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

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# 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)  $35 \cdot 29 \pm 5 \cdot 75 \text{ kg/m}^2$ ] and 100 lean (BMI  $21 \cdot 57 \pm 0 \cdot 29 \text{ kg/m}^2$ ) 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.

(Received 16 November 2005; returned for revision 16 December 2005; finally revised 8 February 2006; accepted 1 March 2006)

#### Introduction

The leptin-regulated melanocortin circuit is the best-characterized and most clinically relevant system that regulates energy homeostasis.<sup>1,2</sup> 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  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH, the endogenous agonist of the MC4R), inhibits feeding in mice when administrated intracerebroventricularly.<sup>3,4</sup> Co-administration of SHU9119, a melanocortin receptor antagonist, blocks the inhibitory effect of  $\alpha$ -MSH on feeding.3 Moreover, mice over-expressing agouti-related protein (AgRP), the endogenous antagonist of the MC3/4R, are hyperphagic, hyperinsulinaemic and obese.<sup>5,6</sup> On the other hand, the MC4R knockout mice<sup>7</sup> 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 homozygousnull littermates, suggesting a gene dosage effect.<sup>7</sup>

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<sup>8,9</sup> 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<sup>10–29</sup> (for a recent review, see Tao<sup>30</sup>). 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 Tao<sup>30</sup>).

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This paper has been presented in part at the 86th Endocrine Society Annual Meeting held in New Orleans, LA, in 2004.

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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)  $\leq 25\cdot0$  kg/m<sup>2</sup>].<sup>31</sup> 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/m<sup>2</sup>, range 30.0-74.1 kg/m<sup>2</sup>) 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/m<sup>2</sup>, range  $18.5-22.8 \text{ kg/m}^2$ ) 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/m<sup>2</sup>, range 30.0-74.1 kg/m<sup>2</sup>) and 82 lean controls (39 men and 43 women, aged  $46.09 \pm 12.69$  years; BMI  $21.81 \pm 0.46$  kg/m<sup>2</sup>, range 21.1-22.8 kg/m<sup>2</sup>) 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/m<sup>2</sup>, range 18.5-22.6 kg/m<sup>2</sup>) 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.<sup>32</sup> 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 (m<sup>2</sup>). 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.<sup>32</sup>

# 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.<sup>12</sup> Primers to amplify the MC4R promoter were forward (5'-CAGGTATTTTAACTGAAC-CACTACTG-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 µl and included 5 µl 10× PCR buffer, 0.08 mм of each dNTP, 0.3 µм 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.*<sup>33</sup> 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'-GTCCCTTGGAAAAGGCTGCTCTGATGGAG-GGTG-C3') and reverse (5'-GCACCCTCCATCAGAGCAGCCTTT-TCCAAGGGAC-3'); C40R forward (5'-CGCTACTCTGATGGA-GGGCGCTACGAGCAACTTTTTGTC-3') and reverse (5'-GACAAAAA-GTTGCTCGTAGCGCCCTCCATCAGAGTAGCG-3'); and M218T forward (5'-GTCCACATGTTCCTGACGGCCAGGCTTCAC-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-GTGAACTCCACCCACCGTG-3', and reverse 5'-GCTGAGGAT-CCCGGTAATATCTGCTAGACAAGTCAC-3' as primers. The PCR products were cloned in pEGFP-N1 (Clontech, Palo Alto, CA, USA) using the *Eco*RI and *Bam*HI 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% CO<sub>2</sub>. All transfections were performed with Lipofectamine reagent (Invitrogen).

# $[Nle^4 D-Phe^7]-\alpha$ -MSH (NDP-MSH) binding to intact cells

The binding assay was performed as described in detail previously.<sup>34</sup> 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 <sup>125</sup>I-NDP-MSH in 50 µl (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 µl 0·5 m NaOH, collected using cotton swabs, and counted in a  $\gamma$ -counter. Binding capacity and IC<sub>50</sub> 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  $\alpha$ -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.<sup>34,35</sup> All determinations were done in triplicate. EC<sub>50s</sub> 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 phosphatebuffered saline for immunohistochemistry (PBS-IH, consisting of 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 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  $32.7 \text{ kg/m}^2$  and  $30.5 \text{ kg/m}^2$ , 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/m<sup>2</sup>) who became increasingly obese in her 20s. M218T was detected in a 47-year-old obese woman (BMI 33.2 kg/m<sup>2</sup>) who was fat as a child, became slimmer in her teens, and developed increasing obesity after childbirth,

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

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

\*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  $(BMI > 30 \text{ kg/m}^2 \text{ or bodyweight})$ above the 97th percentile) or overweight (BMI 25-30 kg/m<sup>2</sup> or bodyweight above the 90th percentile) with the other one having BMI near  $25 \text{ kg/m}^2$ , 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 MC4Rs associated with obesity were frequently observed, we investigated the cellular localization of the variant MC4Rs. 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 ( $\alpha$ -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  $IC_{50}s$ .

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 MC4Rs. The other is examining the reporter gene expression driven by the cAMP concentration (reviewed in Tao<sup>30</sup>). Two agonists can be used in stimulating cAMP generation. One is the natural agonist,  $\alpha$ -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  $\alpha$ -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 MC4Rs. Using direct radioimmunoassay to measure the intracellular cAMP levels, we could not find any significant difference between the wt and mutant MC4Rs 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.<sup>36</sup> Therefore we also measured the basal cAMP levels in cells transfected with wt or variant MC4Rs. The results showed that there were no differences in the basal activities of the wt and variant MC4Rs (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.<sup>37</sup> The *MC4R* gene belongs to the second category.<sup>13</sup> 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<sup>22,38</sup> 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  $(25 \le BMI < 30 \text{ kg/m}^2 \text{ or bodyweight 90th-97th}$ percentile). The filled symbols denote obese subjects (BMI  $\le 30 \text{ kg/m}^2 \text{ or bodyweight > 97th}$ 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,<sup>13,20</sup> 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<sup>2</sup>) 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,<sup>12</sup> whereas a study by a French group found that obligate carriers were not always obese.<sup>13</sup> 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.<sup>14,16</sup> 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,<sup>34</sup> 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 signallling properties of wild-type and variant MC4Rs. In (A), HEK293 cells transiently transfected with wild-type or mutant MC4Rs were assayed for binding to <sup>125</sup>I-NDP-MSH as described in Materials and methods. Increasing concentration of unlabelled NDP-MSH (left panel) or AgRP<sup>83–132</sup> (right panel) was used to displace the radiolabelled NDP-MSH. Results are expressed as mean ± 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 <sub>50</sub> (nm) NDP-MSH	IC <sub>50</sub> (nm) AgRP	$B_{\max}$ (% wt)	Basal cAMP (pmol/10 <sup>6</sup> cells)	EC <sub>50</sub> (nm)	$R_{\rm max}$ (% wt)
wt	7·59 ± 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	$17.32 \pm 7.48$	$8.59 \pm 2.04$	$99 \pm 10$	$12.53 \pm 5.90$	$0.65 \pm 0.13$	$93\pm0$

Data shown are the mean  $\pm$  SEM of three experiments. The  $B_{\text{max}}$  of cells expressing wt MC4R was  $0.038 \pm 0.007$  nmol NDP-MSH bound/ $10^{\circ}$  cells and the  $R_{\text{max}}$  was  $825.2 \pm 69.4$  pmol cAMP/ $10^{\circ}$  cells (mean  $\pm$  SEM of three experiments).

variants associated with obesity or binge-eating disorder do not cause overt functional defects.<sup>34,39</sup> 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<sup>23,34,35,39</sup> (reviewed in Tao<sup>30</sup>). 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.<sup>1</sup> 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.

# Acknowledgements

This study was supported by the Sun Chieh Yeh Heart Foundation (K. S-L. Lam) and start-up funds from Auburn University (Y-X. T.). R. Rong was supported by a PhD studentship from the University of Hong Kong.

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