

CLINICAL STUDY

Mutation analysis of the *MCHR1* gene in human obesity

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Abstract

Objective: The importance of the melanin-concentrating hormone (MCH) system for regulation of energy homeostasis and body weight has been demonstrated in rodents. We analysed the human MCH receptor 1 gene (*MCHR1*) with respect to human obesity.

Design: This consisted of genomic screening of 13.4 kb encompassing the *MCHR1* in extremely obese German children and adolescents and association analyses for two coding single nucleotide polymorphisms (SNPs). To confirm initial positive association results, additional association studies and transmission disequilibrium tests in further German, Danish, French and American samples were conducted. Selected SNPs were investigated using functional *in vitro* studies and reporter gene assays.

Methods: Single-stranded conformation polymorphism analysis, re-sequencing, PCR-restriction fragment length polymorphism analyses, tetra-primer amplification refractory mutation systems, matrix-assisted laser desorption/ionization time of flight mass spectrometry and reporter gene assays were carried out as well as measuring inositol phosphate formation, inhibition of cAMP formation and activation of p42/44 MAP kinase.

Results: We identified 11 infrequent variations and two SNPs in the *MCHR1* coding sequence and 18 SNPs (eight novel) in the flanking sequence. Association and transmission disequilibrium with obesity were detected for several SNPs in independent study groups of German obese children and adolescents and controls. In two German samples, encompassing 4056 and 295 individuals, trends towards association with obesity were detected. Findings in a second epidemiological German sample and in Danish, French and American samples were negative. Functional *in vitro* studies as well as reporter gene assays revealed no significant results.

Conclusion: Our initial association of *MCHR1* alleles/haplotype detected might be related to juvenile-onset obesity, conditional on a particular genetic and/or environmental background. Alternatively, we could not exclude the possibility that the initially detected association represented a false positive finding.

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Introduction

The melanin-concentrating hormone (MCH) system is involved in the regulation of energy intake and expenditure. In rodents, acute and chronic intracerebroventricular MCH injection as well as

overexpression of the pro-MCH gene (*Pmch*) result in increased food intake and body weight (1–6). Fasting induces an upregulation of orexigenic MCH (1) and of the MCH receptor-1 (*MCHR1*) (7), one of the two G protein-coupled receptors that bind MCH (8–10). SNAP-7941, an *MCHR1* antagonist, inhibits food intake

stimulated by central administration of MCH, reduces consumption of palatable food and, after chronic administration to rats with diet-induced obesity, results in a marked, sustained decrease in body weight (11).

Both *Pmch*^{-/-} and *Mchr1*^{-/-} mice have a reduced fat mass and an increased metabolic rate (12–14). Furthermore, *Mchr1*^{-/-} mice were described to either have a similar (13) or a reduced (14) body weight compared with wild-type mice and to be less susceptible (13) or resistant to diet-induced obesity (14). *Pmch*^{-/-}; *ob/ob* mice (double null) had a significantly reduced body weight and fat content compared with *ob/ob* mice caused by an increased metabolic rate and increased physical activity but not by a reduced food intake (15).

MCHR1 (official gene symbol: *GPR24*) is expressed peripherally and centrally in hypothalamic nuclei and hippocampal regions involved in olfaction and regulation of feeding behaviour and body weight (7–9).

Recently, two infrequent missense variations (Y181H and R248Q) were identified in a mutation screen of *MCHR1* in 106 individuals with severe, early onset obesity (16); however, *in vitro* studies showed no evidence for functional implications. Furthermore, no association for single nucleotide polymorphisms (SNPs) rs133072 and rs133073 with obesity-related phenotypes in 541 white adult probands with type 2 diabetes and related disorders was detected (16).

The human reference genome at the University of California Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>) (hg17) currently includes seven human *MCHR1* mRNA entries (AF490537, AB063174, AY562945, BC001736,

BC021146, BT006725 and CR456497). Each harbours three potential translational start codons (Met¹, Met⁶ and Met⁷⁰) corresponding to proteins of 422 amino acids (aa) (17), 417 aa and 353 aa respectively (Fig. 1a). In these entries as well as in the reference protein (Q99705), an open reading frame corresponding to a 422 aa protein is stated. The translational start codon of orthologous *Mchr1* genes in mice (AF498248; Fig. 1b) (18) and rats (AF008650) (19), however, corresponds to human Met⁷⁰. Therefore, MCHR1 proteins longer than 353 aa seem to be specific to humans.

This study was aimed at identifying sequence variations relevant for body weight regulation in the human *MCHR1* by mutation screening. The relevance of *MCHR1* variations was determined by functional studies and genotyping in different German, French, American and Danish study groups.

Materials and methods

Study subjects

The association and linkage disequilibrium studies were based on six study groups in Marburg (study group 1), Berlin, Munich, Copenhagen, Paris and Philadelphia (study groups 2–6) respectively:

Study group 1

- (a) 620 (359 females) unrelated obese children and adolescents with a body mass index (BMI) ≥ 90 th percentile (20; mean BMI 33.4 ± 6.6 kg/m²; mean

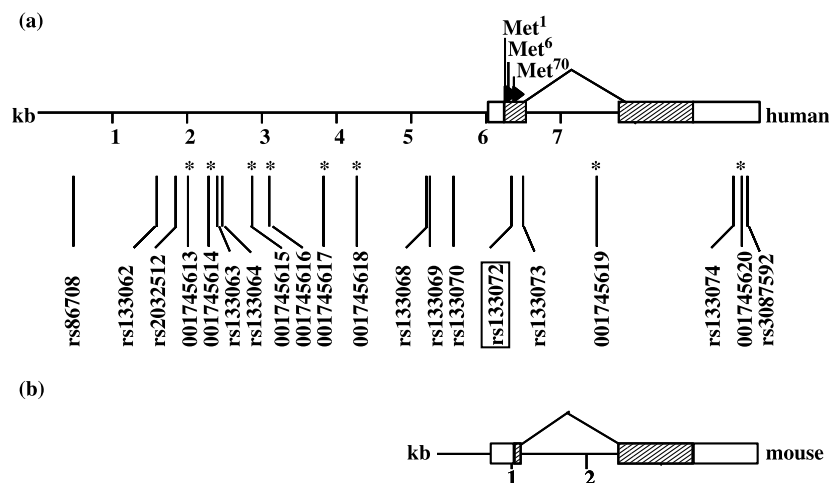


Figure 1 Human and murine *MCHR1* locus. (a; upper section) Part of the re-sequenced genomic region including *MCHR1* on human chromosome 22q13.2. The gene is depicted according to alignment of mRNA (AF490537) to genomic DNA (Z86090). Three potential start codons contained in AF490537 are marked. The coding sequence beginning with Met¹ corresponds to a 422 aa MCHR1 protein (JC7080). (b; lower section) SNPs determined by re-sequencing of 13 379 bp including *MCHR1* in 19 individuals. *Novel SNPs; unmarked, SNPs previously reported in the Single Nucleotide polymorphism database (dbSNP; <http://www.ncbi.nlm.nih.gov/SNP/>); boxed, SNP leading to a non-conservative aa exchange. (b) *Mchr1* locus on mouse chromosome 15. All ESTs (74) of UniGene cluster Mm.221060 (<http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Mm&CID=221060>) were downloaded and assembled using gap4 (<http://www.mrc-lmb.cam.ac.uk/pubseq/>). The resulting consensus sequence was aligned to genomic DNA (AY049011) and exons were defined accordingly. The coding sequence was inferred from GenBank entry AF498247.

BMI percentile 99.3; mean age 14.0 ± 2.7 years). For the initial mutation screen we chose a subgroup of 215 extremely obese children and adolescents (127 females: mean BMI 39.8 ± 5.3 kg/m²; mean age 15.3 ± 2.4 years). For transmission disequilibrium tests (TDT; 21) both parents of 525 of the children and adolescents (304 females: mean BMI 32.2 ± 6.0 kg/m²; mean age 13.7 ± 2.8 years) were genotyped for four SNPs (sample described in 22); TDTs for these and 12 other SNPs were also based on a smaller subgroup of 61 families who are comprised of one obese index patient and both biological parents (trios).

- (b) 230 (110 females) healthy underweight students (BMI ≤ 15 th percentile; mean BMI 18.3 ± 1.1 kg/m²; mean age 25.2 ± 3.7 years).
- (c) 96 (49 females) healthy normal weight students (BMI ≥ 40 th and ≤ 60 th percentile; mean BMI 21.9 ± 1.1 kg/m²; mean age 24.7 ± 2.6 years).
- (d) 99 (51 females) healthy overweight students (BMI ≥ 90 th percentile; mean BMI 29.1 ± 3.4 kg/m²; mean age 25.3 ± 3.7 years).

Study group 2 196 (98 females) unrelated German obese children (BMI ≥ 90 th percentile) and 99 (57 females) unrelated normal weight infants with inborn endocrine disorders were collected in the Department of Paediatric Endocrinology of the Charité Children's Hospital, Berlin.

Study group 3

- (a) 4261 German individuals (2171 females) who were included in the epidemiological study 'Cooperative Health Research in the Region of Augsburg' (KORA S4; 23) from 1999 to 2001 and who were representative of the population within the age range of 25–74 years in the city and region of Augsburg (Bavaria, Germany). The DNA of 4228 individuals was available. 4107 individuals have been successfully genotyped for rs133072 (call rate: 97%). 4056 genotyped individuals with complete phenotypic data (BMI, age and gender) were incorporated in association analyses. Initial analyses were based on comparison of allele and genotype frequencies in individuals with an age-specific BMI > 90 th percentile and ≤ 50 th percentile.
- (b) A second sample from the epidemiological survey 'Cooperative Health Research in the Region of Augsburg' (KORA S3) performed in 1994/1995 which has a comparable design to KORA S4 including 4856 German individuals (2451 females). The DNA of 4443 individuals was available. 4098 individuals have been successfully genotyped for rs133072 (call rate: 92%). 4074 genotyped individuals with complete phenotypic data (BMI, age and gender) were incorporated

in association analyses. Comparisons of allele and genotype frequencies were performed in individuals with an age-specific BMI > 90 th percentile and ≤ 50 th percentile.

Study group 4 231 men (BMI ≥ 31 kg/m²) with juvenile-onset obesity and 318 male controls (BMI < 31 kg/m²) from the draft board examinations carried out at 18–26 years of age (mean age 19.9 ± 1.9 years) in the eastern part of Denmark between 1953 and 1977 (24, 25). Within the draft board population, the criterion for the obese group corresponded approximately to the 99.5th percentile. DNA was sampled at a follow-up examination in 1998–2000.

Study group 5 468 obese (353 females: mean BMI 47.8 ± 7.8 kg/m²; mean age 43.3 ± 12.2 years) adults and 322 controls (166 females: mean BMI 21.5 ± 1.8 kg/m²; mean age 51.9 ± 10.8 years) were collected in the Laboratoire de Nutrition, Hotel Dieu in Paris, France.

Study group 6 187 trios (153 female index cases) based on 160 extremely obese cases of European American origin and 27 extremely obese cases of African American origin (mean BMI 50.5 ± 9.4 kg/m²; mean age 37.2 ± 9.3 years) and their parents associated in the Center for Neurobiology and Behavior of the University of Pennsylvania, Philadelphia, PA, USA.

Written informed consent was given by all participants and, in the case of minors, by their parents. The ethics committee of the University of Marburg and the local ethics committees of the participating investigators approved genotyping of obese subjects, their parents and controls.

Mutation screen

Single-stranded conformation polymorphism analysis (SSCP) was performed as described previously (26). PCR products of primers corresponding to amplicon 1 of exon 2 (see supplementary Table 1) were digested by both *AluI* and *MspI* (Fermentas, St. Leon-Rot, Germany). For analysis of the second amplicon of exon 2 *CrfI* 31 (Fermentas) was used. All amplicons were electrophoresed on 21% acrylamide gels (37.5:1; Q Biogene, Heidelberg, Germany). Gels were run at room temperature for 17 h at 4007 V and additionally at 4 °C for 18 h at 500 V. Gels were silver stained. PCR products of individuals showing aberrant SSCP patterns were sequenced bi-directionally on a LiCor 4200-2 automatic sequencer using the Base ImagIR 4.0 software (MWG Biotech, Ebersberg, Germany) as described previously (26) and by Sequence Laboratories (Seq Lab, Göttingen, Germany) respectively.

Association studies

For initial and confirmatory comparison of genotype and allele frequencies we used the Cochran–Armitage trend test and the asymptotic Pearson Chi-square test respectively.

TDTs

The initial, confirmatory and post hoc TDTs in the German and American families were carried out using the program Genehunter, version 2.0 beta (<http://www.fhcrc.org/labs/kruglyak/Downloads/index.html> (27)). Initially, association studies based on the index patients of the German trios had been performed, so that the TDT results are not independent. We performed haplotype TDTs with up to four markers for all 525 trios (study group 1a), where phase can be determined unambiguously.

Sequencing

A genomic region of 13 379 bp encompassing *MCHR1* was covered by eight overlapping PCR products named regions A–H (see supplementary Table 2). For each region four to eight nested/semi-nested PCRs were performed. PCR products were sequenced using PCR primers and BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Weiterstadt, Germany). Sequencing reactions were electrophoresed on ABI 377 automated sequencers. Base calling was performed using phred (28, 29). Sequence assembly was done using phrap (<http://www.phrap.org/phrap.docs/phrap.html>). Trace files were inspected visually in gap4 (30).

Genotyping of SNPs

Thirteen SNPs (see supplementary Tables 3 and 4) were genotyped by restriction fragment length polymorphism analyses (RFLP) or tetra-primer amplification refractory mutation system (ARMS; 31). SNP rs133072 was genotyped in study groups 1, 2 and 4–6 by PCR-RFLP (see supplementary Table 3). SNP rs133072 was genotyped in study group 3 by PCR followed by a MassEXTEND reaction and subsequent matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI–TOF MS) analysis according to the manufacturer's protocol (hME; Sequenom, San Diego, CA, USA). Four SNPs (SNP001745614, rs133063, rs133064 and SNP001745615) were genotyped by genomic sequencing as described above (see supplementary Table 5).

Haplotype frequencies

These were estimated for four SNPs (rs133068, rs133072, rs133073 and rs133074) in 525 trios (study group 1a) and linkage disequilibria between these SNPs were tested with the program EH, version 1.11 (<http://linkage.rockefeller.edu/ott/eh.htm>) (32).

Receptor constructs

A nested PCR in a pool containing all tissues of human MTC panel I (BD Clontech, Heidelberg, Germany) was performed (see supplementary Table 6). The PCR product was cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). All recombinant plasmids were re-sequenced as described above. Subsequently, the coding sequence of *MCHR1* starting with Met¹ was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen). To allow for immunological detection, *MCHR1* clones were tagged with an N-terminal 9 aa residue epitope (YPYDVPDYA) derived from influenza virus haemagglutinin protein (HA-tag) after Met¹. We subsequently refer to the numbering of aa according to the 422 aa receptor protein which is in agreement with the annotation in the UCSC genome browser (hg17), the Human Genome Variation database and dbSNP.

Heterologous expression

The *MCHR1* variants D32- and N32-*MCHR1* were expressed in transiently transfected COS-7 and HEK293 cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin, HEK293 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) with the same additives. Transient transfection was performed using metafectene (Biontex, Munich, Germany). Cell surface expression of the receptor variants was estimated using an indirect cellular ELISA as previously described (33). HA-tagged receptor variants were detected with a horseradish peroxidase (HRP)-labelled anti-HA monoclonal antibody (12CA5; Roche Diagnostics, Mannheim, Germany). Visualization was carried out using 3,3',5,5' tetramethylbenzidine as a substrate for HRP (1-Step Turbo TMB-ELISA; Pierce Biotechnology, Rockford, IL, USA).

Inositol phosphate (IP) formation

Transfected COS-7 cells were incubated with 2 µCi/ml myo-[2-³H]inositol (15.0 Ci/mmol; Amersham Pharmacia Biotech, Amersham, Bucks, UK) for 18 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl and stimulated with MCH (10^{-11} to 10^{-5} M) for 1 h at 37 °C. Agonist-induced increases in intracellular IP levels were determined as described (34).

Inhibition of cAMP accumulation

In HEK293 cells, D32- and N32-*MCHR1* were transiently coexpressed with the human β₂-adrenoceptor (β₂-AR). Cells were treated with 10 µM isoproterenol to stimulate cAMP formation and 1 µM MCH. As a positive control, β₂-AR was coexpressed

with the G_i-coupled M2 muscarinic acetylcholine receptor which was stimulated with 100 µM carbachol. HEK293 cells were labelled with 2 µCi/ml [2,8-³H]-adenine and intracellular [³H]cAMP was determined by anion-exchange chromatography (34, 35).

Western blot analysis

Activation of p42/44 MAP kinase (MAPK) was determined by Western blot analysis as previously described (36). Phosphorylated p42/44 MAPK was detected using a phosphospecific anti-p42/44 MAPK rabbit monoclonal antibody (Cell Signalling, Beverly, MA, USA), reblots as loading control were performed using an anti-p42 MAPK mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) binding to phosphorylated and unphosphorylated MAPK. Proteins were revealed by chemiluminescence (ECL; Amersham) using peroxidase-coupled secondary antibodies.

Promoter studies

Genomic fragments of 1181 bp (position 98 943–100 122 in GenBank entry Z86090, i.e. upstream of Met¹) were PCR amplified with primers introducing *NcoI* and *XhoI* restriction sites respectively (see supplementary Table 6) and directionally cloned into pGL3-Basic Luciferase reporter vector (Promega, Mannheim, Germany). PC12 rat pheochromocytoma cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and cultured in RPMI 1640 with 10% horse serum and 5% FCS (Biochrom, Germany) in collagen-coated flasks. Transfections and luciferase assays were performed and calculated as described (37), except for electroporation which was carried out with 3×10^6 cells in 0.15 ml and induction for which 1×10^6 cells/ml per well were cultured in the absence or presence of 500 µM dibutyryl-cAMP (Sigma) for 24 h.

Results

Mutation screen, initial and confirmatory association studies and transmission disequilibrium tests in German obese children and adolescents

Mutation screen SSCP screen of the *MCHR1* coding sequence (CDS) in 215 extremely obese children and adolescents (subgroup of study group 1a) and 230 underweight students (study group 1b) revealed 11 infrequent (allele frequencies <1%; Table 1) sequence variations leading to seven missense aa exchanges, six non-conservative and four silent sequence variations. In addition, two known SNPs were found (Table 1): rs133072 (G > A) introduces the non-conservative aa exchange D32N in the N-terminus of the 422 aa receptor protein whereas SNP rs133073

(T > C) is silent (N82). These SNPs are in tight linkage disequilibrium ($D' = 0.989$, $P < 0.001$).

Infrequent sequence variations All infrequent variations were detected in the heterozygous state. Five of the six non-conservative substitutions affect aa that are conserved between humans, mice and rats (D28V, T305M, R317Q, P377S and T411M). Three obese adolescents each harboured two missense variations (D28V and T411M). Since two of them inherited both variations from their mothers (DNA from the parents of the third patient was not available), these two variations form a haplotype [D28V; T411M]. Apart from the common missense variation D32N (rs133072) only one other missense variation (D28V) was detected in both extremely obese (one female: age 16.6 years; BMI 35.83 kg/m²; two males: age 18.83 years; BMI 44.56 kg/m² and age 15.33 years; BMI 41.33 kg/m²) and underweight individuals (age 22.80 years; BMI 19.15 kg/m² and age 23.16 years; BMI 16.80 kg/m²). Four infrequent missense variations occurred only in extremely obese individuals (BMI ≥ 99th percentile): T25M (one male: age 17.13 years; BMI 39.26 kg/m²), T305M (one female: age 13.11 years; BMI 43.32 kg/m²), R317Q (one male: age 15.33 years; BMI 43.24 kg/m²), T411M (one female: age 16.6 years; BMI 35.83 kg/m²; two males: age 18.83 years; BMI 44.56 kg/m² and age 15.33 years; BMI 41.33 kg/m²). Two infrequent missense variations (R210H and P377S; both affect conserved aa) were identified in underweight students only (R210H: one male: age 21.87 years; BMI 19.15 kg/m²; P377S: one female: age 23.40 years; BMI 16.54 kg/m²). For three of the obese carriers of non-conservative missense variations at conserved aa positions, DNA of one or both parents was also available and genotyped. Only one of the parents with such a variation had a BMI in the normal range (age 34.68 years; BMI 22.58 kg/m²; one of the mothers harbouring the [D28V; T411M] haplotype). The other mother with the [D28V; T411M] haplotype was obese (age 46.51 years; BMI 35.21 kg/m²). The father with the non-conservative missense variation T305M was extremely obese (age 41.86 years; BMI 36.11 kg/m²); a sib of the respective index patient with the same variation was also extremely obese (age 10.69 years; BMI 25.10 kg/m²). The father who transmitted the R317Q variation was also extremely obese (age 42.83 years; BMI 44.66 kg/m²).

Common polymorphisms; initial and confirmatory association studies Comparison of allele and genotype frequencies of both SNPs, rs133072 and rs133073, revealed initial association with obesity in 215 obese probands (subsample of study group 1a) versus 230 underweight probands (study group 1b; all two-sided P values <0.04), which was independently confirmed upon comparison of an independent obesity sample comprising 504 individuals (the remaining 405

Table 1 Infrequent variations and SNPs rs133072 and rs133073 in the *MCHR1* CDS detected by SSCP in 215 German extremely obese children and adolescents and 230 German healthy underweight students.

Study group	Nucleotide position†	Exon	Effect on aa sequence‡	Position within <i>MCHR1</i> *	Frequency of heterozygotes#
Extremely obese children and adolescents (<i>n</i> = 215)	100193 C > T	1	T25M	N-ter ED	0.005
	100202 A > T	1	D28V**	N-ter ED	0.014
	101966 C > T	2	Silent, Y211	IL 2	0.005
	102218 C > T	2	Silent, A295	TM 5	0.009
	102247 C > T	2	T305M	IL 3	0.005
	102283 G > A	2	R317Q	IL 3	0.005
	102491 G > A	2	Silent, T386	C-ter	0.005
	102565 C > T	2	T411M**	C-ter	0.014
	100213 G > A	1	D32N	N-ter ED	0.507
	100365 T > C	1	Silent, N82	N-ter ED	0.535
	100202 A > T	1	D28V	N-ter ED	0.009
Healthy underweight students (<i>n</i> = 230)	101962 G > A	2	R210H	IL 2	0.004
	102462 C > T	2	P377S	TM7	0.004
	102515 G > A	2	Silent, S394	C-ter	0.004
	100213 G > A	1	D32N	N-ter ED	0.465
	100365 T > C	1	Silent, N82	N-ter ED	0.491

†Human bacterial artificial chromosome (BAC) containing *MCHR1*, Z86090; ‡human *MCHR1* JC7080; *according to <http://www.ensembl.org/HomoSapiens/protview?peptide=ENSP00000249016>; #genotype-frequencies are in Hardy–Weinberg equilibrium; **three probands with a haplotype [D28V; T411M]; **bold italics**: SNPs rs133072 (100213 G > A) and rs133073 (100365 T > C); ED: extracellular domain; N-ter: N-terminal; TM: transmembrane domain; IL: intracellular loop; EL: extracellular loop; C-ter: C-terminal.

obese probands of study group 1a and the 99 subjects of study group 1d) with 96 normal weight probands (study group 1c; all one-sided *P* values < 0.03). The final comparison based on all 719 obese (study groups 1a and 1d) and 326 non-obese subjects (study groups 1b and 1c) substantiated association of the A-allele of rs133072 (39.1% in obese and 32.4% in controls) and of the C-allele of rs133073 (41.0% in obese and 33.6% in controls) with obesity (two-sided *P* values < 0.003; Table 2).

Common polymorphisms; TDTs In light of the confirmed association, a TDT based on 525 obese children (study group 1a), all of whom had been included in either the initial or confirmatory association study, and their 1050 parents was performed. Preferential transmissions of the alleles that were shown to be associated with obesity (A-allele of rs133072 and C-allele of rs133073) were observed (two-sided *P* values < 0.0003; Table 2). The transmission rates for the A-allele and the C-allele in the trio sample were 58.5% and 58.3% respectively.

Attempts to re-confirm association/transmission disequilibrium in additional study groups

Pursuing the hypothesis that the A-allele of SNP rs133072 is associated with obesity, we genotyped this SNP in additional German and non-German study groups.

- (1) 196 German obese children and 99 German normal weight controls from Berlin (study group 2). Comparison of these groups revealed both genotype and allele frequencies pointing in the

same direction as obtained in the respective obese and control Marburg study group 1; the one-sided *P* values were 0.076 and 0.085 respectively (A-allele 35.5% in obese and 29.8% in controls; Table 2).

- (2) 4056 adult unrelated German individuals representative of the city and region of Augsburg (study group 3a, KORA S4). Tests based on comparison of allele and genotype frequencies between those 393 individuals with a BMI > 90th percentile and those 2052 subjects with a BMI ≤ 50th percentile were negative; the corresponding one-sided *P* value was 0.26 (A-allele 37.2% in obese and 36.0% in controls). More detailed analyses based on subdivisions for age and BMI decile revealed trends for different allele frequencies between those 124 subjects with BMI > 90th percentile and the 633 subjects with a BMI ≤ 50th percentile in probands ≤ 40 years (A-allele 40.3% in obese and 36.3% in controls; one-sided *P* value 0.12; Table 2).
- (3) A second epidemiological sample including 4074 genotyped subjects (study group 3b, KORA S3). No significant differences in allele and genotype frequencies of SNPs rs133072 in 381 obese (BMI > 90th percentile) and 2055 controls (BMI ≤ 50th percentile) were detected. In contrast to our hypothesis and the finding in study group 3a, the frequency of the A-allele was higher in controls than in obese individuals (34.0% in obese and 35.2% in controls; one-sided *P* value = 1; Table 2).
- (4) 231 obese and 318 normal weight men from Denmark (study group 4). The frequency of the A-allele was higher in controls (35.3% in

Table 2 Association and transmission disequilibrium of SNP rs133072 (G > A).

Association	Genotype frequencies#			Allele frequencies	
	GG genotype	GA genotype	AA genotype	G-allele	A-allele
Study group 1					
Marburg obese individuals (<i>n</i> = 719)	261 (36.3%)	354 (49.2%)	104 (14.5%)	876 (60.9%)	562 (39.1%)
Marburg non-obese individuals (<i>n</i> = 326)	144 (44.2%)	153 (46.9%)	29 (8.9%)	441 (67.6%)	211 (32.4%)
Two-sided <i>P</i> value		0.0026†			0.0016‡
Study group 2					
Berlin obese children (<i>n</i> = 196)	79 (40.3%)	95 (48.5%)	22 (11.2%)	253 (64.5%)	139 (35.5%)
Berlin normal weight controls (<i>n</i> = 99)	46 (46.5%)	47 (47.5%)	6 (6.0%)	139 (70.2%)	59 (29.8%)
One-sided <i>P</i> value		0.076†			0.085‡
Study group 3a					
KORA S4 obese individuals (<i>n</i> = 393)	150 (38.1%)	194 (49.4%)	49 (12.5%)	494 (62.8%)	292 (37.2%)
KORA S4 non-obese individuals (<i>n</i> = 2052)	837 (40.8%)	954 (46.5%)	261 (12.7%)	2628 (64.0%)	1476 (36.0%)
One-sided <i>P</i> value		0.26†			0.26‡
KORA S4 age ≤40; obese individuals (<i>n</i> = 124)	44 (35.5%)	60 (48.4%)	20 (16.1%)	148 (59.7%)	100 (40.3%)
KORA S4 age ≤40; non-obese individuals (<i>n</i> = 633)	251 (39.7%)	304 (48.0%)	78 (12.3%)	806 (63.7%)	460 (36.3%)
One-sided <i>P</i> value		0.11†			0.12‡
Study group 3b					
KORA S3 obese individuals (<i>n</i> = 381)	169 (44.4%)	165 (43.3%)	47 (12.3%)	503 (66.0%)	259 (34.0%)
KORA S3 non-obese individuals (<i>n</i> = 2055)	863 (42.0%)	939 (45.7%)	253 (12.3%)	2665 (64.8%)	1445 (35.2%)
One-sided <i>P</i> value		1†			1‡
Study group 4					
Danish obese individuals (<i>n</i> = 231)	96 (41.6%)	107 (46.3%)	28 (12.1%)	299 (64.7%)	163 (35.3%)
Danish non-obese individuals (<i>n</i> = 318)	123 (38.7%)	149 (46.9%)	46 (14.5%)	395 (62.1%)	241 (37.9%)
One-sided <i>P</i> value		1†			1‡
Study group 5					
French obese individuals (<i>n</i> = 468)	194 (41.4%)	200 (42.8%)	74 (15.8%)	588 (62.8%)	348 (37.2%)
French non-obese individuals (<i>n</i> = 322)	137 (42.5%)	136 (42.3%)	49 (15.2%)	410 (63.7%)	234 (36.3%)
One-sided <i>P</i> value		0.372†			0.366‡
Transmission disequilibrium	Transmitted allele	Transmitted/non-transm.	Transm. rate (%)	<i>P</i> value	
Study group 1a					
Marburg trios (<i>n</i> = 525)	A	290/206	58.5	0.00016*	
Study group 6					
American trios (<i>n</i> = 187)	A	79/84	48.5	1**	

Genotype frequencies are in Hardy–Weinberg equilibrium.

†Asymptotic Cochran–Armitage-trend test; ‡asymptotic Pearson Chi-square test.

*Two-sided *P* value; **one-sided *P* value.

For description of the study groups please see Materials and methods.

obese and 37.9% in controls), which again was in contrast to our hypothesis (one-sided P value = 1; Table 2).

- (5) 468 adult obese individuals and 322 controls from France (study group 5). A slightly increased frequency of the A-allele in obese probands was found (37.2% in obese and 36.3% in controls; one-sided P value 0.37; Table 9).
- (6) A TDT based on 187 American adult obese patients and their 374 parents (study group 6) was negative. The A-allele of rs133072 was less frequently transmitted than the G-allele (79 transmitted and 84 non-transmitted; one-sided P value = 1; Table 9).

Identification of additional SNPs, definition of haplotypes and haplotype-based TDTs

To identify additional SNPs which are in linkage disequilibrium with rs133072 we re-sequenced the *MCHR1* transcribed region according to AF490537 as well as 9807 bp of its 5' region and the intron of 1214 bp in nine obese children and adolescents (subgroup of study group 1a) who were homozygous for A at SNP rs133072 and who had received at least one A-allele from a heterozygous parent, as well as in ten obese children and adolescents homozygous for G. This selection was based on the assumption that these two genotype-based groups should be most divergent for functionally relevant SNPs. Twenty SNPs were identified, 12 of which had been reported previously in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>; rs86708, rs133062, rs2032512, rs133063, rs133064, rs133068, rs133069, rs133070, rs133072, rs133073, rs133074 and rs3087592). The eight newly detected SNPs were submitted to the Human Genome Variation database (<http://hgvsbase.cgb.ki.se/>; 001745613, 001745614, 001745615, 001745616, 001745617, 001745618, 001745619 and 001745620; Fig. 1a).

All nine patients who were selected for homozygosity of the A-allele of rs133072 were also homozygous at 15 of the 20 SNPs; the ten obese individuals selected for homozygosity of the G-allele were homozygous for all SNPs except for 001745613, 001745616 and 001745620. This indicated the existence of two ancestral haplotypes differing in at least 13 sites spanning the re-sequenced region of 13 378 bp.

TDTs for 16 SNPs (including SNPs rs133072 and rs133073; Table 3) were performed in 61 trios (including the 19 individuals selected for homozygosity at rs133072) and/or in the total of 525 trios (subgroup of study group 1a). All but one of these SNPs revealed transmission rates greater than 60%.

Based on parental genotypes, haplotype frequencies were estimated as based on one SNP in the 5' region of *MCHR1* (rs133068), two in the CDS (rs133072,

rs133073) and one SNP in the 3' UTR (rs133074), which were genotyped in all 525 trios. P values for testing association between SNPs were below 10^{-4} for all combinations of two SNPs, and the combination of all four SNPs demonstrated strong linkage disequilibrium between these markers. The haplotype associated with obesity (rs133068/rs133072/rs133073/rs133074: G/A/C/T) was transmitted from 186 heterozygous parents and not transmitted from 117 parents (61.4%; $P = 0.000074$).

Functional in vitro studies

Upon heterologous expression in COS-7 cells, D32- as well as N32-MCHR1 were correctly inserted into the cell membrane and were detectable in equal amounts by cell surface ELISA with $92.5 \pm 18.0\%$ relative expression of N32- vs D32-MCHR1 (means $\pm$ S.E.M., $n \geq 3$, optical density for D32-MCHR1 = 100%). Following stimulation with MCH, both receptor proteins were functionally active as indicated by dose-dependent IP accumulation (Fig. 2), inhibition of isoproterenol-induced cAMP formation (see supplementary Fig. 1), and phosphorylation of p42/44 MAPK (see supplementary Fig. 2). Yet, with regard to IP formation no significant functional differences were detectable (Fig. 2), since both receptor variants showed equal basal activity and ligand potency (EC50 values 1.74 ± 0.45 nM and 3.37 ± 0.97 nM for N32- and D32-MCHR1 respectively). Similar results were obtained using HEK293 cells (data not shown).

To evaluate possible effects of SNPs located in the promoter region of *MCHR1*, genomic fragments encompassing 1.2 kb upstream of the start codon Met¹ were analysed, these represented either the haplotype associated with obesity (rs133068/rs133069/rs133070: G/A/G) or the haplotype not associated with obesity (C/C/A). There were no significant differences in either basal or cAMP-induced luciferase expression driven by the respective regulatory regions.

Discussion

We have detected association of an *MCHR1* haplotype with obesity in German children and adolescents. The identification of this haplotype was based on the association of SNPs rs133072 and rs133073 with obesity in 215 cases and 230 controls, which was independently confirmed in an additional 405 cases and 99 controls. These data are supported by TDT results obtained in up to 525 trios comprising a sub-sample of the 620 obese index patients.

A crucial requirement for positive association studies is confirmation in independent samples (38, 39). To substantiate our initially identified and independently confirmed positive association results we determined SNP rs133072 genotypes in five additional study groups

Table 3 TDTs for 16 adjacent SNPs in 61 and 525 trios respectively and for haplotypes including SNPs rs133072 and rs133073.

SNPs	61 trios (single marker)				525 trios (single marker)				525 trios (haplotypes formed by two ^a , three ^b and four ^c SNPs)			
	Trans allele ^s	Trans/non- trans	Trans rate (%)	Two- sided <i>P</i> value	Trans/non- trans	Trans rate (%)	Two- sided <i>P</i> value		rs133068	rs133072	rs133073	rs133074
rs133062 G>A	A	39/17	69.6	0.0033					^a 229/140 62.1% 0.000004#			
rs2032512 C>A	A	38/18	67.9	0.0075								
001745614 G>A	A	38/17	69.1	0.0046						^a 230/142 61.8% 0.000005		
rs133063 I>C	C	38/18	67.9	0.0075								
rs133064 G>A	A	39/17	69.6	0.0033							^a 188/123 60.4% 0.00023	
001745615 A>G	G	38/18	67.9	0.0075								
001745616 A>G	G	14/9	60.9	0.2971					^b 228/140 62.0% 0.000004			
001745617 C>T	T	25/16	61.0	0.1599								
001745618 T>C	C	28/15	65.1	0.0474						^b 190/117 61.9% 0.000031		
rs133068 C>G	G	39/18	68.4	0.0054	289/205	58.5	0.00016					
rs133069 C>A	A	38/18	67.9	0.0075					^c 186/117 61.4% 0.000074			
rs133072 G>A	A	37/18	67.3	0.0104	290/206	58.5	0.00016					
rs133073 T>C	C	38/17	69.1	0.0046	285/204	58.3	0.00025					
001745619 T>G	G	14/13	51.9	0.8474								
rs133074 C>T	T	27/18	60.0	0.1797	269/229		0.07306					
rs3087592 C>T	T	6/3	66.7	0.3173								

Study group 1a–d were analysed; SNPs are arranged from 5' to 3' of *MCHR1*; *sSNP rs133073; **cSNP rs133072 (D32N); §more frequently transmitted (trans) allele for all single SNP and haplotype-based TDTs; #numbers: transmitted (trans) non-transmitted (non-trans), transmission (trans) rate(%); two-sided *P* value; shaded SNPs form the ancestral haplotypes.

(study groups 2–6) comprised of obese children and adults and controls from within and outside of Germany.

The comparison of allele and genotype frequencies in the Berlin sample comprised of children (study group 2; Table 9) revealed trends similar to those observed in the Marburg sample, but the results were not significant. There was no association in an epidemiological German sample (study group 3a, KORA S4) comprising 393 subjects with a BMI in the highest decile and 2052 subjects with a BMI ≤ 50th percentile (Table 2). However, the initial positive finding was supported upon inclusion of younger probands only (≤ 40 years). In a second German epidemiological sample (study group 3b, KORA S3) there were no significant differences between allele and genotype frequencies of SNP rs133072 in obese and control individuals (Table 2).

In an adult French sample (study group 5), the A-allele of rs133072 was slightly more frequent in obese individuals but there was no significant association (Table 2). In contrast, the A-allele of rs133072 was less frequent in obese Danish probands than in controls (study group 4; Table 2). The TDT was negative in American trios (study group 6; Table 2).

These findings agree with Gibson *et al.* (16) who did not detect association of SNPs rs133072 or rs133073 with any obesity-related trait in adult white individuals (age 40–65 years) with type 2 diabetes mellitus and related metabolic disorders.

As our initial results were positive, we analysed the *MCHR1* in more depth, both genetically and functionally. We re-sequenced ~13.4 kb encompassing the *MCHR1*. Within the re-sequenced segment we identified

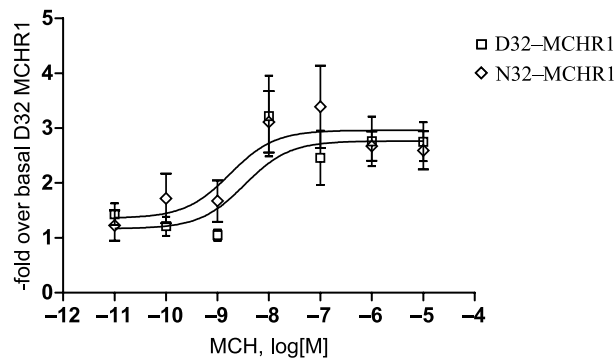


Figure 2 Functional characterization of SNP rs133072 in *MCHR1*. Dose-dependent formation of IPs upon stimulation of transfected COS-7 cells with MCH. Extraction of IP from whole cell lysates was performed by Dowex chromatography. IP formation was quantified by liquid scintillation counting. The value for unstimulated D32-MCHR1-transfected cells was set at 1.0. Each point represents the means $\pm$ S.E.M. of at least eight determinations.

two ancestral haplotypes which were deduced from 13 SNPs (see supplementary Table 7). Each of the ancestral haplotypes harbours seven additional SNPs.

In addition to SNPs rs133072 and rs133073 we detected 11 infrequent sequence variations in the CDS of which five non-conservative missense variations affect aa conserved between humans, mice and rats (Table 1). There was only one variation (D28V) detected in both lean and obese subjects. Because all other variations each occurred in either cases or controls, a role in weight regulation cannot be excluded but remains to be elucidated. We have not characterized the detected infrequent missense variations functionally, so that their role in weight regulation is currently unclear.

Two of the detected non-conservative missense variations (R317Q and T305M) co-segregated with obesity. R317Q was identified in an extremely obese female adolescent and her extremely obese father but not in her extremely obese mother (BMI 40.82 kg/m²; $\geq$ 99th percentile). Gibson *et al.* (16) identified this variation in one severely obese individual, and it co-segregated with obesity across two generations but there was no evidence for a functional relevance. The non-conservative missense variation T305M was detected in one extremely obese female adolescent, her extremely obese sister and her extremely obese father but not in her slightly obese mother (BMI 25.56 kg/m²; 80th percentile). Recently, the functional relevance of a number of aa variations of *MCHR1* was determined; however, none of the studied variations or regions from these studies overlap with the variations identified in this study (40–43).

Altered function of N32-MCHR1, i.e. altered IP formation, inhibition of cAMP formation and p42/44 MAPK activation compared with wild-type D32-MCHR1 was not detectable. Nonetheless, since *MCHR1* couples to multiple G proteins and thus instigates a

plethora of signalling pathways upon stimulation (44, 45), differences in receptor signalling apart from investigated parameters may exist. Also, variations in receptor function might be subtle and not easily discernible with current experimental setups.

In reporter gene assays, we found that *MCHR1* promoter fragments of $\sim$ 1.2 kb comprising either the haplotype associated with obesity or the non-relevant haplotype do not cause differences in either basal or cAMP-induced luciferase expression.

We have concluded that, in accordance with criteria for a solid association study in common disease (38, 39, 46), we have initially detected association for a highly plausible candidate gene (allele) with obesity, which we were able to independently confirm in a second case-control study. These results are supported by family-based studies. However, we cannot exclude the possibility that our original and confirmed association findings represent false positives, since attempts to re-confirm the association and functional studies were negative. It is conceivable that the association applies only to juvenile-onset obesity, conditional on a particular genetic and/or environmental background. In the light of the different results pertaining to *MCHR1* SNP rs133072 in six different study groups we consider epigenetic phenomena (47) as one possible explanation. Several genes are involved in the development of obesity and formal genetic studies indicate that both additive and non-additive gene effects contribute to the disease (48, 49). We suppose that a minor effect of the A-allele of rs133072 in *MCHR1* can, together with supposedly many other minor effects of sequence variations in different genes and/or their regulatory regions, lead to an increased susceptibility for obesity.

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