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Three novel *MC4R* SNPs associated with growth traits in Hu sheep and East Friesian × Hu crossbred sheep

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ABSTRACT

The melanocortin-4 receptor (MC4R) is a G-protein coupled receptor superfamily member, which has been implicated in regulating food intake, energy balance and body weight in mammals. Though the mutations of *MC4R* have been identified as genetic markers for growth traits in many livestock species, little is known about sheep *MC4R* gene. In this study, *MC4R* gene was amplified and sequenced to screen single nucleotide polymorphism sites (SNPs) and three novel SNPs (g.306 G>A and g.706 C>A in the coding region and g.1267 G>A in the 3′-UTR) were detected. The genetic structure of the three SNPs were examined by PCR-RFLP and CRS-PCR-RFLP, respectively, in 298 individuals of two sheep populations (Hu sheep, Hu; East Friesian × Hu crossbred sheep, EH). Association analysis between three SNPs and body traits revealed that two of the SNPs were associated with body weight, except for g.306 G>A mutation; and all three loci were significantly associated with body size. Haplotype blocks showed that there were none loci linked within Hu nor EH *MC4R* gene, but both generated principally seven haplotypes. Moreover, H1H3 (GA-CC-GA) diplotype had better performance than other diplotypes in Hu sheep ($P < 0.05$), while all of diplotypes had no significant difference in EH sheep ($P > 0.05$). These preliminary results indicate that three novel *MC4R* SNPs are related to the growth development, which may be used as genetic markers for sheep breeding and potentially afford a good foundation for further study on *MC4R* gene.

1. Introduction

The melanocortin-4 receptor (MC4R), a G protein-coupled receptor with seven transmembrane domains, is expressed predominantly in the brain. It is involved in the control of energy homeostasis, food intake and body weight (Ellacott and Cone, 2004; Balthasar et al., 2005). It is well known that fed conditions can stimulate the expression of pro-opiomelanocortin (POMC) which modulates energy homeostasis principally through one of its cleavage products, the naturally occurring MC4R agonist, α-melanocyte-stimulating hormone (α-MSH) (Emeson and Eipper, 1986). The MC4R-signaling regulate hypothalamic energy balance by combining to α-MSH or the endogenous antagonist Agouti-related peptide (AgRP) (Tao, 2010; Mul et al., 2012). The balance between the effects of these two neuropeptides on MC4R activation

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is critical. MC4R leads to a reduction of food intake and an elevation of energy expenditure thus resulting in negative energy balance and body weight loss (Chen et al., 2000), emphasizing its importance in controlling body weight.

Huszar et al. (1997) found that *MC4R* gene knockout mice appeared Agouti obesity syndrome, such as maturity onset obesity and insulin hypersecretion. Since the first cases of obesity due to *MC4R* mutations were reported in 1998 (Yeo et al., 1998), more than 150 different *MC4R* variants have been described (Vaisse et al., 2000; Farooqi et al., 2003; Lubrano-Berthelier et al., 2003). Several SNPs in the coding region of bovine *MC4R* gene have been detected by PCR-SSCP or PCR-RFLP (Haegeman et al., 2001; Thue et al., 2001; Valle et al., 2004). Moreover, mutations of *MC4R* gene associated with growth traits in cattle were detected, such as body weight and daily gain (Zhang et al., 2009). In pigs, Kim (2000) found that a *MC4R* missense mutation Asp298Asn significantly associated with feed intake, backfat thickness and growth rates. In Hu sheep, four *MC4R* SNPs in the 3′-UTR of *MC4R* were also identified (Song et al., 2012). Numerous studies also have revealed that SNPs of *MC4R* gene were associated with physical and qualitative traits in chicken (Huo Mingdong and Hui, 2006; Yan et al., 2013) and rabbits (Jiang et al., 2008). The role of MC4R in feed intake and obesity in many livestock species suggests that it may be an important genetic marker for the growth-related traits in the sheep. What is more, sequence analysis presented a high degree of similarity among sheep, cattle, human and mouse *MC4R* gene, which indicates that this gene may have played an important biological role during evolution.

Although sheep *MC4R* gene has been cloned, which is located at chromosomes-23 containing only a single exon and encoding 332 amino acids, relationship between the polymorphism of *MC4R* gene and growth traits related research is still less, except for the research by Song et al. (2012). Hu sheep are widely raised in the Taihu Lake area of China, and they are well known for the excellent traits, e.g., beautiful wavy lambskins, early sexual maturity, immediate growth and high fecundity (lambing rate up to 250%). East Friesian is a long established milking breed originated in the Friesland/Ost Friesland area in the north of Holland and Germany. In China, East Friesian has been applied to promote the milking performance of Hu Sheep by hybridization to form East Friesian × Hu crossbred sheep (EH), a potential dual-purpose (meat- and milk- type) new sheep variety. In the present study, SNPs of *MC4R* gene were detected in 298 individuals including Hu and EH, and their associations with sheep growth traits were analyzed. The results indicated that the three novel *MC4R* SNPs may be used as genetic markers for sheep breeding and potentially afford a good foundation for further study on *MC4R* gene.

2. Materials and methods

2.1. Animals and data collection

Genomic DNA samples ($n = 298$) were collected from two sheep populations (strains): Hu sheep (Hu, $n = 168$); East Friesian × Hu crossbred sheep (EH, $n = 130$). These two groups are reared in Shanghai Yonghui Sheep Industry Co., Ltd. Hu and EH were weaned at 2th month of age and then fed with a concentrate and straw diet *ad libitum*. The body weights were recorded at 2, 4, 6th month of age, and other growth traits, e.g., body height (BH), body length (BL), chest circumference (ChG), chest depth (ChD), hip width (HW), rump height (RH) and cannon circumference (CaC) were recorded at 4th month. Genomic DNA was extracted by the traditional phenol/chloroform method from ear tissue according to standard procedures (Mullenbach et al., 1989), dissolved in TE solution, and stored at −20 °C.

2.2. SNPs identification and genotyping

One pair of primers was designed based on sheep *MC4R* gene (NC_019480) using the Primer 5.0 software (Premier Company, Canada) to amplify *MC4R* gene (1375 bp). The primer sequences were as follows: forward, 5′-TCCAAGTGATGCCGACCAG-3′; and reverse, 5′-CTGGGCACTGCTTCACATC-3′. Polymorphisms were detected by DNA pool sequencing. Briefly, five DNA pools, each containing five Hu DNA samples, were created, respectively to be used as templates for PCR amplification.

PCR were carried out on Gradient Thermal Cycler (Applied Biosysterms, Carlsbad, CA, USA) using 10 μM of each primer 1 μL, 2.5 mM of dNTP mixture 4 μL, 5 × PrimeSTAR Buffer (Mg^{2+} *plus*) 10 μL, 5 μL of genomic DNA as template and 2.5 U/μL of PrimeSTAR HS DNA polymerase (MBI, Fermentas) 0.5 μL, with a final reaction volume of 50 μL. Cycling conditions were as follows: 94 °C for 5 min, 35 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at annealing temperatures (see Supplemental Table 1) and extension for 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. PCR products were sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

Based on the sequencing results, PCR restriction fragment length polymorphism (PCR-RFLP) method was used to genotype g.306 G>A and g.1267 G>A. Created restriction site PCR restriction fragment length polymorphism (CRS-PCR-RFLP) method was utilized to genotype g.706 C>A. The PCR products were digested with restriction enzyme *Msp*I (NEB Company, USA), *Kpn*2I (PureOne Biotechnology, China) and *Nde*I (Beyotime Biotechnology, China), respectively. The reaction system was as follows: 5 μL of each PCR product was mixed with 1.6 μL 10× buffer, 0.5 μL restriction enzyme and 8.9 μL water. Final solution was incubated at 37 °C for 3 h, following the supplier's manual, then separated on 3.0% agarose gel. The sequences obtained were edited and aligned by the DNASTAR Lasergene v7.1 (DNASTAR, Inc., Madison, WI, USA) to search for SNPs.

2.3. Statistical analysis

The allele and genotype frequencies in two populations were estimated according to the standard procedure. The genetic indexes including gene heterozygosity (*He*), effective allele numbers (*Ne*) and polymorphism information content (*PIC*) were calculated by POPGENE software Ver. 32 (Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Canada). The formulas were as follows:

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

$$He = 1 - \sum_{i=1}^{n} P_i^2, \qquad Ne = \frac{1}{\sum_{i=1}^{n} P_i^2}$$

where P_i and P_j is the frequency of the ith and jth allele, respectively, and n is the number of alleles. Haplotype and linkage disequilibrium (LD) block measured by D' and r^2 were performed with the HAPLOVIEW software Ver. 4.2 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Least squares analysis of variance technique was employed to study the effects of genotypes and haplotype associations on growth traits within two sheep populations ($n = 298$). The effects of genotypes of each SNP on the traits were evaluated using a single-marker-lined model association analysis. Statistical analysis was performed using GraphPad Prism software (Ver. 5.0) from GraphPad Software, Inc.

3. Results

3.1. Identification of SNPs polymorphism and genotyping

The whole length of sheep *MC4R* was investigated and three novel SNPs were identified after sequencing