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Melanocortin 4 Receptor Sequence Variations Are Seldom a Cause of Human Obesity: The Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis Cohort

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The prevalence of mutations within and in the flanking regions of the gene encoding the melanocortin 4 receptor was investigated in severely obese and normal-weight subjects from the Swedish Obese Subjects study, the Health, Risk Factors, Exercise Training, and Genetics (HERITAGE) Family study, and a Memphis cohort. A total of 433 white and 95 black subjects (94% females) were screened for mutations by direct sequencing. Three previously described missense variants and nine novel (three missense, six silent) variants were detected. None of them showed significant association with obesity or related phenotypes. In addition, two novel deletions were found in two heterozygous obese women: a −65_−64delTG mutation within the 5′ noncoding region and a 171delC frameshift mutation predicted to result in a truncated nonfunctional receptor. No pathogenic mutations were found among obese blacks or nonobese controls. Furthermore, none of the null mutations found in other populations was present in this sample. In conclusion, our results do not support the prevailing notion that sequence variation in the melanocortin 4 receptor gene is a frequent cause of human obesity. (*J Clin Endocrinol Metab* 87: 4442–4446, 2002)

HUMAN OBESITY IS a complex trait influenced by interacting environmental and genetic factors (1). Several rodent models exist in which obesity is caused by a single mutated gene (2). One such model is the Agouti mouse, the obesity phenotype that is of particular interest because of its resemblance to human nonsyndromic obesity, *i.e.* maturity onset, intact fertility, moderate metabolic aberrations, and adipocyte hypertrophy (3, 4). Characterization of the Agouti phenotype at the molecular level has uncovered the impact of the melanocortin pathway on energy balance (5–7). In short, neurons within the arcuate nucleus and other hypothalamic nuclei project to the lateral hypothalamus and paraventricular nuclei in which they express α-melanocyte stimulating hormone derived from posttranslational processing by enzymes, such as proconvertase-1 and -2, of the hypothalamic/pituitary prohormone proopiomelanocortin. The α-melanocyte stimulating hormone stimulates the melanocortin 4 receptor (MC4R), resulting in inhibition of feeding and increase in energy expenditure (8–10). MC4R is naturally antagonized by the agouti-related protein, which is expressed in neurons distinct from those expressing proopiomelanocortin (6, 8). The melanocortin pathway is targeted by a peripheral feedback signal from adipose tissue mediated by the circulating hormone leptin (11). Disruption of this pathway, at various levels, including the MC4R, has been shown to cause obesity in rodents (7, 12–15). MC4R-deficient mice exhibit an obesity phenotype similar to the Agouti obesity syndrome, albeit with larger weight gain and higher levels of insulin and glucose. Heterozygous MC4R deficiency results in an obesity phenotype intermediate to those of the homozygous mutant and the wild type (7).

Several human studies have reported associations between MC4R mutant variants and early-onset morbid obesity, particularly in females (16–24). Dominant as well as recessive modes of inheritance with variable expressivity or penetrance have been proposed. Based on the high frequencies of mutations found in some populations, it has been suggested that MC4R mutations constitute the most common monogenic cause of human obesity with up to 4% of all patients with morbid obesity attributable to mutations in this gene (22). To verify this hypothesis and establish whether

Abbreviations: AVF, Abdominal visceral fat; BMI, body mass index; FFM, fat-free mass; FM, fat mass; HERITAGE, Health, Risk Factors, Exercise Training, and Genetics Family study; MC4R, melanocortin 4 receptor; RMR, resting metabolic rate; SOS, Swedish Obese Subjects study; UTR, untranslated region.

these results could be replicated, we sequenced the MC4R gene for mutations in samples of severely obese and normal-weight blacks and whites from three different cohorts.

Subjects and Methods

Subject characteristics are shown in Table 1. The obese and control subjects were defined as having body mass index (BMI) of at least 30 kg/m^2 or BMI less than 30 kg/m^2, respectively. Approximately three-quarters (n = 196) of all obese individuals had a current or reported maximum BMI of 40.0 kg/m^2 or more, with 77 patients above 45 and 22 above 50 kg/m^2.

The Swedish Obese Subjects (SOS) project is a national obesity intervention study primarily designed to investigate obesity-associated morbidity and mortality rates following sustained weight loss as induced by bariatric surgery. SOS outcomes are compared with those in conventionally treated obese controls and a randomly selected reference population. A detailed description of the SOS design has been published elsewhere (25, 26). In the present study, a subset of 144 obese white women and 140 nonobese white women from the reference population were included. In addition, we analyzed 149 (33 males) white subjects from 61 Swedish siblingships, each characterized by the presence of a pair of siblings, very discordant for BMI. A BMI difference of at least 10 BMI units was used as the criterion to define discordance. The rationale for investigating extreme-discordant sibling pairs was that the phenotypic difference within pairs of siblings, who share a considerable part of their environment and approximately half their genes, is more likely to be attributable to sequence variation in fewer genes with major effects. From the Swedish cohorts, self-reported age of onset of weight problems was available in 214 obese subjects. The age-of-onset distribution was: infancy 2%, childhood 23%, adolescence 17%, and adulthood 58%. Mean BMI in subjects whose onset occurred during infancy or childhood was 41.9 kg/m^2.

To investigate the role of MC4R sequence variation in obesity across ethnic groups, black females from the Health, Risk Factors, Exercise Training, and Genetics (HERITAGE) Family Study and a Memphis cohort were included.

The HERITAGE Family Study is designed to study the genetics of the variability in response of cardiovascular disease and type 2 diabetes mellitus risk factors to endurance training in sedentary North American individuals. Details of the HERITAGE study have been published elsewhere (27). For the present study, 18 obese and 48 nonobese black women from this cohort were analyzed. The sample included seven pairs of relatives: four mother-daughter pairs (all nonobese controls) and three obesity-discordant sibling pairs (mean BMI difference 16.4 kg/m^2). All obese subjects were unrelated. This cohort was ascertained based on sedentary lifestyles rather than obesity.

The Memphis cohort consists of extremely obese patients who underwent bariatric surgery in a surgical obesity clinic. From this cohort, 29 obese black women were included in the present study.

For all study groups, data collected before any interventions were used. This study was conducted in accordance with the guidelines proposed in The Declaration of Helsinki and was formally approved by the appropriate institutional review boards. Informed consent was obtained from all subjects.

TABLE 1. Subject characteristics

Cohort	No.	Age (yr)	BMI (kg/m^2)
Blacks			
HERITAGE			
Controls	48	28.5 ± 9.4	21.6 ± 1.7
Obese	18	31.1 ± 9.0	38.7 ± 2.4
Memphis			
Obese	29	35.4 ± 9.4	48.4 ± 8.4
Whites			
SOS			
Controls	140	47.6 ± 6.8	22.4 ± 1.6
Obese	144	47.0 ± 6.0	43.1 ± 3.4
Sibships			
Controls	76	37.3 ± 7.1	22.7 ± 2.2
Obese	73	40.2 ± 6.3	35.9 ± 5.5

All subjects were females except among the white sibships where 16 controls and 17 obese were males. Data represent means ± SD.

Obesity-related phenotypes

Measurements were performed after an overnight fast. Body mass was measured on calibrated balances or electronic scales to the nearest 0.1 kg. Body height was measured to the nearest centimeter. BMI was calculated as body mass (kilograms) divided by body height (meters) squared. The subjects among the Swedish siblingships underwent more extensive phenotyping: fat-free mass (FFM) was calculated from total body potassium, as derived from whole-body ^{40}K counting, and fat mass (FM) was obtained by subtracting FFM from measured body mass. Abdominal visceral fat (AVF) was estimated from a computed tomography scan at the level of the iliac crest. Intraabdominal adipose tissue area was determined as previously described (28). Resting metabolic rate (RMR) was obtained from indirect calorimetry measured in the supine position for 30 min in a ventilated hood system after a 15-min rest. RMR was expressed as kilocalories per 24 h.

Statistical analyses

Descriptive statistics were expressed as means and SD. Differences in genotype frequencies (obese *vs.* controls, by race) were analyzed using Fisher's exact test, and P values smaller than 0.05 were considered significant. FM was adjusted for the effects of age and sex. AVF was log transformed to obtain approximate normality and was adjusted for sex, age, and FM. RMR was adjusted for sex, age, age^2, age^3, body height, FM and FFM. Genotype associations with FM, AVF, and RMR were assessed using general linear models, and genotype effect size was defined as the type III sum of squares to the corrected-total sum of squares ratio. Measures of nucleotide diversity were calculated as described (29). The SAS statistical software package (Windows version 8.1, SAS Institute Inc., Cary, NC) was used for all statistical analyses.

Sequencing analysis of the MC4R gene

MC4R is a 332 amino acid seven-transmembrane G protein-coupled receptor, translated from a single exon. The entire 999-bp coding region was sequenced along with 393 and 280 bp of the 5′ and 3′ flanking regions, respectively. Genomic DNA was isolated from either whole blood or permanent lymphoblastoid cell lines. Coding and noncoding regions of the gene were amplified by PCR using four primer pairs, thus generating four overlapping fragments, in which the fragment generated by primer pair 1 covered the 5′ untranslated region (UTR), 2 and 3 the coding region, and 4 the 3′ UTR. Oligonucleotide sequences modified from reference (30) were: 1 forward (f) 5′-AGCTTCCGAGAGGCAGCCGA-3′; 1 reverse (r) 5′-CGGTTCCAGAGGTGCAGAGAAG-3′; 2(f) 5′-ATCAATTCAGGGGGACACTG-3′; 2(r) 5′-GACAGCACTACTATCTGAGT-3′; 3(f) 5′-ATGCTCTCCAGTACCATAAC-3′; 3(r) 5′-TGCAGAAGTACAATATTCAGG-3′; 4(f) 5′-CACTCCGGAGTCAAGAACTG-3′; 4(r) 5′-AAGCTTTTAATAAGGACTTTTCT-3′.

The initial PCR amplification was carried out in a volume of 60 μl containing 250 ng DNA, 200 μM of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech Inc., Piscataway, NJ), 0.3 μM of each primer, and 1.25 U Taq polymerase with 6 μl of the manufacturer's PCR buffer 10x (QIAGEN, Valencia, CA). PCR was performed in a thermocycler (Eppendorf Mastercycler Gradient, New York, NY) at 95 C for 3 min, 55 C for 60 sec and 72 C for 2 min, followed by 40 cycles at 95 C for 30 sec , 55 C for 30 sec, and 72 C for 75 sec terminated by one cycle at 72 C for 10 min. Bidirectional sequencing of the PCR products was performed using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. The primers used in the initial PCR were also used for sequencing. The purified PCR products were directly sequenced using an ABI3700 automated sequencer (Applied Biosystems) as previously described (31). Sequences were assembled and examined using the Sequencer software (Gene Codes, Ann Arbor, MI). Sequences suggesting heterozygous deletions were verified after subcloning the PCR products into the pPCR-Script Amp SK+ cloning vector (Stratagene Cloning Systems, La Jolla, CA) with subsequent transformation into Epicurian Coli XL10-Gold ultra-

competent cells, in concordance with the manufacturer's protocol. Ten representative clones were then sequenced as outlined above.

Results

A total of 12 nucleotide substitutions was detected among the 528 subjects of the study. The Val103Ile, Thr112Met, and Ile251Leu polymorphisms have been reported earlier by other groups (18, 19, 21, 22, 30, 32) and have been shown to be equivalent to the wild type in terms of pharmacological properties (18, 22, 33) as well as being equally common among obese and nonobese people. All other variants reported here were previously unknown. Among them, two were silent, three were missense, and four were in noncoding regions. The genotype frequencies in obese subjects and normal-weight people are shown in Table 2. Except for the −178A>C variant, all were in the heterozygous form. There were four cases of compound heterozygosity: −184G>A + Thr102Ile (black normal-weight), −178A>C + Ile251Leu (white obese), Val103Ile + Thr112Met (white obese), and Val103Ile + 1123G>A (white obese).

Sequence variation was detected among 22 of the 61 white siblingships included in this study. Five variants in heterozygous form (−178A>C, Val103Ile, Thr102Ile, Ile251Leu, 1123G>A) were found to be equally common among obese and nonobese siblings. Among the black discordant sibling pairs, one obese −178A>C heterozygote was detected. In a recent report, there was suggestive evidence for negative association between the Val103Ile polymorphism and abdominal fat as inferred from waist to hip ratio and abdominal sagittal diameter (34). Among the white siblingships of the present study, 8.0% of the subjects were Val103Ile heterozygotes. Associations between Val103Ile and adjusted AVF and between Val103Ile and RMR were tested. Genotype effect sizes on AVF and RMR were 0.2% and 0.003%, respectively, and associations were nonsignificant ($P > 0.14$). Likewise, the −178A>C polymorphism, present in 6.7% of the siblings, was not associated with any of the measured phenotypes. In conclusion, we found no evidence that the phenotypic variance within the discordant siblingships was attributable to sequence variation in MC4R.

There was a tendency for the −178A>C variant, after pooling blacks and whites, to cluster in the uppermost parts of the BMI distribution: 10 of the 77 subjects with BMI of 45 or more and 5 of the 22 subjects with BMI of 50 or more were carriers. Unadjusted *P* values for frequency differences between normal-weight controls and subjects with BMIs of

TABLE 2. Genotype frequencies in black and white obese and controls

Base change	Effect on amino acid sequence	Blacks		Whites	
		Controls[a] (n = 48)	Obese[a] (n = 47)	Controls[b] (n = 216)	Obese[b] (n = 217)
−184G>A	5′ UTR	2 (4.2%)			
−178A>C (AC)	5′ UTR	7 (14.6%)	11 (23.4%)	9 (4.2%)	14 (6.5%)
−178A>C (CC)	5′ UTR	1 (2.1%)	1 (2.1%)		
−65_−64delTG	5′ UTR				1 (0.5%)
171delC	Leu64STP				1 (0.5%)
305T>C	Ile102Thr	1 (2.1%)			
307G>A	Val103Ile	2 (4.2%)		14 (6.5%)	9 (4.1%)
335C>T	Thr112Met			1 (0.5%)	3 (1.4%)
468G>A	Silent		1 (2.1%)		
606C>A	Phe202Leu	1 (2.1%)			
636T>C	Silent		1 (2.1%)		
719A>G	Asn240Ser	1 (2.1%)			
751A>C	Ile251Leu			8 (3.7%)	6 (2.8%)
1101G>A	3′ UTR		1 (2.1%)		
1123T>C	3′ UTR			4 (1.9%)	7 (3.2%)

Numbers do not represent population frequencies, because sample included a few related individuals.
[a] $P > 0.49$; [b] $P > 0.29$ for all obese-control comparisons (Fisher's exact test).

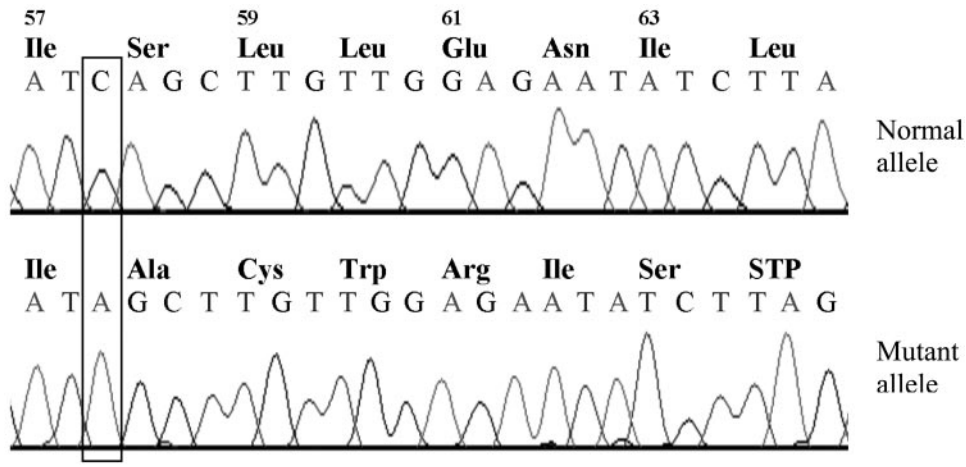

FIG. 1. Chromatogram showing plasmid sequence and predicted amino acid sequences of subcloned alleles from a 171delC heterozygote. Mutation site is indicated by a *vertical box*.

either 45 or more or 50 or more were 0.09 and 0.02, respectively. One of the two −178A>C homozygotes in the black cohort was a normal-weight control. Taken together and considering its 5′ UTR location, we consider the −178A>C association a chance finding for the moment.

By and large, there were no significant associations with obesity for any of the missense variants found in the whole sample, neither when the analyses were done separately by race (Table 2) nor after pooling the data.

In addition to base substitutions, we detected two novel deletions in the heterozygous state. A −65_−64delTG mutation was found in one morbidly obese white female, 40 yr of age. Self-reported onset of weight problems was late (34 yr), and there were no comorbidities or metabolic aberrations except hyperinsulinemia. The reported BMIs of her mother and father was 29.3 and 26.2 kg/m^2, respectively. Because this mutation is located in the 5′ UTR, any effect on gene function would be at the transcription or expression level, which cannot be predicted and will require further study. A heterozygous 171delC mutation in codon 57 was found in an obese white female, 41 yr of age. This deletion is predicted to cause a frameshift, yielding aberrant sequence of six consecutive amino acids, culminating in a premature stop codon at position 64 (Fig. 1). This truncation is within the first transmembrane domain and is presumably incompatible with a functional protein because domains closer to the C terminus are essential for receptor signaling and cell surface localization (33). The BMI of the carrier was 40.0 kg/m^2, body height above average (1.73 m), and blood pressure was 155/100 mm Hg. In addition, she exhibited hyperinsulinemia and hypertriglyceridemia. Notably, self-reported age of onset of weight problems, 15 yr, was not as early as has been reported in other null allele heterozygotes. There was no history of obesity in her parents (neither of which were genotyped, however).

Finally, and consistent with previous reports (18, 30), we found disagreements with the originally published sequence data (32, 35): First, Val103 (GTC) was the more common allele and no Ile103 homozygotes were found. Second, whereas Mountjoy *et al.* (32) reported Ser169 (AGC) as the major allele, all our subjects were Ile169 (ATC) homozygotes. Last, there was an additional adenine nucleotide within the 3′ UTR following nucleotide 1114.

Discussion

It has been proposed that MC4R mutations are a major cause of monogenic human obesity, accounting for up to 4% of all patients with morbid obesity (22). We tested this hypothesis by mutation screening of severely obese and normal-weight individuals from three cohorts. Whereas sequence variation was not uncommon, none of the previously reported null alleles was present in this relatively large sample of 528 individuals. Moreover, no significant associations were found between any of the detected missense variants and obesity. It could be argued that the sample size of the black obese group was too small to allow rejection of a common-allele hypothesis. However, the binomial probability of finding zero carriers in 47 unrelated individuals, assuming a prevalence of 4%, is less than 0.15. This corresponds to a risk of type II error comparable with that of studies in which a power of 80–90% is deemed sufficient. The corresponding probability for the white obese group (considering only unrelated individuals) was 0.0002. Therefore, we conclude that MC4R mutations are not as frequent a cause of obesity as has been proposed.

An interesting observation was the high frequency of variant sites at the MC4R locus. We compared raw and normalized variant frequencies and average heterozygosity in MC4R coding and noncoding regions to those from a recent report based on mutation screening of 106 genes in a sample of 114 chromosomes (36) (Table 3). The most striking difference was the more than 3-fold higher frequency ($\hat{\theta}$) of nonsynonymous substitution sites in MC4R, notwithstanding missense variants reported by other groups. The observed lower average heterozygosity per base pair (π) in our sample is due in part to the difference in sample sizes. Although the two estimates of genetic variation, ($\hat{\theta}$) and π, are expected to be of similar magnitude under the assumption that all mutations are neutral, it has been shown that the correlation between them decreases with increasing sample size (37). Conversely, the normalized difference between $\hat{\theta}$ and π (Tajima's D statistic) has been implemented as a means of testing whether the assumption of mutation neutrality is valid. In the present study, D values for noncoding, synonymous, and nonsynonymous variants were −1.17, −1.09, and −1.51, respectively. Based on the β distribution of D (mean, ≈0; variance, ≈1), none of these values is significantly smaller than zero [95% confidence interval −1.715 to 2.150 (37)]. Thus, the hypothesis of mutation neutrality was not rejected, although there was a nonsignificant trend ($P < 0.1$) for nonsynonymous variants toward significant negative divergence, which could suggest the presence of selection against deleterious mutations. Consistently, noncoding and synonymous variants are likely to be selectively neutral, as suggested by the nonsignificant divergence of their D values.

We identified a novel frameshift mutation in one obese woman. The pathogenicity of this mutation has not been established in terms of functional characteristics or cosegregation with obesity. However, given the fact that other truncated variants have proven nonfunctional, it is very likely

TABLE 3. Rates of different base substitution classes in coding and noncoding regions of MC4R (1056 alleles)

	bp screened	No. of variant sites	Frequency of sites	$\hat{\theta}$ ($\times 10^4$)	π ($\times 10^4$)
Noncoding	673	4	1/168 (1/354)	7.88 ± 4.16 (5.30 ± 1.33)	1.60 ± 2.92 (5.19 ± 2.47)
Coding	999	8	1/125 (1/346)	10.62 ± 4.17 (5.43 ± 1.36)	0.89 ± 1.78 (5.00 ± 2.38)
Synonymous		2	1/499 (1/656)	2.65 ± 1.93 (2.86 ± 0.72)	0.04 ± 0.36 (3.05 ± 1.45)
Nonsynonymous		6	1/167 (1/734)	7.96 ± 3.52 (2.56 ± 0.64)	0.86 ± 1.74 (1.96 ± 0.93)
Total	1672	12	1/139 (1/348)	9.52 ± 3.19 (5.39 ± 1.36)	1.18 ± 1.63 (5.05 ± 2.40)

Frequencies compared to those (within *parentheses*) obtained from 106 genes in Ref. 36. $\hat{\theta}$, normalized frequency scores ± SD, adjusted for fragment length and number of chromosomes screened; π, heterozygosity per base pair.

that this variant represents a null allele. Thus, the prevalence of pathogenic mutations in the Swedish sample was at most 0.5%. The corresponding prevalence in the white morbidly obese subgroup with childhood onset of the disease was zero, despite a power of 80–90% to detect mutations at a 3–4% prevalence. Likewise, no such mutations were found in the black sample, which was small but sufficiently powered to detect common mutations. Our findings are consistent with two previous publications. Sequencing of the MC4R in a sample of obese white British males revealed no presence of pathogenic mutations (30). More recently, MC4R mutation screening in a Mediterranean sample of obese children and adolescents showed a mutation prevalence of less than 0.5% (38). It is possible that the contribution of MC4R mutations to obesity is confined to certain ethnic groups. In addition, we conclude that unless ascertainment is restricted to early-onset cases with very extreme obesity, as has generally been the case in previous studies, chances of finding MC4R mutations are rather low.

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References

1. **Bouchard C** 1991 Current understanding of the etiology of obesity: genetic and nongenetic factors. Am J Clin Nutr 53(Suppl 6):1561S–1565S
2. **Chagnon YC, Bouchard C** 1996 Genetics of obesity: advances from rodent studies. Trends Genet 12:441–444
3. **Herberg L, Coleman DL** 1977 Laboratory animals exhibiting obesity and diabetes syndromes. Metabolism 26:59–99
4. **Johnson PR, Hirsch J** 1972 Cellularity of adipose depots in six strains of genetically obese mice. J Lipid Res 13:2–11
5. **Bultman SJ, Michaud EJ, Woychik RP** 1992 Molecular characterization of the mouse agouti locus. Cell 71:1195–1204
6. **Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD** 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385:165–168
7. **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** 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141
8. **Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO** 1994 Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 371:799–802
9. **Chen AS, Metzger JM, Trumbauer ME, Guan XM, Yu H, Frazier EG, Marsh DJ, Forrest MJ, Gopal-Truter S, Fisher J, Camacho RE, Strack AM, Mellin TN, MacIntyre DE, Chen HY, Van der Ploeg LH** 2000 Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. Transgenic Res 9:145–154
10. **Butler AA, Marks DL, Fan W, Kuhn CM, Bartolome M, Cone RD** 2001 Melanocortin-4 receptor is required for acute homeostatic responses to increased dietary fat. Nat Neurosci 4:605–611
11. **Cheung CC, Clifton DK, Steiner RA** 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 138:4489–4492
12. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432
13. **Naggert JK, Fricker LD, Varlamov O, Nishina PM, Rouille Y, Steiner DF, Carroll RJ, Paigen BJ, Leiter EH** 1995 Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. Nat Genet 10:135–142
14. **Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J** 1995 Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263–1271
15. **Yaswen L, Diehl N, Brennan MB, Hochgeschwender U** 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat Med 5:1066–1070
16. **Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S** 1998 A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet 20:111–112
17. **Vaisse C, Clement K, Guy-Grand B, Froguel P** 1998 A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet 20:113–114
18. **Gu W, Tu Z, Kleyn PW, Kissebah A, Duprat L, Lee J, Chin W, Maruti S, Deng N, Fisher SL, Franco LS, Burn P, Yagaloff KA, Nathan J, Heymsfield S, Albu J, Pi-Sunyer FX, Allison DB** 1999 Identification and functional analysis of novel human melanocortin-4 receptor variants. Diabetes 48:635–639
19. **Hinney A, Schmidt A, Nottebom K, Heibult O, Becker I, Ziegler A, Gerber G, Sina M, Gorg T, Mayer H, Siegfried W, Fichter M, Remschmidt H, Hebebrand J** 1999 Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J Clin Endocrinol Metab 84:1483–1486
20. **Sina M, Hinney A, Ziegler A, Neupert T, Mayer H, Siegfried W, Blum WF, Remschmidt H, Hebebrand J** 1999 Phenotypes in three pedigrees with autosomal dominant obesity caused by haploinsufficiency mutations in the melanocortin-4 receptor gene. Am J Hum Genet 65:1501–1507
21. **Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S** 2000 Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J Clin Invest 106:271–279
22. **Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P** 2000 Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. J Clin Invest 106:253–262
23. **Dubern B, Clement K, Pelloux V, Froguel P, Girardet JP, Guy-Grand B, Tounian P** 2001 Mutational analysis of melanocortin-4 receptor, agouti-related protein, and alpha-melanocyte-stimulating hormone genes in severely obese children. J Pediatr 139:204–209
24. **Mergen M, Mergen H, Ozata M, Oner R, Oner C** 2001 A novel melanocortin 4 receptor (MC4R) gene mutation associated with morbid obesity. J Clin Endocrinol Metab 86:3448–3451
25. **Sjöström L, Larsson B, Backman L, Bengtsson C, Bouchard C, Dahlgren S, Hallgren P, Jonsson E, Karlsson J, Lapidus L** 1992 Swedish obese subjects (SOS). Recruitment for an intervention study and a selected description of the obese state. Int J Obes Relat Metab Disord 16:465–479
26. **Lissner L, Sjöström L, Bengtsson C, Bouchard C, Larsson B** 1994 The natural history of obesity in an obese population and associations with metabolic aberrations. Int J Obes Relat Metab Disord 18:441–447
27. **Bouchard C, Leon AS, Rao DC, Skinner JS, Wilmore JH, Gagnon J** 1995 The HERITAGE family study. Aims, design, and measurement protocol. Med Sci Sports Exerc 27:721–729
28. **Kvist H, Sjöström L, Tylen U** 1986 Adipose tissue volume determinations in women by computed tomography: technical considerations. Int J Obes 10:53–67
29. **Li W-H** 1997 Molecular evolution. Sunderland, MA: Sinauer Associates
30. **Gotoda T, Scott J, Aitman TJ** 1997 Molecular screening of the human melanocortin-4 receptor gene: identification of a missense variant showing no association with obesity, plasma glucose, or insulin. Diabetologia 40:976–979
31. **Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K** 1987 A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. Science 238:336–341
32. **Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD** 1994 Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8:1298–1308
33. **Ho G, MacKenzie RG** 1999 Functional characterization of mutations in melanocortin-4 receptor associated with human obesity. J Biol Chem 274:35816–35822
34. **Rosmond R, Chagnon M, Bouchard C, Björntorp P** 2001 A missense mutation in the human melanocortin-4 receptor gene in relation to abdominal obesity and salivary cortisol. Diabetologia 44:1335–1338
35. **Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, Yamada T** 1993 Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 268:15174–15179
36. **Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ, Lander ES** 1999 Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nat Genet 22:231–238
37. **Tajima F** 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595
38. **Miraglia DG, Cirillo G, Nigro V, Santoro N, D'Urso L, Raimondo P, Cozzolino D, Scafato D, Perrone L** 2002 Low frequency of melanocortin-4 receptor (MC4R) mutations in a Mediterranean population with early-onset obesity. Int J Obes Relat Metab Disord 26:647–651