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SEVERAL MUTATIONS IN THE MELANOCORTIN-4 RECEPTOR GENE INCLUDING A NONSENSE AND A FRAMESHIFT MUTATION ASSOCIATED WITH DOMINANTLY INHERITED OBESITY IN HUMANS

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ABSTRACT: The melanocortin-4 receptor gene (*MC4-R*) has been implicated in weight regulation. Recently, two independent groups reported frameshift mutations associated with a dominant form of obesity (1, 2). We screened the coding region of the *MC4-R* in 306 extremely obese children and adolescents (mean body mass index: BMI 34.4 ± 6.6 kg/m^2), 25 healthy underweight students (mean BMI 17.1 ± 0.8 kg/m^2), 52 normal weight individuals (mean BMI 22.0 ± 1.0 kg/m^2), 51 inpatients with anorexia nervosa (AN, DSM IV criteria, mean BMI 14.3 ± 1.5 kg/m^2) and 27 patients with bulimia nervosa (BN, DSM IV criteria, mean BMI 21.7 ± 5.8 kg/m^2) by single strand conformation polymorphism analysis (SSCP). Several mutations were identified, including the frameshift mutation described (1). The mutations were as follows: a) The deletion of 4 bp (Δ of CTCT at codon 211) results in a frameshift, thus rendering a truncated protein. This mutation has been assumed to be associated with dominantly-inherited morbid obesity in humans (1). Both the index patient (BMI 42.06 kg/m^2, height 171 cm, age 19.6 years) and her mother (BMI 37.55 kg/m^2, height 164 cm, age 42.5 years) were heterozygous for the deletion. b) A nonsense mutation at position 35 of the MC4-R was detected in two obese probands (BMI 31.29 kg/m^2 and BMI 45.91 kg/m^2). This mutation leads to a truncated protein that encompasses the N-terminal extracellular domain. Both carriers additionally showed (c) a missense mutation (Asp-37-Val). In both of these cases Tyr-35-Stop and Asp-37-Val were maternally transmitted, thus these variations form a haplotype. d) e) A male obese proband harbored two missense mutations (Ser-30-Phe, Gly-252-Ser). f)-i) Four different missense mutations (Pro-78-Leu, Thr-112-Met, Arg-165-Trp, Ile-317-Thr) were detected in four different male probands, respectively. All of these mutations (a to i) were found solely in extremely obese individuals whose BMIs were all above the 99th percentile. j) A silent mutation (C-579-T, Val-193-Val) was detected in a male underweight individual. k) A previously described polymorphism (Val-103-Ile; 3) was detected with similar frequencies in all different study groups. l) We identified a novel polymorphism (Ile-251-Leu) with similar allele frequencies in all groups under study. In conclusion, our data indicate that mutations in the *MC4-R* are not uncommon. Whereas our data support the evidence for dominantly inherited obesity as revealed by the three obese probands with haplo-insufficiency, the functional significance of the missense mutations remains to be determined.

INTRODUCTION

The MC4-R is a G protein-coupled, seven-transmembrane receptor that is found in every nucleus reported to bind α-MSH in the adult rat brain. It is highly expressed in the hypothalamus, a brain region that is involved in weight regulation (4, 5). Recently, two dominant forms of obesity were described to be conferred by frameshift mutations in the human *MC4-R* leading to haplo-insufficiency (1, 2): i) A 4-bp insertion at nt 732 of the coding sequence leads to a nonfunctional truncated receptor lacking the sixth and seventh transmembrane domains. The mutation co-segregated with severe obesity in the proband's family over three generations (lod score: 1.5; 2). ii) A 4-bp deletion at codon 211 results in a protein truncated from the fifth transmembrane domain (1).

Apart from these findings, several lines of evidence suggest an involvement of this receptor in weight regulation.

1) In mice, inactivation of both copies of the *MC4-R* results in a maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia, hyperglycemia and increased linear growth. A body weight intermediate between these homozygotes and wildtype mice was described for heterozygotes (6).

2) The MC4-R deficiency phenotype recapitulates several of the characteristic features of the obesity in lethal-yellow mice (A^y/a), which is caused by ectopic and constitutive expression of the endogenous antagonist for melanocortin receptors, the agouti protein. Melanocortinergic neurons exert a tonic inhibition of feeding behavior. In A^y/a mice this inhibitory signal is chronically disrupted (7). Ubiquitous expression of human agouti-related protein (ART/AGRP) cDNA in transgenic mice causes obesity without altering pigmentation. Thus, ART/AGRP is a neuropeptide implicated in the normal control of body weight downstream of leptin signaling (8). ART/AGRP is an antagonist of the human MC-3 and MC-4 receptors (9).

3) α-MSH, the natural ligand of the MC4-R, may mediate physiological satiety signals. Thus, impairment in production, processing, or responsiveness to α-MSH may lead to obesity. α-MSH is derived of pro-opiomelanocortin (POMC). In humans, rare recessive mutations in the POMC gene lead to early onset extreme obesity (10). These mutations impair the α-MSH signaling. Several other mutations that do not affect the α-MSH action are seemingly not involved in weight regulation (11).

4) Feeding induced by weakening of the MC4-R-ergic tone may be mediated through activation of the NPY-ergic system. Physiological feeding response evoked by MC4-R blockage is influenced by NPY signaling (12). An increase in daytime food intake in free-feeding animals is caused by a MC4-R active agent (13).

Family and twin studies have implied that genetic factors play a significant role in both the etiology of obesity and AN (14). Psychopathological features and extremely low body

weight are inseparable in AN, so that AN might be considered as an extreme weight condition. Hence, genes involved in weight regulation might be considered as candidate genes for AN.

MATERIAL AND METHODS

Study Subjects: We screened 306 (143 males) extremely obese German children and adolescents (mean BMI 34.4 ± 6.6, mean age 14.3 ± 2.4, for detail see 15), 25 (10 males) healthy underweight students (mean BMI 17.1 ± 0.8 kg/m^2, mean age 25.6 ± 4.6 years, for detail see 15), 52 (16 male) healthy normal weight students (mean BMI 22.0 ± 1.0 kg/m^2, mean age 24.4 ± 2.7 years), 51 inpatients with AN (2 males, DSM IV criteria, mean BMI 14.3 ± 1.5 kg/m^2, mean age 16.1 ± 2.1 years, 74.5 % of the restricting type), 27 female patients with BN (DSM IV criteria, mean BMI 21.7 ± 5.8 kg/m^2, mean age 23.9 ± 4.9 years, all of the purging type) by SSCP for mutations in the *MC4-R*. All individuals were independently ascertained and hence are presumably unrelated. 76.8 % of the obese children and adolescents had an age and gender specific BMI percentile ≥ 100 as previously determined in a representative German population sample (16). The BMI of the underweight students was below the 15th percentile (16).

We screened an additional 113 underweight students (64 males, mean BMI 18.4 ± 1.1 kg/m^2, mean age 25.4 ± 3.9 years) for mutations in the PCR fragment (MC4R-2F/2R) of the *MC4-R* in which both the insertion (2) and deletion (1) are localized.

Written informed consent was given by all participants and in the case of minors, their parents. This study was approved by the Ethics Committee of the University of Marburg.

SSCP and Sequencing: PCR was performed with primers amplifying the 5' region of the *MC4-R*: MC4R-1F 5`ATCAATTCAGGGGGACACTG-3' and MC4R-1R 5` GACAGCACTACTATCTGAGT-3' (615 bp); and primers amplifying the 3' region of the gene: MC4R-2F 5`ATGCTCTCCAGTACCATAACA-3' and MC4R-2R 5`-TGCAGAAGTACAATATTCAGG-3' (622 bp) according to standard protocols. For subsequent sequencing reactions, artificial M13 sequences (AGGGTTTTCCCAGTCACGAC GTT for both F primers, and GAGCGGATAACAATTTCA CACAGG for both R primers) were added at the 5' ends of each primer. Products of MC4R-1F/1R were digested by both *Eco*81I (recognition sequence: CC↓TNAGG; Fermentas, St. Leon Rot, Germany) and *SSP* (recognition sequence: AAT↓ATT, Fermentas, St. Leon Rot, Germany) and products of MC4R-2F/2R were digested by *Msp*I (recognition sequence: C↓CGG Eurogentec, Seraing, Belgium), prior to SSCP. The digested PCR fragments were diluted in formamide containing buffer and electrophoresed on 15 % acrylamide gels (49:1, Pharmacia, Freiburg, Germany) in 0.5 x TBE buffer (45 mM Tris-HCl; 45 mM Borate and 1.1 mM EDTA). Gels were 16 cm in length and run at 4°C for 22 h at 200 V. Gels were silver stained.

Bi-directional sequencing of PCR products, that were extracted from low-melting agarose (NuSieve GTG Agarose, FMC, Rockland, ME, USA) and re-amplified, of all individuals that showed an aberrant SSCP pattern and of two individuals who showed the wild-type SSCP pattern was performed with fluorescently labeled primers (primer sequences complementary to the underlined M13 sequences, F-primers labeled with IRD 700 and R-primers labeled with IRD 800; MWG-Biotech; Ebersberg, Germany). The 'Thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP' (Amersham, Braunschweig, Germany) was used for cycle-sequencing according to the manufacturer. The sequencing reactions were analyzed on a LiCor 4200 automatic sequencer with the Base ImagIR 4.0 software (MWG-Biotech, Ebersberg, Germany).

Statistical Analyses: To test for association of the two polymorphisms (Val-103-Ile; Ile-251-Leu) to different weight extremes or AN or BN, Fisher's exact test was used.

RESULTS

We screened the entire coding region of the *MC4-R* by SSCP. By sequencing of PCR products showing an aberrant SSCP pattern we identified several heterozygous carriers of mutations and polymorphisms (Table 1):

a) A deletion of 4 bp (Δ CTCT at codon 211) results in a frameshift that introduces five aberrant amino acids culminating in a stop codon in the region encoding the fifth transmembrane domain, thus leading to a truncated protein. This mutation is likely to result in a non-functional receptor. This deletion was previously described and is assumed to result in dominantly-inherited morbid obesity in humans (1).

Both the index patient (BMI 42.06 kg/m^2, height 171 cm, age 19.6 years) and her mother (BMI 37.55 kg/m^2, height 164 cm, age 42.5 years) were heterozygous for the deletion.

b) A nonsense mutation at amino acid position 35 of the MC4-R leads to a receptor that is truncated at the N-terminal extracellular domain. It is hardly conceivable that this receptor molecule can function. Both female index patients (BMI 31.29 kg/m^2, height 152 cm, age 10 years and BMI 45.91 kg/m^2, height 165 cm, age 16.6 years) had a BMI above the 100th population based percentile.

c) In addition to this nonsense mutation both probands harbored a Asp-37-Val mutation. Both, Asp-37-Val and the Tyr-35-Stop were inherited from the respective mothers who are also obese (BMI 48.2 kg/m^2, height 171 cm, age 31.2 years and BMI 38.6 kg/m^2, height 165 cm, age 36 years), thus these variations form a haplotype. The Asp-37-Val mutation is downstream of the stop codon, hence it is not translated. The allelic co-occurrence of two mutations in two separate families suggests a common ancestor of both probands. However, as already pointed out the families were ascertained independently.

d) A missense mutation (Ser-30-Phe) in the N-terminal part of the *MC4-R* was detected in an obese male proband (BMI 34.1 kg/m^2, height 180 cm, age 15.4 years).

e) This same proband harbored another missense mutation (Gly-252-Ser) in the sixth transmembrane domain.

f) Within the first intracellular loop of the receptor a missense mutation (Pro-78-Leu) was detected in an obese male proband (BMI 26.5 kg/m^2, height 132 cm, age 9.6 years).

Table 1: Mutations and polymorphisms in the *MC4-R* in extremely obese children and adolescents, healthy normal weight or underweight students and in patients with anorexia nervosa or bulimia nervosa

Study group	Base position+	Effect on amino acid sequence+	Position within the MC4-R#	Frequency of heterozygotes*
Extremely obese children and adolescents (n = 306)	**Δ of CTCT at nt 631-34**	**frameshift truncated protein**	**TM 5**	**0.003**
	C-105-A	**Tyr-35-Stop truncated protein**	**N-ter ED**	**0.007**
	C-89-T	Ser-30-Phe	N-ter ED	0.003
	A-110-T	Asp-37-Val	N-ter ED	0.007
	C-233-T	Pro-78-Leu	IL 1	0.003
	C-335-T	Thr-112-Met	EL 1	0.007
	C-493-T	Arg-165-Trp	IL 2	0.003
	G-754-A	Gly-252-Ser	TM 6	0.003
	T-950-C	Ile-317-Thr	C-ter	0.003
	A-307-G	Val-103-Ile'	TM 2	0.013
	A-751-C	Ile-251-Leu	TM 6	0.013
Healthy normal weight and underweight students (n = 77)	C-579-T	silent	EL 2	0.013
	A-307-G	Val-103-Ile'	TM 2	0.051
	A-751-C	Ile-251-Leu	TM 6	0.039
Patients with anorexia nervosa (n=51)	A-307-G	Val-103-Ile'	TM 2	0.039
	A-751-C	Ile-251-Leu	TM 6	0.020
Patients with bulimia nervosa (n=27)	A-307-G	Val-103-Ile'	TM 2	0.037
	A-751-C	Ile-251-Leu	TM 6	0.000

+ See (1, 2) for numbering of genomic sequences and amino acid positions. # According to (17). ' Nomenclature according to (3). *Genotype-frequencies are not different from Hardy-Weinberg equilibrium. Polymorphisms are shown in shaded boxes. ED: extracellular domain, TM: transmembrane domain, N-ter: N-terminal, IL: intracellular loop, EL: extracellular loop, C-ter: C-terminal

g) Another male obese proband (BMI 30.1 kg/m^2, height 168 cm, age 13.3 years) harbored a missense mutation (Thr-112-Met) within the first extracellular loop of the MC4-R.

h) A missense mutation (Arg-165-Trp) within the second intracellular loop of the receptor was found in an obese male proband (BMI 26.8 kg/m^2, height 158 cm, age 11.7 years).

i) Within the C-terminal part of the MC4-R another missense mutation (Ile-317-Thr) was detected in a male proband (BMI 36.5 kg/m^2, height 184 cm, age 19.6 years).

Mutations a) to i) were found solely in extremely obese individuals whose BMIs were all above the 99th percentile.

j) A silent mutation (C-579-T, Val-193-Val) in the second extracellular loop of the receptor was detected in a male underweight individual (BMI 20.6 kg/m^2, height 180 cm, age 26.7 years).

k) A previously described (3) polymorphism (Val-103-Ile) was detected with similar frequencies in all study groups (all p-values > 0.06). Similar to Gotoda et al. (3) we did not identify a single individual homozygous for the initially independently described 103-isoleucine allele (17, 4). Therefore we adhered to Gotoda's nomenclature (Val-103-Ile) as valine is the more common allele. The heterozygote frequencies in our study groups (1.3 to 5.1 percent) are in the range of the frequencies previously described (3).

l) We identified a novel polymorphism (Ile-251-Leu) with similar allele frequencies in all groups under study (p-values > 0.3).

Additionally to the SSCP screen of the entire coding region of the *MC4-R* we screened the PCR fragment of the *MC4-R* (MC4R-2F/2R) gene in which both the functionally relevant insertion (2) and deletion (1) are localized, in 113 additional underweight students. No mutations within this fragment were detected in this subgroup.

DISCUSSION

Previously, monogenic forms of human obesity have been identified via associated phenotypical abnormalities (10, 18, 19, 20, 21). Here we describe two mutations (Δ of CTCT at codon 211 and Tyr-35-Stop) in the *MC4-R* that are identical or functionally equivalent to the ones previously reported (1, 2). Our results underscore the previous assumption (1, 2) that these haplo-insufficiency mutations underly a dominant form of obesity. We are currently ascertaining additional family members of the three identified index patients with haplo-insufficiency to investigate cosegregation of the respective mutations with the obesity phenotype. The presently identified individuals are not characterized by any readily detectable phenotypical abnormalities other than obesity.

A reliable estimation of an increased body length reported for the knock-out mice (6) and for humans (1) is not yet possible in our index patients with haplo-insufficiency, because they have not reached adult height. The transmitting mothers have heights in the normal range. A statistical comparison with controls will have to be based on more individuals with haplo-insufficiency of the *MC4-R*.

An exact 95 percent confidence interval for the extremely obese individuals screened in our study group to harbor one of the two mutations leading to haplo-insufficiency (deletion/nonsense mutation) is between 0.2 percent and 2.8 percent. Including our study, up to now four groups (1, 2, 3,

this study) have independently screened the *MC4-R* for mutations in the entire coding region in a total of 617 individuals, 452 of whom were extremely obese (n=63, 1; n=43, 2; n=40, 3; n=306 this study). The three different haplo-insufficiency mutations (insertion, deletion and nonsense) were detected in a total of five independently ascertained extremely obese probands. Thus, approximately one percent of individuals with extreme obesity might have a haplo-insufficiency mutation in the *MC4-R*. Presently, it is unknown whether less obese individuals might also have such mutations. Theoretically, carriers of haplo-insufficiency might benefit from MC4-R agonists.

Prior to the aforementioned screens (1, 2, this study) Gotoda et al. (3) identified a Val-103-Ile substitution with similar allele frequencies in obese (BMI > 35 kg/m2) and lean (BMI < 18 kg/m2) white British males. This finding was confirmed in our study. By Southern blot analysis Chagnon et al (22) detected an association between a non-specified *MC4-R* polymorphism and fat mass ($p = 0.002$) and percent body fat ($p = 0.004$) in females. As the molecular basis of the polymorphism has not been determined, the data cannot readily be compared with the other studies.

Functional studies are warranted to analyze the consequences of the detected missense mutations. They were solely found in the obese study group (see Table). Their low frequencies in addition to the limited number of non-obese individuals (n=155) screened for mutations in the whole coding region preclude any prediction of their possible etiological role in obesity.

In conclusion, our data indicate that mutations in the *MC4-R* are not uncommon. Whereas our data support the evidence for dominantly inherited obesity as revealed by the three obese probands with haplo-insufficiency, the functional significance of the identified missense mutations remains to be determined.

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