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Do Common Variants Separate between Obese Melanocortin-4 Receptor Gene Mutation Carriers and Non-Carriers? The Impact of Cryptic Relatedness

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Key Words
Obesity · *MC4R* · Mutations · Haplotypes · Cryptic relatedness

Abstract
Background/Aims: Genome-wide association studies revealed associations of single nucleotide polymorphisms (SNPs) flanking *MC4R* with body mass index variability and obesity. We genotyped 28 SNPs, covering *MC4R*, and searched for haplotypes discriminating between obese mutation carriers and non-carriers. ***Methods:*** We analyzed all three-marker haplotype combinations of the 28 SNPs to discriminate between obese mutation carriers and non-carriers – overall and in functional categories for 25 different *MC4R* mutations: (a) 'like wild type', (b) 'partial loss of function', and (c) 'complete loss of function'. We checked for the possible impact of 'cryptic relatedness' by sensitivity analyses including only 1 randomly selected patient per mutation. ***Results:*** Overall analyses revealed a haplotype of 3 SNPs downstream of the *MC4R* discriminating between obese mutation carriers and obese non-carriers. However, sensitivity analyses showed that the finding is most likely due to cryptic relatedness. ***Conclusion:*** Given a mutation prevalence of 1–5%, the sample size of 62 obese mutation carriers with overall 25 different *MC4R* mutations represents a unique feature of our study. Taking *MC4R* as an example, we demonstrate the impact of cryptic relatedness when trying to link non-coding SNPs to functionally relevant mutations. Hence, a thorough mutation screen can currently not be guided by SNP genotyping.

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Introduction

Genome-wide association studies (GWAS) have contributed to a growing number of robustly confirmed genetic association signals for common single nucleotide polymorphisms (SNPs) and complex traits such as obesity [1, 2]. The latest meta-analysis of the GIANT (Genetic Investigation of ANthropometric Traits) consortium evaluated approximately 250,000 individuals for genetic variants that are associated with body mass index (BMI) [3]. The analyses revealed 14 known and 18 new genetic loci. These loci imply the existence of regulatory or coding variants in nearby genes. Prominent examples of these GWAS-based candidate genes for obesity are *FTO* (fat mass and obesity-associated gene) [4–6], *TMEM18* (transmembrane protein 18 gene) [7, 8], and *MC4R* (melanocortin-4 receptor gene) [8–12]. With regard to functional characterization and biological pathways, the *MC4R* represents one of the most promising candidate genes. The *MC4R* codes for a G-protein-coupled receptor which is involved in the hypothalamic regulation of appetite and energy balance [13]. Moreover, to date, more than 150 different *MC4R* mutations have been identified and more than half of these mutations modify receptor expression and/or function in in vitro assays [14]. GWAS analyses also revealed association of several common variants outside the *MC4R* coding region with BMI variability and obesity. Interestingly, initial analyses detected the SNPs rs17782313 [9] and rs12970134 [10] 150 and 188 kb downstream of the *MC4R* coding region, whereas more recent analyses also implied common variants in the upstream region [3, 15].

Here we investigated the genomic region flanking the *MC4R* for an association between common non-coding SNP alleles (and haplotypes thereof) and 25 different, naturally occurring and functionally characterized *MC4R* mutations. We performed comparisons of SNP allele and haplotype frequencies of obese individuals who are carriers or non-carriers of *MC4R* mutations. We analyzed the mutations in subgroups according to functional categories and explored the impact of so-called 'cryptic relatedness' (i.e. kinship among cases or controls that is not known to the investigator) on our results. The classification of the mutations into functional categories and the subsequent analyses according to these categories was driven by both statistical and biological considerations: (i) from a statistical point of view, we aimed to avoid problems of misclassification by comparing obese carriers of *MC4R* mutations that were functionally characterized as 'like wild type' in the used in vitro system to obese carriers without *MC4R* mutations which could contribute to a diluted overall effect if present; (ii) from a biological point of view to discriminate between mutations having a relevant effect on receptor function in comparison to wild type (WT) and *MC4R* mutations without a relevant functional effect in comparison to WT. For some disease-causing genes a haplotype was reported to be associated with several mutations [16–18]. In this context, cystic fibrosis can serve as an example, as it has been shown that certain risk microsatellite alleles are indicative of disease-causing mutations in the cystic fibrosis transmembrane conductance regulator gene *(CFTR)* suggesting a single origin for most *CFTR* mutations [16, 17].

Correspondingly, we explored the possibility that a common risk haplotype may indicate a functionally relevant *MC4R* mutation.

Methods

Study Groups

The analyzed mutation carriers derived from a large study group of (extremely) obese German young and adult individuals (Hebebrand group in Essen and formerly Marburg, Germany); individuals were screened for *MC4R* mutations within the coding region. A number of different non-synonymous, frameshift and nonsense mutations, silent variants and two polymorphisms in the *MC4R* (p.Val103Ile; p.Ile251Leu) were identified; most of them were detected just once, but some in several individuals (up to 17) [12, 19–22]. Here we describe 25 different *MC4R* mutations in a total of 62 (extremely) obese mutation carriers and 28 screened obese non-carriers. Descriptive statistics of all 90 obese individuals are displayed in table 1. The study was reviewed and approved by all local IRB boards and conducted in accordance to the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all subjects and, in case of minors, their parents.

SNP Selection and Genotyping

In total, 28 SNPs were chosen for genotyping in the 90 obese individuals (fig. 1). 23 of the 28 SNPs are located between 60 kb upstream and 40 kb downstream of the *MC4R* coding region on chromosome 18q21, reaching from chromosome position 56,148,765 (dbSNP [23], genome build 36.3, rs6567164) 3′ of the *MC4R* to chromosome position 56,247,636 (dbSNP [23], genome build 36.3, rs12966035) 5′ of the gene. SNP selection was performed using HapMap Genome Browser release #22, phase 2 [24], requiring a minimal observed heterozygosity of 40% (calculated with Haploview 4.2 [25]). Three SNPs were selected because of their close proximity to the *MC4R* coding region despite a lower expected heterozygosity: rs35748167 (chromosome position 56,188,413; dbSNP [23], genome build 36.3), rs17066842 (chromosome position 56,191,604; dbSNP [23], genome build 36.3), and rs34114122 (chromosome position 56,190,740; dbSNP [23], genome build 36.3). Moreover, another two SNPs, rs17782313 and rs17700633, located 188 kb (chromosome position 56,002,077; dbSNP [23], genome build 36.3) and 110 kb (chromosome position

Table 1. Descriptive statistics of the 90 obese individuals analyzed

Sample	Mutation subgroup	n total (female)	Median age, years (Q1; Q3)	Median BMI (Q1; Q3)	Median BMI-SDS[a] (Q1; Q3)
Obese mutation carriers	total	62 (38)	13.25 (11.60; 16.80)	31.48 (28.05; 36.12)	3.49 (2.87; 4.52)
	like wild type	19 (12)	13.64 (13.05; 41.95)	32.49 (29.89; 35.60)	3.31 (2.35; 3.67)
	partial loss of function	18 (12)	11.75 (10.92; 23.50)	29.06 (26.58; 33.01)	3.62 (2.82; 4.13)
	complete loss of function	25 (14)	13.20 (9.77; 15.32)	31.93 (28.81; 40.96)	4.41 (3.19; 6.79)
Obese non-carriers	total	28 (22)	16.13 (15.30; 16.84)	38.50 (35.11; 43.39)	6.32 (4.91; 8.85)

[a] BMI-standard deviation score (BMI-SDS) is a normalized version of BMI expressed as standard deviation score that includes information on age and sex based on a reference population (National Nutrition Survey I).

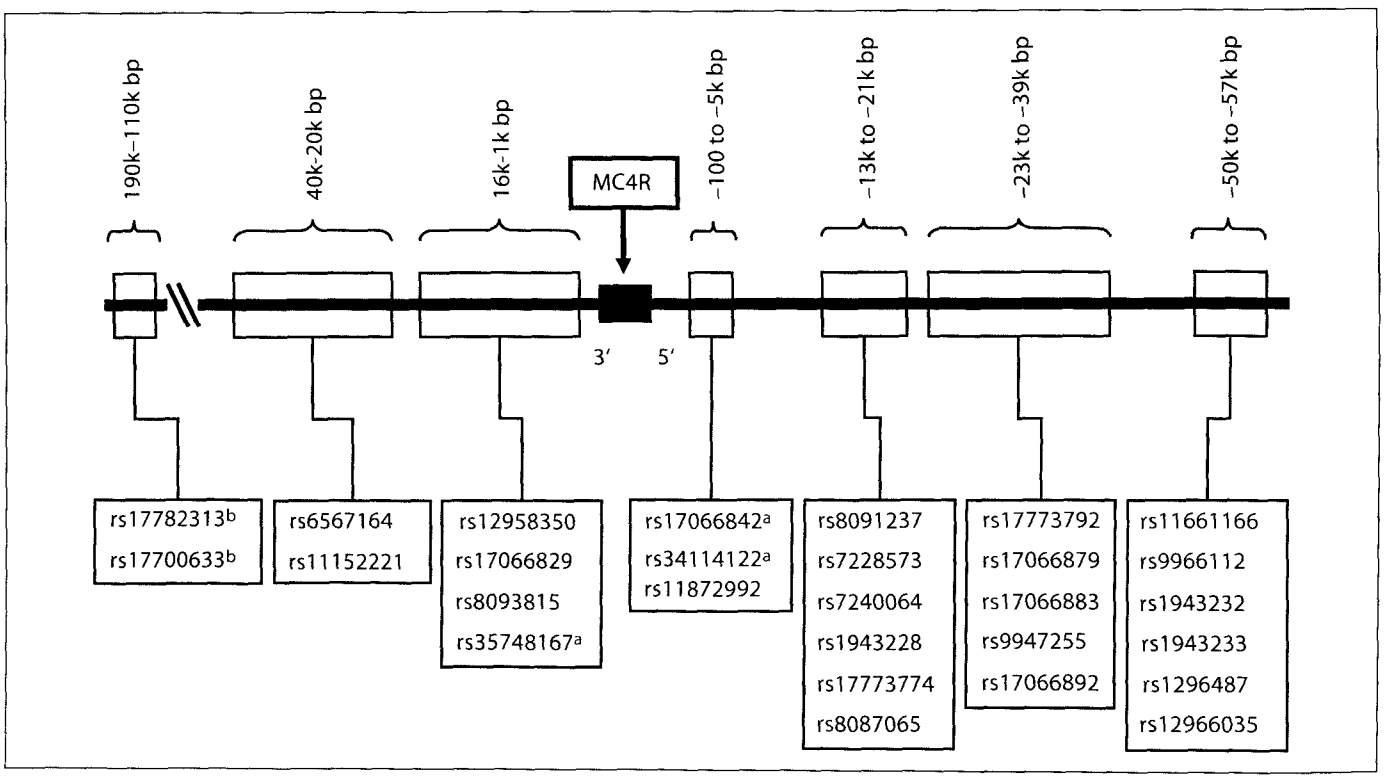

Fig. 1. SNPs that were selected for de novo genotyping and analyses. Their relative physical chromosomal positions in relation to the *MC4R* coding region on chromosome 18q21 are provided in base pairs (bp) according to dbSNP (genome build 36.3). See Methods section for more details. [a]SNP included based on proximity to the *MC4R* coding region. [b]SNP included based on previous reports.

56,080,412; dbSNP [23], genome build 36.3) downstream of *MC4R* based on previous reports [3, 5, 9] (fig. 1). For SNP analyses, polymerase chain reaction assays and iPLEX® reaction assays were designed by using the spectroDESIGNER® software (Sequenom Inc., San Diego, Calif., USA). DNA samples of 90 individuals were genotyped using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc.) as described previously [26].

Functional Characterization of MC4R Mutations

Functional characterization was performed for those *MC4R* mutations which were new at the time of detection in the patients (table 2). The genomic DNA of the respective mutated MC4Rs was directly cloned into the eukaryotic expression vector pcDps. For cell surface expression assays, the respective mutated MC4Rs were amplified from genomic DNA using a forward primer containing the Kozak sequence and an N-terminal hemagglutinin

Table 2. Classification of *MC4R* mutations into three categories, according to own findings and data from literature (as indicated) in the context of cell surface expression and signaling properties in vitro

MC4R mutation	Cases, n	References
(a) Like wild type		
p.Thr112Met[a]	10	20, 43, 48–52
p.Pro230Leu[b]	1	20, 43, 53, 54
p.Val95Ile[a]	1	27
p.Met200Val[a]	1	48
p.Ala175Thr[a]	2	27, 43, 52
p.Thr178Met	1	20, 43
p.Val166Ile[a]	1	55
p.Pro48Ser[a]	1	43, 56
p.Asn274Ser[a]	1	27, 43, 56
(b) Partial loss of function		
p.Ile317Thr	2	57, 58
p.Arg165Trp[b]	2	20, 43
p.Arg305Gln	2	52
[p.Ser127Leu; p.Val103Ile][a]	3	own data, unpubl.
p.Ala244Glu[b]	1	20, 43, 53, 57
p.Ile121Thr[b]	1	20, 59
p.Glu308Lys[b]	1	59, 60
p.Pro275Ser[a]	2	own data, unpubl.
p.Gly252Ser[b]	3	43, 57
p.Met281Val[a]	1	22
(c) Complete loss of function		
[p.Tyr35Stop; c.110 A>T][a]	17	20, 43, 57
p.Leu211fsX15[c]	4	52
[p.Gly181Asp] + [p.Ile251Leu]	1	20, 43, 50, 59
p.Glu61Stop[a]	1	61
p.Leu250fsX34[d]	1	20
p.Gln307Stop[a]	1	22

'Cases, n' is the available number of samples for each *MC4R* mutation.

[a] Own functional data available.

[b] Divergent functional data in the literature, finally classification according to the predominantly obese phenotype as partial loss of function (see Methods section for more details).

[c] Previous nomenclature: L211fsX216.

[d] Previous nomenclature: L250fsX284.

(HA) tag after the start codon. These constructs were directly cloned into the eukaryotic expression vector pcDps.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, Deisenhausen, Germany) supplemented with 5% fetal calf serum and 20 mM glutamine. Cells were incubated at 37°C in humidified air containing 5% CO_2. For all functional assays, COS-7 cells were seeded into 48-well plates (2.5 × 105 cells/well). Transient transfections with WT and mutant MC4Rs, respectively, were carried out by using Metafectene® (Biontex, Munich, Germany) according to the manufacturer's protocol.

The MC4R is a Gs/adenylyl cyclase coupling G-protein-coupled receptor, therefore in order to characterize receptor signaling properties of the mutated MC4R, cAMP accumulation was measured. For cAMP measurements, 2 days after transfection, cells were incubated for 30 min at 37°C with the endogenous MC4R ligands α-MSH and β-MSH as well as with the highly potent ligand NDP-α-MSH respectively. Intracellular cAMP was measured via AlphaScreen® technology (Perkin-Elmer Life Science, Inc., Boston, Mass., USA) as described elsewhere [27]. AlphaScreen measurements were carried out by using a Mithras LB 940 multimode reader (Berthold Technologies, Bad Wildbad, Germany).

For measurement of the cell surface expression, a cell surface ELISA was performed. Therefore, 2 days after transfection with HA-tagged WT and mutant *MC4R* constructs, cells were fixed with 4% formaldehyde. After incubation with 1 μg/ml biotin-labeled anti-HA monoclonal antibody (Roche, Mannheim, Germany), bound anti-HA antibodies were detected with streptavidin-labeled peroxidase (Dianova, Hamburg, Germany) as described elsewhere [28]. Colorimetry was accomplished by using an Anthos Reader 2001 (Anthos Labtech Instruments, Salzburg, Austria).

Haplotype Sequence Analyses for Carriers of Two Different MC4R Mutations

For determination whether individuals who harbor two different *MC4R* mutations are compound heterozygous or whether both mutations are located on a haplotype, we performed family-based analyses where family data were available (for carriers of the *MC4R* mutations p.Tyr35Stop and p.Asp37Val, p.Gly181Asp and p.Ile251Leu) [19–21, 29–35]. In case of index cases without families (carriers of the *MC4R* mutations p.Val103Ile and p. Ser127Leu), we performed genetic analysis via TOPO cloning and automatic resequencing according to the manufacturer's protocol (Invitrogen, Carlsbad, Calif., USA).

Classification of the MC4R Mutations

We classified the 25 *MC4R* mutations according to our own functional data and data from literature into three functional categories: (a) 'like wild type' for all variants that do not differ from the WT receptor in cell surface expression and signaling properties in vitro, (b) 'partial loss of function' for *MC4R* mutations showing partial loss of function, and (c) 'complete loss of function' for *MC4R* mutations with complete loss of receptor function in vitro (table 2). In the case of the carriers of the two mutations p.Gly181Asp and p.Ile251Leu which emerged to be compound heterozygous [20], functional characterization was performed for each mutation separately. The classification was based on the mutation with the stronger functional effect. In the case of the carriers of the haplotypes [p.Val103Ile; p. Ser127Leu] and [p.Tyr35Stop; c.110 A>T] [19–21, 29–35], functional analyses were performed for the haplotypes. Heterozygous (partial) loss of function *MC4R* mutations with a dominant-negative effect could have a higher functional relevance than complete loss of function mutations. To date, only two *MC4R* mutations with a dominant-negative effect were described (Asp90Asn [36] and Ser136Phe [37]). As these *MC4R* mutations were not present in this study, dominant-negative effects were not relevant for the classification.

Statistical Analyses

Initially we screened this region for haplotype combinations containing three SNPs of the 28 SNPs genotyped which discrim-

inate best (as judged by p value) between obese *MC4R* mutation carriers and obese individuals without mutation in the *MC4R* coding region (neither singles SNPs nor two-SNP haplotypes had smaller p values). We performed multiple marker combination tests using Monte-Carlo simulations (50,000 simulations) as implemented in FAMHAP (version 18) [38, 39] to derive empirical p values and p values adjusted for multiple testing. Afterwards, the three-SNP haplotype combination with the lowest p value was explored in subgroups of the obese mutation carriers according to the mutation classification (a) 'like wild type', (b) 'partial loss of function', and (c) 'loss of function' (table 2). Haplotype frequencies were estimated with and without omitting haplotypes with ≥5 estimated counts in mutation carriers and non-carriers. We performed these analyses in the total sample of all mutation carriers and non-carriers and in 100 random draws each with just one randomly selected case for each *MC4R* mutation (overall 25 different mutations as displayed in table 2; both omitting or not omitting rare haplotypes – see table 4) to explore so-called 'cryptic relatedness' (i.e., kinship among mutation carriers or non-carriers that is not known to the investigator). In addition, we estimated the proportion of alleles shared identical by state (IBS) in three groups of (1) individuals with the p.Thr112Met mutation (n = 10), in (2) individuals with the [p.Tyr35Stop; c.110 A>T] mutation (n = 17), and (3) in all individuals not belonging to either of these groups (n = 62). We compared each of the groups (1) and (2) to (3) to support the idea of 'cryptic relatedness' using all 28 SNPs by a permutation test.

Results

Signal Transduction Properties and Functional Characterization of the MC4R Mutations

Twenty-five different *MC4R* mutations were included into statistical calculations and differentiated in the context of their relevance for receptor function. The function was represented by signaling properties in vitro. Carriers of the two mutations p.Gly181Asp and p.Ile251Leu emerged to be compound heterozygous [20] and the mutations p.Val103Ile, p. Ser127Leu and p.Tyr35Stop, p.Asp37Val formed a haplotype in all analyzed carriers as shown previously [19–21, 29–35].

We formed three categories (a) 'like wild type', (b) 'partial loss of function', and (c) 'complete loss of function', according to data from literature and own experiments (13 cases, table 2). In case of diverging characterizations of 'partial loss of function' and 'like wild type' between literature and own data (6 cases, table 2), our classification was first guided by the predominant grouping. If the data still remained ambiguous, we considered own data and finally evolutionary aspects like the degree of conservation of the respective amino acid. In the case of a highly conserved amino acid, we determined the category as 'partial loss of function'. The compound heterozygous carrier of the two mutations p.Gly181Asp and p.Ile251Leu was classified according to the functional characterization of p.Gly181Asp ('loss of function'). This classification is supported by the fact that the p.Ile251Leu has a protective effect against obesity [40] but there is no solid evidence yet that the p.Ile251Leu affects receptor signaling [41–43].

Finally, 9 mutations (19 obese patients) were assigned to 'like wild type', 10 mutations (18 obese patients) to 'partial loss of function', and 6 *MC4R* mutations (25 patients) were assigned to the category 'complete loss of function' (table 2).

Haplotype Analyses

The analyses revealed a set of three SNP (rs17782313, rs12958350, rs17066829) downstream of the coding region of the *MC4R* that discriminates best between obese *MC4R* mutation carriers and obese non *MC4R* mutation carriers (estimated $p < 0.0001$ – 0 out of 50,000 Monte-Carlo simulations lead to more extreme results; table 3). This marker combination was next explored for the three categories of *MC4R* mutations (a) 'like wild type', (b) 'partial loss of function', and (c) 'complete loss of function'. These analyses in the different categories suggested that the overall effect is most likely due to *MC4R* mutations which result in a complete loss of function ($p = 0.001$; table 3). Next, we explored the corresponding haplotypes and their frequencies. In all individuals, the largest discrepancy in haplotype frequencies was observed for the haplotype CGA (for the SNPs rs17782313, rs12958350, rs17066829) with an estimated frequency of 27.6% in obese *MC4R* mutation carriers and 0.7% in obese *MC4R* mutation-free controls. This difference was similarly present in the category 'complete loss of function' (31.9% in obese *MC4R* mutation carriers vs. 1.7% in obese *without MC4R* mutation).

Next, we explored the robustness of our finding in sensitivity analyses addressing 'cryptic relatedness' as individuals with the same infrequent mutation might be remotely related (the patients and the investigators not being aware of their relationship). We used just one randomly selected case for each *MC4R* mutation (overall 25 different mutations as displayed in table 2), performed the same analyses as before and repeated this procedure 100 times (100 random draws). The impact on the p value distributions is shown in table 4. Moreover, we observed no strong or moderate Spearman correlations (all ρ between –0.3 and 0.3) between the results indicating no direct relationship between the overall results and the findings in the categories. Irrespective of omitting or not

Table 3. The three-SNP haplotype combination and the corresponding haplotypes discriminating best between obese individuals with *MC4R* mutation and obese individuals without *MC4R* mutation

Group comparison	Haplotype (rs17782313 rs12958350 rs17066829)	Estimated frequency in cases	Estimated frequency in controls	p value
All mutation carriers vs. non-carriers	C G A	0.276	0.007	<0.0001 (0.006 when adjusted for multiple testing)
	T G T	0.285	0.353	
	T A T	0.188	0.127	
	C A T	0.027	0.080	
	T G A	0.081	0.100	
	C G T	0.027	0.140	
	C A A	0.027	0.153	
	T A A	0.089	0.040	
(a) Like wild type: mutation carriers vs. non-carriers	T G T	0.385	0.363	0.201
	T G A	0.195	0.090	
	C G T	0.030	0.143	
	T A T	0.109	0.126	
	T A A	0.124	0.040	
	C A A	0.103	0.166	
(b) Partial loss of function: mutation carriers vs. non-carriers	T A T	0.298	0.130	0.353
	T G T	0.272	0.360	
	T G A	0.062	0.092	
	C A A	0.111	0.166	
	C G A	0.081	0.143	
(c) Complete loss of function: mutation carriers vs. non-carriers	C G A	0.319	0.017	0.001
	T G T	0.269	0.353	
	C A A	0.104	0.156	
	C G T	0.087	0.136	
	T A T	0.050	0.141	
	T G A	0.137	0.094	

Only haplotypes with ≥5 counts in mutation carriers and non-carriers were concerned. Additionally, the best three-SNP haplotype combination was checked for its effects on the three categories of mutation carriers based on the mutation classification shown in table 2.

omitting rare haplotypes (see section Statistical Analyses), we found no evidence for group differences.

Finally, we compared the median proportions of all alleles shared identity by state (IBS) for all 28 SNP and all pairwise comparisons among all 10 p.Thr112Met mutation carriers, among all 17 [p.Tyr35Stop; c.110 A>T] mutation carriers and among all 62 individuals not belonging to either of these groups. For the 10 p.Thr112Met mutation carriers the medians proportions for the IBS states (0; 1; 2) were (0.00; 0.68; 0.09), for the 17 [p.Tyr35Stop; c.110 A>T] mutation carriers they were (0.00; 0.68; 0.04) and (0.45; 0.30; 0.00) for the 62 individuals not belonging to these groups. Running two permutation tests for each of the groups with carriers of the same mutations also indicated that they are similar to each other as compared to the 62 individuals, also underlining the closer relationship within the groups of carriers of the same mutations (both $p < 0.01$).

Discussion

We aimed to detect SNP alleles or haplotypes which co-segregate with *MC4R* mutations. Ultimately, if truly existent, such a haplotype would be a first step towards an enrichment procedure for clinical practice to more easily detect *MC4R* mutations in obese patients.

Table 4. The impact of choosing just 1 randomly selected case for each *MC4R* mutation (100 times) on the p value distributions for either omitting or not omitting rare haplotypes (for a definition see Methods section)

Group comparison	p value distribution (min., 1st, 2nd, 3rd quartile, max.)	
	without rare haplotypes	with rare haplotypes
All mutation carriers vs. non-carriers	0.03 0.08 0.13 0.29 0.64	0.05 0.10 0.14 0.18 0.36
(a) Like wild type: mutation carriers vs. non-carriers	0.08 0.15 0.16 0.29 0.36	0.05 0.07 0.08 0.14 0.16
(b) Partial loss of function: mutation carriers vs. non-carriers	0.15 0.36 0.65 0.94 0.99	0.07 0.36 0.62 0.94 1.00
(c) Complete loss of function: mutation carriers vs. non-carriers	0.54 0.81 0.89 0.97 1.00	0.06 0.21 0.31 0.39 0.57

We identified a triplet of SNP alleles (rs17782313, rs12958350, rs17066829 [C; G; A]) which discriminated best between all obese mutation carriers and obese non-mutation carriers. SNP rs17782313 which showed a robustly replicated association with obesity in several studies [3, 5, 9] is included in this haplotype combination. Subsequent analyses by mutation categories showed that the overall effect was largely due to an effect for the category c) 'complete loss of function'.

However, this observation is debatable. First, functional characterization represents a general problem in mutation classification (i) because obesity is a highly polygenic trait variation [44] and for melanocortin receptors a higher rate of naturally occurring mutations is known for which no significant effect on receptor function could be detected [45] compared to mutations in other G-protein-coupled receptors, and (ii) because of discrepancies between the situations in vitro and in vivo. In case of the *MC4R* the applied cell systems in vitro differ from the physiological reality and are mostly characterized by overexpression of the receptor. Different readout systems are used and have different sensitivities. This could lead to additional discrepancies and could account for divergent results in receptor function throughout literature. Furthermore, *MC4R* mutations causing dominant-negative effects seem to be rare [35, 37, 46]. The mutation [p.Tyr35Stop; c.110 A>T] is suggested to lead to haploinsufficiency because the short truncated protein is presumed to be rapidly degraded upon translation [32, 35]. The quantitative difference between carriers of the WT allele and the [p.Tyr35Stop; c.110 A>T] in BMI is rather low compared to other *MC4R* missense mutation carriers [35]. Nevertheless, this may be due to the fact that the nonsense mutation is rapidly degraded and thereby cannot affect the function of other proteins which might be the case for a missense mutation within the *MC4R*.

Second, 'cryptic relatedness' had a large impact on our findings which had previously been shown for classical genetic case-control studies of complex traits [47]. When re-running our analysis but only including one patient per mutation, we could not confirm our initial results. This apparent discrepancy was due to a majority of 'complete loss of function' mutation carriers being of the p.[Tyr35Stop; c.110 A>T] type (17 of 25 individuals in this category). For these 17 carriers a distant relationship is very likely and has been supported by our IBS analysis and has also previously been suggested [19, 32]. Moreover, our analysis also indicated evidence for 'cryptic relatedness' between the 10 p.Thr112Met mutation carriers.

Third, our analysis is limited by the number of patients genotyped and characterized for mutations. Limiting our analysis to just one patient per mutation of course further adds to this sample size constraint. However, given our study design, obese individuals with *MC4R* mutations need to be identified and functionally characterized. One should take into consideration that the access to a large cohort is a prerequisite for the availability of a sufficiently large number of well-characterized *MC4R* mutation carriers (given an estimated *MC4R* mutation rate of 1–5% in obese individuals [21, 31]). Having access to such a large number of mutation carriers represent, after all, a unique feature of our study.

For another study design, focusing on individuals from GWAS samples, it has recently been shown that common variants both up- and downstream of the *MC4R* coding region show an obesogenic effect [15]. Removing individuals with a known functionally relevant *MC4R* mutation had little or no impact on the findings, thus contradicting the idea of a 'synthetic association' at the *MC4R* [38].

In sum, we first observed evidence for a haplotype of SNP alleles downstream of the *MC4R* which discriminated between obese *MC4R* mutation carriers and obese non-mutation carriers. Although a replication effort of our data is absolutely necessary, upon closer examination our findings were most likely due to 'cryptic relatedness' effects between carriers of the same mutation. Our data underscore challenges that do arise when common SNP variants and mutations are analyzed jointly in a well-characterized but small study. However, our findings are of great relevance even for much larger sample sizes whenever SNP information from GWAS is linked to rare variant resequencing data.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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