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Prevalence, Spectrum, and Functional Characterization of Melanocortin-4 Receptor Gene Mutations in a Representative Population-Based Sample and Obese Adults from Germany

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Context: Autosomal dominant inheritance of mutations in the melanocortin-4 receptor gene (*MC4R*) is currently regarded as the most relevant genetic cause for extreme obesity and affects 2–4% of extremely obese individuals.

Objective: Our objective was to assess the relevance of *MC4R* mutations in a German population-based sample.

Design and Setting: We conducted a mutation screen of the *MC4R* gene by capillary electrophoresis-based single-strand conformation polymorphism analysis and denaturing HPLC.

Participants: Subjects included 4068 individuals of a German population-based study group [Kooperative Gesundheitsforschung im Raum Augsburg, Survey 4 (KORA-S4); *i.e.* Cooperative Health Research in the Region of Augsburg] and 1003 German obese adults (body mass index $\geq$ 30 kg/m^2).

Main Outcome Measures: Samples with aberrant capillary electrophoresis-based single-strand conformation polymorphism analysis/denaturing HPLC patterns were resequenced. Functional studies including agonistic receptor stimulation (Nle-D-Phe-α-, α-, and β-MSH) and cell surface expression assays were performed.

Results: Sixteen (six novel) coding nonsynonymous mutations were detected in 27 heterozygous individuals of KORA-S4. Four of the mutation alleles led to impaired receptor function *in vitro*; however, none of these six heterozygous mutation carriers was obese (body mass index $\geq$ 30 kg/m^2). In the obese adults, six coding nonsynonymous and a nonsense mutation were detected in 13 individuals. Only the nonsense mutation allele entailed impaired receptor function.

Conclusions: Our study depicts prevalence, spectrum, and functional characterization of *MC4R* mutations in the German population-based sample KORA-S4. In this epidemiological study group, individuals heterozygous for nonsynonymous *MC4R* mutation alleles entailing impaired function were not obese. Furthermore, nonsynonymous *MC4R* mutations causing impaired receptor function were rare in German obese adults (two in 1003 = 0.2%). **(*J Clin Endocrinol Metab* 91: 1761–1769, 2006)**

THE MELANOCORTIN-4 RECEPTOR (MC4R) is involved in central regulation of energy homeostasis and body weight (1–3). To date, 72 coding nonsynonymous (n = 57), nonsense (n = 5), and frameshift (n = 10) mutations were detected in 140 of 6134 extremely obese individuals from different study samples (4–8). The combined frequency for all nonsynonymous, nonsense, and frameshift mutations in these extremely obese individuals was 2.28% [95% confidence interval (CI), 1.92–2.69%). *In vitro*, most of the mutation alleles led to partial or total loss of receptor function (reviewed in Ref. 7). In contrast, only six nonsynonymous *MC4R* mutations were found in six heterozygous individuals of 2731 normal-weight controls (0.22%; 95% CI, 0.08–0.48%) (9–11). Two of these mutation alleles entailed a loss of function (12); another two were reported to cause impaired function (13, 14). Therefore, loss of function mutations in the *MC4R* gene do not necessarily lead to obesity (12).

Nonetheless, a significantly increased transmission for mutation alleles leading to impaired MC4R function to obese children and adolescents was found in 520 obesity trios (11). The quantitative effect of these *MC4R* mutation alleles on body weight was determined in a family-based setting (15). Individuals who were heterozygous for *MC4R* mutation alleles differed by almost two body mass index (BMI) SD scores

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Abbreviations: BMI, Body mass index; CE-SSCP, capillary electrophoresis-based single-strand conformation polymorphism analysis; CI, confidence interval; d, denaturing; KORA-S4, Kooperative Gesundheitsforschung im Raum Augsburg, Survey 4, *i.e.* Cooperative Health Research in the Region of Augsburg; MC4R, melanocortin-4 receptor; SDS, SD score; SNP, single-nucleotide polymorphism.

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(SDS) from homozygotes for the respective wild-type allele. The index patients were not included in the statistical analyses to avoid a bias that would have increased the effect size estimation. In females, the allelic effect was nearly twice as strong as that in males (15).

The observation that relatives of extremely obese heterozygous *MC4R* mutation allele carriers were also often obese but homozygous for the wild-type allele complicates the assessment of the effect size of individual alleles (15). Additional genetic and/or environmental factors contributed to obesity in these individuals. There are also reports about single relatives who harbored the same *MC4R* mutation as the index patient but were only moderately overweight or even lean (15–18). In some cases, this was because of an underlying medical condition (16). Farooqi *et al.* (1) found complete penetrance of early-onset obesity in heterozygous *MC4R* mutation carriers. However, only 68% of individuals heterozygous for mutated *MC4R* alleles in families of homozygotes for these mutations displayed early-onset obesity. In general, individuals homozygous for *MC4R* mutations were more obese than heterozygous mutation carriers within these families (1).

The aforementioned quantitative results apply only to relatives of extremely obese heterozygotes for a mutated *MC4R* allele who are living in our current (German) obesogenic environment (15). A completely unbiased estimation of phenotypic effects of mutation alleles on body weight is only possible within a large representative population-based sample.

Hence, we performed a mutation screen in 4068 individuals from a representative population-based sample [Kooperative Gesundheitsforschung im Raum Augsburg, Survey 4 (KORA-S4); *i.e.* Cooperative Health Research in the Region of Augsburg] that had not been ascertained for obesity to allow for the calculation of the combined frequency of *MC4R* mutations in an epidemiological sample for the first time. Functional studies were performed for novel mutations. We hypothesized that heterozygotes for mutations that lead to an impaired receptor function should have an increased BMI-SDS compared with the rest of the study group. In addition, we screened 1003 German obese adults to evaluate *MC4R* mutations.

Subjects and Methods

Study groups

KORA-S4 is an epidemiological study group including 4261 German adult individuals representative of the population within the age range of 25–74 yr in the city and region of Augsburg (Bavaria, Germany); probands were recruited from 1999–2001 (19). Phenotypic data (for males, mean BMI = 27.51 ± 4.04 kg/m^2 and mean age = 49.60 ± 14.06 yr; for females, mean BMI = 26.95 ± 5.29 kg/m^2 and mean age = 48.79 ± 13.83 yr) were available for all individuals. DNA was available for 4068 individuals (2051 females), and all subsequent analyses are based on this number.

The Marburg obese adults represent a study group of 1003 (635 female) German obese adults (for males, mean BMI = 35.14 ± 4.73 kg/m^2 and mean age = 46.45 ± 14.20 yr; for females, mean BMI = 36.55 ± 5.66 kg/m^2 and mean age = 46.12 ± 15.07 yr) ascertained in the city and region of Marburg (Hessia, Germany) by general practitioners. The sole inclusion criterion was a BMI of at least 30 kg/m^2.

Written informed consent was given by all participants. The study was approved by the Bavarian Ethics Committee and the Ethics Committees of the Universities of Munich and Marburg and conducted in accordance with the guidelines of The Declaration of Helsinki.

Phenotypic measurements

KORA-S4. All probands underwent a standardized interview, physical examination, and blood withdrawal by trained staff. For determination of body weight and height, participants were asked to remove shoes and heavy clothing. Weight measurements were done with a calibrated body weight scale (SECA 709) at an accuracy of ±0.1 kg. The height was read to the nearest 0.5 cm. All participants contributing DNA were screened for mutations in the *MC4R*. For a graphical representation of the BMI distribution of individuals heterozygous for mutated *MC4R* alleles, BMI-percentile curves were derived as the least-square estimates of empirical quantiles for the age range of 25–74 yr for both sexes (see Fig. 1).

Marburg obese adults. Local physicians provided data about age, gender, body weight, and height.

Methods

Mutation screen of the *MC4R* in the representative population-based sample (KORA-S4) was performed by semiautomated fluorescent capillary electrophoresis-based single-strand conformation polymorphism analysis (CE-SSCP). The following primers were used to generate overlapping PCR products: 1) GGAGGTTAAATCAATTCAGG and GGTGAATGCAGATTCTTGT (product size, 278 bp), 2) CATCAGCTTGTTGGAGAAT and ACCCGCTTAACTGTCATAA (product size, 330 bp), 3) ATTGCAGTGGACAGGTACT and CAGCAATCCTCTTAATGTGA (product size, 256 bp), 4) CCTCATCACCATGTTCTTC and GAGACATGAAGCACACACA (product size, 260 bp), and 5) CGTCTTTGTTGTCTGCTG and CCTATATTGCGTGCTCTGT (product size, 269 bp). The sensitivity of CE-SSCP was 95% and the specificity 97%; the method was shown to be highly reproducible (20). DNA of probands heterozygous for 11 different known *MC4R* mutations (11) ([Tyr35Stop; 110A→T], Ser30Phe, Pro78Leu, Ile121Thr, Ser127Leu, Arg165Trp, Gly181Asp, Ala244Glu, and Gly252Ser) and for known single-nucleotide polymorphisms (SNPs) (Val103Ile and Ile251Leu) was used to establish and validate the CE-SSCP method. At one specific temperature (25 C), all variants but one (Pro78Leu) were detectable.

All Marburg obese adults were screened for mutations by denaturing (d)HPLC as described previously (11).

Resequencing of all samples with aberrant CE-SSCP or dHPLC patterns (see Tables 1–4) was performed either commercially (SeqLab, Göttingen, Germany) or in house. For the latter, a genomic region of 2697 bp encompassing *MC4R* was PCR amplified (GCACAGATTCGTCTCCCAAT and TGTTACGAAAGCACGCAAAG). The coding sequence of *MC4R* and flanking regions (5′, 339 bp; 3′, 100 bp) were amplified in four nested, overlapping PCRs: 1) GCATGGCAGCTTCAAGGA and GCACCTCCATCAGAGTAGC (product size, 450 bp), 2) GCACACTTCTCTGCACCTC and CCAACCCGCTTAACTGTCAT (product size, 475 bp), 3) TAGCTCCTTGCTTGCATCC and CGGAGTGCATAAATCAGAGG (product size, 526 bp), 4) GGCCAGGCTTCACATTAAGA and ACGGAAGAGAAAGCTGTTGC (product size, 333 bp). PCR products were resequenced using PCR primers and the BigDye Terminator Cycle Sequencing version 2.0 kit (Applied Biosystems, Darmstadt, Germany) on ABI 3700 automated sequencers. Base calling was performed using phred (21). Sequence assembly was done using phrap (http://www.phrap.org/phrap.docs/phrap.html). Trace files were inspected visually in gap4 (22).

Functional classification of nonsynonymous MC4R mutations. When we initiated our functional analyses, we evaluated all published information on *MC4R* mutation alleles and used the data to complement our functional classification scheme (see Tables 1, 3, and 5). Before or in parallel to our functional *in vitro* studies, 14 mutations were characterized (see Tables 1 and 3).

We analyzed nine mutation alleles by the following *in vitro* assays (see Table 5). Respective alleles were amplified from the DNA of the proband and inserted into the expression vector. Mutated and wild-type receptors were transiently transfected into COS-7 cells, and cAMP accumulation assays were performed as described previously (23, 24). To

investigate cell-surface expression, receptor constructs were hemagglutinin tagged at the NH_2 terminus, and cell-surface ELISAs were performed as previously described (24, 25).

The assays described in the literature measured different properties of the receptor (*e.g.* direct or indirect cAMP measurement and different agonists/antagonists). Because the distinction between loss of function and reduced function is arbitrary, we use the general term impaired function for both groups. Accordingly, for the main statistical analyses, they were regarded as equivalent.

Statistical analyses

We hypothesized that *MC4R* mutation alleles entailing impaired function are associated with (extreme) obesity. Consequently, differences in BMI-SDS of probands heterozygous for the respective mutated allele *vs.* 3823 probands without a mutation/polymorphism were analyzed by the independent-sample *t* test. For validation, the data were also analyzed by Mann-Whitney *U* test and permutation tests and for BMI itself (similar results; data not shown). To assess the effect of allele classification problems on statistical analyses, we combined all 27 heterozygous probands for nonsynonymous *MC4R* mutations and compared those with the 3823 controls in a second test. All tests for significance and resulting *P* values were two sided, with a level of nominal significance of 0.05. Descriptive 95% CIs for frequency estimates were obtained by the method of Clopper and Pearson (26).

Results

MC4R mutations

In 4068 German individuals of the representative population-based sample KORA-S4, we identified 1) 16 nonsynonymous mutations (six of which are novel) in 27 individuals and 2) four synonymous mutations in eight individuals (Tables 1 and 2 and Fig. 1); all probands were heterozygous for the respective mutation allele. Two known SNPs (Val103Ile and Ile251Leu) were also detected.

In the Marburg obese adults (BMI $\geq$ 30 kg/m^2), we identified 1) a nonsense mutation ([Tyr35Stop; 110A$\rightarrow$T]) in two individuals, 2) six different nonsynonymous mutations in 11 individuals, and 3) three synonymous mutations each in a single individual (Tables 3 and 4), again all in the heterozygous state. The two known SNPs were also detected.

TABLE 1. Mutations detected in the *MC4R* within 4068 representative population-based samples of the KORA-S4 study group

Type of mutation: classification	Prevalence[a]		No. of heterozygotes (%)	Change on amino acid level	Function
	n	% (95% CI)			
Coding, nonsynonymous: impaired function	6	0.15 (0.05–0.32%)	2 (0.05)	Ser127Leu	Reduced function [constitutively active (11); reduced cAMP response (12, 18, 29)]
			1 (0.02)	Arg165Gln[b]	Reduced function [partial activity (1); reduced cAMP response and cell surface expression (13)]
			2 (0.05)	Gly181Asp	Loss of function as measured in cAMP response (11, 27)
			1 (0.02)	Val253Ile	Partial activity (14)
Coding, nonsynonymous: uncertain impaired function	13	0.32 (0.17–0.55%)	1 (0.02)	Arg7Cys	Reduced function (reduced constitutive activity) (40); like wild type (cAMP response and cell surface expression) (this study)
			1 (0.02)	Arg18Cys	Reduced function (reduced constitutive activity) (40); like wild type (17)
			2 (0.05)	Ser30Phe	Reduced function (29); like wild-type (cAMP response) (11, 41)
			9 (0.22)	Thr112Met	Reduced function [reduced cell surface expression (41%) and ligand binding] (13); like wild type (cAMP response) (12, 18, 42)
Coding, nonsynonymous: like wild type	8	0.20 (0.08–0.39%)	1 (0.02)	Ala70Thr[c]	Like wild type (cAMP response and cell surface expression) (this study)
			1 (0.02)	Thr112Lys[c]	Like wild type (cAMP response and cell surface expression) (this study)
			1 (0.02)	Gln156Arg[c,d]	Like wild-type (cAMP response and cell surface expression) (this study)
			1 (0.02)	His158Arg[c]	Like wild type (cAMP response and cell surface expression, constitutively active) (this study)
			1 (0.02)	Val166Ile[c]	Like wild type (cAMP response and cell surface expression) (this study)
			1 (0.02)	Met200Val	Like wild type (cAMP response and cell surface expression (this study and Ref. 12)
			1 (0.02)	Ile226Thr	Like wild type (cAMP response and cell surface expression) (18)
			1 (0.02)	Arg236His[c]	Like wild type (cAMP response and cell surface expression) (this study)
Coding, synonymous	8	0.20 (0.08–0.39%)	5 (0.12)	Thr5	Not tested
			1 (0.02)	Val67	Not tested
			1 (0.02)	Ile198	Not tested
			1 (0.02)	Ala244	Not tested

[a] According to classification type.
[b] Also heterozygous for the 103Ile allele.
[c] Novel mutation.
[d] Also a homozygous for the 251Ile allele.

TABLE 2. Gender, age, and BMI of heterozygotes for *MC4R* mutations in the KORA-S4 study group

Variation	Age (yr)	BMI	BMI percentile[a]	SDS value[b]
Male				
Arg7Cys[c]	65	28.45	55	0.13
Arg18Cys[c]	68	25.77	24	−0.72
Ser30Phe[c]	40	23.77	21	−0.80
Ser30Phe[c]	64	25.92	21	−0.81
Ala70Thr	45	24.88	29	−0.55
Thr112Met[c]	70	26.31	26	−0.64
Ser127Leu[d]	56	28.34	71	0.55
His158Arg	45	28.44	60	0.24
Arg165Gln[d]	44	26.25	39	−0.28
Gly181Asp[d]	53	27.86	47	−0.07
Gly181Asp[d]	53	23.47	12	−1.16
Met200Val	68	24.30	15	−1.03
Val253Ile[d]	67	27.41	35	−0.39
Female				
Thr112Lys	48	28.48	47	−0.08
Thr112Met[c]	66	19.25	5	−1.63
Thr112Met[c]	44	22.82	23	−0.75
Thr112Met[c]	49	24.25	28	−0.58
Thr112Met[c]	35	22.32	32	−0.48
Thr112Met[c]	47	28.13	53	0.07
Thr112Met[c]	68	32.12	64	0.35
Thr112Met[c]	37	27.14	70	0.53
Thr112Met[c]	53	38.57	95	1.67
Ser127Leu[d]	26	20.74	30	−0.52
Gln156Arg	27	29.00	87	1.11
Val166Ile	29	19.02	12	−1.16
Ile226Thr	55	25.56	37	−0.34
Arg236His	67	28.24	28	−0.57

[a] BMI percentiles were estimated as the fraction of the number of observations with less extreme BMI compared with the case divided by all observations in the age- and gender-matched category.

[b] SDS values were derived from normalized case BMI with normalization based on means and SD of the case's age- and gender-matched category.

[c] Mutation alleles with uncertain impaired function.

[d] Mutation alleles with impaired function.

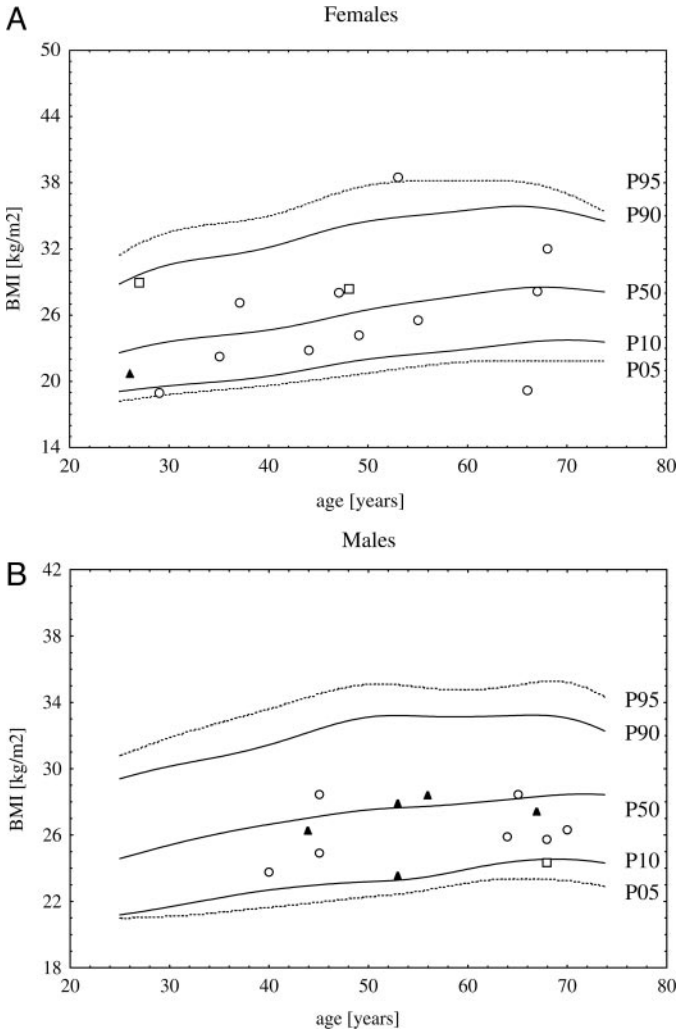

FIG. 1. BMI percentile plots for the representative population-based study group (KORA-S4) indicating heterozygotes for different coding nonsynonymous *MC4R* mutations. The graphics show the BMI distribution of detected heterozygotes for *MC4R* mutations within the KORA-S4 sample (A, females; B, males). Different mutations (▲, impaired function; ○, uncertain impaired function and like wild type; and □, potential gain of function) are depicted. We derived BMI percentile curves as the least-square estimates of empirical quantiles for the age span 25–74 yr for both sexes within the KORA-S4 population. Note that the individual quantiles in Table 2 slightly deviate from those depicted in these figures, which is because of the least square fit. Individual BMI percentiles were derived by calculating the relative proportion of subjects in the age- and gender-matched group who had BMI smaller than the one observed for the individual. Consequently, BMI SDS values were derived by standardization to this group. The 5–95th percentiles are displayed (P95, P90, P50, P10, and P05 refer to the 95th, 90th, 50th, 10th, and 5th percentile, respectively).

Classification of MC4R mutations

To evaluate the functional relevance of the nonsynonymous *MC4R* mutation alleles, we studied receptor signal transduction properties by measurement of agonist-induced intracellular cAMP accumulation. Based on these *in vitro* studies as well as on published data, we defined three main categories to classify *MC4R* mutations: 1) impaired function, 2) uncertain impaired function, and 3) like wild type.

Impaired function (loss of function and reduced function). All available functional data indicate an impaired receptor function. Four of the nonsynonymous mutations identified in KORA-S4 led to an impaired MC4R function (Table 1). In the obese adults from Marburg, the nonsense mutation [Tyr35Stop; 110A→T] entailed a complete loss of function (27).

Uncertain impaired function. Available functional data were ambiguous. Five of the nonsynonymous mutations (Tables 1 and 3) detected in either one or both study groups were previously reported to either lead to functional impairment or to be indistinguishable from the wild-type receptor. In our overexpression system, one of these MC4R constructs (Thr101Ala) was highly expressed on the cell surface. However, maximal stimulation after agonist challenge did not correlate with cell surface expression, indicating altered signal transduction properties (Table 5).

Like wild type. None of the functional tests showed an impaired receptor function. This applied to 11 *MC4R* mutations (Tables 1 and 3). Of these, the His158Arg mutation entailed constitutive receptor activity (Table 5) characterized by increased basal cAMP levels when compared with the wild-type receptor (28). Agonist stimulation resulted in a cAMP response similar to or slightly higher than wild-type MC4R.

TABLE 3. Mutations detected in the *MC4R* within 1003 German obese adults (BMI ≥ 30 kg/m^2)

Type of variation: classification	Prevalence[a]		No. of heterozygotes (%)	Change on amino acid level	Function
	n	% (95% CI)			
Coding, nonsense: impaired function	2	0.20 (0.024–0.72%)	2 (0.20)	[Tyr35Stop; 110A→T]	Loss of function (27)
Coding, nonsynonymous: uncertain impaired function	5	0.50 (0.16–1.16%)	1 (0.10)	Thr101Ala[b]	Reduced function due to increased cell surface expression (this study); like wild type (cAMP response) (this study)
			4 (0.40)	Thr112Met	Reduced function [reduced cell surface expression (41%) and ligand binding] (13); like wild type (cAMP response) (12, 18, 42)
Coding, nonsynonymous: like wild type	6	0.60 (0.22–30%)	1 (0.10)	Val166Ile	Like wild type (cAMP response and cell surface expression) (this study)
			3 (0.30)	Gly252Ser	Like wild type (11)
			1 (0.10)	Pro275Ser	Like wild type, potentially reduced cell surface expression (43)
			1 (0.10)	Ile317Thr	Like wild type (11)
Coding, synonymous	3	0.30 (0.06–0.87%)	1 (0.10)	Val67	Not tested
			1 (0.10)	Gly252	Not tested
			1 (0.10)	Gly324	Not tested

[a] According to classification type.
[b] Novel mutation.

However, we consider His158Arg to be like wild type, because the response to agonist stimulation was not deviant or varied only slightly from the wild-type receptor. We are aware that Vaisse *et al.* (17) reported a constitutively active MC4R receptor, which was subsequently found to be partially retained intracellularly (29). Hence, we cannot exclude that His158Arg entails an impaired function.

Gain of function. Reduced EC_{50} values after agonist stimulation compared with wild type was shown for three mutations: Thr112Lys, Gln156Arg, and Met200Val (Table 5). However, this group was only tentatively assigned and hence included in the like wild-type group for the statistical analyses; the number of tests currently precludes a definitive classification.

TABLE 4. Gender, age, and BMI of heterozygotes for *MC4R* mutations within 1003 German obese adults (BMI ≥ 30 kg/m^2)

Variation	Age (yr)	BMI	SDS value[a]	SDS value[b]
Male				
[Tyr35Stop; 110A→T][c]	42	30.90	1.13	−1.11
[Tyr35Stop; 110A→T][c]	30	38.42	3.60	1.47
Thr101Ala[d]	72	30.00	0.45	−0.94
Thr112Met[d]	51	35.06	1.74	0.14
Ile317Thr	45	38.15	2.41	0.29
Val166Ile	54	37.72	2.33	0.08
Female				
Thr112Met[d]	75	31.22		−0.71
Thr112Met[d]	55	32.47	0.96	−1.11
Thr112Met[d]	50	32.11	0.95	−0.59
Gly252Ser	38	38.95	2.62	0.11
Gly252Ser	43	33.66	1.28	−0.15
Gly252Ser	28	32.79	1.81	−0.73
Pro275Ser	19	31.10		−0.79

[a] SDS values were derived from normalized case BMI with normalization based on the KORA-S4 means and SD of the case's age- and gender-matched category (missing values indicate that these individuals were out of the KORA-S4 age range).
[b] SDS values obtained from the 1003 German obese adults.
[c] Mutation alleles with impaired function.
[d] Mutation alleles with uncertain impaired function.

Prevalence of MC4R mutations

The estimated prevalence of all nonsynonymous/nonsense mutations was 0.66% (95% CI, 0.44–0.96%) in the population-based sample KORA-S4 and 1.30% (95% CI, 0.69–2.21%) in the obese adults from Marburg. Whereas the estimated prevalence of mutations causing impaired function was 0.15% (95% CI, 0.05–0.32%) in KORA-S4 and 0.2% (95% CI, 0.02–0.72%) in the Marburg obese adults (Tables 1 and 3).

Mutations in obese and overweight individuals in the epidemiological sample

Among the participants in KORA-S4, 23.4% were obese (BMI ≥ 30 kg/m^2; n = 950; 499 females); 66.1% were overweight (BMI ≥ 25 kg/m^2; n = 2688; 1203 females). None of the obese probands carried an impaired function *MC4R* mutation allele; two obese females were heterozygous for a mutation allele causing uncertain impaired function (Thr112Met; prevalence among the obese was 0.21%; 95% CI, 0.03–0.76%). Of the 2688 overweight individuals of KORA-S4, four males were heterozygous for mutations with impaired function (Table 2; prevalence, 0.15%; 95% CI, 0.04–0.39%); four males and four females were heterozygous for mutations with uncertain impaired function (Table 2; prevalence, 0.30%; 95% CI, 0.13–0.59%).

Statistical analyses for mutations in the population-based sample

We analyzed initially the six individuals heterozygous for mutation alleles entailing impaired function (Table 1) and compared their BMI-SDS (mean SDS, −0.311; 95% CI, −0.901 to 0.280%) with gender-matched BMI-SDS values of homozy-

TABLE 5. Functional *in vitro* assays pertaining to the previously not functionally analyzed mutation alleles within the *MC4R*

Construct	Basal (basal/basal WT)	cAMP accumulation						Cell surface expression (% of WT MC4R)
		Nle-D-Phe-α-MSH		α-MSH		β-MSH		
		E_{max}/E_{maxWT} (%)	EC_{50} (nM)	E_{max}/E_{maxWT} (%)	EC_{50} (nM)	E_{max}/E_{maxWT} (%)	EC_{50} (nM)	
Wild type	1	100	0.81 ± 0.33	100	5.1 ± 2.4	100	16 ± 8.7	100
Arg7Cys[a]	1.33 ± 0.53	75 ± 21	0.14 ± 0.02	79 ± 17	1.3 ± 0.23	83 ± 18	48 ± 22	100 ± 8.9
Ala70Thr	1.42 ± 0.52	120 ± 27	0.40 ± 0,37	115.4 ± 8.4	1.37 ± 0.59	103 ± 20	12.4 ± 2.1	90 ± 18
Thr101Ala[b]	1.01 ± 0.34	107 ± 49	1.6 ± 0.37	75 ± 32	27.7 ± 16.5	89 ± 12	28.4 ± 16	169 ± 31
Thr112Lys	1.72 ± 0.45	120 ± 5.8	0.15 ± 0.05	97 ± 13	0.74 ± 0.50	112 ± 24	6.4 ± 3.7	76 ± 9.4
Gln156Arg	1.5 ± 0.41	79 ± 17	0.51 ± 0.2	82 ± 25	0.98 ± 0.85	92 ± 20	9.1 ± 7.50	110 ± 12
His158Arg	6.32 ± 0.69	135 ± 22	0.30 ± 0.18	142 ± 23	2.6 ± 1.5	122 ± 15	1.8 ± 1.1	99 ± 8.4
Val166Ile	1.2 ± 0.29	122 ± 8.6	0.31 ± 0.07	87 ± 8.2	3.8 ± 1.1	105 ± 20	29 ± 14	49.9 ± 14
Met200Val[a]	1.1 ± 0.38	78 ± 9.3	0.23 ± 0.02	84 ± 2.7	1.8 ± 0.86	91 ± 27	4.3 ± 3.1	96 ± 1.6
Arg236His	1.4 ± 0.43	105 ± 10	1.5 ± 0.28	97 ± 7.6	3.0 ± 1.2	96 ± 21	18 ± 5.8	104 ± 11

WT, Wild type.
[a] As functional analyses were performed *in vitro*, assays pertaining to these receptor mutations were published (12, 40).
[b] Mutation identified in an obese adult from Marburg.

gotes for the wild-type allele (mean SDS, 0.003; 95% CI, −0.028 to 0.035%). There was no difference between those groups ($P > 0.4$). To control for consistency of this result, we compared all 27 heterozygotes for nonsynonymous mutation alleles (mean SDS, −0.293; 95% CI, −0.578 to −0.008%) with homozygotes for the wild-type allele. Again, there were no differences between the groups ($P > 0.1$).

Heterozygotes for mutation alleles had a slightly reduced BMI-SDS in comparison with homozygotes for the wild-type allele, this nonsignificant effect was opposite to our hypothesis. Explorative comparisons substantiated this effect: 1) 19 heterozygotes for eight mutation alleles that cause either impaired or uncertain impaired function (Table 1; mean SDS, −0.291; 95% CI, −0.646 to 0.064%); and 2) eight heterozygotes for mutation alleles that are functionally like wild type (mean SDS, −0.297; 95% CI, −0.909 to 0.315%) were each compared with 3823 individuals homozygous for the wild-type allele, and all comparisons were negative (global and all pairwise $P > 0.2$). To illustrate gender effects, the BMI distribution of the probands heterozygous for nonsynonymous mutation alleles was graphically displayed for males and females (Fig. 1).

Polymorphisms

Two known nonsynonymous SNPs in the *MC4R* (Val103Ile and Ile251Leu) were each detected with low frequencies in both cases and controls. The allele 103Ile was recently shown to be negatively associated with obesity in the KORA-S4 sample (30, 31). For the 251Leu allele, no allelic effect on body regulation was detected previously: 1) Ile251Leu occurs with a similar frequency (0.5–2%) both in obese and normal-weight subjects in different populations (9–11, 17, 18, 32); 2) there was no transmission disequilibrium in 520 obesity trios (11); and 3) in cAMP assays, 251Leu-MC4R was like wild type (17). Therefore we did not analyze the SNPs further.

Discussion

Autosomal dominantly inherited *MC4R* mutations that lead to an impaired function have consistently been viewed as a risk factor for the development of extreme obesity (2, 3, 7). All those mutations are individually infrequent; the combined frequency for all mutations in extremely obese cases is 10 times higher than in normal-weight controls. Evidence for a strong allelic effect of these mutations on obesity mainly stems from family studies (15).

Here we determined the prevalence and spectrum of *MC4R* mutations in a large German representative population-based sample (KORA-S4) and in a study group of obese adults (Marburg obese adults). Novel mutations were functionally characterized. We hypothesized that individuals heterozygous for mutation alleles leading to an impaired function should have a higher BMI-SDS than probands homozygous for the wild-type allele. We did not identify mutations that led to an impaired function in the 950 obese individuals of the 4068 KORA-S4 probands. Although 23.4% of the individuals were obese, only two heterozygous probands for a nonsynonymous mutation allele with uncertain impaired function were identified (0.21%; 95% CI, 0.03–0.76%). We did, however, detect mutations entailing impaired function in overweight and normal-weight individuals in KORA-S4. In the Marburg obese adults, the frequency of mutations with impaired function was rather low (0.2%).

Age effects and severity of obesity

The prevalence of heterozygotes for mutation alleles leading to impaired function was higher in 808 extremely obese German children and adolescents (1.86%; 95% CI, 1.04–3.04%) whose *MC4R* we screened previously (11) than in 950 obese adults of KORA-S4 (0.00%; 95% CI, 0.00–0.39%; $P = 0.00000804$, explorative Fisher's exact test, two sided). The same holds true for the 1003 obese adults from Marburg (0.2%; 95% CI, 0.02–0.72%; $P = 0.000228$, explorative Fisher's exact test, two-sided). There are several possible explanations for this: 1) the effect size of *MC4R* mutation alleles on the severity of obesity possibly decreases with age (1, 15); 2) the proportion of obesity attributable to *MC4R* mutations decreases because different and additional (partially genetic) causes for obesity apply later in life (33); 3) the current environment might be more obesogenic for carriers of mutation alleles leading to an impaired function; or 4) the severity of

the analyzed phenotype is different because the children are more obese than the screened adults (65.5% of the extremely obese children and adolescents had an age- and gender-specific BMI percentile of 99 or above) (11).

Functional studies

Heterozygotes for *MC4R* mutation alleles leading to an impaired function had BMI-SDS similar to individuals without these mutations in the population-based group KORA-S4. *In vitro* assays of most of the previously described mutations showed that they lead to total or partial loss of function (7). Nonsynonymous mutations had to be grouped for the statistical analyses, because most of them are individually too infrequent to draw meaningful conclusions (34). Three main categories were formed to classify the *MC4R* mutations (see *Results*). We decided to include all mutation alleles leading to an impaired function in one group, although different mutation alleles might exert different quantitative effects *in vivo*. Divergent and sometimes inconsistent results on the degree of functional impairment of specific mutations have previously been described (7). One also has to bear in mind that the functional tests pertained to an artificial homozygous situation, because cotransfection with the wild-type receptor was not performed. *In vivo*, the mutations were detected in the heterozygous state. Dominant negative and haploinsufficiency effects could well lead to a different classification of the severity of functional implications of the different mutations (7). Additionally, most mutations have not yet been fully tested for all experimentally accessible functions, so that a final classification based on more detailed *in vitro* results is not yet possible. Hence, some of the mutations that were functionally characterized as wild type might also lead to an impaired receptor function. In this context, it is noteworthy that variations in genes underlying differences in plasma levels of high-density lipoprotein cholesterol (35) predict functional data better than vice versa. Finally, albeit unlikely, functional alterations detected in *in vitro* assays do not necessarily imply that the respective mutations are also functionally deviant *in vivo*.

For some *MC4R* mutations, even a slight gain of function could be assumed. The Val103Ile SNP is associated with a mean BMI reduction of 0.5 kg/m^2 in heterozygotes (30, 31), implying that in principle variants entailing a gain of function exist in MC4R, although functional assays have not yet been able to substantiate this effect. If indeed other mutations also lead to a substantial gain of function, it would not be too surprising to find these mutations mainly in individuals with a BMI in the normal or even at the bottom of the range.

Impact of ascertainment

Previously, we had shown descriptively a systematic trend within families for a stronger decrease of rates and degree of current and lifetime obesity among homozygotes for the wild-type allele than among heterozygotes for *MC4R* nonsynonymous/nonsense/frameshift mutation alleles with respect to the degree of relationship to the index patient (15). Because this decline is considerably less pronounced among relatives that are themselves heterozygotes, the relevance of *MC4R* mutations for obesity was underscored. However, the analysis also revealed that there were presumably large variations in allelic effect sizes of single mutations. It turned out that in some families, heterozygotes for the mutated alleles had even lower BMI-SDS than homozygotes for the wild-type allele, again underscoring the influence of other factors on body weight regulation (15).

In an attempt to understand why the BMI-SDS of heterozygotes for mutation alleles leading to impaired function in the population-based sample was not increased in comparison with homozygotes for the wild-type allele, we compared our results with other genetic epidemiological analyses. An example for the effect of ascertainment on parameter estimates was previously shown for the penetrance estimation of the breast cancer 1 gene (*BRCA1*) on breast cancer (36); in studies of high-risk families (at least four affected individuals), the highest penetrance estimates were obtained (37), and lower estimates were derived from studies based on cases ascertained independent of family history (38). In accordance with our results, the lowest estimates were obtained in a population-based study (39).

Methodological considerations

If, although unlikely, one or more specific mutations escaped detection, this might affect our results. However, the frequency of 0.66% heterozygotes of nonsynonymous mutation alleles in the *MC4R* was not dramatically distinct from frequency estimates previously reported for normal-weight individuals (0.22%; 95% CI, 0.08–0.48% (9–11, 18). Even if some mutations were missed, it seems improbable this would have occurred solely in obese individuals. Hence, the main message of the current analysis would remain unaltered.

Conclusion

Our results do not indicate that adults from a population-based sample who are heterozygous for *MC4R* nonsynonymous mutation alleles leading to an impaired function have increased BMI-SDS compared with homozygotes for the wild-type allele. Because long-term information on the weight history of the screened individuals is not available, it cannot be excluded that mutation carriers were previously (severely) obese.

Furthermore, we found a lower prevalence of *MC4R* mutations with impaired function in study groups of obese adults compared with our and other previous screens in extremely obese children and adolescents. This might imply an age-dependent prevalence effect.

Otherwise it might indicate that we will be able to detect such mutations only in groups of more extreme phenotypes. Even though the population-based sample is very large, it must be kept in mind that six heterozygotes of mutation alleles leading to an impaired function are not enough to exclude moderate allele effects for obesity with certainty. We demonstrated that the presence of *MC4R* mutations does not automatically lead to an increase in BMI-SDS.

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