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

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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

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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

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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 (Ethylenediamine 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 gladularis, 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: AGCCTGGATTGCTTCTGTCC	861	60.00	qRT-PCR
GAPDH	F: GCACTGTCAAGGCTGAGAATG R: GCAGGTCAGGTCCACGACA	569	59.53	House keeping gene
AKT3-P1	F: AGCTCGAAGATGAAACTCAGCA R: CGCGGCGGCATCGAC	827	60.03	SNPs detection
AKT3-P2	F: GTGTGTGTTGGGGTCTGAATTTT R: AGGTGGGATGAGTGTGTTCAAAT	1847	57.55	SNPs detection
AKT3-P3	F: GTAGTGATGACCTACTGAAAGGA R: ACGTTTCCCAAGCAAATTCCAG	851	57.08	SNPs detection
AKT3-SNP	F: GTAGTGATGACCTACTGAAAGGA R: ACGTTTCCCAAGCAAATTCCAG	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

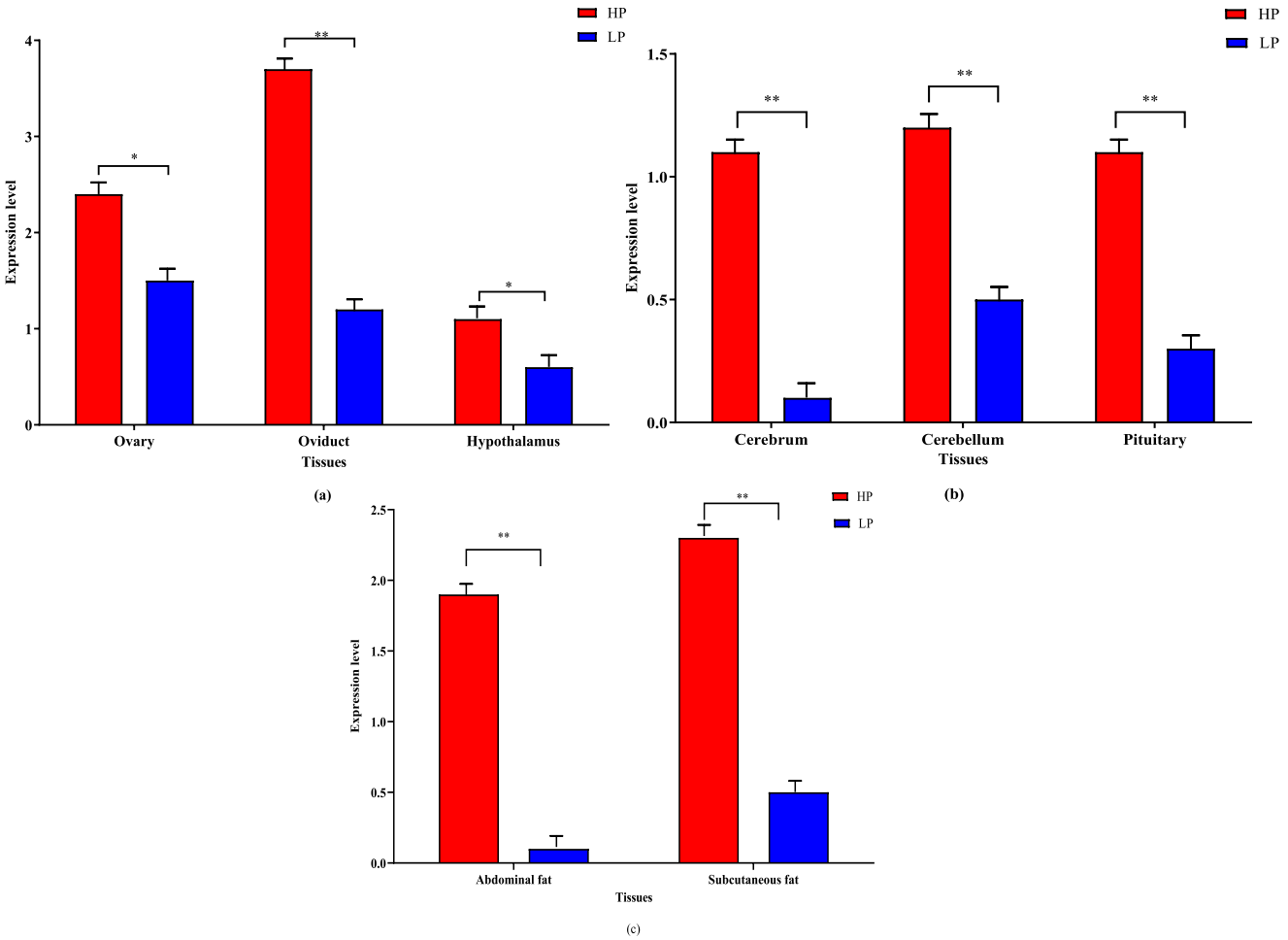

Figure 1. Expression level of the *AKT3* gene in (a) ovary, oviduct, and hypothalamus (b) cerebrum, cerebellum, and pituitary (c) abdominal fat and subcutaneous fat of HP and LP White Muscovy ducks. $^{*}P < 0.05$ and $^{**}P < 0.01$ means there is a significant difference between the expression level of HP and LP Muscovy duck.

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

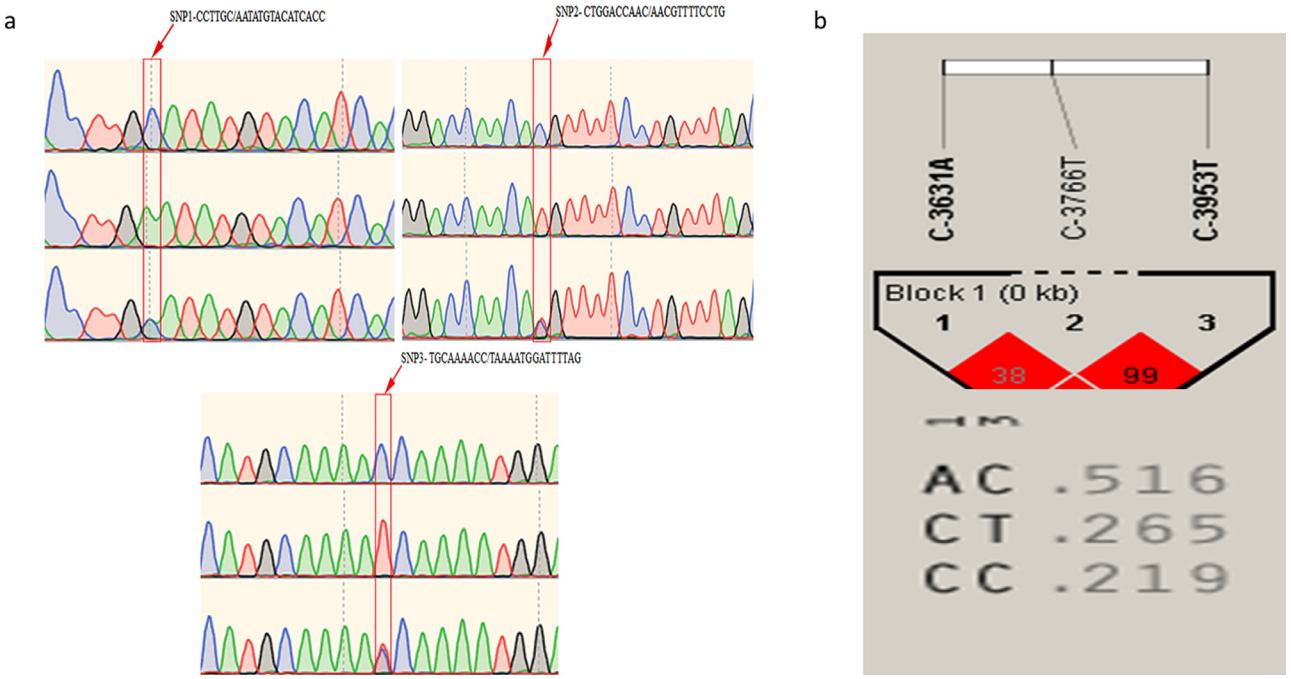

Figure 2. (a) The aligned sequences showing the SNPs sites of Muscovy duck *AKT3*. (b) Haplotype block showing the linkage status of three identified SNPs in the *AKT3* gene. The color of the block indicates the LD status of SNPs; deep red means high linkages between 3 SNPs.

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

SNPs	Egg production traits	Least square mean ± SEM			*P*-value
		CC (121)	CA (289)	AA (139)	
C-3631A	AFE	196.02 ± 0.61[a]	194.80 ± 0.38[a]	196.09 ± 0.60[a]	0.0890
	N300D	96.82 ± 0.92[a]	98.02 ± 0.63[a]	97.63 ± 0.88[a]	0.3120
	N59W	174.60 ± 2.29[a]	175.66 ± 1.76[a]	179.39 ± 2.48[a]	0.1460
		CC (295)	CT (215)	TT (39)	
C-3766T	AFE	195.22 ± 0.38[a]	195.79 ± 0.47[a]	194.51 ± 1.06[a]	0.2500
	N300D	97.62 ± 0.62[b]	96.90 ± 0.73[b]	102.10 ± 1.94[a]	0.0250
	N59W	174.59 ± 1.65[b]	176.47 ± 2.10[b]	189.28 ± 1.06[a]	0.0020
		CC (297)	CT (213)	TT (39)	
C-3953T	AFE	195.23 ± 0.38[a]	195.79 ± 0.48[a]	194.51 ± 1.06[a]	0.2090
	N300D	97.65 ± 0.61[b]	96.85 ± 0.74[b]	102.10 ± 0.97[a]	0.0300
	N59W	174.67 ± 1.64[b]	176.38 ± 2.12[b]	189.28 ± 2.19[a]	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,b]Values within the same row with different superscript differ significantly at $P < 0.05$.

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.

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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.

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