Brief Genetics Report

Association of Melanin-Concentrating Hormone Receptor 1 5′ Polymorphism With Early-Onset Extreme Obesity

Christopher G. Bell,[1,2] David Meyre,[3] Chantal Samson,[3] Cliona Boyle,[1] Cécile Lecoeur,[3] Maïte Tauber,[4] Béatrice Jouret,[4] Delphine Jaquet,[5] Claire Levy-Marchal,[5] Marie Aline Charles,[6] Jacques Weill,[7] Fernando Gibson,[1] Charles A. Mein,[8] Philippe Froguel,[1,3] and Andrew J. Walley[1]

Murine models have been highly effective in identifying the monogenic forms of human obesity discovered to date. Melanin-concentrating hormone receptor 1 (MCHR1) has been shown to be significant in the downstream orexigenic activity of the leptin-melanocortin pathway by such models. In this study, the human MCHR1 gene was extensively characterized by sequencing 3.5 kb of coding, untranslated and intronic regions plus 1 kb of putative promoter region in 180 morbidly obese adults and 87 morbidly obese children, a total of >2.4 Mb of sequencing. Thirty-nine single nucleotide polymorphisms (SNPs) were found, seven of which encode an amino acid change. One mutation, R248Q, appeared to cosegregate with the obesity trait in one pedigree but was also found to be a rare polymorphism in control samples. To investigate the possible polygenic role of MCHR1, the six common SNPs (minor allele frequency >5%) found in the sequenced regions were then screened in 557 morbidly obese adults, 552 obese children, and 1,195 nonobese nondiabetic control subjects. The plausible promoter SNP, rs133068, was found to be associated with protection against obesity in obese children only (allele frequency $P = 0.006$ and genotype frequency $P = 0.004$). Most significant results were found when using a dominant model ($P = 0.001$, odds ratio 0.695 [95% CI 0.560–0.863]). However, similar associations were found when both adults and children were analyzed together ($P = 0.006$, 0.783 [0.658–0.930]), suggesting that severe forms of obesity with early onset may be associated with SNPs in MCHR1. *Diabetes* 54:3049–3055, 2005

From the [1]Section of Genomic Medicine, Faculty of Medicine, Imperial College, Hammersmith Hospital, London, U.K.; the [2]Department of Haematology and Genetics, South Eastern Area Laboratory Services, Prince of Wales Hospital, Sydney, Australia; the [3]Centre National de la Recherche Scientifique, UMR 8090, Pasteur Institute, Lille, France; [4]INSERM U563, Children's Hospital, Toulouse, France; [5]INSERM U457, Robert Debre Hospital, Paris, France; [6]INSERM, U258-IFR69, Paris South Faculty of Medicine, Villejuif, France; the [7]Pediatric Endocrine Unit, Jeanne de Flandre Hospital, Lille, France; and the [8]Bart's and the London Genome Centre, Queen Mary's School of Medicine, London, U.K.

Address correspondence and reprint requests to Prof. Philippe Froguel, Section of Genomic Medicine, Faculty of Medicine, Imperial College, Hammersmith Hospital, Du Cane Rd., London, W12 0NN, U.K. E-mail: p.froguel@imperial.ac.uk.

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MAF, minor allele frequency; MC4R, melanocortin 4 receptor; MCH, melanin-concentrating hormone; MCHR1, melanin-concentrating hormone receptor 1; SNP, single nucleotide polymorphism.

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The discovery of a monogenic form of obesity led to the first insight into human obesity's molecular cause (1). The finding of a leptin-deficient child who mimicked in phenotype the leptin-lacking *ob/ob*$^{-/-}$ mouse was followed by the discovery of further Mendelian forms, all of which were components of the leptin-melanocortin pathway. These include mutations in genes for the leptin receptors, prohormone convertase 1, proopiomelanocortin, and melanocortin 4 receptor (MC4R) (2). These results highlight the significant role of this axis in the hypothalamus in the regulation of food intake and energy expenditure. The first-order, leptin-responsive, arcuate neurons project fibers to further hypothalamic nuclei such as the ventromedial and paraventricular nuclei, as well as the lateral hypothalamic area (3). In the lateral hypothalamic area, a rich supply of fibers innervate melanin-concentrating hormone (MCH) neurons, which have significant orexigenic activity (4).

The action of MCH was determined by its injection into the lateral ventricles of rats, which were then found to become hyperphagic (5). Also, the *ob/ob*$^{-/-}$ mouse was found to have increased mRNA levels of the preprohormone of MCH, and this was similar to the increase in the mRNA of the established orexigenic hormone neuropeptide Y (6). Subsequent investigations proved that MCH and the anorexogenic α-melanin–stimulating hormone functionally counteracted each other in their effect on food intake, although MCH did not act as an antagonist to the α-melanin–stimulating hormone receptor, MC4R (7). Viable homozygous MCH knockout mice were generated, and these MCH-deficient animals were found to have a reduced body weight and were lean because of a combination of hypophagia and an increased metabolic rate (8). MCH's role in energy homeostasis was also demonstrated by the MCH gene being a requirement for the *ob/ob*$^{-/-}$ mouse to express its extreme obesity phenotype (9). A double-null mouse, negative for both the leptin and MCH genes, was found to have a reduction in body fat. Although the hyperphagia of the *ob/ob*$^{-/-}$ persisted, this was offset by a marked increase in energy expenditure at rest and increased locomotor activity.

The orphan human G-protein–coupled receptor number 24, previously known as somatostatin receptor–like protein 1, was identified as the receptor for MCH (MCHR1) (10). Homozygous MCHR1 knockout mice were generated and found to be lean, hyperactive, hyperphagic on normal diet, less prone to dietary-induced obesity, and to have an altered neuroendocrine profile (11). Hyperactivity ap-

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peared to be the cause of the leanness and the reduced susceptibility to obesity. A selective high-affinity antagonist for the MCHR1 receptor was then reported, called SNAP-7941 (12). Administration of this molecule to rats inhibited the orexigenic effect of centrally administered MCH and produced a decrease in body weight and food intake in both normal and diet-induced obese animals. In addition to the anorectic effects of antagonizing this receptor, experimental evidence of antidepressant and anxiolytic actions were also found (12). Due to their ubiquitous role in the communication between a cell and its environment, the G-protein–coupled receptor family has been a successful drug target for a plethora of diseases from cardiovascular to psychiatric disorders (13). A possible role of less severe variants of genes responsible for the monogenic forms of obesity has been hypothesized for polygenic common obesity, as has been found in type 2 diabetes with the monogenic mature-onset diabetes of the young–1 hepatocyte nuclear factor 4-α gene's involvement in susceptibility for the polygenic disease (14). Evidence for this with initial candidates in obesity such as leptin, leptin receptor, and MC4R is not strong, though a rare single nucleotide polymorphism (SNP) in the proopiomelanocortin gene has been found to be associated with childhood obesity in various populations (15).

The significant downstream role of MCHR1 from the arcuate nucleus in mammalian energy and appetite regulation and the phenotypes of the related knockout mice make it a strong candidate gene for both monogenic and polygenic human morbid obesity. To investigate a possible Mendelian role, a cohort of 180 morbidly obese adults and 87 morbidly obese children were fully sequenced for the gene. Those individuals found to have coding changes had their pedigrees genotyped for this mutation to see if the trait cosegregated with the obesity phenotype. To identify a possible role in polygenic obesity, SNPs of minor allele frequency (MAF) >5% were genotyped in a further 377 morbidly obese adults and 465 obese children to make a total of 557 and 552, respectively. For case-control analysis alleles, genotype and haplotype frequencies were compared with frequencies in 1,195 nonobese and nondiabetic adult control subjects.

The location and frequency of SNPs found in the MCHR1 gene are displayed in Table 1. A total of 39 SNPs were found in the sequenced region. Fourteen of these had an MAF >1%, and six were found to have an MAF >5%. These six frequent SNPs were confirmed as present on dbSNP (available from www.ncbi.nlm.nih.gov/SNP). Eleven were located in the coding region of the gene, and seven resulted in a change in the amino acid produced (one conservative and six nonconservative). The positions of the changes within the structure of the protein are shown in Fig. 1. None of the mutations were found in the highly conserved DRY triplet motif crucial for signal transduction (16), nor were any of the variants found in the critical binding and activation site of the receptor located at the 123rd residue (aspartic acid), which lies within the third transmembrane domain (17).

The seven pedigrees of the individuals with the coding changes were subsequently sequenced for the relevant mutation. One pedigree showed cosegregation with the obesity phenotype in the family, with an obese father (BMI = 30.07) and proband (Z BMI = 4.95), both possessing the R248Q mutation. This amino acid lies within the third intracellular loop of the receptor, and both the G allele and its corresponding arginine amino acid are highly evolutionarily conserved across human, monkey, dog, mouse, rat, opossum, zebra fish, and fugu genomes. The proband had an onset of obesity at the age of 2.5 years, and the occurrence of the adiposity rebound was at the age of 2 years. BMI in early childhood usually decreases until around the age of 5 or 6 years and then begins to increase through to adolescence. The adiposity rebound is the point where the BMI begins to increase again after its initial decline. An early, or lack of this occurrence has been reported as a risk factor for obesity (18). The affected child's brother had normal weight with a Z BMI of 1.41 and adiposity rebound at 6 years and did not possess the mutation. This variant was then screened for in a further 353 morbidly obese adults, 289 obese children, and 547 control individuals. The MAF was 0.61% (n = 11) in the cases and not significantly different at 0.55% (n = 6) in the control subjects (P = 0.846).

The six SNPs with MAF >5% were then genotyped in an additional 842 individuals in the case group (making a total of 557 adults and 552 children, respectively; a total of 1,109 cases) and 1,195 control individuals. The locations of these SNPs in the gene are shown in Fig. 2, and the results are in Table 2. The three cohorts of French Caucasians that comprised the control group showed no significant difference in allele or genotype frequencies (P > 0.05). Adult control subjects were used for case-control comparison in both the adult and child cases because these are superior to matched age-group control subjects and because they have received longer environmental exposure but remain nonobese. One 5′ SNP, rs133068, found 1,249 bp upstream from the start methionine codon was shown to be significantly different in allele frequency in the child cases (P = 0.006) but not adults. However, the combined case group remained significant (P = 0.010). The rare G allele was found to be protective. Although the adult results are not significant, the allele frequencies do lie in an intermediary position (G allele frequency = 0.353) between those of the child cases (0.326) and control subjects (0.378). The genotype frequencies were then analyzed, and this same SNP rs133068 was found to be significant in the childhood group (P = 0.004), not adults but again overall (P = 0.021). To further investigate the positive allelic effect, the genotypes were then analyzed using either a dominant and recessive model. The recessive model found no significant difference for any SNP (data not shown); however, the dominant model was significant for the rs133068 SNP in the child cases (P = 0.001, odds ratio 0.695 [95% CI 0.560–0.863]) and in the overall group (P = 0.006, 0.783 [0.658–0.930]). One other SNP, rs133072, which resides in the untranslated region of exon 1, was also found to be significant for the total group of cases versus control subjects (P = 0.044).

Linkage disequilibrium analysis (D′) of the six common (MAF >5%) SNPs revealed two blocks of strong linkage disequilibrium, between the second, third, and fourth SNPs and between the last two SNPs. However, the lowest linkage disequilibrium throughout the whole gene was found to be a D′ = 0.763 between the first, rs133068, and last SNP, rs3087592, a distance of 3.9 kb. Three major haplotypes plus 35 minor (<5%) haplotypes were identified using Phase 2.0 (29), using all six SNPs. None of these three common haplotypes showed any significant difference between the frequency of case versus control subjects, adult case versus control subjects, or child case versus control subjects (data not shown).

This study found one coding polymorphism, R248Q,

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TABLE 1
Location of the SNPs found within the sequenced region of MCHR1

SNP	Chromosome position (NCBI)	Gene position	SNP change	Amino acid change	Frequency
rs133068	39317446	5′	c→g	—	0.357
MCHR1_17781	39317781	5′	g→a	—	0.002
MCHR1_17802	39317802	5′	g→a	—	0.021
rs133070	39317812	5′	a→g	—	0.417
MCHR1_17885	39317885	5′	t→a	—	0.021
MCHR1_18071	39318071	5′	t→c	—	0.021
rs6002015	39318148	5′	t→c	—	0.019
MCHR1_18188	39318188	5′	t→c	—	0.002
rs133071	39318271	5′	c→t	—	0.009
MCHR1_18276	39318276	Exon 1 (untrans)	g→a	—	0.002
MCHR1_18562	39318562	Exon 1 (untrans)	c→t	—	0.006
MCHR1_18571	39318571	Exon 1 (untrans)	a→t	—	0.013
rs133072	39318582	Exon 1 (untrans)	g→a	—	0.352
rs133073	39318734	Exon 1(coding)	t→c	N13N (synonymous)	0.387
MCHR1_18761	39318761	Exon 1(coding)	t→c	D22D (synonymous)	0.002
MCHR1_18772	39318772	Exon 1(coding)	c→t	S26L	0.002
MCHR1_18854	39318854	Intron	t→c	—	0.002
rs6002017	39318907	Intron	t→a	—	0.021
MCHR1_19171	39319171	Intron	c→t	—	0.002
MCHR1_19711	39319711	Intron	t→g	—	0.007
MCHR1_20041	39320041	Exon 2 (coding)	t→g	P43P (synonymous)	0.004
MCHR1_20105	39320105	Exon 2 (coding)	g→a	V66M	0.002
MCHR1_20382	39320382	Exon 2 (coding)	c→t	S158F	0.002
MCHR1_20588	39320588	Exon 2 (coding)	g→a	A227T	0.002
MCHR1_20652	39320652	Exon 2 (coding)	g→a	R248Q	0.002
MCHR1_20934	39320934	Exon 2 (coding)	c→t	T342M	0.006
MCHR1_20935	39320935	Exon 2 (coding)	g→a	T342T (synonymous)	0.002
MCHR1_20942	39320942	Exon 2 (coding)	g→a	E345K	0.002
rs2071827	39321019	Exon 2 (untrans)	c→t	—	0.002
MCHR1_21059	39321059	Exon 2 (untrans)	g→a	—	0.011
MCHR1_21387	39321387	Exon 2 (untrans)	c→t	—	0.006
MCHR1_21493	39321493	Exon 2 (untrans)	g→t	—	0.004
rs133074	39321512	Exon 2 (untrans)	c→t	—	0.450
MCHR1_21572	39321572	Exon 2 (untrans)	a→g	—	0.039
MCHR1_21665	39321665	Exon 2 (untrans)	g→a	—	0.002
rs3087592	39321704	Exon 2 (untrans)	c→t	—	0.052
MCHR1_21757	39321757	Exon 2 (untrans)	t→c	—	0.009
MCHR1_21804	39321804	Exon 2 (untrans)	c→g	—	0.002
MCHR1_21871	39321871	3′	t→g	—	0.002

dbSNP rs number is given if known; location within gene (NCBI chromosome position), base change, and amino acid changes are given if occurring. Novel SNPs are named MCHR1 with a suffix of the last five digits of their chromosome position. Frequency is determined from the 534 chromosomes sequenced. Untrans, untranslated.

which appeared to cosegregate with obesity but was subsequently found to also be present in control subjects at a similar (rare) frequency. Due to the very low MAF, it was not possible to test whether this variant may be contributing to the phenotype but with variable penetrance. Even though these data in themselves are limited because of unavailability of further samples from the pedigree, they are of considerable interest because during the course of the study reported here, another group working in the U.K. Caucasian population reported this same variant cosegregating with obesity (19). No functional differences between the wild-type and variant were identified. Although these functional investigations were not successful, it is possible that further second messengers may be involved, differing tissue environments may cause the response, or the effects may occur during a specific developmental stage. R248Q, although found in control subjects, may confer an inherited susceptibility to obesity but with changing phenotype over time, a similar phenotypic history to that of MC4R mutations, where the maximal obesity effect occurs in childhood and lessens with age (20), which could lead to a low rate being found in "normal " adults. Finally, the polymorphism may be in linkage disequilibrium with a true functional polymorphism.

This study also investigated a possible role of MCHR1 SNPs in human polygenic morbid obesity and found association with one plausible promoter SNP, rs133068. This SNP was found to be significant after correction for multiple testing with a conservative adjustment for the linkage disequilibrium present within the gene. Noncoding sequence variations can have many unpredictable effects that can be difficult to discern (21). A possible hypothesis for its action may be that this variant causes reduced activity of the promoter of the gene, leading to reduced expression of the receptor and thereby reduces the orexigenic effect of MCH. This may have long-term effects due to a reduced quantity of receptor overall in specific regions of the hypothalamus, or reduced numbers during a critical

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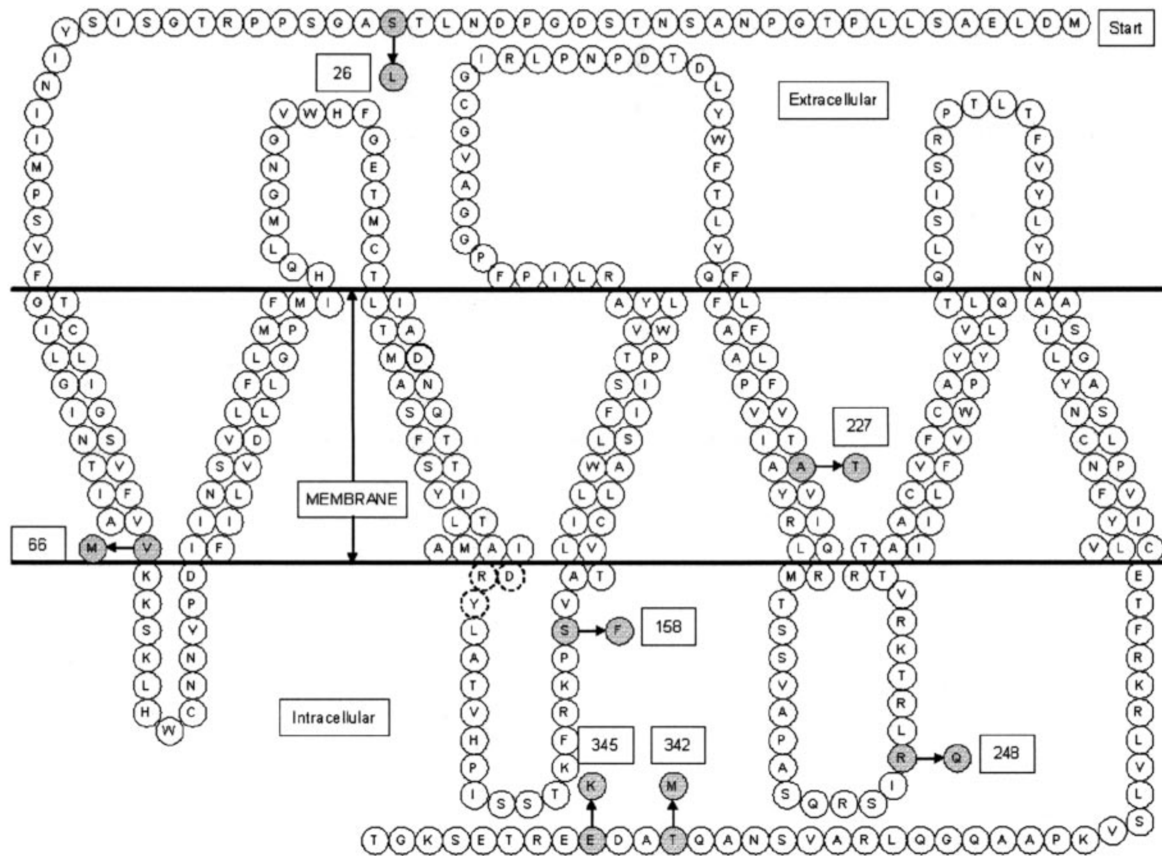

FIG. 1. Cellular locations of amino acids in the MCHR1 seven-helix transmembrane protein. Location of the seven amino acid changes identified in this study are shown shaded with boxed numbers indicating their position. The bold outline amino acid in the third transmembrane domain indicates the location of residue 123 aspartic acid, which is the critical binding and transduction site. The highly conserved DRY triplet is shown with dashed lines at the start of the second intracellular loop.

developmental stage may lead to long-term effects with regard to any neurotrophic role of receptor signaling (22). The dominant effect of the rare allele suggests that two fully functioning MCHR1 genes are required to produce enough active receptor for optimal orexigenic activity. The protective nature of the nonancestral allele (the allele not present in *Pan troglodytes* [23]) is consistent with the reduced selection for obesity-promoting pathways and "thrifty" obesity-promoting genes in the more recent portion of human history (24). Likewise, this polymorphism may be in linkage disequilibrium with the true functional variant possibly further upstream from the sequenced region. The childhood cohort has a stronger genetic component to its obesity because of reduced environmental time of impact in the pathogenesis of their obesity, and this may explain the association being found only in this group and not in the adult case group.

To confirm no effect of age due to using adult control subjects, a separate control group of 198 French Caucasian lean nondiabetic children was genotyped for the

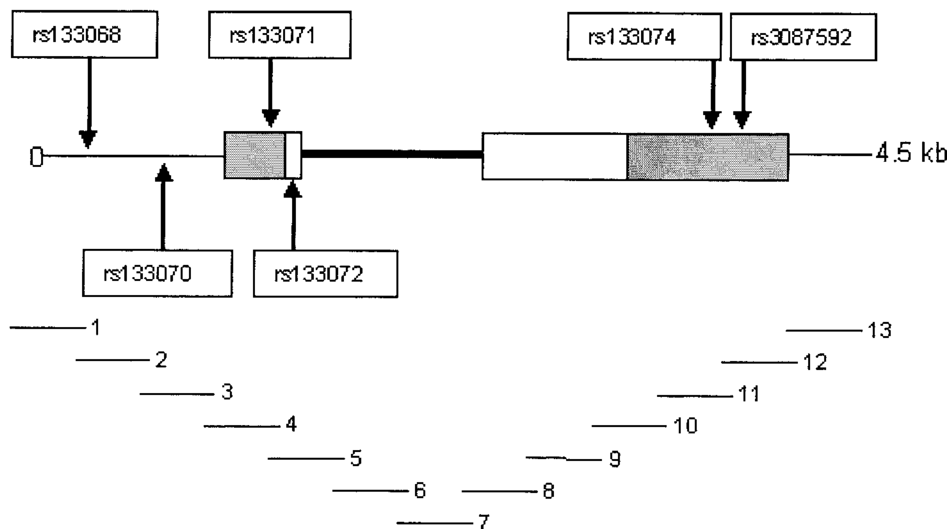

FIG. 2. Structure of the MCHR1 gene and location of frequent SNPs. ■ indicates exons, with shaded region indicating untranslated regions. Bold line indicates the single intron. □ indicates SNPs with their National Institutes of Health dbSNP rs numbers. Below the gene structure, the 13 overlapping sequencing fragments are indicated.

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TABLE 2
SNP allele frequencies of cases (adults and children) and control subjects for the six common SNPs

SNP	n	Genotype frequency 11	12	22	Allele frequency P value	Genotype frequency P value	Dominant model P value
rs133068							
Controls subjects	1,127	0.382	0.479	0.138	—	—	—
Children	469	0.471	0.405	0.124	0.006*	0.004*	0.001*
Adults	518	0.415	0.463	0.122	0.162	0.386	0.208
Total cases	987	0.442	0.436	0.123	0.010*	0.021*	0.006*
rs133070							
Controls subjects	1,086	0.417	0.466	0.117	—	—	—
Children	483	0.447	0.42	0.133	0.685	0.233	0.266
Adults	521	0.424	0.443	0.132	0.813	0.57	0.788
Total cases	1,004	0.435	0.432	0.132	0.948	0.256	0.402
rs133072							
Controls subjects	1,076	0.415	0.461	0.124	—	—	—
Children	459	0.464	0.399	0.137	0.363	0.08	0.078
Adults	489	0.456	0.425	0.119	0.224	0.315	0.132
Total cases	948	0.46	0.412	0.128	0.185	0.08	0.044*
rs133073							
Controls subjects	1,115	0.406	0.472	0.122	—	—	—
Children	479	0.438	0.43	0.132	0.518	0.31	0.233
Adults	525	0.413	0.453	0.133	0.876	0.715	0.786
Total cases	1,004	0.425	0.442	0.132	0.748	0.382	0.375
rs133074							
Controls subjects	1,009	0.295	0.494	0.211	—	—	—
Children	488	0.305	0.477	0.217	0.938	0.829	0.673
Adults	537	0.285	0.486	0.229	0.455	0.703	0.679
Total cases	1,025	0.295	0.482	0.223	0.666	0.797	0.993
rs3087592							
Controls subjects	1,129	0.895	0.103	0.003	—	—	—
Children	470	0.909	0.091	0	0.379	0.418	0.401
Adults	528	0.902	0.095	0.004	0.739	0.817	0.666
Total cases	998	0.905	0.093	0.002	0.404	0.722	0.435

Genotype frequencies are given with 11 representing homozygous common allele, 12 representing the heterozygous state, and 22 for homozygous rare allele. Genotype frequency compares all three groups. Dominant model analysis combines 12 with 22 and compares these with 11. P values are given for the χ^2 test for cases versus control subjects and are also given for adults and children separately versus control subjects. *$P < 0.05$.

rs133068 SNP. This demonstrated no significant difference in the allele frequencies between the adult (0.378) and child control subjects (0.358), and using the child control subjects alone still supported the dominant model genotype association seen in the child cases ($P = 0.039$). This result cannot completely exclude an age cohort effect due to the small sample size of the lean children; however, it does reach the nominal level of significance for a replication ($P < 0.05$) even if it does not reach the calculated, more stringent, study-wide significant P value ($P < 0.004$). A further replication in a larger French Caucasian cohort would be required to confirm this result. Subsequent testing for quantitative differences within the case or within the control group was not found for normalized Z BMI values and rs133068 genotype (data not shown).

The linkage disequilibrium analysis revealed linkage disequilibrium throughout the gene with a strong block that contained the SNPs rs133072 and rs133073. This is consistent with the U.K. Caucasian study, which found linkage disequilibrium with these two SNPs; however, that study did not examine any of the other frequent SNPs within the region (19). Furthermore, as in the U.K. study, this investigation found no association with the obesity trait for the synonymous coding SNP rs133073 but did find a weak association with SNP rs133072.

This investigation has extensively characterized the genetic variation found in the MCHR1 gene. No strong evidence for a role in monogenic human obesity was found, but one plausible promoter SNP, rs133068, was found to be significantly associated with obesity. This result indicates that MCHR1 may be involved in the polygenic form of severe human obesity with early age of onset, and this will require both further investigation in different populations and functional work on the promoter region variant.

RESEARCH DESIGN AND METHODS

Subjects were all French Caucasian and were recruited using a multimedia campaign run by the Centre National de la Recherche Scientifique, the Department of Nutrition of the Paris Hôtel-Dieu Hospital, the Institut Pasteur de Lille, the Department of Pediatric Endocrinology of Jeanne de Flandres Hospital, and the Toulouse Children's Hospital. For this study, a cohort of 557 unrelated, morbidly obese adults (BMI >40), and 552 unrelated obese children (age <18 years, BMI >97th percentile for age and sex) were used giving a total of 1,109 cases. All subjects had been previously screened for MC4R mutations. Additionally, a total of 1,195 nonobese, nondiabetic adult control subjects were utilized for genotyping for the association study. The control subjects were unrelated adult nonobese, nondiabetic, French Caucasians pooled from three separate studies. The Supplementation en Vitamines et Minéraux Antioxidant study (25) included 246 male and 300 female control subjects (mean BMI 22 ± 1.8, mean age 55 ± 6 years). Regular weight measurements were taken so that those who had never been obese during the 8-year course of the study were chosen. The TAF control subjects consisted of 97 men and 155 women (BMI 23.02 ± 2.20, age 42.47 ± 4.53 years) gathered from the

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Fleurbaix Laventie Ville Sante Study (26). The LORAINC cohort consisted of 174 men and 191 women (BMI 21.28 ± 2.00, age 23.23 ± 3.89 years) who were gathered from maternity registry data for a study investigating the adult outcome of those born small or appropriate for gestation age gathered from northeastern France, with only the latter used here as control subjects (27).

For the independent testing of child control subjects to exclude any age-cohort effect, 198 children from the Fleurbaix Laventie Ville Sante Study cohort (26) were used. These children were all French Caucasian, lean, and nondiabetic (age 14.6 ± 2.2 years, BMI 18.3 ± 2.2, male-to-female ratio 1:1). The ethical committee of Hôtel-Dieu in Paris and Centre Hospitalier Régional Universitaire in Lille approved the genetic study. Weight was measured in a nonpostprandial state and with an empty bladder and was determined to the nearest 0.1 kg on a standard physician's beam scale with the subject dressed only in light underwear and without shoes. Height was measured to the nearest 0.5 cm on a standard height board, again without shoes. BMI was calculated as weight (in kilograms) divided by the square of height (in meters).

DNA isolation. Genomic DNA was extracted from peripheral blood cells using PURE-GENE D50K DNA isolation kits (Gentra Systems).

Sequencing. For the sequencing of the MCHR1 gene, a random subset of 180 morbidly obese adults from the adult morbid obesity set described above and an extreme subset of 87 morbidly obese children from the obese children set selected for having a Z BMI >4.5 and onset of obesity before the age of 5 years were utilized. Thirteen overlapping PCR fragments were designed to cover the ~4.5-kb region including the exons, the single intron, and a plausible promoter region of 1 kb upstream from the start of exon 1 (Fig. 1). The fragments were sequenced in a forward and reverse direction, leading to a total of >2.4 Mb of sequence. Primers were designed for these fragments using Primer 3 (available at www.genome.wi.mit.edu/genome_software/other/primer3.html). Primer details are available from the authors. Primers were optimized with control DNA reactions to specify the temperature and $MgCl_2$ concentration. A total of 0.5 units AmpliTaq Gold (Applied Biosystems), 1.5 μl 10× PCR Buffer II (Applied Biosystems), 0.3 μl deoxy-nucleoside triphosphate 10 mmol/l, and a range of 25 mmol/l MgCl2 between 0.9 and 2.4 μl, specified by optimization, was added to 20 ng of DNA. A total of 30 ng of both forward and reverse primer was added to this reaction volume and then made up to a volume of 15 μl with H_2O. DNA was subsequently amplified by PCR using MJ research PTC-225 Peltier Thermal Cycler tetrad machines. Cycling conditions employed were 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 50–65°C for 30 s, 72°C for 30 s, and then a final step of 72°C for 10 min.

Cleanup of the amplified product for sequencing then required 2 μl EXOSAP IT (USB) to be added to each 5 μl of PCR product. The mixture was then incubated at 37°C for 15 min, at 80°C for 15 min, and then cooled to 4°C. The sequencing reaction required 4 μl DNA template to be added to 4 μl Big Dye Terminator Master Mix (Applied Biosystems), 1 μl of the forward or reverse primer at a concentration of 5–10 pmol/μl, and 1 μl H_2O to make a 10-μl volume total. The reaction plate was placed in the thermal cycler machine and underwent a temperature cycling protocol of 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. Following this reaction, the sequencing products were purified by ethanol precipitation. For each 96-well plate, 200 μl of 7.5M ammonium acetate and 6 ml of 95% (vol/vol) ethanol were added together, and 62 μl of this mix were then added to each well. An adhesive lid was used to seal the plate; then, it was inverted to mix and spun in a centrifuge for 30 min at 3,100*g* and 4°C. The supernatant was then removed immediately, and the plate was drained upside down on tissue. A total of 100 μl chilled 70% (vol/vol) ethanol was then added to each well and was spun again at 3,100*g* for 2 min. The supernatant was again removed, 100 μl chilled 70% ethanol was added again to each well, and it was spun at 3100*g* for 2 min. The final supernatant was removed, and the plate was spun upside down on tissue at 250*g* for 1 min. The plate was then allowed to air dry before the precipitated sequencing products were resuspended in 10 μl H_2O.

This sample was then run on an automated ABI 3700 DNA Analyzer (Applied Biosystems). Sequences were analyzed using PhredPhrap software (www.genome.washington.edu). The sequence of MCHR1 from the UCSC database (http://genome.ucsc.edu/) was converted into a trace file for reference. SNPs found in one primer direction were confirmed by viewing the opposite strand. SNP allele frequencies were calculated from this sequencing data.

Genotyping. The six SNPs with an MAF of >5% from the sequencing analysis were then genotyped in the total case and control groups using the Sequenom MALDI-TOF technology (28) (http://www.sequenom.com). Primers and extension primers were designed using the MassARRAY Assay Design software (Sequenom), and details can be obtained from the authors. For a 4-plex PCR, 2.5 ng of each DNA sample were mixed with 2.18 μl H_2O, 0.5 μl Quiagen Hot Star Buffer, 0.2 μl $MgCl_2$, and 0.02 μl Hot Star *Taq* (5 units/μl); 0.1 μl of 10 mmol/l dNTP; and 0.5 μl of 1 μmol/l primer mix. A PCR was then carried out at 95°C for 15 min, then four cycles of 95°C for 20 s, 65°C for 30 s, 72°C for 1 min, followed by four cycles of 95°C for 20 s, 58°C for 30 s, 72°C for 1 min, followed by 38 cycles of 95°C for 20 s, 53°C for 30 s, 72°C for 1 min, and ending with 72°C for 3 min. Shrimp alkaline phosphatase was then added to samples as 0.3 μl SAP, 0.17 μl hME buffer (10×), and 1.53 μl H_2O to make a total of 2 μl per single reaction. Samples were incubated for 20 min at 37°C and then at 87°C for 5 min. The hME reaction solution was then added, which comprised 1.732 μl H_2O, 0.2 μl hME 10× termination mix, 0.05 μl hME primer (100 μmol/l), and 0.018 μl MassExtend enzyme (32 units/μl) for each single reaction to make a total volume of 2 μl. The hME thermocycle reaction was 94°C for 2 min, followed by 55 cycles of 94°C for 5 s, 52°C for 5 s, 72°C for 5 s, and then cooled to 4°C. The reaction products were purified by dispensing 3 mg clean resin into each well, followed by 6 μl of H_2O. The plate was rotated for 5 min and then centrifuged for 5 min at 300*g*. Approximately 15 nl of the hME reaction products were then transferred onto a SpectroCHIP. The mass-extended products were then analyzed by mass spectrometry using the Sequenom MALDI-TOF instrument (28). Based upon the expected masses of the products, SNP alleles were called by the Sequenom software. Plates were considered successful if >75% of genotypes could be called and were not significantly different from Hardy-Weinberg equilibrium ($P > 0.05$).

Statistics. Frequencies were calculated and analyzed using the SPSS statistical package version 11.0.0 (SPSS). The χ^2 test was performed, testing all cases against all control subjects as well as adult and children separately against the control group. This was done using allele frequencies, as well as genotype frequencies. Genotype frequencies were then analyzed using dominant and recessive models. The dominant model compared the combined group of the heterozygotes and homozygotes for the rare allele with the homozygotes for the common allele, and the recessive model compared the homozygote rare allele group versus the rest.

To confirm that there was no difference between the three groups that comprised the control subjects, allele and genotype frequencies for each group were also compared by the χ^2 test. Haplotypes were constructed with the six common SNPs using the program Phase 2.0 (29). Those who had missing data for any of the six SNPs were removed from this analysis, giving a total of 1,779 individuals. Linkage disequilibrium was calculated using HaploXT from the result from all cases and control subjects and graphically displayed using the program Gold (30). Quantitative analysis of normalized Z BMI within case and control groups was performed by the ANOVA test using the SPSS program.

Uncorrected *P* values of 0.05 were regarded as significant. However, in consideration of the number of statistical tests carried out, a modified Bonferroni correction was attempted. The six SNPs within the gene are all in strong linkage disequilibrium with each other, so the SNP tests were not completely independent. Conservatively, using the minimum value of correlation between SNPs, i.e., the lowest linkage disequilibrium D′ value of 0.763, the corrected threshold of significance for the SNP tests was $P = 0.018$ (31). When the haplotype tests were also factored in, this led to a global significance level of $P = 0.004$. Certain results were still significant if this value was taken into account. However, the uncorrected *P* values are presented in the tables and text because of the continuing debate about the validity of the Bonferroni correction (32).

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APPENDIX

Electronic database information: dbSNP (www.ncbi.nlm.nih.gov/SNP), Ensembl (www.ensembl.org), National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov), Obesity Gene Map (obesitygene.pbrc.edu), PhedPhrap software (www.genome.washington.edu), Primer 3 (www-genome.wi.mit.edu/genome_software/other/primer3.html), Sequenom (www.sequenom.com), and UCSC genome browser (http://genome.ucsc.edu/).

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