Research Note: Association of single nucleotide polymorphism of AKT3 with egg production traits in White Muscovy ducks (Cairina moschata)

Semiu Folaniyi Bello,*,† Haiping Xu,*,† Kan Li,*,† Lijin Guo,*,† Siyu Zhang,*,† Ridwan Olawale Ahmed,‡ Endashaw Jebessa Bekele,*,† Ming Zheng,*,† Mingjian Xian,*,† Bahareldin Ali Abdalla,*,† Adeniyi Charles Adeola,‖ Adeyinka Abiola Adetula,§ Raman Akinyanju Lawal,¶ Weijian Zhu,‖ Dexiang Zhang,*,†|| Xiquan Zhang,*,† Congliang Ji,‖ and Qinghua Nie*,†‖

*Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, South China Agricultural University, Guangzhou 510642, Guangdong, China; †Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding and Key Lab of Chicken Genetics, Breeding and Reproduction, Ministry of Agriculture, Guangzhou 510642, Guangdong, China; ‡Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA; ¶State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, 650223 Yunnan, China; §Reproductive Biotechnology, Department of Molecular Life Sciences, TUM School of Life Sciences, Technical University Munich, 85354 Freising, Germany; ‖The Jackson Laboratory, Bar Harbor, ME 04609, USA; and ‖Wens Foodstuff Group Co. Ltd., Yunfu 527400 Guangdong, China

ABSTRACT Prior studies on transcriptomes of hypothalamus and ovary revealed that AKT3 is one of the candidate genes that might affect egg production in White Muscovy ducks. The role of AKT3 in the uterus during reproductive processes cannot be overemphasized. However, functional role of this gene in the tissues and on egg production traits of Muscovy ducks remains unknown. To identify the relationship between AKT3 and egg production traits in ducks, relative expression profile was first examined prior to identifying the variants within AKT3 that may underscore egg production traits [age at first egg (AFE), number of eggs at 300 d (N300D), and number of eggs at 59 wk (N59W)] in 549 ducks. The mRNA expression of AKT3 gene in high producing (HP) ducks was significantly higher than low producing (LP) ducks in the ovary, oviduct, and hypothalamus (P < 0.05 or 0.001). Three variants in AKT3 (C-3631A, C-3766T, and C-3953T) and high linkage block between C-3766T and C-3953T which are significantly (P < 0.05) associated with N300D and N59W were discovered. This study elucidates novel knowledge on the molecular mechanism of AKT3 that might be regulating egg production traits in Muscovy ducks.

Key words: AKT3, mRNA expression, egg production traits, variation

INTRODUCTION

Muscovy ducks are widely reared due to their unique adaptation to local environments, high fertility rate, and increasing meat productivity (Cui et al., 2019). However, improvement of egg production remains a major concern among duck breeders.

Egg production is a major economic trait of poultry species which declines with ovarian aging as a result of a decrease in the levels of secreted reproductive hormones. Molecular techniques are one of the significant methods to improve egg production (Sato et al., 2016). Specifically, the identification of a candidate single nucleotide polymorphism (SNP) within a gene can be adopted to understand the relationship that exist between a specific gene and quantitative trait loci (QTL) (Bello et al., 2022).

AKT3 was identified as one of the candidate genes responsible for egg production in White Muscovy ducks (Bello et al., 2021). Although, several candidate genes have been identified as essentials in egg production but the role of AKT3 in the uterus during reproductive processes can not be overemphasized. This forms the basis for further investigation of AKT3.

AKT3 (AKT serine/threonine kinase 3) is one of the three associated serine/threonine-protein kinases (AKT1, AKT2, and AKT3) called AKT kinase. It plays an important role in...
many cytokines- and hormone-driven processes, AKT3 functions in the uterus (Fabi and Asselin, 2014).

Studies revealed that SNPs within AKT3 are related with pig litter size (Getmantseva et al., 2020) and on myofiber characteristics (indispensable indicators of meat quality) in broiler chicken (Chen et al., 2013). Despite many efforts to understand AKT3 variants and their association with reproductive tissues, their expression level in these tissues and relationship with egg production traits in these ducks are not yet investigated. This finding elucidates the significance of AKT3 polymorphisms in molecular breeding for egg production in ducks.

**MATERIALS AND METHODS**

**Experimental Animals**

One thousand five hundred thirty-seven Muscovy laying ducks raised in breeding farm of Guangdong Wenshi Southern Poultry Breeding Co., Ltd Renma Farm were used. These ducks have also been utilized in the previous study (Bello et al., 2021). Egg recording was done from 28 wk to 59 wk of age. Age at the first egg (days) (AFE), number of egg at 300 d (N300D) and number of egg at 59 wk (N59W) were recorded for all experimental ducks. The consent of South China Agricultural University Institutional Animal Care and Use Committee (Guangzhou, People’s Republic of China) was obtained prior to sample collection. All experimental animals were handled with maximum care during blood collection and euthanization for tissue collection.

**Blood Sampling**

Two milliliter of blood was collected from 1,467 individual ducks through their wing-web into EDTA (Ethylene-diamine tetraacetic acid) tubes at 56 wk of age and stored at −20°C till further use.

**DNA Extraction and Quality Check**

Genomic DNA was extracted from 10 μL whole blood of individual ducks using E.Z.N.A. NRBC Blood DNA kit (OMEGA, Bio-tek, Norcross, GA) according to the manufacturer’s instructions. The quality and integrity of genomic DNA samples were checked using Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). All DNA samples were diluted to a working concentration of 50 ng/μL and stored at −20°C for further use.

**Collection of Tissue Samples**

Based on N59W, 4 lowest (LP) and 4 highest producing (HP) ducks within the same egg number from each group were euthanized for tissues collection. Eighteen tissue samples that include reproductive (hypothalamus, pituitary, ovary [excluding both the white and yellow follicles], and oviduct), and non-reproductive organs (brain, cerebrum, cerebellum), fat (abdominal and subcutaneous), (heart, kidney, gizzard, stomachus glandula, lung, liver, spleen, small intestine, breast muscle, and leg muscle) were collected. However, only 8 tissue samples relating to egg production were used for the expression profile analysis. All tissues were washed with RNA-free water, wrapped in nylon polybags, frozen in liquid nitrogen, and then stored at −80°C.

**Primer, RNA Extraction, Synthesis of cDNA and Quantitative Real Time-PCR (qRT-PCR)**

The primer used for qRT-PCR was designed according to NCBI database sequences of AKT3 with Accession number XM_027453844.2 via Primer premier version 5.0 software (Applied Biosystems, Norcross, GA) (Table 1). RNA extraction, cDNA synthesis, and qRT-PCR conditions were similar to those used in our previous study (Bello et al., 2021). The 2−ΔΔCT method was used to calculate target gene expression.

**Primer Design, PCR Amplification, Sequence Alignment, and SNP Detection**

AKT3 is located on chromosome 3 (28648609-28801623bp) (Accession number of 101804556) of *Anas platyrhynchos* (mallard) genome and possess 15 exons. Three pairs of primers were designed to amplify different regions of AKT3 (Table 1). DNA mixed pool was constructed with 10 μL DNA sample of 20 randomly selected individuals with equal concentration. The PCR

**Table 1.** Details of the six (6) pairs of primers used in this study.

| Primer name | Sequence (5’ to 3’) | Product size (bp) | Tm (°C) | Purpose |
|-------------|---------------------|-------------------|---------|---------|
| **AKT3 E1** | F: TGACGTCGGGAGTTTTCCTG R: AGCCTGGAATTGCTTCCTGCC | 861 | 60.00 | qRT-PCR |
| **GAPDH**  | F: GCACGTCTCAAGCCTGAGAATG R: GCAGGGTCAGGTCCACGACA | 569 | 59.53 | House keeping gene |
| **AKT3 P1** | F: AGCTCGAAGATGAAACTCAGCA R: CCGGCGGCGATCGAC | 827 | 60.03 | SNPs detection |
| **AKT3 P2** | F: GTGTTGGTGTGGGTCTGAATTTTT R: AGCCTGGAGTAGATTTGCAGCAAT | 1847 | 57.55 | SNPs detection |
| **AKT3 P3** | F: GTAGTTGACCTAATCAGAAGGA R: ACGTTTCCCCAAGCAAATTTCCAG | 851 | 57.08 | SNPs detection |
| **AKT3 SNP** | F: GTAGTTGACCTAATCAGAAGGA R: ACGTTTCCCCAAGCAAATTTCCAG | 851 | 57.08 | Genotyping |

Notes. Primers: AKT3 E1 was used for qRT-PCR. AKT3 P1, AKT3 P2, and AKT3 P3 were used for detection of SNPs, and AKT3 SNP was used for genotyping.
products were performed in 35 μL volume consisting of 31.5 μL Golden Star T6 Super PCR Mix (Tsingke Biological Technology, China), 1 μL (10 μmol/L) each of forward and reverse primers and 1.5 μL of DNA mixed pool using T100 Thermal Cycler (BioRad, Singapore). The PCR reactions were performed on two steps conditions. First, an initial denaturation at 98°C for 3 min was done, followed by 15 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 10 sec and extension at 10°C. Second, 30 cycles of denaturation at 98°C for 10 sec, annealing at 50°C for 10 sec, extension at 72°C for 15 sec, and final extension at 72°C for 3 min were done. The quality of PCR products was checked on 1% agarose gel electrophoresis before sequencing. All PCR products were sequenced directly using ABI-3730XL DNA analyzer (USA) by Sangon Biotech Company (Guangzhou, China). Trimming, alignment of sequences and SNP discovery were conducted using SnapGene 4.3.6 software.

**PCR Amplification of SNP Sites and Genotyping by DNA Sequencing**

The region containing SNPs was amplified using the same thermocycler with necessary reagents through one pair of primer (Table 1). Considering dam effect of duck population based on available breeding records, only 549 individuals were selected for amplification of SNP sites. The PCR protocols were similar to those used in SNP discovery. All primers were synthesized by Tian Yi Hui Yuan Gene Technology Company (Guangzhou, China).

**Haplotype and Statistical Analyses**

Linkage disequilibrium was measured using Haploview software version 4.2 (BROAD, Cambridge UK). The significant differences between average expression of LP and HP tissues were examined with a t-test using SPSS 19.0 statistical software (IBM, Chicago, IL). Association analyses of SNPs with egg production traits of 549 Muscovy laying ducks were analyzed using the GLM procedure in SPSS 21.0. For each egg production trait, the least-squares mean and standard error of means (SEM) were calculated. Differences between the genotypes were analyzed. The difference with *P*-value ≤0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Relative Expression Level of AKT3 Gene in Tissues of White Muscovy ducks**

In the 3 tissues (ovary, oviduct, and hypothalamus), mRNA expression of *AKT3* gene in HP ducks is higher.
than LP, with AKT3 expression being the highest in oviduct (Figure 1a). There was significant difference ($P < 0.05$) in expression level of AKT3 in ovary of HP and LP ducks while its level is lowest in their Hypothalami (Figure 1a). Interestingly, cerebrum, cerebellum, and pituitary tissues had similar expression trends of AKT3 gene in HP ducks, while cerebrum and pituitary of LP ducks showed low expression levels of AKT3 (Figure 1b). There was significant higher expression of AKT3 in HP’s subcutaneous and abdominal fats compared to LP counterpart ($P < 0.001$). Although, abdominal fat of LP had a lower expression of AKT3 when compared to its subcutaneous fat (Figure 1c). This result reveals that there is variation in AKT3 expression in the 8 selected tissues of HP and LP. The expression of AKT3 in human fetal brain was higher than other tissues sampled, emphasizing its important role in brain development (Wu et al., 2009). The high expression of AKT3 in ovary, oviduct, and hypothalamus of HP corroborates a report by Bionaz and Loor (2011). In cerebrum, cerebellum and pituitary, mRNA expression of AKT3 was higher in HP than LP duck which ratifies a finding that expression of AKT3 is the most expressed isoform in the brain (Yang et al., 2006).

**Variation at AKT3 Gene and Association Analyses of AKT3 with Egg Production Traits**

We identified three significant SNP sites when aligned with reference genome of *Anas platyrhynchos* (mallard) (Figure 2a). The three SNPs (C-3631A, C-3766T, and C-3953T) identified in intron 15 of AKT3 are on an average of 150bp apart. Moreover, no synonymous amino acid substitution was observed at these SNPs sites. Haplotype analysis showed a high linkage block between C-3766T and C-3953T of AKT3 suggesting that SNPs might have been inherited together (linkage disequilibrium) (Figure 2b). There is a significant difference ($P < 0.05$) in C-3766T genotypes with TT recording values of 102.10 ± 1.94 and 189.28 ± 1.06 in N300D and N59W, respectively than CC and TT genotypes (Table 2).

The TT genotype individuals of C-3953T laid five to six eggs more than individuals with genotypes CC and CT at 300 days of laying. Furthermore, the number of eggs laid in individuals with TT genotype at N59W was more than their CT and CC genotypes with 13 and 14 eggs, respectively. The wide difference between N300D and N59W by TT genotype individuals might be due to smaller number of individual ducks with TT genotype at these SNP sites. It was observed that high linkage sites of C-3766T and C-3953T are significantly ($P < 0.05$) associated with N300D and N59W. This justifies the findings on polymorphic sites at A-1864G and C-1704G of *IGF2* in ducks having high linkage disequilibrium (Ye et al., 2017). Although, TT genotype individuals in the 2 SNPs sites (C-3766T and C-3953T) had the highest N300D and N59W.

In the C-3631A polymorphic site, there is no significant difference ($P > 0.05$) in three egg production traits considered across three genotypes (CC, CA, and AA). This finding is similar with previous studies that had no significant difference ($P > 0.05$) on association analysis of SNPs at C-1704G and A-1864G of *IGF2* with FEA and E300D (Ye et al., 2017), A-227G and C-320T of *FSHR* associated with E33W and E59W, and AFE and E33W, respectively (Ye et al., 2017), and g.3270 A > G of *GH* with AFE (Wu et al., 2014) in ducks.

The identified molecular markers which were significantly related to egg production parameters could be used
by Muscovy duck breeders to improve egg production. However, due to limitation of data from antibodies for protein expression of AKT3 gene, further conclusions could not be made from the quantitative real-time PCR result. Therefore, future studies should incorporate antibodies for protein expression of AKT3 gene in Muscovy ducks.

ACKNOWLEDGMENTS

This research was funded by the Local Innovative and Research Teams Project of Guangdong Pearl River Talents Program (2019BT02N630) and the Construction Project of Modern Agricultural Science and Technology Innovation Alliance in Guangdong Province (2021KJ128).

Data and model availability statement: All nucleotide sequences used in this study have been deposited in BankIt of NCBI with accession numbers of OL616434–OL617008.

DISCLOSURES

No conflict of interest.

REFERENCES

Bello, S. F., A. C. Adeola, and Q. Nie. 2022. The study of candidate genes in the improvement of egg production in ducks- a review. Poult. Sci. 101:101850.

Bello, S. F., H. Xu, L. Guo, K. Li, M. Zheng, Y. Xu, S. Zhang, E. J. Bekele, A. A. Bahareldin, W. Zhu, D. Zhang, X. Zhang, C. Ji, and Q. Nie. 2021. Hypothalamic and ovarian transcriptome profiling reveals potential candidate genes in low and high egg production of white Muscovy ducks (Cairina moschata). Poult. Sci. 100:101310.

Biomaz, M., and J. Loor. 2011. Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinform. Biol. Insights 5:83–98.

Chen, S., J. An, L. Lian, L. Qu, J. Zheng, G. Xu, and N. Yang. 2013. Polymorphisms in AKT3, FIGF, PRKAG3, and TGF-β genes are associated with myofiber characteristics in chickens. Poult. Sci. 92:325–330.

Cui, Y. M., J. Wang, Z. Hai-Jun, J. Feng, S. Geng Wu, and G. Hai Qi. 2019. Effect of photoperiod on ovarian morphology, reproductive hormone secretion, and hormone receptor mRNA expression in layer ducks during the pullet phase. Poult. Sci. 98:2439–2447.

Fabi, F., and E. Asselin. 2014. Expression, activation, and role of Akt isoforms in the uterus. Reproduction 148:R85–R95.

Getmanseva, L., S. Bakoev, V. Shevtsova, A. Kolosov, N. Bakoev, and M. Kolosova. 2020. Assessing the effect of SNPs on litter traits and their relationships with egg production traits of White Muscovy ducks (Cairina moschata). Hereditas 151:14–19.

Sato, S., Y. Uemoto, T. Kikuchi, S. Egawa, K. Kohira, T. Saito, H. Sakuma, S. Miyashita, S. Arata, T. Kojima, and K. Suzuki. 2016. SNP- and haplotype-based genome-wide association studies for growth, carcass, and meat quality traits in a Duroc multigenerational population. BMC Genet. 17:60.

Wu, C., C. Orozco, J. Boyer, M. Leglise, J. Goodale, S. Batalov, C. Hodge, J. Haase, J. Janes, and J. Huss. 2009. BioGPS: an extendable and customizable portal for querying and organizing gene annotation resources. Genome Biol 10:R130, doi:10.1186/gb-2009-10-11-r130.

Wu, X., M. Yan, S. Lian, X. Liu, and A. Li. 2014. GH gene polymorphisms and expression associated with egg laying in muscovy ducks (Cairina moschata). Hereditas 151:14–19.

Yang, Z.-Z., O. Tschopp, N. Di-Poï, E. Bruder, A. Baudry, B. Dümmler, W. Wahli, and B. Hemmings. 2006. Dosage-dependent effects of Akt1/Protein Kinase B (PKBα) and Akt3/PKBβ on thymus, skin, and cardiovascular and nervous system development in Mice. Mol. Cell. Biol. 25:10407–10418.

Ye, Q., J. Xu, X. Gao, H. Ouyang, W. Luo, and Q. Nie. 2017. Associations of IGF2 and DRD2 polymorphisms with laying traits in Muscovy duck. PeerJ 5:e4803.

Table 2. Association analysis of the three SNPs of AKT3 with egg production traits of White Muscovy ducks.

| SNPs     | Egg production traits | CC (121) | CA (280) | AA (139) | P-value |
|----------|-----------------------|---------|---------|---------|---------|
| C-3631A  | AFE                   | 196.02 ± 0.61a | 194.80 ± 0.38a | 196.09 ± 0.60a | 0.0890 |
| N300D    |                      | 98.62 ± 0.92a  | 98.02 ± 0.63a  | 97.63 ± 0.88a  | 0.3120 |
| N59W     |                      | 174.60 ± 2.29a | 175.66 ± 1.76a | 179.39 ± 2.48a | 0.1460 |
|          |                      | CC (295)   | CT (215)  | TT (39)  |         |
| C-3766T  | AFE                   | 195.22 ± 0.38a | 195.79 ± 0.47a | 194.51 ± 1.06a | 0.2500 |
| N300D    |                      | 97.62 ± 0.62a  | 96.90 ± 0.73a  | 102.10 ± 1.94a | 0.0250 |
| N59W     |                      | 174.59 ± 1.65a | 176.47 ± 2.10a | 189.28 ± 1.06a | 0.0020 |
|          |                      | CC (287)   | CT (213)  | TT (39)  |         |
| C-3953T  | AFE                   | 195.23 ± 0.38a | 195.79 ± 0.48a | 194.51 ± 1.06a | 0.2090 |
| N300D    |                      | 97.65 ± 0.61a  | 96.85 ± 0.74a  | 102.10 ± 0.97a | 0.0300 |
| N59W     |                      | 174.67 ± 1.64a | 176.38 ± 2.12a | 189.28 ± 2.19a | 0.0020 |

Abbreviations: AFE, age at first egg laid; N300D, number of eggs at 300 days of age; N59W, number of eggs at 59 wk of age; SNPs, single nucleotide polymorphisms; SEM, standard error of mean.

a,bValues within the same row with different superscript differ significantly at $P < 0.05$. 