Identification and Functional Analysis of Novel Human Melanocortin-4 Receptor Variants

Wei Gu, Zhiming Tu, Patrick W. Kleyn, Ahmed Kissebah, Laura Duprat, John Lee, Wendy Chin, Sanchit Maruti, Nanhua Deng, Stewart L. Fisher, Lucia S. Franco, Paul Burn, Keith A. Yagaloff, Julie Nathan, Steven Heymsfield, Jeanine Albu, F. Xavier Pi-Sunyer, and David B. Allison

Inactivation of the melanocortin-4 receptor (MC4-R) by gene-targeting results in mice that develop maturity-onset obesity, hyperinsulinemia, and hyperglycemia. These phenotypes resemble common forms of human obesity, which are late-onset and frequently accompanied by NIDDM. It is not clear whether sequence variation of the MC4-R gene contributes to obesity in humans. Therefore, we examined the human MC4-R gene polymorphism in 190 individuals ascertained on obesity status. Three allelic variants were identified, including two novel ones, Thr^{112}Met and Ile^{137}Thr. To analyze possible functional alterations, the variants were cloned and expressed in vitro and compared with the wild-type receptor. One of the novel variants, Ile^{137}Thr, identified in an extremely obese proband (BMI 57), was found to be severely impaired in ligand binding and signaling, raising the possibility that it may contribute to development of obesity. Furthermore, our results also suggest that sequence polymorphism in the MC4-R coding region is unlikely to be a common cause of obesity in the population studied, given the low frequency of functionally significant mutations. *Diabetes* 48:635–639, 1999

Genetic factors contribute substantially to the development of obesity, but the precise genetic determinants in humans remain unknown (1). The identification of the genes underlying the monogenic models of obesity in mice (*obese*, *diabetes*, *fat*, *tubby*, and *agouti*) has provided a framework for studying pathogenesis of obesity in humans (2). However, genetic evidence that directly links these genes with human obesity is lacking; to date, few mutations of functional significance have been identified in the human homologs of these genes (3–5).

Obesity in one of the monogenic murine models, the *agouti* mice, is caused by a dominantly inherited promoter rearrangement at the *agouti* locus that results in widespread constitutive expression of the agouti protein (6,7). The ectopically expressed agouti protein induces obesity primarily by antagonizing the hypothalamic melanocortin-4 receptor (MC4-R) (8–10). Inactivation of this receptor by gene-targeting recapitulates some characteristic features of the *agouti* obesity syndrome, including maturity-onset obesity (in contrast to the early-onset, massive obesity of the lep^{ob} and $lepr^{db}$ mice), hyperphagia, hyperinsulinemia, and hyperglycemia (10). These phenotypes resemble the common forms of human obesity, which are late-onset and frequently accompanied by NIDDM. Therefore, it is possible that sequence variation at the human MC4-R locus may contribute to genetic susceptibility to development of obesity in humans. In this study, we examined MC4-R sequence polymorphism in 190 individuals ascertained on obesity status, and assessed the significance of the sequence variants by in vitro functional analysis and association studies.

RESEARCH DESIGN AND METHODS

Subjects. The DNA samples were obtained from two collections. Informed consent was obtained from each participating individual. The first 94 subjects screened were from a large family collection ascertained by the Medical College of Wisconsin in collaboration with Millennium Pharmaceuticals (Cambridge, MA). The proband in each of these families was a member of the TOPS (Take Off Pounds Sensibly) Club, an international nonprofit weight-loss support group with over 300,000 members in North America and throughout the world. The vast majority of TOPS members are women between 30 and 50 years of age. This collection included families selected on the basis of BMI and family structure from eight midwestern and southern states. Families met inclusion criteria if an obese proband, with a BMI 30 (kg/m^2), had a female sibling also with a BMI 30 and had at least one living and one nonobese (BMI <27) parent. Additional siblings to the proband were included, when available, and regardless of BMI status or sex. The subjects chosen for our screening from this collection were selected on the basis of BMI only and could be any member in one of the hundreds of families. All subjects screened in this collection were European-Americans. The subjects were classified into four BMI categories: extremely obese (BMI 50), n = 14 (100% women); obese (50 > BMI 30), n = 30 (97% women, 3% men); intermediate (30 > BMI 25), n = 3 (66% women, 33% men); lean (BMI <25), n = 47 (60% women, 40% men).

The 96 individuals screened next were from a cohort of subjects collected at the Obesity Research Center at St. Luke's/Roosevelt Hospital in New York and in collaboration with Millennium Pharmaceuticals. This collection consists of singletons, without ascertainment of relatives. The criterion for inclusion was a current or, in a few cases, a past BMI 50. A BMI of 50 is well above the 95th percentile for the adult U.S. population. Subjects ranged in age from 10 to 67 years.

From Millennium Pharmaceuticals (W.G., Z.T., P.W.K., L.D., J.L., W.C., S.M., N.D.), Cambridge, Massachusetts; the Obesity Research Center (J.N., S.H., J.A., F.X.P., D.B.A.), St. Luke's/Roosevelt Hospital and Columbia University College of Physicians and Surgeons, New York, New York; the Division of Endocrinology, Metabolism, and Clinical Nutrition (A.K.), Medical College of Wisconsin, Milwaukee, Wisconsin; and the Department of Metabolic Diseases (S.L.F., L.S.F., P.B., K.A.Y.), Hoffmann-LaRoche, Nutley, New Jersey.

Address correspondence and reprint requests to Wei Gu, Millennium Pharmaceuticals, 640 Memorial Dr., Cambridge, MA 02139. E-mail: gu@mpi.com.

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W.G. and Z.T. contributed equally to this work.

EC$_{50}$, effective concentration of agonist at 50% maximum activity; MC4-R, melanocortin-4 receptor; MSH, melanocyte–stimulating hormone; NDP-α-MSH, [Nle^4, D-Phe^7]α-MSH; PCR, polymerase chain reaction; SAP, shrimp alkaline phosphatase; SSCP, single-stranded conformational polymorphism; TOPS, Take Off Pounds Sensibly.

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With respect to ethnicity, 42.3% were African-American, 39.4% were European-American, 10.6% were Hispanic, and 6.7% were others. The sex breakdown was 29% men and 71% women.

Leptin assay. Serum leptin levels of the subjects were determined by radioimmunoassay (Linco, St. Charles, MO).

SSCP and sequencing. Primers for polymerase chain reaction (PCR) amplification were designed based on the GenBank sequence S77415 (11) in such a way that the entire 999-bp coding region could be amplified in five overlapping fragments ranging from 300 to 425 bp. The five PCR primer pairs were as follows:

MC4Rafl: 5′-TGTAAAACGACGGCCAGTCTGACCCAGGAGGTTAAATC-3′
MC4Rarl: 5′-CAGGAAACAGCTATGACCGCTGCAGATGAAAAAGTACATG-3′
MC4Rbfl: 5′-TGTAAAACGACGGCCAGTTGCTACGAGCAACTTTTTCTC-3′
MC4Rbrl: 5′-CAGGAAACAGCTATGACCGGTACTGGAGAGCATAGAAG-3′
MC4Rcfl: 5′-TGTAAAACGACGGCCAGTTGGTGAGCGTTTCAAATGGAT-3′
MC4Rcrl: 5′-CAGGAAACAGCTATGACCGAGCCAGCATGGTGAAGAAC-3′
MC4Rdfl: 5′-TGTAAAACGACGGCCAGTATCTTCTATGCTCTCCAGTAC-3′
MC4Rdrl: 5′-CAGGAAACAGCTATGACCTTCTGAGGACAAGAGATGTAG-3′
MC4Refl: 5′-TGTAAAACGACGGCCAGTTTCATCTCTATGTCCACATGTTC-3′
MC4Rerl: 5′-CAGGAAACAGCTATGACCGAGTGAAAAAGTCTCTTATGCATG-3′

The following PCR amplification conditions were used: the 25 µl PCR reactions contained 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 100 µmol/l each dNTP, 1 µmol/l each primer, 0.5 U *Taq* polymerase, and 50–100 ng genomic DNA. The touchdown PCR cycles consisted of 94°C for 30 s, 65–55°C for 30 s (–1°C for cycle), and 72°C for 40 s. The amplicons were treated with Exonuclease I (*Exo*I) to remove residual single-stranded primers and shrimp alkaline phosphatase (SAP) to remove unincorporated nucleotides. The purified fragments were then digested with restriction enzymes *Msp*I and *Hinf*I to yield fragments smaller than 250 bp, better suited to analysis by single-stranded conformational polymorphism (SSCP). The digested PCR fragments were diluted in a ratio of 1:9 in loading buffer (95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heat-denatured at 98°C for 2 min, and snap-cooled in ice. 2–3 µl (of this mixture) were loaded on a 10% acrylamide gel (50:1 acrylamide:*bis*-acrylamide) with 10% glycerol and run at 25 W for 4 h at 4°C. The gel was stained with SYBR Green I and II to detect both single- and double-stranded DNA fragments and visualized on MD575 fluorimager (Molecular Dynamic, Sunnyvale, CA). Variants detected by SSCP were confirmed by direct sequencing of the *Exo*I/SAP–treated PCR products on an ABI 377 fluorescent sequencer.

Cloning and expression of MC4-R gene variants. The variant and wild-type genes were amplified from genomic DNA and cloned into pCDNA3 expression vector. All clones were confirmed by sequencing. Stable cell lines were established by transfecting 293 cells using Lipofectamine (Gibco, Grand Isle, NY) and then selected in G418 (Gibco). To avoid clonal variations in MC4-R expression level, pools of stable transformants were used in the assays. For transient expression, 293 T-cells (293 cells transformed by SV40 T-antigen gene) were transfected by Lipofectamine and used for assays 60–72 h after transfection. For both cAMP and binding assays, β-galactosidase expression vector was cotransfected to monitor efficiency of transient expression, and protein content was determined in cell extracts to normalize cell number per well.

cAMP assays. Ligand-stimulated receptor activity was measured by increase of intracellular cAMP content. Cells cultured in 96-well plates were incubated with melanocortin peptides (Bachem, Marina Del Ray, CA, or Peninsula Laboratories, Belmont, CA) in the presence of 0.25 mmol/l 3-isobutyl-1-methyl-xanthine (Sigma, St. Louis, MO) in culture media. After 1 h, the media was replaced with 70% ethanol. An aliquot of the 70% ethanol supernatant was used to determine cAMP content by scintillation proximity assay (Amersham, Arlington Heights, IL). The 96-well plates were counted in MicroBeta plate reader (Wallac, Gaithersburg, MD). Effective concentration of agonist at 50% maximum activity (EC_{50}) values were calculated using Prism2 software (GraphPad, San Diego, CA).

Binding assays. Ligand binding studies were performed using ^{125}I-labeled [Nle^4, D-Phe^7]α-melanocyte–stimulating hormone (MSH) (NDP-α-MSH) (Amersham). NDP-α-MSH is a potent synthetic analog of α-MSH, and can be iodinated while retaining biological activity. Binding was carried out on transfected cells in 24-well culture plates. Each well was added the amount of 0.6 ml binding buffer (50 mmol/l HEPES, 100 mmol/l NaCl, 10 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 0.05% sodium azide, 0.25 mmol/l 1,10-phenanthroline, 0.2% BSA [pH 7.4]) containing ^{125}I-labeled NDP-α-MSH. After 3.5 h of incubation, cells were washed with binding buffer, and 0.4 ml of 0.1 N NaOH was added. Membrane-bound counts per minute was determined in a MicroBeta plate reader using cross-talk elimination inserts and Super-Mix scintillation fluid (Wallac). K_d and B_{max} were obtained through nonlinear regression using Prism2 software. Binding studies were also performed on membrane preparations made from transfected cells using a scintillation proximity assay (Amersham).

RESULTS

Human MC4-R gene variants. The subjects (n = 190) were classified into four categories based on BMI: 110 subjects were extremely obese (BMI 50 kg/m^2), 30 were obese (50 > BMI 30), 3 were intermediate (30 > BMI 25), and 47 were lean (BMI < 25). Among these subjects, SSCP analysis identified a total of three MC4-R gene variants. Upon sequencing, all three variants were found to contain a single nucleotide substitution resulting in an amino acid change: $A^{304}G$ ($Val^{103}Ile$), $C^{335}T$ ($Thr^{112}Met$), and $T^{410}C$ ($Ile^{137}Thr$).

As shown in Table 1, the $Val^{103}Ile$ allele was found in 7 subjects in heterozygous state: 4 of 110 who were extremely obese (3.6%), 1 of 30 who was obese (3.3%), none of 3 who were intermediates (0%), and 2 of 47 who were lean subjects (4.3%). The $Thr^{112}Met$ allele was found in a very lean subject (BMI 18), and the $Ile^{137}Thr$ allele was found in an extremely obese subject (BMI 57). Both were heterozygotes.

Functional studies. To assess possible functional alterations, the variants were cloned and expressed in both transiently and stably transfected 293 cells and compared with the wild-type receptor for their ability to be activated by melanocortin peptides, as measured by increase in intracellular cAMP concentration (Fig. 1*A* and *B*). Both MC4-R ($Val^{103}Ile$) and MC4-R ($Thr^{112}Met$) exhibited dose-response patterns that were not significantly different from that of wild-type MC4-R. However, the third variant, MC4-R ($Ile^{137}Thr$), identified in an extremely obese subject, demonstrated a markedly attenuated response to the agonists. It differed from the wild-type receptor in two respects (Table 2). First, it exhibited a much higher EC_{50} (37.1 nmol/l) for α-MSH,

TABLE 1
Profiles of the nine individuals with MC4-R variant alleles

Variant	Sex	Race	Age (years)	BMI (kg/m^2)	Leptin (ng/ml)
Val103Ile	F	EA	77	21	NA
	F	EA	42	23	6.1
	F	EA	46	43	47.1
	M	AA	31	50	42.8
	M	AA	30	74	68.7
	F	AA	51	50	87.0
	F	H	19	52	76.2
Thr112Met	F	EA	26	18	4.9
Ile 137Thr	F	EA	43	57	87.7

AA, African-American; EA, European American; H, Hispanic. The serum leptin level of these subjects were well within the expected range given their respective BMIs.

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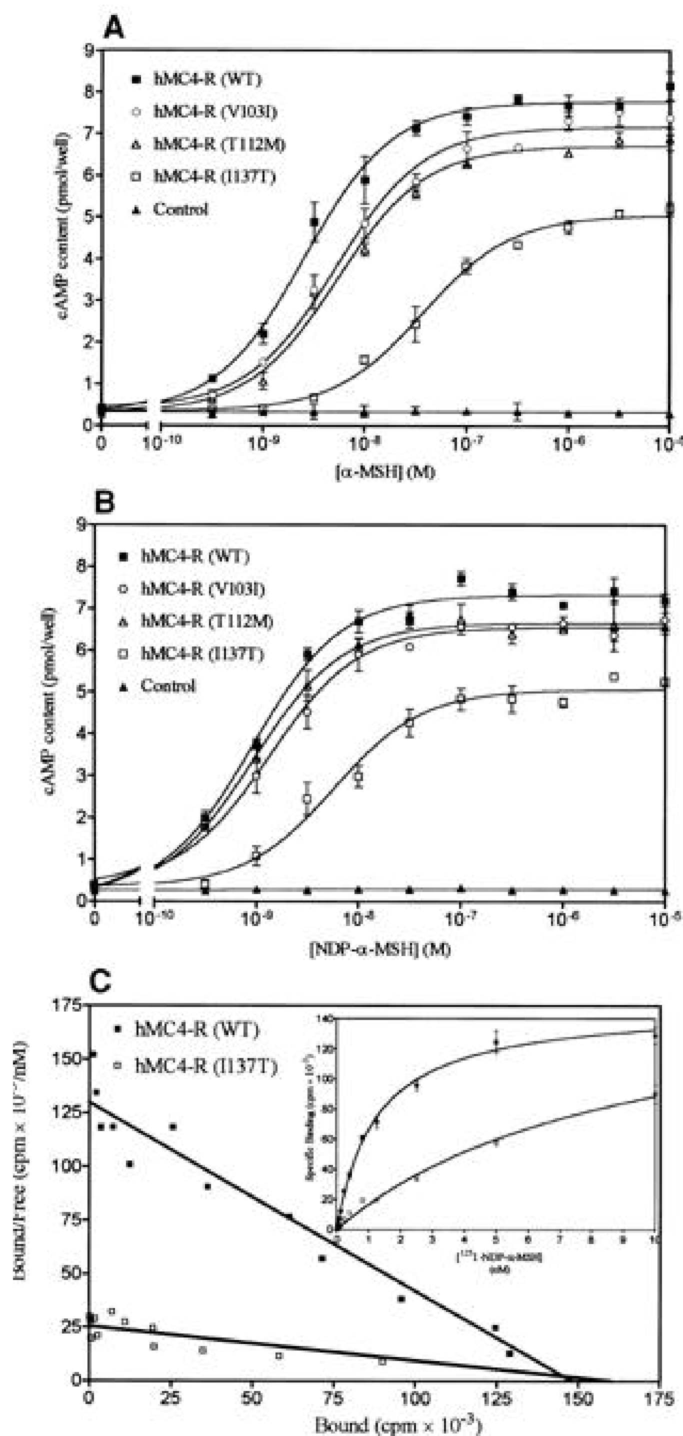

FIG. 1. Activation of transfected human MC4-R, as measured by increase of intracellular cAMP content, by α-MSH, the endogenous ligand (*A*), and by NDP-α-MSH, a potent synthetic analog of α-MSH (*B*). Data were obtained from 293 T-cells transiently transfected with hMC4-R cDNAs. In the control, cells were transfected with the vector pcDNA3. In separate experiments, agonist-stimulated receptor activity was also assayed in stably transfected 293 cells and was found to be consistent with that obtained from transient transfection (data not shown). *C*: The Scatchard plot of ^{125}I-labeled NDP-α-MSH binding to hMC4-R with binding isotherm (inset). Binding was performed on transiently transfected 293 T-cells. In separate experiments, binding was also determined on membrane preparations made from stably transfected 293 cells, and similar results were obtained (data not shown).

~15-fold of that of the wild-type receptor (2.5 nmol/l), indicating a lower affinity for the ligand. Second, it achieved a maximum agonist-stimulated activity, only 65% of that of the wild-type MC4-R, suggesting poor expression on cell surface and/or impaired activation upon ligand binding. When the assay was repeated with four additional endogenous melanocortins, β, γ1, γ2-MSHs, and ACTH, similar dose-response curves were observed for the Ile^{137}Thr variant (data not shown).

To examine ligand affinity and surface expression of the Ile^{137}Thr variant, binding studies were carried out using radioactively labeled ligand (Fig. 1*C*). The affinity of the Ile^{137}Thr variant (K_d, 9.0 nmol/l) was found to be greatly reduced, compared with the wild-type MC4-R (K_d, 1.2 nmol/l). However, its level of surface expression, as reflected in maximum binding (B_{max}), was comparable to that of the wild-type receptor in this system (Table 2). Taken together, these findings suggest that the apparent functional impairment of the Ile^{137}Thr variant results not from poor expression on cell surface, but from a combination of lower ligand affinity and impaired signaling upon ligand binding.

Family studies. To assess the possible association of the Ile^{137}Thr allele with obesity, nine additional members of the proband's extended family were screened, and four were found to be heterozygotes for the Ile^{137}Thr allele (Fig. 2). A simple association analysis was conducted within the pedigree. BMI was regressed on genotype at the *MC4-R* locus using ordinary least-squares regression, while controlling for age and sex. The effect of the heterozygous Ile^{137}Thr genotype was not statistically significant ($P = 0.609$). Although this simple analysis did not take the potential residual correlation among relatives into account, an analysis that did take this into account would most likely produce an even higher P value (12) and, therefore, not change our conclusion of a nonstatistically significant association. However, given the complexity of BMI, including its polygenic origin, this small sample size would not have a sufficient power to detect small to moderate effects. Therefore, these results cannot be used to rule out any effect of this hMC4-R variant. Similarly, we should not rule out the possibility that this variant could have a discernible effect on a more refined phenotype than BMI (e.g., fat mass).

DISCUSSION

We screened the entire human MC4-R coding region in 190 subjects of a wide BMI range and identified three allelic variants, including two novel ones. Among the three variants, the Val^{103}Ile allele is the most prevalent one. In fact, when the human *MC4-R* gene was first cloned, the published sequence contained an isoleucine, rather than valine, at codon 103 (11). As we found in the present study, the valine^{103} allele appeared to be the common allele, while the isoleucine^{103} allele was found in <4% of the subjects in a heterozygous state. This variant involves a conservative substitution in the second transmembrane domain of this G-protein–coupled receptor, and does not appear to differ significantly from the wild-type receptor in agonist-stimulated receptor activity. Consistent with the functional data, this variant was found in both lean and obese groups with no significant difference in allele frequency. While this work was in progress, a study on human MC4-R coding sequence polymorphism was published in which Val^{103}Ile was the only allelic variant identified (13). In that study, ~4.4% of the British subjects studied were found to be heterozygous for the Val^{103}Ile allele, and the allele

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TABLE 2
Summary of functional data for wild-type and variant hMC4-Rs

hMC4-R	Wild-type	Val103Ile	Thr112Met	Ile137Thr
EC_{50} (nmol/l)	2.5 ± 0.5	5.2 ± 1.2	5.4 ± 0.4	37.1 ± 4.1
Maximum activation (%)	100	92	86	65
K_d (nmol/l)	1.2 ± 0.1	NA	NA	9.0 ± 1.5
B_{max} (cpm)	149,000 ± 3,400	NA	NA	168,000 ± 17,200

Data are means ± SD or %. EC_{50} and maximum activation values are for α-MSH and were calculated based on intracellular cAMP accumulation. K_d and B_{max} values are for NDP-α-MSH, a potent synthetic analog of α-MSH that can be iodinated. The values were obtained through nonlinear regression. B_{max} is expressed in counts per minute per well; 150,000 cpm represents ~10,000 receptors per cell.

was not found to be associated with BMI or other obesity-related phenotypes. While these findings on the Val[103]Ile variant are consistent with our results, the study differs from ours in several respects. First, a relatively small sample, consisting of 50 white British men, was screened for the entire MC4-R coding sequence. Second, Val[103]Ile was the only variant identified. Third, no functional studies were conducted.

Both Thr[112]Met and Ile[137]Thr variants are novel and are reported here for the first time. The Thr[112]Met variant, found in a lean individual, contains a nonconservative substitution in the first extracellular loop of the receptor. This substitution does not appear to have a major effect on receptor activity. However, it remains possible that the receptor function is affected in a manner not detectable by our assays, and additional studies may be required to assess if there are any subtle functional alterations.

The proband of the third allelic variant, Ile[137]Thr, is an extremely obese woman with a BMI of 57, which is above the 99.9th percentile for adult women. This variant causes a nonconservative amino acid substitution in the third transmembrane domain, resulting in a receptor that is severely impaired in both ligand binding and signaling. Therefore, the Ile[137]Thr allele is unlikely to contribute full MC4-R activity in vivo as does a wild-type allele.

Interestingly, in mice in which the MC4-R gene is inactivated by gene targeting, the heterozygous mutant mice (+/–) consistently develop body weight and other obesity-associated phenotypes that are intermediate between those seen in the homozygous mutant (–/–) and wild-type mice (+/+) (10). Moreover, in mice carrying various dominant *agouti* alleles or *agouti* transgenes, the level of ectopic *agouti* expression, hence the extent of MC4-R antagonization, positively correlates with expressivity of the obese phenotype, suggesting that partial MC4-R antagonization can lead to obesity (14–17). Thus, at least in mice, the effect of MC4-R on body weight appears to be gene dosage-dependent; the two wild-type alleles of MC4-R are nonredundant. In humans, the functional impairment of the Ile[137]Thr variant raises the possibility that it may contribute to development of obesity. While it is likely that Ile[137]Thr homozygosity may lead to obesity, heterozygosity for this allele does not statistically account for all the BMI differences in the pedigree analyzed. Nevertheless, the statistical power of this association analysis is probably limited both by its small sample size and by the complexity of BMI. Human obesity is predominantly a polygenic disorder (1), arising from interaction of multiple genes that each alone may not be sufficient to give rise to obesity. It is therefore possible that Ile[137]Thr heterozygosity may have an effect on body weight, but its penetrance and phenotypic expression may depend on other genetic and environmental factors. It is also possible that Ile[137]Thr heterozygosity could have a measurable effect on some intermediary phenotypes, such as fat mass, which are less complex than BMI and more likely to be directly influenced by a given gene. Further studies will be required to assess these possibilities.

In addition, among the 190 individuals screened, a total of 9 were found to be heterozygous for the MC4-R allelic variants, including 1 for the severely impaired Ile[137]Thr variant. Given the low frequency of functionally significant mutations, our results suggest that sequence variation in the MC4-R coding region does not appear to be a common cause of obesity in the population studied.

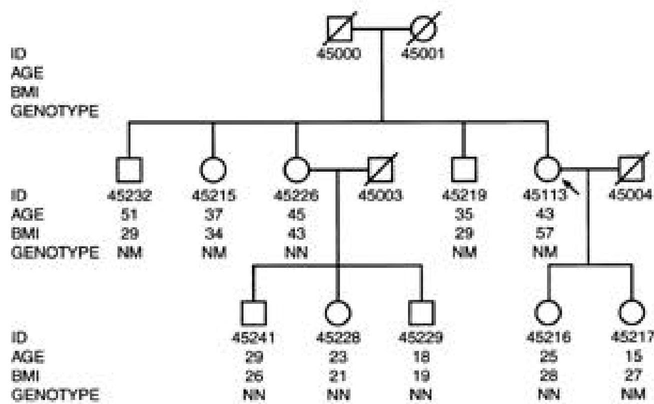

FIG. 2. Pedigree of the family with hMC4-R Ile[137]Thr variant. The arrow indicates the proband. The age, BMI, and MC4-R genotype of the subjects, if known, are indicated below the symbol. MC4-R genotype: N, normal allele; M, mutant allele (Ile[137]Thr).

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