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Ghrelin Receptor Gene: Identification of Several Sequence Variants in Extremely Obese Children and Adolescents, Healthy Normal-Weight and Underweight Students, and Children with Short Normal Stature

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GH secretagogue receptor (GHSR, ghrelin receptor) is involved in regulation of body weight and GH secretion. We initially analyzed two single-nucleotide polymorphisms of the *GHSR* in up to 184 extremely obese children and adolescents and up to 184 healthy underweight students. The frequency of the 171T allele of rs495225 was higher in our obese samples (75.0%) than in the underweight individuals (70.2%; nominal $P = 0.14$). This trend could not be substantiated in an additional association study in 270 obese and 145 underweight and normal weight individuals and in a transmission disequilibrium test based on 387 obesity trios (transmission rate of 171T, 51.8%; nominal $P = 0.53$). Additionally, the coding region of *GHSR* was systematically screened, and seven sequence variants were identified in 93 obese, 96 normal weight, and 94 underweight individuals and 43 children with short normal stature (SNS). Five silent single-nucleotide polymorphisms showed similar genotype frequencies in the different weight groups and SNS children (all nominal $P > 0.3$). Two novel missense variants were detected only in one obese carrier and one SNS child, respectively. In conclusion, we did not obtain conclusive evidence for an involvement of the ghrelin receptor gene in body weight regulation or SNS in our study groups. (*J Clin Endocrinol Metab* 89: 157–162, 2004)

GH SECRETAGOGUE (GHS) receptor (GHSR, ghrelin receptor), the target of GHSs and ghrelin, is involved in regulation of food intake and body weight. Intracerebroventricular administration of synthetic GHS stimulated food intake in free-feeding rats (1). Ghrelin, a novel peptide isolated from rat stomach and subsequently found also in humans, was recently identified as the endogenous ligand for GHSR (2). Both intracerebroventricular and peripheral administration of ghrelin caused adiposity by increasing food intake and decreasing fat oxidation in rodents (3–5). Ghrelin enhances appetite and increases food intake in healthy men (6). Human ghrelin plasma levels are inversely correlated with body mass index (BMI); extremely high levels are observed in patients with anorexia nervosa (7, 8), and obese individuals have reduced levels (9). The expression of GHSR mRNA was observed at high levels in the arcuate nucleus and ventromedial nucleus of the hypothalamus and in the pituitary (10). Transgenic rats with impaired GHSR function in the arcuate nucleus had lower body weight and less adipose tissue than control rats (11).

GHSR, located on chromosome 3q26.31, was cloned from pituitary and hypothalamus of humans and swine in 1996 (12). The gene is highly conserved between humans, chimpanzees, pigs, cows, rats, and mice. Sequencing revealed that two types of GHSR cDNAs, 1a and 1b, originate from alternative mRNA processing of a single gene. The human full-length GHSR 1a cDNA consists of two exons, encoding 366 amino acids with seven transmembrane domains, a typical feature of G protein-coupled receptors. GHSR 1b, 289 amino acids with only five transmembrane domains, is encoded by an unspliced transcript. The codons 1–265 in *GHSR* 1b are identical with the first exon of 1a. After 265Leu, the 24 carboxy-terminal amino acids of *GHSR* 1b are encoded by the intron of 1a. GHSR 1a was demonstrated to confer high-affinity, specific binding of GHSs, whereas GHSR 1b failed to respond to GHSs (13). It is unknown whether *GHSR* 1b encodes a functional protein (14).

The human ghrelin gene has been analyzed in several association studies for obesity. Ukkola *et al.* (15) reported

Abbreviations: BMI, Body mass index; DHPLC, denaturing HPLC; GHS, GH secretagogue; GHSR, GHS receptor; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; SNS, short normal stature; SSCP, single-strand conformational polymorphism; TDT, transmission disequilibrium test.

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three variations in the genomic sequence of the *preproghrelin/ghrelin* in 96 Swedish obese female subjects and 96 controls. The *ghrelin* variant Arg51Gln was identified in six (6.3%) obese subjects but not at all in the controls, implying a role in the etiology of obesity (15). By screening 215 extremely obese German children and adolescents and 93 normal-weight controls, we previously identified four sequence variants in the coding region of the *ghrelin precursor*. However, none of the variants seemed to be involved in weight regulation (16). Additionally, association studies were performed in 3004 subjects by three different study groups, in which the Arg51Gln polymorphism could not be confirmed as associated with obesity. For a second polymorphism (Leu72Met), carriers of the Met72 allele seemed to be protected against fat accumulation and associated metabolic comorbidities (17). The inconsistent results obtained from the different association studies can possibly be attributed to factors such as sample size, stratification, subgroup analysis, multiple testing, selection of controls, *etc.* (18).

The GHSs/ghrelin-GHSR system not only is involved in weight regulation but also plays an important role in the regulation of GH secretion. GHSs stimulate GH secretion via GHSR distinctly from GH-releasing factor and somatostatin (12). Ghrelin was also demonstrated to stimulate GH secretion in rats (2, 19). GH secretion and plasma IGF-I levels were reduced in female transgenic rats with impaired GHSR function (11). Human stature is known to be highly heritable, with heritability estimates ranging from 75–90% (20). Genetic variation is involved in some syndromes that comprise short stature as one symptom (21). Short normal stature (SNS) without any pathological cause is a variant of normal growth rather than a disorder. Genes underlying normal variation in height are yet unknown.

Thus, *GHSR* is a candidate gene for both obesity and short normal stature in humans. We assumed that loss-of-function mutations in *GHSR* would potentially entail underweight and/or decrease GH secretion and therefore be involved in leanness or SNS. Alternatively, gain-of-function mutations could lead to obesity. We therefore investigated two single-nucleotide polymorphisms (SNPs) of this gene; based on the initial results, we performed an additional association study and a transmission disequilibrium test (TDT) (22) in 387 trios based on an obese index case for one of the SNPs. In parallel, the coding region of *GHSR* was screened for sequence variants in 93 extremely obese children and adolescents, 96 normal-weight students, 94 underweight students, and 43 children with SNS.

Subjects and Methods

Subjects

The ascertainment strategy for extremely obese, normal-weight, and underweight groups was described in detail previously (23). All extremely obese children and adolescents had an age- and gender-specific BMI $\geq$ 90th percentile as previously determined in a representative German population sample (24). The BMIs of the underweight students were below the 15th percentile and those of the normal-weight students between the 40th and 60th percentiles. We classified children as having SNS when their body height was <5th percentile (25) and no underlying illness or endocrinopathy could be detected.

Study groups. Altogether we studied 746 obese German children and adolescents [423 female; mean BMI, 32.9 $\pm$ 6.2 kg/m^2; mean height percentile, 63.4 (age- and sex-adjusted percentiles); mean age, 13.9 $\pm$ 2.6 yr], 232 underweight students (111 female; mean BMI, 18.3 $\pm$ 1.1 kg/m^2; mean age, 25.6 $\pm$ 3.8 yr), 96 normal-weight students (49 female; mean BMI, 21.9 $\pm$ 1.1 kg/m^2; mean age, 24.7 $\pm$ 2.6 yr), and 43 children with SNS (10 female; mean height percentile, 1.1; mean BMI, 17.4 $\pm$ 2.7 kg/m^2; mean age, 11.7 $\pm$ 3.1 yr) in first- and second-association studies, TDTs, and mutation screen.

Written informed consent was given by all participants and, in the case of minors, their parents. This study was approved by the Ethics Committees of the Universities of Marburg and Giessen.

Molecular genetic methods

High-throughput genotyping of two SNPs with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Assays for two SNPs (rs495225 and rs572169, dbSNP; National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/SNP) in the coding region of *GHSR* were designed by the SpectroDesigner software (Sequenom, San Diego, CA), and genotyping was done by MALDI-TOF MS, employing the homogeneous mass extension protocol as provided by the manufacturer of the system (Sequenom).

Genotyping of SNP rs495225 with PCR-RFLP. PCR with subsequent diagnostic restriction fragment length polymorphism analyses (RFLP) was performed for genotyping SNP rs495225. Primers were derived from the genomic sequence (AF369786; NCBI: http://www.ncbi.nlm.nih.gov) as follows: GHSRrs495225-F 5′-CGGGGTTCAACCTCACACT-3′ and GHSRrs495225-R 5′-AGAGCGCACCGCAAACTC-3′ (593 bp). PCR was performed according to standard protocols. The PCR amplicons were digested by *Lwe*I, with the 171T allele cut into two fragments (567 and 26 bp). Restriction patterns were visualized on 2.5% agarose gels stained with ethidium bromide.

Screen for sequence variants. 1) For PCR, as two transcripts of *GHSR*, 1a and 1b, originate from alternative mRNA processing of a single gene, we designed three primer pairs to amplify the entire coding region of both

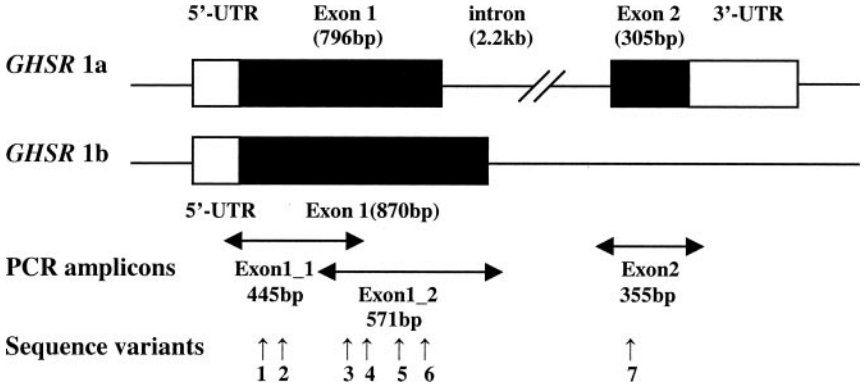

FIG. 1. Coding region of the human *GHSR* and position of PCR amplicons and identified sequence variants: 1, rs2232165 (60C>T); 2, rs495225 (171C>T); 3, rs2232169 (447C>G); 4, rs572169 (477G>A); 5, rs4988509 (531C>A); 6, SNP002901685 (611C>A); 7, SNP002901686 (837C>A).

GHSR 1a and 1b (Fig. 1): pair 1, exon 1_1-F 5′-GTCCCAGAGCCTGTTCAGC-3′ and exon 1_1-R 5′-GCTCAGCGCTGTGATGGT-3′ (445 bp); pair 2, exon 1_2-F 5′-CAGTGAGAGCTGCACCTACG-3′ and exon 1_2-R 5′-GAGAGACAGAGGCCCAGAGA-3′ (571 bp); pair 3, exon 2-F 5′-TCTCTCTCCCATTGTCCTTTT-3′ and exon 2-R 5′-CGGTGACTGTACTCGCAATG-3′ (355 bp). PCRs of exon 1_1 and exon 2 were performed according to standard protocols. The AmpliTaq Gold polymerase was used for PCR of exon 1_2. 2) Single-strand conformational polymorphism (SSCP) analysis was performed as described previously (26). Before SSCP, exon 1_1-amplicon, exon 1_2-amplicon, and exon 2-amplicon were digested by *Fsp*I, *Hpy*188III, and *Ear*I, respectively. The SSCP gels were run at both room temperature and 4 C. 3) To increase sensitivity of the screening, we reanalyzed exon 1_1-amplicons with denaturing HPLC (DHPLC) by using the WAVE system (Transgenomic, Cheshire, UK). Deduced from the WAVEMAKER 4.1 software, the oven temperature for optimal separation of homo- and heteroduplexes was set to 63 C. 4) All PCR amplicons with SSCP or DHPLC patterns deviant from the wild-type were sequenced as described previously (26). 5) For genotyping, PCR-RFLPs were performed for all different variants in the GHSR. *Bse*NI was used for RFLP of rs2232165 in exon 1_1 (fragments of 60T allele, 364 and 81 bp). *Hpa*II, *Fau*I, *Apa*I, and *Bpl*I were used, respectively, for four SNPs in exon 1_2 (fragments of 447G, 412 and 159 bp; 477A, 457 and 114 bp; 531C, 406 and 165 bp; 611A, 335 and 236 bp). *Afl*III was used for 837C>A in exon 2 (fragments of 837A, 291 and 64 bp). For validity of the genotypes, allele assignments were made by at least two experienced individuals independently. Discrepancies were solved unambiguously either by reaching consensus or by repeating.

Statistics

Cochran-Armitage trend tests were used to investigate differences in the genotype distribution between the study groups. Additionally, Pearson's χ^2 tests were used to study differences on the allele level. A TDT (22) for rs495225 was performed in 387 trios. Because the initial analyses of SNPs (rs495225 and rs572169) were carried out within a high-throughput genotyping of 39 SNPs in 26 candidate genes, several of which have been analyzed in independent samples, and no correction for multiple testing was done, all *P* values have to be considered as nominal.

Results

We initially analyzed genotype and allele distributions of two SNPs in *GHSR* (Table 1). Whereas no *P* values ≤ 0.05 were obtained for the comparison of extremely obese children and adolescents with underweight students for rs572169 (nominal $P = 0.65$) and rs495225 (nominal $P = 0.14$), the latter SNP revealed a trend in that the frequency of the 171T allele of rs495225 was higher in extremely obese children and adolescents (75.0%) than in underweight students (70.2%). To follow up we 1) performed an additional association study in 270 obese individuals and 145 normal-weight and underweight students and 2) investigated transmission disequilibrium in 387 trios for rs495225. We were not able to substantiate the initial trend with the additional association study (Table 1; nominal $P = 0.39$) and the TDT (the T-allele was transmitted to the obese offspring from 51.8% of the heterozygous parents, 161 transmissions *vs.* 150 nontransmissions; nominal $P = 0.53$).

In parallel, we performed a mutation screen of the entire coding region of the *GHSR* with SSCP or DHPLC in 93 extremely obese children and adolescents, 96 normal-weight and 94 underweight students, and 43 children with SNS. By sequencing of PCR amplicons showing aberrant SSCP or DHPLC pattern, we identified seven sequence variants (Fig. 1). We detected five SNPs that were in the database but not yet validated (NCBI: http://www.ncbi.nlm.nih.gov/locuslink/), including the two initially analyzed SNPs (rs495225 and rs572169). For these SNPs, no differences of genotype frequencies were found between obese and normal-weight, underweight, or SNS individuals (Table 2; all $P > 0.3$). Additionally, two novel variants [611C>A and 837C>A; these two novel sequence variants have been submitted to the HGVbase database (http://hgvbase.cgb.ki.se/) under accession nos. SNP002901685 and SNP002901686] were identified in one obese carrier and one child with SNS, respectively. The obese carrier harbors a C>A transition leading to amino acid exchange from Ala to Glu at codon 204. The 837C>A mutation in the child with SNS leads to Phe279Leu.

In a *post hoc* analysis we combined all genotypes for rs495225 from the investigated study groups (Table 3) and compared obese and nonobese study groups. There were no differences detected (all *P* values >0.2).

Discussion

To our knowledge, this is the first genetic study to elucidate the relationship between sequence variants in the *GHSR* and body weight regulation or SNS in humans. We initially investigated association with obesity for two SNPs (rs495225 and rs572169) within *GHSR*. The genotype frequencies of SNP rs495225 showed a trend toward association between 171T and obesity. Subsequent association studies were performed in additional samples, but the initial trend could not be substantiated. In parallel, we performed a TDT in 387 trios (obese index probands and both parents) for this SNP. The

TABLE 1. Genotype distributions[a] (percentages in *parentheses*) of two SNPs within *GHSR*

		rs 495225 (171T>C)				rs 572169 (477G>A)			
		N	TT	TC	CC	N	GG	GA	AA
Initial association study	Extremely obese children and adolescents	182[b]	101 (55.5)	71 (39.0)	10 (5.5)	180[b]	95 (52.8)	63 (35.0)	22 (12.2)
	Underweight students	183[b]	90 (49.2)	77 (42.1)	16 (8.7)	177[b]	82 (46.3)	79 (44.6)	16 (9.0)
Additional association study	Extremely obese children and adolescents	270	149 (55.2)	106 (39.3)	15 (5.5)				
	Normal-weight and underweight students	145	79 (54.5)	51 (35.2)	15 (10.3)				

N, Numbers of the samples with genotype data.

[a] Genotype frequencies were in Hardy-Weinberg Equilibrium except for the obese group for rs572169 ($P = 0.03$).

[b] These numbers are deviant from the initial numbers (184 extremely obese children and adolescents and 184 underweight students) because the call rates of the MALDI-TOF MS were slightly below 100%.

TABLE 2. Sequence variants within the *GHSR* in 93 extremely obese children and adolescents, 96 normal-weight and 94 underweight students, and 43 children with SNS

Accession no. in NCBI[a]	Base position[b]	Effect on amino acid sequence[b]	Position within the *GHSR*[c]	Genotype distribution in study groups[d]									
				Obese			Underweight			Normal-weight			Short normal stature		
				11[e]	12[f]	22[g]	11[e]	12[f]	22[g]	11[e]	12[f]	22[g]	11[e]	12[f]	22[g]
rs2232165	60C>T	Silent	N-ter ED	87 (93.6)	6 (6.5)	0 (0.0)	88 (93.6)	6 (6.4)	0 (0.0)	91 (94.8)	5 (5.2)	0 (0.0)	42 (97.7)	1 (2.3)	0 (0.0)
rs495225	171T>C	Silent	TM1	46 (49.5)	39 (41.9)	8 (8.6)	44 (46.8)	41 (43.6)	9 (9.6)	53 (55.2)	33 (34.4)	10 (10.4)	28 (65.1)	12 (27.9)	3 (7.0)
rs2232169	447C>G	Silent	IL2	88 (94.6)	5 (5.4)	0 (0.0)	88 (93.6)	6 (6.4)	0 (0.0)	91 (94.8)	5 (5.2)	0 (0.0)	42 (97.7)	1 (2.3)	0 (0.0)
rs572169	477G>A	Silent	IL2	49 (52.7)	32 (34.4)	12 (12.9)	40 (42.5)	42 (44.7)	12 (12.8)	44 (45.8)	43 (44.8)	9 (9.4)	22 (52.2)	16 (37.2)	5 (11.6)
rs4988509	531C>A	Silent	TM4	92 (98.9)	1 (1.1)	0 (0.0)	93 (98.9)	1 (1.1)	0 (0.0)	96 (100.0)	0 (0.0)	0 (0.0)	42 (97.7)	1 (2.3)	0 (0.0)
SNP002901685	**611C>A**	**Ala204Glu**	**EL2**	**One carrier with obesity**											
SNP002901686	**837C>A**	**Phe279Lcu**	**TM6**	**One carrier with short normal stature**											

N-ter, N-terminal; ED, extracellular domain; TM, transmembrane domain; IL, intracellular loop; EL, extracellular loop; C-ter, C-terminal. Two novel variants are shown in *bold*.

[a] Nomenclature according to Ref. 27.

[b] See Ref. 13 *GHSR* 1a for numbering of genomic sequence and amino acid position.

[c] Positions within the *GHSR* are deduced from Ref. 12.

[d] Genotype distributions (percentages in *parentheses*) are in Hardy-Weinberg equilibrium. None of the comparisons among groups rendered a P value < 0.3.

[e] Homozygotes for the more frequent allele; [f] Heterozygotes; [g] homozygotes for the less frequent allele.

TABLE 3. Genotype distribution[a] of rs495225 in all study groups

	Genotype frequencies		
	TT	TC	CC
Extremely obese children and adolescents (n = 746)	397 (53.2)	296 (39.7)	53 (7.1)
Underweight students (n = 232)	116 (50.0)	95 (41.0)	21 (9.0)
Normal-weight students (n = 96)	53 (55.2)	33 (34.4)	10 (10.4)
Children with short normal stature (n = 43)	28 (65.1)	12 (27.9)	3 (7.0)

[a] Genotype frequencies are in Hardy-Weinberg Equilibrium; none of the comparisons among groups rendered a P value <0.2.

171T allele was transmitted only slightly more frequently to obese offspring. The test result was not statistically significant so that we could not confirm the initial trend.

Additionally, we screened the entire coding region of the *GHSR* in individuals belonging to the different weight groups and children with SNS for sequence variants. Besides the two SNPs (rs495225 and rs572169) tested initially, we identified five additional variants, including three SNPs and two novel variants (SNP002901685 and SNP002901686). All sequence variants were identified in the shared region of *GHSR* 1a (exon 1) and 1b, except for the SNP002901686 (837C>A) which is located in exon 2 of *GHSR* 1a. The novel variants appeared in only one obese carrier (Ala204Glu) and one child with SNS (Phe279Leu). We did not detect differences in genotype frequencies of the identified SNPs between obese and normal-weight or underweight individuals, indicating that none of them seems to be associated with obesity in Germans. Despite not finding any significant association, identification and validation of these variants in the ghrelin receptor gene might be important for additional studies in other populations and be helpful for future phamocogenomics analyses.

Based on the previous studies, the GHSs/ghrelin-GHSR system may be important in weight regulation by influencing food intake and energy balance (3–6). Recent studies indicated ghrelin and GHSR play an important role on rat adipogenesis (27), and antagonism of ghrelin receptor reduces food intake and body weight in mice (29). However, the results of association studies pertaining to the human ghrelin gene were conflicting (15–17). Additionally, our group performed an association study of a *ghrelin* promoter polymorphism (rs27647), in which similar genotype frequencies were detected in 186 obese and 170 underweight individuals (unpublished data). Therefore, the relevance of *GHSR* and *ghrelin* for obesity awaits further clarification.

The novel variant Ala204Glu (SNP002901685) leads to a nonconservative amino acid exchange in the obese carrier. The 204Ala is located in the second extracellular loop, highly conserved among humans, swine, and rats. Any functional relevance of this nonconservative variant could be detected only in functional studies. Additionally, we detected a child with SNS with a novel variant (Phe279Leu, SNP002901686) leading to a conservative amino acid exchange in the 6th transmembrane domain of GHSR. The Phe279 is one of the essential amino acid residues in the GHSR with high con-

servation among humans, swine, and rats. Coincidentally, this mutant has been studied by site-directed mutagenesis. The 279Leu variant was described to exert decreased specific binding properties for a GHSR agonist ([^{35}S]MK-0677) compared with wild type (30). It is likely that this variant causes reduced function of the GHSR and might hence play a role in the etiology of SNS in the respective carrier. Interestingly, we found the mother of this SNS child, who also harbors the variant, is both short (height, 1.57 m) and obese (BMI, 34.6 kg/m^2). Although these two variants have been observed only in one obese carrier and one SNS child, respectively, it is possible that association may be found with either an obese or short stature phenotype in an additional study with a different population or with a larger number of individuals.

No significant differences of allele or genotype frequencies were detected for all SNPs in *GHSR* between children with SNS and any other study group. Hence, no evidence for an association of any of the polymorphic sites with SNS could be detected. However, considering there were only 43 patients in our SNS group, this study is not a comprehensive analysis of the relationship between SNS and *GHSR*.

In conclusion, we identified seven sequence variants in the coding region of *GHSR* in different weight groups or SNS children. None of the SNPs seems to influence weight regulation or GH secretion. The two novel variants, which were detected in one obese carrier and one child with SNS only, await additional studies. We did not get conclusive evidence for an involvement of the *GHSR* in body weight regulation or SNS in our study groups.

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