Association analysis and expression level of ACE polymorphisms with egg-laying trait in Taihang chicken

Peng Wang,*,1 Kaiyang Li,† Yekai Fan,* Hui Zhang,* Yifan Zhang,* Ziyi Liu,* Wentao Li,* Haiyin Han,* Yahui Gao,* Jiannan Liu,‡ and Yufang Liu*,2

*College of Life Sciences and Food Engineering, Hebei University of Engineering, Handan 056021, China; †Beijing General Station of Animal Husbandry, Beijing 100107, China; and ‡School of Landscape and Ecological Engineering, Hebei University of Engineering, Handan 056021, China

ABSTRACT The number of egg-laying is an important indicator of reproduction performance in poultry breeding. To investigate the relationship between the function of Angiotensin-converting enzyme (ACE) and egg-laying performance of Taihang chicken, the mRNA and protein expression and single nucleotide polymorphism (SNP) of ACE were detected. Analysis of ACE bioinformatics and association analysis of polymorphisms were then performed. The polymorphisms analysis of ACE showed that three SNP loci (g.5066812A>C, g.5080076G>A, and g.5072728A>G) were detected in 800 Taihang chickens with egg-laying records. Association analysis of egg-laying found that ACE g.5066812A>C mutation was significantly associated with the egg-laying performance of Taihang chickens (P < 0.05), and the individuals with the g.5066812A>C mutation showed significantly increasing egg-laying. The mRNA expression was significantly higher in individuals with the AA genotype mutation than those with the AC and CC genotypes (P < 0.01), and the expression of ACE protein levels was consistent with the mRNA expression. Bioinformatics analysis indicated that these mutations affected the secondary and tertiary structure of ACE. This study provides new insights into ACE affecting chicken egg production and some basis for improving the egg production rate of Taihang chickens.

Key words: SNPs, gene expression, ACE, reproductive trait, Taihang chicken

INTRODUCTION In recent years, the demand for egg products has changed due to rising living standards. The quantity of eggs is no longer the focus of attention, but the quality of eggs is starting to be a concern (Filipiak-Florkiewicz et al., 2017). Taihang chicken is an important local chicken breed for both meat and eggs in China, produced in Hebei and Henan near the Taihang mountain. It’s characterized by strong disease resistance, adaptability, and excellent egg quality, with a large proportion of yolk, clarified egg white, transparent and clear egg liquid, sticky texture and rich egg flavor, and which is loved by consumers (Qiaoxian et al., 2020; Guo et al., 2021). However, its low egg production rate and high feed-to-egg ratio are shortcomings that seriously affect the development of Taihang chicken breeding. The estimation of local chicken genetic parameters can be used as a reference for early breeding data and provide a basis for rapid improvement in egg production of local breeds.

Angiotensin-converting enzyme (ACE) belongs to peptidyl dipeptide hydrolase, a dipeptidyl carboxypeptidase commonly found in invertebrates, with the physiological role of converting decapeptide angiotensin (Ang-I) into active octapeptide angiotensin (Nakai et al., 1995; Bezerra et al., 2019; Ghafoori-Fard et al., 2020). It was found that ACE acts as a membrane-bound extracellular enzyme, but also plays an important role in the physiological role of converting decapetide angiotensin (Ang-I) into active octapeptide angiotensin (Nakai et al., 1995; Bezerra et al., 2019; Ghafoori-Fard et al., 2020). It was found that ACE acts as a membrane-bound extracellular enzyme, but also plays an important role in the physiological role of converting decapetide angiotensin (Ang-I) into active octapeptide angiotensin (Nakai et al., 1995; Bezerra et al., 2019; Ghafoori-Fard et al., 2020). The function of ACE is mainly to maintain the stability of blood pressure and electrolytes in the body (Danilov et al., 2016). Studies have shown that ACE has been found to play an important role in the variety of tissues. As one of the important regulators, ACE plays an important function in lung tissues, and its homolog ACE2 is highly expressed in endothelial cells of the lung, contributing to the maintenance of normal function and development of nonadherence (Gintoni et al., 2022). In the carotid artery, ACE is involved in...
the mechanism that regulates carotid stenosis and affects the regulation of the carotid artery to the brain (He et al., 2019). In cardiac tissue, ACE alters the homeostasis of the local renin-angiotensin system and has a protective effect on the heart (Bukowska et al., 2017). Mutations in ACE are associated with angiotensinase expression and contribute strongly to the maintenance of homeostasis in the kidney during transcription (Li et al., 2017). ACE is found in the bone marrow and plays an important role in the immune response of cells by influencing key steps in blood cell production, such as hematopoiesis, myelopoiesis, and the development of other cell lines (Fang et al., 2018). Along with the study of the mechanism of action of ACE in different tissues and organs throughout the body, important functions of ACE have been discovered (Sharma et al., 2020). Currently, ACE research is mainly focused on mammals and rodents, while in poultry, as long as it is focused on for-

In this study, we analyzed the regulatory role of ACE in poultry egg production by comparing the expression patterns of ACE in ovarian tissues of 34-wk-old high- and low-laying Taihang chickens. SNPs in the coding region of ACE were detected and their correlation with reproductive traits (number of egg-laying) in Taihang chickens was analyzed. In addition, RT-qPCR and western blot were used to detect the expression levels of ACE in the ovaries with different mutation genotypes of Taihang chickens. The changes in physicochemical properties and structures of the mutated ACE protein were predicted and analyzed. The correlation between the ACE and reproductive traits in Taihang chickens was elucidated. Therefore, this study provides a scientific basis for the subsequent selection and breeding of high-fertility Taihang chicken strains and lays a theoretical foundation for further research on the function of ACE in the process of fertility enhancement in Taihang chickens.

**MATERIALS AND METHODS**

**Animal Management**

This study was approved by the Ethics Committee of Hebei Engineering University (approval number: AEEI-16015). The test material for this study was selected from a farm in Xingtai City, Hebei Province of China, and 800 Taihang chickens (hens) were randomly selected as the study material. The rearing environment was homogeneous (sufficient light time, same rearing environment, adequate feed nutrition, and sufficient water). Phenotypic data such as several egg-laying and body-weight of Taihang chickens from 34 wk of age were recorded. A single-cage rearing pattern was used to facilitate accurate egg counts.

Blood was drawn from 800 chickens, the fresh blood samples were collected in 2 mL cryotubes, and subsequently taken to the laboratory for storage at −20°C. Three each were selected according to genotype AA, AC, and CC for dissection and collection of ovarian tissue. Stored in liquid nitrogen tanks and subsequently sent to the laboratory to be stored at −80°C.

**DNA Extraction, RNA Extraction, and cDNA Synthesis**

DNA extraction kits (Tiangen, Beijing, China) were used to extract DNA from the blood samples of 800 Taihang chickens. extracted DNA samples were tested for concentration and quality using a NanoDrop2000 spectrophotometer (Thermo, Shanghai, China) and 1.5% agarose gel electrophoresis imaging. After evaluation, the qualified DNA samples were sent to Novogene Technology Company for sequencing and genotyping.

Total RNA extraction was performed using the Trizol kit (Tiangen, Beijing, China). After testing the quality and concentration of RNA samples with a Nanodrop 2000 spectrophotometer (Thermo, Shanghai, China), the qualified samples were reverse transcribed into cDNA using a reverse transcription kit (Takara Bio, Japan) and stored at −20°C.

**Primer Design, LDR-SNP Sequencing, and Typing**

Primers were designed and synthesized using Primer Premier5 and Oligo (Novogene Technology, Beijing, China) according to the sequences published in GenBank. The primer sequence information of ACE is shown in Table 1.

LDR-SNP sequencing of monitored mutant loci was performed using multiplex PCR (MJP/TC-200 and Gene Amp PCR system 9600 Norwalk) with a final reaction system of 20 μL: 2.0 mL buffer; 0.6 mL 3 mmol/L Mg2+, 1.0 mL 50 ng/mL DNA template, 2.0 mL 2.0 mmol/L dNTP, 0.2 mL 1U Taq enzyme, 12.2 mL ddH2O and 2 mL 0.5 pmol/μL primer mixture. The reaction proceeds as follows: 95°C for 2 min; 40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min; then 72°C for 10 min. After the reaction was completed, the amplified products were amplified using 3% agarose gel and observed for electrophoresis. The products were then sent to DynaSys for SNP sequencing (DynaSys, Tianjin, China).

| SNP loci | Primer sequences | Tm(°C) |
|----------|------------------|-------|
| g.5066812A>C | F: CTCGCAACCCCTTCCCCCT | 59 |
| R: CACGACCTTTGGGCCAC |
| g.5072728A>G | F: CGTGGGCTCTCATGTCCTGC | 59 |
| R: GGAGTGGAAGGGTCTCGTGGTTG |
| g.5080076G>A | F: AGAAGGGCAGGATGAGGAAAG | 59 |
| R: GGAAAGGGTCAGGCTGGGGTTA |
Analysis of ACE SNPs

The Excel software was used to calculate genotype frequency, allele frequency, heterozygosity, effective allele number, and polymorphism information content. The chi-square test for Hardy-Weinberg equilibrium was performed, and the significance level was set at 0.05. Correlation between the reproductive traits of chicken and the SNPs of ACE were analyzed using the general linear model in SPSS21.0, as follows:

\[ Y_{ij} = \mu + G_i + e_{ij} \]

where \( Y_{ij} \) represents the phenotypic value of the trait or the reproductive level, \( \mu \) represents the overall mean, \( G_i \) represents the genotype fixed effect, and \( e_{ij} \) represents the random error.

RT-qPCR Validation

The primers used were designed according to the chicken ACE coding region sequence, synthesized by DynaScience (Tianjin, China). RT-qPCR analysis was performed using the TaKaRa SYBR Premix Ex Taq II kit (TaKaRa Bio.). The 10 mL of the reaction system included 1.0 mL of cDNA template, 5 mL of SYBR PremixEx Taq, 0.4 mL of forward and reverse primers, 0.2 mL of ROX II reference dye, and 3 mL of sterile water. Three replicates of each sample were performed simultaneously. The RT-qPCR protocol was as follows: 40 cycles of 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s. The expression of GAPDH was used as the standard mRNA expression level and calculated using the \( 2^{-\Delta\Delta CT} \) method (Liu et al., 2021). The primers of RT-qPCR are shown in Table 2.

Bioinformatics Analysis of the ACE in the Taihang Chicken

RNAFOLD software was used to predict the mRNA secondary structures of ACE with different genotypes. TMHMMServer (v.2.0) software was used to analyze the transmembrane region of the target protein. Phyre2 software was used for the tertiary structure analyses of the target protein.

Statistical Analysis

Statistical analysis was performed on the collected data by using SPSS21.0 statistical software, and the average of 3 replicates was evaluated and displayed as the mean ± standard error (SE). P-value calculation adopts a t-test. * \( P < 0.05 \) and ** \( P < 0.01 \)

RESULTS

Identification and Genotyping of SNPs in the ACE in Taihang Chickens

The OD values of the Taihang chicken genomic DNA were all between 1.8 and 2.0. PCR amplification of the ACE was performed using designed primers. Three mutant loci were identified by LDR genotyping, including g.5080076G>A in intron 17, g.5072728A>G in intron 6, and g.5066812A>C in exon 1. All 3 loci were reported in the Ensembl database (Reference genome: ENSGALG00010023369).

The genotype, allele frequencies, and population estimates (heterozygosity, allele, and polymorphism information) are shown in Table 3. All 3 SNPs identified in this study occurred with a frequency greater than 95%. The chi-square test showed that only the g.5066812A>C mutation was consistent with Hardy-Weinberg equilibrium.

Association Analysis Between ACE SNPs and Egg-Laying Number in Taihang Chickens

The analysis of the association between ACE genotypes and the egg-laying number was shown in Table 4. The results showed that ACE g.5066812A>C mutation was significantly associated with the number of egg-laying in 34 wk in Taihang chickens, while the other 2 loci were not significant.
Relative Expression Levels of ACE mRNA and Protein in Ovaries of Taihang Chicken

RT-qPCR was used to detect the relative expression of ACE mRNA with g.5066812A>C mutation in three different genotypes in the ovarian tissues of Taihang chickens. The results showed that the expression of ACE with AA genotype was significantly higher than that in AC and CC genotypes (P < 0.05), and the expression of ACE with AC genotype was significantly higher than that in CC genotype (P < 0.01; Figure 1). The protein expression levels of ACE with g.5066812 A>C mutation in 3 different genotypes in ovarian tissues of Taihang chickens were detected by western blot. The results showed that a distinct band was found at 117 KDa, consistent with the size of the ACE protein (as shown in Figure 2A). The protein expression level of ACE with AA genotype was significantly higher than that with AC and CC genotypes, and the protein expression level of ACE with AC genotype was significantly higher than that with CC genotype (Figure 2B), which is consistent with the expression of ACE mRNA with different genotypes. Together, all the results further verified our speculation that the ACE mutation could affect the egg-laying number in Taihang chicken.

Bioinformatics Analysis of ACE in Taihang Chicken

To further investigate the effect of the g.5075082A>C mutation site on the structure of ACE, the amino acid prediction was performed using the sequence before and after the ACE mutation. It was found that when the ACE g.5075082A>C site was mutated, the structure of mRNA was changed and its minimum structural free energy was reduced from -1,792.13 KJ/mol to -1,791.88 KJ/mol. Figure 3 showed the predicted secondary structure of the ACE g.5075082A>C wild-type and mutant-type proteins.

The homology modeling of the ACE protein was used by SPDBV software (Figure 4). The function of the

Table 3. Genotypes, allele frequencies, and diversity parameters of ACE SNPs in Taihang chickens.

| SNP         | Quantity | Genotype frequencies (n) | Allelic frequencies | PIC  | He  | Ne  | P-Value |
|-------------|----------|--------------------------|--------------------|------|-----|-----|---------|
| g.5066812A>C | TH (n = 774) | AA 0.386 (n = 298) AC 0.569 (n = 416) CC 0.004 (n = 60) | C 0.68 A 0.32 | 0.35 0.44 1.8 0.0017 |
| g.5080076G>A | TH (n = 774) | GG 0.152 (n = 378) AG 0.358 (n = 249) AA 0.489 (n = 147) | G 0.65 A 0.35 | 0.35 0.46 1.3 0.718 |
| g.5072728A>G | TH (n = 774) | GG 0.432 (n = 312) GA 0.297 (n = 250) AA 0.269 (n = 212) | A 0.58 C 0.42 | 0.37 0.49 1.95 0.236 |

Table 4. Results of association analysis between ACE different genotypes and egg-laying number in Taihang chickens.

| SNPs        | Genotypes | Number of egg-laying at 34 wk |
|-------------|-----------|------------------------------|
| g.5066812A>C | AA        | 67.48 ± 0.716*               |
|             | AC        | 68.78 ± 0.915                |
|             | CC        | 67.00 ± 4.503                |
| g.5080076G>A | GG        | 68.04 ± 1.995                |
|             | GA        | 68.64 ± 0.884                |
|             | AA        | 68.15 ± 0.549                |
| g.5080076G>A | GG        | 69.32 ± 0.848                |
|             | AG        | 69.70 ± 0.945                |
|             | AA        | 69.32 ± 0.845                |

*abThe different lowercase letters represented the significant differences P < 0.05.

Figure 1. The expression of ACE with g.5066812A>C different genotypes of individuals. *P < 0.05; **P < 0.01.

Figure 2. (A) ACE protein expression levels with g.5066812A>C different genotypes. (B) Gray value analysis. *P < 0.05; ** P < 0.01.
protein was closely related to its spatial structure, so whether the function of ACE was affected needs further investigation. A total of 34 protein transmembrane structural domains were identified in the predicted results for the tertiary structure of the ACE protein. The ACE 5066812A>C mutation leads to the conversion of glycine to BO3, which changes from one linking hydrogen bond to 5, increasing the stability of linkage between protein structures, which in turn affects the enzyme activity (Figure 4).

**DISCUSSION**

Egg-laying performance has always been an important factor limiting the development of excellent local chicken breeds in China. The egg-laying trait in chickens is a complex trait controlled by a variety of factors that are highly correlated, either promoting each other or constraining each other (Wongngam et al., 2020). The generation and development of molecular genetic marker-assisted selection methods provide a good means to shorten the generation intervals and accelerate the genetic progress (Xu et al., 2017). In the present study, three mutations in the ACE of Taihang chickens were identified and which were stably inherited and significantly associated with the egg-laying number. After quality control and data filtering, was consistent with the Hardy-Weinberg equilibrium. Association analysis showed that ACE g.5066812A>C mutation was significantly associated with 34 wk egg-laying number in Taihang chickens ($P < 0.05$). This result suggested that the ACE might be a potential molecular marker for improving reproductive traits in Taihang chickens.

In chickens suffering from pulmonary hypertension, ACE expression is significantly upregulated in response to high-speed blood transport (Wheeler-Schilling et al., 2001). In canine ACE polymorphism assays, individuals with ACE polymorphism mutations were found to promote aldosterone production in vivo compared to individuals without the mutation, resulting in activation of the renin-angiotensin-aldosterone system (RAAS) pathway and inhibition of the classical RAAS pathway (Adin et al., 2020). In the whole-genome sequencing of the silkworm, four different ACE types were identified, resulting in altered nucleotide sequences of ACE genes,
and causing diverse intestinal functions in the silkworm (Adin et al., 2020). This possibility was that ACE is very efficient at trimming paired basic residues from the C-terminus of peptides that resemble processing intermediates of the leukocyte splitting hormone family of mammalian neuropeptides, suggesting a possible novel role for angiotensin-converting enzymes in prohormone processing (Isaac et al., 1997; Almadi et al., 2016). Some studies show ACE plays a crucial role in vivo homeostasis process such as oocyte maturation, ovulation, luteal maturation, and steroidogenesis in mammals (Chen et al., 2022). We hypothesized that during egg production, an angiotensin-converting enzyme in the blood of laying hens after feeding could all be explained by the conversion of angiotensin-converting enzyme in the ovary, but could not reveal the principle of angiotensin-converting enzyme synthesis in ovarian tissues, which was studied in adult female mosquitoes (Ekbote et al., 1999). It is possible that the angiotensin-converting enzyme is synthesized in the adipose outside the ovary and is later transferred to the oocyte, where deposition occurs during oocyte differentiation, thus affecting ovarian function (Nazeer et al., 2021). Therefore, ACE may be important for poultry, mammals, and developing embryos alike (Sun et al., 2009; Nazeer et al., 2021). In our study, the ACE g.5066812A>C mutation was found to exist in Taihang chickens. The expression levels of mRNA and protein of ACE with three different genotypes (AA, AC, and CC) were significantly different in the ovarian tissues of Taihang chickens. Individuals with the AA genotype in the ACE g.5066812A>C mutation had a higher egg-laying number at 34 wk of age than that with the other two genotypes (AC and CC). And further study showed that ACE g.5066812A>C mutation is located in the exon1. Hence, we speculated that the ACE g.5066812A>C mutation may lead to altered protein expression by affecting amino acid changes, resulting in functional changes.

The accumulation of ACE in the mature ovary may be important in regulating the activity of peptides that control oocyte development, and the transfer of maternal angiotensin-converting enzyme to the egg suggests that this enzyme may produce regulatory peptides during embryogenesis (Maroney et al., 2021). Like mammals, poultry removes C-terminal dipeptides from the C-terminus of oligopeptides, a process that can confer or eliminate biological activity and requires ACE to function. Poultry is known to possess a large number of peptides that may be involved in controlling the synthesis and release of digestive enzymes, the cyclic synthesis of yolk-producing proteins, fluid and electrolyte homeostasis, and behavior (Terashima et al., 2010). In addition, bioinformatics has shown that the α-helix is the most abundant origin of protein secondary structure and random coiling usually builds into the active site or characteristic functional site of the enzyme (Yoshioka et al., 2017). In this study, when the ACE g.5066812A>C was mutated, the secondary structure of the mRNA was changed and the minimum free energy was also changed making the protein structure altered. The function of a protein is closely related to its spatial structure, so whether its function is affected needs to be further investigated. Transmembrane structural domains of the ACE protein are highly conserved among different species and are involved in the activation of adenylate cyclase, ligand binding, and G protein expression (Baumgartner et al., 2017). In the predicted results of the tertiary structure of the ACE protein, a total of 34 protein transmembrane structural domains g.5066812A>C was found to exist in ACE leading to glycine mutations in ACE protein, causing changes in the length and folding direction of the nascent peptide chain, affecting the rate of protein translation and thus the enzyme activity. But no coiled-coil structure was predicted in ACE protein, which corresponded to the tertiary structure of the protein. The transmembrane structural domains of the ACE protein are highly conserved in different species and play important roles in adenylate cyclase activation, ligand binding, and G protein expression. Therefore, the changes in mRNA secondary structure and protein tertiary structure of ACE caused by the g.5066812A>C mutation may be one of the important reasons affecting egg-laying numbers in Taihang chickens.

**CONCLUSIONS**

In summary, the ACE g.5066812A>C mutation is a potential molecular marker for screening egg-laying numbers of Taihang chickens during the early egg-laying period. This study provides a theoretical basis for further research into the molecular mechanisms by which ACE regulates reproductive traits in chickens.

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Institutional review board statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee at Hebei University of Engineering (AEEI-16015) (Hebei, China).

Author contributions: Conceptualization, Y.L., and K.L.; methodology, H.H.; software, Y.F.; validation, P.W., K.L., W.L., Z.L., Y.G., J.L. and H.Z.; formal analysis, P.W. resources, H.H.; data curation, P.W.; writing—original draft preparation, Y.Z.; writing—review and editing, Y.L.; project administration, Y.L.; funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

Ethical approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee at Hebei University of Engineering (AEEI-16015) (Hebei, China).
DISCLOSURES

The authors declare that they have no competing interests.

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