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«Research Note»

A Nonsynonymous Single-nucleotide Polymorphism in *SLC24A5* Regulates Feather Pigment Deposition in Chinese Yellow Quail

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Plumage color in birds is determined by melanin, whose synthesis and transport are affected by many genes, including specific solute carriers (SLCs). The main objective of this study was to detect polymorphisms in the *SLC24A5* gene of the Chinese yellow quail (*Coturnix japonica*) and analyze their effect on tyrosinase activity in skin tissue and melanin content in down feathers. The cDNA of the *SLC24A5* gene was cloned by RT-PCR and subjected to Sanger sequencing. Potential single-nucleotide polymorphisms (SNPs) were screened using multiple sequence alignment. The screened nonsynonymous SNPs were genotyped across 265 Chinese yellow quails using the kompetitive allele-specific PCR method. The association of genotypes with tyrosinase activity in the skin and melanin content in down feathers was analyzed. The g.8884145A/G SNP was identified in exon 9 of the *SLC24A5* gene, resulting in an Asp396Ala mutation. The mutant residue was predicted to be located inside the eighth transmembrane helix of the SLC24A5 protein, which is primarily responsible for recognizing Na^+/Ca^{2+} ions. Mutant individuals had significantly lower total melanin content in the feathers and tyrosinase activity in dorsal skin, in spite of no significant difference in *SLC24A5* mRNA expression in the same tissues. This study indicates that the g.8884145A/G mutation reduced tyrosinase activity by affecting the function of the SLC24A5 protein, which in turn decreased melanin content of down feathers in Chinese yellow quail.

Key words: Chinese yellow quail, nsSNP, plumage color, *SLC24A5*, tyrosinase activity

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Introduction

China has the largest egg quail population in the world, estimated at 360 million individuals in 2022. Most birds are hybrids derived from Korean quails and Chinese yellow quails (both *Coturnix japonica*). The Chinese yellow quail was derived from the Korean quail in the 1990s, and both originated from the Japanese quail (*C. japonica*)[1]. The gender of egg quails can be distinguished based on plumage color at birth; therefore, the consistency and stability of plumage color are very important for this species. Plumage color is mainly influenced by melanin content and is regulated by several multi-gene interactions[2].

Solute carriers (SLCs) are membrane transport proteins, whose main function is to facilitate the transport of various small molecules into cells. Mutations in these genes may affect melanin synthesis and transport[3]. Mutations in the *SLC45A2* gene affect melanin production in the feathers of alexandrine parrots and plum-headed parakeets, and account for the sex-linked yellow phenotype of rose-ringed parakeets[4]. SLC24A5 is an eleven transmembrane domain-containing V-ATPase-dependent Na^+/Ca^{2+} pump that maintains the pH of organelles via Ca^{2+} uptake. In melanocytes, SLC24A5 mediates the transport of one Ca^{2+} and one K^+ ion to the melanosome in exchange for four Na^+ ions towards the cytoplasm[5]. Ion and pH stability of melanosomes have important implications for melanin biosynthesis.

SLC24A5 affects melanin synthesis in animals and is present mainly in stage III–IV melanosomes and the trans-Golgi network. Mutations in this gene are the main cause of iris color changes in quails[6] and pigeons[7]. The *SLC24A5* gene is also among differentially expressed genes determining plumage color in chickens[8] and geese[9]. Mutations in pigmentation-associated genes are the main causes of feather color variation. Single-nucleotide polymorphisms (SNPs) in the *HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2) -oculocutaneous albinism II (OCA2)*[10] locus and the *premelano-*

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some protein (PMEL) gene[11] have been reported to be the main determinants of plumage color variation in quails. In the present study, we identified a novel nonsynonymous SNP (nsSNP) in the *SLC24A5* gene and analyzed its effect on tyrosinase activity in skin tissues and melanin content of feathers in a population of Chinese yellow quail.

Materials and Methods

Ethical statement

All animal experiments were conducted according to the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China) and were approved by the Faculty Animal Policy and Welfare Committee of Henan University of Science and Technology (approval number HAUST 2023-047).

Animals and collection of tissue samples

Chinese yellow quails were raised at the Poultry Research Center of Henan University of Science and Technology. A total of 265 blood samples, dorsal skin samples, and down feathers were collected from three-day-old Chinese yellow quails. Skin tissue samples were frozen in liquid nitrogen for 2 h and then stored at -80 °C.

Genomic DNA and total RNA isolation

Genomic DNA was extracted from blood samples using a TaKaRa MiniBEST Whole Blood Genomic DNA Extraction Kit (TaKaRa, Dalian, China); whereas total RNA from the dorsal skin was extracted using TRIzol reagent (#15596026; Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's recommendations. The concentrations of genomic DNA and total RNA were detected using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and their quality was assessed by 1% agarose gel electrophoresis. Genomic DNA was diluted to approximately 25 ng/μL and then stored at -20 °C.

Variant detection in the SLC24A5 gene

Reverse transcription was performed using PrimeScript^{TM} RT Reagent Kit (TaKaRa). The cDNA of *SLC24A5* mRNA was cloned by RT-PCR using gene-specific primers (5′-TAAGCCTGGTCGCCTCCT-3′ and 5′-AAGAGTCCAATGGAAGTCTTGG-3′) and then sequenced. Sequences from 10 individuals (five individuals of each plumage color) were aligned using SeqMan 7.0 (DNAStar, Madison, WI, USA) to screen for SNPs based on sequence discrepancies.

Bioinformatics analysis of SNPs

The effect of SNPs on the amino acid sequence of the encoding protein was analyzed using DNAStar 7.0, and nsSNPs were selected for biological functional prediction. The deleterious effects of nsSNPs on the coding proteins were predicted using PROVEAN (http://provean.jcvi.org/seq_submit.php) and SNAP2 (https://service.rostlab.org/snap). Protein stability was analyzed using I-Mutant (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi). The transmembrane helix of SLC24A5 was predicted using TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). The secondary structure of SLC24A5 was predicted using SOSUI (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/).

Variant genotyping using the kompetitive allele-specific PCR (KASP) method

The nsSNP was genotyped in 265 Chinese yellow quails using KASP technology. The KASP reaction system (10 μL) consisted of 1× KASP reaction mix, 0.17 μM KASP assay mix (5′-GGTATTCCCAGACACAGCATAT-FAM-3′, 5′-GGTATTCCCAGACACAGCATAC-HEX-3′, and 5′-GGGTGTTTCACATGAAGTTG-3′), and 25 ng of genomic DNA. PCR amplification was performed using a CFX-96 thermal cycler (Bio-Rad, Hercules, CA, USA). KASP reaction conditions were as follows: predenaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s; and a final extension step at 72 °C for 10 min. End-point fluorescence signals were collected, and fluorescent SNP genotyping was analyzed using the CFX-96 manager software (Version 3.1).

Analysis of SLC24A5 mRNA expression

Total RNA from the dorsal skin was reverse-transcribed into the first strand of cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). A quantitative PCR was performed using a CFX-96 thermal cycler. All quantitative PCR assays were performed in triplicate, and *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used for data normalization. The fold change in relative gene expression was calculated using the standard $2^{-\Delta\Delta Ct}$ method.

Melanin analysis by spectrophotometry

Dried feathers were homogenized in water and diluted to 15 mg/mL. Aliquots of 100 μL were dissolved in 900 μL of Soluene-350. Absorbance at 500 nm (A500) and 650 nm (A650) was measured using a Multiskan SkyHigh spectrophotometer (Thermo Fisher Scientific). The A500 value was used to estimate total melanin content; whereas the A650/A500 ratio was calculated to estimate the relative proportion of eumelanin to pheomelanin in dried feather samples.

Skin tyrosinase activity

Approximately 0.1 g of skin tissue was weighed into 1 mL of extraction solution and homogenized with an electric homogenizer. After incubation on ice for 30 min, 200 μL of the mixture was transferred to a 96-well plate, followed by adding 50 μL L-DOPA (2 mg/mL). The absorbance of the mixture was immediately monitored at 475 nm using a multi-mode plate reader (Cytation^{TM} 5; BioTek, Winooski, VT, USA). The mixtures were incubated for another 20 min at 37 °C and absorbance was measured again at 475 nm. The tyrosinase relative activity (U/g) was calculated as 90.09 × ΔA / W, where ΔA represents the absorbance value at 20 min minus the absorbance value at 0 min, and W represents the weight of the skin sample.

Statistical analysis

The relative expression of *SLC24A5* mRNA in different genotypes was analyzed by one-way ANOVA followed by Duncan's test for pairwise comparisons using SPSS 20.0 (IBM, Chicago, IL, USA). Polymorphic information content (PIC) was calculated using the PIC_Calc software. General linear model procedures were used to determine associations between the different geno-

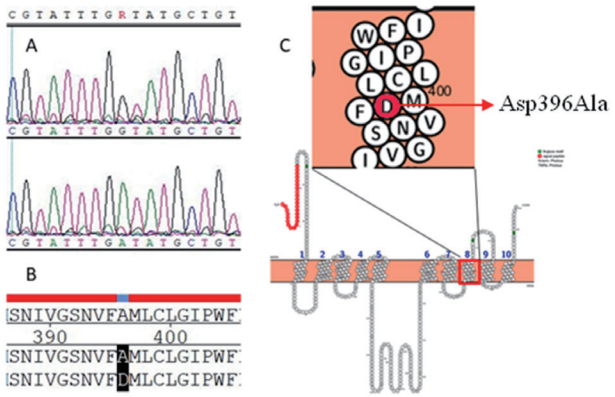

Fig. 1. **The g.8884145A/G mutation of *SLC24A5* gene.** (A) The g.8884145A/G mutation was identified by Sanger sequencing. (B) The g.8884145A/G mutation causes an amino acid change (Asp396Ala) in the SLC24A5 protein. (C) The Asp396Ala mutation is located on the eighth transmembrane helix (TM8) of the SLC24A5 protein.

types with the content of melanin and tyrosinase activity according to the following model, $Y_{ij} = \mu + G_i + \varepsilon_{ij}$, where Y_{ij} is melanin content plus tyrosinase activity, μ is the population mean, G_i is the genotype value, and ε_{ij} is the random error.

Results and discussion

Polymorphism of SLC24A5

A 1612-bp cDNA of the *SLC24A5* gene was cloned, including a 44-bp 5′UTR, a 1461-bp full-length coding sequence, and a 107-bp 3′UTR. Only one SNP (g.8884145A/g) was found in the *SLC24A5* coding region following multiple alignments of PCR sequences from different individuals (Fig. 1A). This SNP is located at 1187 bp within the open reading frame and causes an Asp396Ala mutation (Fig. 1B). Three genotypes were detected at the g.8884145A/G locus in 265 Chinese yellow quails. Locus polymorphisms were considered high, medium, or low if PIC > 0.5, PIC > 0.25, or PIC < 0.25, respectively[10]. In the present study, the g.8884145A/G locus showed moderate polymorphism (PIC = 0.44).

Association of the g.8884145A/G locus with tyrosinase activity and feather melanin content

The g.8884145A/G locus was significantly associated with skin tyrosinase activity and feather melanin content in Chinese yellow quail. Tyrosinase activity was significantly higher in skin samples from individuals with the AA genotype (wild-type) than in those with the AB (mutant heterozygote) and BB (mutant homozygote) genotypes. Accordingly, total melanin and pheomelanin were also significantly higher in individuals with the AA genotype than in those with the AB and BB genotypes (Table 1). Eumelanin content in the feathers showed no significant variation across individuals with different genotypes. Distinct levels of total melanin and ratios of pheomelanin/eumelanin in the feathers resulted in different dorsal plumage color among individuals (Fig. 2). Genetic variation in *SLC24A5* is responsible for a significant degree of skin color variability from zebrafish to mammals. Five pathogenic nsSNPs (A111T, A115E, R174K, S182R and W197X) have been identified in human *SLC24A5*; of which A111T is associated with light skin pigmentation in Europeans and East Asians[12]. Another study showed that mutations in the *SLC24A5* gene were strongly associated with diluted iris pigmentation in horses[13]. These studies suggest that the *SLC24A5* gene plays an important regulatory role in melanin biosynthesis and that its disruption can affect pigmentation in many organisms.

Expression of SLC24A5 mRNA in skin tissues with different genotypes

SLC24A5 mRNA levels did not vary significantly in skin tissues from individuals with different genotypes (Table 1). *SLC24A5* is a Na^+/K^+ exchange pump[14] and has been detected in pigmentation-related tissues by next-generation sequencing. The expression of *SLC24A5* mRNA in feather follicles correlates significantly with feather color in chickens[15], ducks[16], and geese[17], with lighter feather color correlating with lower *SLC24A5* expression. Abundant *SLC24A5* mRNA promotes melanin deposition in chicken skin tissues[18]. In this study, the g.8884145A/G SNP resulted in a significant decrease in melanin content in feathers, without significant changes in *SLC24A5* mRNA, indicating that the g.8884145A/G SNP altered mainly the function of the SLC24A5 protein.

Bioinformatics analysis of the Asp396Ala mutation

Secondary structure analysis revealed that the Asp396Ala mutation was located in the eighth transmembrane domain (TM8) of SLC24A5 (Fig. 1C). The predicted PROVEAN score was -7.522, which indicated the Asp396Ala mutation was deleterious. I-Mutant was used to evaluate protein stability and the predicted DDG value was -0.90 Kcal/mol, which indicated that the Asp396Ala mutation could lower protein stability. A 3D structural model of SLC24A5 constructed using NCX_Mj (PDB No.3V5U) as template revealed a set of 10 transmembrane domains and two additional regions[19]. The N-terminus of the SLC24A5 protein contained a transmembrane domain (TM0) that was removed from the mature peptide. The core substrate-binding sites were located in the middle of TM2-3 and TM7-8, where the Na^+/Ca^{2+} ions were bound and transported across the membrane. A111T, A115E, R174K, and S182R were predicted to be located on TM4 and TM6, which are located in the outer part of core substrate-binding transmembrane domains. W197X was predicted to be located outside TM7. The position of the mutated residues likely explains why these nsSNPs caused only slight color changes in hair and skin[20]. The Asp396Ala mutation was predicted to be located inside TM8; therefore, this mutation may severely disturb substrate recognition by SLC24A5 and negatively affect Na^+/Ca^{2+} transport. This could explain the significant decrease in tyrosinase activity in the skin of mutant individuals, along with the significant drop in total melanin content in feathers observed in the present study.

Table 1. **Association of genotypes with melanin content (μg/mL) and tyrosinase activity (U/g).**

Parameter	Genotypes			*F*-Value	*p*-Value
	AA	AB	BB		
Melanin	1139 ± 82^{A}	1014 ± 144^{B}	986 ± 150^{B}	10.19	0.00
Eumelanin	329 ± 62	314 ± 68	320 ± 72	0.44	0.65
Pheomelanin	810 ± 84^{A}	698 ± 137^{B}	665 ± 130^{B}	11.06	0.00
Tyrosinase activity	1.73 ± 0.19^{a}	1.01 ± 0.24^{b}	0.94 ± 0.19^{b}	4.86	0.03
SLC24A5 expression	0.0462 ± 0.0033	0.0416 ± 0.0051	0.0426 ± 0.0044	1.47	0.28

Different lowercase letters in the same row indicate significant differences; whereas different capital letters in the same row indicate highly significant differences.

Melanin, eumelanin, and pheomelanin levels were measured in 21 individuals with the AA genotype, 131 individuals with the AB genotype, and 113 individuals with the BB genotype. Five individuals were selected for each genotype to analyze tyrosinase activity and *SLC24A5* mRNA expression.

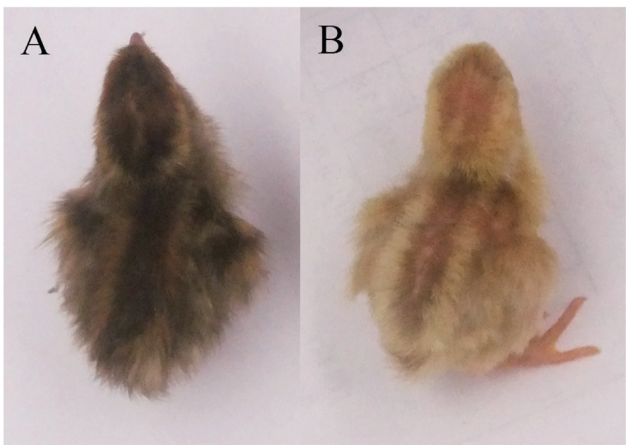

Fig. 2. **Dorsal feather coloration in individuals of different genotypes.**

(A) Individual with the AA genotype. (B) Individual with the BB genotype.

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Author Contributions

Xiaohui Zhang and Yanxia Qi designed the study; Lingyun Fan and Yifei Wang collected the samples and performed the experiments; Fanghu Wu and Yuanyuan Shang analyzed and interpreted the data; Xiaohui Zhang, Yanxia Qi, and Yuanyuan Shang drafted the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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