Association of MC4R Gene Polymorphisms with Growth and Body Composition Traits in Chicken

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ABSTRACT : Genetic and pharmacological studies in mice have demonstrated a complementary role for the melanocortin 4 receptor (*MC4R*) in the control of food intake, energy balance and body weight. This study was designed to investigate the associations of a *MC4R* gene polymorphism on chicken growth and body composition traits in broiler lines divergently selected for abdominal fat. A SNP (G54C) was found in CDS region of chicken *MC4R* gene. The analysis of the least squares and variance revealed a significant association between the G54C SNP and BW, CW and SL at 7 wk of age, and there were significant differences in different genotypes ($p<0.05$). The results from protein secondary structure prediction and tertiary structure prediction showed that it appeared a helix in 13^{th} amino acid and two strands at 14^{th} and15^{th} amino acid in mutant protein, respectively. It maybe induce the change of the activity or function of *MC4R* gene in poultry. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 6 : 763-768)***

Key Words : Melanocortin 4 Receptor, SNP, Body Weight, Body Composition, Chicken

INTRODUCTION

The central melanocotinergic system in rodent is an important regulator of feeding behavior and energy homeostasis, and consists of pro-opiomelanocortin (*POMC*), agouti-related protein (*AGRP*), neuropeptide Y (*NPY*) and melanocortin receptors (*MCRs*) (Cone, 1999; Schwartz et al., 2000). This unique system is comprised of peptides synthesized by two distinct populations of neurons in the arcuate nucleus of the hypothalamus that collectively exert both agonistic and antagonistic effects on hypothalamic melanocortin receptors that participate in food intake and body weight regulation (Strader et al., 2003). All melanocortin receptors belong to the G protein coupled receptor superfamily. *MCRs* have seven transmemebrane domains and mainly couple with the cAMP signal transduction pathway (Roger et al., 1996). Five melanocortin receptor subtypes (*MCR1~5*) have been identified in rat and human (Vergoni and Bertolini, 2000; MacNeil et al., 2002) thus far. The melanocortin pathways have functions of inhibiting food intake, increasing the energy expenditure and decreasing the energy homeostasis (Baskin et al., 1999; Mizuno et al., 1999). Evidences suggest that the hypothalamic melanocortin system is directly influenced by *leptin* to regulate food intake and body weight (Hwa et al., 2001). Genetic and pharmacological studies in mice have demonstrated a complementary role for the melanocortin 4 receptor (*MC4R*) in the control of food intake, energy balance and body weight (Schwartz et al., 1996). The development of obesity in *MC4R* -deficient mice and the stimulation of feeding upon blockade of the *MC4R* suggest that the *MC4R* could be an important mediator of *leptin*'s effects on food intake (Kask et al., 1998). The animals, having the deficiencies of *leptin* or *leptin*-signaling pathways, showed the reduction of mRNA expression of *POMC* gene and elevation of mRNA expression of *AGRP* gene. *POMC*- and *AGRP*-containing neaurons have been demonstrated to regulate energy homeostasis through modulation of melanocortin receptors. The *MC4R* appears critical to normal body weight homeostasis because its targeted deletion results in obesity and hyperphagia (Hagan et al., 1999). Null mutations in the pro-opiomelanocortin (*POMC*) gene and the *MC4R* gene, or overexpression of the melanocortion receptor antagonists agouti and agouti-related protein (*AGRP*) caused a severe obesity syndrome in mice and humans (Vaisse et al., 1998, 2000; Hinney et al., 1999; Ho et al., 1999; Butler et al., 2000).

There are two basic methods of Quantitative trait loci (QTLs) identification: the candidate gene approach and whole-genome linkage-disequilibrium scanning (Rothschild and Soller, 1997; Ikeobi et al., 2002; Kim et al., 2005). The candidate gene approach is a powerful method for finding QTLs responsible for genetic variation in the traits of interest in agricultural animal species and determining whether specific genes are related to economic traits in farm animals (Rothschild and Soller, 1997; Li et al., 2003). Many studies have been involved in the fields of association analysis between candidate gene SNPs with animal growth and body composition traits (Jiang et al., 2002b; Li et al., 2003; Zhang et al., 2005; Chung et al., 2005; Meng et al., 2005; Xu et al., 2005). The objectives of the present study were to identify the SNPs of the chicken *MC4R* gene, develop the PCR-SSCP methods to genotype the polymorphisms of the individuals in the broiler lines that

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Received August 18, 2005; Accepted January 9, 2006

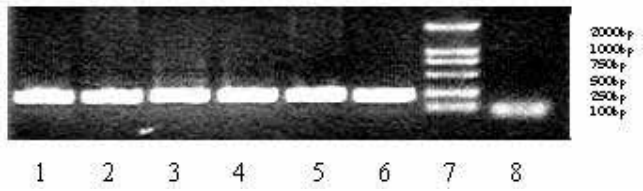

Figure 1. PCR Products. Lane 1, 2, 3, 4, 5 and 6: PCR products of different individuals; Lane7: DL 2,000 marker; Lane 8: Negative control.

divergent selection for abdominal fat and evaluate the association between *MC4R* gene polymorphism and growth and body composition traits.

MATERIALS AND METHODS

Experimental animals

The Northeast Agricultural University divergent lines of lean and fat broilers, derived from a commercial Arbor Acres grandsire line, were used. The population of the sixth generation of those two lines was used in the present study, and totally 214 birds were investigated.

Birds management and trait measurements

Birds were raised according to the conventional program of commercial broiler. Body weight and body composition traits were measured at 7 wk of age. These traits included body weight (BW), carcass weight (CW), shank length (SL), abdominal fat weight (AFW), heart weight (HW), liver weight (LW), spleen weight (SW), muscular stomach weight (MSW) and glandular stomach weight (GSW). Abdominal fat percentage (AFP) was calculated (abdominal fat weight expressed as percentage of AFW to BW at 7 wk of age).

Development of PCR-SSCP assays and screening the population

Genomic DNA was isolated from venous blood and stored in ethylenediaminetetraacetic acid (EDTA). The PCR primers (MC4R1 F 5'-GAA TTT CAC CCA GCA TCG-3', MC4R1 R 5'-GAG GTT CTT GTT TTG GCT AT-3') were designed to amplify a 220 bp fragments in the CDS region according to the chicken *MC4R* DNA sequence (Accession No. AB012211). PCR conditions were 94°C for 7 min, 35 cycles of at 94°C for 30 sec, 55°C for 30sec, 72°C for 45 sec, and an extension at 72°C for 10 min. The 10 μl reaction volume included 25 ng of template, 1×PCR reaction buffer, 2.5 pmol of each primer, 20 mM dNTP and 0.5 U Taq polymerase. A PCR of DNA from each bird was performed according to the condition described above. Mix 3 μl PCR products with 8 μl loading buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA, 10% glycerol), then denature the mixture in 98°C for 10 min, place on ice for 5 min, then electrophoresed for 17 h at 10 V/cm on a 16% PAG with 3% glycerol. Silver stain method was developed to display the bands. Individual PCR-SSCP banding patterns were determined under visible light.

Statistical analysis

The association between the genotypes of *MC4R* gene and the traits was analyzed using the GLM program of JMP (SAS Institute Inc., 2002). The model was fitted with the genotype (G), line (L) as fixed effects; sire nested within line (s (L)), dam nested within line and sire (d (L (S))) as random effects; and BW at 7 wk of age (BW7) as a covariate. The full model was as follows:

$$Y = \mu+G+L+BW7+s\ (L)+d\ (L\ (S))+e \qquad (1)$$

Where Y is the dependent variable, μ is population mean and e is the random error. The interaction of G and L was not significant for all traits, therefore was not included in the model. Significant differences between least squares means of the different genotypes were calculated using a contrast test (JMP 4.0, SAS Institute Inc. 2000). Significant level was set to be 0.05. The additive and dominance effects of genotype were estimated according to formulas as follow:

$$\text{Additive} = (BB-AA)/2$$

$$\text{Dominance} = AB-(AA+BB)/2$$

Here AA, AB and BB are the least squares means of AA, AB and BB genotype groups.

The percentage contribution of *MC4R* gene to the total phenotypic variance of traits was estimated by using MTDFREML package (Boldman et al., 2002). The full model was as follows:

$$Y = \mu+a+G+L+BW7+s\ (L)+d\ (L\ (S))+e \qquad (2)$$

In this model, a is the residual minorpolygene effects, other effects are same to those of model (1).

RESULTS

PCR-SSCP analysis

The PCR-SSCP method was developed successfully for screening the individuals of the population. PCR products (220 bp) were same as expected (Figure 1). The polymorphism resulted in three genotypes defined as AA, AB and BB (Figure 2).

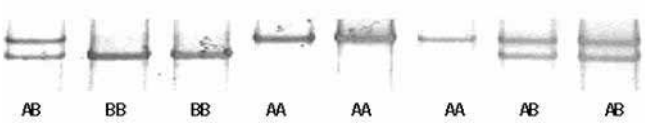

Figure 2. SSCP analysis on PCR amplification with primer in different individuals.

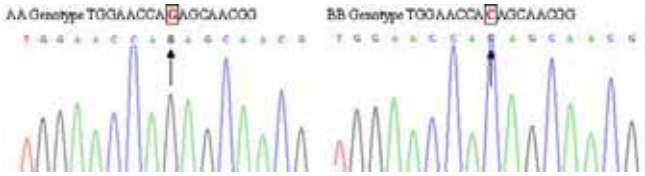

Figure 3. The mutant in CDS 54 nucleotide acid between AA and BB genotype.

Cloning and sequencing

The homozygous individuals of different genotypes were cloned and sequenced (Figure 3). Results showed that there was a G54C mutation in CDS region of *MC4R* gene (Accession No. AB012211). This base mutation is a sense mutation and leads to transforming Gln into His at 18th amino acid of mutant protein.

Association of MC4R gene SNP with growth and the body composition traits

The least square analysis showed that the *MC4R* gene SNP was mainly related to growth and skeletal traits such as BW, CW and SL ($p<0.05$). However, there were no significant associations between the genotype and other body composition traits, such as AFW, AFP, HW, LW, SW, MSW and GSW ($p>0.05$) (Table 1).

Birds with BB genotype had significantly higher BW and CW than birds with AB genotype ($p<0.05$). Birds with BB genotype had significantly higher SL than AA genotype birds ($p<0.05$) (Table 2).

Table3. Effects of MC4R gene on BW, CW and SL

Contribute ratio	Traits[1]		
	BW	CW	SL
Heredity contribute ratio (%)	12.02	11.90	26.97
Phenotype contribute ratio (%)	2.80	2.87	4.47

[1] BW = body weight; CW = carcass weight; SL = shank length.

The contribution of QTL marked by MC4R SNP to phenotypic variance of traits

The percentage of genetic and phenotypic variance in the divergent lines and fat broilers population that was determined by *MC4R* gene polymorphisms was calculated for growth and skeletal traits (BW, CW and SL) (Table 3). The results indicated that *MC4R* gene had effects to genetic and phenotypic variance of BW, CW and SL. The effects of G54C polymorphism accounted for 12.02%, 11.90% and 26.97% of genetic variance, respectively. And the effects of G54C polymorphism accounted for 2.80%, 2.87% and 4.47% of phenotypic variance, respectively. It was speculated that the *MC4R* gene was a potential marker for use in molecular-assisted selected programs to obtain more genetic advance in BW, CW and SL.

Protein secondary structure prediction caused by G54C mutant

A sense mutant of G54C in CDS of *MC4R* gene induced the changes of amino acids codon resulting in transforming Gln (Q, AA genotype) into His (H, BB genotype) at 18th amino acid of mutant protein. The protein secondary structure prediction was analyzed by nnPredict software (www.cmpharm.ucsf.edu/~nomi/nnpredict.html), and the results showed that a helix and two strands appeared at 13th and 14th, 15th amino acids of mutant protein, respectively (Figure 4). The spatial configuration of protein was changed in mutant, which maybe resulted in different biological function between wild and mutant. The multifunction sites play very important roles related to the gene function. The

Table1. Effects of genotypes on growth and body compositions traits in chicken

Traits[1]	BW (g)	CW (g)	SL (cm)	AFW (g)	AFP (%)	HW (g)	LW (g)	SW (g)	MSW (g)	GSW (g)
P	0.0291	0.0302	0.0061	NS[2]	NS	NS	NS	NS	NS	NS

[1] BW = body weight; CW= carcass weight; SL = shank length; AFW = abdominal fat weight; AFP = abdominal fat weight expressed as percentage of AFW to BW at 7 wk of age; HW = heart weight; LW = live weight; SW = spleen weight; MSW = muscular stomach weight; GSW = glandular stomach weight.

[2] NS: No significant at $p>0.05$.

Table2. Effects of different genotypes on BW, CW and SL

Genotype	Number	Traits[1]		
		BW	CW	SL
AA	41	2,557.8±39.59 $^{a\,b}$	2,313.5±36.4$^{a\,b}$	9.146±0.071 ^{b}
AB	101	2,497.1±25.7 ^{b}	2,254.6±23.8 ^{b}	9.168±0.046 $^{a\,b}$
BB	72	2,589.4±32.1^{a}	2,337.0±29.7 ^{a}	9.365±0.057^{a}
Additive		15.8	11.75	0.219
Dominance		-76.5	-70.65	-0.0875

[a, b] Means within a column with no common superscript are different ($p<0.05$).

[1] BW = body weight; CW = carcass weight; SL = shank length.

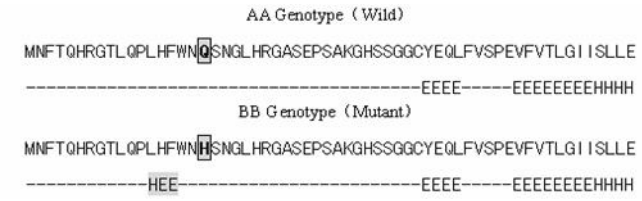

Figure 4. The results of protein secondary structure prediction of wild and mutant.

protein motifs were analyzed by PredictProtein software (www.embl-heidelberg.de/predictprotein/predictprotein.html), and the results showed no difference in protein between wild and mutant. The prosites in current study include Asn_Glycosylatio (NFTQ, N**Q**SN/N**H**SN, NGSE), Protein kinase C phosphorylation site (SAK, TVK, TFK), Casein kinase II phosphorylation site (SLLE, SIID, TFKE), N-myristoylation site (GLHRGA, GVIITC), Prokaryotic membrane lipoprotein lipid attachment site (GVIITCIWAAC), Microbodies C-terminal targeting signal (GKY) and Protein coupled receptors signature (ASICSLLSIAVDRYFTI).

Protein tertiary structure prediction of G54C mutant

The protein tertiary structure prediction of wild and mutant were analyzed by Swiss-Model software (www.expasy.ch/swissmod/SWISS-MODEL.html), and the results showed that a helix and two strands appeared at 13th (white arrow), 14th and15th (green arrow) amino acids of mutant protein, respectively (Figure 5).

DISCUSSION

Effects of MC4R gene genotypes on traits

Body weight : Growth is under complex genetic control, and uncovering the molecular mechanism of growth will contribute to more efficient selection for growth in broiler chickens (Deeb and Lamont, 2002). Hypothalamic *MC4R* is responsible for food intake and body weight. Some studies have proved the association between SNPs of *MC4R* gene and growth traits in mammals. Frameshift and nonsense mutations resulting in the expression of a nonfunctional *MC4R* are associated with a dominant form of obesity in humans (Vaisse et al., 1998). The hypothalamic *MC4R* system is tonically active as *MC4R* knockout mice and a frame-shift mutation in the *MC4R* in humans lead to both hyperphagia and obesity (King et al., 2000). The current study was aimed to analyze the association between SNPs of *MC4R* gene with growth and body composition traits. The results revealed significant associations between the G54C SNP and body weight and carcass weight of broiler at 7 wk of age, and there were significant differences in different genotypes ($p<0.05$). Birds with BB genotype had significantly higher body weight and carcass weight than

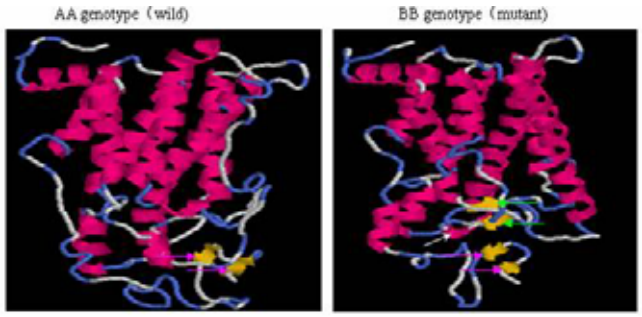

Figure 5. The comparison of protein tertiary structure prediction of MC4R gene between wild and mutant. White arrow denotes helix and green arrow denotes strands in mutant protein; Pink arrow denotes strands at same position in wild and mutant protein.

birds with AB genotype at 7 wk of age ($p<0.05$), and the effects of *MC4R* gene accounted for 12.02% and 11.9% of the genetic variance of BW and CW, respectively. The results point to the possible identification of *MC4R* gene as a marker for the selection of body weight and carcass weight in chickens.

Skeleton : Leg problems are a serious issue in current broiler commercial production, resulting from the lack of coordination of development and growth between whole body mass and the skeleton system (Julian et al., 1998). Increasing bone strength and keeping proper skeletal proportions could increase bird welfare and production efficiency in breeding of heavy-bodied chickens. Several studies proved that *MC4R* gene had effects on bone formation in mammals. The *MC4R* knockout mouse exhibits increased linear growth (Huszar et al., 1997) and mutations resulting in defective *MC4R* alleles in humans result in increased bone mineral density (Steppan et al., 2000). An obese animal model, the *MC4R*-/- mouse, in which the *CART* (Cocaine Amphetamine Related Transcript) pathway is intact, exhibits a high bone mass phenotype and a reduction in osteoclast surface (Robert, 2005). Expression of *MC4R* mRNA in developing rat limb buds, teeth, and skull bone first indicated a possible role for *MC4R* in bone metabolism (Dumont et al., 2005). The results from the current study revealed significant associations between the G54C SNP and shank length of broiler at 7 wk of age, and there were significant differences in different genotypes ($p<0.05$). Birds with BB genotype had significantly higher shank length than birds with AA genotype at 7 wk of age, and the effects of *MC4R* gene accounted for 26.97% of the genetic variance of SL. Considering the current results, *MC4R* gene might affect the bone development of chicken, and maybe a good candidate QTL controlling skeleton length in chickens.

Protein structure prediction

A base mutant in CDS region of a gene maybe result in the alteration of protein configuration, and subsequently the

function of the gene may be changed. *MC4R* gene belongs to G protein coupled receptors and plays a very important role in signal conduction (Roger, 1996; Vergoni, 2000; Macneil, 2002). A SNP (Asp298Asn) causing an amino acid substitution in the porcine *MC4R* gene was significantly associated with increased levels of back fat and influenced body weight and food intake (Kim et al., 1998). The results showed that a mutant of G54C in CDS of *MC4R* gene leads to transforming Gln into His at 18th amino acid of mutant protein. The results from protein secondary structure prediction (Figure 4) and tertiary structure prediction (Figure 5) showed that it appeared a helix at 13th amino acid and two strands at 14th and15th amino acid in mutant, respectively. Although there were no differences in the predict results of protein motifs between wild and mutant, the change of the protein secondary structure prediction and tertiary structure prediction of *MC4R* gene maybe affect the protein's activity or function of *MC4R* gene in chicken.

In summary, according to the results of current research, we presumed that *MC4R* gene maybe have important role in the regulation of body weight and skeletal development in chicken. The *MC4R* gene may be a major gene influencing the development of body weight and shank length or linked to QTLs affecting these traits, and is therefore as a potential marker for using in molecular MAS programs.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the members of the Poultry Breeding group of the college of Animal Science & Technology in the Northeast Agricultural University for help in managing the birds and collecting the data. This research was supported by National Natural Science Foundation (No. 30270950), National Natural Science Foundation Key Project (30430510) and the National High Technology Research and Development Project of China (No. 2002AA211021).

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