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# The SNPs of Melanocortin 4 Receptor (*MC4R*) Associated with Body Weight in Beagle Dogs

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**Abstract:** Melanocortin 4 receptor (*MC4R*), which is associated with inherited human obesity, is involved in food intake and body weight of mammals. To study the relationships between *MC4R* gene polymorphism and body weight in Beagle dogs, we detected and compared the nucleotide sequence of the whole coding region and 3'- and 5'- flanking regions of the dog *MC4R* gene (1214 bp). In 120 Beagle dogs, two SNPs (A420C, C895T) were identified and their relation with body weight was analyzed with RFLP-PCR method. The results showed that the SNP at A420C was significantly associated with canine body weight trait when it changed amino acid 101 of the *MC4R* protein from asparagine to threonine, while canine body weight variations were significant in female dogs when *MC4R* nonsense mutation at C895T. It suggested that the two SNPs might affect the *MC4R* gene's function which was relative to body weight in Beagle dogs. Therefore, *MC4R* was a candidate gene for selecting different size dogs with the *MC4R* SNPs (A420C, C895T) being potentially valuable as a genetic marker.

**Key words:** beagle dogs, body weight, *MC4R*, SNP

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## Introduction

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Melanocortin-4 receptor (*MC4R*) plays an important role in the regulation of feeding behavior, body weight and energy homeostasis [2]. As one of five receptors in the melanocortin system, it is encoded by a single exon and expressed in the brain, predominantly in the hypothalamus. Mouse and human genetic studies showed that the mice of *MC4R* knockout displayed extreme obesity [8], and human *MC4R* gene mutations can cause childhood simple obesity [1, 14], which is the most common cause of hereditary human obesity [4]. Over 50 mutations affecting the amino acid sequence of the human *MC4R* gene have been recorded [12], and some mutations were

related to obesity that affect body weight. Body weight is an important economic trait. It has been reported that many significant associations of *MC4R* genotypes with backfat, growth rates and food intake in a number of breeds and lines of the pigs [3, 5, 6, 13]. Recently, some single nucleotide polymorphisms (SNPs) in the canine *MC4R* gene were identified in red fox, Chinese raccoon dog and arctic fox [16], too. However, no SNP that affect the *MC4R* gene function was found in Beagle dogs. In this study, we found two SNPs in *MC4R* genes in the Beagle dogs, and analyzed their relation with dog's body weight.

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**Table 1.** Primers used for PCR amplification of four canine *MC4R* fragments

Primer symbol	Primer sequence (5'-3')	SNP	Amino acid change	Length
MC4R-1	F: CCTAGACTAAAGTTAAGGTGGGA R: AGGACTCAGTGGCGTTGC	NO	NO	432 bp
MC4R-2	F: ACGCCACTGAGTCCCT R: TGTCCGAGTAAATGATGAAC	A420C	N101T	492 bp
MC4R-3	F: CATCATTTACTCGGACAG R: CACCCAGAGGATAGCA	C895T	NO	419 bp
MC4R-4	F: ACTCCATCATCGACCT R: TCTCAACTCCTGCCTAT	NO	NO	232 bp

## Materials and Methods

### Animals

Six beagle dogs from litter mate were housed as a group (males or females) in an air conditioned room (space: 5×5 m, temperature: 22 ± 2°C, relative humidity: 55 ± 10%, light period: 7:00 AM to 7:00 PM) in Laboratory Animal Center of Academy of Military Medical Sciences. Feeding was conducted twice a day at 8:00 AM and at 5:00 PM by providing abundant dog foods. Heparinized blood of 120 beagles (60 females and 60 males, 12–13 months) was collected for isolation of genomic DNA that was used to amplify the *MC4R* gene. The body weight of the dogs was recorded as while as taking blood.

### SNP identification

Primers were designed based on the canine *MC4R* DNA sequence (GenBank BD181636) and the canine *MC4R* gene sequence (GenBank DQ084210). Four sets of PCR primers were designed to cover the whole coding sequence and part of the 3' and 5' flanking regions. The sequences and the length of PCR products were shown in the Table 1. Each PCR reaction contained 100 ng of genomic DNA, 0.2 μM of each primer, 0.2 mM dNTPs, 1× PCR buffer with 1.5 mM MgCl<sub>2</sub> and 1 U Taq polymerase in a total volume of 20 μl. PCR was performed using a Biometra thermocycler. The amplification conditions were 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 52°C (primer pairs 1), 50°C (primer pairs 2), 57°C (primer pairs 3) and 48°C (primer pairs 4) respectively, and 1 min at 72°C, followed by a 10-min final extension at 72°C (Table 1).

The PCR products in two individual dogs with 6.5 and 16 kg were isolated and purified from agarose gel with the use of a Agarose Gel DNA Fragment Recovery Kit (TaKaRa, DaLian, China) and then sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The two single

nucleotide polymorphisms (SNPs) were identified in the sequenced fragments using BLAST, situated in *Eco*0109 I and *Psh*A I restriction enzyme recognition site, respectively (Fig. 1).

### PCR-RFLP

To genotype all the studied animals at two polymorphic sites, PCR-RFLP analyses were performed. *Eco*0109 I and *Psh*A I endonucleases were used. All restriction enzyme digestions were performed in 10 μl reaction mixture containing 4 μl of the PCR products, 1× digestion buffer and 2 U of the respective endonuclease. All digested products were detected by 2% agarose gel electrophoresis.

### Statistical analyses

Allele and genotype frequencies were calculated by a simple allele counting method. The association between the genotypes of *MC4R* candidate gene and body weight traits was evaluated with the least square method (GLM procedure of the SAS software package; SAS Institute, USA) using the following statistical linear model:

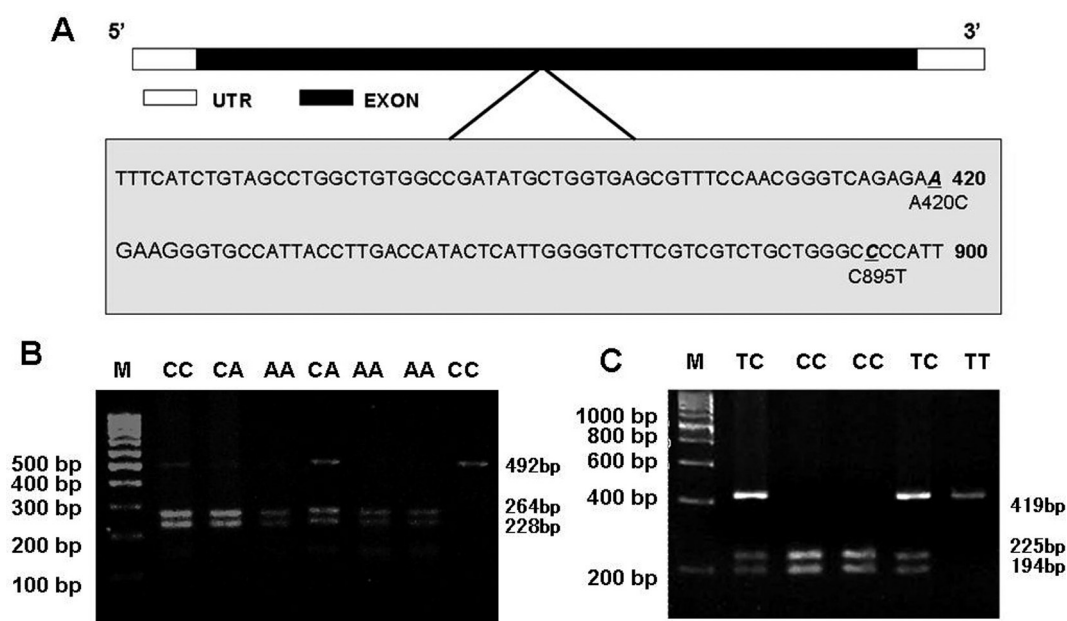
$$Y_{ij} = \mu + X_j + b_{ij} + e_{ij}$$

$Y_{ij}$  is the observation of the carcass traits,  $\mu$  is the mean for the trait,  $b_{ij}$  is the regression coefficients of the weight and  $e_{ij}$  is residual effect.

## Results and Discussion

### Identification of two SNPs in canine *MC4R* gene

Two SNPs were identified by DNA sequencing in the *MC4R* sequences from two individual beagles with minimum and maximum body weight (Table 2), a missense substitution A420C that changes asparagine to threonine at position 101 of the amino acid sequence, and a silent substitution C895T (Fig.1A).



**Fig. 1.** Identification and PCR-RFLP analysis of two SNPs in canine MC4R gene. A. The site of two new SNPs in canine MC4R gene. B. The electrophoresis results of PCR-RFLP test in MC4R A420C site. After *PshAI* digestion, it divided into gene types with CC (492 bp, 1 bands), CA (228 bp, 264 bp, 492 bp, 3 bands) and AA (228 bp, 264 bp, 2 bands). C. The electrophoresis results of PCR-RFLP test in MC4R C895T site. Three genotypes with TT (419 bp, 1 band), TC (194 bp, 225 bp, 419 bp, 3 bands) and CC (194 bp, 225 bp, 2 bands) were identified by *Eco0109* I restriction enzyme.

**Table 2.** Descriptive Statistics of dog body weight

Gender	Number	Body weight (kg)			<i>t</i> -test*
		Minimum	Maximum	Mean $\pm$ SD	
Female	60	6.5	14	8.76 $\pm$ 2.09	<i>P</i> =0.052
Male	60	6.6	16	9.65 $\pm$ 2.84	
Total	120	6.5	16	9.21 $\pm$ 2.53	

\*Difference in female and male dog's body weight.

As one of G protein-coupled receptors (GPCRs), the highly conserved residues in MC4R protein may play important roles for ligand binding or intracellular signal transmission [11]. A multiple alignment of the amino acid sequences of the MC4R proteins from different species showed that the threonine found at the position 101 of the second transmembrane domain was very highly conserved in the MCR proteins (Fig. 2). Although a silent mutation doesn't affect the amino-acid sequence of a protein, it can affect protein folding and function. Kimchi-Sarfaty *et al.* found a such silent mutation that was caused by a synonymous SNP in MDR1 (Multidrug Resistance 1), which encodes the protein pump P-gp (P-glycoprotein) [7]. Therefore, either mis-sense mutation or synonymous mutation in CDS (Coding DNA

Sequence) region of MC4R gene may affect its function, which would present some variant traits among dogs.

#### Association mis-sense SNP of MC4R with canine body weight trait

To investigate the relationships between the two SNPs of MC4R with canine body weight trait, we analyzed different MC4R genotypes in 120 Beagle dogs. Estimated MC4R alleles and genotype frequencies for the Beagles were shown in the Table 3. In A402C SNP site, the frequency for allele A was higher than that for allele C, and 11% and 55% displayed the CC and AA genotype. The C895T frequency for allele C was higher than that for allele T, and 3% and 67% displayed the TT and CC genotype, respectively. In general, body weight of fe-

MC4R_DOG	FI CSLA VADML VSVSNGSET I V I TL	106*
MC4R_HUMAN	FI CSLA VADML VSVSNGSET I V I TL	106
MC4R_MOUSE	FI CSLA VADML VSVSNGSET I V I TL	106
MC4R_BOVIN	FI CSLA VADML VSVSNGSET I V I TL	106
MC4R_PIG	FI CSLA VADML VSVSNGSET I V I TL	106
MC4R_RAT	FI CSLA VADML VSVSNGSET I V I TL	106
MC4R_CHICKEN	FI CSLA VADML VSVSNGSET I V I TL	105
MC1R_HUMAN	FI CCLALSDL L VSGSNVLETAV I LL	100
MC2R_HUMAN	FI CSLA I SDML GSLYKI LEN I L I L	86
MC3R_HUMAN	FL CSLA VADML VSVSNALET I M I AI	137
MC5R_HUMAN	FVCSLA VADML VSMSSAWET I T I YL	98

**Fig. 2.** Multiple amino acid alignments of the putative second transmembrane domain of canine MC4R with other MCRs. \* represented the predicted sequence positions for canine MC4R. The other amino acid sequences were obtained from the GenBank database (Accession Numbers P32245, P56450, Q9GLJ8, O97504, P70596, AAT73771, AAP23196, AAH80622, AAO67714, P41968). The missense variant in canine MC4R substituted amino acid N (Asn) for T (Thr) in the position marked with an arrow. The Thr was highly conserved among different MC4R proteins, and it changed to Asn only in Human MC2R protein.

males was lower than that of males in Beagle dogs. Therefore, statistical analysis in different sex group was also operated in this study, separately.

Association of different *MC4R* genotypes with body weight trait were evaluated with the least square method, and the overall means  $\pm$  standard deviation of the analyzed traits were summarized in the Tables 4 and 5. In the overall analysis, the dogs with homozygous CC genotypes were on average significantly weightier than others, while insignificant differences between the CA and AA genotype dogs. The results suggested that the mis-sense substitution in A402C of *MC4R* gene affected the phenotype variation of body weight in 120 Beagle dogs. Meanwhile, insignificant differences for body weight were found between the TT, TC and CC genotype dogs on the whole for the C895T mutation although female dog with heterozygous gene have lighter body weight compared with homozygous gene. It indicated that silent mutation of *MC4R* genes in female dogs could affect the modulation of canine body weight, and the estrogen could be an important effective factor of *MC4R* function to regulate body weight of Beagle dogs. Recently, some SNPs of canine *MC4R* gene, such as G637T, T777C and C+33G, were found from 19 different dog breeds [16, 18]. For the SNPs G637T and T777C, no association of the variant with canine body weight,

**Table 3.** Genotype frequencies and allele frequencies of *MC4R* gene

SNPs	Gender	% of genotype frequency (No. of samples)			% of allele frequency	
		CC	CA	AA	C	A
A420C	Female	8 (5)	33 (20)	58 (35)	25	75
	Male	13 (8)	35 (21)	52 (31)	31	69
	Total	11 (13)	34 (41)	55 (66)	28	72
C895T	Female	7 (4)	30 (18)	63 (38)	22	78
	Male		30 (18)	70 (42)	15	85
	Total	3 (4)	30 (36)	67 (80)	18	82

length, height, or body index score was found in 187 Golden Retrievers [18]. However, the associations between polymorphisms of *MC4R* with obesity factors [10, 15], and birth weight [17] have been verified in other animals. The studies suggested that the variations of body weight among dogs with *MC4R* mis-sense mutation may indicate functional defect of *MC4R in vitro*.

The change of amino acid in A402C sites might affected second transmembrane domain (Transmembrane 2, TM2) that construct a zinc finger structure of the human *MC4R* receptor [9]. However, a mutation does not always influence a gene function, Jin Yan *et al* found that the natural variant *cMC4R* V213F (G637T) did not have any functional defects by measuring ligand binding and signaling properties in Human embryonic kidney (HEK) 293T cells [19]. *MC4R* functional abnormality may induce excess food intake and lead to obesity. In our study, the dogs were on a condition they ate what they could because excess food was fed. Therefore, the difference in body weights observed in Beagle dogs might be addressed to food intake and the difference in body fat content although the relationship of *MC4R* genotypes with obesity was not directly indicated.

To explore the effect of the SNPs (A420C, C895T) in canine *MC4R* gene, the following researches should be done in the future. The difference of food intake and obesity should be analysed among the dogs with the different *MC4R* SNPs. Whether would the pharmacological characterization of canine melanocortin-4 receptor in MDCK cells be influenced by the variant N101T receptor or C895T mutation? Whether does the estrogen affect canine *MC4R* gene function *in vivo* and *in vitro*?

In conclusion, the analysis of the *MC4R* gene polymorphism on the genotypes in beagle dogs showed the *MC4R* gene was a candidate modifier gene for canine body weight and the moderate *MC4R* SNP (A420C,

**Table 4.** Body weight of different *MC4R* (A420C) genotypes in Beagle dogs

Body weight (kg)	SNP Genotype			P value
	CC (mean ± SD)	CA (mean ± SD)	AA (mean ± SD)	
Female	7.52 ± 0.55 <sup>B</sup>	8.21 ± 1.81 <sup>B</sup>	9.26 ± 1.6 <sup>A</sup>	0.021
Male	7.40 ± 0.30 <sup>B</sup>	8.95 ± 2.08 <sup>B</sup>	10.72 ± 3.21 <sup>A</sup>	0.004
Total	7.44 ± 0.41 <sup>B</sup>	8.58 ± 1.96 <sup>B</sup>	9.94 ± 2.59 <sup>A</sup>	0.000

<sup>A, B</sup>Different superscripts within columns differ significantly ( $P < 0.05$ ).

**Table 5.** Body weight of different *MC4R* (C895T) genotypes in Beagle dogs

Body weight (kg)	SNP Genotype			P value
	TT (mean ± SD)	TC (mean ± SD)	CC (mean ± SD)	
Female	10.75 ± 2.30 <sup>A</sup>	7.76 ± 1.33 <sup>B</sup>	9.02 ± 2.16 <sup>A</sup>	0.013
Male		9.18 ± 3.49 <sup>A</sup>	9.86 ± 2.54 <sup>A</sup>	
Total	10.75 ± 2.30 <sup>A</sup>	8.47 ± 2.72 <sup>A</sup>	9.46 ± 2.39 <sup>A</sup>	

<sup>A, B</sup>Different superscripts within columns differ significantly ( $P < 0.05$ ).

C895T) might be applied in selecting different size dogs as a genetic marker.

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