Research Journal of Animal Sciences 2 (2): 45-49, 2008
ISSN: 1993-5269

Study on Association of Melanocortin 1-Receptor (MC1R) Mutations with Melanin Trait in Chinese Domestic Chickens

Z.Q. Yang, Z.R. Zhang, M. Xu and Q. Zhu
College of Animal Science and Technology, Sichuan Agricultural University,
Ya'an, Sichuan, 625014, P.R. China

Abstract: Most of the standardized chicken breeds have a plumage color that often allows their unique identification and preferred by different client in the living chicken market place among most of the place in China. This study was aimed at investigating the effect of MC1R gene on plumage color and skin trait in chicken populations. Primer pairs for code region in MC1R were designed from database of chicken genomic sequence. Polymorphisms were detected using DNA sequencing. Sequence analysis indicated that 20 mutations were detected and 6 SNPs were dominated in all plumage and skin color chickens including: T69C, C212T, A274G, G636A, T637C and A644C. Lastly, the relations between SNPs and chicken melanin traits were analyzed, the results showed that there was significant association ($p < 0.05$) between the T69C, C212T, A274G, G636A, T637C and A644C mutation and chicken plumage color, between the T69C, C212T and A274G mutation and chicken skin color. These results were useful for studying the molecular mechanism that influences plumage and skin color and were used as the base of molecular-assisted selection to plumage and skin color trait. So, MC1R gene may be a potential candidate gene affecting the plumage and the skin color trait of chickens.

Key words: Chinese domestic chicken, MC1R , melanin trait, SNPs

INTRODUCTION

In birds and mammals, there are several different loci controlling plumage or coat color. Domestic animal species displayed a wide variety of plumage colors under artificial selection. In standardized domestic breeds, color is a basic ethnic character used in the morphological evaluation during early period selection. Thus, the study of polymorphisms in genes involved in pigmentation could provide genetic markers useful for their identification (Crepaldi *et al*., 2003). The plumage color variability within and between breeds is common and it makes domesticated species unique for studying gene function and gene regulation of loci affecting pigmentation (Klungland and Vage, 2003). At present, more than 120 genes have been identified and shown to regulate pigmentation, one of the key genes is Melanocortin 1-Receptor (MC1R) that it is a seven-transmembrane G protein-coupled receptor expressed on the surface of melanocytes.

Takeuchi *et al.* (1996) first cloned MC1R gene in chicken and pointed out the regulatory mechanism of MC1-R function was possible shared in chickens and mammals. Kijas *et al.* (1998) detected MC1R gene polymorphism in pig and pointed out mutation of MC1R was association with coat color. Classic genetics have established 8 alleles at the Extended black locus in chicken, which is assumed to correspond to the Extension locus in mammals. Kerje *et al.* (2003) investigated the co-segregation of plumage color and sequence polymorphism in MC1R gene using an intercross between the red jungle fowl and White Leghorn chickens. The results provided compelling evidence that the Extended black (E) locus controlling plumage color is equivalent to MC1R .

In wild species, three unrelated bird species has been reported that their MC1R locus was responsible for melanin polymorphisms, they were the bananaquit (Theron *et al*., 2001), the snow goose and the arctic skua (Mundy, 2004). Beside the domestic and wild animals, MC1R is also a key determinant of pigmentary phenotype in human. MC1R gene has been identified to explain variation in the normal population such as that leading to red hair, freckling and sun-sensitivity (Schioth *et al*., 1999).

MC1R locus also was studied for its role in evolution, especially in birds. Evolutionary changes in patterns and coloration of plumage are likely to represent a major

Corresponding Author: Prof. Q. Zhu, Laboratory of Animal Genetic and Breeding, College of Animal Science and Technology, Sichuan Agricultural University, Xinkang Road 46#, Ya'an, Sichuan Province, 625014, P. R. China

mechanism for speciation among birds. The role of MC1R in plumage patterning is surprisingly diverse among different species. The conserved molecular basis for the evolution of melanism in birds and several other vertebrates is probably related to low pleiotropic effects at the MC1R (Mundy *et al*., 2005). In chicken, 7 alleles of the chicken MC1R were cloned into expression vectors, expressed in mammalian cells and pharmacologically characterized. The E and E (R) alleles are associated with black feather color in chicken. The 3 constitutively active receptors share a mutation of Glu to Lys in position 92. The results are discussed in relationship to feather color in chicken, molecular receptor structures and evolution. We suggest that properties for the E92K switch mechanism may have evolved in an ancestor common to chicken and mammals and were maintained over long time periods through evolutionary pressure, probably on closely linked structural features (Ling *et al*., 2003).

The objective of this study is to identify polymorphisms of MC1R gene; to develop DNA sequencing methods to detect those DNA polymorphisms in different Chinese domestic populations and to evaluate associations between MC1R polymorphisms and plumage color trait in chicken populations.

MATERIALS AND METHODS

Animal material and phenotyping: In this study, Blood samples of 128 individuals were collected from pure indigenous chicken breeds from small remote villages or from participating farms and stored at -20°C. The genomic DNA was isolated from the blood using phenol extraction method and the genomic DNA was used for DNA sequencing assays. Samples used in this study consisted of Chinese domestic chicken breeds including Fengkai Xinghua chicken, Shandi Wugu chicken, white and black silkie, Cao Ke chicken, Huiyang Huxu chicken, black Rock Cornish, Qingyuan Ma chicken, Guangxi Xiayan chicken, S01 and S06 lines (developed in form of pureline selection by Sichuan Dahen Poultry Breeding Company using local breeds in Sichuan and Guangdong provinces of China).

Sequence analysis and detection of Single Nucleotide Polymorphism (SNP): The PCR primer pairs were designed to amplify the target fragments in MC1R gene using OLIGO 6.0 according to chicken genomic sequence in the GenBank database (Accession No: D78272). Primer synthesis was completed by Shanghai Ying Jun Biology Technique Corporation, China. The primer pairs were designed to detect the polymorphism. Primer pair F374 and R905 were used for amplify the former part of MC1R gene code region; Primer pair F840 and R1412 were used for amplify the later part of MC1R gene code region. F374 primer 5'-GCA CTG GTG GGG CTG GTT GGG CG-3' and R905 primer 5'-GCG TCA TGA TGC TGT GGT AG-3'; F840 primer 5'-CTC ATC TGC AGC TCC GTC GTG T-3' and R1412 primer 5'-CCA TCC ATC CAT CCT CCT GTC TGT-3'.

The PCR conditions were as follows: 94°C for 8 min, 35 cycles at 94°C for 50 sec, 60°C (58°C for primer F840 and R1412) for 50 s, 72°C for 1 min and an extension at 72°C for 8 min. The 50 μL reaction volume included 4.0 μL (50 ng uL^{-1}) template, 25 μL 2X Taq PCR MasterMix (Beijing Tianwei Biology Technique Corporation, China), 18 μL ddH_2O and 1.5 μL primers (10 pmol μL-1) of each. PCR amplification was detected by 1% agarose gel and gels were both visualized on Gel DocTMEQ170-8060 and photographed. The PCR products were purified and were sequenced by Shanghai Ying Jun Biology Technique Corporation, China.

The polymorphism information content (PIC): The PIC was calculated according to Bolstein:

$$PIC = 1 - (\sum_{i=1}^{n} P_i^2) - (\sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2)$$

where, i and j were the allele gene, Pi and Pj were the frequency of allele gene and n was the number of allele genes. We regarded PIC > 0.5 as indicating a high level of polymorphism, 0.25PIC < 0.5 as indicating a medium level of polymorphism and PIC < 0.25 as indicating a low level of polymorphism.

Statistical analysis: Sequences were edited and aligned by DNAstar package (DNASTAR Inc.) and the nucleotide variations in the analyzed segments were exported by using MEGA 2.1. Data were analyzed with chi-squared test of SAS (Statistics Analysis System Inst. Inc., Cary NC, USA).

RESULTS

The direct DNA sequencing method was used for the detection of nucleotide sequence polymorphism in code region of the chicken MC1R gene. The target fragment of gene was amplified and denatured. Twenty mutations were detected in this study, including site 27, 28, 46, 69, 85, 159, 212, 274, 376, 398, 409, 427, 636, 637, 649, 659, 834, 837, 906, 919 in DNA sequence of chicken (Accession No: D78272). C27G and G28C were only existed in black Rock Cornish, 46R was founded in red jungle fowl, a C906T was only existed in Cao Ke chicken, T69C, C212T, A274G, G636A, T637C and A644C were major mutations in MC1R gene and existed nearly all

Table 1: The relations between the MC1R genotype distributions with plumage color trait among domestic chickens[1]

SNPs	Geno type	Plumage color class: Black	Spot-yellow	Yellow	Wild	White	Total	Chi-Square test
T69C	TT	21(0.55)	11(0.31)	1(0.10)	4(0.17)	14(0.64)	51(0.40)	X^2=20.8232
	TC	6(0.16)	9(0.26)	5(0.50)	8(0.35)	5(0.23)	33(0.26)	p=0.0076
	CC	11(0.29)	15(0.43)	4(0.40)	11(0.48)	3(0.13)	44(0.34)	
C212T	CC	24(0.63)	17(0.49)	1(0.10)	3(0.125)	7(0.32)	52(0.41)	X^2=37.9681
	CT	4(0.11)	8(0.23)	0(0.00)	0(0.000)	4(0.18)	16(0.12)	p<0.0001
	TT	10(0.26)	10(0.28)	9(0.90)	20(0.875)	11(0.50)	60(0.47)	
A274G	AA	30(0.79)	28(0.80)	2(0.20)	8(0.35)	21(0.95)	89(0.70)	X^2=37.6601
	AG	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	p<0.0001
	GG	8(0.21)	7(0.20)	8(0.80)	15(0.65)	1(0.05)	39(0.30)	
G636A	GG	25(0.66)	35(1.00)	4(0.40)	9(0.39)	16(0.72)	89(0.70)	X^2=44.0924
	GA	8(0.21)	0(0.00)	6(0.60)	5(0.22)	3(0.14)	22(0.17)	p<0.0001
	AA	5(0.13)	0(0.00)	0(0.00)	9(0.39)	3(0.14)	17(0.13)	
T637C	TT	23(0.60)	35(1.00)	4(0.40)	10(0.43)	15(0.68)	87(0.68)	X^2=42.5558
	TC	9(0.24)	0(0.00)	6(0.60)	4(0.17)	3(0.14)	22(0.17)	p<0.0001
	CC	6(0.16)	0(0.00)	0(0.00)	9(0.40)	4(0.18)	19(0.15)	
A644C	AA	32(0.84)	12(0.34)	9(0.90)	21(0.92)	15(0.68)	89(0.69)	X^2=31.9956
	AC	3(0.08)	11(0.32)	1(0.10)	1(0.04)	3(0.14)	19(0.15)	p<0.0001
	CC	3(0.08)	12(0.34)	0(0.00)	1(0.04)	4(0.28)	20(0.16)	

[1]: The figures in table indicate the number of chickens

Table 2: The relations between the MC1R genotype distributions with skin color trait among domestic chickens[1]

SNPs	Geno type	Skin color class: Black	White -yellow	Total	chi-square test
T69C	TT	34(0.61)	17(0.24)	51(0.40)	X^2=29.2668
	TC	15(0.27)	13(0.18)	28(0.22)	p<0.001
	CC	7(0.12)	42(0.58)	49(0.38)	
C212T	CC	29(0.52)	23(0.32)	52(0.41)	X^2=8.6779
	CT	9(0.16)	7(0.10)	16(0.12)	p=0.0131
	TT	18(0.32)	42(0.58)	60(0.47)	
A274G	AA	50(0.89)	40(0.56)	90(0.70)	X^2=17.1688
	AG	0(0.00)	0(0.00)	0(0.00)	p<0.0001
	GG	6(0.11)	32(0.44)	38(0.30)	
G636A	GG	39(0.70)	50(0.69)	89(0.70)	X^2=2.4622
	GA	12(0.21)	10(0.14)	22(0.17)	p=0.2920
	AA	5(0.09)	12(0.17)	17(0.13)	
T637C	TT	38(0.68)	49(0.68)	87(0.68)	X^2=4.4508
	TC	13(0.23)	9(0.13)	22(0.17)	p=0.1080
	CC	5(0.09)	14(0.19)	19(0.15)	
A644C	AA	40(0.71)	49(0.68)	89(0.69)	X^2=0.7749
	AC	9(0.16)	10(0.14)	19(0.15)	p=0.6788
	CC	7(0.13)	13(0.18)	20(0.16)	

[1]: The figures in table indicate the number of chickens

Table 3: Average polymorphism information content of each locus

Sites	Plumage color class: Black	Spot-yellow	Yellow	Wild	White	PIC
T69C	0.494	0.565	0.774	0.776	0.367	0.5952
C212T	0.443	0.525	0.174	0.218	0.535	0.3790
A274G	0.321	0.309	0.309	0.774	0.093	0.3612
636A	0.356	0.000	0.422	0.625	0.309	0.3424
T637C	0.400	0.000	0.422	0.605	0.367	0.3588
A644C	0.203	0.625	0.093	0.127	0.367	0.2830

Note: PIC>0.5 as indicating a high level of polymorphism, 0.25 < PIC < 0.5 as indicating a medium level of polymorphism, and PIC<0.25 as indicating a low level of polymorphism

domestic breeds. Forty-five populations contained heterozygosis sites. Three kinds of genotypes were observed in each site. Amino acid sequence analysis showed that 13 amino acid substitutions were found.

The MC1R polymorphism was mainly related to the plumage color trait. According to plumage color of chicken, total samples was divided into 5 types including Black, Spot-yellow, Wild, Yellow and White chickens. The MC1R genotype distribution with each class of plumage color was investigated to evaluate the role of this gene for plumage color. A chi-squared test on the allele frequencies for five plumage color class was performed using SAS ver. 8.1. The result showed that there was significant difference in plumage color trait among the three genotypes ($p < 0.05$) for site T69C, C212T, A274G, G636A, T637C and A644C (Table 1). In site T69C, the frequency of TT genotype in black plumage color chicken was higher than that in spot-yellow, yellow and wild populations and was lower than that in white chicken. In site C212T, the frequency of CC genotype of black plumage color chicken was higher than that in others plumage color. In site A274G, the frequency of AA genotype in black plumage color chicken was higher than that in yellow and wild populations and was lower than that of spot-yellow and white chicken. In site G636A, the frequency of GG genotype in black plumage color chicken was higher than that in yellow and wild populations and was lower than that in spot-yellow and white chicken. In site T637C, the frequency of TT genotype in black plumage color chicken was higher than that in yellow and wild populations and was lower than that in spot-yellow and white chicken. In site A644C, the frequency of AA genotype in black plumage color chicken was higher than that in spot-yellow and white chicken and was lower than that in yellow and wild populations.

According to skin color, total samples was divided into 2 types including Black and White (contain Yellow) chicken. The MC1R genotype distribution with

each class of skin color was investigated to evaluate the role of this gene for skin color. A chi-squared test on the allele frequencies for two skin color class showed that there was significantly difference in the skin color traits among three genotypes ($p < 0.05$) for site T69C, C212T and A274G and there was no significant in the skin color traits among three genotypes for site G636A, T637C and A644C (Table 2). In site T69C, the frequency of TT genotype in black skin chicken was higher than that in white and yellow chickens. In site C212T, the frequency of CC genotype in black skin chicken was higher than that in white and yellow chicken. In site A274G, the frequency of AA genotype in black skin chicken was higher than that in white and yellow chicken population.

According to plumage color of chicken, 5 plumage color classes were obtained in this study, the polymorphism information content was calculated in every plumage color class for each site (Table 3). Statistic result showed that T69C belong to high level of polymorphism, C212T, A274G, G636A, T637C and A644C were regarded as at a medium level of polymorphism.

DISCUSSION

The study of candidate genes is one of the primary methods to determine whether specific genes are related to economic traits in farm animals. In addition, the population used for the candidate gene approach does not need specified design. The MC1R as a candidate gene for plumage color and skin traits in chicken was examined by DNA sequencing and twenty SNPs were detected in code region. Amino acid sequence analysis showed that Gly29Arg (site 85) was only existed in silkie. It guessed this site was relation with the activity of MC1R and the forms of melanin. Multi-amino acid substitution could affect the structure and the activity of MC1R gene. Therefore, the functions of the MC1R gene needs further study.

Skin pigmentation is a polygenic multi-factorial trait determined by the cumulative effects of multiple genetic variants and environmental factors. Melanocortin-1 Receptor (MC1R) is one of the genes involved in pigmentation and has been implicated in the red hair and pale skin phenotype in human Caucasoid individuals (Niamh *et al*., 2000). Classic genetics have established 8 alleles at the Extended black locus in chicken, which is assumed to correspond to the Extension locus in mammals. In animals, there was no single MC1R allele that was found consistently carrying dominant black and neither MC1R nor a cosegregated with dominant black (Kerns *et al*., 2003). Hosoda *et al*. (2005) reported that independent nonframe shift deletions in the MC1R gene were not associated with melanistic coat coloration in 3 Mustelid lineages. After mass SNP analyze, MC1R gene was found that it wasn't the principal factor affecting the coat color differences of Chinese native pig breeds (Shi *et al*., 2004).

PIC was an ideal index for detecting the allele polymorphism and it was also a reflection for the level of gene mutation. This study showed that the PIC of C212T, A274G, G636A, T637C and A644C was regarded as at a medium level of polymorphism and the PIC of T69C was considered as at a high level of polymorphism. Higher PIC indicates higher heterozygosis within animal population, resulting in more genetic variation, which is favorable for the genetic improvement of the relevant traits. So, it was possible that these sites are used as genetic markers in broiler breeding programs in form of molecular-assisted selection.

MC1R polymorphism was mainly correlated with the plumage and skin color traits. This study showed that there was significant difference in plumage color trait among the three genotypes ($p < 0.05$) for site T69C, C212T, A274G, G636A, T637C and A644C and there was significant different in skin color trait among the 3 genotypes for site T69C, C212T and A274G ($p < 0.05$). The frequency of TT genotype in site T69C, the frequency of AA genotype in site A274G, the frequency of GG genotype in site G636A, the frequency of TT genotype in site T637C and the freguency of AA genotype in site A644C in black plumage color were higher. Although, it in white plumage color chicken was highest in site T69C and A274G and it in spot-yellow plumage color chicken was highest in site G636A and T637C and it in wild plumage color chicken was highest in site A644C respectively, the number of black plumage color population of 6 genotypes in 6 sites was the biggest in all plumage color class. The frequency of CC genotype in black plumage color population was highest in C212T. The frequency of TT genotype in site T69C, the frequency of CC genotype in C212T and the frequency of AA genotype in site A274G in black skin population were higher that in white-yellow populations, meanwhile, the number of black skin population of 3 genotypes in 3 sites was the biggest in all skin class, respectively. So, it concluded this 3 mutations (T69C, C212T and A274G) may be association with the function of MC1R and C212T mutation was a relatively ideal molecular marker in selection scheme. AG genotype in all plumage color class populations was absent in site A274G, CT genotype in yellow and wild chickens was absent site C212T, AA genotype in spot-yellow and yellow chickens and GA genotype in spot-yellow chickens were absent in site G636A, CC genotype in spot-yellow and yellow chickens

and TC genotype in spot-yellow chickens were absent in site T637C, CC genotype in yellow chickens was absent in site A644C. The absent of these genotype maybe the result of nature selection or the small number of samples or the biological function of MC1R gene, it needs further study.

CONCLUSION

Based on the results obtained in this study, the authors of this study concluded that the plumage and the skin color traits of the chickens were affected by the single nucleotide variation in code region of MC1R gene. The MC1R gene is, therefore, useful as a potential marker in the marker-assisted selection programs. However, before MC1R gene could be applied in commercial chicken breeding practice, further study using selection experiment is needed.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Bo Xia for his help in managing the birds and collecting the data. This work was financially supported by Bio-Tech Programs of Sichuan Province, China (05NG002-017).

RERERENCES

Crepaldi, P., M. Marilli, D. Meggiolaro, F. Fornarelli, C. Renieri, E. Milanesi and P. Ajmone-Marsan, 2003. The MC1R gene polymorphism in some cattle breeds raised in Italy. Pigm. Cell Res., 16 (5): 578.

Hosoda, T., J.J. Sato, T. Shimada, K.L. Campbell and H. Suzuki, 2005. Independent Nonframeshift Deletions in the MC1R Gene Are Not Associated with Melanistic Coat Coloration in Three Mustelid Lineages. J.Hered., 96 (5): 607-613.

Kerje, S., J. Lind, K. Schutz, P. Jensen and L. Andersson, 2003. Melanocortin 1-Receptor (MC1R) mutations are associated with plumage colour in chicken. Anim. Genet., 34(4): 241-248.

Kerns, J.A., M. Olivier, G. Lust and G.S. Barsh, 2003. Exclusion of melanocortin-1 Receptor (MC1R) and agouti as candidates for dominant black in dogs. J. Hered., 94 (1): 75-79.

Kijas, J.M.H., R. Wales, A. Tornsten, P. Chardon, M. Moller and L. Andersson, 1998. Melanocortin Receptor 1 (MC1R) Mutations and Coat Color in Pigs. Genetics, 150: 1177-1185.

Klungland, H. and D.I. Vage, 2003. Pigmentary switches in domestic animal species. Ann. N. Y. Acad. Sci., 94: 331-338.

Ling, M.K., M.C. Lagerstrom, R. Fredriksson, R. Okimoto, N.I. Mundy, S. Takeuchi and H.B. Schioth, 2003. Association of feather colour with constitutively active melanocortin 1 receptors in chicken. Eur. J. Biochem., 270 (7): 1441-1449.

Mundy, N.I., N.S. Badcock, T. Hart, K. Scribner, K. Janssen and N.J. Nadeau, 2004. Conserved genetic basis of a quantitative plumage trait involved in mate choice. Science, 303: 1870-1873.

Mundy, N.I., 2005. A window on the genetics of evolution: MC1R and plumage colouration in birds. Proc. Biol. Sci., 272 (1573): 1633-1640.

Niamh, F., H. Eugene, R. Amanda, P. Sion, T. Carole, J.J. Lan, A. Mark and L.R. Jonathan, 2000. Pleiotropic effects of the Melanocortin 1 Receptor (MC1R) gene on human pigmentation. Hum. Mol. Genet., 9 (17): 2531-2537.

Schioth, H.B., S.R. Phillips, R. Rudzish, M.A. Birch-Machin, J.E.S Wikberg and J.L. Rees, 1999. Loss of function mutations of the human melanocortin 1 receptor are common and are associated with red hair. Biochem. Biophys. Res. Commun., 260: 488-491.

Shi, K., A. Wang, N. Li and X. Deng, 2004. Single nucleotide polymorphism analysis on Melanocortin Receptor 1 (MC1R) of Chinese native pig. Sci. (China) C., 47 (3): 287-292.

Takeuchi, S., H. Suzuki, S. Hirose, M. Yabuuchi, C. Sato, H. Yamamoto and S. Takahashi, 1996. Molecular cloning and sequence analysis of the chick melanocortin 1-receptor gene. Biochim. Biophys. Acta., 1306: 122-126.

Theron, E., K. Hawkins, E. Bermingham, R. Ricklefs and N.I. Mundy, 2001. The molecular basis of an avian plumage polymorphism in the wild: a point mutation in the melanocortin-1 receptor is perfectly associated with melanism in the bananaquit (Coereba flaveola). Curr. Biol., 11: 550-557.