Short stature in two siblings heterozygous for a novel bioinactive GH mutant (GH-P59S) suggesting that the mutant also affects secretion of the wild-type GH

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Abstract

Objective: Short stature caused by biologically inactive GH is clinically characterized by lack of GH action despite normal-high secretion of GH, pathologically low IGF1 concentrations and marked catch-up growth on GH replacement therapy.

Design and methods: Adopted siblings (girl and a boy) of unknown family history were referred for assessment of short stature (−4.5 and −5.6 SDS) at the age of 10 and 8.1 years respectively. They had delayed bone ages (6.8 and 4.5 years), normal GH peaks at stimulation tests, and severely reduced IGF1 concentrations (−3.5 and −4.0 SDS). Genetic analysis of the \textit{GH1} gene showed a heterozygous P59S mutation at position involved in binding to GH receptor (GHR).

Results: Isoelectric focusing analysis of secreted GH in patient serum revealed the presence of higher GH-P59S peak compared with that of wt-GH. Furthermore, computational simulation of GH-P59S binding to GHR suggested problems in correct binding of the mutant to the GHR. \textit{In vitro} GHR binding studies revealed reduced binding affinity of GH-P59S for GHR (IC\textsubscript{50}, 30 ng/ml) when compared with the wt-GH (IC\textsubscript{50}, 11.8 ng/ml) while a significantly decreased ability of the mutant to activate the Jak2/Stat5 signaling pathway was observed at physiological concentrations of 25–100 ng/ml.

Conclusions: The clinical and biochemical data of our patients support the diagnosis of partial bioinactive GH syndrome. The higher amount of GH-P59S secreted in their circulation combined with its impact on the wt-GH function on GHR binding and signaling may alter GHR responsiveness to wt-GH and could ultimately explain severe short stature found in our patients.
GH-responsive element that drives transcriptional regulation of multiple GH-responsive genes leading to the biological effects of GH (8).

One of the causes of growth failure is a disorder in the GH–insulin-like growth factor 1 (IGF1) axis. In most cases with sporadic isolated GH deficiency (GHD), the genetic cause is unknown. The estimated incidence of GHD is 1/4000–10 000 live births (9, 10, 11), and much lower of GH insensitivity and reduced bioactivity of the GH. The diagnosis of ‘syndrome of bioinactive GH’ has often been discussed and suggested in short children with the phenotype resembling isolated GHD with normal or even slightly elevated basal GH levels, low IGF1 concentration, and normal catch-up growth on GH replacement therapy. Short stature associated with bioinactive GH was first described by Kowarski et al. (12) while additional cases were reported in the 1980s on clinical basis (13, 14, 15, 16, 17). Takahashi et al. (18) described a heterozygous point mutation in the GH1 gene (D112G) found in a Japanese patient with short stature. The D112G mutant involved a single nucleotide substitution within the GH binding site 2 for the GHR, which interfered with a correct binding to GHR/GH binding protein (GHBP) additionally preventing dimerization of GHR (19). Further, six GH variants found in the heterozygous state were suggested to be bioinactive by Millar et al. (20), but no clear correlation between laboratory/clinical phenotype and patient genotype was demonstrated. Moreover, in one of the more convincing cases of bioinactive GH reported to date, a homozygous missense mutation C53S in the GH1 gene led to disruption of the disulfide bridge between Cys-53 and Cys-165 in a short Serbian boy (21). Functional studies demonstrated that both GHR binding and Jak2/Stat5 signaling activity were significantly reduced in the GH-C53S compared with wt-GH.

Here, we describe two siblings with severe short stature carrying a heterozygous P59S mutation in GH1 gene. Clinical data of patients, which included normal GH peaks after stimulation, delayed bone ages and severely reduced IGF1 concentrations, suggested the diagnosis of bioinactive GH. The data of functional analysis combined with the clinical data of our patients support the diagnosis of partial bioinactive GH, which seems to be caused by the GH-P59S mutation also affecting the secretion of the endogenous wt-GH.

Subjects and methods

Patients

Two siblings, a sister and her brother, adopted Roma children from Hungary, were referred to our clinic for short stature at the age of 9.9 and 8.0 years respectively. They had come to The Netherlands at the age of 6 and 4 years. At the first physical examination, the girl presented with a height of 113.8 cm (−4.55 SDS) and a bone age of 6.8 years (22) (Fig. 1A). Weight was 16.6 kg (−2.64 SDS), head circumference 49.7 cm (−1.75 SDS), and she was prepubertal. She had a strabismus of the left eye, had learning difficulties, and was developmentally retarded by 3 years. The boy presented with a height of 100.7 cm (−5.50 SDS) and a bone age of 4.5 years (Fig. 1B). Weight was 13.3 kg (−2.18 SDS), head circumference 50.4 cm (−1.25 SDS), and he was also prepubertal. He was born prematurely and had been admitted to hospital after birth but details about their medical history and their family history are missing. Furthermore, genetic analysis revealed a heterozygous P59S mutation in the GH1 gene in both patients (based on the HGVS nomenclature: NM_000515.3, c.254C>T; NP_000506.2, p.P85S).

At referral, laboratory assessment showed normal thyroid function, liver and renal function, screening for celiac disease was negative, and no anemia or signs of chronic infection were present. IGF1 of the girl was 70 ng/ml (−2.77 SDS) and that of the boy was 39 ng/ml (−3.01 SDS) while IGF-binding protein 3 (IGFBP3) of the girl was 1.69 mg/l (−1.5 SDS) and that of the boy was 1.15 mg/l (−2.45 SDS). A GH stimulation test (clonidine) showed a GH peak of 17 and 11 ng/ml respectively (23). Repeated analysis of IGF1 and IGFBP3 showed similar results. Further, IGF1 generation tests were performed. After 2 weeks of rhGH (0.7 mg/m² per day), IGF1 increased in the girl and the boy from −2.61 and −3.16 SDS to −1.94 and +0.29 SDS respectively. IGFBP3 increased from −1.22 and −2.09 SDS to −0.55 and +0.30 SDS respectively according to age and sex.

Genetic analysis

Genomic DNA was isolated from peripheral blood samples using the Autopure LS Instrument (Gentra
Cell culture and treatment

Mouse pituitary (AtT-20/D16v-F2) cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (4.5 g/l glucose) supplemented with 10% heat-inactivated FCS (Life Technologies, Invitrogen AG) and 100 U/l penicillin/streptomycin. This F2 subclone was developed from the original AtT-20 cells, an ACTH-secreting cell line established from a murine pituitary tumor.

Chinese hamster ovary (CHO-K1) cells were a gift from Prof. U Wiesmann (Inselspital, Bern, Switzerland) and were cultured in Ham’s F12 medium (Biochrom AG, Seromed, Berlin, Germany) supplemented with 10% FCS, 100 U/l penicillin/streptomycin (Biochrom AG), and 2 mM 1-glutamine (Gibco-BRL, Life Technologies).

Human embryonic kidney (HEK) 293 cells stably expressing hGHR (293GH) were a gift from Prof. R Ross (Northern General Hospital, Sheffield, UK) and were grown in DMEM Nut F12 (Gibco), supplemented with 10% FCS, 100 U/l penicillin/streptomycin, 2 mM 1-glutamine, and 400 µg/ml geneticin G418 (Promega Corp.).

Production of GH peptides

Wild-type GH was cloned in pcDNA3.1 (-) neo vector as described previously (24). GH mutant (GH-P59S) was made by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit from Stratagene AG (Basel, Switzerland). In order to produce GH variants, stable clones of wt-GH or GH-P59S were generated in CHO-K1 cells by transfection with FuGene 6 (Roche Diagnostics AG).

Concentrations of GH produced by CHO cells during 3 days in serum-free Ham’s F12 medium were measured by the DSL-GH ELISA Kit (DSL, Webster, TX, USA). To confirm that the mutation P59S does not affect the affinity of the antibody used in DSL-ELISA, two different GH assays were performed on two samples of CHO supernatant and the results were compared (21).

Isoelectric focusing

AtT-20 cells were cultured in DMEM+5% FCS in six-well plates and transfected using FuGene 6 (Roche Diagnostics AG). The cells were transiently transfected with 1 µg plasmid in total, containing either 1 µg wt-GH or 0.5 µg of wt-GH and GH-P59S. After 24-h incubation, cells were stimulated with 50 µM forskolin (Sigma–Aldrich) for an additional 24 h when aliquots of culture medium were collected. Isoelectric focusing was performed as described (25). Patient serum or culture media samples (200–300 µl) were electrofocused in a buffer containing 1% hydroxypropyl methylcellulose and 4% ampholine (pH gradient, 3.5–8.0) at 200 V for 12 h and then at 500 V for 12 h. The fractions were collected and assayed for immunoreactive GH.

Hormonal measurement

The serum GH was measured by the DSL-10-1900 Active hGH ELISA assay kit (26) as described previously (21).

3D protein model and in silico mutagenesis of wt-GH

The 3D structural model of wt-GH (NP_000506.2, P01241) was based on previously reported crystal structure of hGH isoform 1 structure (PDB ID, 1HGU), which was chosen based on structure quality and coverage of hGH sequence. Secondary structure features of the wt-GH structures were taken into account for the structure alignment. Amino acids 27–217 of human wt-GH molecule were aligned with an X-ray crystal template structure to generate the structural alignments of full wt-GH sequence. We performed model building to repair the gaps in the X-ray crystal structure and enable the molecule to undergo molecular dynamic simulations with the programs YASARA (27) and WHATIF (28).

First, a secondary structure prediction was performed for building the missing parts in the crystal structure of GH with the program DSC (29). The side chains in the newly built parts were optimized first by a steepest descent and then a simulated annealing minimization. At this stage, backbone atoms of aligned residues were kept fixed. The model was then subjected to 1000 ps refinement by molecular dynamic simulation and then checked by the programs WHATCHECK (31), WHATIF (28), Verify3D (32, 33), ERRAT (34), and Ramachandran plot analysis (35, 36). In silico mutagenesis was performed with YASARA and WHATIF and optimized by simulated annealing and a short 500 ps molecular dynamics (MD) simulation. Coordinates of two human hGH crystal structures (PDB IDs, 1HGU and 3HHR chain A) were used for comparative studies. The structural model of wt-GH and P59S mutant of GH in complex with GHR was based on the structure of GHR (amino acids N-49–254) in complex with GH (PDB ID, 1HGU) (2). Structure models were depicted with Pymol (www.pymol.org) and rendered as ray-traced images with POVRAY (www.povray.org). All numbering of amino acids in GHR is according to updated NCBI RefSeq of full-length GHR (NP_000154) containing 638 amino acids, while numbering in older publications is either N-49 (for 3HHR structure) (2) or N-18 (for 1A22 structure) (37).
**MD simulation for model refinement**

The MD simulations were performed using YASARA dynamics using AMBER03 force field (27). The simulation cell was filled with water and the AMBER03 (38) electrostatic potentials were evaluated for all water molecules; the one with the lowest or highest potential was turned into a sodium or chloride counter ion until the cell was neutral. We then ran MD simulations with AMBER03 force field at 298K and 0.9% NaCl in the simulation cell for 1000 ps to refine the model. Simulation trajectories were analyzed with WHATIF functions and snapshots of simulation were captured every 25 ps for further analysis. The best model was selected for analysis and evaluation of mutant amino acids.

**Receptor binding assay**

Receptor binding assays were performed using 293HEK cells stably expressing the hGHR (293GHR) as described previously (21). Four independent experiments were performed in triplicates and IC₅₀ values for the different GH peptides were determined by nonlinear regression analysis, using a single site competition model (GraphPad Prism Software, version 5.0).

**Luciferase reporter gene assay of Stat5 activation**

293GHR cells were used to assay Stat5 activation as described (39, 40). Briefly, cells were transfected with a Stat5-responsive luciferase reporter gene construct (41, 42) and treated with increasing amounts of GH (wt-GH and GH-P59S) for 6 h. Luciferase expression was then measured by the dual-luciferase reporter assay (Promega) on a luminometer (Mediators PhL. Aureon Biosystems, Vienna, Austria).

**Statistical analysis**

The statistical significance of GH secretion was assessed using ANOVA one-way test plus Dunnett’s multiple comparison test, comparing GH-P59S with wt-GH while the statistical analysis for bioassay (testing bioactivity through GHR binding and activation of Jak2/Stat5 pathway) was performed using the nonparametric Mann–Whitney U test.

**Results**

**Analysis of GH in serum of the patients and in culture medium of wt-GH and GH-P59S expressing AtT-20 cells by isoelectric focusing**

At stimulation tests, both patients had normal GH peaks. However, the ELISA-based method used to measure GH concentration cannot distinguish the secretion of wt-GH from that of GH-P59S. Therefore, to determine the specific concentration of each GH variant, we performed isoelectric focusing of GH in the serum of the affected patients (Fig. 2A), which confirmed the presence of an increased GH-P59S peak over the wt-form (area under the curve: P<0.05). In the control sample, only one peak (wt-GH) was detected (Fig. 2B). The same analysis was also performed on culture medium of forskolin-stimulated AtT-20 cells expressing wt-GH or both GH variants. The presence of two peaks was detected in medium of cells co-expressing wt-GH and GH-P59S.

![Figure 2 Isoelectric focusing of GH in serum from the male patient (A), of rhGH (B) and in culture medium of AtT-20 cells co-expressing wt-GH and GH-P59S (C) and expressing only wt-GH (D). The serum (culture medium) fractions were pooled separately and assayed for GH immunoreactivity. The pH gradient formed during isoelectric focusing is indicated. The peaks for wt-GH and GH-P59S are indicated by the open and solid arrows respectively.](image-url)
wt-GH and GH-P59S (Fig. 2C: area under the curve of GH-P59S vs wt-GH: \(P < 0.05\)) while one peak corresponding to wt-GH (Fig. 2D) was detected in culture medium of AtT-20 cells expressing only wt-GH.

**3D-structural model of wt-GH, generation of GH-P59S by in silico mutagenesis, and MD simulation of GHR binding**

Based on its position in the GH molecule located within the segment between residues 54–74 that are tightly in contact with GHR, P59 residue is considered to contribute to the proper binding of GH to GHR through the GH binding site 1. Therefore, a mutation introduced at this position might lead to defects in GHR binding. To investigate this in more detail, the binding of GH-P59S to GHR was analyzed by **in silico** mutagenesis following the MD simulation and compared with wt-GH. In the hGH crystal structure as well as in the repaired model, the P59 is in contact with K70 to stabilize the small helical turn containing R64 residue that interacts with GHR (Fig. 3A and B). Energy analysis showed no drastic changes, and P59S caused no change in protein stability or changes in the core structure of the GH molecule. However, multiple changes in local environment and bond order were observed, which may have an impact on its interaction with the acidic E60, E62, and D182 residues on GHR (Fig. 3D). The interaction of R64 in GH with E62 and D182 residues of GHR has been reported to be important for GH–GHR binding (reported as E44 and D164 based on N-18 numbering of amino acids in GHR) (37). The observed changes affect multiple atomic contacts, but individually, all can be considered to cause minor disturbance in binding of GH-P59S to GHR and may rather lead to moderate than to severe binding defects.

**Functional analysis of the GH-P59S through GHR binding and activation of the JAK/STAT signaling pathway**

As the computational analysis of wt-GH vs GH-P59S binding to GHR performed through **in silico** mutagenesis and MD simulation predicted lower binding of the mutant variant, we performed GHR binding studies in 293GHR cells and compared the binding affinities of wt-GH and GH-P59S (Fig. 4). The IC\(_{50}\) values for the rhGH, wt-GH, and GH-P59S were found to be 13, 12, and 30 ng/ml respectively. No significant difference was observed between the IC\(_{50}\) values from wt-GH and rhGH while a statistically significant difference was found between the IC\(_{50}\) values from wt-GH and GH-P59S (ANOVA, \(P < 0.05\)), confirming the reduced binding affinity of GH-P59S for the GHR.

Improper binding of GH variants to the GHR has an impact on the Jak2/Stat5 signaling cascade downstream of GHR. To investigate whether reduced binding of GH-P59S variant consequently evoked abnormalities in the activation of Jak2/Stat5 signaling pathway, we performed a bioassay using the combination of 293GHR cells stably expressing GHR and Stat5-responsive luciferase reporter gene assay system (23, 24). Using this *in vitro* system, which requires all steps of the
Jak2/Stat5 signaling pathway to be functional, we were able to quantify the signal transduction activity of GH-P59S and to compare it with that of wt-GH. As expected and in line with the binding data, the GH-P59S mutant displayed reduced ability to activate the Jak2/Stat5 pathway when compared with wt-GH (Fig. 5A). Significantly reduced bioactivity of GH-P59S was observed at the physiological dose of 25 ng/ml ($P < 0.05$) as well as at 50 ng/ml (upper normal range) and 100 ng/ml ($P < 0.01$). No significant difference in bioactivity between GH-P59S and wt-GH was found at the doses considered as sub-physiological (5 and 10 ng/ml) and supra-physiological (200 and 400 ng/ml) (Fig. 5A).

As shown in Fig. 5B, co-stimulation of the 293GHR cells with 12.5 ng/ml of wt-GH and GH-P59S (P59S 12.5/wt-GH 12.5) displayed a significantly reduced activation of the Jak2/Stat5 pathway ($P < 0.05$) when compared with that evoked by the wt-GH at 25 ng/ml (wt-GH 25). Moreover, the stimulation of the Jak2/Stat5 signaling pathway induced by both the wt-GH and the GH-P59S at 25 ng/ml (P59S 25/wt-GH 25) and at 50 ng/ml (P59S 50/wt-GH 50) was significantly reduced ($P < 0.01$) when compared with the stimulation evoked only by wt-GH at 50 ng/ml (wt-GH 50) and at 100 ng/ml (wt-GH 100). Three negative controls were used in this experiment: PSA, corresponding to the supernatant of CHO cells transfected with pSecPSA; empty, representing the supernatant of CHO cells transfected with an empty pSec plasmid; and NT, which is the supernatant of non-transfected CHO cells. Furthermore, the size of 22 kDa for both wt-GH and GH-P59S was confirmed in protein extracts from CHO cells by western blot (data not shown).

**Discussion**

A heterozygous missense mutation in GH1 gene converting codon 59 from P (proline) to S (serine) was identified in siblings (girl and a boy) presenting with the clinical symptoms of severe growth retardation (−4.5 and −5.6 SDS) and delayed bone ages (6.8 and 4.5 years) at chronological ages of 10 and 8.1 years and severely reduced IGFI concentrations (−3.5 and −4.0 SDS). GH peaks of 17 and 11 ng/ml at stimulation tests were found to be within normal range and a recently performed IGFI generation test revealed normal increase in IGFI following rhGH injections. Analysis of GH in serum (isoelectric focusing) showed that the abnormal peak (GH-P59S) was higher than the normal one (wt-GH). The area under the peak corresponding to the GH mutant variant was significantly higher when compared with that under wt-GH peak. Isoelectric focusing of GH in culture medium from AtT-20 cells co-expressing wt-GH and GH-P59S (mimicking heterozygous conditions found in patients) detected both GH peaks of the size comparable with that found in the serum of patients. This proved our AtT-20-based cellular model to be suitable and reliable to investigate these events *in vitro*.

Based on the clinical data, the diagnosis of partial bioinactive GH syndrome was suggested. As the patients were adopted siblings of unknown clinical family history, no DNA from biological parents or other family members was available for genetic analysis to check other individuals for the same mutation.

The bioinactive GH syndrome is characterized by genetic defects in the GH1 gene giving rise to GH variants (mutants) that are secreted from the pituitary gland in the ‘classical’ pulsatile manner, reaching normal or slightly high concentrations in circulation. These GH mutants often display either overall structural aberrances or defects that are localized to the binding sites for GHR, each of which affects their correct binding to GHR, impacting the Jak2/Stat5 signaling pathway. Comparative analysis of the protein sequence of hGH
with orthologs from various vertebrate species clearly demonstrated that the P59 residue has been conserved throughout evolution. Substitution of proline (P) by leucine (L) at position 59 in the GH1 gene has been recently reported to cause partial GHD combined with features of bioinactive GH syndrome in a patient presenting with modest growth retardation (43). Comparative analysis of 3D structural models of wt-GH and GH-P59L revealed no difference in protein stability or in core structure between these GH variants. Moreover, MD simulation of wt-GH and GH-P59L binding to GHR suggested reduced binding interactions of GH-P59L with GHR, which was confirmed through competition-binding and signaling studies (bioassay).

In this study, our experimental data obtained in vitro (analysis of GH secreted in serum, GHR binding, and signaling data) complement the clinical data of our patients making them well in line with the suggested diagnosis of partial bioinactive GH syndrome. The mutation identified in these patients was at the same position (P59) in GH1 gene, as in the study mentioned earlier, but with the difference that serine (S) was the substituting amino acid. Moreover, the patients carrying GH-P59S mutation presented with severe growth failure (based on SDS for age and height) as opposed to a patient with GH-P59L mutation reported with modest growth retardation. In order to explain such a broad difference in phenotype (short stature) evoked by S vs L substitution at position 59, we decided to investigate in more detail the impact of GH-P59S mutation at the molecular and cellular level. The crystallographic model of the hGH/GHBP complex (44) revealed that the P59 is located in the long crossover loop between helices 1 and 2 of the GH molecule. Furthermore, the studies of homolog- and alanine-scanning mutagenesis identified the segment between residues 54 and 74 to be tightly in contact with GHR, in which the residues are strictly close to P59 (namely F54, E56, I58, and R64) were confirmed to be important in in vitro receptor binding (3, 4). Similar to the case of GH-P59L, comparative analysis of 3D structural models of wt-GH and GH-P59S showed that protein stability and the core structure of GH molecule were not affected by P59S mutation and that the overall 3D structure of the mutant variant does not differ from that of the wt-GH. Analysis of GH binding to GHR performed by MD simulation takes into consideration the influence of local factors (pH, temperature, and water), the nature of amino acid substitution introduced (serine for proline), and its position in GH/GHR complex. This approach proved effective to predict the behavior and interaction of amino acid replacement with other residues helping us to anticipate GHR binding abnormalities. Our analysis of wt-GH and GH-P59S binding to GHR yielded reduced binding interactions of GH-P59S with GHR. Competition-binding studies confirmed these predictions revealing significantly lower affinity of GH-P59S for the GHR when compared with wt-GH, which was comparable to that of GH-P59L mutation in the previous report (43). The Jak2/Stat5 pathway is thought to be the most important signaling pathway attributed to the growth-promoting effects of GH (45), and improper binding of GH to GHR may lead to subsequent Jak2/Stat5 signaling abnormalities. The data we obtained in the bioassay clearly demonstrated significantly lower capability of GH-P59S to activate Jak2/Stat5 pathway when compared with wt-GH and the absence of a synergistic effect between these GH variants in Jak2/Stat5 activation. In addition, these data also revealed that GH-P59S was slightly more potent in Jak2/Stat5 pathway activation than GH-P59L, as 25 ng/ml GH-P59S was the lowest concentration at which statistically significant reduction in bioassay was observed as opposed to 50 ng/ml in the case of GH-P59L (43). Taken together, the results generated in the functional analysis of GH-P59S mutant are quite similar to those obtained through characterization of GH-P59L mutation previously reported. However, of importance and new is that GH-P59S is able to have an impact on secretion of the wt-GH as demonstrated by the isoelectric focusing data. This fact may explain the dramatic influence on height and height velocity when compared with GH-P59L.

In conclusion, the biochemical data of GH-P59S functional analysis are in line with clinical data supporting the diagnosis of partial bioinactive GH syndrome. GH secretion (based on GH stimulation tests) seems to be normal in these patients while the analysis of GH in serum from the patients revealed a significantly higher amount of the mutant variant compared with wt-GH. Hence, the secretion data combined with effects of GH-P59S on GHR binding and signaling may explain severe short stature found in these patients. Alternatively, the mutant variant might alter responsiveness of GHR to either wt-GH and/or rhGH treatment ultimately having an impact on normal growth. Finally, as reported in this study, even a slight alteration at a same position (like amino acid replacement) in GH has an impact on its function and highlights that not only genetic studies but also functional analysis is of importance in defining the mechanism of action of any novel GH mutations also heterozygously inherited.

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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