The Role of Melanocortin 3 Receptor Gene in Childhood Obesity

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OBJECTIVE—Melanocortin 3 receptor (MC3R) plays a critical role in weight regulation of rodents, but its role in humans remains unclear. The objective of this study was to identify genetic variants of the *MC3R* gene and determine its association with childhood obesity.

RESEARCH DESIGN AND METHODS—We screened 201 obese children for *MC3R* gene mutations with anthropometric measurements, blood tests, feeding behavior, and body composition assessment. We identified three novel heterozygous mutations (Ile183Asn, Ala70Thr, and Met134Ile) in three unrelated subjects, which were not found in 188 control subjects, and two common polymorphisms Thr6Lys and Val81Ile.

RESULTS—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 with obese control subjects, 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 with wild-type 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 in which heterozygotes had an intermediate phenotype compared with homozygotes.

CONCLUSIONS—*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. ***Diabetes*** **56:2622–2630, 2007**

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*$^{-/-}$ 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*$^{-/-}$ mice were hypophagic with hyperleptinemia compared with wild-type littermates (2). Mice lacking both MC3R and MC4R have exacerbated obesity, which supports the notion that both are important and nonredundant (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 study, 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, and the association of the common *MC3R* variants with obesity-related phenotypes in our cohort of obese children.

RESEARCH DESIGN AND METHODS

The *MC3R* gene was analyzed 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 >140% and onset of obesity before 6 years of age. There were 128 boys (63.7%); the ethnic distribution was as follows: Chinese 105 (52.2%), Malay 69 (34.3%), Indian 21 (10.4%), and others 6 (3%). The age (mean ± SD) was 11.1 ± 3.0 years, WFH 170.5 ± 22.7%, BMI 31.9 ± 5.5 kg/m^2, and body fat 40.7 ± 5.2% by dual-energy X-ray absorptiometry (DEXA) or 45.7 ± 9.1% by bipedal bioimpedance analysis (BIA) for body fat composition. This research was approved by research and ethics committee of our hospital, 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, Webster, TX). Insulin resistance was calculated using the homeostasis model assessment (HOMA) (14), and insulin sensitivity was calculated by quantitative insulin sensitivity check index (QUICKI) (15). Bone density and body fat composition were assessed using DEXA (model XR-36; Norland, Fort Atkinson, WI). BIA was performed using the portable Tanita Body Composition Analyzer (TBF-300GS; Tanita, Tokyo).

Qualitative food-intake evaluation was performed using the Three-Factor Eating Questionnaire, a psychometric instrument to study eating behavior, measuring three dimensions: restraint, disinhibition, and hunger (16). Restrained eating is defined as the tendency to restrict food intake 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: 1, inactive; 2,

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BIA, bioimpedance analysis; DEXA, dual-energy X-ray absorptiometry; DMEM, Dulbecco's modified Eagle's medium; HOMA, homeostasis model assessment; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptor; MSH, melanocyte-stimulating hormone; NDP, [Nle4, D-Phe7]; POMC, proopiomelanocortin; QUICKI, quantitative insulin sensitivity check index; TRF, time-resolved fluorometry; WFH, weight for height.

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. Genomic DNA samples (n = 188) from 99 healthy children with normal height and weight (age [mean ± SD] 7.1 ± 4.6 years, BMI 16.8 ± 3.4 kg/m^2, 63 males) and 89 healthy adults (mean BMI <25 kg/m^2, 30 males) were analyzed as normal control subjects. The three novel mutations were not found in these 188 normal control subjects. Allele-specific PCR for Ile183Asn was performed as previously described (13). The other methods used are restriction enzyme digest using *Hha*I for Ala70Thr (G208A) and allele-specific PCR for Met134Ile.

The two common variants Thr6Lys and Val81Ile were always found together, and we confirmed that 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 *Bse*DI. 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 (Ala70Thr, Met134Ile, and Ile183Asn) *MC3R* were directly amplified from individual genomic DNA using Pfx DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA). Forward and reverse primers were designed with *Hin*dIII and *Xba*I enzyme restriction sequences at the 5′ end. The *Renilla* luciferase reporter gene was amplified from the phRG-TK reporter vector (Promega, Madison, WI) with a pair of primers containing the *Kpn*I and *Xho*I restriction sites at the 5′ end. All generated amplicons were TA cloned into the pDrive vector using the QIAGEN PCR Cloning Plus kit (Qiagen, Hilden, Germany).

All *MC3R* inserts were excised from the pDrive/*MC3R* constructs using *Hin*dIII and *Xba*I and ligated into the pBudCE4 mammalian dual-expression vector (Invitrogen Life Technologies) under the control of the cytomegalovirus promoter while the *Renilla* luciferase reporter gene was ligated into the *Kpn*I and *Xho*I site of the vector under the control of the elongation factor-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).

One day before transfection, subconfluent Griptite human embryonic kidney cells (Invitrogen Life Technologies) were trypsinized and seeded in 96-well plates at a density of 1×10^4 cells/well. Transient transfection was performed using the Effectene transfection reagent (Qiagen) 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) 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 9:1. After 48 h, the cells were trypsinized and seeded into T75 flasks containing complete Dulbecco's modified Eagle's medium (DMEM) and 100 μg/ml hygromycin B for selection.

MC3R stimulation, luciferase assay, and dimerization. Twenty-four hours after transient transfection, cells were washed with PBS and stimulated with α-MSH (Sigma-Aldrich, St. Louis, MO) with concentrations ranging from 1 pmol/l to 1 μmol/l in DMEM containing 0.2% BSA for 6 h. Cells were then washed and lysed with 1× passive lysis buffer and then assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega) in the TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA). 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 (nonstimulated transfected cells). Data points were fitted by nonlinear regression analysis using GraphPad Prism software (version 4.00 for Windows; GraphPad, San Diego, CA).

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, and then resuspended in 2 ml ice-cold homogenization buffer (20 mmol/l HEPES, pH 7.0, 1 mmol/l $MgCl_2$, 1 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride). The cell suspension was homogenized in Dounce glass homogenizer on ice. The homogenate was centrifuged at 1,700g for 10 min at 4°C, and the supernatant was then collected and centrifuged at 40,000g for 40 min 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 Protein Assay reagent (Bio-Rad, Hercules, CA) with BSA as the standard.

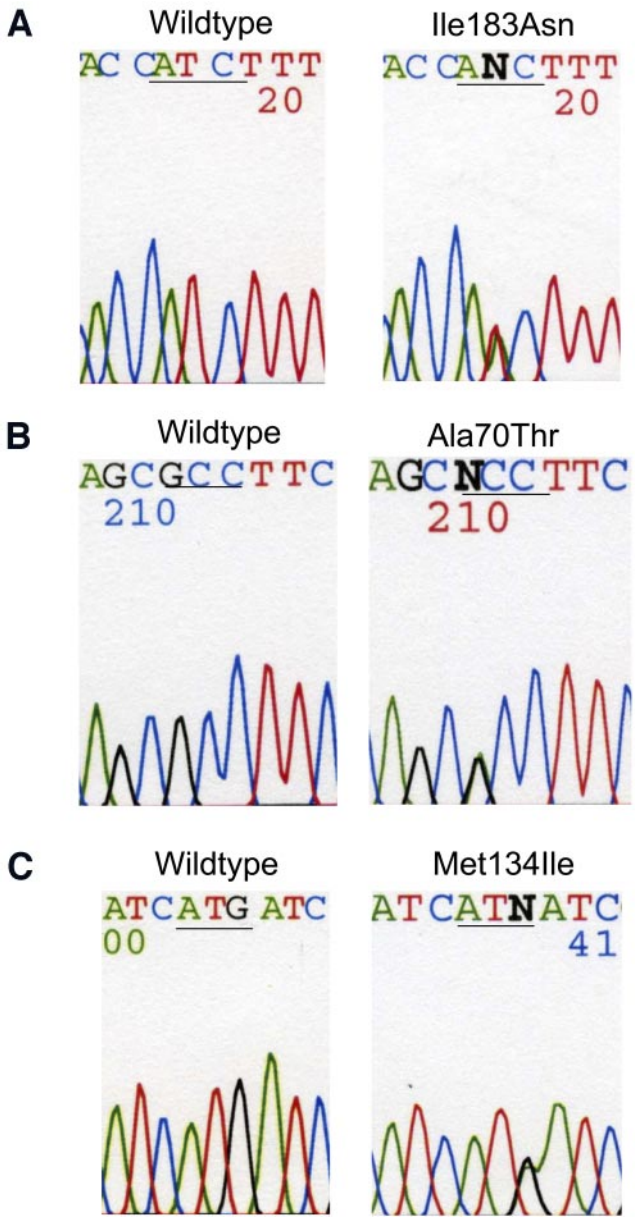

FIG. 1. *A*: Sequencing of the *MC3R* gene in the proband revealed T to A transition in heterozygous state at nucleotide position 548 (N), leading to the substitution of isoleucine by asparagine at codon position 183. *B*: G to A change in heterozygous state at nucleotide position 208 causing change 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.

Receptor binding studies. Binding studies were performed using time-resolved fluorometry (TRF) technology with europium-labeled ligand. Twenty-four hours after transient transfection in 96-well plates, cells were washed once in PBS and incubated for 2 h with increasing concentrations of europium-labeled [Nle4, D-Phe7] (NDP)-αMSH (PerkinElmer, Boston, MA) in binding buffer (25 mmol/l HEPES, pH 7.0, 1.5 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 100 mmol/l NaCl, 25 μmol/l EDTA, and 0.2% BSA). Nonspecific binding was determined in the presence of 3 μmol/l unlabeled NDP-αMSH. After incubation, cells were washed four times with wash buffer (50 mmol/l Tris-Cl, pH 7.5, and 5 mmol/l $MgCl_2$) and incubated with 100 μl/well DELFIA Enhancement Solution (PerkinElmer) at room temperature for 15 min. Fluorescence were measured in the Wallac Victor^2-V 1420 multilabel HTS counter (PerkinElmer) fitted with filters for europium TRF measurements. Saturation studies using crude cell membranes were performed similarly in 100 μl HEPES binding buffer using 2 μg membrane protein, in Acrowell filter plates (Pall, East Hills, NY). Reactions

TABLE 1
Additive effect of the 6K/81I variants on the adiposity, leptin levels, and insulin resistance indices

	Wild type	Heterozygous for 6K/81I	Homozygous for 6K/81I	*P* value*	*P* value†
All subjects					
n	121	70	7	—	—
Age (years)	11 ± 3	11.2 ± 3	12.2 ± 3.5	—	0.371 (NS)
WFH (%)	168.5 ± 18.6	172 ± 24.8	174.1 ± 21	0.495 (NS)	0.222 (NS)
Leptin (μg/l)	45.4 ± 31.3	54.9 ± 37.9	82.6 ± 64.8	0.041	<0.001
Body fat (%)	44.4 ± 9	48.7 ± 8.7	54.1 ± 14.5	0.002	<0.001
HOMA	5.180 ± 1.914	5.076 ± 2.397	2.707 ± 2.060	0.031	0.042
QUICKI	0.303 ± 0.025	0.306 ± 0.035	0.331 ± 0.035	0.016	0.047
Insulin-to-glucose ratio	6.48 ± 4.11	8.71 ± 14.09	3.81 ± 2.77	0.074 (NS)	0.276 (NS)
Chinese subjects					
n	64	38	3	—	—
Age (years)	11.5 ± 3	11.6 ± 2.7	12 ± 2.4	—	0.71
WFH (%)	167.3 ± 18	165 ± 19	166 ± 15.4	0.841	0.818
Leptin (μg/l)	41.5 ± 31.5	45.1 ± 32.7	48.8 ± 4	0.07	0.091
Body fat (%)	44.4 ± 8.9	48.8 ± 8.7	55.3 ± 13.3	0.002	<0.001
HOMA	5.975 ± 1.851	5.701 ± 1.944	2.494 ± 1.576	0.018	0.069
QUICKI	0.297 ± 0.023	0.299 ± 0.025	0.334 ± 0.023	0.009	0.038
Malay subjects					
n	44	24	—	—	—
Age (years)	10.1 ± 2.9	10 ± 3.1	—	—	NA
WFH (%)	168.6 ± 17.3	181.3 ± 31	—	0.04	NA
Leptin (μg/l)	43.7 ± 23.5	49.7 ± 30	—	0.064	NA
Body fat (%)	44.5 ± 7.8	48.5 ± 7.5	—	0.048	NA
HOMA	4.219 ± 1.904	3.778 ± 3.204	—	0.731	NA
QUICKI	0.312 ± 0.027	0.321 ± 0.048	—	0.361	NA
Indian subjects					
n	8	7	4	—	—
Age (years)	10.3 ± 2.5	12 ± 3.4	11.4 ± 5	—	0.603 (NS)
WFH (%)	164.8 ± 16.6	179.9 ± 21.4	179.7 ± 30.3	0.552 (NS)	0.325 (NS)
Leptin (μg/l)	66.8 ± 34.5	113.3 ± 28.5	127 ± 67.6	0.046	0.035
Body fat (%)	51.2 ± 10	57.8 ± 7.8	69.2 ± 10	0.157	0.058
HOMA	5.654 ± 2.045	5.587 ± 2.004	2.937 ± 2.827	0.228	0.309
QUICKI	0.300 ± 0.024	0.294 ± 0.026	0.330 ± 0.050	0.118	0.213

Data are means ± SD. *General linear model with age, sex, and race as covariates (where appropriate). †One-way ANOVA trend analysis. NA, not applicable; NS, not significant.

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 HEPES binding buffer containing 2 μg crude membranes, europium-labeled ligand at a concentration close to the kilodalton 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 pBudCE4/*MC3R*/*Renilla* using the Effectene reagent. After 48 h of incubation, the cells were washed once in PBS and fixed with 3.7% paraformaldehyde for 15 min at room temperature. The cells were washed three times with PBS and then incubated with blocking buffer (5% BSA and 2% fetal bovine serum) for 30 min 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 h at room temperature. Cells were washed four times with PBS and then incubated with goat anti-rabbit IgG conjugated with Alexa fluor 488 dye (Molecular Probes, Eugene, OR) in 1% BSA for 1 h at room temperature. Cells were then subjected to five washes with PBS, and coverslips were mounted with ProLong Gold Antifade reagent (Molecular Probes). Fluorescence was visualized with the Carl Zeiss Axioskop microscope (×100).

Statistical analysis. Comparison of parameters was performed with Student's *t* test; general linear model with covariates analyses to adjust for age, sex, and ethnicity; one-way ANOVA trend analysis; Mann-Whitney *U* test; Fisher's exact test; and χ^2 test where appropriate (SPSS, Chicago, IL). Graph fit curves and half-maximal effective concentration (EC_{50})/half-maximal inhibitory concentration (IC_{50}) were obtained and performed using GraphPad Prism 4 for Windows (v. 4.02 GraphPad software), and curves were fitted using the logistical equation. K_i values were determined from IC_{50} 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 (Fig. 1*A*–*C*) and two common variants, Thr6Lys and Val81Ile. The three potentially novel mutations Ile183Asn, Ala70Thr, and Met134Ile were not found in 188 nonobese normal control subjects.

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 control subjects. Of 198 subjects (excluding the three with novel variants), 121 subjects were homozygous for the wild-type allele, 70 subjects were heterozygous, and 7 subjects were homozygous for the allele (haplotype) with common variants. Eighty-eight DNA samples from the 99 nonobese children (control subjects) were randomly selected for genotyping for the common variants, and five DNA samples failed to yield satisfactory PCR products or sequence readings. Among these 83 nonobese 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 with the obese group ($P = 0.843$).

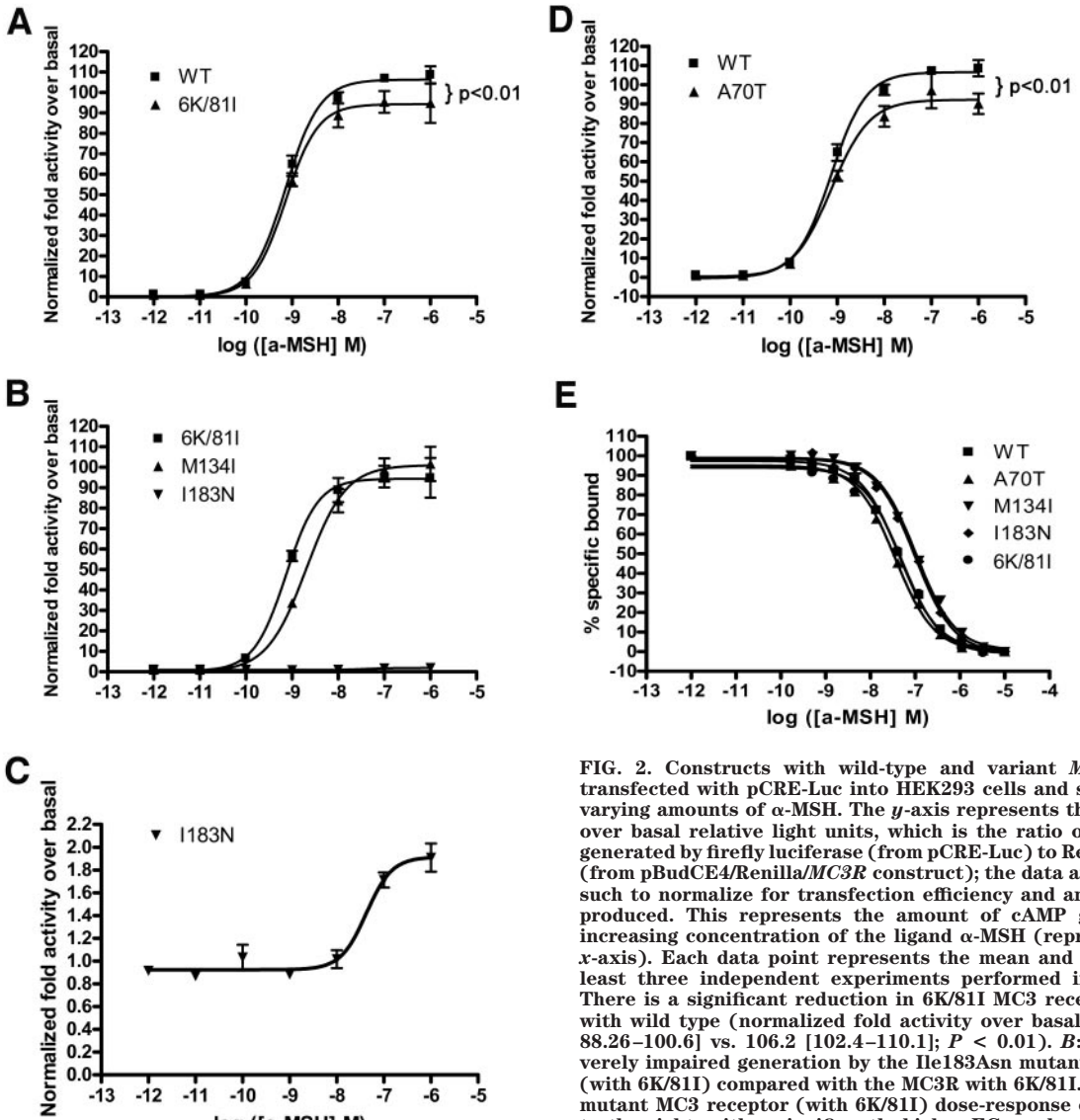

FIG. 2. Constructs with wild-type and variant *MC3R* were co-transfected with pCRE-Luc into HEK293 cells and stimulated with varying amounts of α-MSH. The *y*-axis represents the fold increase over basal relative light units, which is the ratio of luminescence generated by firefly luciferase (from pCRE-Luc) to Renilla luciferase (from pBudCE4/Renilla/*MC3R* construct); the data are expressed as such to normalize for transfection efficiency and amount of MC3R produced. This represents the amount of cAMP generated with increasing concentration of the ligand α-MSH (represented by the *x*-axis). Each data point represents the mean and SE range of at least three independent experiments performed in triplicate. *A*: There is a significant reduction in 6K/81I MC3 receptor compared with wild type (normalized fold activity over basal 94.42 [95% CI 88.26–100.6] vs. 106.2 [102.4–110.1]; $P < 0.01$). *B*: There was severely impaired generation by the Ile183Asn mutant MC3 receptor (with 6K/81I) compared with 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 with receptor with 6K/81I (2.051×10^{-9} [95% CI 1.358×10^{-9} to 3.097×10^{-9}] vs. 7.408×10^{-10} [5.008×10^{-10} to 1.096×10^{-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 with wild-type MC3R (normalized fold activity 92.7 [95% CI 86.0–99.3] vs. 106.2 [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 kilodalton values and competitor αMSH. Data are expressed as a percentage of the maximum counts of europium-labeled NDP-αMSH binding to MC3R. Each point represents the mean and SE range of at least six independent experiments in triplicate.

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 with homozygotes (Table 1). In addition, the obese subjects heterozygous and homozygous for 6K/81I had significantly lower triglyceride levels (1.19 ± 0.50 vs. 1.41 ± 0.73 mmol/l, $P = 0.012$) and lower fasting glucose levels (4.4 ± 0.5 vs. 4.8 ± 0.8 mmol/l, $P = 0.024$) compared with obese subjects with wild-type *MC3R* alleles. We did not detect any significant differences in other parameters, including insulin and C-peptide levels, insulin-to-glucose ratios, frequency of diabetes/impaired glucose tolerance, physical activity pattern, or feeding behavior 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 with wild-type MC3 receptor (Fig. 2*A*), without any significant difference in binding affinity to α-MSH or NDP-MSH (Fig. 2*E*). 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 (Fig. 3*A*). Four years later, at 17 years of age, the proband had developed morbid obesity

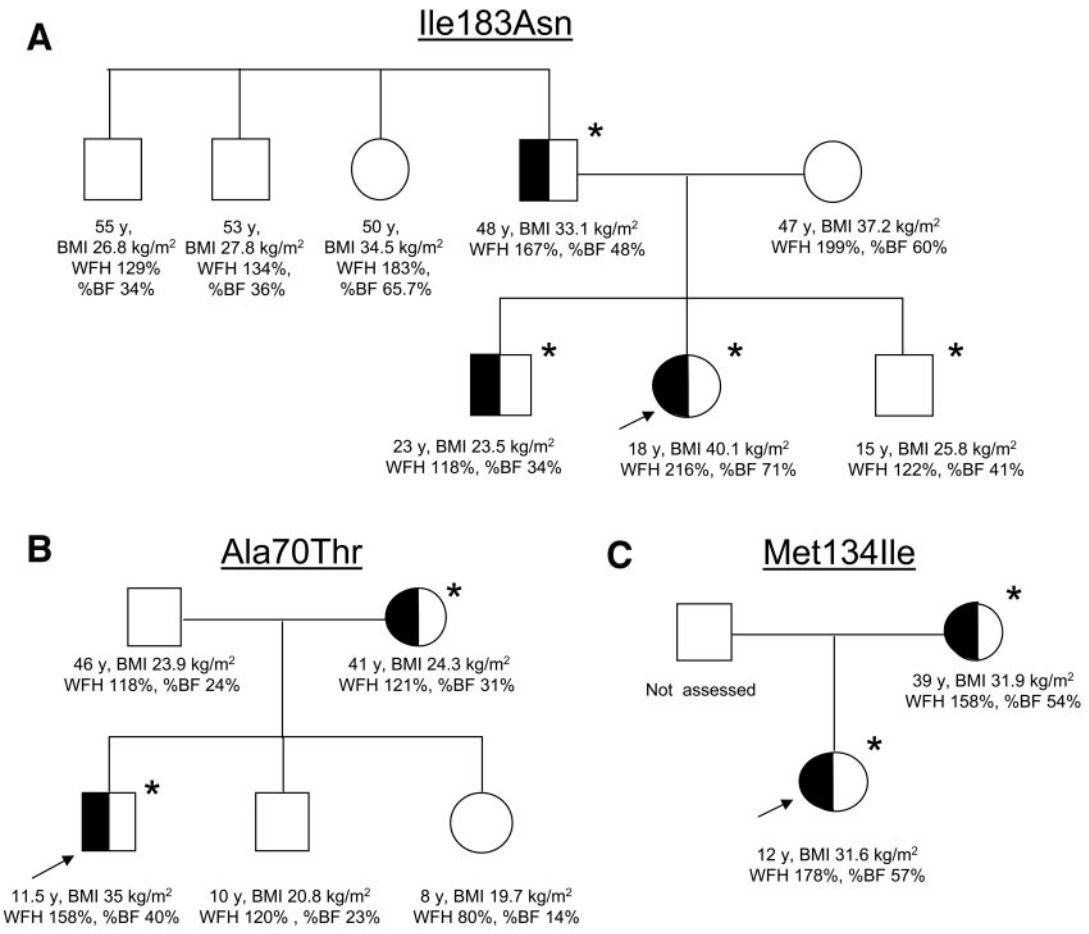

FIG. 3. Family pedigrees of affected subjects with *MC3R* mutations. The age (in years), BMI, percentage of ideal WFH, and percentage body fat (%BF) are listed. Heterozygotes are half-shaded. *Subjects with history of childhood or early-onset obesity. *A*: Family with Ile183Asn mutation. *B*: Family with Ala70Thr mutation. *C*: Family with Met134Ile mutation.

with high percentage ideal body WFH of 216% and percent body fat of 71%. Her heterozygous father's WFH has increased to 167%, and percent body fat was 48%. The proband was the most overweight of the family with the highest percent body fat. 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/m^2. 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 2 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 wild-type *MC3R* alleles. An interesting observation was the cosegregation of early-onset childhood obesity with the mutant *MC3R*

TABLE 2
Comparison of the age, BMI, ideal WFH, waist-to-hip ratio, and body fat of each proband with a group of control subjects of similar age, same sex, and 6K/81I carrier status

	Met134Ile	Control subjects*	Ala70Thr	Control subjects†	Ile183Asn	Control subjects‡	Probands	Control subjects	*P*
n		25		37		4	3	110	
Age (years)	11.9	11.1 ± 3.2	11.7	11.1 ± 1.3	13.1	13.6 ± 1.9	12.3 ± 0.7	12.3 ± 1.7	0.929
BMI (kg/m^2)	31.6	30.7 ± 3.6	35	32 ± 3.6	36.6	33.1 ± 5.7	34.4 ± 2.6	33.5 ± 5.1	0.769
WFH (%)	178	166.1 ± 19.8	173	166.5 ± 15.8	182	180.5 ± 22.9	177.7 ± 4.5	171 ± 23.2	0.633
Waist-to-hip ratio	0.990	0.890 ± 0.072	0.972	0.950 ± 0.062	0.954	0.870 ± 0.035	0.981 ± 0.013	0.926 ± 0.072	0.281
Body fat (%)	57.3	47 ± 6.7	40	41.1 ± 3.9	61.3	58.4 ± 12.5	52.2 ± 12.5	47.8 ± 9.4	0.428
Leptin (μg/l)	116.4	53.7 ± 36.4	71.9	45.8 ± 29.6	142.8	100.9 ± 75.1	110.4 ± 35.8	54.7 ± 39.7	0.018*

Data are means ± SD. *Females selected based on age 3 years above or below 11.9 years, and heterozygous for 6K/81I, because subject with Met134Ile is heterozygous for 6K/81I. †Males selected based on age 2 years above or below 11.7 years and with wild-type *MC3R* alleles 6T/81V, because subject with Ala70Thr is homozygous for wild-type allele. ‡Females selected based on age 2 years above or below 13.1 years and homozygous for 6K/81I, because subject with Ile183Asn is homozygous for 6K/81I. All control subjects that fulfilled age criteria are included in the analysis.

TABLE 3
Feeding behavior assessment of the three probands and obese heterozygote parents compared with obese control subjects without *MC3R* mutations

	Obese subjects without *MC3R* mutations (n = 161)	Proband Ile183Asn	Father Ile183Asn	Proband Ala70Thr	Proband Met134Ile	Mother Met134Ile	All heterozygotes
Restraint	9(0–20)	11	16	7	9	11	11(7–16)
Disinhibition	7(0–14)	2	8	6	4	6	6 (2–8)
Hunger	7(0–14)	3	4	7	3	2	3(2–7)*

Data are median (range). *P = 0.026.

allele (Fig. 3*A*). 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/m^2. The other three sibs were overweight only after 30 years of age: the eldest uncle was 64 kg at 18 years of age, and the second uncle was 60 kg at 20 years of age.

Ala70Thr. The Ala70Thr mutation was found in an 11-year-old boy and his mother (Fig. 3*B*). The proband was overtly obese, whereas his heterozygous mother had mild obesity (WFH 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, because 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 (Fig. 3*C*).

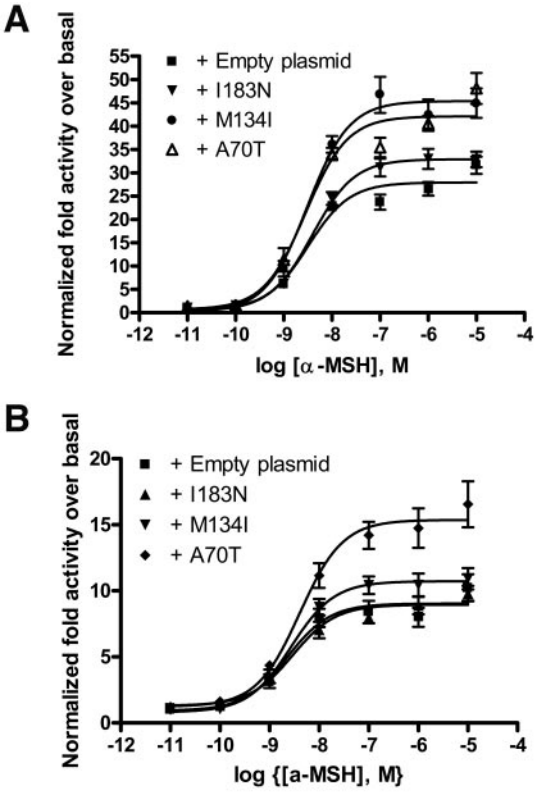

FIG. 4. Mutant MC3 receptors were transfected into stable cell lines expressing wild-type MC3R (*A*) or MC3R with 6K/81I (*B*). There was no significant reduction in cAMP generation by these cells to suggest dimerization as a result of concomitant expression of the mutant receptors.

The proband was asymptomatic but was diagnosed by abnormal oral glucose tolerance test.

The three probands were compared with groups of obese control subjects of similar age and same sex as shown in Table 2. The control subjects 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 control subjects 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 appeared to have higher BMI, WFH, and percent body fat, although these did not reach statistical significance. We also observed that heterozygotes had significantly lower Hunger scores (less subjective feeling of hunger) compared with that of the obese control subjects (P = 0.026) (Table 3). The scores for restrained eating and disinhibition were not significantly different between the heterozygotes and the obese control subjects without *MC3R* mutations ($P > 0.05$ for each factor analyzed). 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, insulin-to-glucose ratio, or physical activity levels.

Impaired signaling activities of the mutant receptors. Because wild-type MC3R and MC3R with common variants 6K/81I (haplotype) showed significant differences in response to α-MSH stimulation (11), we compared the signaling properties of the mutant MC3Rs with 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 with MC3R with 6K/81I (haplotype) in our transfection studies. This was determined by cloning each of the two *MC3R* alleles from the subjects' DNA samples 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 MC3R Ile183Asn showed negligible response to increasing α-MSH concentration (Fig. 2*B* and *C*). Mutant receptor Met134Ile demonstrated a modest but significantly higher EC_{50} compared with MC3R with 6K/81I (Fig. 2*B*). The mutant Ala70Thr receptor also demonstrated significantly reduced cAMP response to MSH stimulation (Fig. 2*D*). Our competitive ligand binding studies did not find any significant differences in IC_{50} between the wild-type, 6K/81I, Ala70Thr, Met134Ile, and Ile183Asn

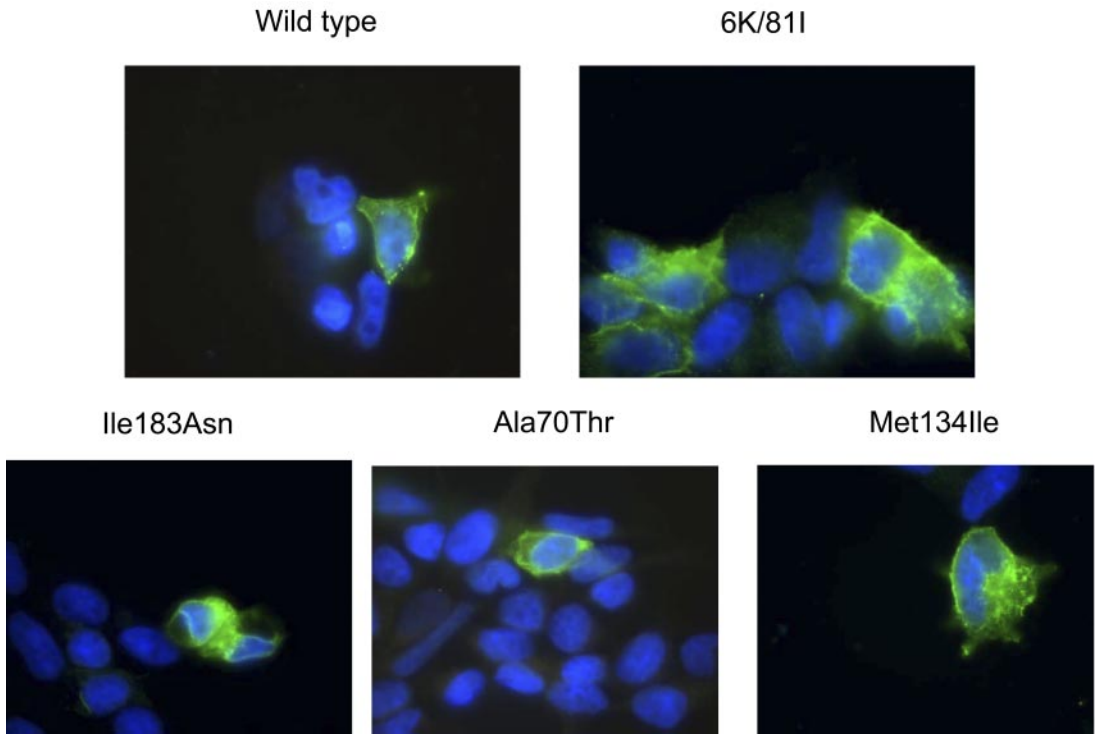

FIG. 5. Cell surface expression of MC3R detected by immunofluorescence staining. Griptite 293 cells were grown in coverslips and transiently transfected with indicated wild type and variants. Cells were not permeabilized and were stained with anti-MC3R antibody followed by secondary antibody conjugated to Alexa 468 dye. (Please see http://dx.doi.org/10.2337/db07-0225 for a high-quality digital representation of this figure.)

MC3Rs (Fig. 2*E*). The three mutant MC3 receptors did not exert any dimerization effect on wild-type or 6K/81I MC3 receptors (Fig. 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 MC3Rs revealed good level of cell surface expression comparable with wild-type MC3R qualitatively (Fig. 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. Although *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 that contributes to increased adiposity and that 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 proopiomelanocortin (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, whereas the phenotype of the heterozygous *Mc3r*$^{+/-}$ mice did not appear to differ significantly from wild-type mice (1,2). In comparison, the *Pomc*$^{-/-}$ mice also exhibit an obese phenotype, whereas the heterozygous *Pomc*$^{+/-}$ mice appeared to have a similar phenotype to wild-type 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 that 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 wild-type 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* 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 (Fig. 3*A*–*C*), although the significance of this is uncertain.

There were varying reports of increased insulin-to-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 with wild-type MC3R (12). Tao and Segaloff (27), however, reported that there was no difference in receptor activity and ligand binding affinity for these two variants. 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 et al. (12), 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 nos.: zebrafish, NP_851303; chicken, BAA32555; mouse, AAI03670; rat, NP_001020441; and human, NP063941). This hydrophobic isoleucine was substituted by hydrophilic asparagine in Ile183Asn MC3R. The transmembrane domain directly interacts with G-proteins and control subjects 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 and Segaloff (27) and Rached et al. (28) subsequently reported that the Ile183Asn MC3R had totally abolished cAMP response to MSH, and Rached et al. (28) also reported a dominant-negative effect on wild-type 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 wild-type 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 no. 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 Met134Ile 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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REFERENCES

1. Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, Cone RD: A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 141:3518–3521, 2000
2. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van der Ploeg LHT: Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 26:97–102, 2000
3. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141, 1997
4. Gantz I, Konda Y, Tashiro T, Shimoto Y, Miwa H, Munzert G, Watson SJ, DelValle J, Yamada T: Molecular cloning of a novel melanocortin receptor. *J Biol Chem* 268:8246–8250, 1993
5. Roselli-Rehfuss L, Mountjoy KG, Robbins LS, Mortrud MT, Low MJ, Tatro JB, Entwistle ML, Simerly RB, Cone RD: Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A* 90:8856–8860, 1993
6. Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Frogeul P: Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest* 106:253–262, 2000
7. Farooqi IS, Yeo GSH, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S: Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J Clin Invest* 106:271–279, 2000
8. Schalin-Jantti C, Valli-Jaakola K, Oksanen L, Martelin E, Laitinen K, Krusius T, Mustajoki P, Heikinheimo M, Kontula K: Melanocortin-3-receptor gene variants in morbid obesity. *Int J Obes Relat Metab Disord* 27:70–74, 2003
9. Li WD, Joo EJ, Furlong EB, Galvin M, Abel K, Bell CJ, Price RA: Melanocortin 3 receptor (MC3R) gene variants in extremely obese women. *Int J Obes Relat Metab Disord* 24:206–210, 2000
10. Hani EH, Dupont S, Durand E, Dina C, Gallina S, Gantz I, Froguel P: Naturally occurring mutations in the melanocortin receptor 3 gene are not associated with type 2 diabetes mellitus in French Caucasians. *J Clin Endocrinol Metab* 86:2895–2898, 2001
11. Yiannakouris N, Melistas L, Kontogianni M, Heist K, Mantzoros CS: The Val81 missense mutation of the melanocortin 3 receptor gene, but not the 1908c/T nucleotide polymorphism in lamin A/C gene, is associated with hyperleptinemia and hyperinsulinemia in obese Greek Caucasians. *J Endocrinol Invest* 27:714–720, 2004
12. Feng N, Young SF, Aguilera G, Puricelli E, Adler-Wailes DC, Sebring NG, Yanovski JA: Co-occurrence of two partially inactivating polymorphisms of *MC3R* is associated with pediatric-onset obesity. *Diabetes* 54:2663–2667, 2005
13. Lee YS, Poh LKS, Loke KY: A novel melanocortin 3 receptor gene (*MC3R*) mutation associated with severe obesity. *J Clin Endocrinol Metab* 87: 1423–1426, 2002
14. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412–419, 1985
15. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ: Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 85:2402–2410, 2000
16. Stunkard AJ, Messick S: The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. *J Psychosom Res* 29:71–83, 1985
17. Aaron DJ, Kriska AM, Dearwater SR, Cauley JA, Metz KF, LaPorte RE: Reproducibility and validity of an epidemiologic questionnaire to assess past year physical activity in adolescents. *Am J Epidemiol* 142:191–201, 1995

18. Schmidt GJ, Walkuski JJ, Stensel DJ: The Singapore Youth Coronary Risk and Physical Activity Study. *Med Sci Sports Exerc* 30:105–113, 1998
19. Cheng Y, Prusoff WH: Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. *Biochem Pharm* 55:3099–3108, 1973
20. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U: Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 5:1066–1070, 1999
21. Challis BG, Coll AP, Yeo GS, Pinnock SB, Dickson SL, Thresher RR, Dixon J, Zahn D, Rochford JJ, White A, Oliver RL, Millington G, Aparicio SA, Colledge WH, Russ AP, Carlton MB, O'Rahilly S: Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36). *Proc Natl Acad Sci U S A* 101:4695–4700, 2004
22. Krude H, Biebermann H, Schnabel D, Tansek MZ, Theunissen P, Mullis PE, Gruters A: Obesity due to proopiomelanocortin deficiency: three new cases and treatment trials with thyroid hormone and ACTH4–10. *J Clin Endocrinol Metab* 88:4633–4640, 2003
23. Farooqi IS, Drop S, Clements A, Keogh JM, Biernacka J, Lowenbein S, Challis BG, O'Rahilly S: Heterozygosity for a POMC-null mutation and increased obesity risk in humans. *Diabetes* 55:2549–2553, 2006
24. Challis BG, Pritchard LE, Creemers JW, Delplanque J, Keogh JM, Luan J, Wareham NJ, Yeo GS, Bhattacharyya S, Froguel P, White A, Farooqi IS, O'Rahilly S: A missense mutation disrupting a dibasic prohormone processing site in pro-opiomelanocortin (POMC) increases susceptibility to early-onset obesity through a novel molecular mechanism. *Hum Mol Genet* 11:1997–2004, 2002
25. Lee YS, Challis BG, Thompson DA, Yeo GS, Keogh JM, Madonna ME, Wraight V, Sims M, Vatin V, Meyre D, Shield J, Burren C, Ibrahim Z, Cheetham T, Swift P, Blackwood A, Hung CC, Wareham NJ, Froguel P, Millhauser GL, O'Rahilly S, Farooqi IS: A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. *Cell Metab* 3:135–140, 2006
26. Lembertas AV, Perusse L, Chagnon YC, Fisler JS, Warden CH, Purcell-Huynh DA, Dionne FT, Gagnon J, Nadeau A, Lusis AJ, Bouchard C: Identification of an obesity quantitative trait locus on mouse chromosome 2 and evidence of linkage to body fat and insulin on the human homologous region 20q. *J Clin Invest* 100:1240–1247, 1997
27. Tao YX, Segaloff DL: Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation. *J Clin Endocrinol Metab* 89:3936–3942, 2004
28. Rached M, Buronfosse A, Begeot M, Penhoat A: Inactivation and intracellular retention of the human I183N mutated melanocortin 3 receptor associated with obesity. *Biochim Biophys Acta* 1689:229–234, 2004
29. Schioth HB, Petersson S, Muceniece R, Szardenings M, Wikberg JE: Deletions of the N-terminal regions of the human melanocortin receptors. *FEBS Lett* 410:223–228, 1997