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Melanocortin-4 Receptor Gene: Case-Control Study and Transmission Disequilibrium Test Confirm that Functionally Relevant Mutations Are Compatible with a Major Gene Effect for Extreme Obesity

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We initially performed a mutation screen of the coding region of the *MC4R* in 808 extremely obese children and adolescents and 327 underweight or normal-weight controls allowing for a case-control study. A total of 16 different missense, nonsense, and frameshift mutations were found in the obese study group; five of these have not been observed previously. *In vitro* assays revealed that nine [the haplotype (Y35X; D37V) was counted as one mutation] of the 16 mutations led to impaired cAMP responses, compared with wild-type receptor constructs. In contrast, only one novel missense mutation was detected in the controls, which did not alter receptor function. The association test based on functionally relevant mutations was positive ($P = 0.006$, Fisher's exact test, one-sided). We proceeded by screening a total of 1040 parents of 520 of the aforementioned obese young index patients to perform transmission disequilibrium tests. The 11 parental carriers of functionally relevant mutations transmitted the mutation in 81.8% ($P = 0.033$; exact one-sided McNemar test). These results support the hypothesis that these *MC4R* mutations represent major gene effects for obesity. (*J Clin Endocrinol Metab* 88: 4258–4267, 2003)

WORLDWIDE, DIFFERENT GROUPS have found frameshift, nonsense, and missense mutations in the melanocortin-4 receptor gene (*MC4R*) (1–15). The frameshift and nonsense mutations are presumed to result in haploinsufficiency of the MC4R; original studies suggested a major gene effect resulting in autosomal dominantly inherited obesity (2, 4, 5). In this context, the term, major gene effect, implies that the respective *MC4R* mutations have a high penetrance and explain a high percentage of the phenotypic variance. *MC4R* mutations have been detected in up to 6% of obese individuals (15).

Currently, five frameshift mutations and one nonsense mutation have been reported (2–5, 9, 14, as based on studies published before March 2003). Their combined frequency amounts to 0.59% in 1853 (extremely) obese individuals in whom the coding region of the *MC4R* was completely screened for mutations. None of these mutations were detected in 826 unrelated normal weight controls, who were also completely screened.

The functional implications of the previously identified 32 missense mutations (2–6, 9–15) are seemingly less clear: Two missense variants (V103I and I251L) occur in approximately 2–3% and 1% of both obese and nonobese subjects from European and North American populations (3, 5, 9, 14), respectively; functional studies revealed no differences between these two variants and the wild-type receptor (3, 6, 16). A third missense variant (T112M) has been shown to occur infrequently in both normal-weight controls and obese individuals (5, 6, 9, 14); compatible with these data, a functional effect of this variant was not detected in an *in vitro* assay system (6). Recently three novel missense variants were detected in normal-weight individuals (I102T, F202L, N240S) (14). Unfortunately, functional studies were not performed for these variants. All of the other missense mutations have been found mainly or exclusively in extremely obese index patients encompassing children, adolescents, and adults. For some of the missense mutations, functional studies (3, 6, 9, 12, 16) have shown that the variants were either only partially active (N62S, S127L, I137T, T150I, R165W, I170V, A244E, I301T, I317T) or showed no activity (P78L, G98R, C271Y, P299H), compared with wild-type receptors. Surprisingly, one mutation that was identified in an extremely obese index patient leads to a constitutively active receptor molecule (L250Q) (3). Recently it was shown (15) that the mutated receptor molecule is partially retained intracellularly.

In family studies, relatives who are carriers of the same functionally relevant *MC4R* mutation as the respective index patient have mostly been shown to be obese (2–5, 8–10, 12,

Abbreviations: BMI, Body mass index; CI, confidence interval; SSCP, single-strand conformation polymorphism; TDT, transmission disequilibrium test; TEAA, triethylammonium acetate.

13), thus substantiating a major gene effect. However, single carriers within these families were not overweight (3, 6, 8–10). Whereas one relative with a nonsense mutation (Y35X) (8) had been obese before a heart attack, Vaisse *et al.* (3) identified three lean family members with a frameshift mutation (47–48insG) ascertained via an extremely obese carrier; the respective mutation leads to a truncation in the N terminus of the protein. In the three lean relatives, obesity had never occurred; no explanation for their leanness was found. Farooqi *et al.* (9) detected homozygous carriers of a missense mutation (N62S) in a consanguineous family. The five homozygous mutation carriers of that family were extremely obese, whereas the heterozygous relatives were nonobese, leading the authors to conclude that this *MC4R* mutation confers a recessively transmitted form of obesity. Kobayashi *et al.* (12) reported a missense mutation (G98R) leading to a loss of function. The extremely obese Japanese girl was homozygous for the mutation; the parents and the sister, all heterozygous for the mutation, were overweight. This mutation is seemingly associated with a codominant form of obesity.

On the basis of these aforementioned findings, it is currently unclear to what extent mutations in *MC4R* indeed influence body weight. In the original two publications (2, 4), based on a single family each, the investigators had concluded that *MC4R* mutations are compatible with dominant inheritance of obesity. Ho and MacKenzie (16), who performed functional studies, presumed that *MC4R* mutations lead to haploinsufficiency of the receptor. However, individuals with large deletions in the chromosomal region (18q) that harbors the *MC4R* are not obese. Thus, obesity might result as a consequence of a dominant-negative effect of *MC4R* mutations (17). Nonsense-mediated decay inhibits the accumulation of nonsense- or frameshift-mutated mRNA and thus minimizes the synthesis of truncated proteins with potential dominant-negative effects. However, it was recently shown that *MC4R* mRNA is not susceptible to nonsense-mediated decay (18). Hence, it remains uncertain whether the effect of the functionally relevant mutations is due to haploinsufficiency or whether the mutated alleles exert a dominant-negative effect. Very recently Lubrano-Berthelier *et al.* (15) showed that approximately 80% of the mutations identified in children and adolescents cause partial or complete intracellular retention of the receptor.

The identification of lean carriers with frameshift mutations (3) suggests that other genetic and/or specific environmental factors are potentially prerequisites for the manifestation of obesity in *MC4R* mutation carriers. Jacobson *et al.* (14) have since questioned the relationship between *MC4R* mutations and severe obesity. *A priori*, obesity, which represents the right end of the body mass index (BMI) distribution, is caused by both environmental and genetic factors. Among the latter, both additive and nonadditive factors contribute to this quantitative phenotype (19). Most likely, monogenic forms of obesity are infrequent; thus, in genetic terms oligogenic or polygenic inheritance of obesity should be much more common.

In the current study, we addressed the question of whether functionally relevant mutations in the *MC4R* (excluding the known polymorphisms) are indeed compatible with an autosomal dominant major gene effect on the phenotype extreme obesity. For this purpose, we performed a mutation screen in 808 extremely obese children and adolescents, 231 underweight (≤15th BMI percentile) and 96 normal-weight (40–60th percentile) adult controls to enable a case-control study. Functional studies were performed to determine whether newly identified variants (especially missense mutations) lead to impaired receptor function or even loss of function of the MC4R. Up to this point, we adhered to the procedure recently proposed for analyses of common diseases (20). To further substantiate our findings, we screened a total of 1040 parents of 520 of the aforementioned 808 extremely obese index patients. If indeed functionally relevant *MC4R* mutations represent major gene effects, we hypothesized that 1) parents should not or only infrequently harbor additional functionally relevant mutations other than those transmitted to the offspring and 2) that parents with relevant mutations are obese.

Study subjects

We screened a total of 808 (456 female) extremely obese German children and adolescents not known to be related (mean BMI 32.5 ± 6.3, mean age 13.9 ± 2.7 yr) 231 healthy underweight students (mean BMI 18.3 ± 1.1 kg/m^2, BMI ≤ 15th percentile; mean age 25.3 ± 3.8 yr) and 96 healthy, normal-weight students (mean BMI 21.9 ± 1.1 kg/m^2, BMI between 40th and 60th percentile, mean age 24.7 ± 2.6 yr). A total of 65.5% of the extremely obese children and adolescents had an age- and gender-specific BMI percentile of 99 or greater as previously determined in a representative German population sample (21). Additionally, the 1040 parents (mothers: mean BMI 30.4 ± 6.7 kg/m^2, mean age 41.5 ± 5.5 yr; fathers: mean BMI 30.4 ± 5.7 kg/m^2, mean age 44.5 ± 6.2 yr) of those 520 obese children and adolescents were screened, for whom both biological parents had been ascertained before the *MC4R* mutation screen of the index patient, thus guaranteeing a nonbiased transmission disequilibrium test (TDT). Because we had excluded trios not compatible with Mendelian inheritance in previous single nucleotide polymorphism and microsatellite analyses, nonpaternity should be a rare event only. Written informed consent was given by all participants and in the case of minors, their parents. The study was conducted in accordance with the guidelines proposed in the Declaration of Helsinki and was approved by the Ethics Committee of the University of Marburg. The current analyses are based on a total of 2175 individuals, who were either newly screened for *MC4R* mutations (n = 1606) or had contributed to previous studies (5, 8).

Phenotypic measurements

Body weights and heights of all probands were measured. To illustrate the BMI distribution of both carriers and noncarriers of different kinds of mutations in relationship to the BMI distribution observed in the general population, we plotted BMIs of the ascertained 808 obese children and adolescents and of 1040 parents of a subgroup of 520 of these index patients into BMI percentile curves, which were calculated using quantile regression for the age span 8–70 yr

based on the large and representative German National Nutrition Survey (21).

Materials and Methods

Molecular genetic method

PCRs were performed with primers amplifying the *MC4-R* in two overlapping parts with the primer pairs MC4R-1 F/MC4R-1R (F1; 615 bp) and MC4R-2 F/MC4R-2R (F2, 622 bp; sequences have been described previously) (5). Subsequently, single-strand conformation polymorphism (SSCP) analyses for mutations in the *MC4-R* were performed at 4 C as described previously (5).

Additionally, all samples that revealed SSCP patterns indistinguishable from those of either one of the two known polymorphisms (V103I, I251L) were reanalyzed with denaturing HPLC by using the WAVE system (Transgenomic, Cheshire, UK). Melt temperatures for optimal separation of homo- and heteroduplexes were deduced from the melting profile of each amplicon's DNA sequence using the WAVEmaker software (version 4.0, Transgenomic). The PCRs were performed according to standard protocols using 12 ng DNA (primers and PCR conditions as described) (5). Five microliters of unpurified denatured-renatured PCR products were loaded onto a preheated specific column (DNASep column, Transgenomic) and eluted from the column by a linear acetonitrile gradient in a 0.1-M triethylammonium acetate (TEAA) buffer, pH 7, and at a constant flow rate of 0.9 ml/min. The gradient was created by mixing eluents A (0.1 M TEAA) and B (0.1 M TEAA, 25% acetonitrile). For analyses of the *MC4R* F1 fragment, PCR products were eluted from the column (61 C) by increasing buffer B from 54–59% within 30 sec and from 59–68% within 4.5 min. The oven temperature for analyzing the *MC4R* F2-fragment was set to 59 C and 60 C, respectively. For elution of the *MC4R* F2 products, buffer B was increased from 54–59% within 10 sec and from 59–68% within 4.5 min. PCR amplicons with more than one peak or differences in peak appearance were sequenced. All PCR amplicons with SSCP or WAVE patterns deviant from the wild-type pattern or the patterns created by the two polymorphisms were sequenced as described previously (5). One presumed I251L polymorphism (by SSCP) turned out to be a missense mutation (G181D). Another individual turned out to harbor a frameshift mutation leading to a deletion of GA at codon 250 in addition to the I251L polymorphism.

PCR-restriction fragment length polymorphism analyses

To reconfirm all identified variants (mutations and polymorphisms), PCRs with subsequent diagnostic restriction fragment length polymorphism analyses were performed. For alleles that did not alter restriction sites, specific primers were designed to either introduce artificial restriction sites or perform allele-specific PCR reactions. The [Y35X, D37V] haplotype alleles were each detected by allele-specific PCR as described previously (8); V103I was detected as described by Gotoda *et al.* (1). All mutations and polymorphisms were reconfirmed by an independent mutation analysis in every carrier of a variant.

Isolation and characterization of genomic DNA encoding the MC4R

The gDNA of the human wild-type and mutant *MC4R* was amplified by PCR using the primer pairs EcoRI-Koz-MC4r (forward primer), 5′-GAA TTC CCC ACC ATG GTG AAC TCC ACC CAC-3′ and BamHI-Stop-MC4r (reverse primer), 5′-GGA TCC TTA ATA TCT GCT AGA CAA GTC-3′. The PCR fragment was inserted into the eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA). PCR products were further characterized by restriction endonuclease digestion and subsequent dideoxy sequencing (Perkin-Elmer, Boston, MA; Sequencer 310, PE Biosystems).

Transient expression of wild-type and mutant MC4Rs and functional assays

COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C in a humidified 5% CO_2 incubator. For transient transfections COS-7 cells were split into 12-well plates (2×10^5 cells/well) and transfected with 1 μg plasmid DNA per well using Fugene reagent (Roche, Mannheim, Germany). For cAMP measurements, cells were prelabeled with [2,8-^{3}H]adenine (2 μCi/well) (Perkin-Elmer). Assays were performed 3 d after transfection. For cAMP assay, cells were washed once in serum-free DMEM, followed by a preincubation with the same medium containing 1 mM 3-isobutyl-1-methylxanthine (Sigma, Deisenhofen, Germany) for 20 min at 37 C in a humidified 5% CO_2 incubator. Subsequently cells were stimulated with appropriate concentrations of α-MSH (Sigma) for 1 h. Reactions were terminated by aspiration of the medium and addition of 1 ml 5% trichloric acid. The cAMP content of the cell extracts was determined by anion-exchange chromatography as described (22): EC_{50} and E_{max} values were obtained from α-MSH concentration-response curves (from 1 nM to 10 μM α-MSH/ml), using the computer program GraphPad Prism (GraphPad Inc., San Diego, CA). Data are indicated as means $\pm$ SEM of three to six independent experiments, each carried out in duplicates.

The wild-type receptor showed E_{max} values approximately 10-fold over wild-type basal; EC_{50} values were approximately 50 nM α-MSH (Table 1). We used the following criteria to define functional groups: (1) loss of function was defined via mean E_{max} values less than 2-fold over wild-type basal and undeterminable EC_{50} values; (2) reduced function was defined via EC_{50} values that were more than 2.5-fold increased, compared with wild-type with E_{max} values similar or slightly reduced (P78L) to the wild-type; (3) like wild-type was defined by E_{max} and EC_{50} values similar to wild-type MC4R. Because the distinction between loss of function and reduced function is arbitrary, we refer to the general term, impaired function, for both groups (1 and 2). Two *MC4R* constructs (S127L and P230L) showed constitutively active receptors, which were characterized by increased basal cAMP levels, compared with the wild-type receptor (see Table 1).

We used previous data to complement our functional classification (see Table 1). When we initiated our functional analyses, two of our detected mutations (T112M and R165W) had been functionally characterized by others (Refs. 3 and 6, respectively). Very recently the functional analyses of some more of the mutations identified in our study were published (15); these data, which are consistent with our own data, were not implemented in Table 1.

The nonsense mutation leading to a stop codon at amino acid position 35 of the *MC4R* is compatible only with a complete loss of function (15). *In vitro* tests for this mutation were regarded as unnecessary. Because the missense mutation at codon 37 (D37V) is always found on a haplotype with the nonsense mutation, we did not perform functional assays pertaining to this mutation.

Statistics, confirmatory analyses

The hypothesis of the study was the association of functionally relevant mutations of the *MC4R* with extreme obesity. Therefore, the difference in the numbers of carriers of mutations leading to functional impairment between 808 obese and 327 nonobese individuals were investigated using a one-sided Fisher's exact test. Subsequently TDTs were conducted in trios based on those 520 of the 808 obese index patients whose parents had also been ascertained. Here we also combined the mutations leading to functional impairment to allow for a one-sided TDT [(23); exact McNemar test] based on a larger number of informative trios. The grouping of the mutations was based on the results of functional assays as recommended by Hirschhorn and Altshuler (20).

Exploratory analyses

Further association tests for the obese and nonobese groups and TDTs were performed for other groupings of the observed mutations. Differences in age, BMI, and SD scores for BMI between carriers of mutations leading to an impaired function *vs.* all other probands were analyzed with two-sample *t* tests.

Odds ratio and genotype relative risk

Further analyses were conducted to estimate the risk to develop extreme obesity because of a mutation leading to impaired function. We estimated the odds ratio and a 95% confidence interval (CI) from the 808 obese cases and 327 nonobese controls. The genotype relative risk and

TABLE 1. Functional characterization of the wild-type melanocortin-4 receptor in comparison to receptors harboring frameshift or missense mutations or polymorphisms as based on cAMP production induced via binding of α-MSH

Study group	Variant	X	Base position	Effect on amino acid sequence	N[a]	N[b]	Basal (fold over wt basal)	E_{max} (fold over wt basal)	EC_{50} (nM α-MSH)	Interpretation
	Wild type						1[a]	9.8 ± 0.9	51 ± 18	
808 Obese children and adolescents[b]	Frameshift, nonsense and missense mutations	9	89C>T	S30F	2	1	1.4 ± 0.5	15.4 ± 3.8	23 ± 14	Like wild type
		6	**[105C>A, 110A>T[b]]**	**[Y35X;** D37V[b]]	**5**	**2**	**NT**	**NT**	**NT**	**Loss-of-function** (15); ND[b]
		10	233C>T	P78L	1	0	0.8 ± 0.3	3.5 ± 1.6	570 ± 182	Reduced function
		12	283G>A	V95I	1	1	1.2 ± 0.8	1.7 ± 0.6	ND	Loss-of-function
		8	335C>T	T112M	1	0	NT	NT	NT	Like wild type (6)
		17	362T>C	I121T	1	1	0.4 ± 0.02	7.7 ± 1	518 ± 308	Reduced function
		13	380C>T	S127L	2	2	2.1 ± 0.3	20.2 ± 3.4	4003 ± 615	Constitutively active
		5	493C>T	R165W	2	1	NT	NT	NT	Reduced function (3)
		26	542G>A	G181D	1	0	0.7 ± 0.1	1.1 ± 0.1	ND	Loss-of-function
		2	**631_634del**	**L211fsX216**	**2**	**2**	**0.5 ± 0.3**	**0.5 ± 0.1**	**ND**	**Loss-of-function** (9)
		16	689C>T	P230L	1	0	3.1 ± 1.6	27 ± 10	107 ± 20	Constitutively active
		15	731C>A	A244E	1	1	0.5 ± 0.1	10 ± 1.5	425 ± 146	Reduced function
		25	**750_751del**	**L250fsX284**	**1**	**1**	**0.4 ± 0.2**	**1.3 ± 0.6**	**ND**	**Loss-of-function**
		3	754G>A	G252S	1	0	1.1 ± 0.6	9.7 ± 2.7	37 ± 20	Like wild type
		11	950T>C	I317T	1	1	1.3 ± 0.4	12 ± 3	57 ± 37	Like wild type
	Polymorphisms		307G>A	V103I	15	10	0.95 ± 0.1	8.9 ± 1.3	29 ± 15	Like wild type
			751A>C	I251L	9	6	NT	NT	NT	Like wild type (3)
1,040 Parents of 520 of the obese children and adolescents[c]	Frameshift and missense mutations	22	282C>G	S94R	0	1	1 ± 0.2	1.2 ± 0.3	ND	Loss-of-function
		24	**960delT**	**Y320fsX354**	**0**	**1**	**1.4 ± 0.4**	**13.3 ± 1.2**	**149 ± 105**	Reduced function
	Polymorphisms		307G>A	V103I	8	27	0.95 ± 0.1	8.9 ± 1.3	29 ± 15	Like wild type
			751A>C	I251L	6	5	NT	NT	NT	Like wild type (3)
327 Controls[d]	Missense mutation		533C>T	T178M	1	0	1.5 ± 0.7	14.1 ± 5.7	90 ± 39	Like wild type
	Polymorphisms		307G>A	V103I	8	0	0.95 ± 0.1	8.9 ± 1.3	29 ± 15	Like wild type
			751A>C	I251L	7	0	NT	NT	NT	Like wild type (3)

Bold, Frameshift and nonsense mutations. *Boxed areas* indicate mutations leading to an impaired function. ND, Not determinable; NT, not tested; X, nomenclature for the different mutations also used in the figures; N[a], total number of carriers of respective mutations in the total study group (n = 808 obese and 327 controls, respectively); N[b], number of carriers of respective mutations, whose parents were coanalyzed (n = 520); wt, wild-type.

[a] Basal value of the wild type, 233 + 87 cpm.

[b] This missense variant forms a haplotype with Y35X, thus not analyzed separately.

[c] Only (novel) mutations not transmitted to obese offspring.

[d] Healthy, normal, and underweight students.

a 95% exact binomial CI were estimated from those trios in which only one of the four parental alleles harbored a mutation. The frequency of the functionally relevant mutations and a 95% exact CI were estimated from the nontransmitted parental alleles in the 520 trios. This estimate is valid under the assumption of random mating and Hardy-Weinberg equilibrium.

Results

Extremely obese children and adolescents

A total of 18 different nonsilent *MC4R* gene variants were detected in 43 of the 808 obese children and adolescents in whom the coding region of the *MC4R* was screened for mutations (Table 1). Based on the underlying mutation on the DNA level, these 18 variants can be classified as frameshift (n = 2), nonsense (n = 1), and missense (n = 15) mutations, respectively. The frameshift and nonsense mutations are expected to interrupt the reading frame in the domains encoding the N terminus (Y35X) and the fifth and sixth transmembrane domains [codon 211 deletion of CTCT (L211fsX216) and codon 250 deletion of GA (L250fsX284)], respectively. Nine of the missense mutations (S30F, D37V, I121T, S127L, R165W, G181D; G252S, A244E, I317T) code for nonconservative amino acid changes; six missense variants (P78L, T112M, P230L, V95I; polymorphisms V103I and I251L) were conservative. Two silent variants were detected (405A>G, 972C>T).

The two most common variants, V103I and I251L, which have previously been identified as polymorphisms without detectable functional implications (3, 6, 15), were observed in 13 (1.61%) and nine (1.11%) of the obese individuals, respectively.

The third most common mutations, Y35X and D37V, were each observed in the same five (0.62%) individuals; these two variants form a haplotype as revealed by parental transmission patterns. The missense mutations S30F, S127L, and R165W and the frameshift mutation L211fsX216 were detected in two individuals each, the remaining 10 variants, one frameshift and nine missense mutations, were observed only once in the study group (Table 1). Three index patients were compound heterozygous for two missense variants each (a: S127L and R165W; b: S30F and G252S; c: G181D and I251L), as revealed by parental transmission patterns. The haplotype S127L and V103I was identified in an index patient and her mother.

The results of the heterologous *in vitro* studies based on the ability of α-MSH to stimulate cAMP production in addition to previous findings in the literature (3, 6) are illustrated in Table 1. Nine [the haplotype (Y35X; D37V) was counted as one mutation] of the 16 different nonsilent mutations showed an impaired function. In the applied assay, the single nonsense, two frameshift (L211fsX216, L250fsX284), and two missense mutations (V95I, G181D) each led to a loss of function. Four missense mutations (P78L, I121T, R165W, A244E) resulted in an altered receptor function, which we crudely classified as reduced in function. MC4R harboring either one of the remaining four missense mutations (S30F, T112M, G252S, I317T) or the polymorphisms I251L and V103I were functionally indistinguishable from the wild-type receptor. The missense mutations S127L and P230L showed elevated basal cAMP levels, indicating a constitutively active receptor.

Adult underweight and normal-weight controls

Among the 231 underweight and 96 normal-weight students, a total of eight carriers of the 103I allele were identified. The second polymorphism (I251L) was detected in seven individuals, one of whom also harbored allele 103I. No frameshift or nonsense mutations were detected among these 327 students. A single conservative missense mutation was found in a normal-weight individual (T178M). The cAMP assay (Table 1) revealed that the T178M missense mutation seemingly does not affect receptor function. Finally, a single silent mutation (579C>T) was detected in an underweight proband.

Association analysis

Association analyses (Table 2) were complicated by the fact that the detected *MC4R* variants were rare and had different functional implications. The analyses were based on our own and previously (3, 6) generated functional data. Because of uncertainties as to their functional implications, the two mutations leading to a constitutively active receptor (S127L and P230L) were excluded from these analyses. When all mutations leading to impaired (loss of or reduced function) function were combined, the association test was significant (one sided $P = 0.006$). *Post hoc* analyses revealed that separate analyses of the frameshift plus nonsense mutations and the missense mutations show trends for association within each group (descriptive, two-sided: $P = 0.114$ and $P = 0.124$, respectively; Table 2).

TDTs

Our subsequent analyses were based on trios recruited via those 520 of the 808 extremely obese index patients whose

TABLE 2. Association tests for carriers of at least one mutation (the polymorphisms V103I and I251L are not included) of the melanocortin-4 receptor gene (percentage of respective study group in *parentheses*) in 808 obese children and adolescents and 327 healthy underweight and normal weight controls

	No. of individuals with at least one functionally relevant mutation[a]	Frameshift and nonsense mutations	Missense mutations
808 Obese children and adolescents	15 (1.86)	8 (0.99)	12 (1.49)
327 Controls	0 (0)	0 (0)	1 (0.31)
P value[b]	0.006[c]	0.114[d]	0.124[d]

Carriers of mutations that form a haplotype ([Y35X; D37V]) and compound heterozygotes (compound 1: S127L and R165W; compound 2: S30F and G252S) were each counted once.

[a] See Table 1.

[b] Fisher's exact test: [c] one-sided; [d] two-sided.

parents had also been ascertained. We also combined the mutations leading to functional impairment to allow for a TDT (23) based on a larger number of informative trios. A total of 11 heterozygous carriers of mutations leading to a functional impairment were detected among the 1040 parents (the two mutations leading to constitutively active receptors were again excluded from the analyses). All parents were completely screened by SSCP; non-Mendelian transmissions were not detected. The confirmatory test revealed nine transmissions and two nontransmissions (P = 0.033 exact McNemar test, one-sided; Table 3). The two nontransmitted mutations (the nonconservative missense mutation S94R and the frameshift mutation Y320fsX354) both lead to an impaired function (Table 1). The results of all *post hoc* TDT analyses are shown in Table 3.

Odds ratio and genotype relative risk

For the combined set of mutations leading to impaired function, we estimated the odds ratio, genotype relative risk, and allele frequency. Because no functionally relevant mutation was observed in the 327 controls and 15 of the 808 obese individuals were carriers of mutations associated with an impaired function, the odds ratio estimate results in $+\infty$, the exact 95% CI ranges from 1.47 to $+\infty$. Based on the nontransmitted parental alleles (which were found to result in an impaired function in the *in vitro* assays), the estimate for the frequency of functionally relevant mutations in the general population is 0.19% (95% CI: 0.02%–0.69%). Based on the observed nine transmissions and two nontransmissions of mutations associated with an impaired function in our 520 trios, we estimated the genotype relative risk of heterozygous carriers of functionally relevant mutations as 4.5; the 95% CI ranges from 0.9 to 42.8.

Percentile plots: extremely obese children and adolescents

Figure 1, A and B, illustrates BMI of those 21 extremely obese young index patients with frameshift, nonsense, and/or missense mutations (excluding the two polymorphisms) in relationship to both the 787 extremely obese nonmutation carriers (including carriers of one or the other polymorphism, in whom no other nonsilent mutation was present) and population-based gender-specific BMI percentiles. Three mutations (Y35X, R165W, S30F) occurred in both genders. Except for two variants (S30F and S127L), all mutations in the females led to a receptor with impaired function (Fig. 1A). BMI of the eight male mutation carriers clustered in the middle to upper range of the BMI distribution formed by all male obese subjects (Fig. 1B); four of the respective mutations were associated with an impaired receptor function. The variants detected in the other four male carriers led to receptors compatible with unaltered MC4R function; one coded for a constitutively active receptor molecule.

The extremely obese patients harboring mutations leading to an impaired function were heavier than noncarriers (mean SD scores = 3.1 ± 0.6 in carriers *vs.* 2.6 ± 0.6 in noncarriers, descriptive P = 0.01, t test, two-sided). The 22 carriers of either one of the two polymorphisms skewed over the whole BMI and age range in both males and females (data not shown).

Percentile plots: parental study group

All 1040 parents of 520 of the total of 808 obese children and adolescents were also screened for *MC4R* mutations (Fig. 2, A and B). Fifteen different nonsilent (including the two polymorphisms) and two different silent (405C>A, 972C>T) variants were detected in these 1040 parents.

Five of the seven mutations found among the mothers lead to an impaired function (Table 1, Fig. 2A) and all seven mutation carriers had BMI in the 85th centile or greater. Among the fathers, two of the nine mutation carriers had a BMI below the 85th percentile, one of these below the 50th percentile. Our *in vitro* assays revealed that the two variants of the normal weight fathers are seemingly functioning like the wild-type receptor. Finally, parents with mutations associated with an impaired function (n = 11) all had BMI 85th BMI percentile or greater.

Discussion

We aimed to further clarify the role of *MC4R* mutations in obesity. We detected association of functionally relevant *MC4R* mutations with extreme obesity by genotyping the largest obese study group as of today and normal-weight controls. Whereas it has been common practice to extend pedigrees only upon detection of a mutation carrier, we systematically screened all 1040 parents of 520 obese children and adolescents irrespective of their *MC4R* genotype. Thereby we were also able to identify parental variants not transmitted to their offspring. The major advantage of this procedure is that we were able to perform a TDT and use a family-based approach to estimate the epidemiological relevance of *MC4R* mutations leading to functional impairment. Both our case-control study and the TDT are compatible with the notion that *MC4R* mutations, which are associated with an impaired function in *in vitro* assays, are compatible

TABLE 3. Transmission disequilibrium tests for melanocortin-4 receptor gene mutations in 520 obese children and adolescents and both of their parents

	All mutations leading to impaired function[a]	All identified frameshift and nonsense mutations	All identified missense mutations
Transmitted	9	5	8
Nontransmitted	2	1	2
Transmission rate	81.8	83.3	80.0
P value[b]	0.033[c]	0.219[d]	0.109[d]

If within a trio both parents harbored a variant/haplotype, all transmissions and nontransmissions were counted. The polymorphisms V103I and I251L were excluded.

[a] See Table 1.

[b] Exact McNemar test: [c] one-sided, [d] two-sided.

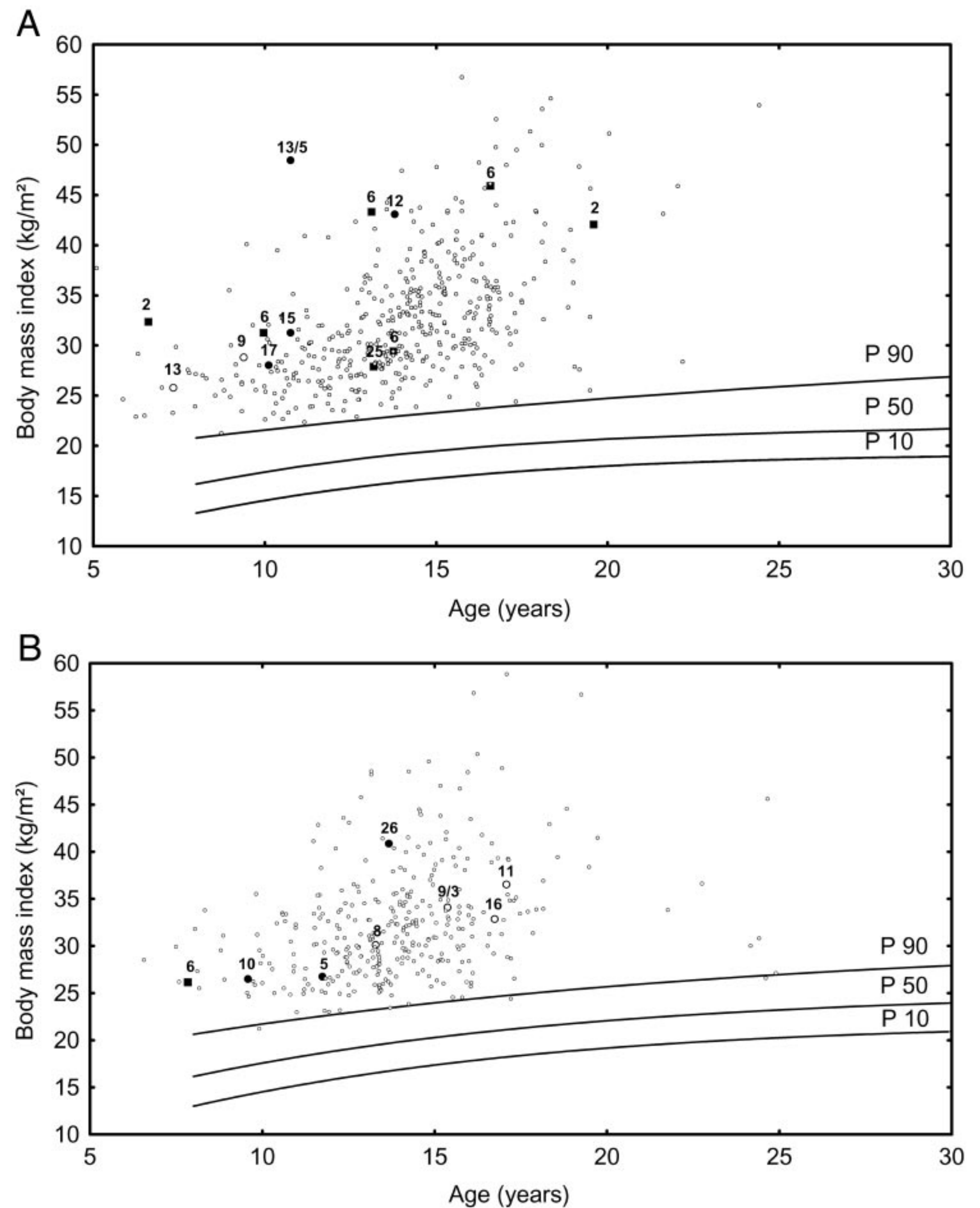

FIG. 1. A and B, BMI percentile curves, age 5–30 yr. To illustrate the BMI distribution of both carriers and noncarriers of different kinds of mutations in relationship to the BMI distribution observed in the general population, BMI of the ascertained 808 obese children and adolescents (A, 456 females; B, 352 males) were plotted into BMI percentile curves (P10, 10th percentile; P50, 50th percentile; P90, 90th percentile), as determined from the German National Nutrition Survey (21). *Black symbols*, carriers of mutations leading to a reduced function or mutations leading to loss of function in *in vitro* assays; *small circles*, wild-type carriers; *large unfilled circles*, carriers of mutations with functional properties not distinguishable from wild type; *squares*, carriers of nonsense and frameshift mutations; *large circles*, carriers of missense mutations. *Numbers* indicate different mutations (see Table 1).

with a major gene effect. Whereas the original reports had already suggested that *MC4R* mutations lead to extreme obesity (2, 4), observations of lean carriers without a known cause for their leanness (3) cast doubt on the original assumption. Very recently the role of *MC4R* mutations in human obesity has even been questioned (14).

To evaluate the functional relevance of the identified *MC4R* mutations, COS-7 cells were transiently transfected with different receptor cDNAs, and functional studies were performed. To assess receptor function at a proximal step of the cellular signaling cascade, we directly measured second-messenger production and determined agonist-induced intracellular cAMP accumulation. Based on these *in vitro* studies and published data (3, 6), we formed two main non-overlapping categories (for criteria see *Materials and Methods*) to classify the mutations: (1) impaired function (loss of function and reduced function) and (2) like wild-type. This categorization excludes the two mutations that turned out to lead to constitutively active receptors (P230L and S127L, Table 1). Vaisse *et al.* (3) also described a constitutively active receptor in an obese individual; the respective receptor proteins are partially retained intracellularly (15). This result is in line with previous data indicating that expression of constitutively active receptors is often reduced (24). Additionally, such receptors have been described to be structurally unstable (25). Hence, we cannot exclude that both S127I and P230L entail an impaired function. Nevertheless, based on the uncertain functional classification of the two identified constitutively active receptor variants, we excluded them from the association and TDT analyses.

We chose the umbrella term, impaired function, for both loss of function and reduced function because our subcategorization is somewhat arbitrary. Our *in vitro* results are compatible with the previous data (9, 15). Because only one

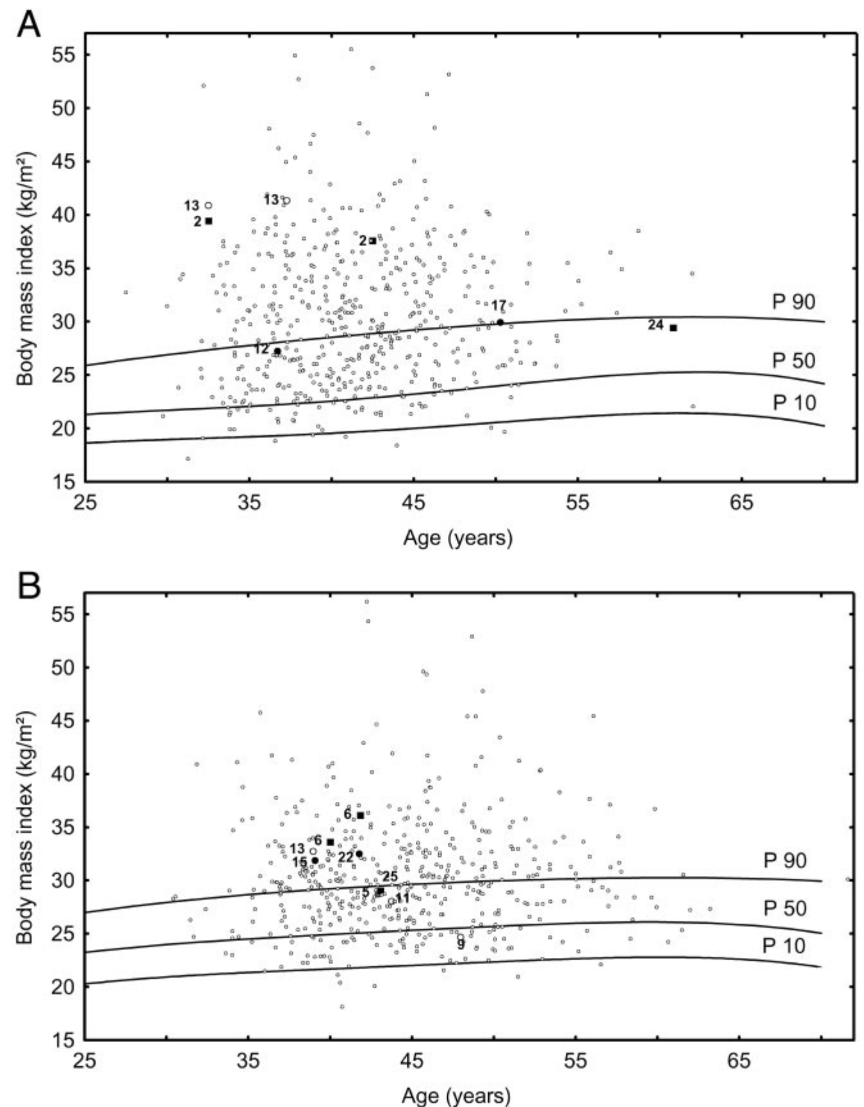

FIG. 2. A and B, BMI percentile curves, age 25–65 yr. To illustrate the BMI distribution of both carriers and noncarriers of different kinds of mutations in relationship to the BMI distribution observed in the general population, BMI of the 1040 parents (A, 520 mothers, and B, 520 fathers) of a subgroup of 520 of the 808 index patients were plotted into BMI percentile curves (P10, 10th percentile; P50, 50th percentile; P90, 90th percentile), as determined from the German National Nutrition Survey (21). *Black symbols*, carriers of mutations leading to a reduced function mutations leading to loss of function in *in vitro* assays; *small circles*, wild-type carriers; *large unfilled circles*, carriers of mutations with functional properties not distinguishable from wild type; *squares*, carriers of nonsense and frameshift mutations; *large circles*, carriers of missense mutations. *Numbers* indicate different mutations (see Table 1).

functional test was performed, we obviously cannot exclude the possibility that single mutations associated with a normal cAMP response could show impaired function in other assays. Indeed, recently Lubrano-Bertehlier *et al.* (15) showed that one of the mutations (I317T), which we had classified as like wild-type, showed impaired cell surface expression. However, because these data are not available for all of our identified mutations, we did not include this information in the biometrical analyses. Furthermore, we did not test binding of MC4R antagonists. Finally, albeit unlikely, functional alterations detected in *in vitro* assays do not necessarily imply that the respective variants are also functionally deviant *in vivo*.

The nine mutations leading to impaired function occurred in 15 of the 808 obese individuals (1.9%; 95% CI 1.0–3.0%). The frequency of mutation carriers among obese study groups ranged from 0.5% to 6.3% in previous reports (2–6, 9–15). All currently known mutations and polymorphisms in the coding region are seemingly randomly distributed; there is no evidence for a mutational hotspot in DNA segments coding for any of the domains of the receptor molecule.

The observation of the same haplotype formed by the nonsense mutation Y35X and the missense mutation D37V in five obese probands suggests a common founder for 0.62% of our study population. Indeed, we have previously shown that two index patients ascertained in two different German states (Bavaria and Hessia) are second-degree cousins (8). Because all other mutations occurred only once or twice, the thrifty genotype hypothesis (26) is not readily substantiated. Only a single female index patient with a BMI of 48.5 kg/m^2, one of the highest in the total study group, was compound heterozygous for two functionally relevant mutations (Fig. 1A), indicating that compound heterozygotes are not detected disproportionately in our sample.

Our confirmatory analyses, which substantiate association between *MC4R* mutations and extreme childhood and adolescent obesity, were based on pooling of functionally relevant mutations, an approach recently recommended by Hirschhorn and Altshuler (20). The significant ratio of 15 carriers of functionally relevant mutations in 808 obese individuals to 0 in 327 nonobese individuals is suggestive of a major gene effect of the respective mutations on the pheno-

type extreme obesity (one-sided Fisher's exact test: $P = 0.006$, lower confidence limit of the odds ratio: 1.47). Obviously, large-scaled epidemiological studies are required to obtain more exact estimates of the genetic effects and the population frequencies of the different *MC4R* mutations. Based on our data, we predict that they will be detectable almost only in the uppermost BMI strata. Indeed, among our index patients, carriers of mutations leading to impaired function had a higher BMI than patients carrying either wild-type alleles or alleles not associated with an impaired function.

Our approach to pool functionally relevant mutations for TDT analyses was also based on the recommendation for association studies (20). The mutations leading to an impaired function (loss of function and reduced function) had a transmission rate of 81.8% ($P = 0.033$, Table 3). All related *post hoc* TDTs (Table 3) also showed that all mutations (nonsense, frameshift, and missense) were preferentially transmitted to the obese offspring. The two nontransmissions need to be interpreted in the light of the frequent occurrence of obesity in both parents of our obese index patients. In our study group approximately 25% of the index patients have two obese (BMI $\geq 30\ kg/m^2$) parents. Assortative mating (27, 28) and common environmental effects as well as the sampling procedure in itself (Dempfle, A., unpublished data) need to be considered as underlying this observation. If both parents are obese, obese offspring can genetically result from a single or more than one gene transmitted from either one or both of the parents.

Estimation of the genotype relative risk for heterozygous carriers of mutations leading to functional impairment indicates a 4.5-fold increased genotype relative risk (95% CI 0.9–42.8) to become obese (BMI $\geq$ 90th centile) for carriers of mutations leading to functional impairment, compared with noncarriers. However, the large 95% CI encompasses 1, and the upper confidence limit indicates that the relative risk might be substantially greater than 4.5. Because of the high percentage of bilinear parental obesity, the genotype relative risk may be underestimated in our study. As a result of the low frequency of functionally relevant mutations in the *MC4R*, these are evidently not relevant for the majority of obese individuals.

In conclusion, we tested for association and transmission disequilibrium of *MC4R* mutations in early-onset extreme obesity. Both approaches critically depend on the joint analyses of the functionally relevant mutations and their correct functional classification (see Ref. 20). Based on the significantly higher frequency of functionally relevant mutations in obese, compared with nonobese, individuals and the subsequent significant TDT, we conclude that *MC4R* mutations are compatible with a major gene effect for the development of obesity. This hypothesis is further supported by the genotype relative risk estimate of 4.5 (95% CI 0.9–42.8). In combination with the small population frequency of mutation carriers, the results indicate that *MC4R* mutations have a low epidemiological but a high individual relevance.

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