The role of Melanocortin 3 Receptor Gene in Childhood Obesity

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Short running title: Role of MC3R mutations in obese children

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ABSTRACT

Introduction: Melanocortin 3 receptor (MC3R) plays a critical role in weight regulation of rodents, but its role in humans remains unclear.

Objective: To identify genetic variants of the MC3R gene and determine its association with childhood obesity.

Methods: We screened 201 obese children for MC3R gene mutations, with anthropometric measurements, blood tests, feeding behaviour and body composition assessment.

Results: We identified three novel heterozygous mutations (Ile183Asn, Ala70Thr, and Met134Ile) in three unrelated subjects, which were not found in 188 controls, and two common polymorphisms Thr6Lys and Val81Ile. In-vitro functional studies of the resultant mutant receptors revealed impaired signaling activity but normal ligand binding and cell surface expression. The heterozygotes demonstrated higher leptin levels and adiposity, and less hunger, compared to obese controls, reminiscent of the MC3R knockout mice. Family studies showed that these mutations may be associated with childhood or early onset obesity. The common variants Thr6Lys and Val81Ile were in complete linkage disequilibrium, and in-vitro studies revealed reduced signaling activity compared to wildtype MC3R. Obese subjects with the 6Lys/81Ile haplotype had significantly higher leptin levels, percentage body fat, and insulin sensitivity, and the causative role of the 6Lys/81Ile variants is supported by the presence of an additive effect, where heterozygotes had an intermediate phenotype compared to homozygotes.

Conclusion: MC3R mutations may not result in autosomal dominant forms of obesity, but may contribute as a predisposing factor to childhood obesity, and exert an effect on the human phenotype. Our report supports the role of MC3R in human weight regulation.
The melanocortin pathway mediates leptin action and regulates energy balance by inhibiting feeding, increasing energy expenditure and reducing energy storage. The Melanocyte Stimulating Hormone (MSH) is the principal agonist of the neuronal melanocortin receptors, melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R), both of which are critical for weight regulation in rodents (1-3).

MC3R is a seven-transmembrane G-protein coupled receptor(4) expressed in hypothalamic nuclei known to regulate energy homeostasis. It exhibits a more restricted distribution than MC4R in the central nervous system (5), and has a dominant role in inhibition of energy storage (1,2). Mc3r<sup>-/-</sup> mice homozygous for knockout mutations of MC3R gene had increased body fat (1,2) not caused by increased food intake but by increased feed efficiency. The Mc3r<sup>-/-</sup> mice were hypophagic with hyperleptinemia compared to wildtype littermates (2). Mice lacking both MC3R and MC4R have exacerbated obesity, which supports the notion that both are important and non-redundant (2).

MC4R mutations causing human obesity are well described (6, 7), but the search for human MC3R mutations has been largely unsuccessful (8-12). We previously described a novel MC3R gene mutation Ile183Asn in two obese members of a Singaporean family (13). In this paper, we report the complete genotype and phenotype of the extended pedigree, our in-vitro analysis of the Ile183Asn mutant MC3R, and more importantly two other novel missense mutations in two families, as well as the association of the common MC3R variants with obesity related phenotypes in our cohort of obese children.

**METHODS**

**Study subjects and assessment**

The MC3R gene was analysed in 201 unrelated children and adolescents with early onset severe obesity, recruited from the general clinics of the nationwide School Health Service. Inclusion criteria were percentage ideal weight for height (WFH) of more than 140%, and onset of obesity before 6 years of age. There were 128 boys (63.7%); ethnic distribution was as follows: Chinese 105 (52.2%), Malay 69 (34.3%), Indian 21 (10.4%) and Others 6 (3%). The mean (SD) age was 11.1 (3.0) years, WFH 170.5 (22.7) %, BMI 31.9 (5.5) kg/m<sup>2</sup>, and % body fat 40.7 (5.2) % by dual energy x-ray absorptiometry (DEXA) or 45.7 (9.1) % by bipedal bioimpedance analysis for body fat composition (BIA). This research was approved by our hospital’s Research and Ethics Committee, and informed written consent was obtained from all subjects and parents.

Blood samples were obtained in the fasted state. Leptin was measured using DSL-10-23100 Human Leptin Enzyme-Linked Immunosorbent Kit (Diagnostic Systems Laboratories, Texas). Insulin resistance was calculated using the homeostasis model assessment (HOMA) (14), and insulin sensitivity by Quantitative Insulin Sensitivity Check Index (QUICKI) (15). Bone density and body fat composition were assessed using DEXA (Norland DEXA model XR-36, Fort Atkinson, Wisconsin). BIA was performed using the portable Tanita Body Composition Analyzer (TBF-300GS, Tanita Corporation, Tokyo).

Qualitative food-intake evaluation was performed using the Three-Factor Eating Questionnaire, a psychometric instrument to study eating behaviour, measuring three dimensions: restraint,
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disinhibition and hunger (16). Restrained eating is defined as the tendency to restrict food intake in order to control body weight. Disinhibition is the inability to resist emotional and social eating cues. Hunger is the subjective feeling of hunger. Numeric scores for each of the three factors were derived for each subject. Data concerning estimation of their physical activities were also obtained by using a physical activity questionnaire modified from the Modified Activity Questionnaire for Adolescents and validated in our local population (17, 18), and each subject was categorized depending on physical activity for the past month: categories 1 (inactive), 2 (relatively inactive), 3 (light physical activity), 4 (moderately active), and 5 (vigorous physical activity).

**DNA analysis**

Genomic DNA was extracted from peripheral leukocytes. The single exon was amplified by PCR and sequenced as previously described (13).

Three novel mutations, Ile183Asn, Ala70Thr, and Met134Ile were found during the screening process. 188 genomic DNA samples from 99 healthy children with normal height and weight [mean age (SD) = 7.1 (4.6) years; mean BMI (SD) = 16.8 (3.4) kg/m$^2$; 63 males], and 89 healthy adults (mean BMI < 25 kg/m$^2$, 30 males) were analysed as normal controls. The three novel mutations were not found in these 188 normal controls. Allele-specific PCR for Ile183Asn was performed as previously described (13). The other methods used are restriction enzyme digest using HhaI for Ala70Thr (G208A) and allele-specific PCR for Met134Ile.

Two common variants Thr6Lys and Val81Ile were always found together, and we confirmed they resided on the same allele (haplotype) in the heterozygotes. Allele specific PCR of Thr6 and Lys6 alleles were performed using selective forward primers, and amplified DNA was then incubated with BseDI. Only amplicons with Val81 (triplet GTT) would be cut.

**In-vitro receptor function studies**

**Construction of MC3R plasmids and expression**

Wild type, variant (Thr6Lys and Val81Ile) and mutant MC3R (Ala70Thr, Met134Ile and Ile183Asn) were directly amplified from individual genomic DNA using Pfx DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA). Forward and reverse primers were designed with Hind III and XbaI enzyme restriction sequences at the 5’ end. The Renilla luciferase reporter gene was amplified from the pHRG-TK reporter vector (Promega Corp, Madison, WI) with a pair of primers containing the KpnI and XhoI restriction sites at the 5’ end. All generated amplicons were TA cloned into the pDrive vector using the QIAGEN PCR Cloning Plus Kit (Qiagen GmbH, Hilden, Germany).

All MC3R inserts were excised from the pDrive/MC3R constructs using Hind III and XbaI, and ligated into the pBudCE4 mammalian dual expression vector (Invitrogen Life Technologies, Carlsbad, CA) under the control of the CMV promoter while the Renilla luciferase reporter gene was ligated into the KpnI and XhoI site of the vector under the control of the EF-1α promoter. For creating stable cell lines expressing the receptors, amplified MC3R gene was TA cloned into the pcDNA5/FRT/V5-His vector (Invitrogen Life Technologies, Carlsbad, CA).

One day prior to transfection, subconfluent Griptite human embryonic
kidney cells (Invitrogen Life Technologies, Carlsbad, CA) were trypsinized and seeded in 96-well plates at a density of 1x10^4 cells/well. Transient transfection was performed using the Effectene transfection reagent (Qiagen GmbH, Hilden, Germany) with 90 ng pCRE-Luc (Stratagene, La Jolla, CA) and 10 ng pBudCE4/MC3R/Renilla construct per well.

Stably expressed MC3R cells were created with the Flp-In System (Invitrogen Life Technologies, Carlsbad, CA) as recommended by the manufacturer. Briefly, pcDNA5/FRT/V5-His/MC3R and pOG44 vectors were transfected into Flp-In 293 cells at a ratio of 9 to 1. After 48 hours, the cells were trypsinized and seeded into T75 flasks containing complete DMEM media and 100 ug/ml hygromycin B for selection.

MC3R stimulation, luciferase assay, and dimerisation

24 hours after transient transfection, cells were washed with PBS and stimulated with α-MSH (Sigma-Aldrich, St. Louis, Missouri, USA) with concentrations ranging from 1 pM to 1 μM in DMEM media containing 0.2% BSA for 6 hours. Cells were then washed and lysed with 1x passive lysis buffer, then assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corp, Madison, WI) in the TD-20/20 luminometer (Turner Biosystems Inc, Sunnyvale, USA). cAMP induced firefly luciferase readings from pCRE-Luc expression were normalized with Renilla luciferase readings expressed from the pBudCE4/MC3R/Renilla plasmid construct. Normalized luciferase readings were then expressed as fold activity over basal (non-stimulated transfected cells). Data points were fitted by non-linear regression analysis using GraphPad Prism software (version 4.00 for Windows, GraphPad, San Diego, California, USA).

To investigate effects of receptor dimerization between wild type and mutant receptors, stable cells expressing wild type receptors were transfected with pBudCE4/mutant constructs, pCRE-Luc and phRG-TK reporter vector. Receptor stimulation and luciferase assay were then performed similarly as above.

Membrane preparation

Stable cell lines expressing receptors were grown in selective media until subconfluent. Cells were washed with ice cold PBS and scraped off, pelleted, then resuspended in 2 mls of ice cold homogenization buffer (20 mM HEPES, pH 7.0, 1 mM MgCl2, 1 mM EDTA, 1 mM PMSF). The cell suspension was homogenized in Dounce glass homogenizer on ice. The homogenate was centrifuged at 1700xg for 10 minutes at 4°C, the supernatant was then collected, and centrifuged at 40,000xg for 40 minutes at 4°C. The pellet containing crude cell membranes was then resuspended in homogenization buffer, aliquoted and stored at –80°C immediately. Protein concentration of crude membranes was determined using Bio-Rad’s Protein Assay reagent (Bio-Rad, Hercules, CA) with BSA as the standard.

Receptor binding studies

Binding studies were performed using time-resolved fluorometry (TRF) technology with europium labeled ligand. 24 hours after transient transfection in 96-well plates, cells were washed once in PBS and incubated for two hours with increasing concentrations of europium labeled NDP-αMSH (PerkinElmer, Boston, MA) in binding buffer (25 mM HEPES, pH 7.0, 1.5 mM CaCl2, 1 mM
MgSO₄, 100 mM NaCl, 25 uM EDTA, 0.2% BSA). Non-specific binding was determined in the presence of 3 µM unlabeled NDP-αMSH. After incubation, cells were washed four times with wash buffer (50 mM Tris-Cl, pH 7.5, 5 mM MgCl₂) and incubated with 100 µL/well of DELFIA Enhancement Solution (PerkinElmer, Boston, MA) at room temperature for 15 minutes. Fluorescence were measured in the Wallac Victor²-V 1420 multilabel HTS counter (PerkinElmer, Boston, MA) fitted with filters for europium TRF measurements.

Saturation studies using crude cell membranes were performed similarly in 100 µl of HEPES binding buffer using 2 µg of membrane protein, in Acrowell filter plates (Pall Corp, East Hills, NY). Reactions were terminated by rapid filtration using a vacuum manifold with four washes of wash buffer before the addition of the DELFIA enhancement solution.

Competitive ligand binding studies were performed in 100 µl of HEPES binding buffer containing 2 µg of crude membranes, europium labeled ligand at a concentration close to the Kd values and competitor ligands (NDP-αMSH and α-MSH). Incubation conditions were similar as for saturation studies and plates were processed as described above.

**Immunofluorescence staining of MC3R**

Griptite 293 cells grown on glass coverslips were transiently transfected with 200 ng of pBudCE4/MC3R/Renilla using the Effectene reagent. After 48 hours incubation, the cells were washed once in PBS and fixed with 3.7% paraformaldehyde for 15 mins at room temperature. The cells were washed three times with PBS and then incubated with blocking buffer (5% BSA, 2% FBS) for 30 minutes at room temperature. After rinsing in PBS, cells were incubated with polyclonal rabbit anti-MC3R IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA for 2 hours at room temperature. Cells were washed 4 times with PBS and then incubated with goat anti-rabbit IgG conjugated with Alexa fluor 488 dye (Molecular Probes Inc, Eugene, OR) in 1% BSA for 1 hour at room temperature. Cells were then subjected to 5 washes with PBS and coverslips were mounted with ProLong Gold Antifade reagent (Molecular Probes Inc, Eugene, OR). Fluorescence was visualized with the Carl Zeiss Axioskop microscope (100x).

**Statistical analysis**

Comparison of parameters was performed with Student’s t test, General Linear Model with covariates analyses to adjust for age, gender and ethnicity, One way ANOVA trend analysis, Mann Whitney U test, Fisher’s exact test and chi-square test where appropriate (SPSS Inc., Chicago, Ill., USA). Graph fit curves and EC₅₀/IC₅₀ were obtained and performed using GraphPad Prism 4 for Windows (ver 4.02 GraphPad software, San Diego, California, USA), and curves were fitted using the logistical equation. Ki values were determined from IC₅₀ values using the equation of Cheng and Prusoff (19).

**RESULTS**

We found three rare variants or potentially novel missense mutations, Ile183Asn (T548A), Ala70Thr (G208A), and Met134Ile (G402A), in the heterozygous state in three unrelated subjects (Figures 1a, b and c), as well as two common variants Thr6Lys and Val81Ile. The three potentially novel mutations Ile183Asn, Ala70Thr, and Met134Ile were not found in 188 non-obese normal controls.
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Common variants
We confirmed the Thr6Lys (17C>A) and Val81Ile (241G>A) variants were in complete linkage disequilibrium and located on the same allele (haplotype) in our cohort and normal controls. Of 198 subjects (excluding the three with novel variants), 121 were homozygous for the wildtype allele, 70 subjects were heterozygous, and 7 subjects were homozygous for the allele (haplotype) with common variants. 88 DNA samples from the 99 non-obese children (controls) were randomly selected for genotyping for the common variants, and 5 DNA samples failed to yield satisfactory PCR products or sequence readings. Among these 83 non-obese children, 31 were found to be heterozygous and 2 homozygous for the 6Lys/81Ile haplotype (henceforth identified as 6K/81I). No significant difference in genotypic frequencies was detected when compared to the obese group ($p=0.843$).

Obese subjects with 6K/81I had significantly higher leptin levels, percentage body fat, and insulin sensitivity index QUICKI, with lower insulin resistance index HOMA. The causative role of the 6K/81I variants is further supported by the presence of an additive effect, where the heterozygotes had an intermediate phenotype compared to homozygotes (table 1). In addition, the obese subjects heterozygous and homozygous for 6K/81I had significantly lower triglyceride levels ($1.19 + 0.50$ mmol/L vs. $1.41 + 0.73$ mmol/L, $p=0.012$) and lower fasting glucose levels ($4.4 + 0.5$ mmol/L vs. $4.8 + 0.8$ mmol/L, $p=0.024$) compared to obese subjects with wildtype MC3R alleles. We did not detect any significant differences in other parameters, including insulin and c-peptide levels, insulin-glucose ratios, frequency of diabetes mellitus/impaired glucose tolerance, physical activity pattern, or feeding behaviour scores. Our clinical findings are supported by in-vitro studies which revealed a modest but significant decrease ($p<0.01$) in maximal activity of the MC3 receptor with variants 6K/81I compared to wildtype MC3 receptor (figure 2a), without any significant difference in binding affinity to α-MSH or NDP-MSH (figure 2e). Based on the MC3R knockout mouse model, reduced MC3 receptor activity is expected to lead to increased body fat (feed efficiency), and leptin levels. (1, 2)

Ile183Asn
This novel MC3R mutation was found in an Indian family, and only part of the pedigree has been described previously (11). We have further examined the extended pedigree in an attempt to better define the effects of this mutation, and also completed our in-vitro studies of the Ile183Asn mutant MC3 receptor. A 13-year-old Indian girl (proband) with early onset severe obesity was heterozygous for this mutation (figure 3a). Four years later, at 17 years of age, the proband had developed morbid obesity with high percentage ideal body weight for height (WFH) of 216%, and percentage body fat (%BF) of 71%. Her heterozygous father’s WFH has increased to 167%, and %BF was 48%. The proband was the most overweight of the family with the highest %BF. The eldest sibling (not reported previously) was recently found to be heterozygous for this mutation. At 23 years old, he was not obese, with BMI of 23.5 kg/m². However, he was overweight as a child. At 7 years old, his weight was documented as 27 kg with WFH of 125% (>120% defined as mild obesity). He only started to lose weight in his teenage years as he was physically active, and underwent army training for two years.
He has since maintained a very active lifestyle with regular exercise and sports, and is careful with his diet. The paternal uncles and aunt had late onset obesity or overweight phenotypes, and genotyping revealed wildtype MC3R alleles. An interesting observation was the cosegregation of early onset childhood obesity with the mutant MC3R allele (figure 3a). Among the four siblings (paternal uncles and aunt), the proband’s father was the only one who was obese since early childhood. His documented weight and BMI at 16 years were 86 kg and 33 kg/m². The other three sibs were overweight only after 30 years of age: the eldest uncle was 64 kg at 18 years of age, and second uncle was 60 kg at 20 years old.

Ala70Thr

The Ala70Thr mutation was found in an 11-year-old boy and his mother (figure 3b). The proband was overtly obese, whereas his heterozygous mother had mild obesity (% wt for ht 121%). His mother was overweight as a child, but documented weight and height measurements were unavailable. She has been careful with her diet and exercises regularly, as she is very conscious of her weight and body image.

Met134Ile

The heterozygous Met134Ile mutation was found in a 12-year-old Indian girl and her obese mother, and both mother and child have type 2 diabetes mellitus (figure 3c). The proband was asymptomatic but was diagnosed by abnormal oral glucose tolerance test.

The 3 probands were compared to groups of obese controls of similar age and same gender as shown in table 2. The controls selected were 2-3 years younger or older than the subjects with rare variants. This age range was chosen arbitrarily to include a sizeable number of controls for this comparison. All subjects who fulfilled the criteria are included in the analysis. The probands had significantly higher leptin levels ($p=0.018$), and also appeared to have higher BMI, W/FH and %BF, although these did not reach statistical significance. We also observed that heterozygotes had significantly lower Hunger scores (less subjective feeling of hunger) compared to that of the obese controls ($p=0.026$) (table 3). The scores for Restricted Eating and Disinhibition were not significantly different between the heterozygotes and the obese controls without MC3R mutations ($p >0.05$ for each factor analysed). This is reminiscent of the MC3R knockout mice which were hypophagic with high leptin levels and body fat mass (1-2). We did not detect any significant difference in HOMA or insulin/glucose ratio, or physical activity levels.

Impaired signaling activities of the mutant receptors

As wildtype MC3R and MC3R with common variants 6K/81I (haplotype) showed significant differences in response to $\alpha$-MSH stimulation (11), we compared the signaling properties of the mutant MC3Rs to the corresponding MC3R with or without 6K/81I. This is also in consideration of possible conformational changes induced by interaction of these variants. The Ile183Asn and Met134Ile mutant alleles of our two subjects carried the 6K/81I (haplotype) concomitantly, and were thus compared to MC3R with 6K/81I (haplotype) in our transfection studies. This was determined by cloning each of the two MC3R alleles from the subject’s DNA sample into pDrive vector followed by direct sequencing, and also
by allele-specific PCR for Lys6 followed by direct sequencing as described above.

Both transient and stable transfection studies revealed severely impaired signaling response of the Ile183Asn mutant receptor to α-MSH. The cells transiently expressing the mutant MC3 receptor Ile183Asn showed negligible response to increasing α-MSH concentration (figure 2b and c). Mutant receptor Met134Ile demonstrated a modest but significantly higher EC$_{50}$ compared to MC3R with 6K/81I (figure 2b). The mutant Ala70Thr receptor also demonstrated significantly reduced cAMP response to MSH stimulation (figure 2d). Our competitive ligand binding studies did not find any significant differences in IC$_{50}$ between the wildtype, 6K/81I, Ala70Thr, Met134Ile and Ile183Asn MC3 receptors (figure 2e). The three mutant MC3 receptors did not exert any dimerisation effect on wildtype or 6K/81I MC3 receptors (figure 4). The findings were similar using NDP-MSH instead of α-MSH (data not shown). Immunofluorescence staining of HEK cells transiently transfected with these variant MC3 receptors revealed good level of cell surface expression comparable to wildtype MC3R qualitatively (figure 5).

DISCUSSION

Common obesity is a polygenic trait resulting from interaction of multiple genetic loci with the environment. Sequence variants in a large set of genes implicated in energy regulation could predispose an individual to excessive weight gain in a given environment. While MC3R mutations are unlikely to result in an autosomal dominant form of monogenic obesity, this study provides evidence that MC3R can be one of the predisposing genes which contributes to increased adiposity, and the wide variation in the adiposity of the individuals with common and rare variants may be due to other modifying genetic and environmental factors.

MSH, a derivative of pro-opiomelanocortin (POMC), acts on MC4R and MC3R to reduce feeding and feed efficiency (1-3). The Mc3r$^{-/-}$ mice demonstrated mild obesity but increased body fat and leptin, while the phenotype of the heterozygous Mc3r$^{+/-}$ mice did not appear to differ significantly from wildtype mice (1, 2). In comparison, the Pomc$^{-/-}$ mice also exhibit an obese phenotype while the heterozygous Pomc$^{+/-}$ mice appeared to have a similar phenotype to wildtype mice on standard chow (20, 21). However, Pomc$^{+/-}$ mice developed obesity when put on a high-fat diet (21), exhibiting an intermediate obese phenotype between wild-type and Pomc$^{-/-}$ mice, demonstrating that a single functional copy of the POMC gene is not sufficient for maintaining normal energy homeostasis under certain environmental conditions, and haploinsufficiency can interact with dietary factors to increase body weight. There is evidence to suggest that loss of one POMC allele, and even genetic variants with subtle effects on POMC function, could influence susceptibility to obesity in humans in our modern ‘obesogenic’ environment. Heterozygous parents of obese children homozygous for POMC null mutations had high normal to high BMI (22), and other family members heterozygous for POMC null allele were more overweight than those with wildtype alleles (23). Heterozygous partially inactivating POMC mutations Arg236Gly and Tyr221Cys were also found more frequently in obese children, and the phenotype is reminiscent of MC4R deficiency (24, 25). Therefore, partially inactivating genetic variants of MC3R
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may likewise exert a significant effect on the phenotype even in the heterozygous state, in the ‘obesogenic’ environment. This notion was supported by the linkage of a locus encoding MC3R on human chromosome 20q13.2 to the regulation of BMI, subcutaneous fat mass and fasting insulin (26) We report common and rare novel variants at the MC3R locus which result in partially reduced activity of the MC3R in response to MSH, and demonstrate that the common variants, and possibly the rare variants, are associated with increased body fat and leptin levels (with additive effect), and perhaps decreased hunger, in human subjects, congruous with the phenotype of the Mc3r-/- mice. Of note is the presence of childhood or early onset obesity in all the carriers of the rare mutations (figures 3a-c), although the significance of this is uncertain.

There were varying reports of increased insulin-glucose ratio, HOMA, leptin, BMI, and body fat in humans with 6K/81I (8,11,12). Our report reinforced the evidence that these two variants contribute significantly to the human phenotype by demonstrating a significant additive effect of 6K/81I on adiposity and leptin. Feng et al reported that the MC3R with 6K/81I resulted in reduced receptor activity, binding affinity, and protein expression compared to wildtype MC3R (12). Tao et al however reported there was no difference in receptor activity and ligand binding affinity for these two variants (27). Our report has furthered this debate; we found that MC3R with 6K/81I had reduced receptor activity in response to MSH as reported by Feng, but we did not detect any difference in ligand binding, and there was qualitative evidence that the variant MC3R was well expressed on the cell surface.

The three rare variants were not found in other similar studies (8-12). Isoleucine residue 183 of MC3R is located in the second intracytoplasmic loop of this G-protein coupled receptor (4). Isoleucine at codon 183 is a highly conserved residue, present in the MC3R sequence of many other species, from teleost fish through to mammals (accession numbers: Zebrafish NP_851303, Chicken BAA32555, Mouse AA103670, Rat NP_001020441, Human NP063941). This hydrophobic isoleucine was substituted by hydrophilic asparagine in Ile183Asn MC3R. The transmembrane domain directly interacts with G proteins and controls cAMP production; this mutation may cause partial reduction in receptor function resulting from abnormal G protein interaction. After our first communication of this mutation (13), Tao et al (27) and Rached et al (28) subsequently reported that the Ile183Asn MC3R had total abolished cAMP response to MSH, and Rached also reported a dominant negative effect on wildtype MC3R, and complete intracellular retention with no cell surface expression. Our studies demonstrated near total but not complete loss of cAMP generation in response to MSH stimulation, with normal cell surface expression, normal ligand binding affinity, and absence of dominant negative activity on wildtype MC3R.

The Ala70Thr mutation affects the extracellular domain (4), within the critical region for binding activity (29), where the hydrophobic alanine residue was changed to hydrophilic threonine. The hydrophobic alanine residue at codon 70 is replaced by another hydrophobic residue Glycine in the MC3R of chicken, mouse, rat and spiny dogfish (accession number: AAS66720); thus it may be possible that the substitution by a
hydrophilic residue (threonine) may result in a significant change in conformation and function of the receptor. The met134lle mutation is located at the second transmembrane region of the seventh transmembrane domain (4). The hydrophobic methionine is conserved in chicken, mouse, and rat.

The three novel rare mutations were not found in the population sample, nor reported elsewhere, and the clinical characteristics of our subjects coupled with the in-vitro studies support their pathogenic role. We believe that our report has shed light on the human MC3R mutation phenotype, and further studies with bigger numbers will continue to unravel the phenotype and support the role of MC3R in human weight regulation and pathogenesis of obesity.

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**Table 1.** Additive effect of the 6K/81I variants on the adiposity, leptin levels and insulin resistance indices in Table A. all subjects, B. Chinese subjects, C. Malay subjects, and D. Indian subjects.

Table 1A. All subjects

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<th>Wildtype</th>
<th>Heterozygous for 6K/81I</th>
<th>Homozygous for 6K/81I</th>
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<td>121</td>
<td>70</td>
<td>7</td>
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<td>-</td>
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<tr>
<td>Age (years)</td>
<td>11.0 (3.0)</td>
<td>11.2 (3.0)</td>
<td>12.2 (3.5)</td>
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<td>WFH (%)</td>
<td>168.5 (18.6)</td>
<td>172.0 (24.8)</td>
<td>174.1 (21.0)</td>
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<td>0.222 (ns)</td>
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<td>Leptin (mcg/L)</td>
<td>45.4 (31.3)</td>
<td>54.9 (37.9)</td>
<td>82.6 (64.8)</td>
<td>0.041</td>
<td>&lt;0.001</td>
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<td>body fat (%)</td>
<td>44.4 (9.0)</td>
<td>48.7 (8.7)</td>
<td>54.1 (14.5)</td>
<td>0.002</td>
<td>&lt;0.001</td>
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<td>HOMA</td>
<td>5.180 (1.914)</td>
<td>5.076 (2.397)</td>
<td>2.707 (2.060)</td>
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<td>QUICKI</td>
<td>0.303 (0.025)</td>
<td>0.306 (0.035)</td>
<td>0.331 (0.035)</td>
<td>0.016</td>
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<td>Insulin/glucose ratio</td>
<td>6.48 (4.11)</td>
<td>8.71 (14.09)</td>
<td>3.81 (2.77)</td>
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<td>0.276 (ns)</td>
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Table 1B. Chinese subjects

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<td>Age (years)</td>
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<td>-</td>
<td>0.71</td>
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<tr>
<td>WFH (%)</td>
<td>167.3 (18.0)</td>
<td>165.0 (19.0)</td>
<td>166.0 (15.4)</td>
<td>0.841</td>
<td>0.818</td>
</tr>
<tr>
<td>Leptin (mcg/L)</td>
<td>41.5 (31.5)</td>
<td>45.1 (32.7)</td>
<td>48.8 (4.0)</td>
<td>0.07</td>
<td>0.091</td>
</tr>
<tr>
<td>body fat (%)</td>
<td>44.4 (8.9)</td>
<td>48.8 (8.7)</td>
<td>55.3 (13.3)</td>
<td>0.002</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA</td>
<td>5.975 (1.851)</td>
<td>5.701 (1.944)</td>
<td>2.494 (1.576)</td>
<td>0.018</td>
<td>0.069</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.297 (0.023)</td>
<td>0.299 (0.025)</td>
<td>0.334 (0.023)</td>
<td>0.009</td>
<td>0.038</td>
</tr>
</tbody>
</table>
Table 1C. Malay subjects

<table>
<thead>
<tr>
<th></th>
<th>Wildtype</th>
<th>Heterozygous for 6K/811</th>
<th>Homozygous for 6K/811</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>44</td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>10.1 (2.9)</td>
<td>10.0 (3.1)</td>
<td>-</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>WFH (%)</strong></td>
<td>168.6 (17.3)</td>
<td>181.3 (31.0)</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Leptin (mcg/L)</strong></td>
<td>43.7 (23.5)</td>
<td>49.7 (30.0)</td>
<td>-</td>
<td>0.064</td>
</tr>
<tr>
<td><strong>body fat (%)</strong></td>
<td>44.5 (7.8)</td>
<td>48.5 (7.5)</td>
<td>-</td>
<td>0.048</td>
</tr>
<tr>
<td><strong>HOMA</strong></td>
<td>4.219 (1.904)</td>
<td>3.778 (3.204)</td>
<td>-</td>
<td>0.731</td>
</tr>
<tr>
<td><strong>QUICKI</strong></td>
<td>0.312 (0.027)</td>
<td>0.321 (0.048)</td>
<td>-</td>
<td>0.361</td>
</tr>
</tbody>
</table>
Table 1D. Indian subjects

<table>
<thead>
<tr>
<th></th>
<th>Wildtype</th>
<th>Heterozygous for 6K/81I</th>
<th>Homozygous for 6K/81I</th>
<th>p-value #</th>
<th>p-value +</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.3 (2.5)</td>
<td>12.0 (3.4)</td>
<td>11.4 (5.0)</td>
<td>-</td>
<td>0.603 (ns)</td>
</tr>
<tr>
<td>WFH (%)</td>
<td>164.8 (16.6)</td>
<td>179.9 (21.4)</td>
<td>179.7 (30.3)</td>
<td>0.552 (ns)</td>
<td>0.325 (ns)</td>
</tr>
<tr>
<td>Leptin (mcg/L)</td>
<td>66.8 (34.5)</td>
<td>113.3 (28.5)</td>
<td>127.0 (67.6)</td>
<td>0.046</td>
<td>0.035</td>
</tr>
<tr>
<td>body fat (%)</td>
<td>51.2 (10.0)</td>
<td>57.8 (7.8)</td>
<td>69.2 (10.0)</td>
<td>0.157</td>
<td>0.058</td>
</tr>
<tr>
<td>HOMA</td>
<td>5.654 (2.045)</td>
<td>5.587 (2.004)</td>
<td>2.937 (2.827)</td>
<td>0.228</td>
<td>0.309</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.300 (0.024)</td>
<td>0.294 (0.026)</td>
<td>0.330 (0.050)</td>
<td>0.118</td>
<td>0.213</td>
</tr>
</tbody>
</table>

All values are expressed as mean (standard deviation).

# General linear model with age, gender and race as covariates (where appropriate).

+ One-way ANOVA trend analysis.
Table 2. Comparison of the age, body mass index (BMI), percentage ideal weight for height (WFH), waist-hip ratio (WHR), and percentage body fat (%BF) of each proband with a group of controls of similar age, same gender, and 6K/81I carrier status.

<table>
<thead>
<tr>
<th></th>
<th>Met134Ile</th>
<th>Controls† N=25</th>
<th>Ala70Thr Controls‡ n=37</th>
<th>Ile183Asn Controls§ n=4</th>
<th>All 3 probands</th>
<th>Controls n=110</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11.9</td>
<td>11.1 (3.2)</td>
<td>11.7</td>
<td>11.1 (1.3)</td>
<td>13.1</td>
<td>13.6 (1.9)</td>
<td>12.3 (0.7)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.6</td>
<td>30.7 (3.6)</td>
<td>35.0</td>
<td>32.0 (3.6)</td>
<td>36.6</td>
<td>33.1 (5.7)</td>
<td>34.4 (2.6)</td>
</tr>
<tr>
<td>WFH (%)</td>
<td>178.0</td>
<td>166.1 (19.8)</td>
<td>173</td>
<td>166.5 (15.8)</td>
<td>182</td>
<td>180.5 (22.9)</td>
<td>177.7 (4.5)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.990</td>
<td>0.890 (0.072)</td>
<td>0.972</td>
<td>0.950 (0.062)</td>
<td>0.954</td>
<td>0.870 (0.035)</td>
<td>0.981 (0.013)</td>
</tr>
<tr>
<td>%BF</td>
<td>57.3</td>
<td>47.0 (6.7)</td>
<td>40.0</td>
<td>41.1 (3.9)</td>
<td>61.3</td>
<td>58.4 (12.5)</td>
<td>52.2 (12.5)</td>
</tr>
<tr>
<td>Leptin (mcg/L)</td>
<td>116.4</td>
<td>53.7 (36.4)</td>
<td>71.9</td>
<td>45.8 (29.6)</td>
<td>142.8</td>
<td>100.9 (75.1)</td>
<td>110.4 (35.8)</td>
</tr>
</tbody>
</table>

Values expressed as mean (standard deviation).

† Females selected based on age 3 years above or below 11.9 years, and heterozygous for 6K/81I, as subject with Met134Ile is heterozygous for 6K/81I.
‡Males selected based on age 2 years above or below 11.7 years, and with wildtype \textit{MC3R} alleles 6T/81V, as subject with Ala70Thr is homozygous for wildtype allele.

§Females selected based on age 2 years above or below 13.1 years, and homozygous for 6K/81I, as subject with Ile183Asn is homozygous for 6K/81I.

All controls that fulfilled age criteria are included in the analysis.
Table 3. Feeding behaviour assessment of the three probands and obese heterozygote parents, compared to obese controls without MC3R mutations.

<table>
<thead>
<tr>
<th></th>
<th>Obese subjects without MC3R mutations (n=161)</th>
<th>Proband Ile183Asn</th>
<th>Father Ile183Asn</th>
<th>Proband Ala70Thr</th>
<th>Proband Met134Ile</th>
<th>Mother Met134Ile</th>
<th>All heterozygotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restraint</td>
<td>9 (0-20)</td>
<td>11</td>
<td>16</td>
<td>7</td>
<td>9</td>
<td>11</td>
<td>11 (7-16)</td>
</tr>
<tr>
<td>Disinhibition</td>
<td>7 (0-14)</td>
<td>2</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>6 (2-8)</td>
</tr>
<tr>
<td>Hunger</td>
<td>7 (0-14)</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>3 (2-7)*</td>
</tr>
</tbody>
</table>

Scores expressed as median (range).
**Figure Legends**

**Figure 1 a.** Sequencing of the MC3R gene in the proband revealed T to A transition in heterozygous state at nucleotide position 548 (N), leading to the substitution of isoleucine by asparagine at codon position 183. **b.** G to A change in heterozygous state at nucleotide position 208 causing change of alanine to threonine at codon position 70. **c.** G substitution by A in heterozygous state at nucleotide position 402, leading to change of methionine to isoleucine at codon 134.

**Figure 2.** Constructs with wildtype and variant MC3R were co-transfected with pCRE-Luc into HEK293 cells and stimulated with varying amounts of α-MSH. Y axis represents the fold increase over basal Relative Light Units (RLU), which is the ratio of luminescence generated by Firefly luciferase (from pCRE-Luc) to Renilla luciferase (from pBudCE4/Renilla/MC3R construct); the data is expressed as such to normalize for transfection efficiency and amount of MC3R produced. This represents the amount of cyclic AMP generated with increasing concentration of the ligand α-MSH (represented by x-axis). Each data point represents the mean and standard error range of at least 3 independent experiments performed in triplicates. **a.** There is a significant reduction in 6K/81I MC3 receptor compared to wildtype (normalized fold activity over basal 94.42 [95% CI 88.26 – 100.6] vs. 106.2 [95% CI 102.4-110.1]; p<0.01) **b.** There was severely impaired generation by the Ile183Asn mutant MC3 receptor (with 6K/81I) compared to the MC3R with 6K/81I. The Met134Ile mutant MC3 receptor (with 6K/81I) dose response curve is shifted to the right, with a significantly higher EC$_{50}$ value compared to receptor with 6K/81I (2.051x10$^{-9}$ [95% CI 1.358x10$^{-9}$ – 3.097x10$^{-9}$] vs. 7.408x10$^{-10}$ [95% CI 5.008x10$^{-10}$ – 1.096x10$^{-9}$]; p=0.0005). **c.** Ile183Asn mutant receptor actually exhibits a small response to MSH stimulation. Note magnified scale of y-axis. **d.** Ala70Thr mutant receptor has significantly reduced response to MSH stimulation compared to wildtype MC3R (normalized fold activity 92.7 [95% CI 86.0-99.3] vs. 106.2 [95% CI 102.4-110.1]; p<0.01). **e.** Competitive ligand binding studies using crude membranes and europium labeled NDP-MSH at a concentration close to the Kd values and competitor αMSH. Data is expressed as a percentage of the maximum counts of europium labeled NDP-αMSH binding to MC3R. Each point represents the mean and standard error range of at least 6 independent experiments in triplicates.

**Figure 3.** Family pedigrees of affected subjects with MC3R mutations. The age (in years), body mass index (BMI), percentage of ideal weight for height (WFH), and percentage body fat (%BF) are listed. Heterozygotes are half-shaded. Subjects with history of childhood or early onset obesity are highlighted with asterisk (*). **a.** Family with Ile183Asn mutation. **b.** Family with Ala70Thr mutation. **c.** Family with Met134Ile mutation.

**Figure 4** Mutant MC3 receptors were transfected into stable cell lines expressing **a.** wildtype MC3R. **b** MC3R with 6K/81I. There was no significant reduction in cAMP generation by these cells to suggest dimerisation as a result of concomitant expression of the mutant receptors.
Figure 5. Cell surface expression of MC3R detected by immunofluorescence staining. Griptite 293 cells were grown in coverslips and transiently transfected with indicated wild type and variants. Cells were not permeabilized and stained with anti-MC3R antibody followed by secondary antibody conjugated to Alexa 468 dye.
Role of MC3R mutations in obese children
Role of MC3R mutations in obese children

Fig 2a

Normalized fold activity over basal

log ([a-MSH] M)

WT
6K/81l

Fig 2b

Normalized fold activity over basal

log ([a-MSH] M)

6K/81l
M134l
I183N

25
Role of MC3R mutations in obese children

**Fig 2c**

![Graph showing normalized fold activity over basal for I183N mutation](image)

**Fig 2d**

![Graph showing normalized fold activity over basal for WT and A70T](image)
Role of MC3R mutations in obese children

Fig 2e

% specific bound

log ([α-MSH] M)

WT
A70T
M134I
I183N
6K/81I
Role of MC3R mutations in obese children

**Fig 3a**

**Ile183Asn**

55 y, BMI 26.8 kg/m², %BF 34%
53 y, BMI 27.8 kg/m², %BF 36%
50 y, BMI 34.5 kg/m², %BF 65.7%
48 y, BMI 33.1 kg/m², %BF 48%
47 y, BMI 37.2 kg/m², %BF 60%

23 y, BMI 23.5 kg/m², %BF 34%
18 y, BMI 40.1 kg/m², %BF 71%
15 y, BMI 25.8 kg/m², %BF 41%

**Fig 3b**

**Ala70Thr**

46 y, BMI 23.9 kg/m², %BF 24%
41 y, BMI 24.3 kg/m², %BF 31%

11.5 y, BMI 35 kg/m², %BF 40%
10 y, BMI 20.8 kg/m², %BF 23%
8 y, BMI 19.7 kg/m², %BF 14%

**Fig 3c**

**Met134Ile**

Not assessed

39 y, BMI 31.9 kg/m², %BF 54%

12 y, BMI 31.6 kg/m², %BF 57%
Role of MC3R mutations in obese children

Mutant receptors transfected into stable cell lines expressing wild type MC3R

Mutant receptors transfected into stable cells expressing MC3R with 6K/81I

Fig 4a

Normalized fold activity over basal

Fig 4b

Normalized fold activity over basal
Role of MC3R mutations in obese children

Fig 5

Wild type 6K/81I

Ile183Asn Ala70Thr Met134Ile