

ORIGINAL ARTICLE

Identification and functional characterization of three novel human melanocortin-4 receptor gene variants in an obese Chinese population

Rong Rong*‡, Ya-Xiong Tao§, Bernard M. Y. Cheung*†, Aimin Xu*†, Grace C. N. Cheung* and Karen S. L. Lam*†

*Department of Medicine and †Research Centre of Heart, Brain, Hormone and Healthy Ageing, Li Ka Shing Medical Faculty, The University of Hong Kong, Pokfulam, Hong Kong, China, ‡Shanghai Clinical Centre for Endocrine & Metabolic Diseases, Rui Jin Hospital, Shanghai Second Medical University, Shanghai, China, §Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, AL 36849, USA

Summary

Objective Mutations in the melanocortin-4 receptor gene (*MC4R*) are the most common monogenic form of human obesity. However, the contribution of *MC4R* mutations to obesity in Chinese has not been investigated. We studied the frequency of *MC4R* mutations in an obese southern Chinese population and the functional consequences of the novel variants identified.

Methods We screened for *MC4R* mutations in 227 obese [body mass index (BMI) 35.29 ± 5.75 kg/m²] and 100 lean (BMI 21.57 ± 0.29 kg/m²) southern Chinese subjects using PCR-direct sequencing. *In vitro* functional studies, including cell surface expression, ligand binding, and cyclic adenosine monophosphate (cAMP) accumulation, were performed to examine the functional properties of three novel missense mutations.

Results Apart from two previously reported polymorphisms, V103I and -176 A > C, three novel missense heterozygous variants (Y35C, C40R and M218T) were identified. The polymorphisms -176 A > C and Y35C were detected in both obese and normal subjects with similar frequency. C40R was identified only in an obese subject. Pedigree analysis revealed M218T carriers in both lean and obese subjects. The prevalence of V103I carriers in normal-weight controls was significantly higher than that in obese subjects (5.3% vs. 1.3%, $P < 0.05$). *In vitro* functional studies showed that all three novel missense variants have normal functions.

Conclusions Two known polymorphisms and three novel variants of the *MC4R* were identified. No overt functional defects were observed for the three novel *MC4R* variants, suggesting that they might not be the cause of obesity in variant carriers.

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Introduction

The leptin-regulated melanocortin circuit is the best-characterized and most clinically relevant system that regulates energy homeostasis.^{1,2} Several lines of evidence, including anatomical, pharmacological and mouse genetic, suggest that the melanocortin-4 receptor (*MC4R*) is a critical component. Anatomically, the *MC4R* is abundantly expressed in the hypothalamic paraventricular nucleus, which is involved in the central control of food intake and energy balance. The *MC3/4R* agonist melanotan II, an analogue of α -melanocyte stimulating hormone (α -MSH, the endogenous agonist of the *MC4R*), inhibits feeding in mice when administered intracerebroventricularly.^{3,4} Co-administration of SHU9119, a melanocortin receptor antagonist, blocks the inhibitory effect of α -MSH on feeding.³ Moreover, mice over-expressing agouti-related protein (*AgRP*), the endogenous antagonist of the *MC3/4R*, are hyperphagic, hyperinsulinaemic and obese.^{5,6} On the other hand, the *MC4R* knockout mice⁷ exhibit a maturity-onset obesity syndrome associated with increased food intake, hyperinsulinaemia and hyperglycaemia. Of particular interest was the observation that heterozygous mice lacking one allele of the *MC4R* gene had an intermediate body weight between wild-type and homozygous-null littermates, suggesting a gene dosage effect.⁷

In 1998, two heterozygous frameshift mutations in the *MC4R* gene were reported in obese patients by two independent groups, highlighting the importance of the *MC4R* in the regulation of body weight in humans.^{8,9} So far, more than 70 different variants have been identified in several ethnic obese and lean populations including German, French, British, Swedish, Spanish, Caucasian American, African American, and Japanese^{10–29} (for a recent review, see Tao³⁰). Functional studies demonstrated the existence of a variety of deficiencies, including cellular localization, ligand binding, and the response of the receptor to ligands (reviewed in Tao³⁰).

R. Rong and Y-X. Tao contributed equally to this work and should be considered as co-first authors.

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Correspondence, Karen S. L. Lam, Department of Medicine, The University of Hong Kong, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong. Tel.: +852 2855-4769; Fax: +852 28162187; E-mail: kslam@hkucc.hku.hk

In China, the prevalence of obesity has dramatically increased in the recent decades. A recent large survey showed that the prevalence of overweight was 26.9% in men and 31.1% in women, according to the World Health Organization global criteria [body mass index (BMI) ≤ 25.0 kg/m²].³¹ However, MC4R deficiency, the most common genetic cause of obesity, has not been systematically investigated in Chinese. The major objective of this study was to define the role of MC4R deficiency in a Chinese obesity population. We determined the frequency of MC4R mutations in this cohort, and evaluated the clinical phenotypes of subjects with novel MC4R mutations and their available family members. Finally, *in vitro* functional studies were performed to assess the potential contribution of these genetic variants to human obesity.

Materials and methods

Subjects

A total of 227 unrelated obese southern Chinese subjects (103 men and 124 women aged 44.93 \pm 12.34 years; BMI 35.29 \pm 5.75 kg/m², range 30.0–74.1 kg/m²) and 100 lean southern Chinese controls (47 men and 53 women aged 42.42 \pm 11.99 years; BMI 21.57 \pm 0.29 kg/m², range 18.5–22.8 kg/m²) were enrolled to screen for mutations in the coding region of the MC4R gene using direct sequencing of PCR products. Of these subjects, 88 obese subjects (40 men and 48 women, aged 44.49 \pm 12.45 years; BMI 37.50 \pm 7.12 kg/m², range 30.0–74.1 kg/m²) and 82 lean controls (39 men and 43 women, aged 46.09 \pm 12.69 years; BMI 21.81 \pm 0.46 kg/m², range 21.1–22.8 kg/m²) were also screened for variants in the 5' untranslated region of the MC4R gene. An additional 128 lean subjects (60 men and 68 women, aged 51.81 \pm 12.78 years; BMI 21.12 \pm 1.09 kg/m², range 18.5–22.6 kg/m²) were recruited to screen for the V103I and Y35C variants using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) to better define the allele frequencies of these two polymorphisms in non-obese subjects. Obese subjects were recruited from the Diabetes Clinic and the Endocrine Clinic for adults at the Queen Mary Hospital, University of Hong Kong. Age at onset of obesity was estimated from subjective recall, and not confirmed with health records. Based on such imprecise assessment, less than half of the obese subjects claimed to have been 'fat' before leaving primary school (usually at around 12 years of age). All normal-weight controls were recruited from subjects who participated in the population-based Hong Kong Cardiovascular Risk Factors Prevalence Study.³² All subjects signed written informed consent before participation. The protocol was approved by the Ethics Committee of the University of Hong Kong.

Bodyweight and height were assessed as the mean of two measurements (DETECTO, Webb City, MO, USA) on the same day. BMI was calculated as bodyweight (in kilograms) divided by the square of the body height (m²). Waist circumference were assessed as the mean of two measurements, and blood samples were drawn for DNA analysis and serum glucose, insulin, triglycerides, total cholesterol, HDL-cholesterol measured as previously described.³²

Direct nucleotide sequencing of the MC4R gene

Four pairs of primers were used to amplify the putative promoter and coding region of the MC4R. Three of the primer pairs for the coding region have been described previously.¹² Primers to amplify the MC4R promoter were forward (5'-CAGGTATTTTAACTGAACCACTACTG-3') and reverse (5'-AGCCTGAGCGTTGCTTTGAG-3'), which amplified a 300 bp in the 5' untranslated region of the MC4R. PCR was carried out in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA). The reaction volume was 50 μ l and included 5 μ l 10 \times PCR buffer, 0.08 mM of each dNTP, 0.3 μ M of each primer, and 1 unit of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR conditions were hotstart at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing for 1 min at 63 °C and extension at 72 °C for 1 min, with a final extension of 10 min. Bidirectional sequencing was performed using an Amersham DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA). The electrophoresis was performed on an ABI PRISM 3700 DNA sequencer (Applied Biosystems), and data were analysed using Sequencing Analysis software version 2.121.

PCR-RFLP analysis

Genotyping of the V103I and Y35C was carried out by PCR-RFLP analysis. V103I was detected as described by Gotoda *et al.*³³ For Y35C, genomic DNA was amplified with PCR with forward primer (5'-AGTTACAGACTGCACAGCAATG-3') and reverse primer (5'-TACATGGGTGAATGCAGATTC-3') and then digested overnight with Fnu4HI (New England BioLabs, Beverly, MA, USA).

Site-directed mutagenesis

Wild-type (wt) human MC4R cDNA cloned in the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was kindly provided by Dr Giles S.H. Yeo (Cambridge Institute for Medical Research, University of Cambridge, UK). The mutations were introduced into wt MC4R vector by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primer pairs were designed to introduce the desired mutations in the coding region of MC4R: Y35C forward (5'-GTCCCTTGAAAAAGGCTGCTCTGATGGAGGGTG-C3') and reverse (5'-GCACCCTCCATCAGAGCAGCCTTTTCCAAGGGAC-3'); C40R forward (5'-CGCTACTCTGATGGAGGGCGCTACGAGCAACTTTTTTGTC-3') and reverse (5'-GACAAAAA-GTTGCTCGTAGCGCCCTCCATCAGAGTAGCG-3'); and M218T forward (5'-GTCCACATGTTCTGACGGCCAGGCTTCAC-3') and reverse (5'-GTGAAGCCTGGCCGTCAGGAACATGTGGAC-3'). Briefly, primers incorporating the desired mutations replaced the wt sequence by PCR using MC4R in pBlueScript as the template and *pfu* Turbo DNA polymerase. *DpnI* was used to digest the parental methylated DNA in pBlueScript in the reaction product (37 °C for 1 h), and the digested reaction product was transformed into the supercompetent XL1-Blue *Escherichia coli* cells so the nicked mutated plasmids were repaired. Individual colonies were grown and sequenced by automated DNA sequencing. The plasmids with the correct mutation and without any spurious mutation introduced

during PCR were ligated back into pcDNA3.1. The final constructs in pcDNA3.1 were prepared with a Midiprep kit (QIAGEN, Valencia, CA, USA) and were sequenced again to verify the presence of desired mutations and the absence of unwanted mutations introduced during the PCR before being used for transfections.

Construction of tagged receptors

The wt and the three mutant receptors tagged at the N termini with a Flag tag were fused to the enhanced green fluorescent protein (EGFP) using PCR. PCR was conducted using wt and mutant MC4Rs cloned into pcDNA3.1 as template and the oligonucleotides: forward 5' -GACTGAATTCATGGACTACAAGGACGACGACGACAAG-GTGAACCTCCACCCACCGTG-3', and reverse 5'-GCTGAGGAT-CCCGGTAATATCTGCTAGACAAGTCAC-3' as primers. The PCR products were cloned in pEGFP-N1 (Clontech, Palo Alto, CA, USA) using the *EcoRI* and *BamHI* sites and subsequently verified by direct sequencing.

Cell culture and transfection

HEK293 cells (obtained from American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10^5 u/l penicillin, 100 mg/l streptomycin (all from Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO₂. All transfections were performed with Lipofectamine reagent (Invitrogen).

[Nle⁴ D-Phe⁷]- α -MSH (NDP-MSH) binding to intact cells

The binding assay was performed as described in detail previously.³⁴ Briefly, 48 h after transfection, cells were washed twice with warm Waymouth/BSA (Waymouth's MB752/1 media modified to contain 1 g/l BSA and 50 mg/l gentamicin). Fresh Waymouth/BSA was added to each well, together with 100 000 cpm of ¹²⁵I-NDP-MSH in 50 μ l (obtained from PerkinElmer Life and Analytical Sciences, Boston, MA, USA), with or without different concentrations of unlabelled NDP-MSH or AgRP (83–132) (purchased from Phoenix Pharmaceuticals, Belmont, CA, USA). After 1 h of incubation at 37 °C the cells were placed on ice and washed twice with ice-cold Hank's balanced salt solution containing 1 g/l BSA. The cells were then lysed with 100 μ l 0.5 M NaOH, collected using cotton swabs, and counted in a γ -counter. Binding capacity and IC₅₀ were calculated using PRISM 4 (GraphPad, San Diego, CA, USA).

Signalling properties of mutant MC4Rs

HEK293 cells were stably transfected with pCRE (BD Biosciences, Palo Alto, CA, USA) and pcDNA3.1 vector using LipofectAMINE under serum-free conditions. Selection was performed with 1.0 g/l of geneticin (G418) (Invitrogen). Functional colonies were obtained by testing the luciferase activity. After having been transfected with wt or mutant MC4Rs and internal control plasmid, pRL-CMV (Promega, Madison, WI, USA) and overnight incubation, HEK293 cells were stimulated with different concentrations of α -MSH (Bachem, San Carlos, CA, USA) for 16 h. The cells were lysed and

cyclic adenosine monophosphate (cAMP) generation was assessed by luciferase activities using Dual-Luciferase Reporter Assay System (Promega) measured on Lumat LB 9507 (EG & G BERTHOLD, Germany) according to manufacturer's protocols.

Alternatively, the signalling properties of the MC4Rs were assessed by directly measuring intracellular cAMP levels in response to NDP-MSH stimulation. Forty-eight hours after transfection, cells transfected with wt or mutant MC4Rs were stimulated with NDP-MSH for 1 h in the presence of 0.5 mM isobutyl methylxanthine, and intracellular cAMP levels measured with radioimmunoassay.^{34,35} All determinations were done in triplicate. EC_{50s} and maximal responses (R_{max}) were calculated using PRISM 4.

Confocal imaging of transiently transfected cells

HEK293 cells transiently transfected with MC4R tagged at the C terminus with EGFP were washed three times with phosphate-buffered saline for immunohistochemistry (PBS-IH, consisting of 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, 4.3 mM Na₂HPO₄, pH 7.4), fixed in 4% paraformaldehyde (prepared in PBS-IH) for 30 min, and washed again with PBS-IH for five times, and sealed in Vectashield Mounting Media (Vector Laboratories, Burlingame, CA, USA). Images were collected with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) excited with a 488 nm argon laser and detected with a 530–560 nm filter.

Statistical analysis

Data were presented as the mean and SD unless otherwise stated. Fisher's exact test was applied to test differences in genotype frequencies. All statistical analyses were done using PRISM 4. *P*-values were two-sided and a *P*-value of less than 0.05 was considered significant.

Results

Identification of three novel MC4R variants

A total of 227 unrelated obese subjects and 100 lean controls were screened for mutations in the coding region and putative promoter of the MC4R gene by direct DNA sequencing. Two previously described single nucleotide polymorphisms (SNPs), V103I and -176 A > C, were found in both obese and normal subjects. The allelic distributions of these two SNPs were in Hardy–Weinberg equilibrium. For V103I, the frequency in normal controls is significantly higher than in obese subjects (5.3% vs. 1.3%; *P* = 0.032) as detected by PCR-RFLP (Table 1). Three novel heterozygous missense variants, Y35C, C40R and M218T, were identified in obese subjects. Y35C was identified in two unrelated obese males with BMI of 32.7 kg/m² and 30.5 kg/m², respectively. One was apparently obese at primary school entry (6–7 years of age) but the other allegedly became obviously obese only in his 30s. C40R was identified in a 31-year-old obese woman (BMI 33.1 kg/m²) who became increasingly obese in her 20s. M218T was detected in a 47-year-old obese woman (BMI 33.2 kg/m²) who was fat as a child, became slimmer in her teens, and developed increasing obesity after childbirth,

Table 1. *MC4R* mutation screening in obese subjects and normal weight controls

Sequence variant	Obese group (no. with variants/total number)	Normal weight controls (no. with variants/total number)
-176 A > C	A/C: 5/88 (5.7%)	A/C: 6/82 (7.3%) C/C: 1/82 (1.2%)
V103I	3/227 (1.3%)	12/228 (5.3%)*
Y35C	2/227 (0.8%)	4/228 (1.7%)
C40R	1/227 (0.4%)	0/100
M218T	1/227 (0.4%)	0/100

**P* = 0.032.

reaching peak weight in her mid-30s. All three novel variants were not detected in the 100 normal controls. To exclude the possibility that Y35C might be an SNP, a further 128 normal subjects were subsequently screened for this variant using PCR-RFLP. A total of four subjects with Y35C were identified in 228 normal subjects. Therefore, the prevalence of Y35C was similar in obese and normal groups (Table 1).

Variable penetrance and expressivity of human *MC4R* variants

Two family trees of probands with Y35C and M218T were available for evaluating the penetrance and expressivity of the *MC4R* missense mutations (the family of the proband with C40R refused investigations following her suicide). In the case of Y35C, 12 family members were screened for the variant using PCR-RFLP. Six of seven Y35C variant-carriers were either obese (BMI > 30 kg/m² or bodyweight above the 97th percentile) or overweight (BMI 25–30 kg/m² or bodyweight above the 90th percentile) with the other one having BMI near 25 kg/m², while three subjects without this variant were overweight (Fig. 1A). Waist circumference exceeding the normal range was present in all but one adult with the variant. The significance of this missense variant was subsequently disproved as it was found at a similar frequency in normal subjects in a more extensive case-controlled study (Table 1). For M218T, 15 family members were screened for the variant using direct DNA sequencing. Obesity, based on BMI, weight percentile, or waist circumference, did not segregate with M218T genotype. Of the six carriers of M218T, one was obese, two were overweight and three were lean (Fig. 1B).

Functional characterization of the novel variants

Y35C and C40R are both located in the N-terminal extracellular domain and M218T is located in the third intracellular loop of the *MC4R*. Since intracellular retention of the mutant *MC4R*s associated with obesity were frequently observed, we investigated the cellular localization of the variant *MC4R*s. HEK293 cells were transiently transfected with wt or the missense variants tagged at the C terminus with EGFP. Cellular localization images were obtained from a laser scanning confocal microscope. Representative results are shown in Fig. 2. All three variants (Y35C, C40R and M218T) were expressed well on the cell surface.

Next we studied whether the variants could bind to radiolabelled agonist. Since the signalling balance between the agonist (α -MSH)

and the antagonist (AgRP) is important to maintain the function of the *MC4R*, we used both NDP-MSH and AgRP as competitors. As shown in Fig. 3A and Table 2, all variants bound to NDP-MSH and were displaced by NDP-MSH and AgRP with similar IC₅₀s.

The *MC4R* transduces its signal by coupling to the heterotrimeric Gs protein and activates adenylyl cyclase resulting in intracellular cAMP accumulation. There are two approaches to assess the cAMP generation. One is measuring cAMP levels directly in cells transfected with wt or mutant *MC4R*s. The other is examining the reporter gene expression driven by the cAMP concentration (reviewed in Tao³⁰). Two agonists can be used in stimulating cAMP generation. One is the natural agonist, α -MSH. The other is the superpotent agonist NDP-MSH.

In the present study, the aforementioned two approaches were used to characterize the signalling properties of the three novel variants. Different concentrations of α -MSH were used to stimulate the production of cAMP. The luciferase activity was assessed by Dual-Luciferase reporter assay. As shown in Fig. 3B (left panel), there was no significant difference between the wt and mutant *MC4R*s. Using direct radioimmunoassay to measure the intracellular cAMP levels, we could not find any significant difference between the wt and mutant *MC4R*s either [Fig. 3B (right panel) and Table 2].

It was recently reported that the basal (constitutive) activity of the *MC4R* is important for maintaining energy homeostasis.³⁶ Therefore we also measured the basal cAMP levels in cells transfected with wt or variant *MC4R*s. The results showed that there were no differences in the basal activities of the wt and variant *MC4R*s (Table 2).

Discussion

To date, investigations into the genetic contribution of obesity have been based on two main hypotheses. One is the common disease/common variant hypothesis. The other focuses on the effects of a large number of rare genetic variants with substantial allelic heterogeneity at disease-causing loci.³⁷ The *MC4R* gene belongs to the second category.¹³ In the present study, two previously reported SNPs in the *MC4R* (V103I and -176 A > C) were found in both obese and normal-weight subjects. V103I was found at a higher frequency in normal controls than in obese subjects, in agreement with previous reports^{22,38} Y35C, which was first identified in two obese subjects but not detected in 100 normal subjects, was found on more extensive screening to be present also in normal subjects, suggesting that it is a new SNP. The prevalence of Y35C was similar in normal and obese subjects (Table 1). C40R was identified in an

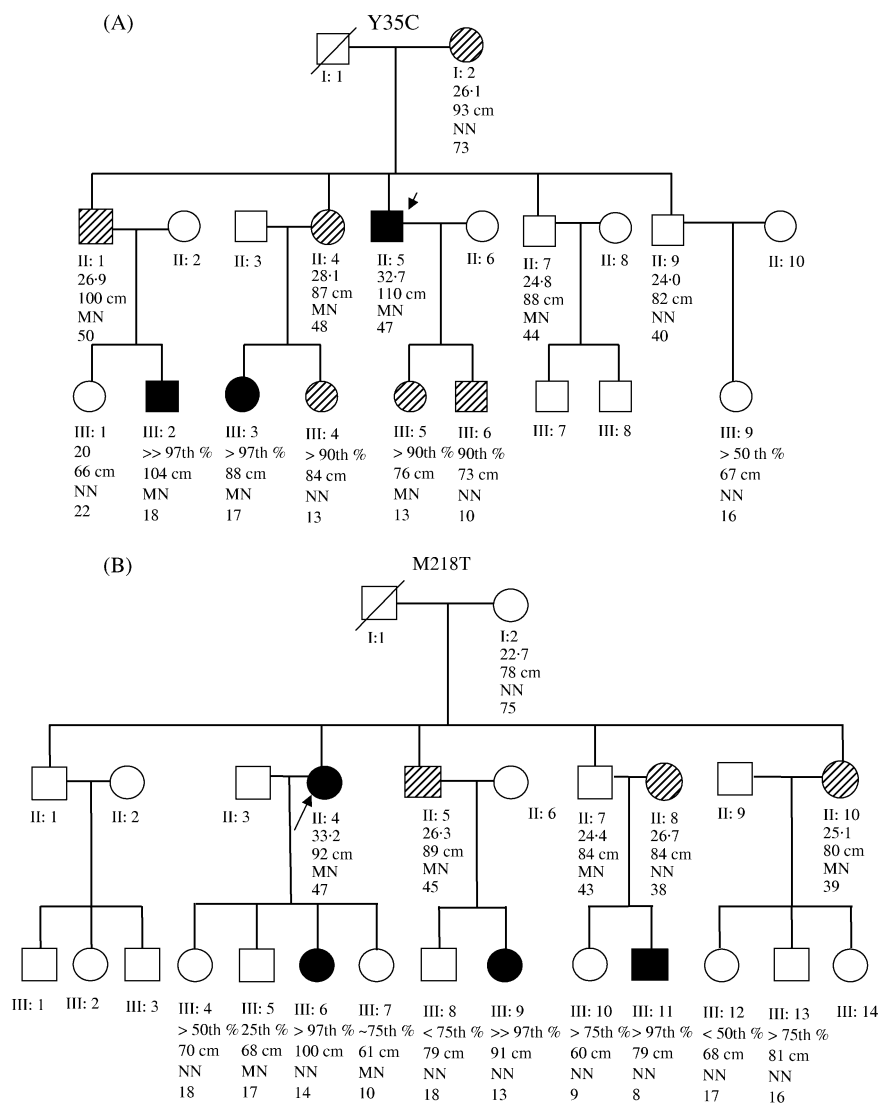


Fig. 1 Pedigrees and phenotypes in the family members of the Y35C (A) and M218T (B) carriers. The arrows indicate the proband. The hatched symbols represent the overweight individuals ($25 \leq \text{BMI} < 30 \text{ kg/m}^2$ or bodyweight 90th–97th percentile). The filled symbols denote obese subjects ($\text{BMI} \leq 30 \text{ kg/m}^2$ or bodyweight > 97th percentile). BMI and waist circumference are stated in the second and third line, respectively. Normal waist circumference for Asian men and women are < 90 and < 80 cm, respectively (IDF 2005 criteria). The fourth line denotes genotype, and the fifth line, age of the subject. N: normal (wt) allele; M: mutant allele; %, percentile.

obese subject. We did not have access to family members of the proband. The third novel missense variant (M218T) was identified in both obese and lean subjects in the family (Fig. 1B). Compared with the reported prevalence of *MC4R* mutations in early-onset morbidly obese populations in France and the UK,^{13,20} the prevalence of *MC4R* mutations in our cohort was relatively low. The most likely reason is the different recruitment criteria of the cohorts. Both the French and UK groups recruited early-onset morbidly obese subjects. Although the mean BMI of the present study (35 kg/m^2) was not significantly different from the previous studies, less than half of our subjects had apparent onset of obesity in childhood. It is possible that a higher frequency may be found in Chinese with morbid obesity of early onset. Whereas our controls were recruited from a population-based study, obese subjects recruited from the diabetes and endocrine clinics may not be representative of the general population. Furthermore, although endocrine causes of obesity were excluded, the weight-increasing effect of antidiabetic drugs such as sulphonylurea and insulin could be present, and provide another explanation for the low frequency of *MC4R* mutations.

To determine the inheritance patterns, two large families (13 in Y35C and 16 in M218T) were available for study. A study conducted in the UK showed a 100% penetrance of early-onset obesity in heterozygous probands,¹² whereas a study by a French group found that obligate carriers were not always obese.¹³ We showed that Y35C and M218T did not segregate with obesity, suggesting the presence of a modulated expressivity and penetrance. Variants in modifier genes, intrinsic and extrinsic to the melanocortin pathway, have been proposed to contribute to such an inheritance pattern.^{14,16} This possibility remains to be addressed by further studies on potential modifier genes such as *proopiomelanocortin* or *AgRP*. On the other hand, based on our study data, we cannot exclude the possibility that M218T and C40R, like Y35C, are also rare polymorphisms.

For the three novel missense variants (Y35C, C40R and M218T), the properties of the variants including cell surface expression, receptor binding, and cAMP accumulation (both basal and stimulated) were similar to wt *MC4R*. According to the classification scheme proposed by Tao and Segaloff,³⁴ the missense variants identified in our population belong to class V, variants with no known defects. It has been repeatedly shown that some

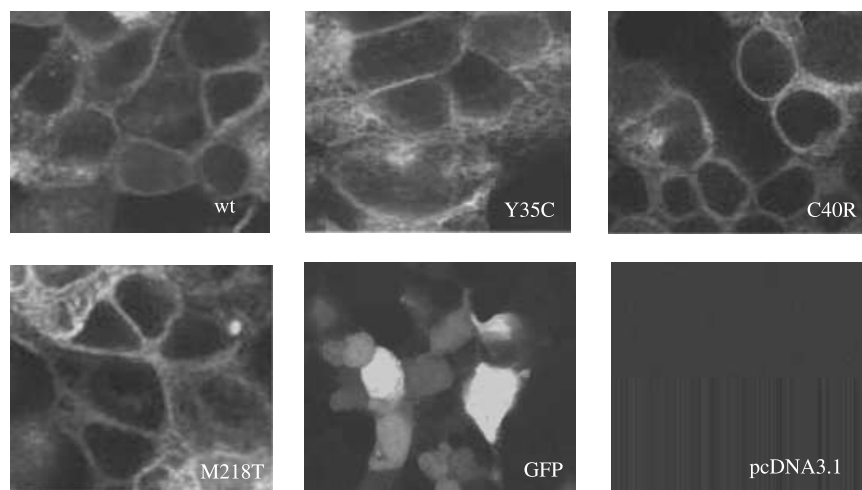


Fig. 2 Confocal imaging of the wild-type and variant MC4Rs tagged at the C terminus with GFP.

Fig. 3 Ligand binding and signalling properties of wild-type and variant MC4Rs. In (A), HEK293 cells transiently transfected with wild-type or mutant MC4Rs were assayed for binding to ^{125}I -NDP-MSH as described in Materials and methods. Increasing concentration of unlabelled NDP-MSH (left panel) or AgRP $^{83-132}$ (right panel) was used to displace the radiolabelled NDP-MSH. Results are expressed as mean \pm range within one experiment. The experiments were performed three times with similar results. In (B), signalling properties of wild-type and variant MC4Rs as determined by cAMP-responsive luciferase assay (left panel) or direct measurement of cAMP levels (right panel). Results shown are mean \pm SEM from three experiments (left panel) or a representative experiment performed in triplicate (right panel) repeated three times with similar results.

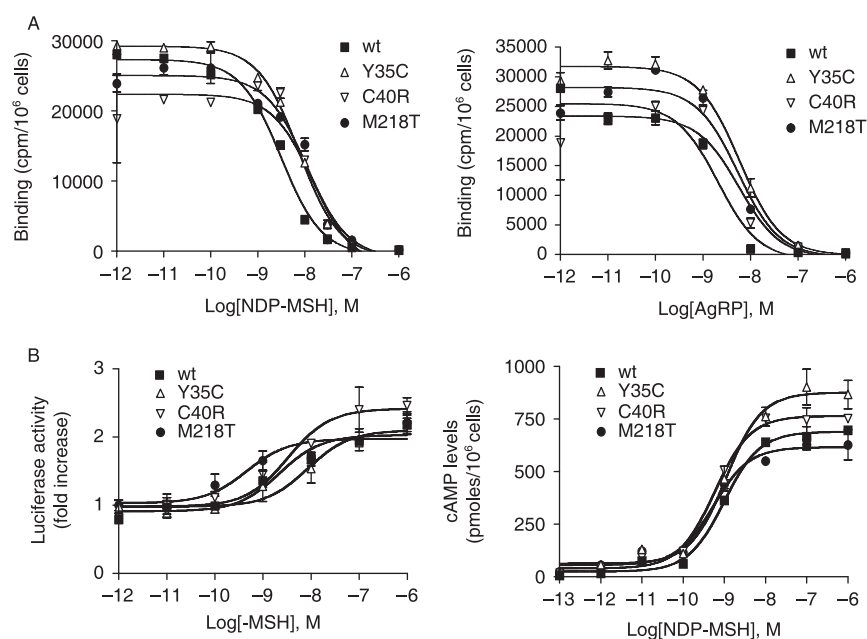


Table 2. Ligand binding, basal and agonist-stimulated cAMP production of wild-type (wt) and mutant MC4Rs

MC4R	NDP-MSH binding			NDP-MSH-stimulated cAMP		
	IC ₅₀ (nM) NDP-MSH	IC ₅₀ (nM) AgRP	B _{max} (% wt)	Basal cAMP (pmol/10 ⁶ cells)	EC ₅₀ (nM)	R _{max} (% wt)
wt	7.59 \pm 3.93	4.46 \pm 2.42	100	6.86 \pm 0.50	0.63 \pm 0.15	100
Y35C	6.28 \pm 1.26	2.34 \pm 1.35	109 \pm 3	11.77 \pm 4.58	0.72 \pm 0.12	105 \pm 13
C40R	7.95 \pm 2.57	6.89 \pm 1.56	72 \pm 4	10.76 \pm 3.06	0.48 \pm 0.01	87 \pm 12
M218T	17.32 \pm 7.48	8.59 \pm 2.04	99 \pm 10	12.53 \pm 5.90	0.65 \pm 0.13	93 \pm 0

Data shown are the mean \pm SEM of three experiments. The B_{max} of cells expressing wt MC4R was 0.038 \pm 0.007 nmol NDP-MSH bound/10⁶ cells and the R_{max} was 825.2 \pm 69.4 pmol cAMP/10⁶ cells (mean \pm SEM of three experiments).

variants associated with obesity or binge-eating disorder do not cause overt functional defects.^{34,39} On the other hand, using the same techniques, we have identified mutants that are defective in cell surface expression, ligand binding and signalling in both

MC4R and the related MC3R^{23,34,35,39} (reviewed in Tao³⁰). We cannot exclude the possibilities that the variants, whereas functioning normally *in vitro*, might affect MC4R functions in neurons *in vivo*.

The *in vitro* functional data are consistent with the genetic data from pedigree analysis, highlighting the importance of functional studies. For example, Y35C and M218T have normal functions suggesting that they are likely to be polymorphisms. From the identification of the variants from obese subjects, it might be concluded that they were the cause of obesity in these subjects. Pedigree analysis and functional characterizations both disproved that tentative conclusion.

It is well known that obesity is caused by interactions of environmental and genetic factors. Environmental factors such as diet, physical activity, and psychosocial factors are also important in contributing to obesity.¹ One limitation of the present study was the lack of information related to lifestyle factors.

In summary, two previously reported single nucleotide polymorphisms (V103I and -176 A > C) and three novel missense variants (Y35C, C40R and M218T) were identified in an obese Chinese cohort. The association of the single nucleotide polymorphism V103I with adiposity, reported in other ethnic groups, was also found in this population.

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References

- Spiegelman, B.M. & Flier, J.S. (2001) Obesity and the regulation of energy balance. *Cell*, **104**, 531–543.
- Cone, R.D. (2005) Anatomy and regulation of the central melanocortin system. *Nature Neuroscience*, **8**, 571–578.
- Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J. & Cone, R.D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, **385**, 165–168.
- Thiele, T.E., van Dijk, G., Yagaloff, K.A., Fisher, S.L., Schwartz, M., Burn, P. & Seeley, R.J. (1998) Central infusion of melanocortin agonist MTH in rats: assessment of c-Fos expression and taste aversion. *American Journal of Physiology*, **274**, R248–R254.
- Graham, M., Shutter, J.R., Sarmiento, U., Sarosi, I. & Stark, K.L. (1997) Overexpression of Agtr leads to obesity in transgenic mice. *Nature Genetics*, **17**, 273–274.
- Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I. & Barsh, G.S. (1997) Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. *Science*, **278**, 135–138.
- Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P. & Lee, F. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*, **88**, 131–141.
- Vaisse, C., Clement, K., Guy-Grand, B. & Froguel, P. (1998) A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nature Genetics*, **20**, 113–114.
- Yeo, G.S., Farooqi, I.S., Aminian, S., Halsall, D.J., Stanhope, R.G. & O'Rahilly, S. (1998) A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nature Genetics*, **20**, 111–112.
- Gu, W., Tu, Z., Kleyn, P.W., Kissebah, A., Duprat, L., Lee, J., Chin, W., Maruti, S., Deng, N., Fisher, S.L., Franco, L.S., Burn, P., Yagaloff, K.A., Nathan, J., Heymsfield, S., Albu, J., Pi-Sunyer, F.X. & Allison, D.B. (1999) Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes*, **48**, 635–639.
- Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H. & Hebebrand, J. (1999) Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *Journal of Clinical Endocrinology and Metabolism*, **84**, 1483–1486.
- Farooqi, I.S., Yeo, G.S., Keogh, J.M., Aminian, S., Jebb, S.A., Butler, G., Cheetham, T. & O'Rahilly, S. (2000) Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *Journal of Clinical Investigation*, **106**, 271–279.
- Vaisse, C., Clement, K., Durand, E., Hercberg, S., Guy-Grand, B. & Froguel, P. (2000) Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *Journal of Clinical Investigation*, **106**, 253–262.
- Dubern, B., Clement, K., Pelloux, V., Froguel, P., Girardet, J.P., Guy-Grand, B. & Tounian, P. (2001) Mutational analysis of melanocortin-4 receptor, agouti-related protein, and α -melanocyte-stimulating hormone genes in severely obese children. *Journal of Pediatrics*, **139**, 204–209.
- Kobayashi, H., Ogawa, Y., Shintani, M., Ebihara, K., Shimodahira, M., Iwakura, T., Hino, M., Ishihara, T., Ikekubo, K., Kurahachi, H. & Nakao, K. (2002) A novel homozygous missense mutation of melanocortin-4 receptor (MC4R) in a Japanese woman with severe obesity. *Diabetes*, **51**, 243–246.
- Miraglia Del Giudice, E., Cirillo, G., Nigro, V., Santoro, N., D'Urso, L., Raimondo, P., Cozzolino, D., Scafato, D. & Perrone, L. (2002) Low frequency of melanocortin-4 receptor (MC4R) mutations in a Mediterranean population with early-onset obesity. *International Journal of Obesity*, **26**, 647–651.
- Jacobson, P., Ukkola, O., Rankinen, T., Snyder, E.E., Leon, A.S., Rao, D.C., Skinner, J.S., Wilmore, J.H., Lonn, L., Cowan, G.S. Jr, Sjostrom, L. & Bouchard, C. (2002) Melanocortin 4 receptor sequence variations are seldom a cause of human obesity: the Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis cohort. *Journal of Clinical Endocrinology and Metabolism*, **87**, 4442–4446.
- Marti, A., Corbalan, M.S., Forga, L., Martinez, J.A., Hinney, A. & Hebebrand, J. (2003) A novel nonsense mutation in the melanocortin-4 receptor associated with obesity in a Spanish population. *International Journal of Obesity*, **27**, 385–388.
- Lubrano-Berthelie, C., Durand, E., Dubern, B., Shapiro, A., Dazin, P., Weill, J., Ferron, C., Froguel, P. & Vaisse, C. (2003) Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. *Human Molecular Genetics*, **12**, 145–153.
- Farooqi, I.S., Keogh, J.M., Yeo, G.S., Lank, E.J., Cheetham, T. & O'Rahilly, S. (2003) Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *New England Journal of Medicine*, **348**, 1085–1095.
- Yeo, G.S., Lank, E.J., Farooqi, I.S., Keogh, J., Challis, B.G. & O'Rahilly, S. (2003) Mutations in the human melanocortin-4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms. *Human Molecular Genetics*, **12**, 561–574.
- Hinney, A., Hohmann, S., Geller, F., Vogel, C., Hess, C., Wermter, A.K., Brokamp, B., Goldschmidt, H., Siegfried, W., Remschmidt, H., Schafer, H., Gudermann, T. & Hebebrand, J. (2003) Melanocortin-4

- receptor gene: case-control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. *Journal of Clinical Endocrinology and Metabolism*, **88**, 4258–4267.
- 23 Donohoue, P.A., Tao, Y.X., Collins, M., Yeo, G.S.H., O'Rahilly, S. & Segaloff, D.L. (2003) Deletion of codons 88–92 of the melanocortin-4 receptor gene: a novel deleterious mutation in an obese female. *Journal of Clinical Endocrinology and Metabolism*, **88**, 5841–5845.
 - 24 Biebermann, H., Krude, H., Elsner, A., Chubanov, V., Gudermann, T. & Gruters, A. (2003) Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. *Diabetes*, **52**, 2984–2988.
 - 25 Santini, F., Maffei, M., Ceccarini, G., Pelosini, C., Scartabelli, G., Rosellini, V., Chiellini, C., Marsili, A., Lisi, S., Tonacchera, M., Agretti, P., Chiovato, L., Mammoli, C., Vitti, P. & Pinchera, A. (2004) Genetic screening for melanocortin-4 receptor mutations in a cohort of Italian obese patients: description and functional characterization of a novel mutation. *Journal of Clinical Endocrinology and Metabolism*, **89**, 904–908.
 - 26 Valli-Jaakola, K., Lipsanen-Nyman, M., Oksanen, L., Hollenberg, A.N., Kontula, K., Bjorbaek, C. & Schalin-Jantti, C. (2004) Identification and characterization of melanocortin-4 receptor gene mutations in morbidly obese Finnish children and adults. *Journal of Clinical Endocrinology and Metabolism*, **89**, 940–945.
 - 27 Lubrano-Berthelie, C., Le Stunff, C., Bougneres, P. & Vaisse, C. (2004) A homozygous null mutation delineates the role of the melanocortin-4 receptor in humans. *Journal of Clinical Endocrinology and Metabolism*, **89**, 2028–2032.
 - 28 Ma, L., Tataranni, P.A., Bogardus, C. & Baier, L.J. (2004) Melanocortin 4 receptor gene variation is associated with severe obesity in Pima Indians. *Diabetes*, **53**, 2696–2699.
 - 29 Larsen, L.H., Echwald, S.M., Sorensen, T.I., Andersen, T., Wulff, B.S. & Pedersen, O. (2005) Prevalence of mutations and functional analyses of melanocortin 4 receptor variants identified among 750 men with juvenile-onset obesity. *Journal of Clinical Endocrinology and Metabolism*, **90**, 219–224.
 - 30 Tao, Y.X. (2005) Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early-onset obesity. *Molecular and Cellular Endocrinology*, **239**, 1–14.
 - 31 Gu, D., Reynolds, K., Wu, X., Chen, J., Duan, X., Reynolds, R.F., Whelton, P.K. & He, J. (2005) Prevalence of the metabolic syndrome and overweight among adults in China. *Lancet*, **365**, 1398–1405.
 - 32 Janus, E.D., Watt, N.M., Lam, K.S., Cockram, C.S., Siu, S.T., Liu, L.J. & Lam, T.H. (2000) The prevalence of diabetes, association with cardiovascular risk factors and implications of diagnostic criteria (ADA 1997 and WHO 1998) in a 1996 community-based population study in Hong Kong Chinese. Hong Kong Cardiovascular Risk Factor Steering Committee. American Diabetes Association. *Diabetic Medicine*, **17**, 741–745.
 - 33 Gotoda, T., Scott, J. & Aitman, T.J. (1997) Molecular screening of the human melanocortin-4 receptor gene: identification of a missense variant showing no association with obesity, plasma glucose, or insulin. *Diabetologia*, **40**, 976–979.
 - 34 Tao, Y.X. & Segaloff, D.L. (2003) Functional characterization of melanocortin-4 receptor mutations associated with childhood obesity. *Endocrinology*, **144**, 4544–4551.
 - 35 Tao, Y.X. & Segaloff, D.L. (2004) Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation. *Journal of Clinical Endocrinology and Metabolism*, **89**, 3936–3942.
 - 36 Srinivasan, S., Lubrano-Berthelie, C., Govaerts, C., Picard, F., Santiago, P., Conklin, B.R. & Vaisse, C. (2004) Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *Journal of Clinical Investigation*, **114**, 1158–1164.
 - 37 Comuzzie, A.G. & Allison, D.B. (1998) The search for human obesity genes. *Science*, **280**, 1374–1377.
 - 38 Geller, F., Reichwald, K., Dempfle, A., Illig, T., Vollmert, C., Herpertz, S., Siffert, W., Platzer, M., Hess, C., Gudermann, T., Biebermann, H., Wichmann, H.E., Schafer, H., Hinney, A. & Hebebrand, J. (2004) Melanocortin-4 receptor gene variant I103 is negatively associated with obesity. *American Journal of Human Genetics*, **74**, 572–581.
 - 39 Tao, Y.X. & Segaloff, D.L. (2005) Functional analyses of melanocortin-4 receptor mutations identified from patients with binge eating disorder and non-obese or obese subjects. *Journal of Clinical Endocrinology and Metabolism*, **90**, 5632–5638.