

## Identification of single nucleotide polymorphisms in Melanocortin 1 Receptor Gene of Iraqi chicken breeds and their effects on protein structure

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**Abstract:** Melanocortin 1 Receptor (*MC1R*) gene plays important roles in regulating biosynthesis of eumelanin (black/brown) and pheomelanin (red/yellow) pigments in a wide range of mammals, reptiles, and birds. In this study, the sequence diversity of a partial coding region of *MC1R* was investigated in Iraqi chicken breeds. After DNA extraction from blood samples of 95 chickens, PCR amplification was performed for a 681-bp fragment of *MC1R* and the products were sequenced to detect single nucleotide polymorphisms (SNPs). A total of four SNPs were detected including two synonymous (D78272:g.1094A>G and D78272:g.1292C>T) and two nonsynonymous mutations (D78272:g.915A>G and D78272:g.1095C>T). The A/G allele frequencies were 0.92/0.08 and 0.95/0.05 in D78272:g.915A>G and D78272:g.1095C>T mutations, respectively. In addition, the C/T allele frequencies were 0.94/0.06 and 0.96/0.04 in D78272:g.1095C>T and D78272:g.1292C>T mutations, respectively. The amino acid substitution of isoleucine to valine in D78272:g.915A>G SNP had no considerable effect on physicochemical properties of *MC1R* protein. However, a significant effect of cysteine to arginine substitution in D78272:g.1095C>T mutation was predicted on some physicochemical properties (isoelectric point, net charge, and hydrophobic index) of the translated protein. These results imply that the mutation D78272:g.1095C>T probably affect the function of *MC1R* protein and that this polymorphism can be used as a useful molecular marker for identification of plumage color in Iraqi native chickens.

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### Introduction

Identification of genetic markers associated with phenotypic differences is important for chicken breed identification (Hoque et al., 2013). The melanocortin 1 receptor gene (*MC1R*) play important roles in regulating biosynthesis and distribution of eumelanin (black/brown) and pheomelanin (red/yellow) in a wide range of mammal, reptile, and avian species (Cheviron et al., 2006; Dobson et al., 2012). Sequence variation in *MC1R* is associated with plumage polymorphism in several species of birds including the domestic chicken, *Gallus Gallus* (Andersson, 2003). In the chicken, the *MC1R* gene is located on chromosome 11 and encodes a 314 amino acid protein in only one exon (Kerje et al., 2003). *MC1R* encodes a seven pass transmembrane G protein coupled receptor for  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH). After binding to ligands of  $\alpha$ -MSH and, *MC1R* causes a change of the G-protein coupling the receptor from inactive guanosine diphosphate (GDP) to active form guanosine triphosphate (GTP). GTP subsequently stimulates Adenylate Cyclase to raise the level of the second messenger cAMP. The cAMP finally stimulates production of the melanogenic enzyme

tyrosinase and increased production of eumelanin. When *MC1R* is in its inactive state, the biogenesis of tyrosinase reduces resulting in expression of pheomelanin in melanocytes (Huang et al., 2014).

During the last two decades, the study and knowledge of *MC1R* polymorphism and its role in plumage color has increased considerably, because this gene provides a useful genetic marker identification of breeds/subspecies in mammals and birds (Dávila et al., 2014). The human cutaneous pigmentation (e.g. skin, hair and eye) has been reported to be controlled by about 120 genes and *MC1R* has a role key in this process (Dessinioti et al., 2011). By 2013, a total of 16 polymorphisms for *MC1R* have been identified in seven species, including pig (5), dog (3), sheep (2), cattle (2), arctic fox (2), horse (1), and red fox (1), that are located in extracellular (6), transmembrane (5) or intracellular (5) domains (Switonski et al., 2013). Single nucleotide polymorphisms (SNPs) that result in amino acid substitutions in *MC1R* have been identified in association with color polymorphisms in several wild and domestic species of mammals and birds (Guernsey et al., 2013). The association between *MC1R*

polymorphism and plumage coloration has been also reported in pigeons, where three SNPs (namely G199A, G225A, and A466G) were detected from *MC1R* gene, one of which was synonymous and the two others were identified to result in amino acid substitution (Asp67Asn and Thr156Ala)(Ran et al., 2016). The genetic variation of a 741 bp fragment of the *MC1R* gene domestic geese with five plumage color patterns have been investigated and five synonymous SNPs have been identified by Huang et al. (2014). Rahayu et al. (2015) used SNP to study the genetic variation in *MC1R* gene sequence in Magelang duck populations and found two nonsynonymous SNPs on a 256-bp fragment of the gene(Rahayu et al., 2015). In mice, chicken and the bananaquit (*Coereba flaveola*), one SNP, causing a guanine (G) to adenine (A) transition at site 274, and consequently, a glutamate to lysine substitution at amino acid position 92 of *MC1R* is responsible for plumage/peleage pigmentation (Takeuchi et al., 1996; Theron et al., 2001). In Korean native chickens, nine SNPs have been detected from *MC1R* gene, three and six of which were synonymous and nonsynonymous, respectively (Hoque et al., 2013). A total of 11 SNPs, including nine nonsynonymous and two synonymous variants, have been detected from *MC1R* gene of Spanish chicken breeds (Dávila et al., 2014). In other studies, six nonsynonymous mutations were identified in association with eumelanin and pheomelanin pigmentation in chicken (Kerje et al., 2003).

In this study, we sequenced partial coding region (a 681-bp fragment) of the *MC1R* gene in Iraqi native chicken breeds to test for possible diversity. Iraqi native breeds have a diverse plumage color patterns and polymorphism in the *MC1R* gene was hypothesized to be the major cause of this phenotypic diversity. We also studied the potential impact of amino acid substitutions, resulted from synonymous polymorphisms, on the structure and physicochemical properties of MC1R protein.

## Materials and methods

### *Animals*

Blood samples were collected from the external jugular vein of chickens belonging to Iraqi native chicken breeds. All samples used in this study were obtained from Abu Ghraib's research station (Baghdad, Iraq) and stored at -20 °C for DNA extraction. This study was approved by the Institutional Animal Care and Use Committee of Tehran University.

### *DNA extraction and amplification*

Genomic DNA was extracted from whole blood samples using Phenol method (Miller et al., 1988) and stored at -20 °C until being used for PCR amplification. Based on the chicken *MC1R* gene

sequence present in the Genbank with accession number of D78272.1, one pair of primers (F:5'-TGTCATCGACATGCTCATCTGC-3' and R:5'-CATCCACCCATCTGTTTGTCCATC-3') was designed to amplify a 681-bp fragment of the coding region of chicken *MC1R* gene using polymerase chain reaction (PCR). The PCR was performed in a 30 µL reaction mixture, containing 15 µL of PCR Master Mix, 1 µL of each primer, 2 µL of DNA and 11 µL of nuclease free water. The PCR temperature profiles consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 63 °C for 30 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. Electrophoresis of PCR products was performed in 1% (w/v) agarose gel in parallel with 100 bp DNA marker, in 1x TAE buffer at a fixed voltage of 90 V for 30 min. After ethidium bromide staining, the products were visualized by ultraviolet transillumination.

### *Sequencing and Analysis*

All PCR products were subjected to sequence analysis using ABI 3730 XL DNA Analyzer (BioNer, Daejeon, South Korea). The chicken *MC1R* nucleotide sequences were aligned using ClustalW program available in Bioedit software and then, SNPs were determined and each animal was genotyped for SNPs. Genotypic, allelic frequencies and Hardy-Weinberg equilibriums were estimated with use of GenA1Ex 6.41 software.

### *Protein sequence and structural analysis*

In order to analyze the sequence and structure of proteins, the sequence of chicken mRNA translated from *MC1R* was recaptured from NCBI GenBank databases (GenBank accession No. D78272.1). BioEdit software was used for inducing the mutations and nucleotide sequences of translated proteins. Also, we used GPMW and ProtParam tools available in ExPASy website for analyzing the possible impact of amino acid substitutions on physicochemical parameters of the chicken MC1R proteins. Furthermore, I-TASSER software was used for designing the three-dimensional (3D) structure of the MC1R protein.

## Results and Discussion

The PCR resulted in amplification of a 681-bp DNA fragment as a partial coding region of the *MC1R* gene, indicating that our selected primers had appropriate quality and specificity for amplification of this fragment in domestic chicken. Sequences of PCR amplicons from the whole samples were analyzed and compared to NCBI reference sequence (accession no. D78272.1) using Bioedit software. We detected four single nucleotide mutations in *MC1R* gene of Iraqi chicken breeds, including D78272:g.915A>G, D78272:g.1094A>G, D78272:g.1095C>T and

D78272:g.1292C>T (Fig. 1). The allele frequency of these mutations in the studied population has been shown in Table 1.



Fig. 1. Comparative alignment of conceptualized nucleotide sequence of MC1R gene in Iraqi native chicken with NCBI reference sequence D78272.1.

These results showed that none of them had high genetic diversity within chicken MC1R gene in analyzed populations (Table 1). However, the two SNPs, D78272:g.915A>G and D78272:g.1095C>T had slightly more genetic diversity in comparison with D78272:g.1094A>G and D78272:g.1292C>T mutations (Table 1). A/G allele frequencies were 0.92/0.08, and 0.95/0.05 in D78272:g.915A>G and D78272:g.1095C>T mutations, respectively. In addition, C/T allele frequencies were 0.94/0.06 and 0.96/0.04 in D78272:g.1095C>T and

D78272:g.1292C>T mutations, respectively (Table 1). The Chi square ( $\chi^2$ ) test of Hardy-Weinberg equilibrium (HWE) showed that the D78272:g.1094A>G and D78272:g.1095C>T mutations were in HWE ( $P>0.05$ ), whereas, D78272:g.915A>G and D78272:g.1292C>T mutations showed significant deviation from HWE ( $P<0.01$ ) (Table 1). The chicken *MC1R* gene has only one exon and is located on chromosome 11 and encodes a 314 amino acid protein (Kerje et al., 2003; Takeuchi et al., 1996).

Table 1: Allele frequencies of the MC1R gene and the test Hardy-Weinberg for level of significance of the deviation within population

The position of SNP			Allele						value (HWE)
	A	G	A	G	C	T	C	T	
<b>D78272:g.915A&gt;G</b>	0.92	0.08							p<0.05
<b>D78272:g.1094A&gt;G</b>			0.95	0.05					p<0.05
<b>D78272:g.1095C&gt;T</b>					0.94	0.06			p<0.05
<b>D78272:g.1292C&gt;T</b>							0.96	0.04	p<0.05

This gene influences the induction of tyrosinase, an enzyme that stimulates the synthesis of pheomelanin and eumelanin pigments (Hearing and Tsukamoto, 1991). The eumelanin synthesis will increase upon the activation of the *MC1R* receptor,

while pheomelanin is produced when the expression of *MC1R* is low (Robbins et al., 1993; Schiöth, 2001; Schiöth et al., 1999). It has been reported that *MC1R* gene is mainly involved in melanogenesis and has crucial key in hair and skin color in humans and

hair/plumage color in animals. Given the close association between the genetic diversity of *MC1R* and plumage color of birds, the genetic diversity of *MC1R* has been the subject of several studies in recent years. Several SNPs in the chicken *MC1R* gene are associated with different phenotypes, ranging from the dominant extended black to the recessive yellow (Kerje et al., 2003; Takeuchi et al., 1996). One of the SNPs of *MC1R* gene (69T>C) has different genotype distribution in different breeds; TT genotype is prevalent in black Korean native chicken (56.7%) and black silky (80%), while the CC genotype is common in yellow Korean native chicken (80%), red Korean native chicken (66.7%), and white Leghorn (100%)

chicken breeds (Hoque et al., 2013). In this SNP, black feathered chicken breeds (black Korean native chicken and black silky breed) have high frequencies of the T allele, while the C allele was in high frequency in the other breeds that have other feather colors (red and yellow Korean native chicken and white Leghorn breed). According to our sequence analysis, two out of four SNPs detected in Iraqi chicken *MC1R* were nonsynonymous (Table 2). They include D78272:g.915A>G and D78272:g.1095C>T in which, an isoleucine to valine substitution and a cysteine to arginine substitution was detected, respectively (Table 2).

Table 2: The effect of nucleotide substitutions on amino acid of chicken *MC1R* protein.

Nucleotide substitution	Position	Changed amino acid	The type of SNP
A/G	D78272:g.915A>G	Isoleucine converted into a valine	nonsynonymous
A/G	D78272:g.1094A>G	Alanine	synonymous
C/T	D78272:g.1095C>T	Cysteine converted into an arginine	nonsynonymous
C/T	D78272:g.1292C>T	Asparagine	synonymous

The two other SNPs (D78272:g.1094A>G and D78272:g.1292C>T) were synonymous. We simulated the three-dimensional structure of *MC1R* translated protein (Fig. 2) and predicted the possible impacts of amino acid substitution on physicochemical parameters of the chicken *MC1R* gene protein using GPMW and ProtParam tools.

According to the results, one of the detected SNPs (D78272:g.915A>G) did not have significant impacts on physicochemical parameters of *MC1R* including isoelectric point, net charge, and hydrophobic index, probably because these amino acids belong to a non-polar group (Table 3). Indeed, substitution of cysteine, as a polar and uncharged amino acid with arginine, as a polar and charged amino acid, can result in increased isoelectric point (from 9.68 to 9.79) and net charge (from +6 to +7) and decreased hydrophobic index (from 0.88 to 0.85) (Table 3). Therefore, these results imply that polymorphism probably affects the function of *MC1R* protein, and suggest that it can be used as a molecular marker for identification of plumage color in chicken.

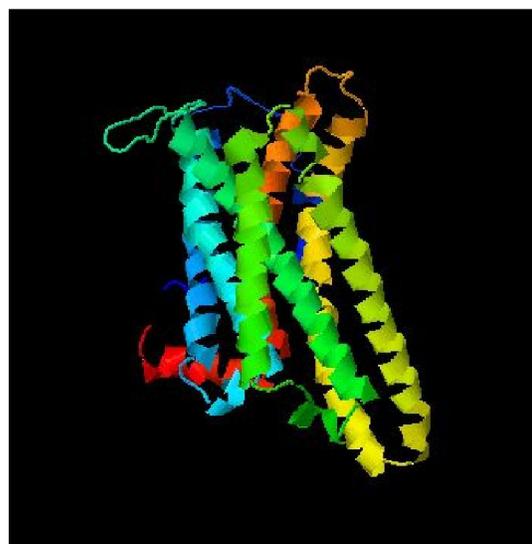


Fig. 2. the three-dimensional (3D) structure of the *MC1R* protein.

Table 3: The effect of amino acid substitutions on physicochemical parameters of chicken *MC1R* protein

Physicochemical parameters	Amino acid substitutions					
	D78272:g.915A>G		D78272:g.1095C>T			
	Isoleucine	>	Valine	Cysteine	>	Arginine
Isoelectric point	9.68	>	9.68	9.68	>	9.79
Net charge	+6	>	+6	+6	>	+7
Hydrophobic index	0.88	>	0.87	0.88	>	0.85

Certain amino acids with hydrophobic side (R) group tend to pack in the interior of globular protein in order to avoid the aqueous environment at exterior surface. Hoque et al., (2013) identified two non-synonymous SNPs in chicken (Val126Ile and Ala143Thr). Dávila et al., (2014) found 11 SNPs for *MC1R* gene in Spanish breeds of chickens, two of which were synonymous (C69T and C834T) and others were nonsynonymous (T212C, G274A, G376A, T398AC, G409A, A427G, C637T, A644C, and G646A). The nonsynonymous mutations correspond to amino acid changes of Met72Thr, Glu92Lys, Val126Ile, Leu133GlnPro, Ala137Thr, Thr143Ala, Arg213Cys, His215Pro, and Val216Ile. Takeuchi et al. (1996a, b) determined a nonsynonymous SNP (Glu92Lys) in *MC1R* that leads an active receptor to produce eumelanin. According to Kerje et al. (2003), this SNP is necessary, and of course sufficient, to discriminate between the extended black and the buttercup phenotypes in domestic chicken.

### Conclusion

Altogether, results of the current study revealed four single nucleotide polymorphisms in *MC1R* gene of Iraqi chicken breeds. The two SNPs D78272:g.1094A>G and D78272:g.1292C>T were synonymous, while the two others D78272:g.915A>G and D78272:g.1095C>T were nonsynonymous. The cysteine to arginine amino acid substitution resulted from D78272:g.1095C>T SNP had significant effect on some physicochemical properties (isoelectric point, net charge, and hydrophobic index) of MC1R protein. These results imply that this SNP probably affects the function of MC1R protein, suggesting that this polymorphism can be used as a molecular marker for plumage color in chicken. A more detailed study is required to investigate the genetic diversity of Iraqi chickens and its association with plumage color.

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