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Original Article

Identification of Polymorphism in the MC1R Gene and Its Association with the Melanin Content in Feathers of Chinese Yellow Quails

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ABSTRACT

MC1R plays a crucial role in controlling the type of melanin synthesized in the melanocytes, which greatly affects plumage color in birds. One g.16796362G/T SNP was found in the MC1R gene coding region, which caused a Met120Ile mutation in the amino acid sequence. The Met120Ile mutation was located in the third transmembrane domain of the MC1R protein and decreased protein stability. The g.16796362G/T locus achieved medium polymorphism and had significant association with feather melanin content in Chinese yellow quails. The contents of total melanin and pheomelanin with AA genotype were significant lower than those with AB or BB genotypes in skin tissues, while the expression levels of MC1R mRNA had no significant difference in feathers with different genotypes. This experiment indicated that the Met120Ile mutation could affect the function of the MC1R protein and change the biosynthesis of melanin in Chinese yellow quails.

INTRODUCTION

The total number of egg quails raised in China was around 350 million in 2020, and most of them are hybrids of male Chinese yellow quail × female Korean quail or male Chinese yellow quail × female Beijing white quail. Chinese yellow quails came from Korean quails in the 1990s, which had been proved to be a sex chromosome mutation (Wu *et al.*, 2018). The hybrids of male Chinese yellow quails with female Korean quails could be autosexing by plumage colors at birth, and the egg production performance was higher than purebred Korean quails (Pang & Zhao, 2003). This led Chinese yellow quails to be widely raised in China, especially in the north. Young Chinese yellow quails could not be distinguish between male and female only according to feather color, because they all had light yellow plumage with brown stripes on the back and wings (Zhang *et al.*, 2013), while the shadings were different between individuals. This phenomenon indicated that gene mutations outside the sex chromosome can also affect feather color, and result in different melanin contents in the feathers.

The MC1R (melanocortin-1 receptor) is a kind of G-protein coupled receptor and mainly located in the plasma membrane of the melanocytes (Herraiz *et al.*, 2017). Activation of MC1R leads to an increased biosynthesis of eumelanin, whereas an inhibition of MC1R usually leads to an increased biosynthesis of pheomelanin (Wolf Horrell *et al.*, 2016). The ratio of eumelanin to pheomelanin can also change plumage color in birds (Jeon *et al.*, 2021), and this indicates that the MC1R gene plays an important role in the regulation of feather color. The level of MC1R activation was mainly regulated by three factors: the concentration of extracellular MC1R agonists such as α-MSH (alpha melanocyte stimulating hormone), the concentration of extracellular MC1R antagonists/inverse agonists such as ASIP (agouti signaling

protein), and the intrinsic basal level of activity of the receptor itself (Bourgeois *et al.*, 2016). So, in this study, the main aim was to test the hypothesis that the mutation in the MC1R gene could also cause different shades of plumage color between individuals in Chinese yellow quails.

MATERIALS AND METHODS

Animals and collection of tissue samples

Chinese yellow quails were raised in the Poultry Research Center of HAUST (Henan University of science and technology). A total of 211 blood samples of Chinese yellow quails were collected. Skin samples were collected from the back of quails at embryo developmental stages E14 (just before hatching out), then whole body feathers were collected. All experimental and surgical procedures were approved by the Biological Studies Animal Care and Use Committee, Henan Province, China.

Melanin analysis by spectrophotometry

The dried feather samples of the quails were homogenized in ultrapure water using an electric homogenizer at a concentration of 15 mg/ml. Aliquots of 100 uL were additionally dissolved in 900 uL of Soluene-350. The A500 value was measured using a Multiskan SkyHigh spectrophotometer (Thermo Fisher Scientific, Waltham, MA), in order to estimate the total melanin content. The absorbance ratio of A650/A500 was calculated to estimate the relative ratio of eumelanin to pheomelanin in the samples (Del Bino *et al.*, 2015).

Genomic DNA and total RNA isolation

Genomic DNA was extracted from the blood samples using a TaKaRa MiniBEST Whole Blood Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, 15596026) in accordance with the manufacturer's recommendation. The integrity and yield of genomic DNA and total RNA were assessed and verified using agarose gel electrophoresis and a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), respectively. Stock DNA samples at a concentration of approximately 25 ng/μL were stored at -20°C for KASP analysis.

RT-qPCR analysis

Reverse transcription was done using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Real-time quantitative PCR was done on the BioRad CFX-96 thermal cycler (BioRad, California, USA) using TB Green® Premix DimerEraser™ kit (TaKaRa, Dalian, China). All qPCR assays were performed in triplicate and the GAPDH gene was used as an internal control (yuan). The designed primers are listed in Table 1. The fold change in relative gene expression was calculated using the standard $2^{-\Delta\Delta Ct}$ method.

Variant detection in MC1R gene

The genomic sequence (NC_029526.1:16795988-16800696) of quail MC1R gene was downloaded from NCBI genomes datasets (https://www.ncbi.nlm.nih.gov/datasets/genomes/?taxon=93934). Read pairs (PRJNA756792) were aligned to the MC1R reference genome sequence using the Hisat2 algorithm with the default parameters, and mapped reads were sorted using SAMtools (version: 1.6). Variants were called using VCFtools (version: 11.1) and filtered with parameters "QUAL > 30, DP>10". A search for sequence variants within the duplicated region that may not be called correctly by VCFtools was conducted by visualizing the alignment files in the integrative genomics viewer (IGV, version: 2.4.3). The nsSNP (nonsynonymous single nucleotide polymorphisms) were selected according to the annotation information in the VCF file.

Bioinformatics analysis of SNPs

The deleterious effect of nsSNP was predicted using online bioinformatics tools PROVEAN (http://provean.jcvi.org/seq_submit.php) and SNAP2 (https://

Table 1 – qPCR and KASP primers.

Primer name	Primer sequence (5'-3')	Tm (°C)	Product length (bp)	Purpose
M1	CATGGACAACGTCATCGACATG-FAM CACATGGACAACGTCATCGACATT-HEX AGAAGATGGTGATGTAGCGGTCC	64	98	KASP
MC1R	CAGAAGCAGCCCACCATC GAAGAAGACTCCCAGCAGG	55	78	qPCR
GAPDH	TGCCGTCTGGAGAAACC CAGCACCCGCATCAAAG	55	160	qPCR

Note: The underline indicates the polymorphic site.

www.rostlab.org/services /snap/). Protein stability was analyzed using the online tool I-Mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi). The transmembrane region of the MC1R protein was predicted using online bioinformatics tool TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). The secondary structure of the MC1R protein was predicted using online bioinformatics tool SOSUI (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/).

Variant genotyping using KASP method

The selected nsSNP was genotyped by KASP in 211 Chinese yellow quails. The PCR amplification was performed in a BioRad CFX-96 thermal cycler in a reaction mixture of 10 μL containing 1× KASP reaction mix, 0.17 μM KASP assay mix (two forward allele-specific upstream primers and a common downstream primer, Table 1) and 25 ng of genomic DNA. The PCR reaction was performed with the following cycling conditions: an initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 60 s, extension at 72 °C for 15 s; and a final extension at 72 °C for 10 min. An end-point fluorescent reading of the PCR products was done, and fluorescent SNP genotyping was conducted using the CFX-96 manager software.

Statistical analysis

The gene expression in different groups was analyzed by one-way ANOVA followed by *Bonferroni* test, and the level of significant difference was set at $p<0.05$. PIC (polymorphic information content) was calculated using PIC_Calc software.

General linear model (GLM) procedures were used to determine associations between different genotypes and melanin contents in feathers, according to the following model: $Y_{ij} = \mu + G_i + \varepsilon_{ij}$, where Y_{ij} was the observed value of melanin content, μ was the population mean, G_i was the genotype value, and ε_{ij} was the random error.

RESULTS

Polymorphism of the MC1R gene

Only one G/T nsSNP (g.16796362G/T) was found in the MC1R gene coding region and identified by DNA sequencing (Figure 1 A). This nsSNP was located at 360 bp within the ORF (Open Reading Frame) and caused a Met120Ile mutation in the amino acid sequence (Figure 1 B).

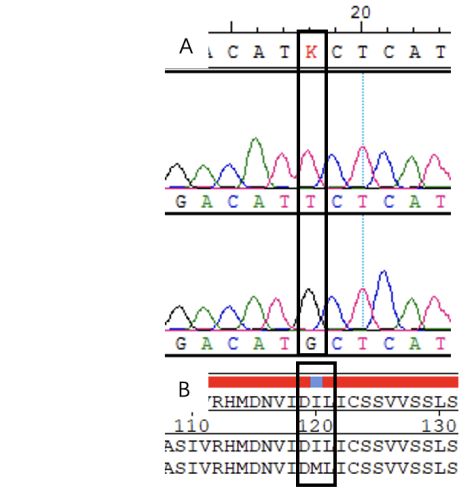

Figure 1 – The mutation of the MC1R gene.

Bioinformatics analysis of Met120Ile mutation

The Met120Ile mutation was located in the third transmembrane domain (TM-III) of the MC1R protein by secondary structure analysis (Figure 2). The PROVEAN (Protein Variation Effect Analyzer) tool was used to analyze the deleteriousness of Met120Ile mutation, and the predicted score was 0.125, which indicated that the Met120Ile mutation was neutral (A score below or equal to -2.5 is considered "deleterious", while those above -2.5 are neutral). The DDG value predicted by I-Mutant was -1.09 Kcal/mol, which indicated the Met120Ile mutation could decrease protein stability (positive DDG values indicate increased protein stability, while negative DDG values indicate protein destabilization).

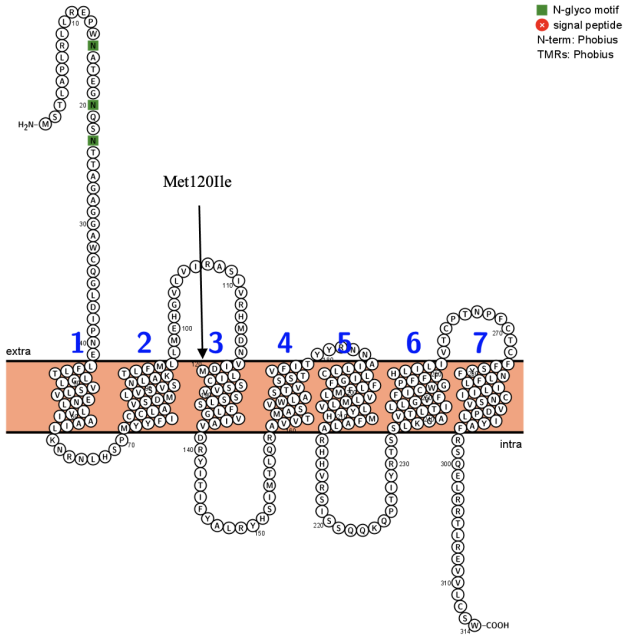

Figure 2 – The 2D structure of quail MC1R protein with the identified amino acid change.

The results of genotyping by KASP

Three genotypes were detected in this SNP locus in 211 Chinese yellow quails (Figure 3). Locus polymorphism is considered high, medium, or low if PIC > 0.5, PIC > 0.25, or PIC < 0.25, respectively (Chen *et al.,* 2021). In this study, the g.16796362G/T locus achieved medium polymorphism and preserved the *Hardy-Weinberg* equilibrium (x^2=0.99, *p*=0.61) (table 2).

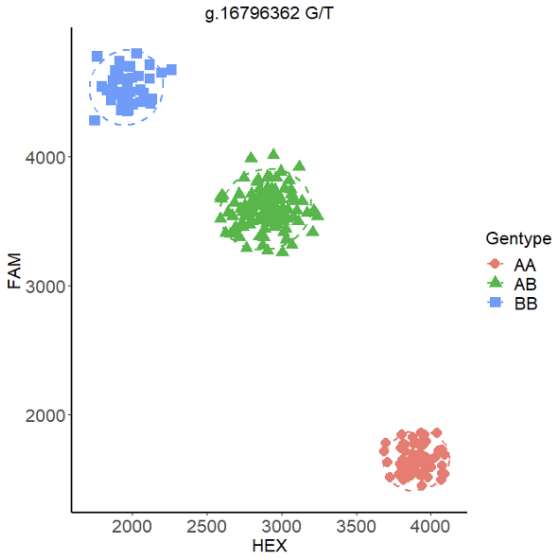

Figure 3 – The genotyping by KASP.

Association analysis of MC1R polymorphism with melanin content in feathers

The g.16796362G/T locus had significant association with the melanin content of feathers in Chinese yellow quails. The contents of total melanin and pheomelanin in quails with AA genotype were significant lower than in those with AB or BB genotypes (table 3). The contents of eumelanin in feather samples had no significant difference among groups with different genotypes.

The expression of MC1R mRNA in skin tissues with different genotypes

The expression of MC1R mRNA had no significant difference in skin tissues with different genotypes (Figure 4).

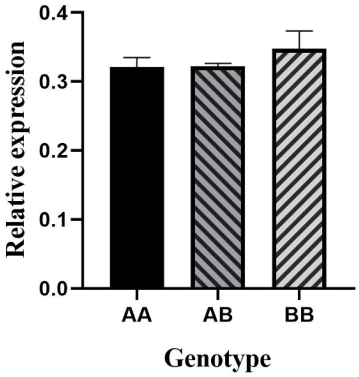

Figure 4 – The expression of MC1R mRNA in skin tissues with different genotypes.

DISCUSSION

The contents of eumelanin and pheomelanin in skin, hair, or feather usually showed great difference between individuals within the same breed or group. The main degradation product of pheomelanin is TTCA (thiazole-2,4,5-tricarboxylic acid), whereas the main degradation product of eumelanin is PTCA (pyrrole-2,3,5-tricarboxylic acid). The contents of TTCA and PTCA can be measured using the HPLC (high-performance liquid chromatography) method (Ito *et al.*, 2020). The coefficient variations of TTCA and PTCA in swallow feathers have been reported to be 27.32% and 35.51%, respectively (Arai *et al.*, 2019). In this study, the range of concentrations of eumelanin and pheomelanin in quail feathers were 157~468 and 416~953, respectively, and the corresponding coefficient variations were 22.46% and 20.84%, respectively. The coefficient variations of TTCA and

Table 2 – Distributions of allele frequencies and genotypes.

Loci	numbers	Frequencies of each genotype			Allele frequencies		PIC	x^2 value	*p* value
		AA	AB	BB	A	B			
g.16796362G/T	215	63	111	37	0.56	0.44	0.49	0.99	0.61

PIC: polymorphism information content.

Table 3 – Association of genotypes with melanin content.

pigments	genotypes			F-Value	*p*-Value
	AA	AB	BB		
melanin	919±129^A	1052±143^B	1043±163^B	18.53	0.00
eumlanin	329±71	321±73	303±68	1.57	0.21
pheomelanin	590±104^A	731±139^B	740±133^B	27.68	0.00

Melanin was the total of eumelanin and pheomelanin.

Different capital letters in the same line indicate significant differences.

PTCA in dark hair samples from three ethnic origins were 36.83% and 31.80%, respectively (Ito *et al.*, 2018), which indicated that the contents of melanin in these hair samples were significantly different. These experiments indicated that the concentrations of melanin in skin or feather were very different among individuals, even in the same group. This phenomenon was also reported in young quails, and the shadings of feather colors were different between individuals. The melanin biosynthesis pathway played important roles in the formation and regulation of feather colors. At present, at least 636 genes have been reported to be associated with biosynthesis of melanin in mammals and birds (Jeon *et al.*, 2021, Zhou *et al.*, 2021), out of which MC1R played a key regulatory role.

The association of plumage color with MC1R gene has been studied widely in birds. The intrinsic basal activity of MC1R could be affected by nsSNPs, and some of them have been reported to result in gross changes in coloration over the body of birds (Mundy, 2005, Ng & Li, 2018), mammals, and other vertebrates (Zhou *et al.*, 2018). In particular, the E locus (Extension genotype locus) was located in chromosome 1, which contained the locus of MC1R gene in birds. Variation in the E locus was an important factor in determining plumage color in chickens (Yang *et al.*, 2019) and some mammalian species (Gebreselassie *et al.*, 2020, Jiang *et al.*, 2021). The mutations in the MC1R gene could affect various feather colors in Korean native chicken breeds (Nam *et al.*, 2021). The same phenomena have been reported in Chinese native chicken breeds (Yang *et al.*, 2019, Zhang *et al.*, 2020), native Japanese chicken (Kabir *et al.*, 2020), black-bone chicken (Khumpeerawat *et al.*, 2021), and so on. Lots of nsSNPs in MC1R gene have been detected in chicken. The Arg213Cys mutation could increase the function of the receptor of producing pheomelanin, while the Glu92Lys mutation could be responsible for the deactivation of the receptor for pheomelanin production (Dávila *et al.*, 2014). The Glu92Lys mutation was located in the second transmembrane domain (TM-II) of the MC1R protein, and close to the outside of the melanocytes membrane as Met120Ile mutation, which indicated that the mutations of Glu92Lys and Met120Ile had similar effects on the intrinsic basal activity of the MC1R protein. This may be the main reason for individuals with the Met120Ile mutation having lower contents of total melanin and pheomelanin in feathers.

The density of the MC1R receptor on melanocytes also plays a crucial role in determining the melanin type. Experimental up-regulation of MC1R using the pcDNA3.1-MC1R eukaryotic expression vector could increase pigmentation in feather of quail embryos (Li *et al.*, 2019). This indicated the expression level of MC1R could affect feather color in birds. In our prior experiment, the expression level of MC1R mRNA in skin tissue was higher in black quails than in Beijing white quails (Zhang *et al.*, 2013). The same phenomenon was found in Korean native chickens (Nam *et al.*, 2021) and Silky Fowl chickens (Li *et al.*, 2011). In this study, the expression of MC1R mRNA had no significant difference between individuals, which indicated that the differences of total melanin and pheomelanin in feathers were not caused by the expression of the MC1R gene, but by the mutation in the MC1R gene.

CONCLUSION

The Met120Ile mutation could affect the function of the MC1R protein and change the biosynthesis of melanin in Chinese yellow quails. This was the main reason for different contents of total melanin and pheomelanin in feathers of Chinese yellow quails.

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AUTHOR CONTRIBUTIONS

Yanxia Qi drafted and prepared the manuscript; and Xiaohui Zhang conceived the study and participated in its design and coordination; and Youzhi Pang collected and analyzed data; Bingjie Yuan and Jiayao Cheng performed most of the experiments. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

No potential conflict of interest was reported by the author(s).

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