Brief Genetics Report

A Novel Homozygous Missense Mutation of Melanocortin-4 Receptor (*MC4R*) in a Japanese Woman With Severe Obesity

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The melanocortin-4 receptor (MC4R) is a member of the seven membrane–spanning G protein–coupled receptor superfamily and signals through the activation of adenylyl cyclase. The *MC4R* mutations are the most common known monogenic cause of human obesity. However, no such mutations have been found in Japanese obese subjects. Here we report a novel homozygous missense mutation of *MC4R* (G98R) in a nondiabetic Japanese woman with severe early-onset obesity, which is located in its second transmembrane domain. Her birth weight was 3,360 g, and she gained weight progressively from 10 months of age. At 40 years of age, her weight reached 160 kg and a BMI of 62 kg/m^2. Her parents, who are heterozygous for the mutation, have BMIs of 26 and 27 kg/m^2. In vitro transient transfection assays revealed no discernable agonist ligand binding and cAMP production in HEK293 cells expressing the mutant receptor, indicating a severe loss-of-function mutation. This study represents the first demonstration of a pathogenic mutation of *MC4R* in Japan and will provide further insight into the pathophysiologic role of the hypothalamic melanocortin system in human obesity. *Diabetes* 51:243–246, 2002

Obesity is a multifactorial disease that arises from complex interactions between genetic predisposition and environmental factors (1). It increases the risk of cardiovascular and metabolic diseases such as diabetes, hypertension, and hyperlipidemia, thus contributing to a marked increase in atherosclerotic disorders in Westernized countries. Recent molecular genetic studies have disclosed at least five monogenic forms of obesity in humans. They include leptin (*LEP*) (2,3), leptin receptor (*LEPR*) (4), prohormone convertase 1 (*PC1*) (5), proopiomelanocortin (*POMC*) (6), and melanocortin-4 receptor (*MC4R*) (7–13). The MC4R is a seven-transmembrane G protein–coupled receptor that is expressed in the hypothalamic nuclei implicated in the regulation of food intake and body weight (14,15). It signals through the activation of adenylyl cyclase in response to its endogenous agonist ligand, α-melanocyte-stimulating hormone (α-MSH), a well-established satiety neuropeptide produced upon cleavage from POMC (14,16). The *MC4R* mutations are remarkable in that they have been identified at a relatively high frequency of 3–4% in severe early-onset obesity in France and the U.K. (12,13). All the mutations reported to date have occurred in an autosomal-dominant fashion, except for a single unique pedigree in the U.K. (13). In this context, Ohshiro et al. (17) found no obesity-causing mutations of *MC4R* in 50 Japanese patients with obesity/diabetes. Here, we report a novel homozygous missense mutation of *MC4R* in a nondiabetic Japanese woman with severe early-onset obesity. The mutant receptor does not bind to and respond to α-MSH, indicating a loss-of-function mutation. This study represents the first demonstration of a pathogenic mutation of *MC4R* in Japan.

The proband is a 40-year-old obese Japanese woman who is the second child of two siblings born of nonconsanguineous parents. The pedigree is illustrated in Fig. 1. Her weight is 160 kg, her height is 161 cm, and her BMI is 62 kg/m^2. Her birth weight was 3,360 g, and she began to gain weight progressively at 10 months of age. She weighed 15 kg at 1 year of age. Figure 2 shows her photographs at 2, 3, and 23 years of age. She had normal mammary glands and pubic and axillary hair. There was a history of continuous nocturnal carbohydrate hyperphagia with food seeking and distress when food was not provided. She passed through puberty normally, with the onset of menstruation at 15 years of age. Her menstrual cycles had been irregular, and she had never been pregnant. Dual-energy X-ray absorptiometry scanning showed that her bone mineral density of lumbar vertebrae is greater than that expected from the age-adjusted popula-

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MC4R, melanocortin-4 receptor; α-MSH, α-melanocyte-stimulating hormone; PCR, polymerase chain reaction; POMC, proopiomelanocortin; RFLP, restriction fragment–length polymorphism; TCA, trichloroacetic acid.

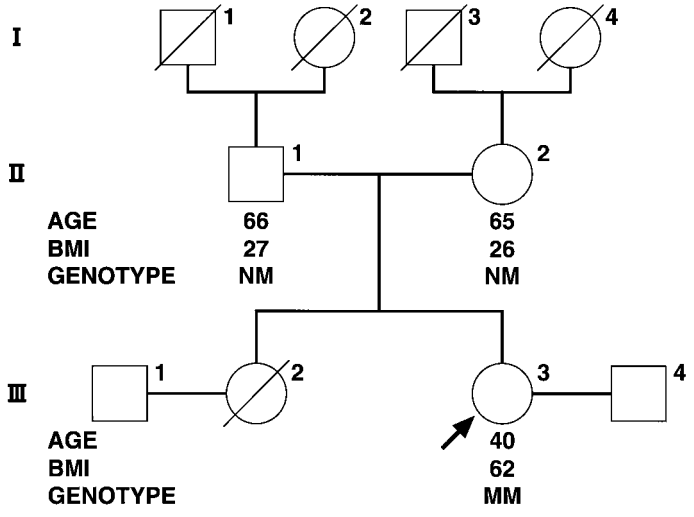

FIG. 1. Pedigree of the family. The arrow indicates the proband. The age, BMI, and *MC4R* genotype of the family members, if available, are shown below each symbol. M, mutant allele (G98R); N, normal allele.

tion range (Z score: 2.02). The proband had an average record during her school days, but she was slightly mentally retarded at 22 years of age as judged by the Weschler Adult Intelliegnce Scale (WAIS) test. Table 1 summarizes her metabolic and endocrine measurements on admission. Except for slightly decreased levels of cortisol, the values of all other anterior pituitary–derived hormones were within normal limits. The serum leptin concentration was in proportion to the degree of adiposity and appeared to reflect body fat mass. She was normoglycemic, with high levels of fasting serum insulin, consistent with hyperinsulinemia and insulin resistance seen in *Mc4r*-deficient mice (15) and humans with *MC4R* mutations (12,13). Further analysis revealed a normal karyotype (46, XX), normal computerized tomography of the brain, and no clinical features of Prader-Willi syndrome.

Her parents and her elder sister (who died of liver cirrhosis at 38 years of age) did not exhibit such severe obese phenotype, but they all had been overweight or even obese according to the criteria of the Japan Society for the Study of Obesity (the cutoff for obesity is BMI >25 kg/m^2) (Fig. 1). Her paternal grandparents (I-1, I-2) had a history of being overweight or obese, whereas her maternal grandfather (I-3) did not.

To examine whether a *MC4R* mutation might be involved in the proband's morbid obesity, the coding sequences of *MC4R* were amplified by polymerase chain reaction (PCR) using genomic DNA extracted from peripheral leukocytes and subjected to direct sequencing. We identified a novel homozygous missense mutation in the second transmembrane domain of MC4R (G98R [*G*GA → *A*GA transition]) (Fig. 3*A*). The G98R mutation was not detected in 100 healthy Japanese volunteers using PCR analysis combined with *Alw* I restriction fragment–length polymorphism (RFLP) (data not shown). Her parents proved to be heterozygous for the mutation (Fig. 3*B*).

To explore the pathogenic implication of the G98R mutation, the wild-type and mutant receptors were expressed in HEK 293 cells and assayed for their ability to bind and respond to α-MSH (Fig. 4*A*). Cells expressing the wild-type receptor showed a sigmoidal dose response to

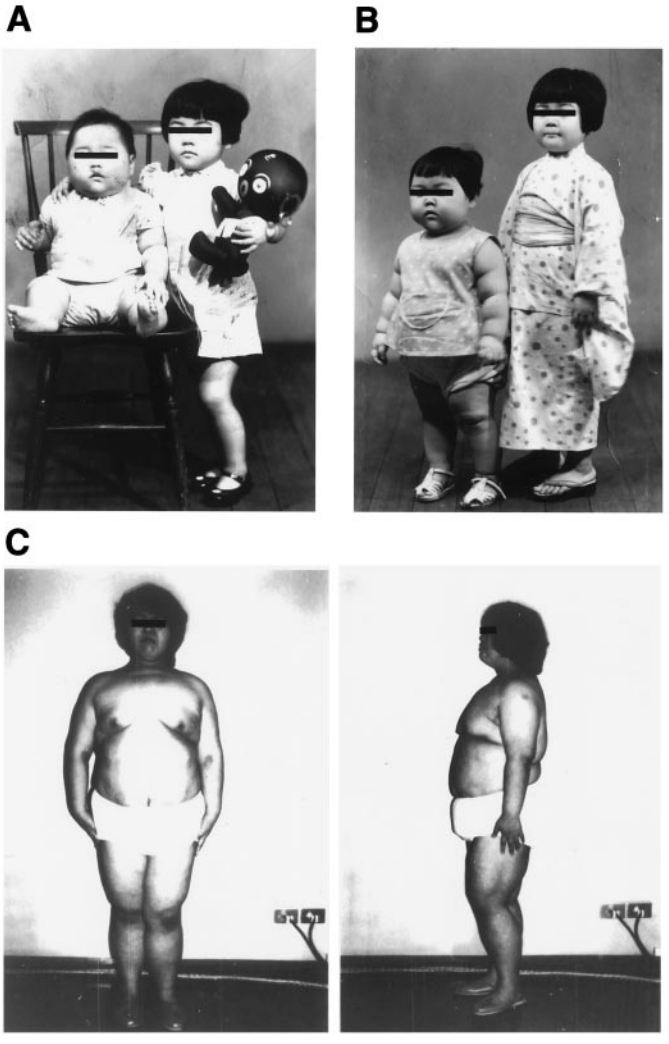

FIG. 2. Photographs of the proband at 2 (*A*) and 3 (*B*) years of age (with her elder sister at 4 and 5 years of age, respectively) and 23 years of age (*C*) (she weighed 115 kg). The photographs are reproduced with the informed consent of the proband and her mother.

α-MSH. By contrast, cells expressing the G98R mutant receptor showed no response. They were unable to bind to α-MSH in a competitive-binding assay (Fig. 4*B*), indicating a loss-of-function mutation. We could not examine whether the mutant receptor is expressed on the cell surface by Western blot analysis because an antibody specific for human MC4R is currently unavailable. In this context, a previous study showed that rat prostaglandin $F_{2\alpha}$ receptor with amino acid substitutions of several kinds in its second transmembrane domain, when transfected, is expressed on the cell surface, although the level is slightly reduced relative to the wild-type receptor (18). Given that the basic Arg residue at position 8 of α-MSH may interact with the acidic residues in the second and third transmembrane domains of MC4R (19), it is conceivable that α-MSH does not bind to the G98R mutant receptor because of the alteration from the neutral Gly to basic Arg residues in its second transmembrane domain.

The G98R mutation reported herein is the second homozygous mutation of *MC4R* described in human obesity. The homozygous N62S mutation previously described in the U.K. was the first to be associated with a recessive

TABLE 1
Hormonal profile of the proband

	Proband	Normal range
Leptin (ng/ml)	58.4	6.3–10.0
Glucose (mmol/l)	4.8	3.5–5.5
Insulin (pmol/l)	164	12–48
LH (IU/l)	3.58	0.9–15.5 (follicular phase)
FSH (IU/l)	9.61	3.1–23.9 (follicular phase)
Estradiol (pmol/l)	239	51–826
Testosterone (ng/ml)	0.36	0.36–3.1
GH (μg/l)	1.8	<5.0
TSH (mU/l)	1.3	0.5–4.2
FT4 (pmol/l)	16.4	11.5–21.2
ACTH (pmol/l) at 9:00 A.M.	4.2	<13
Cortisol (nmol/l) at 9:00 A.M.	119	138–673
Prolactin (μg/l)	24.6	<30

FSH, follicle-stimulating hormone; FT4, free thyroxine; GH, growth hormone; LH, luteinizing hormone; TSH, thyrotropin-stimulating hormone.

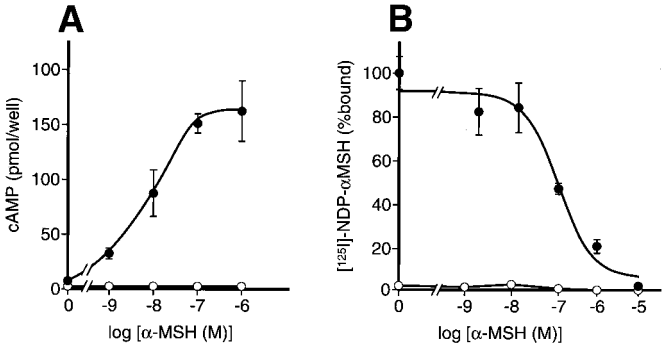

FIG. 4. *A*: Activation of transfected human MC4R by α-MSH. HEK 293 cells transiently transfected with MC4R cDNAs or the vector pcDNA3.1 were stimulated for 1 h with medium only or increasing amounts of α-MSH, after which intracellular cAMP content was measured. All curves are representative of three independent experiments, and each point is the mean triplicate values. Error bars indicate SE. *B*: Competition binding assay. Transiently transfected HEK 293 cells were incubated with [^{125}I]NDP–α-MSH in the presence of an increasing concentration of α-MSH. All curves are representative of three independent experiments, and each point is the mean of triplicate values. Error bars indicate SE. ●, Wild-type allele; ○, G98R mutant allele. The ordinate is expressed as a percentage of total specific binding.

pattern of inheritance and retains some capacity to signal to cAMP generation (13). Thus, this study represents the first description of a homozygous missense mutation of *MC4R* with no signaling capacity, leading to obesity. Her parents, who were heterozygous for the mutation, were overweight. These observations suggest a codominant pattern of inheritance. They all should express one wild-type allele and one functionally null allele, which appears to cause overweight or obesity in this family. It was reported that heterozygous loss-of-function mutations of *MC4R* in humans do not always lead to severe obesity (12,13), as heterozygous *Mc4r*-deficient mice display a broad variety in phenotype, ranging from that of wild-type to that of homozygous *Mc4r*-deficient mice (15). We did not examine the intrafamilial variation in phenotype further because there were no family members available within this pedigree.

In conclusion, we have identified a novel homozygous missense mutation of *MC4R* in a Japanese woman with severe early-onset obesity. This study will provide further

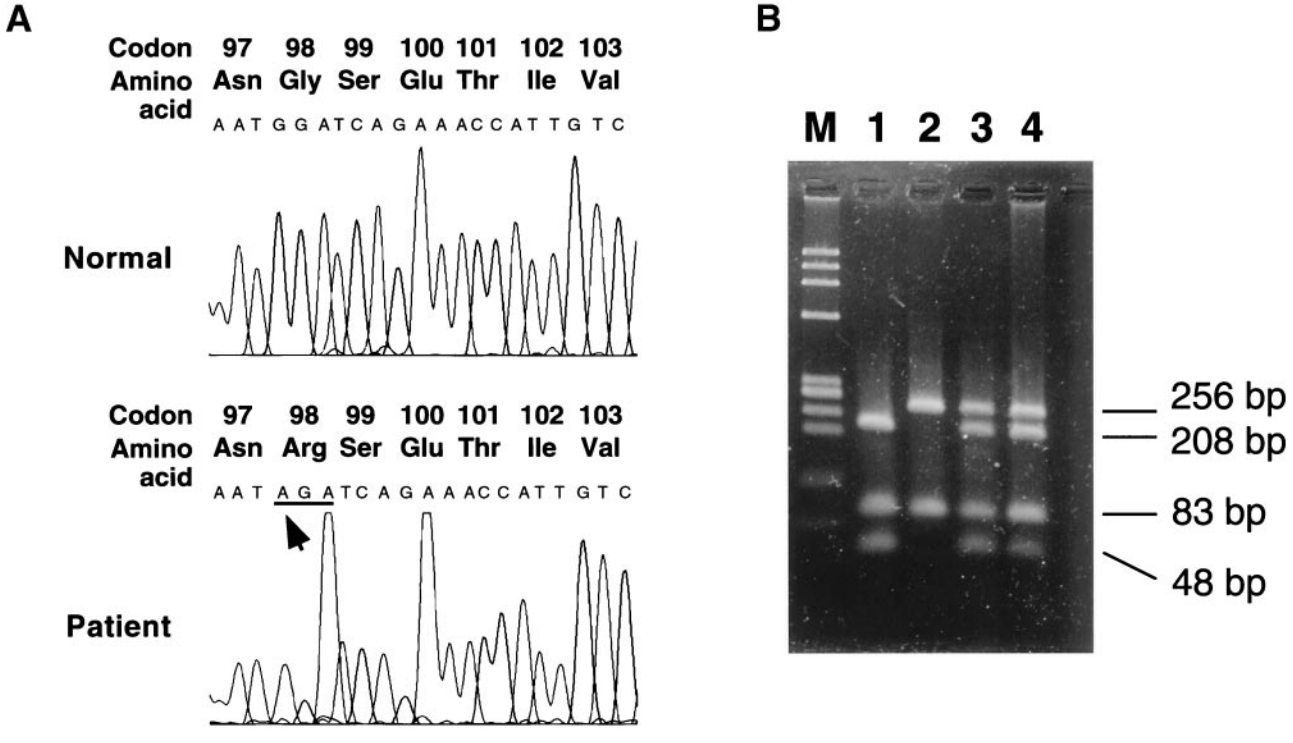

FIG. 3. *A*: PCR direct sequences of *MC4R* from a healthy volunteer and the proband. *B*: Detection of the G98R mutation using PCR analysis combined with *Alw* I RFLP. M, size marker; 1, a healthy volunteer; 2, the proband, with homozygous G98R mutation; 3, the proband's father, with the heterozygous G98R mutation; 4, the proband's mother, with the heterozygous G98R mutation.

insight into the role of the hypothalamic melanocortin system in human obesity.

RESEARCH DESIGN AND METHODS

Patients. This study was conducted with informed consent from the proband and her mother and was approved by the institutional review board of Kobe City General Hospital for genetic analysis and the ethical committee on human research of Kyoto University Graduate School of Medicine.

Genetic studies. Genomic DNA was extracted from peripheral leukocytes by standard techniques. The full-length *MC4R* coding sequences were amplified by PCR using sense and antisense primers (5′-GACTTGGAGGAAATAACTG AGA CG-3′ and 5′-CTACACGGAAGAGAAAGCTGTTGC-3′, respectively). The PCR products thus obtained were subjected to nucleotide sequencing on both strands. The PCR/RFLP analysis of the G98R mutation was performed by PCR-amplified genomic sequences of *MC4R*. An aliquot of the reaction mixture was digested with *Alw* I (Takara, Kusatsu, Japan).

Transfection studies. The wild-type and mutant *MC4R* coding sequences were amplified by PCR using sense and antisense primers (5′-CAGCATGGT-GAACTCCACCCA-3′ and 5′-CTCTGTCCCCATTTAATATCT-3′, respectively) and subcloned into pGEM-T easy vector (Promega, Madison WI). The *Eco*RI-*Spe*I fragment containing MC4R was excised and ligated into the *Eco*RI and *Xba*I restriction sites of pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). All clones were verified by DNA sequencing. HEK293 cells were maintained as previously described (13), transfected with the wild-type or mutant MC4R expression vectors by LipofectAmine Plus (Life Technologies, Rockville, MD), and assayed 24–48 h after transfection. For both ligand binding and cAMP assays, Renilla luciferase expression vector was cotransfected to monitor the efficiency of transient expression. Protein content was determined in cell extracts to normalize the cell number per well.

Ligand-stimulated receptor activity was measured by increased intracellular cAMP content. Cells cultured in 24-well plates were incubated with α-MSH (Peptide Institute, Minoh, Japan) in the presence of 0.25 mmol/l 3-isobutyl-1-methyl-xanthine (Sigma, St Louis, MO) in the culture media. After 1 h, the media was replaced with 6% trichloroacetic acid (TCA). An aliquot of TCA was used to determine cAMP content by the commercially available radioimmunoassay (Yamasa, Chiba, Japan).

Competitive binding experiments were performed as previously described (20). Cells cultured in 24-well plates were washed with 200 μl of the binding medium (1 mg/ml bovine serum albumin in Ca^{+}/Mg^{2+} phosphate-buffered saline) and incubated in 150 μl binding medium containing 40,000–60,000 cpm of [^{125}I]NDP-MSH (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) per well. Series concentrations of unlabeled α-MSH were used to compete with the labeled NDP-MSH. Controls for nonspecific binding contained 10 μmol/l unlabeled α-MSH. After 1 h of incubation, the binding medium was aspirated, cells were washed with 400 μl of binding medium, and 200 μl of 0.1N NaOH was added. Membrane-bound counts per minute were determined in a gamma counter (Aloka, Mitaka, Japan). Total specific binding and IC_{50} values were determined by nonlinear regression analysis from triplicate data points using Prism software (GraphPad Software for Science, San Diego, CA).

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