4H4 had a negative effect on it. Also for EWFE, LWFE, LW and EP, the H1H1 chickens were superior to H4H4 chickens it maybe tell that H1H1 is an advantaged diplotype for chicken reproductive traits.

Chicken *VLDLR* gene is an important carrier for the vitellogenesis process and play a very important role in the process for Vitellogenin (VTG) and Very Low Density Lipoprotein (VLDL) depositing the yolk membrane. Bujo *et al.* (1995) described a naturally occurring mutation in a VLDLR that caused a dramatic abnormal phenotype. Hens of the mutant restricted-ovulator chicken strain carried a single mutation, lack functional oocyte receptors, were sterile and displayed severe hyperlipidemia with associated premature atherosclerosis. The mutation converted a cysteine residue into a serine resulting in an unpaired cysteine and greatly reduced expression of the mutant avian *VLDLR* on the oocyte surface. Extraoocytic cells in the mutant produced higher than normal amounts of a differentially spliced form of the receptor that was characteristic for somatic cells but absented from germ cells. Zhan *et al.* (2009) found that *VLDLR* gene had important effect on the age of first egg, egg production, egg weight and the percentage of yolk. Han *et al.* (2009) using quantitative real-time PCR measured transcriptional expression of VTG/VLDL-R mRNA in various tissues and for different stages of oocyte growth, in individual female zebra finches. They found significant temporal variation in VTG/VLDL-R expression during follicle growth with highest levels in ovary and a gradual decrease from pre-F3 to F1 vitellogenic follicles. Variation in ovary mRNA expression was correlated with inter-individual variation in clutch size and laying interval. Furthermore, variation in F3 follicle VTG/VLDL-R mRNA expression was correlated with inter-individual variation in egg mass and F1 follicle mass, suggesting that VTG/VLDL receptor mRNA expression is a key determinant of inter-individual variation in reproductive phenotype. Wang *et al.* (2011) reported that an association analysis using two completely linked SNP sites (T/C at position 2025 bp of the ORF and G/A in intron 13) and records from two generations demonstrated that the duck *VLDLR* gene was significantly associated with egg production ($p<0.01$), age of first egg ($p<0.01$) and body weight of first egg ($p<0.05$).

In the current study, researchers screened the VLDLR SNPs in a Chinese Indigenous chicken breed by PCR-RFLP Methods. As a result, A12321G (rs14777632) and A13876G (rs13817786) were identified. The associations between the two SNPs and egg production traits showed that the chickens harboring genotype A2A2 had significantly higher EW ($p<0.05$) than that of A1A1 chickens. Also, the EWFE and EP values of A2A2 chickens were higher than A1A1 chickens and A2A2 chickens had lower LWFE, LW and AFE than A1A1 chickens. The EW values of B2B2 chickens were significantly higher than B1B1 chickens ($p<0.05$). For LWFE, LW and WP, B2B2 chickens were superior to B1B1 chickens but no significant difference between them ($p>0.05$). The result of the association analysis were in agreement with the data reported by other researchers on chickens and were in line with the function of *VLDLR* gene described in other species (Bujo *et al.*, 1995; Rodriguez *et al.*, 1996; Schonbaum *et al.*, 2000; Hirayama *et al.*, 2003; Han *et al.*, 2009).

For studying both trait association (marker vs. trait) and linkage disequilibrium (marker vs. marker), haplotype or haplotype block reconstruction was more useful than marker by marker analysis and provided a practical

Table 3: Haplotypes inferred based on the 2 single nucleotide polymorphisms in the chicken *VLDLR* gene

Haplotype	A+12321G	A+13876G	Frequency (%)
H1	G	G	78.41
H2	G	A	0.57
H3	A	G	0.19
H4	A	A	20.83

Table 4: Diplotypes inferred based on the 4 chicken *VLDLR* gene haplotypes in experimental populations

Diplotype	Frequency (%)
H1H1	78.79
H2H2	0.57
H3H3	0.19
H4H4	20.45

Table 5: Associations between diplotypes and the chicken reproductive traits

	Diplotype			
Traits	H1H1	H2H2	H3H3	H4H4
Egg weight at first egg (g)	29.97±0.18	32.33±2.1200	26.00±3.6800	29.90±0.360
Living weight at first egg (g)	1433.60±7.31	1368.33±83.680	1512.00±144.95	1437.19±14.35
Age at first egg (g)	161.08±0.54	159.33±6.2100	169.00±10.760	161.01±1.060
Living weight at 300 days (g)	1494.23±9.49	1385.33±106.58	1260.00±184.61	1498.80±18.55
Egg weight at 300 days (g)*	46.78±0.17^{a}	47.76±1.9700^{ab}	47.85±3.4100^{ab}	45.84±0.350^{b}
Egg production at 300 days	99.91±0.95	104.33±10.760	110.00±18.640	97.74±1.880

Bold values represent the advantageous diplotypes. Underline represents the negative diplotypes. All are represented as least square means±standard error means; *$p \leq 0.05$

solution to resolve these problems (Daly *et al*., 2001; Zhang *et al*., 2002). The associations of haplotypes with phenotypic traits were more accurate than those of single SNP (Zhang *et al*., 2004, 2005; Zhou *et al*., 2010, 2012). In this study, based on 2 SNPs, four haplotypes were identified. Haplotype-GGGG accounted for 78.41% of the observations. Four diplotypes were obtained from these 4 haplotypes. Correlation analysis results showed that significantly dominant effects of diplotypes H1H1 were observed for traits EW whereas H4H4 had a negative effect on it. Also for EWFE, LWFE, LW and EP, the H1H1 chickens were superior to H4H4 chickens and it indicated that H1H1 is an advantaged diplotype for chicken reproductive traits.

CONCLUSION

In this syudy, the A→G mutation at position 12321 nt in exon 6 and the A→G mutation at position 13876 nt in intron 17 were identified. Each SNP was significantly associated with reproductive traits in chickens. The results once again confirmed that the *VLDLR* gene may have a major effect on egg production traits in chickens. The *VLDLR* gene can be a useful marker for molecular marker-assisted selection of reproductive traits in chickens. In order to make the selection schemes more applicable it would be necessary to further definitions of the effect of VLDLR variants on chicken reproductive traits and confirmation of their inheritance in different genetic populations. In particular, this study laid the foundation for the innovative use of local chicken breeds in China.

ACKNOWLEDGEMENTS

The current research was supported by the fund from: Major Agricultural Stock Breeding Project of Shandong Province (2008LZ015-05); Chinese Shandong Province Nature Fund (ZR2009DM004) and Major Agricultural Stock Breeding Project of Shandong Province (2011LZ15). Wang, Z.L. and Y. Lu contributed equally to this research.

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