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Report

Melanocortin-4 Receptor Gene Variant I103 Is Negatively Associated with Obesity

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Several rare mutations in the melanocortin-4 receptor gene (*MC4R*) predispose to obesity. For the most common missense variant V103I (rs2229616), however, the previously reported similar carrier frequencies in obese and nonobese individuals are in line with in vitro studies, which have not shown a functional implication of this variant. In the present study, we initially performed a transmission/disequilibrium test on 520 trios with obesity, and we observed a lower transmission rate of the I103 allele ($P = .017$), which was an unexpected finding. Therefore, we initiated two large case-control studies ($N = 2{,}334$ and $N = 661$) and combined the data with those from 12 published studies, for a total of 7,713 individuals. The resulting meta-analysis provides evidence for a negative association of the I103 allele with obesity (odds ratio 0.69; 95% confidence interval 0.50–0.96; $P = .03$), mainly comprising samples of European origin. Additional screening of four other ethnic groups showed comparable I103 carrier frequencies well below 10%. Genomic sequencing of the *MC4R* gene revealed three polymorphisms in the noncoding region that displayed strong linkage disequilibrium with V103I. In our functional in vitro assays, the variant was indistinguishable from the wild-type allele, as was the result in previous studies. This report on an SNP/haplotype that is negatively associated with obesity expands the successful application of meta-analysis of modest effects in common diseases to a variant with a carrier frequency well below 10%. The respective protective effect against obesity implies that variation in the *MC4R* gene entails both loss and gain of function.

Mutation screens of the melanocortin-4-receptor gene (*MC4R* [MIM 155541]) have been performed by several groups on the basis of samples of obese subjects and lean control individuals (Gotoda et al. 1997; Gu et al. 1999; Ohshiro et al. 1999; Farooqi et al. 2000, 2003; Vaisse et al. 2000; Dubern et al. 2001; Jacobson et al. 2002; Branson et al. 2003; Marti et al. 2003; Hinney et al. 2003). The respective probands are mostly of European origin. The *MC4R* gene is part of the melanocortinergic pathway that controls energy homeostasis. Functional data indicate that many of the missense, nonsense and frameshift mutations reported elsewhere result in either a reduced function or a total loss of function (Yeo et al. 2003). Furthermore, transmission disequilibrium between obesity (MIM 601665) and functionally relevant *MC4R* mutations has recently been demonstrated in 520 trios (Hinney et al. 2003).

The V103I polymorphism, first described in 1997 (Gotoda et al. 1997), is the most common *MC4R* variant, with allele frequencies >1% in almost all studied populations. Pharmacological studies have not detected a functional difference between the receptor comprising an isoleucine at position 103 and the wild-type receptor (Gu et al. 1999; Vaisse et al. 2000; Hinney et al. 2003). Systematic differences in frequencies of the I103 allele

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Table 1

Description of the Study Groups Considered in the Meta-Analysis (Ordered by Geographical Regions)

Study Group/Investigators	Description of Group		No. (%) of Individuals with I103		
	Obese	Nonobese	Obese	Nonobese	OR (95% CI)
Augsburg	German probands from the KORA study (BMI >90th percentile)	German probands from the KORA study (BMI ≤50th percentile)	9/383 (2.3%)	78/1,951 (4.0%)	.58 (.29–1.16)
Essen	Obese German probands (BMI ≥30 kg/m^2)	Nonobese German probands (BMI <25kg/m^2)	16/489 (3.3%)	8/172 (4.7%)	.69 (.29–1.65)
Hinney et al. 2003	German children and adolescents (BMI ≥90th percentile)	Normal-weight and underweight German students	15/808 (1.9%)	9/327 (2.8%)	.67 (.29–1.54)
Branson et al. 2003	Severely obese Swiss probands (BMI >35 kg/m^2)	Normal-weight Swiss probands (BMI ≤25 kg/m^2)	11/469 (2.3%)	1/25 (4.0%)	.58 (.07–4.65)
Vaisse et al. 2000	Morbidly obese French probands (BMI >40 kg/m^2)	Nonobese French probands from two studies	8/209 (3.8%)	11/366 (3.0%)	1.28 (.51–3.25)
Dubern et al. 2001	Severely obese French children (SDS_{BMI} >3)	Never-obese normal-weight French adults	1/63 (1.6%)	8/283 (2.8%)	.55 (.07–6.49)
Gotoda et al. 1997	British white males (BMI >28 kg/m^2)	British white males (BMI <22 kg/m^2)	8/190 (4.2%)	6/132 (4.5%)	.92 (.31–2.73)
Farooqi et al. 2000, 2003	U.K. probands with early-onset obesity	Healthy nonobese U.K. whites	10/500 (2.0%)	1/54 (1.9%)	1.08 (.14–8.62)
Jacobson et al. 2002[a]	Females from the SOS project (Sweden) (BMI ≥30 kg/m^2)	Females from the SOS project (Sweden) (BMI <30 kg/m^2)	5/144 (3.5%)	6/140 (4.3%)	.80 (.24–2.69)
Rosmond et al. 2001[b]	Males from a Swedish 1944-birth cohort (BMI ≥30 kg/m^2)	Males from a Swedish 1944-birth cohort (BMI <30 kg/m^2)	0/38 (.0%)	13/230 (5.7%)	…[c]
Marti et al. 2003	Obese Spanish probands (BMI >30 kg/m^2)	Normal-weight Spanish probands (BMI <25 kg/m^2)	1/159 (.6%)	0/154 (.0%)	…[c]
Gu et al. 1999	European Americans (BMI ≥ 30 kg/m^2)	European Americans (BMI <30 kg/m^2)	1/82 (1.2%)	2/50 (4.0%)	.30 (.03–3.36)
Jacobson et al. 2002	Black females from two studies (United States) (BMI ≥ 30 kg/m^2)	Black females from the HERITAGE study (United States) (BMI <30 kg/m^2)	0/47 (0.0%)	2/48 (4.2%)	…[c]
Ohshiro et al. 1999	Extremely obese Japanese probands (past max-BMI ≥35 kg/m^2)	Healthy nonobese Japanese probands (past max-BMI ≤20 kg/m^2)	1/50 (2.0%)	4/150 (2.7%)	.74 (.08–6.82)
Meta-analysis	Obese individuals from all studies (N = 3,631)	Nonobese individuals from all studies (N = 4,082)	2.1%[d]	3.5%[d]	.69 (.50–.96)

[a] Carrier frequencies of the unrelated individuals, received by personal communication.
[b] Carrier frequencies, according to different BMI categories, received by personal communication.
[c] For study groups with 0 carriers in one group, no OR could be calculated.
[d] Mean of the single-study percentages.

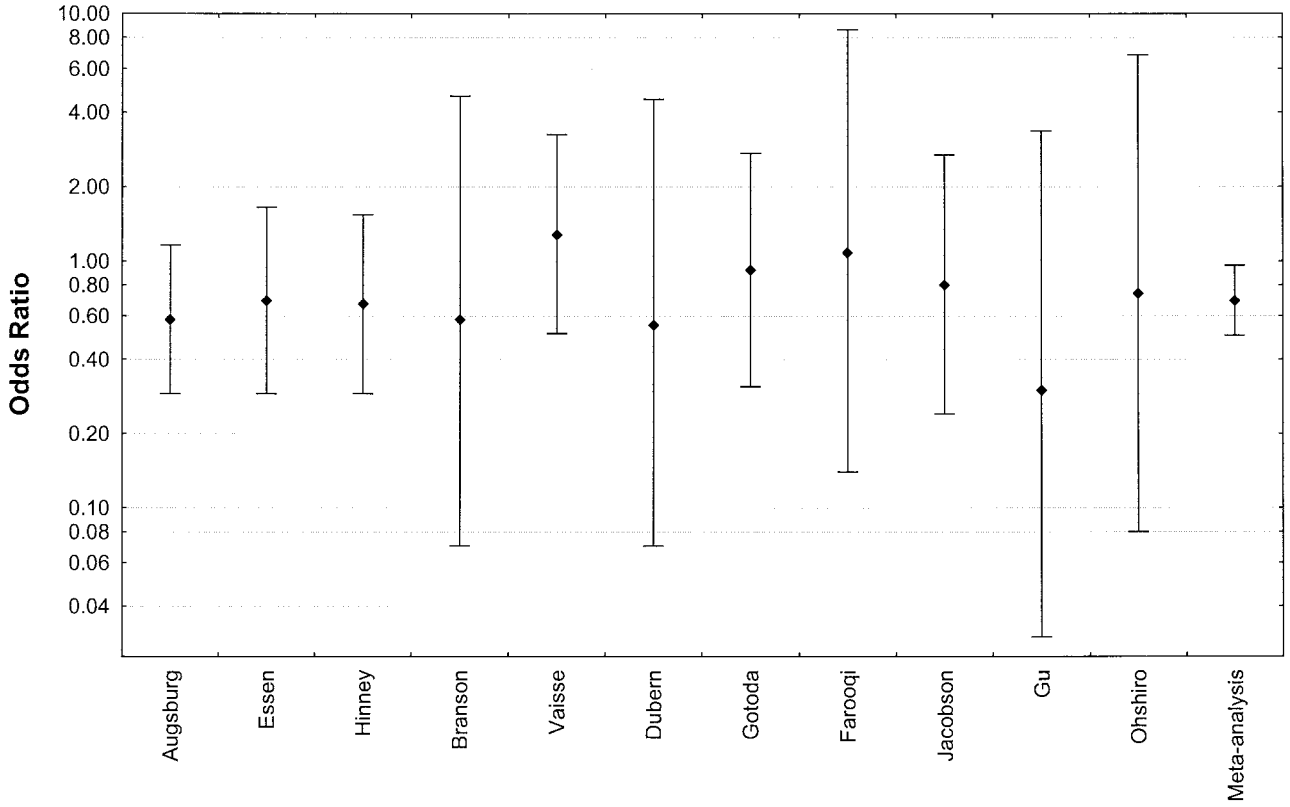

Figure 1 ORs of the single studies and the meta-analysis (logarithmic scale)

between obese subjects and lean control individuals have not been observed. Only one study (Rosmond et al. 2001) has explicitly reported that individuals with the I103 allele have lower body mass indices (BMIs) (24.1 kg/m^2 vs. 26.3kg/m^2; $P = .054$), lower waist-to-hip ratios (0.89 vs. 0.94; $P = .023$), and lower abdominal sagittal diameter (21.0 vs. 22.7; $P = .095$).

Our current study is based on the unexpected observation of a reduced transmission of the I103 allele in 520 obese trios: we detected 35 heterozygous parents, only 10 of whom transmitted the I103 allele to their offspring ($P = .017$), suggesting that this allele is protective against obesity. For heterozygous carriers, we estimated the respective genotype relative risk of developing obesity as 0.40 (exact 95% CI 0.17–0.86). The respective population attributable risk was −0.03—which means that the prevalence of obesity would increase by 3% if no I103 carriers were in the population—which is rather high for the observed carrier frequency, reflecting the relatively large effect estimate of this variant.

On the basis of these results, we screened two large German study groups ascertained in Augsburg, in southern Germany, and Essen, in the Ruhr area, for the V103I polymorphism, and we combined all available information on obese and nonobese individuals in a meta-analysis. The two studies showed consistent results for the I103 allele, with odds ratios (ORs) of 0.58 (95% CI 0.29–1.16) and 0.69 (95% CI 0.29–1.65), respectively (table 1); the moderate effect and the imbalance in the number of obese and nonobese individuals resulted in P values >.05 in these quite large studies. For the meta-analysis, we combined these data with all previously published data on V103I status in obese and nonobese individuals of the same respective ethnicity (table 1). Whereas 11 of the 14 studies display a higher percentage of I103 carriers in the nonobese group, none of the 95% CIs for the OR excludes 1. The meta-analysis results in an OR of 0.69 (95% CI 0.50–0.96) with a P value of .03, thus providing evidence for a negative association between the I103 allele and obesity. Figure 1 shows the large CIs of the ORs for every single study and the small CI for the meta-analysis. The consistency of these results is underlined by the fact that all CIs overlap and contain the OR estimate of the meta-analysis. Additionally, we performed the Breslow-Day test (Breslow and Day 1980) for homogeneity of the ORs, where the null hypothesis of homogeneity was not rejected ($P = .88$). We also analyzed the subgroup of whites, who account for 12 of the 14 study groups and an even larger proportion of subjects; the results were very similar (OR 0.71; 95% CI 0.50–0.99).

A drawback of our study is the use of different criteria to define obese and nonobese individuals in the different studies (table 1). Some studies (e.g., Gotoda et al. [1997], Jacobson et al. [2002], and Marti et al. [2003]) were based on rather low BMI thresholds for obesity, whereas others (e.g., Vaisse et al. [2000] and Dubern et al. [2001]) included only morbidly obese patients. Nevertheless, most of the studies with weaker inclusion criteria mainly pertain to rather severely obese probands, as indicated by mean BMI well over 35 kg/m^2 (Jacobson et al. 2002; Marti et al. 2003); similar differences apply to the definitions of nonobese individuals. Another limitation of this meta-analysis is that age and sex effects cannot readily be addressed, because the respective phenotypic information was not provided for V103I status in all studies. In our large Augsburg sample, the distribution of I103 carriers does not differ considerably by sex or age (table 2). We do not perceive that publication bias, a usual concern in meta-analysis, has an impact on our analysis, because almost all of the included studies analyzed and reported all variants in the *MC4R* gene and did not specifically investigate the association between the V103I polymorphism and obesity.

Given the population-based sample from Augsburg, we were able to estimate the effect of the I103 allele, in terms of BMI. In the total sample of 3,872 individuals, 139 (67 females) were I103 carriers (mean BMI 26.71 ± 4.08 kg/m^2; mean age 48.48 ± 14.04 years), including one homozygous male (BMI 23.89 kg/m^2; age 33 years); 3,733 (1,886 females) individuals were homozygous for V103 (mean BMI 27.23 ± 4.73 kg/m^2; mean age 49.14 ± 13.88 years). Sex- and age-adjusted regression analysis resulted in an effect estimate of −0.48 kg/m^2 for I103 carrier status ($P = .22$), which is equivalent to a reduction of 1.6 kg in a 1.8-m–tall individual. BMI is a complex trait influenced by many genes and environmental factors; we assume that, similar to the predictive testing for venous thrombosis (Yang et al. 2003), knowledge of such polygenes will enable prediction of BMI.

Because of the few studies based on nonwhite populations, we determined the carrier frequencies for I103 in Tanzanians (5.2%; 95% CI 1.7%–11.7%), African Americans (6.3%; 95% CI 2.3%–13.1%), Chinese (6.5%; 95% CI 2.4%–13.7%), and Japanese (0.0%, 95% CI 0.0%–3.8%). Since I103 carriers in the Japanese population have been reported elsewhere (Ohshiro et al. 1999), the results indicate that carriers are present at a frequency well below 10% in all these populations. Sequence comparison of human MC4R to its rodent, pig, and cattle homologues reveals that, in those species, MC4R encompasses a valine at position 103 (Alvaro et al. 1996; Kim et al. 2000; Dumont et al. 2001; Haegeman et al. 2001).

To address functional implications of the observed association, two approaches were taken:

1. To detect other potentially functionally relevant SNPs that are in linkage disequilibrium with the V103I polymorphism, we screened the *MC4R* coding region, as well as 1,676 bp upstream and 1,330 bp downstream, by use of genomic sequencing in 10 individuals who were heterozygous and 2 individuals who were homozygous for either the I103 or the wild-type allele. Six SNPs (five novel) were identified, three of which (SNP001745710, SNP001745711, and SNP002901655) were completely genotyped in 501 trios. Transmission/disequilibrium test (TDT) analysis revealed that none of these SNPs showed substantially stronger transmission disequilibrium than did V103I (table 3). I103 always formed a haplotype with their less-frequent alleles. When we consider the results for this haplotype, we can almost rule out that the observed transmission disequilibrium was caused by undetected heterozygous offspring in the trios, which can result in an overtransmission of the common allele

Table 2

BMI Percentiles P50 and P90 and I103 Carrier Frequencies for the KORA Study Group from Augsburg, Germany

	Finding in Females				Finding in Males			
Age category (years)	P50 (kg/m^2)	P90 (kg/m^2)	Proportion (%) of Probands with I103 and BMI ≤P50	Proportion (%) of Probands with I103 and BMI >P90	P50 (kg/m^2)	P90 (kg/m^2)	Proportion (%) of Probands with I103 and BMI ≤P50	Proportion (%) of Probands with I103 and BMI >P90
25–29	22.6	30.0	4/79 (5.1%)	0/17 (.0%)	24.8	29.3	1/70 (1.4%)	0/14 (.0%)
30–34	24.0	31.8	1/114 (.9%)	0/22 (.0%)	25.5	30.4	6/118 (5.1%)	3/24 (12.5%)
35–39	24.4	31.3	8/113 (7.1%)	1/23 (4.4%)	26.6	31.0	5/102 (4.9%)	0/21 (.0%)
40–44	24.2	32.3	3/97 (3.1%)	1/20 (5.0%)	26.6	31.4	4/92 (4.4%)	0/17 (.0%)
45–49	26.1	34.0	2/106 (1.9%)	0/22 (.0%)	27.2	32.9	5/100 (5.0%)	0/20 (.0%)
50–54	26.7	34.6	4/101 (4.0%)	0/17 (.0%)	27.9	34.3	2/104 (1.9%)	2/21 (9.5%)
55–59	27.4	35.5	7/92 (7.6%)	0/15 (.0%)	27.2	32.4	1/107 (.9%)	0/20 (.0%)
60–64	28.1	34.8	6/107 (5.6%)	1/22 (4.6%)	28.0	33.5	3/103 (2.9%)	0/18 (.0%)
65–69	29.5	36.2	1/99 (1.0%)	0/19 (.0%)	28.1	34.4	8/89 (9.0%)	0/19 (.0%)
70–74	28.6	34.3	3/72 (4.2%)	0/14 (.0%)	28.6	33.0	4/86 (4.7%)	1/18 (5.6%)

Table 3

TDT Analysis in 501 Trios

Marker	No. of Probands with Allele		
	Transmitted	Not Transmitted	*P*
V103I: A allele	9	24	.014
SNP001745710: T allele	9	25	.009
SNP001745711: T allele	34	43	.362
SNP002901655: G allele	9	24	.014
Haplotype V103I-001745710-001745711-002901655:			
G-C-C-C	43	33	.302
G-C-T-C	24	18	.441
G-T-C-C	0	1	1
A-T-T-G	9	24	.014

(Mitchell et al. 2003). The functional role of the detected SNPs remains unclear. Each of the four SNPs, further SNPs outside of the resequenced segment, or combinations thereof could underlie the observed association. None of the SNPs located upstream of the *MC4R* coding sequence resides in the essential promoter region defined in mice (Dumont et al. 2001). However, a basal promoter element within position −430 to −1,600 from the translation start of *MC4R* has been reported (Lamar and Kesterson 2002); all upstream SNPs are located within this region.

2. We performed functional studies. For functional characterization, COS-7 cells were transiently transfected with MC4R-V103 and I103 in two independent labs. Intracellular cAMP accumulation was determined after stimulation with α-MSH in both labs and additionally with NDP-α-MSH and β-MSH in one lab. Basal cAMP values were found to be indistinguishable in cells expressing either the wild-type allele or I103. Stimulation of V103 and I103 with all three agonists did not result in consistent differences in maximal stimulation and EC50 values. Furthermore, binding properties of both variants are indistinguishable. In conclusion, we found no consistent differences in the in vitro test systems; our findings are in line with previous studies (Gu et al. 1999; Vaisse et al. 2000). However, these results obtained in transient overexpression systems do not rule out quantitatively minor differences in the employed systems, differences in receptor expression, and signaling properties in other test systems and/or under physiological conditions.

In this study, we have shown that an *MC4R* polymorphism is negatively associated with obesity. We initially detected a significantly reduced transmission rate of I103 in 520 obese trios. Confirming this effect was possible only by performing two new association studies and incorporating them in a meta-analysis of all published studies, which resulted in an OR of 0.69 (95% CI 0.50–0.96; $P = .03$). It should be noted that none of the individual studies had rendered a P value $\leq .05$. With assumed carrier frequencies of 2% in obese and 3% in nonobese individuals and a resulting moderate effect of OR = 0.66, the mean power of the individual studies was only 10% (minimum 4%; maximum 20%). On the other hand, an unstratified analysis with the number of case and control individuals included in our meta-analysis results in a power of 80%.

On the basis of the total loss of function or the reduced function detected for those *MC4R* mutations involved in obesity, it seems conceivable that the relevant SNP/haplotype causes a gain of function of MC4R. Thus, variants in this gene would cover a functional spectrum ranging from loss to moderate gain of function. Single loss-of-function mutations often result in BMIs >40 kg/m^2 and are mainly reported in only one or two pedigrees worldwide. On the other hand, the effect of the more common variant I103 is rather low; clearly, carriers do not present with an extremely lean phenotype.

To our knowledge, only three large-scale meta-analyses for obesity have been performed to date; the minor allele frequencies of the investigated SNPs were all >15%. The first meta-analysis pertaining to the Trp64Arg polymorphism in the β_3 adrenergic receptor (Allison et al. 1998) was not significant (BMI difference between Trp/Trp homozygotes and Trp/Arg heterozygotes: 0.19 kg/m^2; $P = .07$), whereas a later analysis (Fujisawa et al. 1998) that included more individuals showed a significant BMI difference, between carriers and noncarriers of the Arg allele, of 0.30 kg/m^2 (95% CI 0.13–0.47 kg/m^2). For leptin receptor (*LEPR*) polymorphisms (Heo et al. 2002), meta-analysis revealed no association. There is an ongoing debate as to whether common (allele frequency >1%) or rare variants are more likely to be involved in complex disorders (Pritchard 2001; Lohmueller et al. 2003). Our study, which was based on an average I103 carrier frequency of 2.1% in 3,631 obese subjects and 3.5% in 4,082 nonobese control individuals (table 1), is a good example that meta-analysis is a powerful tool for investigating even moderate effects of common variants on sus-

ceptibility to common disease. The potential contribution of rare variants with a moderate effect to common disorders has to be challenged in even-larger studies.

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Appendix A

Primers for Genomic Sequencing of 4.005 bp of the *MC4R* Locus*

Upstream:
- Primers in PCR1:
  - F: GAAGGGAGTTGGAGGTGTGA
  - R: GCACCCTCCATCAGAGTAGC
- Primers in PCR2:
  - 1F: GATATGGTCCTGCCACCTG
  - 1R: GGTGGTTTGAAAGATGGCAG
  - 2F: GAAAGAGGATACATTCCCAACC
  - 2R: ATGAATGGGTTCAAAAGGGG
  - 3F: TGTCGGTTCAACTTACGATACG
  - 3R: AAGCTATCTTCAGGCAGCCA
  - 3S: TTTCCTGAGTTCAGTGTAAGCA
  - 4F: TGAACCACTACTGGCTGGGT
  - 4R: GCTTTGAGTGTTAGGGGCTG
  - 5F: CAGTCTCTTATCCGGCTTGC
  - 5R: CCATGCCTGCTGTGAGTAAA
  - 6F: GCACAGATTCGTCTCCCAAT
  - 6R: GCCTTTTCCAAGGGACTCAC

Coding sequence:
- Primers in PCR1:
  - F: GCACAGATTCGTCTCCCAAT
  - R: TGTTACGAAAGCACGCAAAG
- Primers in PCR2:
  - 7F: GCATGGCAGCTTCAAGGA
  - 7R: GCACCCTCCATCAGAGTAGC
  - 8F: TGCACACTTCTCTGCACCTC
  - 8R: CCAACCCGCTTAACTGTCAT
  - 9F: GTAGCTCCTTGCTTGCATCC
  - 9R: CGGAGTGCATAAATCAGAGG
  - 10F: GGCCAGGCTTCACATTAAGA
  - 10R: ACGGAAGAGAAAGCTGTTGC

Downstream:
- Primers in PCR1:
  - F: CCGGAGTCAAGAACTGAGGA
  - R: TTGCATATGACTTCTGCCCA
- Primers in PCR2:
  - 11F: CATCTGTTGCTATCCCCTGG
  - 11R: TGTTACGAAAGCACGCAAAG
  - 12F: GCCCAGTCTCTGTATTATTTCCA
  - 12R: CAGGCTGTTTGCTAAGGGAG
  - 13F: AGGTTTGTTCATCCTCCCTG
  - 13R: TTGCCAGAAAACACTTCTGC
  - 14F: GGATTCAGGGTCTGCTGAAG
  - 14R: ACTTCTGCCCATGAGCATTT

* Primer 3S replaced primer 3R in the sequencing reaction of the PCR product obtained with primers 3F and 3R.

Appendix B

Study Groups

Marburg: The 520 obese trios were recruited via obese children and adolescents who had a BMI >90th sex- and age-specific percentile, as reported elsewhere (Hinney et al. 2003).

Augsburg: The KORA Survey 2000 is a representative sample of the adult general population of German nationality and comprises 4,261 individuals aged 25–74 years (Filipiak et al. 2001). Because of the variation in BMI over this age span, sex-specific 5-year groups (from 25–29 years to 70–74 years) were built, and the 50th and 90th percentiles in these age groups (P50 and P90, respectively) were calculated. Individuals were considered to be nonobese if their BMI was ≤P50 and to be obese when their BMI was >P90. This resulted in a total of 1,951 nonobese and 383 obese genotyped individuals; some individuals could not be included, owing to failed genotyping or missing BMI values.

Essen: There were 489 obese and 172 nonobese individuals aged 18–65 years, with BMIs ≥30 kg/m^2 (mean 41.6 ± 8.8 kg/m^2) and <25 kg/m^2 (mean 22.7 ± 1.6 kg/m^2), respectively, as described elsewhere (Herpertz et al. 2003).

Additional ethnicities: Blood from Tanzanians was randomly collected from patients of a local hospital in Daressalam ($N = 96$). Blood from African Americans was obtained at the Blood Bank of San Bernadino and Riverside Counties, CA ($N = 96$). Blood from Chinese subjects was obtained from voluntary students of the Technical College of Wuhan, People's Republic of China ($N = 92$). Blood from Japanese was obtained at Gifu,

Japan ($N = 95$). All blood samples were taken in order to study SNP distributions in different ethnicities (Siffert et al. 1999); anthropometrical data were not available.

Appendix C

Genotyping

Genotyping of the KORA S 2000 study group from Augsburg was performed in the Genome Analysis Center of the GSF. Analyses were performed using the MassARRAY system (Sequenom). In brief, genomic DNAs were amplified by PCR by use of HotStar*Taq* DNA Polymerase (Qiagen). Genotyping assays were performed by use of 5 ng of genomic DNA. PCR primers were used at 167 nM final concentrations, for a PCR volume of 6 μl. The following primers were used: PCR: 5′-ACGTTGGATGGATATGCTGGTGAGCGTTTC-3′ and 5′-ACGTTGGATGACTCTGTGCATCCGTATCTG-3′.

Extension reaction was performed with the following primer: 5′-GTTTCAAATGGATCAGAAACCATT-3′.

The PCR condition was 95°C for 15 min for hot start, followed by denaturing at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min for 44 cycles, and, finally, incubation at 72°C for 10 min. PCR products first were treated with shrimp alkaline phosphatase (SAP) (Amersham) for 20 min at 37°C to remove excess dNTPs and, afterward, for 10 min at 85°C to inactivate SAP. ThermoSequenase (Amersham) was used for the base extension reactions. Extension primers were at a final concentration of 0.54 μM in 10μl reactions. The base extension reaction condition was 94°C for 2 min, followed by 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s for 40 cycles. All reactions (reverse transcription, PCR amplification, and base extension) were performed in a Tetrad PCR thermal cycler (MJ Research). The final base extension products were treated with SpectroCLEAN resin (Sequenom) to remove salts in the reaction buffer. This step was performed with a Multimek 96-channel autopipette (Beckman Coulter), and 16 μl of resin-water suspension was added into each base extension reaction, making the total volume 26 μl. After a quick centrifugation (2,000 rpm, 3 min) in an Eppendorf Centrifuge 5810, 10 nl of reaction solution was dispensed onto a 384-format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid (3-HPA) by use of a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix-assisted laser desorption ionization-time-of-flight SpectroCHIPmass spectrometer (Sequenom) was used for data acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom).

For study groups from Marburg, Essen, and additional ethnicities, genotyping was performed by PCR with subsequent diagnostic RFLP analyses, or, for alleles that did not alter restriction sites, specific primers were designed to perform allele-specific PCR reactions (tetra-ARMS (Ye et al. 2001); V103I was detected as described elsewhere (Gotoda et al. 1997). In detail: SNP001745710, SNP001745712, SNP001745713, and SNP002901655 were genotyped by tetra-ARMS-PCR (Ye et al. 2001). Primers were as follows: (*a*) SNP001745710 C→T; MC4R-710-FiC 5′-GTTACTAGGTATTTGTCGGTTCAACTGAC-3′ and MC4R-710-Ro 5′-TGAGGAGTTTGTGTTATAGCTGAAAAAA-3′ 198-bp amplicon detects the SNP001745710 C-allele; MC4R-710-RiT 5′-TTTAAACCTCCAGAGTTTAACGTAGCA-3′ and MC4R-710-Fo 5′-TTAACCCTGAAATAAGCAGCTACAAACT-3′ 260-bp amplicon detects the SNP001745710 T-allele. Amplicon length of the two outer primers (MC4R-710-Fo and MC4R-710-Ro) was 402 bp. (*b*) SNP001745712 C→T; MC4R-712-FiC 5′-GAAATATGGGATATTAGTGCATTAAACTC-3′ and MC4R-712-Ro 5′-TGCTTTTTGGCTAGGATACTGAATTTAT-3′ 219-bp amplicon detects the SNP001745712 C-allele; MC4R-712-RiT 5′-ATTATGAATGGGTTCAAAAGGGGGTA-3′ and MC4R-712-Fo 5′-GTCGGTTCAACTTACGATACGTTAAACT-3′ 242-bp amplicon detects the SNP001745712 T-allele. Amplicon length of the two outer primers (MC4R-712-Fo and MC4R-712-Ro) was 406 bp. (*c*) SNP001745713 C→T; MC4R-713-FiC 5′-ATCCTCTCTGTTTTTCAGGTATTTTCAC-3′ and MC4R-713-Ro 5′-GCCAGGTTCATTCTTATGTAAAAGACAT-3′ 197-bp amplicon detects the SNP001745713 C-allele; MC4R-713-RiT 5′-TAAGAACCCAGCCAGTAGTGGTTAAA-3′ and MC4R-713-Fo 5′-AGAATGCAGCTTATTATTTTCCTGAGTT-3′ 285-bp amplicon detects the SNP001745711 T-allele. Amplicon length of the two outer primers (MC4R-713-Fo and MC4R-713-Ro) was 428 bp. (*d*) SNP002901655 G→C; MC4R-655-FiG 5′-CCAGAGGATGTCATGCAATAAACTTGG-3′ and MC4R-655-Ro 5′-CATGAGCATTTGAAGACCCTGTAAATCC-3′ 218-bp amplicon detects the SNP002901655 G-allele; MC4R-655-RiC 5′-TACTCTTCAAGAAGTGGCTGTGGATGG-3′ and MC4R-655-Fo 5′-TTCTCATTTCACATGAAAATGACTTGCTG-3′ 256-bp amplicon detects the SNP002901655 C-allele. Amplicon length of the two outer primers (MC4R-655-Fo and MC4R-655-Ro) was 420 bp. PCR-RFLP was performed for genotyping SNP 001745711 C→T. Primers were derived from the genomic sequence rcAC091576 (National Center for Biotechnology Information) as follows: MC4R-711-F 5′-CACCAGTCTTGGCTTGATAAGT-3′ and MC4R-711-R 5′-ACCCAGCCAGTAGTGGTTCA-3′ (amplicon: 385 bp), the SNP001745711 T-allele was digested by *Ear*I (fragment length: 222 bp and 163 bp). All PCR amplicons (RFLP or tetra-ARMS) were visualized on 2.5% agarose gels stained with ethidium bromide. For validity of the genotypes, allele determination

was made independently by at least two experienced individuals. Discrepancies were solved unambiguously either by reaching consensus or by repeating. In addition, we checked for Mendelian inconsistencies in the trios with the program Pedcheck (O'Connell and Weeks 1998).

Appendix D

Genomic Sequencing

We used a nested PCR approach. A genomic region of 4,005 bp including *MC4R* was covered by three overlapping PCR products, referred to as "upstream region" (1.877 bp), "coding sequence" (CDS) (1,697 bp), and "downstream region" (1,447 bp). We performed six nested PCRs on PCR products of the upstream region and four each on the CDS and downstream region. Nested PCR products were sequenced by use of PCR primers and BigDye Terminator Cycle Sequencing, version 2.0 kit (Applied Biosystems). Sequencing reactions were electrophoresed on ABI 377 automated sequencers. Base calling was performed using Phred (Ewing et al. 1998). Sequence assembly was done using Phrap. Trace files were inspected visually in gap4 (Bonfield et al. 1995).

For the SNP001745714 A→C described elsewhere (Jacobson et al. 2002), only the homozygous V103 carrier was heterozygous, whereas all of the I103 carriers were homozygous for the major allele A. Thus, the TDT results for V103I cannot be explained by this SNP, and it was not further investigated. SNPs 001745712 and 001745713 were investigated in a subgroup of 336 trios, in whom their minor alleles occurred more frequently than did I103 (minor allele frequencies of 13.2% and 32.4%, respectively, in the parents), and displayed transmission rates ~50%.

Appendix E

Functional Studies

In our functional studies performed in Berlin and Marburg, the cDNAs of the wild-type MC4R and of the V103I polymorphism were subcloned into the eukaryotic expression vector pSG5 (Stratagene). cDNA inserts in the expression plasmids were characterized by restriction endonuclease digestion and subsequent dideoxy sequencing (Perkin Elmer; Sequencer 310; PE Biosystems). For functional assays, COS-7 cells were grown as described elsewhere (Hinney et al. 2003). For transfections, COS-7 cells were split into 12-well plates (1.5×10^5 cells per well) and were transfected with 0.5 μg of plasmid DNA per well by use of Metafectene (Biontex). cAMP measurements were performed 2 d after transfection, as described elsewhere (Biebermann et al. 1997; Hinney et al. 2003). EC_{50} and E_{max} values were obtained from NDP-α-MSH, α-MSH, and β-MSH concentration-response curves.

For cell surface–binding studies, cells were transfected with Metafectin in 10-cm dishes. Cells were trypsinated 1 d later and were seeded in 48-well plates. Cells were incubated overnight 48 h later, with increasing amounts of NDP-α-MSH and ^{125}I-labeled NDP-α-MSH. After washing, specifically bound NDP-α-MSH was measured.

Appendix F

Statistical Analyses

TDTs: In our analyses of 520 trios and the subgroups of 501 and 336 trios, we performed TDTs for the single SNPs (Spielman et al. 1993) and the haplotypes consisting of several SNPs. *P* values were calculated using the exact two-sided McNemar test.

Genotype relative risk: The genotype relative risk of obesity for I103 carriers, conditional on the parental genotypes and a 95% exact binomial CI, were estimated from the 520 trios (Schaid and Sommer 1994). These estimates are valid under the assumption of random mating and Hardy-Weinberg equilibrium.

Meta-analysis: We combined data from 14 study groups. Special attention was given to the fact that the study groups of Farooqi et al. (total obese group published in 2003, nonobese group in 2000) and Hinney et al. (total obese and nonobese groups published in 2003) were reported more than once, because of enlarged samples in later analyses. The frequency of I103 carriers in similar weight groups varies across studies. Therefore, we did not consider studies that reported V103I status only for one weight group. Origin, sampling criteria, and sample size of the study groups are given in table 1. A stratified analysis was performed in SAS, and we present Mantel-Haenszel estimates of the OR and the respective CIs. In addition, the Breslow-Day test for homogeneity (Breslow and Day 1980) was conducted, to investigate if there are differences between the study groups. Because our previous study (Hinney et al. 2003) included the 520 obese offspring of the trios used in the TDT, we also performed a meta-analysis without those individuals, reducing the number of obese individuals in this single study from 808 to 288 individuals; this meta-analysis also resulted in a significant OR of 0.69 (95% CI 0.49–0.97; $P = .03$).

Electronic-Database Information

The URLs for data presented herein are as follows:

National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *MC4R* and obesity)
Phrap, http://www.phrap.org/phrap.docs/phrap.html

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