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. K6G, c.251G>O (p.Ser84Ile), c.505G>A (p.Ala169Thr), c.545 T>O (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 hetero-
yzous 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 decrea-
sed 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 develop-
ment 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 µg/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 ± 2 °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 GHSR1a (or an empty expression vector), 2 ng/well, ii) a serum-responsive element-luciferase reporter gene (SRE5X-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 SpectraMaxR microplate 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 χ² 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).

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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 ~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).

### 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.Ala169Thr (c.505G>A), p.Val182Ala (c.545 T>C), and p.Ala358Thr (c.1072G>A)) are missense and all of them but p.Ala358Thr 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 physiologically acceptor or donor GHSR splice sites by these allelic variants; in contrast, analysis by PolyPhen (22) suggested that p.Ala169Thr and p.Ala358Thr 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.
 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 μg/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>
<thead>
<tr>
<th>Receptor</th>
<th>Ghrelin pEC50</th>
<th>Basal activity</th>
<th>Surface expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHSR1a wt</td>
<td>8.95±0.07</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Val182Ala</td>
<td>8.71±0.12</td>
<td>52±3*</td>
<td>77±6*</td>
</tr>
<tr>
<td>Ala169Thr</td>
<td>8.70±0.16</td>
<td>103±2</td>
<td>81±6</td>
</tr>
<tr>
<td>Ala358Thr</td>
<td>8.58±0.25</td>
<td>88±4</td>
<td>85±10</td>
</tr>
<tr>
<td>Ser84Ile</td>
<td>8.25±0.19*</td>
<td>3±1*</td>
<td>15±3*</td>
</tr>
</tbody>
</table>

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

*Percentage of basal signaling activity of the wild-type GHSR.

*Percentage of wild-type GHSR surface expression.

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**Table 2 Pharmacological properties of wild-type versus mutant GHSR.** All values represent the mean±S.E.M. from at least five independent experiments.
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 boys.

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

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
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<tbody>
<tr>
<td>GHSR mutation</td>
<td>p.Ser84Ile</td>
<td>p.Val182Ala</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Father’s height SDS</td>
<td>−1.5</td>
<td>+1.0a</td>
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<tr>
<td>Mother’s height SDS</td>
<td>+0.3</td>
<td>−1.2</td>
</tr>
<tr>
<td>Target height in cm (SDS)</td>
<td>158.1 (−0.7)</td>
<td>161.6 (−0.1)</td>
</tr>
<tr>
<td>Birth weight SDS</td>
<td>1.0</td>
<td>−0.9</td>
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<tr>
<td>Birth length SDS</td>
<td>−0.8</td>
<td>−1.3</td>
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<tr>
<td>First evaluation</td>
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<tr>
<td>Age (years)</td>
<td>12.8</td>
<td>13</td>
</tr>
<tr>
<td>Bone age (years)</td>
<td>11</td>
<td>11</td>
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<tr>
<td>Height SDS</td>
<td>−2.4</td>
<td>−2.3</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>−2.6</td>
<td>+1.1</td>
</tr>
<tr>
<td>Puberty</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at start of puberty</td>
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<td>13</td>
</tr>
<tr>
<td>Age at menarche (years)</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Last observation</td>
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<td></td>
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<tr>
<td>Age (years)</td>
<td>22</td>
<td>17.7</td>
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<tr>
<td>Height SDS</td>
<td>−0.8</td>
<td>−1.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>18.4</td>
<td>22.3</td>
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<tr>
<td>Laboratory evaluation</td>
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<tr>
<td>GH peak at stimulation test (ng/ml)</td>
<td>10.3</td>
<td>7.9</td>
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<tr>
<td>IGF1 SDS</td>
<td>−2.3b/−0.5c</td>
<td>−1.3b</td>
</tr>
<tr>
<td>IGFBP3 SDS</td>
<td>−1.6b/−2.2c</td>
<td>+1.1b</td>
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<tr>
<td>LH (UI/l)</td>
<td>&lt;0.65/5.4c</td>
<td>0.1b</td>
</tr>
<tr>
<td>FSH (UI/l)</td>
<td>1.3b/6.4c</td>
<td>2.6b</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>&lt;13b/51c</td>
<td>13b</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>90c</td>
<td>80b</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>7.7c</td>
<td>10b</td>
</tr>
<tr>
<td>Basal acylated ghrelin (pg/ml)d</td>
<td>66.5c</td>
<td>—</td>
</tr>
<tr>
<td>After meal</td>
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<td>—</td>
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<tr>
<td>Acylated ghrelin (pg/ml)d</td>
<td>53.1c</td>
<td>—</td>
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<tr>
<td>Suppression</td>
<td>20%</td>
<td>—</td>
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</tbody>
</table>

*Presence of the mutation in heterozygous state.
Sample collected at the first evaluation.
Sample collected at the last evaluation.
Normal range for basal acylated ghrelin levels: 67.7±33.3 pg/ml.

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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 ± 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.
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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**References**


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