CLINICAL STUDY

Novel inactivating mutations in the GH secretagogue receptor gene in patients with constitutional delay of growth and puberty

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Abstract

Background: A limited number of mutations in the GH secretagogue receptor gene (*GHSR*) have been described in patients with short stature.

Objective: To analyze *GHSR* in idiopathic short stature (ISS) children including a subgroup of constitutional delay of growth and puberty (CDGP) patients.

Subjects and methods: The *GHSR* coding region was directly sequenced in 96 independent patients with ISS, 31 of them with CDGP, in 150 adults, and in 197 children with normal stature. The pharmacological consequences of *GHSR* non-synonymous variations were established using *in vitro* cell-based assays.

Results: Five different heterozygous point variations in *GHSR* were identified (c. – 6 G>C, c.251G>T (p.Ser84Ile), c.505G>A (p.Ala169Thr), c.545 T>C (p.Val182Ala), and c.1072G>A (p.Ala358Thr)), all in patients with CDGP. Neither these allelic variants nor any other mutations were found in 694 alleles from controls. Functional studies revealed that two of these variations (p.Ser84Ile and p.Val182Ala) result in a decrease in basal activity that was in part explained by a reduction in cell surface expression. The p.Ser84Ile mutation was also associated with a defect in ghrelin potency. These mutations were identified in two female patients with CDGP (at the age of 13 years, their height SDS were – 2.4 and – 2.3). Both patients had normal progression of puberty and reached normal adult height (height SDS of – 0.7 and – 1.4) without treatment.

Conclusion: This is the first report of *GHSR* mutations in patients with CDGP. Our data raise the intriguing possibility that abnormalities in ghrelin receptor function may influence the phenotype of individuals with CDGP.

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Introduction

The GH secretagogue receptor (*GHSR*, OMIM *601898) is a member of the G protein-coupled receptor (GPCR) superfamily characterized by a seven transmembrane domain structure. There are two isoforms of *GHSR*: *GHSR1a*, which is active, and *GHSR1b*, which is truncated and has no known biological activity (1). Our manuscript is focused on *GHSR1a* that will subsequently be referred to as '*GHSR*'. This receptor is mainly expressed in the hypothalamus and pituitary (1) and is characterized by a high level of constitutive activity (2). Ghrelin is the endogenous ligand of the *GHSR* and it is primarily secreted by gastric cells (3). Ghrelin has recently emerged as a pleiotropic

neuroendocrine modulator involved in a wide spectrum of biological functions. Through interaction with *GHSR1a*, ghrelin stimulates GH secretion and has a potent orexigenic effect (4). Two major forms of ghrelin have been demonstrated in the circulation: unacylated-ghrelin, which is the main circulating form, and acyl-ghrelin, which is the active form generated by octanoyl incorporation at Ser3, a process mediated by ghrelin O-acyltransferase (5, 6). This acylation is essential for binding to the *GHSR1a* and for most recognized endocrine actions of ghrelin.

Recently, mutations in the *GHSR* have been implicated in the etiology of short stature in humans (7–9). Pantel *et al.* (7) described the missense mutation p.Ala204Glu in the second extracellular loop of the *GHSR1a* in two

unrelated families from Morocco. In the first family, the defect was associated with idiopathic short stature (ISS) and in the second family with isolated GH deficiency (GHD). Wang *et al.* (9) described this same mutation in an obese child. In addition, this group reported another *GHSR* mutation (p.Phe279Leu) in a boy with ISS as well as in his obese, short mother (9). These first reports suggest that *GHSR* inactivating mutations may cause short stature and impairment of GH secretion with variable severity and penetrance (7). In 2009, Pantel *et al.* (8) reported an isolated GHD patient with delayed puberty who was compound heterozygous for two *GHSR* mutations (p.Trp2X and p.Arg237Trp). Interestingly, the patient's father, who was heterozygous for the nonsense mutation, also had delayed puberty (8). More recently, a Japanese group described four novel heterozygous *GHSR* mutations (p. Gln36del, p.Pro108Leu, p.Cys173Arg, and p.Asp246Ala) in a group of patients with GHD or ISS (10). Unfortunately, no clinical and laboratory data from these patients were given. All described *GHSR* missense mutations markedly decreased the constitutive activity of the receptor, but some of these mutations preserved its ability to respond to ghrelin (7, 8, 11), suggesting the importance of *GHSR* basal activity for growth (12). In addition, two recent large genome-wide association studies demonstrated a strong association between *GHSR* loci (3q26.3) and height determination (13, 14).

The objective of this study was to investigate the presence of *GHSR* mutations in a group of ISS patients including a subgroup of patients with constitutional delay of growth and puberty (CDGP).

Patients and methods

Subjects

This study was approved by the local ethics committee, and the patients or guardians gave their written informed consent. Subjects in this study included 96 independent Brazilian patients with ISS (64 males), who fulfilled the following diagnostic criteria: proportional postnatal short stature, height more than 2.5 SDS below the normal mean height for age and sex (15), unremarkable medical history, and absence of abnormal findings on clinical examination or in laboratory tests that could account for short stature (16). Routine laboratory tests included blood cell count, erythrocyte sedimentation rate, electrolytes, albumin levels, kidney and liver function tests, karyotype (in all female patients), celiac disease screening, and free thyroxine and TSH levels. All children had adequate nutritional status, as assessed by interviews with parents or guardians, showed absence of signs of malnutrition, and satisfied normal laboratory parameters. All patients had normal GH secretion as assessed by GH peak after provocative testing with clonidine or insulin (17).

A total of 83 ISS patients (88%) had started puberty prior to the initiation of the genetic studies. According to the age of puberty onset, 31 patients (24 males) were subcategorized as presenting with CDGP. The diagnosis of CDGP was based on lack of breast development (Tanner stage 2) by the age of 13 years in girls and testicular volume <4.0 ml by the age of 14 years in boys, absence of other identifiable causes of delayed puberty, delayed bone age (BA), as well as spontaneous and complete achievement of pubertal development during follow-up (18). The complete pubertal development was established by regular menses in female and normal adult testosterone levels in male CDGP patients.

We also studied as a control group 150 adults (45% males) with normal stature (height SDS of 0.3 ± 1.1) and 197 children (64% males) without growth impairment (mean age of 10.7 ± 1.5 , height SDS of 1.0 ± 1.0) with the same ethnic background.

Hormonal studies

GH was measured by immunofluorometric assay (AutoDELFIA, PerkinElmer, Waltham, MA, USA) with MABs. The cutoff levels used to rule out GHD diagnosis after stimulation test were peak GH levels $> 3.3 \mu\text{g/l}$ (17). IGF1 was measured by chemiluminescence assays (IMMULITE, Diagnostic Products Corporation – DPC, Los Angeles, CA, USA) and expressed as SDS for age and sex according to reference values provided by the assay kit. Active ghrelin (acylated ghrelin) levels were measured using a commercial ELISA kit (Millipore, St Charles, MO, USA). Blood samples were collected from a forearm vein in the morning after overnight fasting and again 60 min after intake of a high carbohydrate meal. Whole blood samples were collected in polypropylene tubes and a dipeptidyl peptidase IV inhibitor (Millipore) at a final concentration of 100 μM was immediately added. The clotted blood was then centrifuged for 15 min at $4 \pm 2^\circ\text{C}$, plasma was separated and acidified by addition of HCl to a final concentration of 0.05 M, and stored at -80°C until being assayed.

Molecular studies

Genomic DNA was extracted from peripheral blood leucocytes, and the entire coding region as well as the exon–intron boundaries of *GHSR* (GenBank accession number NM_198407.2) was PCR amplified in all patients and control group. The *GHSR* proximal promoter region (1 kb) (19) was also amplified in patients if *GHSR* allelic variants in the coding region were identified. Primer sequences and amplification protocols will be sent on request. PCR products were bidirectionally sequenced with the dideoxy chain-termination method using a dye terminator kit and analyzed in an ABI Prism 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA).

In silico prediction of mutation effects

To identify the potential effects of sequence variants identified in *GHSR* on splice and protein function or structure, the wild-type (WT) and variant sequences were submitted to Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) (20), SpliceView (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html) (21), and a new version of the PolyPhen method (<http://genetics.bwh.harvard.edu/pph>) (22).

Functional studies

Materials Ghrelin was purchased from Bachem (Bubendorf, Switzerland). Cell culture media, fetal bovine serum, and lipofectamine reagent were obtained from Invitrogen. Peroxidase-conjugated, anti-hemagglutinin (HA) MAB (3F10) and BM-blue, a peroxidase substrate, were purchased from Roche Applied Science. The plasmid encoding the serum response element (SRE) luciferase reporter gene has been described previously (23).

Construction of human *GHSR* plasmids The constructs encoding the untagged and HA-tagged WT human *GHSR* cDNA (isoform 1a) were reported previously (11). Missense mutations were introduced into both template cDNAs (i.e. untagged and HA-tagged receptors) using oligonucleotide-directed site-specific mutagenesis as described previously (24, 25). For each mutant, the presence of the indicated amino acid change was confirmed by sequence analysis of the full protein coding region of each construct.

Cell culture Human embryonic kidney (HEK) 293 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. The cells were maintained at 37 °C in a humidified environment containing 5% CO₂.

Luciferase reporter gene assay Receptor-mediated signaling was assessed using a luciferase assay as described previously (11, 23, 26). In brief, HEK293 cells were plated at a density of 1000–2000 cells/well onto clear-bottom, white 96-well plates and grown for 2 days to ~80% confluency. Cells were then transiently transfected using LipofectamineR reagent (Invitrogen) with cDNAs encoding i) a WT or mutant *GHSR*1a (or an empty expression vector), 2 ng/ well, ii) a serum-responsive element-luciferase reporter gene (SRE_{5X}-luc), 30 ng/well, and iii) β-galactosidase, 5 ng/well, to enable correction of interwell variability. After 24 h of transfection, cells were stimulated for 4 h with ghrelin diluted in serum-free medium. Ligand potencies were determined by stimulating receptor-expressing cells with increasing concentrations of ghrelin. The medium

was gently aspirated following ligand treatment and luciferase activity was measured using SteadyliteR reagent (PerkinElmer, Boston, MA, USA). A β-galactosidase assay was then performed after adding the enzyme substrate, 2-nitrophenyl β-D-galactopyranoside. Following incubation at 37 °C for 30–60 min, substrate cleavage was quantified by measurement of optical density at 420 nm using a SpectraMaxR microplate reader (Molecular Devices, Sunnyvale, CA, USA). Corresponding values were used to normalize the luciferase data.

Assessment of receptor expression using ELISA

The expression levels of the *GHSR* variants were determined using a procedure described by Fortin *et al.* (27). In brief, HEK293 cells grown in 96-well plates were transiently transfected with a plasmid encoding either an HA-tagged WT or mutant ghrelin receptor, 2 ng/well. After 48 h of transfection, the cells were washed once with PBS, pH 7.4, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS/100 mM glycine, the cells were incubated for 30 min in blocking solution (PBS/20% bovine serum). An HRP-conjugated MAB (Roche; clone 3F10) directed against the HA-epitope was then added to the cells (1:500 dilution in blocking solution). After 1 h, the cells were washed five times with PBS, and BM-blue (3,3',5,5'-tetramethylbenzidine, Roche) solution (50 µl/well) was added. After incubation for 30 min at room temperature, conversion of this substrate by antibody-linked HRP was terminated by adding 2.0 M sulfuric acid (50 µl/well). Converted substrate (which correlates with the amount of receptor) was assessed by measuring light absorbance at 450 nm using a SpectraMaxmicroplate reader (Molecular Devices).

Data analysis GraphPad Prism software version 5.0 (GraphPad, San Diego, CA, USA) was used for non-linear curve fitting of receptor signaling and for calculation of half-maximal effective concentrations (EC₅₀ values). Each EC₅₀ value (expressed as a molar concentration) was transformed to a pEC₅₀ value; pEC₅₀ = –log(EC₅₀). The mean pEC₅₀ values ± S.E.M. are shown. The pEC₅₀ and surface expression values for each of the mutants were compared with the corresponding control values at the WT receptor using one-way ANOVA followed by Dunnett's post-test (GraphPad INSTAT software).

Statistical analysis

Differences between groups were tested by *t*-test or Kruskal–Wallis and χ^2 or Fisher exact test, as appropriate. Statistical analyses were performed using the SIGMA stat statistical software package (Windows version 3.5; Systat Software, Inc., Erkrath, Germany).

Table 1 Clinical characteristics of patients with ISS with or without CDGP selected for the study.

	Idiopathic short stature (ISS)		
	CDGP	Non-CDGP	Total
Number of patients	31	65	96
Males (%)	77	61	67
Target height SDS	-1.2 ± 0.6	-1.3 ± 0.6	-1.3 ± 0.6
Family history of SS (%) ^a	52	49	50
Data on first evaluation			
Chronological age (years)	$13.5 \pm 2.6^*$	9.4 ± 3.6	10.7 ± 3.8
Bone age (years)	$10.7 \pm 2.7^*$	7.2 ± 3.7	8.3 ± 3.7
Bone age delay (years)	$3.1 \pm 1.1^*$	2.3 ± 1.2	2.6 ± 1.3
Height SDS	$-3.1 \pm 0.9^*$	-2.7 ± 0.8	-2.8 ± 0.9
BMI SDS	$-1.7 \pm 1.5^*$	-0.6 ± 1.1	-1.0 ± 1.4
IGF1 SDS	$-1.6 \pm 1.5^*$	-0.8 ± 0.9	-1.0 ± 1.2
Age at start of puberty ^b			
Female	$13.5 \pm 0.6^*$	11.1 ± 1.2	11.7 ± 1.5
Male	$15.3 \pm 1.5^*$	12.7 ± 1.0	13.8 ± 1.8

SS, short stature; * $P < 0.05$ in comparison with non-CDGP group.

^aHeight SDS < -2.0 in any one of the parents.

^bBreast development (Tanner stage 2) in girls and testicular volume > 4.0 ml in boys.

Results

Patients' characteristics

Clinical characteristics of the patients are shown in Table 1. The cohort was characterized by a male predominance, especially in the CDGP group. At the first evaluation for short stature, patients from the CDGP group were older; had shorter height, lower body mass index (BMI) SDS, and lower IGF1 levels; and had more marked delayed BA when compared to patients with normal puberty.

Molecular results

In ISS patients, five different heterozygous variations in *GHSR* were identified, all of them in patients with CDGP (three males and two females). Of the five variations, one is located in the 5'-UTR, 6 bp prior to the initiation codon (c.-6 G>C). The other four variations (p.Ser84Ile (c.251G>T), p.Alal69Thr (c.505G>A), p.Val182Ala (c.545 T>C), and p.Alal358Thr (c.1072G>A)) are missense and all of them but p.Alal358Thr predict amino acid changes in highly conserved residues in *GHSR*. The protein location of the amino acid substitutions is indicated in Fig. 1. No additional variations were identified in the *GHSR* promoter region in these patients. *In silico* analysis did not predict changes in the physiological acceptor or donor *GHSR* splice sites by these allelic variants; in contrast, analysis by PolyPhen (22) suggested that p.Alal69Thr and p.Alal358Thr are benign, whereas p.S84I and p.Val182Ala are predicted to be probably and possibly damaging respectively. All the missense variations were selected for *in vitro* functional evaluation.

These allelic variants were not found in 694 alleles from controls (adults and normal height children). In addition, no other mutations were identified in the entire *GHSR* coding sequence in the normal height children (197 individuals sequenced). Notably, the frequency of mutation observed in the CDGP group was higher than that expected by chance in contrast with ISS children ($P = 0.003$) and control children ($P < 0.001$).

Functional studies

The Ser84Ile *GHSR* missense mutation alters ghrelin potency Ghrelin failed to increase signaling activity in HEK293 cells transfected with the empty plasmid pcDNA1 (Fig. 2), suggesting absence of an endogenous *GHSR*. In contrast, in cells expressing recombinant *GHSR* variants, stimulation with ghrelin triggered a concentration-dependent increase in receptor-mediated signaling. Agonist potency was comparable at the WT, Val182Ala, Ala169Thr, and Ala358Thr receptors (Fig. 2 and Table 2). In contrast, the Ser84Ile variant displayed a significant reduction in ghrelin potency/efficacy.

The Ser84Ile and Val182Ala mutations show decreased basal activity that correlates with reduced cell surface expression Consistent with previous studies using an SRE-luciferase reporter gene assay (2, 11), the WT-*GHSR* exhibited a high level of constitutive activity (i.e. signals in the absence of agonist). The Val182Ala variant showed $\sim 50\%$ reduction in basal activity relative to WT. Trace, if any, residual basal activity was observed for the Ser84Ile mutant. In contrast, the Ala169Thr and Ala358Thr had a basal activity level comparable to the WT *GHSR* (Fig. 2 and Table 2). In parallel experiments, cell surface expression levels of variant receptors were determined by ELISA using HA-tagged versions of the WT and mutant *GHSR* isoforms (Fig. 3

Figure 1 Schematic representation of *GHSR* and the location of missense variations within the receptor protein. The black circles indicate the variants described in this study. The white circles indicate the mutations already described in the literature. The underlined are mutations with proven functional impairment.

Figure 2 Selected GHSR variants show altered basal receptor activity and/or ghrelin potency. HEK293 cells were transiently transfected with a receptor-encoding cDNA, together with a SRE-Luc reporter gene construct. After 24 h of transfection, cells were stimulated for 4 h with media containing either no peptide (basal) or increasing concentrations of ghrelin. Following stimulation, luciferase activity was quantified as described in Patients and methods section. All activity values were normalized relative to the ghrelin-stimulated maximum at the wild-type GHSR. Data represent the mean $\pm$ s.e.m. from at least three independent experiments, each performed in triplicate.

and Table 2). Both tagged and untagged WT receptors showed comparable pharmacological properties (data not shown). These studies revealed that receptors with reduced basal signaling (Val182Ala and Ser84Ile) were also expressed at a significantly lower density relative to the WT GHSR (100%). Consistent with the major loss of signaling observed with the Ser84Ile variant, this receptor also displayed very poor cell surface expression. The Val182Ala mutant showed an intermediate level of basal activity and receptor expression. Pharmacological abnormalities (i.e. decreased basal activity and/or potency) of the Val182Ala and Ser84Ile were detected for both tagged and untagged receptors (data not shown). In contrast, each of the GHSR variants with normal basal activity (Ala169Thr and Ala358Thr) showed surface expression comparable to WT.

Phenotype/genotype relationships

Patient with p.Ser84Ile mutation The mutation p.Ser84Ile was identified in a female patient (patient 1) with CDGP. Clinical and laboratory data are shown in Table 3. At her first appointment at 12.8 years, she had short stature with delayed BA and low BMI (height SDS = -2.4, BA = 11 years, and BMI SDS = -2.6). Her hormonal evaluation showed reduced IGF1 and IGFBP3 levels but a normal GH response to the clonidine test. She started puberty just after this first visit at 13 years and had normal pubertal development with menarche at 16 years. She achieved a normal final height of 157.6 cm (-0.7 s.d.) without GH treatment (her target height was 158.1 (-0.7 s.d.)) but maintained a low BMI of 18.4 kg/m². At adult age, her IGF1 levels increased to normal, but her IGFBP3 levels remained low.

Gonadotropins and estradiol levels were at prepubertal range at the first evaluation and reached adequate levels by the end of puberty (Table 3). She had normal levels of basal ghrelin and adequate suppression after the high-carbohydrate breakfast meal. No abnormalities in glucose homeostasis were observed. At the age of 21 years, she gave birth to a healthy female child. The patient's mother and her daughter had a normal GHSR genotype. In contrast, her two siblings who had normal stature and puberty were also heterozygous for the same mutation. Her father was not available for genetic studies.

Patient with p.Val182Ala mutation Clinical and laboratory data of the CDGP female patient with the p.Val182Ala mutation (patient 2) are shown in Table 3. At her first evaluation, she was 13 years old with a BA of 11 years and she had just started puberty. Her height was compromised (height SDS = -2.5) but her weight was normal (BMI SDS = 1.0). She had IGF1 and IGFBP3 levels within the reference range, a normal GH peak after clonidine stimulation and LH, FSH, and estradiol levels within the prepubertal range. She had normal pubertal development, her menarche occurred at 14 years, and she achieved, without GH treatment, a normal final height of 154 cm (-1.4 s.d.), but below her target height of 161.6 cm (-0.1 s.d.). The patient's father and sister are also heterozygous for the GHSR mutation. Both of these individuals reported short stature during the prepubertal period as well as delayed puberty and reached normal adult height, a growth pattern compatible with CDGP. In addition, the sister of patient 2 was treated with recombinant human GH for GHD (maximum GH peak at stimulation test of 1.8 μ g/l at the age of eleven), reaching a normal adult height of 155 cm (-1.2 s.d.).

Discussion

We report five new GHSR variations in patients with CDGP, all of them absent in a large ethnically matched population. Each amino acid substitution except for p.Ala358Thr occurred at a highly conserved position within the GHSR. Recent studies using whole-genomic

Table 2 Pharmacological properties of wild-type versus mutant GHSR. All values represent the mean $\pm$ s.e.m. from at least five independent experiments.

Receptor	Ghrelin pEC ₅₀	Basal activity ^a	Surface expression ^b
GHSR1a wt	8.95 $\pm$ 0.07	100	100
Val182Ala	8.71 $\pm$ 0.12	52 $\pm$ 3*	77 $\pm$ 6*
Ala169Thr	8.70 $\pm$ 0.16	103 $\pm$ 2	81 $\pm$ 6
Ala358Thr	8.58 $\pm$ 0.25	88 $\pm$ 4	85 $\pm$ 10
Ser84Ile	8.25 $\pm$ 0.19*	3 $\pm$ 1*	15 $\pm$ 3*

*Values differ significantly from the wild-type ($P < 0.01$).

^aPercentage of basal signaling activity of the wild-type GHSR.

^bPercentage of wild-type GHSR surface expression.

Figure 3 Selected GHSR variants show decreased cell surface expression. HEK293 cells were transfected with a plasmid encoding either the wild-type or a mutant HA-tagged GHSR. After 48 h, surface expression was measured by ELISA as described in Patients and methods section. Data were normalized relative to the maximal value observed at the wild-type GHSR and represent the mean $\pm$ S.E.M. from at least three independent experiments, each performed in triplicate. ** $P < 0.01$ expression level of GHSR mutant versus wild-type, ANOVA followed by Dunnett's post-test.

sequencing revealed that individuals tend to differ from a reference genome by putative loss of function mutations in 250–300 genes (28). However, focusing on *GHSR*, no loss-of-function variants were identified by low-coverage whole-genome sequencing of 179 individuals; an initial cohort from the 1000 genome project (<http://browser.1000genomes.org/index.html>) (28). Furthermore, the absence of other *GHSR* mutations in a large group of control children suggests that the association between *GHSR* variations and CDGP phenotype is unlikely to be fortuitous.

Functional studies confirmed the *in silico* prediction by PolyPhen and revealed that p.Ser84Ile and p.Val182Ala are functionally defective and will be designated as mutations. The mutations p.Ser84Ile and p.Val182Ala show a decrease in GHSR basal activity that is at least in part explained by a reduction in cell surface expression. The p.Ser84Ile variant was also associated with a decrease in ghrelin potency. As part of the molecular mechanism underlying the observed loss of function at these variants, it is possible that the mutations also directly or indirectly disrupt receptor coupling to intracellular signaling effectors, including the G-protein. These functional studies were performed in a heterologous cellular expression system. As previously documented for other GPCRs (29), it is possible that in the endogenous cellular microenvironment, the other identified variations might result in impaired GHSR function that is not evident when studied in HEK293 cells. However, it is most likely that the other variations (c. –6 G>C, p.Ala169Thr and p.Ala358Thr) are only rare benign polymorphisms.

The two mutations (p.Ser84Ile and p.Val182Ala) were found in the heterozygous state in two female patients with postnatal short stature during youth due to CDGP. GHD was excluded in both patients; however, IGF1 levels were at or below the lower normal limit of the reference values. Both patients had normal progression of puberty and spontaneously reached normal adult height. The p.Ser84Ile mutation was also found in members of the families that had neither the phenotype of short stature nor the delayed puberty, whereas the p.Val182Ala mutation segregated with CDGP phenotype in the family. These findings suggest a dominant mode of inheritance with incomplete penetrance, consistent with the pattern of inheritance observed in other families with *GHSR* mutations (7, 8) and in families with CDGP (30). Some possibilities to explain the incomplete penetrance and variable expression include interacting environmental factors and/or genetic variants at other loci.

CDGP is among the most commonly diagnosed growth disorders and it is considered a subcategory of ISS, as before the age of 13 years in girls or 14 years in

Table 3 Clinical and laboratorial characteristics of the two patients with functionally significant *GHSR* mutations.

	Patient 1	Patient 2
<i>GHSR</i> mutation	p.Ser84Ile	p.Val182Ala
Sex	Female	Female
Father's height SDS	–1.5	+1.0 ^a
Mother's height SDS	+0.3	–1.2
Target height in cm (SDS)	158.1 (–0.7)	161.6 (–0.1)
Birth weight SDS	1.0	–0.9
Birth length SDS	–0.8	–1.3
First evaluation		
Age (years)	12.8	13
Bone age (years)	11	11
Height SDS	–2.4	–2.3
BMI SDS	–2.6	+1.1
Puberty		
Age at start of puberty (years)	13	13
Age at menarche (years)	16	14
Last observation		
Age (years)	22	17.7
Height SDS	–0.8	–1.4
BMI (kg/m ²)	18.4	22.3
Laboratory evaluation		
GH peak at stimulation test (ng/ml)	10.3	7.9
IGF1 SDS	–2.3 ^b /–0.5 ^c	–1.3 ^b
IGFBP3 SDS	–1.6 ^b /–2.2 ^c	+1.1 ^b
LH (UI/l)	<0.6 ^b /5.4 ^c	0.1 ^b
FSH (UI/l)	1.7 ^b /6.4 ^c	2.6 ^b
Estradiol (pg/ml)	<13 ^b /51 ^c	13 ^b
Glucose (mg/dl)	90 ^c	80 ^b
Insulin (μ U/ml)	7.7 ^c	10 ^b
Basal acylated ghrelin (pg/ml) ^d	66.5 ^c	–
After meal		
Acylated ghrelin (pg/ml) ^d	53.1 ^c	–
Suppression	20%	–

^aPresence of the mutation in heterozygous state.

^bSample collected at the first evaluation.

^cSample collected at the last evaluation.

^dNormal range for basal acylated ghrelin levels: 67.7 $\pm$ 33.3 pg/ml.

boys, this condition cannot be differentiated from ISS (31). This disorder occurs more frequently in males (18, 32), and indeed, in our cohort, the proportion of males was greater in the CDGP group than in the non-CDGP group (77 vs 61%, see Table 1). The fact that on the first evaluation our CDGP patients were older than the non-CDGP patients suggests that they might have reached a height SDS below -2.0 (short stature) later in life, therefore coming in later for medical evaluation. CDGP is characterized by a significant delay in both BA and adolescent growth spurt (31), which underlies the transient short stature stage, which is seen in affected individuals. Calculated measures of heritability suggest that 50–80% of the variance in pubertal onset is genetically controlled (30, 32). CDGP appears to be a multifactorial trait, yet at the same time the inheritance patterns suggests that single genes exert major effects (30). Although previous studies failed to identify mutations in candidate genes in patients with CDGP (33–38), the *GHSR* gene has not been previously investigated in patients with this condition (7–9). We regard our findings as hypothesis generating; it will be of great interest to screen other clinical cohorts with CDGP (as well as family members) to determine the frequency and inheritance pattern of *GHSR* mutations in various populations.

Ghrelin has orexigenic effects mediated by *GHSR1a* (4); therefore, it would be anticipated that mutations that impair ghrelin's receptor function would lead to a lean phenotype. In fact, animal studies showed that transgenic rats with attenuated *GHSR* protein expression in the arcuate nucleus have lower body weight, reduced adipose tissue, and consume less food than control rats (39). However, the patients with *GHSR* mutations described to date have a variable phenotype regarding weight (7–9). It was hypothesized that partial ghrelin system deficiency might be compensated by a host of developmental, genetic, and environmental factors that influence feeding behavior and body weight (8).

The exact mechanisms that trigger the start of puberty are yet unknown. Puberty onset is sensitive to the energy reserves of the organism, especially in females where there is an association between obesity and early puberty (reviewed in (40)). Therefore, we hypothesize that in the presence of *GHSR* mutations, there is a decrease in ghrelin-mediated appetite, resulting in relatively low BMI, which contributes to the delayed onset of puberty. Furthermore, delayed puberty is observed in clinical conditions associated with low IGF1 (41, 42), suggesting that IGF1 also exerts stimulatory, synergistic, or permissive effects on the onset of puberty (43). Thus, low IGF1 levels due to a decrease in GH secretion caused by *GHSR1a* haploinsufficiency may also negatively modulate the timing of puberty onset.

In conclusion, this is the first report of *GHSR* mutations in patients with CDGP, a condition with a significant hereditary component, so far without a recognized genetic cause. Our study raises the

intriguing possibility that there is an association between the observed *GHSR* mutations and the CDGP phenotype. Analyses of larger cohorts (including family members) are needed to explore the nature of this putative link. Such future efforts will better define the role of *GHSR*-mediated signaling on pubertal control.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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