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Gene Polymorphisms Are Associated with Eggshell Ultrastructure Organization in Hens

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ABSTRACT

Background: Eggshell ultrastructure organization, including effective layer thickness, mammillary layer thickness, and average size of mammillary cones, is important for breeding and significantly influences eggshell mechanical properties. Several matrix proteins were known to be important in eggshell formation. However, the proteins and variations that determine eggshell ultrastructure organization are not known.

Results: In this study, 17 single-nucleotide polymorphisms of three major genes in a hen population using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Five single-nucleotide polymorphisms with a very low minor allele frequency (< 1%) were excluded from further analysis. The remaining 12 single-nucleotide polymorphisms in Hardy-Weinberg equilibrium were used for analysis of associations with eggshell ultrastructure organization. Associations were found for (i) ovocleidin-116 with effective layer thickness (EFF), mammillary layer thickness (MAM), and average size of mammillary cones (SMAM); (ii) ovalbumin with eggshell thickness (ESH), effective layer thickness, and density of the mammillary cone (DMAM); and (iii) calmodulin1 with density of the mammillary cone.

Conclusions: The single-nucleotide polymorphisms identified in the present study may be used as potential markers to improve eggshell quality.

INTRODUCTION

The eggshell is a complex bioceramic that provides protection against physical damage and promotes embryo development (Burley & Vadehra, 1989). Eggshell quality is affected by many factors, including genetics, disease, nutrition, and environmental conditions (Roberts, 2004). Previous studies have shown that eggshell ultrastructure organization, including eggshell thickness and mammillary layer thickness, influences both eggshell quality and egg hatchability. In addition, mammillary cone size contributes to the mechanical properties of the eggshell (Liao *et al*., 2013). Thus, appropriate eggshell ultrastructure organization is critical for eggshell quality in hens.

Egg calcification occurs in the uterine fluid over three distinct phases (initiation, active calcification, and termination of shell calcification) (Nys *et al*., 2004). Numerous matrix genes involved in eggshell formation have been intensively studied (Gautron *et al*., 2001; Hincke, 1995; Hincke *et al*., 1999; Nys *et al*., 2004). Ovocleidin-116 (OC-116) is the most abundant eggshell matrix protein. OC-116 is synthesized and secreted by the granular cells of the uterine epithelium and it is widely distributed throughout the palisade region of the calcified eggshell. Thus, OC-116 is a promising candidate molecule for the regulation of

calcite growth during the active calcification phase of shell formation (Hincke *et al*., 1999). Ovalbumin is present in the uterine fluid and is primarily localized in the mammillary knobs of the eggshell (Hincke, 1995). Moreover, ovalbumin is predominant during the initial phase of the eggshell formation process (Panheleux *et al.*, 1999) and it is critical for the maintenance and function of the shell gland (Nys *et al*., 2004). Finally, calmodulin1 (CALM1) is a calcium-binding protein involved in eggshell formation and is thought to play a role in calcium ion transportation (Jonchère *et al.*, 2010). Association studies have shown that specific alleles of these candidate genes are correlated with measurements of eggshell quality (Dunn *et al.*, 2009).

Therefore, in this study, we aimed at determining if single-nucleotide polymorphisms (SNPs) in OC-116, ovalbumin, and CALM1 were associated with characteristics of eggshell ultrastructure organization in chickens.

MATERIAL AND METHODS

Sample collection

Pureline Rhode Island White layers (n = 384) from 40 half-sib families (one cock mating 9 to 10 hens), representing the sixth generation of a pedigree line from Beijing Zhongnongbangyang Poultry Breeding Co. Ltd., China, were used for this study.

All hens were caged individually in an automated environmental control poultry house and managed under conventional conditions. Commercial diets were provided *ad libitum*. The house was automatically ventilated to maintain ambient temperature between 20 and 28°C. A photoperiod of 16 h light: 8 h dark at light intensity of 15 lx was applied. The sexual maturity age of the flock was 136 days (50% laying rate), and average egg weight and laying rate in week 56 were 59.7 g and 77.8%, respectively. When the hens reached 57 weeks of age, eggs were collected during three consecutive days to ensure at least one egg per hen, and 1.5 mL of whole blood was individually collected by venipuncture. This protocol was approved by the Animal Care and Use Committee of China Agricultural University (permit number: SYXK 2007-0023).

Scanning electron microscopy (SEM)

The eggs were broken, and egg whites and yolks were removed. To facilitate membrane removal, eggshells were boiled in 2% NaOH for 10 min. Shell thickness (after removing the eggshell membrane, ESH), effective layer thickness (palisade layer and vertical crystal layer, EFF), and mammillary layer thickness (MAM) were measured by SEM (Panheleux *et al.*, 1999) at the equatorial region of the eggshell of each egg. The average size of mammillary cones (SMAM) was calculated, using two-dimensional images, as s = L/n, where n is the number of mammillary cones at the intersecting line, and L is the length of the intersecting line (DeHoff & Rhines, 1968). The density of the mammillary cone (DMAM) was calculated as d = c/A, where c is the number of mammillary cones within the field of view, and A is the area of the field.

SNPs and genotyping

Genomic DNA was extracted from the blood samples using a standard phenol-chloroform method and then quantified using a NanoDrop spectrophotometer (GE Healthcare Life Sciences, Uppsala, Sweden). The final concentrations ranged from 2 to 10 ng/μL. Seventeen SNPs in three genes (CALM1, ovalbumin, and OC-116) were selected from the UCSC database (http://genome.ucsc.edu/cgi-bin/hgGateway), from the ensemble database at (http://asia.ensembl.org/index.html), and from the preliminary experiment, which included nine registered SNPs and three unregistered SNPs, from the SNP database (Table 1) (five SNPs out of Hardy-Weinberg equilibrium removed). Genotyping of 384 birds was performed using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry on a Mass ARRAY iPLEX Platform (Sequenom, San Diego, CA, USA).

Statistical analysis

Values of the individual records of eggshell ultrastructure organization traits (i.e., ESH, MAM, EFF, SMAM, DMAM) outside the range of the mean ± three standard deviations were discarded. The Hardy-Weinberg equilibrium of genotypes was analyzed using Chi-square (χ^2) tests. SNPs that deviated from the Hardy-Weinberg equilibrium were excluded. SNPs with a genotype call rate of less than 85% and minor allele frequency (MAF) of less than 1% across all individuals were discarded. The association of the remaining SNPs with eggshell ultrastructure organization traits was assessed using the GLM procedure of SAS statistical package (version 9.2, SAS Institute Inc., Cary, NC, USA). The following model was applied:

$$Yij = \mu + Gi + eij$$

where Yij represents the observed values of the traits, μ is the population mean, Gi is the effect of SNP, and eij are the residual errors.

Table 1 – Details of the genes and SNPs examined in this study

Gene name	[1]SNP code	[2]Location in gene	[3]Gene location in chromosome	[4]p-value
OC-116	rs16400775	exon4	chr4:45085851	0.5927
	rs313064671	exon4	chr4:45087409	0.1396
	rs317191671	exon4	chr4:45087709	0.8931
	rs316353058	exon4	chr4:45087750	0.8256
Ovalbumin	rs15113585	3'UTR	chr2:67772086	0.6561
	RS2	3'UTR	chr2:67772323	0.5170
	RS1	3'UTR	chr2:67772356	0.1095
	rs16030727	5'UTR	chr2:67777794	0.9966
CALM1	snp.2.515.5438.S.2	intron3	chr5:43139434	0.7905
	rs315208191	intron3	chr5:43139557	0.8428
	RS20	exon4	chr5:43140343	0.5170
	rs14540325	intron4	chr5:43140580	0.8900

[1]SNPs (single-nucleotide polymorphisms), coded as rs, were selected from the ENSEMBLE database (http://www.ensembl.org/index.html), and SNPs, coded as snp, from the UCSC database (https://genome.ucsc.edu/). SNPs, coded as RS, were newly identified in this flock.

[2]Location in the gene and

[3]gene location in the chromosome were obtained from the UCSC database Assemble: NOV.2011(ICGSC Gallus-4.0/galGal4)

[4]p-value in Hardy-Weinberg equilibrium (HWE) test

RESULTS AND DISCUSSION

Phenotypic analysis and SNP summary

Detailed information on the selected candidate genes and the selected SNPs in the present study is shown in Table 1. Descriptive statistics of the eggshell ultrastructure organization traits are presented in Table 2. Genotype quality control and data filtering resulted in the removal of five SNPs, and the remaining 12 SNPs presented genotype call rates of more than 85% and MAFs of less than 1%, and were further analyzed. Association analysis revealed that 10 SNPs from three genes were significantly associated with eggshell ultrastructure organization traits, as shown in Table 3.

Eggshell ultrastructure organization traits

The coefficients of variation of ESH, MAM, EFF and DMAM were about 10%, and 24.58% for SMAM, which may partially explained by the variation of the SNPs in ovocleidin-116, ovalbumin and calmodulin1 genes.

Ovocleidin-116

OC-116 is a major component of the chicken eggshell matrix and plays an important role in calcite growth during eggshell calcification (Hincke *et al.*, 1999). SNPs in *OC-116* are significantly associated with eggshell elastic modulus and thickness, as well as with egg shape (Dunn *et al.*, 2009). In the current study, the association analysis revealed that four SNPs in the *OC-116* gene were significantly associated with EFF, MAM, and SMAM in chickens, as shown in Table 3. In addition, the four SNPs caused missense mutations in the amino acids in exon 4. For rs313064671, chickens with the AA genotype had significantly thicker EFFs than those with the CA genotype ($p<0.05$); this particular SNP caused an amino acid variation that changed a hydrophilic threonine into a conserved hydrophobic proline. Both rs317191671 and rs316353058 were significantly associated with SMAM ($p<0.05$). For rs317191671, the CC genotype was significantly more frequent than the CA genotype, and the amino acid changed from proline into threonine. For rs316353058, chickens

Table 2 – Descriptive statistics for chicken eggshell ultrastructure organization traits

Trait1	Mean	SD	CV (%)	Maximum	Minimum
ESH (μm)	303.63	25.05	8.25	368.00	245.00
MAM (μm)	90.99	11.15	12.25	121.00	60.50
EFF (μm)	212.15	22.67	10.68	275.50	154.80
SMAM (μm)	170.09	41.82	24.58	284.92	68.45
DMAM(N/μm^2)	74.55	10.77	14.44	105.30	54.48

[1]ESH = eggshell thickness, MAM = mammillary layer thickness, EFF = effective layer thickness, SMAM = average size of mammillary cones, DMAM = density of the mammillary cone.

Table 3 – Means of eggshell traits among SNPs genotypes the evaluated genes

Gene		Genotype	ESH	MAM	EFF	DMAM	SMAM
OC-116	rs16400775	AA	304.55 ± 3.74	94.20 ± 1.65^{a}	212.83 ± 3.46	195.34 ± 7.54	78.67 ± 1.60^{A}
		AG	302.19 ± 1.96	90.66 ± 0.87^{b}	210.63 ± 1.76	211.35 ± 3.99	73.48 ± 0.84^{B}
		GG	305.35 ± 2.43	90.18 ± 1.07^{b}	214.04 ± 2.17	206.81 ± 4.93	74.58 ± 1.02^{AB}
	rs313064671	AA	305.76 ± 2.08	90.94 ± 0.93	215.25 ± 1.88^{a}	207.43 ± 4.31	75.44 ± 0.90
		CC	303.84 ± 3.47	89.82 ± 1.51	211.37 ± 3.06^{ab}	204.67 ± 7.33	75.13 ± 1.49
		CA	300.97 ± 2.28	91.57 ± 1.01	208.82 ± 2.04^{b}	209.58 ± 4.62	73.20 ± 0.98
	rs317191671	AA	304.85 ± 3.58	89.80 ± 1.56	212.21 ± 3.17	204.57 ± 7.54	75.71 ± 1.53^{ab}
		CA	302.22 ± 2.11	91.55 ± 0.93	210.16 ± 1.90	210.38 ± 4.28	73.06 ± 0.91^{b}
		CC	304.96 ± 2.23	90.98 ± 1.00	214.47 ± 2.02	205.78 ± 4.62	75.80 ± 0.96^{a}
	rs316353058	CC	303.64 ± 3.51	89.85 ± 1.53	211.10 ± 3.11	202.30 ± 7.39	75.46 ± 1.50^{a}
		CT	302.41 ± 2.12	91.42 ± 0.94	210.47 ± 1.91	211.50 ± 4.29	73.07 ± 0.91^{b}
		TT	304.96 ± 2.23	90.98 ± 1.00	214.47 ± 2.02	205.78 ± 4.61	75.80 ± 0.96^{a}
OVA	RS1	CC	295.16 ± 5.10	91.24 ± 2.32	202.60 ± 4.60^{b}	200.88 ± 10.96	73.88 ± 2.30
		CT	305.01 ± 1.94	90.12 ± 0.86	213.91 ± 1.75 ^{a}	204.31 ± 4.00	74.65 ± 0.84
		TT	303.43 ± 2.21	92.05 ± 0.98	211.65 ± 1.99^{ab}	213.51 ± 4.50	74.52 ± 0.96
	rs16030727	CC	300.00 ± 2.09^{AB}	90.29 ± 0.94	209.96 ± 1.91^{ab}	209.71 ± 4.45^{ab}	74.22 ± 0.92
		CT	308.25 ± 2.08^{A}	91.65 ± 0.93	215.47 ± 1.87^{a}	201.86 ± 4.25^{b}	75.33 ± 0.90
		TT	299.48 ± 4.18^{B}	91.05 ± 1.91	207.17 ± 3.80^{b}	223.23 ± 8.06^{a}	72.64 ± 1.81
CALM1	snp.2.515.5438.S.2	CC	304.81 ± 2.11	91.79 ± 0.99	213.03 ± 1.84	208.30 ± 4.19^{ab}	73.67 ± 0.87
		TC	303.34 ± 2.13	91.62 ± 1.00	211.73 ± 1.87	204.04 ± 4.24^{b}	74.91 ± 0.88
		TT	301.29 ± 5.49	90.50 ± 2.57	210.79 ± 4.81	229.96 ± 10.92^{a}	76.65 ± 2.26
	rs315208191	CC	304.14 ± 2.37	90.87 ± 1.06	212.46 ± 2.15	211.82 ± 4.85^{ab}	74.18 ± 1.02
		CT	301.97 ± 2.03	91.22 ± 0.90	210.63 ± 1.83	200.41 ± 4.13^{b}	75.59 ± 0.87
		TT	307.20 ± 3.40	90.57 ± 1.53	215.80 ± 3.08	220.97 ± 7.00^{a}	72.29 ± 1.48
	RS20	CC	301.29 ± 5.49	90.50 ± 2.57	210.79 ± 4.80	229.96 ± 10.93^{a}	76.65 ± 2.26
		CT	303.47 ± 2.13	91.56 ± 1.00	211.92 ± 1.87	204.53 ± 4.25^{b}	74.99 ± 0.88
		TT	304.69 ± 2.11	91.85 ± 0.99	212.84 ± 1.84	207.82 ± 4.19^{ab}	73.59 ± 0.87
	rs14540325	CC	306.37 ± 3.26	90.78 ± 1.46	216.09 ± 2.97	220.54 ± 6.72^{a}	72.35 ± 1.42
		TT	303.96 ± 2.38	90.96 ± 1.06	212.19 ± 2.15	211.17 ± 4.85^{a}	74.38 ± 1.02
		CT	302.26 ± 2.07	91.09 ± 0.91	210.58 ± 1.85	200.42 ± 4.19^{b}	75.53 ± 0.89

^{A,B}Among genotypes within each SNP for each trait, means without a common superscript differ ($p<0.01$).
^{a,b}Among genotypes within each SNP for each trait, means without a common superscript differ ($p<0.05$).
ESH = eggshell thickness, MAM = mammillary layer thickness, EFF = effective layer thickness, SMAM = average size of mammillary cones, DMAM = density of the mammillary cone

with the CC and TT genotypes had significantly thicker SMAMs than chickens with the CT genotype. When CC was mutated into CT, the amino acid alanine changed to valine at this position. For rs16400775, chickens with the AA genotype had significantly thicker SMAMs than those with the AG genotype ($p< 0.01$), and AA individuals exhibited significantly thicker MAMs than AG and GG individuals, indicating that the A allele was favorable for mammillary cones. This SNP caused a missense mutation resulting in a change from histidine to arginine. Importantly, these three SNP (rs313064671, rs317191671, and rs316353058) of the *OC-116* gene were in the conserved domain (http://asia.ensembl.org/Gallus_gallus/Transcript/Domains), suggesting that these SNPs may alter the structure of the conserved domain and affect protein function during the initial phase of shell mineralization (Jiang *et al.*, 2010).

Ovalbumin

Ovalbumin was the first egg white protein identified in the shell matrix by N-terminal amino acid sequencing and immunochemistry (Hincke, 1995). This protein is localized in the mammillary knobs of the eggshell (Hincke, 1995) and functions to increase the Ca^{2+} concentration of nucleation centers in the initial steps of mineralization by binding between carboxylate groups of ovalbumin and calcium ions on the $CaCO_3$

surface (Pipich *et al.*, 2008; Schwahn *et al.*, 2004, Wang *et al.*, 2009). Recent studies have shown that polymorphisms in the ovalbumin gene are significantly associated with breaking strength and shell thickness in Rhode Island Red hens (Dunn *et al.*, 2009). In the present study, we found that the CT genotype was significantly more frequent than the CC genotype ($p<0.05$) in RS1, a new SNP that was significantly associated with EFF. In rs16030727, hens with the CT genotype had significantly thicker ESHs ($p<0.01$) and EFFs ($p<0.05$) than those with the TT genotype, whereas hens with the CT genotype had significantly lower DMAMs than those with the TT genotype ($p< 0.05$; Table 3). Burgess *et al*. (1992) demonstrated that ovalbumin does not influence crystal morphology, but slightly accelerates the nucleation of calcium carbonate in an in vitro precipitation assay. In contrast, Pipich *et al*. (2008) reported that the crystallization process starts immediately in the absence of ovalbumin and yields crystallites, with amorphous calcium carbonate particles formed within minutes after initiation. Moreover, ovalbumin may modify the morphology of calcite crystals and retard the transformation of unstable crystalline vaterites into more stable calcites in a concentration-dependent manner (Wang *et al.*, 2009; Wang *et al.*, 2010). Thus, our data further support that ovalbumin influences nucleation and initial mineralization. Because all four SNPs in ovalbumin examined in the study were in the 3' untranslated region (UTR) and 5'UTR, which may influence the regulation of ovalbumin expression, these SNPs may affect ovalbumin function during initial mineralization and alter the morphology of calcite crystals and vaterites.

CALM1

The *CALM1* gene encodes calmodulin (CaM), a ubiquitous eukaryotic calcium-binding protein, which is one of the main mediators of the calcium signal (Carafoli, 1987), and plays an important role in intercellular communication, cell movement, cell differentiation, cell proliferation, and other physiological and biochemical activities (Hanley *et al.*, 1990). In humans, two SNPs (rs12885713 [–16C > T] and rs5871) in the *CALM1* gene have been shown to be predisposing factors for adolescent idiopathic scoliosis (Zhao *et al.*, 2009). However, few studies have examined the role of *CALM1* in poultry.

In the current study, we found, for the first time, that four SNPs in the *CALM1* gene were significantly associated with the DMAM trait in chickens, as shown in Table 3. For rs315208191 and snp.2.515.5438.S.2, individuals with the TT genotype had higher DMAMs than those with the CT genotype. Hens with rs316353058 CC and TT genotypes had significantly greater DMAMs than those with the CT genotype. Additionally, in RS20, chickens with the CC genotype had significantly greater DMAMs than those with the CT genotype. These results indicate that heterozygosity of *CALM1* resulted in lower DMAMs compared with homozygosity.

In proteomic analysis of eggshells, CaM protein has been shown to exhibit moderate expression (Mann *et al.,* 2006). Moreover, it was demonstrated that Ca^{2+}-calmodulin-dependent protein kinase II is expressed in the calcified eggshell of layers during the early stages of eggshell precipitation (Liu *et al.,* 2013; Mann *et al.*, 2007), indicating that the *CALM1* gene may play an important role in mediating eggshell mineralization. Sun *et al*. (2013) reported that CaM is expressed in the uterine fluids of both strong and weak eggs, and it is not expressed in strong or weak eggshells. Taken together, these studies show that the *CALM1* gene may not be abundantly expressed during eggshell mineralization, which would explain why we could not always detect the *CALM1* gene in the eggshells. CALM1 may act as an eggshell structure regulatory protein during eggshell formation. Thus, we hypothesized that the four SNPs in the *CALM1* gene may affect the formation of the eggshell.

In summary, we found that three genes were strongly associated with chicken eggshell ultrastructure organization. The *OC-116* gene was important for ESH, EFF, and SMAM; ovalbumin was important for ESH, EFF, and DMAM; and *CALM1* was important for DMAM. The SNPs identified in the present study may be used as potential molecular genetic markers in layer breeding. Further studies with more birds and different breeding flocks are needed to validate the SNPs and linkage analyses performed to definitively demonstrate the functions of these SNPs in eggshell ultrastructure organization and hatchability. Such studies may reveal potential molecular markers for the selection for hatchability.

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