DNA polymorphisms of the Hu sheep melanocortin-4 receptor (MC4R) gene associated with birth weight and 45d-weaning weight

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ABSTRACT. The melanocortin-4 receptor (MC4R) has important roles in regulating food intake, energy balance, and body weight in mammals. In pigs and cattle, MC4R mutations have been identified as genetic markers for growth and traits. Compared with abundant research conducted on other livestock species, little is known about mutations of the ovine MC4R gene. We investigated the affect of MC4R polymorphisms on birth weight and 45d-weaning weight in 144 Hu sheep. Four single nucleotide polymorphisms (SNPs; g.1016 G/A, g.1240 T/C, g.1264 G/A, and g.1325 A/G) were identified in the 3’-untranslated region (UTR) of Hu sheep MC4R by PCR-single-strand conformation polymorphism and DNA sequencing. A haplotype block, containing g.1240 T/C, g.1264 G/A, and g.1325 A/G, was constructed within the Hu sheep MC4R gene. Four SNPs were found to be significantly associated with 45d-weaning weight, while the haplotype block was significantly associated with birth weight. Hu sheep with the genotypes GG in g.1016 G/A or with the genotype CCAAGG in the haplotype block, had higher 45d-weaning weights. We
conclude that these 4 SNPs of the MC4R gene have potential as genetic markers for early growth traits in Hu sheep.

Key words: MC4R gene; Polymorphism; Early growth traits; Hu sheep

INTRODUCTION

The melanocortin-4 receptor (MC4R), a Rhodopsin-like G protein-coupled receptor, is primarily expressed in the neural system and is involved in the regulation of food intake, energy balance, and body weight in mammals. Reports regarding spiny dogfish and goldfish MC4R have demonstrated that the central melanocortin system evolved early in vertebrate evolution for the regulation of energy homeostasis, implying that MC4R was involved in important early physiological processes (Cerda-Reverter et al., 2003a,b; Ringholm et al., 2003; Govaerts et al., 2005). Therefore, MC4R is of considerable interest among candidate genes for controlling variation in energy metabolism and growth traits. In 2004, over 60 nucleotide variations were identified from various pig-like species separated by several million years of the evolution (Kim et al., 2004). Some of these variations appear to have been preserved during the diversification in the Sus scrofa species. The pattern of these variations provides insight into the genetic diversity of mammalian MC4R (Kim et al., 2004).

To date, 58 frame shift mutations in MC4R significantly associated with human obesity have been reported (MacKenzie, 2006). It has been accepted that the MC4R mutations are the most common single gene that causes non-syndromic human obesity. In livestock species, MC4R mutations have also been identified as genetic markers for growth and fatness traits (Kim et al., 2000; Haegeman et al., 2001; Bruun et al., 2006). The pig quantitative trait locus (QTL) analyses have supported the hypothesis of the MC4R gene as a positional candidate gene for the fatness and fleshiness QTL (Bruun et al., 2006).

Compared with the abundant research in human beings, pigs, dogs, and other mammals, little is reported regarding the details of the ovine MC4R gene (Lubrano-Berthelier et al., 2003a,b; Stinckens et al., 2009; Skorczyk et al., 2007, 2012). Hu sheep, a famous Chinese sheep breed, are distributed in Zhejiang and Jiangsu Provinces and the suburbs of Shanghai in China. As a result of a long process of acclimatization and artificial selection, Hu sheep are well recognized for their beautiful wavy lambskins, early sexual maturity, seasonal breeding, fast growth, prolificacy and the adaptability to a hot and humid climate. Adult Hu sheep weights can be reached at the age of 12 months; the adult rams weigh 47-65 kg, and adult sheep weigh 32-44 kg (Chen, 1984; Pan et al., 1987). In this study, we identified the polymorphisms of the MC4R gene in a Hu sheep meat line used to produce heavy sheep, and evaluated their association with birth weight and 45d-weaning weight of Hu Sheep.

MATERIAL AND METHODS

Experimental animals

The Hu sheep meat line was established from purebred sheep at the Yuhang Hu Sheep Breeding Farm in Hangzhou, China. Venous jugular blood samples (10 mL per sheep, using acid citrate dextrose as an anticoagulant) were collected from 144 Hu sheep. Genomic DNA
was extracted from whole blood by the phenol-chloroform method as described by Sambrook and Russell (2001), then dissolved in TE buffer [10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and kept at -20°C. The birth weight and 45d-weaning weight data from 144 Hu sheep were recorded for further analysis.

**Primers, PCR amplification, genotyping, sequencing, and analysis of mutations**

One pair of primers, SMC4RF and SMC4RR, was designed according to conserved sequences of *MC4R* multiple sequence alignment (GenBank accession Nos. NM_001126370 and NC_007325) to amplify the full-length *MC4R* gene of Hu sheep. Subsequently, 8 pairs of primers for single-strand conformation polymorphism (SSCP) were designed to detect single nucleotide polymorphisms (SNPs) in *MC4R* (primers listed in Table 1). All DNA variants were located by Mutation Surveyor™ v4.0.4 (SoftGenetics, LLC, State College, PA, USA).

PCR was carried out in a 25 µL volume containing approximately 2.5 µL 10X PCR buffer (Mg²⁺), 200 µM of each dNTP, 10 pM of each primer, 2 U TransTaq HiFi DNA polymerase (Transgene, Beijing, China), and 50 ng sheep genomic DNA as a template. Amplification conditions were as follows: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54.8-66°C (listed in Table 1) for 30 s, extension at 72°C for 30 s with extension at 72°C for 10 min, then holding at 4°C on a Mastercycler 5333 (Eppendorf AG, Hamburg, Germany).

A 5-µL volume of PCR product was transferred to an Eppendorf tube, mixed with 10 µL gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mM EDTA, pH 8.0, and 10% glycerol. The mixture was centrifuged and denatured at 99°C for 10 min, then chilled on ice for 30 min and loaded onto 15% neutral polyacrylamide gels (acrylamide:bisacrylamide = 39:1). Electrophoresis was performed in 1X Tris borate (pH 8.3)-EDTA buffer at 9-15 V/cm at 4°C overnight. After electrophoresis, the DNA fragments in the gels were visualized by silver staining, then photographed. Finally, the PCR products with different electrophoresis patterns were purified for sequencing.

**Statistical analysis**

Gene frequencies were determined for each locus by direct counting. Allele frequencies and polymorphism information content (PIC) were derived using the Population Genetic Analysis package (POPGENE; Version 1.31; Molecular Biology and Biotechnology Centre, University of Alberta: Edmonton, Canada) (Krawczak et al., 2006). Haplotype and linkage disequilibrium (LD) blocks analysis were performed by HaploView version 4.2 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The haplotype block definition was followed according to that of Gabriel et al. (2002). They define pairs to be in “strong LD” if the 1-sided upper 95% confidence bound of D’ is > 0.98 (that is, consistent with no historical recombination) and the lower bound is above 0.7.

All loci were tested for deviations from Hardy-Weinberg equilibrium by means of a standard chi squared test. The additive effect of SNPs was estimated as the difference between the mean of the 2 homozygotes divided by 2, and dominance was estimated as the deviation of the heterozygote from the mean of the 2 homozygotes (Falconer and Mackay, 1996).

The relationship between the different genotypes of each SNP and birth weight or 45d-
weaning weight least squares means was evaluated using a single-marker lined-model association analysis. Data were analyzed by fitting a general linear model using the restricted maximum likelihood method in the Statistical Package for the Social Sciences (SPSS; Version 13.0; SPSS Inc., Chicago, IL, USA). The statistical analyses model included fixed effects of SNP genotype, sib number, birth weight, 45d-weaning weight, and sex traits. The following 2 fixed effects models were employed for analysis of birth weight or 45d-weaning weight in Hu sheep, and least squares means were used for multiple comparisons of birth weight or 45d-weaning weight among different genotypes. The general model used for early growth traits was as Equation 1, where:

\[ Y_{ijkl} = \mu + M_j + S_k + N_l + e_{ijkl} \quad \text{(Equation 1)} \]

\[ Y_{ijklm} = \mu + A_j + D_k + S_l + N_m + e_{ijklm} \quad \text{(Equation 2)} \]

where: \( Y_{ijkl} \) or \( Y_{ijklm} \) was the phenotypic value of birth weight or 45d-weaning weight observed value; \( \mu \) was the least square mean; \( M_j \) was the fixed effect of the \( j \)th genotype (3 levels); \( A_j \) was the fixed effect of the \( j \)th additive effect (3 levels); \( D_k \) was the fixed effect of the \( k \)th dominance effect (2 levels); \( S_k \) or \( S_l \) was the fixed is the effect of sex (2 levels); \( N_l \) or \( N_m \) was the effect of sib number (4 levels); \( e_{ijkl} \) or \( e_{ijklm} \) was the random residual effect of each observation. \( A_j = -0.5, 0, \) or \( 0.5 \) if individual \( j \) has genotype AA, AB, or BB, and \( D_k = 1 \) (or -0.5) if individual \( i \) has genotype AB (or others).

RESULTS

PCR-SSCP analysis and genetic polymorphism of the Hu sheep MC4R gene

To identify Hu sheep homolog of the MC4R gene, multiple alignments of sheep (GenBank accession No. NM_001126370) and cow (GenBank accession No. NC_007325) MC4R sequences were carried out (data not shown). Primer pair SMC4R (Table 1) was designed from the conserved regions to amplify the partial sequence of MC4R from genomic DNA of Hu sheep. The amplified products with the expected size were purified and sequenced. Sequence analysis showed that the sequence from Hu sheep (submitted to the National Center for Biotechnology Information (NCBI), GenBank accession No. JQ710684) had high identity with sheep and cow MC4R sequences (99 and 94%, respectively), indicating that the obtained sequence was the MC4R sequence of Hu sheep.

To detect polymorphisms of Hu sheep MC4R, 8 primer pairs (M1-M8) were designed for SSCP analysis. Three genotypes were found when primer pair M6 or M7 was used, but not with other primer pairs, and these genotypes were used for amplification (Figure 1). Among the 3 genotypes, the homozygote AA/CC was defined as the wild genotype, BB/DD as the mutation genotype, while AB/CD was the heterozygous genotype. Each fragment was sequenced and the sequences were analyzed with Mutation Surveyor™ (SoftGenetics, LLC, State College, PA, USA). The results indicated that 4 polymorphisms appeared in the Hu sheep MC4R gene, and all of them were in the MC4R 3′-untranslated region (UTR; Table 2). A G→A mutation, located at +1016 (the first nucleotide of the coding region of p MC4R gene is defined as +1), was found in the PCR fragment amplified by primer pair M6. Three polymorphisms, T→C mutation at +1240, G→A mutation at +1264, and A→G mutation at +1325, were found in the PCR fragments amplified by primer pair M7.
Table 1. Primers used for sequencing and genotyping Hu sheep MC4R gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5ꞌ→3ꞌ)</th>
<th>Location of mutation</th>
<th>Size of PCR products (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC4R</td>
<td>F: 5ꞌ-GCCTAAGATTTCACAGTGATC-3ꞌ</td>
<td>-211-1546</td>
<td>1765</td>
<td>60.0</td>
</tr>
<tr>
<td>M1</td>
<td>F: 5ꞌ-GCCTAAGATTTCACAGTGATGCA-3ꞌ</td>
<td>-211-46</td>
<td>257</td>
<td>60.0</td>
</tr>
<tr>
<td>M2</td>
<td>F: 5ꞌ-GAATCCAAATGAACTCTACCCAGC-3ꞌ</td>
<td>-9-231</td>
<td>240</td>
<td>54.8</td>
</tr>
<tr>
<td>M3</td>
<td>F: 5ꞌ-GGAGAATTATCTGTAGTGCATGGC-3ꞌ</td>
<td>180-449</td>
<td>270</td>
<td>57.5</td>
</tr>
<tr>
<td>M4</td>
<td>F: 5ꞌ-CATTGACTCTTGATCTGACCT-3ꞌ</td>
<td>372-649</td>
<td>278</td>
<td>66.0</td>
</tr>
<tr>
<td>M5</td>
<td>F: 5ꞌ-CATCTGCCCTCATCAACCGTTCT-3ꞌ</td>
<td>582-892</td>
<td>311</td>
<td>64.2</td>
</tr>
<tr>
<td>M6</td>
<td>F: 5ꞌ-CCTGACCTGATCTCCTACCACT-3ꞌ</td>
<td>786-1059</td>
<td>274</td>
<td>62.2</td>
</tr>
<tr>
<td>M7</td>
<td>F: 5ꞌ-GCGAGGCACACCAAGACTCT-3ꞌ</td>
<td>1013-1333</td>
<td>321</td>
<td>62.2</td>
</tr>
<tr>
<td>M8</td>
<td>F: 5ꞌ-CCTGACACATGGATGAAGACT-3ꞌ</td>
<td>1226-1546</td>
<td>321</td>
<td>64.2</td>
</tr>
</tbody>
</table>

Table 2. Sequence comparison among different genotypes.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Genotype</th>
<th>Nucleotide sequence</th>
<th>Location of mutation</th>
<th>Change of nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6</td>
<td>AA</td>
<td>acaactcggctgcaaacac</td>
<td>+1016</td>
<td>G→A</td>
</tr>
<tr>
<td></td>
<td>BB</td>
<td>acaactcggatgcaaacac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>CC</td>
<td>ggtgaTggcactctgatttataaaaaagaa; attacagagacttctgtgaccattt</td>
<td>+1240</td>
<td>T→C</td>
</tr>
<tr>
<td></td>
<td>DD</td>
<td>ggtgaCggactctgatttataaaaaagaa; attacagagacttctgtgacactt</td>
<td>+1264</td>
<td>G→A</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>ggtgaTggcactctgatttataaaaaagaa; attacagagacttctgtgaccattt</td>
<td>+1325</td>
<td>A→G</td>
</tr>
</tbody>
</table>

To determine the genetic characteristics of polymorphisms of the Hu sheep MC4R gene, we calculated the genotypic frequencies, allelic frequencies, and PIC of 4 SNPs in the Hu sheep meat line population. The results are presented in Table 3, which revealed that, in the Hu sheep meat line population, allele A at +1016 locus, and allele TGA at +1240, +1264, and +1325, respectively, were the predominant alleles, which were consistent with the predominant genotypes in the 4 loci. The results of PIC of the 4 loci indicated that the 4 SNPs should be considered as moderate polymorphisms (Table 3). Moreover, the genotype frequencies at all 4 SNPs were in agreement with Hardy-Weinberg equilibrium.
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<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotypes</th>
<th>Genotypic frequencies</th>
<th>Alleles</th>
<th>Allelic frequencies</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.1016 G/A</td>
<td>GG (10)</td>
<td>0.0694</td>
<td>G</td>
<td>0.2639</td>
<td>0.3130</td>
</tr>
<tr>
<td></td>
<td>GA (56)</td>
<td>0.3889</td>
<td>G</td>
<td>0.2639</td>
<td>0.3130</td>
</tr>
<tr>
<td></td>
<td>AA (78)</td>
<td>0.5417</td>
<td>A</td>
<td>0.7361</td>
<td>0.7361</td>
</tr>
<tr>
<td>g.1240 T/C</td>
<td>TTGGAA (78)</td>
<td>0.5417</td>
<td>TGA</td>
<td>0.7292</td>
<td>0.3169</td>
</tr>
<tr>
<td>g.1264 G/A</td>
<td>TCGAAG (54)</td>
<td>0.3750</td>
<td>CAG</td>
<td>0.2708</td>
<td>0.2708</td>
</tr>
<tr>
<td>g.1325 A/G</td>
<td>CCAAGG (12)</td>
<td>0.0833</td>
<td>CAG</td>
<td>0.2708</td>
<td>0.2708</td>
</tr>
</tbody>
</table>

PIC = polymorphic information content.

### Intra-MC4R linkage disequilibrium

To understand the intra-MC4R LD of the 4 SNPs, D', r² and log odds (LOD) scores were estimated for each pair of SNPs in Haploview. The results are shown in schematic Figure 2, where the red diamonds indicate strong LD between pairs of SNPs (D’ > 0.8) with statistical significance (LOD score > 2.0; Barrett et al., 2005). Four SNPs in MC4R were found to be in LD with D’ above 0.75 for each SNP pair combination. Further, r² values between each pair of MC4R SNPs were above 0.5612, while the value between the 3 SNPs in the haplotype block, containing g.1240 T/C, g.1264 G/A, and g.1325 A/G, was 1.

![Figure 2](image.png)

**Figure 2.** Linkage disequilibrium (LD) analyses in the Hu sheep MC4R gene. Pair-wise LD was noted based on r² measurement (number inside the squares).

### Least squares mean and standard error for birth weight or 45d-weaning weight of different MC4R genotypes in Hu sheep

To investigate the effects of Hu sheep MC4R polymorphisms, we analyzed the relation-
DNA polymorphism analysis of Hu sheep *MC4R* gene

The ship between *MC4R* genotypes and their effects on birth weight or weaning weight. The least squares mean and standard error for birth weight or weaning weight of different *MC4R* genotypes in the Hu sheep meat line were calculated with a general linear model (Table 4). The sheep with the GG genotype at +1016 showed higher weaning weight (17.87 ± 2.431 kg) in comparison to the sheep with the AA (14.51 ± 2.214 kg) or GA (14.78 ± 1.964 kg) genotypes (P < 0.01; Table 4); however, the weaning weight of GA genotype and AA genotype sheep showed no significant difference (P > 0.05). These results indicated that the sheep with the GG genotype had a tendency to gain more birth weight or weaning weight than the sheep with GA or AA genotypes.

In other words, 1016G might be the beneficial allele for birth weight or weaning weight. Regarding the haplotype block, the homozygous mutant sheep showed higher weaning weight (17.81 ± 2.172 kg) relative to the homozygous wild type (14.44 ± 2.230 kg) or heterozygous mutant (14.70 ± 1.841 kg) sheep (P < 0.01; Table 4); the weaning weight of homozygous wild type and heterozygous mutant sheep were not significantly different (P < 0.05). These results indicated that homozygous mutant sheep had a tendency to gain more birth weight or weaning weight than the homozygous wild type or heterozygous mutant sheep; the mutant allele in the *MC4R* 3' UTR haplotype block might be a beneficial allele for birth weight or weaning weight.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype means ± SE</th>
<th>Overall P</th>
<th>Additive effect ± SE</th>
<th>Domain effect ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.1016 G/A</td>
<td>GG</td>
<td>3.550 ± 0.946</td>
<td>0.270</td>
<td>0.226 ± 0.145</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>GA</td>
<td>3.072 ± 0.747</td>
<td>14.70 ± 1.841</td>
<td>0.001</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>AA</td>
<td>3.096 ± 0.685</td>
<td>14.51 ± 2.214</td>
<td>0.000</td>
</tr>
<tr>
<td>g.1240 T/C</td>
<td>TTGGAA</td>
<td>17.871 ± 2.431**</td>
<td>0.006</td>
<td>-0.411 ± 0.125*</td>
</tr>
<tr>
<td>g.1264 G/A</td>
<td>TCGAAG</td>
<td>14.776 ± 1.964</td>
<td>17.811 ± 2.172**</td>
<td>0.000</td>
</tr>
<tr>
<td>g.1325 A/G</td>
<td>CCAAGG</td>
<td>14.512 ± 2.214</td>
<td>17.811 ± 2.172**</td>
<td>0.000</td>
</tr>
</tbody>
</table>

According to the definition of presented by Falconer and Mackay (1996), the genetic effects of different *MC4R* genotypes analyzed by a general linear model (Equation 2) are summarized in Table 4. An additive effect was significant or extremely significant at 4 loci, but there was no significant dominance effect. Furthermore, the dominant gene effect was negative, which indicated negative heterosis. The homozygous wild type allele of 1016G and the mutant allele in the haplotype block were significantly associated with an increase in 45d-weaning weight. All absolute values of the additive effect were higher than those of the dominant effect, with the exception of the birth weight of g.1016 G/A. These results indicated that the genetic loci characteristics agreed with the additive-dominance model, and the additive gene effect played a more important role than the dominant gene effect.

**DISCUSSION**

Hu sheep originated from Mongolian sheep; as early as the time of the Song Dynasty.
Mongolian sheep were introduced from the pastoral region of North China to the Taihu lake basin that borders the present provinces of Zhejiang and Jiangsu. As a result of a long process of acclimatization and artificial selection, Hu sheep grow quickly, thus the primary objective of this study was to identify the associations between MC4R SNPs and early growth traits. In the present study, by PCR-SSCP and DNA sequencing, we characterized the 3′-UTR mutations of the MC4R gene in Hu sheep for the first time.

In humans, MC4R represents a compelling biological candidate underlying obesity, as rare coding mutations in the gene are the leading cause of the most common known monogenic obesity disorder (Farooqi et al., 2000). Fifty-eight mutations in the coding sequence (2400 bp) of the human MC4R gene have been demonstrated to be associated with obesity pathogenicity (MacKenzie, 2006). Therefore, this gene has been studied in various mammals, including pig (Kim et al., 2004), cattle (Huang et al., 2010; Liu et al., 2010), dog, and red fox (Skorczyk et al., 2007). Furthermore, the association of MC4R variants with obesity-related quantitative traits has also been extensively studied in many mammals. Several reports presented strong evidence of associations between a porcine MC4R missense mutation, Asp298Asn, and some obesity-related traits, such as fatness, growth rate, and feed intake (Kim et al., 2000; Hernandez-Sanchez et al., 2003; Kim et al., 2004; Jokubka et al., 2006). SNPs of MC4R genes associated with growth traits in cattle were also detected (Zhang et al., 2009). In our study, we reported 4 SNPs in the sheep MC4R gene 3′-UTR (300 bp) for the first time, and demonstrated their significant associations with birth weight and 45d-weaning weight in Hu sheep, which supported the possibility that MC4R SNPs detection could be applied in practical breeding to choose fast-growing individuals to satisfy particular customer requirements.

The 3′-UTR is known to play crucial roles in the post-transcriptional regulation of gene expression, including its nucleo-cytoplasmic transport (Kohler and Hurt, 2007), stability (Borrmann et al., 2001; Kamiyama et al., 2007), translation efficiency (Kindler et al., 2005; Piccone et al., 2009), and subcellular localization (Narsai et al., 2007; Thomsen et al., 2010). Moreover, the binding sequence of molecular regulator miRNA is more often located in the 3′-UTR (Grillo et al., 2010). Hence, we suppose that the mutations in the 3′-UTR of the MC4R gene might modulate the gene expression level by a mechanism that should be addressed further.

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