0021-972X/07/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 92(6):2223–2231

doi: 10.1210/jc.2006-2624

Primary Growth Hormone (GH) Insensitivity and Insulin-Like Growth Factor Deficiency Caused by Novel Compound Heterozygous Mutations of the GH Receptor Gene: Genetic and Functional Studies of Simple and Compound Heterozygous States

Peng Fang,* Stefan Riedl,* Serge Amselem, Katherine L. Pratt, Brian M. Little, Gabriele Haeusler, Vivian Hwa, Herwig Frisch, and Ron G. Rosenfeld

Department of Pediatrics (P.F., B.M.L., K.L.P., V.H., R.G.R.), Oregon Health and Science University, Portland, Oregon 97239-3098; Department of Pediatrics (S.R., G.H., H.F.), University of Vienna, 1090 Vienna, Austria; Institut National de la Santé et de la Recherche Médicale U654 (S.A.), Hopital Henri Mondor, 94010 Creteil Cedex, France; St. Anna Children's Hospital (H.F.), A-1090 Vienna, Austria; Lucile Packard Foundation for Children's Health (R.G.R.), Palo Alto, California 94304; and Department of Pediatrics (R.G.R.), Stanford University, Stanford, California 94305-2038

Context: Primary GH insensitivity (GHI) or Laron syndrome, caused by mutations of the GH receptor (GHR) gene, has a clinical phenotype of postnatal growth failure associated with normal elevated serum concentrations of GH and low serum levels of IGF-I.

Objective: We investigated the clinical and biochemical implications of molecular defects in the GHR gene in an Austrian family with two daughters who were GHI.

Patients: Patient 1 [height, −4.8 SD score (SDS)] and patient 2 (height, −5.0 SDS) had elevated circulating levels of GH, low-normal levels of GH-binding protein, and abnormally low IGF-I (−5.0 SDS and −2.6 SDS, respectively) and IGF-binding protein-3 (−2.6 SDS and −2.0 SDS, respectively).

Results: Both patients carry novel compound, missense, heterozygous GHR mutations, C94S and H150Q. *In vitro* reconstitution experiments demonstrated that whereas each of the mutants could be stably expressed, GHR(C94S) lost its affinity for GH and could neither activate signal transducer and activator of transcription (STAT)-5b nor drive STAT5b-dependent gene transcription in response to GH (1–100 ng/ml). GHR(H150Q) showed normal affinity for GH but impaired capacity for signal transduction. The compound heterozygote and C94S heterozygote, but not the H150Q heterozygote, showed significant deficiency in activating GH-induced gene expression, corroborating diminished GH-induced STAT5b activation in fibroblasts carrying GHR(C94S) as either a compound heterozygote (in the patients) or a simple heterozygote (in one parent).

Conclusions: Each of the compound heterozygous mutations contributed additively to the pathological condition seen in the patients, and the more detrimental of the two mutations, C94S, may cause (partial) primary GHI, even in a heterozygous state. **(*J Clin Endocrinol Metab* 92: 2223–2231, 2007)**

PRIMARY GH INSENSITIVITY (GHI), characterized by postnatal growth retardation, is a pathological condition associated with normal-elevated serum concentrations of GH and low serum levels of IGF-I (reviewed recently in Ref. 1). The clinical phenotypes seen in primary GHI patients have been attributed to a range of genetic defects along the GH-IGF axis, including GH receptors (GHRs) (2), the postreceptor components in the GH signal transduction pathway [such as signal transducer and activator of transcription (STAT)-5b] (3, 4), and the IGF-I gene (5).

The pleiotropic cellular actions of GH are mediated through its cell surface receptor, GHR. Upon GH binding, GHR activates cytosolic Janus kinase 2, which in turn activates multiple cytosolic proteins, including STAT proteins, as well as multiple adaptor proteins leading to activation of the phosphatidylinositol 3-kinase and the MAPK signaling pathways. The activated, tyrosine-phosphorylated STAT proteins homo- or heterodimerize and translocate into the nucleus to function as transcription factors, among which, STAT5b, in particular, activates the transcription of the IGF-I gene in human (3, 4).

The human GHR, the product of a single gene with 10 exons, is a glycosylated protein comprising 620 amino acid residues in its mature form. The GHR protein can be divided into the extracellular domain (encoded by exons 2–7), the single transmembrane domain (encoded by exon 8), and the intracellular domain (encoded by exons 9 and 10). The extracellular domain of the GHR also contains two functional subdomains, subdomain 1, with residues 1–123 (exons 2–5) involved in GH-GHR interaction, and subdomain 2, with residues 128–246, involved in receptor dimerization and GH-induced rotation (6, 7). In addition, a soluble, proteolytic

First Published Online April 3, 2007

* P.F. and S.R. contributed equally to this work.

Abbreviations: F-STAT5b, Flag-tagged human STAT5b; GHBP, GH binding protein; GHI, GH insensitivity or GH insensitive; GHR, GH receptor; GHRd3, exon 3-excluded GHR; GHRfl, full-length GHR; IGFBP, IGF binding protein; pSTAT5b, phosphorylated STAT5b; SDS, SD score; STAT, signal transducer and activator of transcription.

form of the GHR, corresponding to the extracellular domain, circulates as the GH binding protein (GHBP), and provides a convenient means of assessing the affinity of mutant GHRs for GH.

Defects in GHR protein resulting from abnormalities of the GHR gene have been shown to result in the clinical phenotypes of classical GHI or Laron syndrome (8, 9). To date, more than 60 different gene defects, almost all recessively inherited, have been identified, including gene deletions, missense, or nonsense mutations, splice-site mutations, and insertions or deletions (1, 2, 10). The majority of these genetic aberrancies, mostly in their homozygous forms, occur in the region encoding the extracellular domain of the receptor. Unlike homozygous mutations, contributions of heterozygous GHR mutations to GHI are less well defined (11–15). Several compound heterozygous mutations have been reported to be associated with GHI but have mostly involved nonsense mutations in at least one allele (5, 12, 15–18) (Table 1).

We report two female patients with primary GHI from a nonconsanguineous Austrian family. The older of the two patients responded poorly to GH therapy, and subsequent treatment with recombinant human IGF-I for 3 yr moderately accelerated her growth. Genetic analysis revealed two novel compound heterozygous missense mutations, in exons 5 and 6 of the GHR gene, in both patients, and parents were shown to be heterozygous for the mutant alleles. Functional analyses further delineated the effects of the mutations on GH-induced signal transduction and gene expression, thus providing pathophysiological insights into how heterozygous mutations, both simple and compound, may affect GH sensitivity.

Subjects and Methods

Case reports

Patient 1, the fourth child born to nonrelated parents of Austrian origin (Caucasian, from different regions), was normal for birth weight (3100 g) and birth length (47.5 cm). First presenting at 5 yr of age, she had a height of 90.3 cm [−4.8 SD score (SDS), based on Austrian standards], a body weight of 12.6 kg (50th percentile for height), and a delayed bone age (2.5 yr). She had normal hair, mild midfacial hypoplasia, a depressed nasal bridge, moderate frontal bossing, and small hands. No high-pitched voice or hypoglycemic episode was observed. Her plasma GH concentration was 39.1 ng/ml at baseline and greater than 50 ng/ml after stimulation with arginine or insulin. The patient's serum GHBP concentration was 383 pmol/liter (normal range 431-1892). Her serum levels of IGF-I (20 ng/ml, −5.0 SDS) and IGF binding protein (IGFBP)-3 (1720 ng/ml, −2.6 SDS) were markedly low. In addition, her serum IGF-I level failed to increase after an IGF generation test [human (h) GH 0.03 mg/kg daily for 4 d], and she did not respond to a 3-month trial of GH treatment (0.05 mg/kg·d). IGF-I therapy (80 μg/kg twice daily sc) was initiated at age 6.5 yr (19). Her height velocity increased from −3.0 SDS before treatment to +1.1 SDS (first year), +1.4 SDS (second year), and −0.2 SDS (third year). Her height increased from −4.9 SDS to −3.8 SDS, and her body mass index was 13.9 kg/m^2 before and 14.7 kg/m^2 after the 3 yr of IGF-I therapy.

The second affected sibling was born by cesarean section after an uneventful pregnancy at 38 wk of gestation. Her birth length (50 cm) and weight (2950 g) were normal. At 1.8 yr, her height was 71.8 cm (−5.0 SDS), with a delayed bone age of 1.2 yr. She had no conspicuous features of Laron syndrome. Like patient 1, she had an elevated basal GH level (58 ng/ml) and low GHBP (208 pmol/liter), IGF-I (20 ng/ml, −2.6 SDS), and IGFBP-3 concentrations (1540 ng/ml, −2.0 SDS).

The heights of the other family members of the patients were all in the normal range (Table 2).

Serum assays

Serum assays (performed in Portland, OR) were by means of immunoradiometric assays for IGFBP-3 and ELISAs for IGF-I and GHBP (Diagnostic Systems Laboratories, Webster, TX).

For the results from Austria, IGF-I was determined by a RIA developed in the laboratory of one of the investigators (H.F.). Serum samples were extracted by acid-ethanol and diluted 1:20 before assay. IGFBP-3 level was analyzed by commercially available kits (Mediagnost, Tübingen, Germany). Serum hGH was determined by an immunoradiometric assay, using two monoclonal antibodies, one of which is labeled with I^{125} (Bioclone, Sydney, Australia).

Cell culture

Primary fibroblast cultures were established from skin biopsies taken from patient 1 and her father and mother, with consent and in compliance with the institutional review boards of both Oregon Health and Sciences University and the Department of Pediatrics, Medical University of Vienna (Vienna, Austria). Establishment of normal human dermal fibroblasts have been described previously (3). The fibroblasts, as well as HEK293 cells, were maintained in DMEM (Cellgrow; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Inc., Grand Island, NY) at 37 C in 5% CO_2.

Genomic DNA and cDNA

Genomic DNA from either whole blood or primary fibroblast cultures was obtained with the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN). The intronic primers for PCR amplification were: GHR exon 5, forward, GHRx5f (intron 4), 5′-cggcctcgtagcagtcgtca-3′, reverse, GHRx5r (intron 5), 5′-cccctgccctttttcctcttgtt-3′; and exon 6, forward, GHRx6f (intron 5), 5′-tggtcttctgagaagaatgccttccatta-3′, reverse, GHRx6r (intron 6), 5′-gcatacagcatgaacatgtggtattaggtcaaa-3′. Primer GHRx5f and GHRx6f were also used for sequencing. The primers for amplifying other exons are available on request. Genotyping of GHR exon 3 was performed as described previously (20).

Total RNA from primary fibroblast cells was extracted with the RNeasy purification kit (QIAGEN, Valencia, CA), and cDNA from 1 μg total RNA was synthesized with first-strand cDNA synthesis system (Marligen Inc., Ijamsville, MD).

TABLE 1. Reported GHI cases with compound heterozygous GHR mutations

No.	Sex, age (yr)	Height (SDS)	GHBP (pmol/liter)	IGF-I (ng/ml)	IGFBP-3 (ng/ml)	Mutations	Functional studies	Ref.
1	Male, 8.7	−2.9	(−2.8 SDS)	(−4.2 SDS)	N/A	E44K/R161C	No	12
2	Female, 9.1	−8.0	<15.6	<4	300–340	E224X/981del (330X)	No	16
3	11[a]	−6.43	0	N/A	−9.1 SDS	R43X/V144D	No	13
4	Male, 5.0	−6	<15.6	<10	477	Y208C/D244N	No	17
5	Male, 2.8	−4	0	<20	486	Y208C/D244N	No	17
6	Male, 0.5	−5.5	0	<5	<300	W16X/C38X	No	15
7	Female, 17	−5.28	218 (−2.6 SDS)	94 (−2.6 SDS)	1500 (−3.5 SDS)	C83X/1776del-581X	Yes	18
8	Female, 5	−4.8	383	20 (−5.0 SDS)	1720 (−2.6 SDS)	C94S/H150Q	Yes	This study
9	Female, 1.8	−5.0	208	20 (−2.6 SDS)	1540 (−2.0 SDS)	C94S/H150Q	Yes	This study

[a] Gender not provided.

TABLE 2. Clinical and biochemical characteristics of the patients and their family members

Subject	Sex, age (yr)	Height (cm) (SDS)	GHBP (pmol/liter)[a]	IGF-I (ng/ml) (SDS)[b]	IGFBP-3 (ng/ml) (SDS)[c]	GHR genotype
Patient 1	F,5	90.3 (−4.8)	383	20 (−5.0)	1720 (−2.6)	d3(C94S)/fl(H150Q)
Patient 2	F,1.8	71.8 (−5.0)	208	20 (−2.6)	1540 (−2.0)	d3(C94S)/fl(H150Q)
Mother	F	(−1.1)	1511	241	4467	d3/fl(H150Q)
Father	M	(−1.0)	386	284	3434	d3(C94S)/d3
Sister 1[d]	F,18.5	164 (0)	2660	247	4043	d3/fl(H150Q)
Sister 2	F,12.2	170 (−0.6)	147	567	5396	d3(C94S)/d3
Brother	M,13.3	167 (+1.5)	302	660	5310	d3/fl

F, Female; M, male.

[a] Normal range: 431-1892 pmol/liter.

[b] Normal ranges (Diagnostic Systems Laboratories): female, 12 yr, 334–680 (±1 SDS); male, 13 yr, 295–682 (±1 SDS); female, 18 yr, 193–575; older than 30 yr, 100–494.

[c] Normal ranges (Diagnostic Systems Laboratories): female, 12 yr, 1800–8410; male, 13 yr, 2400–7330; female, 18 yr, 2310–7480; older than 30 yr, 1730–5590.

[d] Half-sibling, different father from probands, sister 2, and brother.

Generation of recombinant GHR

To generate exon 3-excluded GHR (GHRd3), the N-terminal part of the cDNA encoding exon 3-deleted hGHR was PCR amplified (forward primer, 5′-attggatccggtcctacaggtatggatctct-3′; reverse primer, 5′-cacacgcgtacttcatattccttat-3′), digested with *Bam*HI and *Mlu*I restriction enzymes, and cloned into the full-length GHR (GHRfl) pcDNA1/AMP expression plasmid (21). GHR carrying C94S or H150Q mutation was then generated with QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with either GHRfl or GHRd3 cDNA as template. The primers for mutagenesis were: C94S, 5′-gctggggaaaacagctcttactttaattcatcg-3′ (sense), 5′-cgatgaattaaagtaagagctgttttccccagc-3′ (antisense); H150Q, 5′-gtttaactgggattcaggcagatatccaagtg-3′ (sense), 5′-cacttggatatctgcctgaatcccagttaaac-3′ (antisense). All resulting GHR variants were confirmed by sequencing.

Generation of adenovirus expressing Flag-tagged STAT5b

A *Not*I/*Hin*dIII digested fragment containing N-terminal Flag-tagged human STAT5b cDNA from a pcDNA3.1 expression plasmid (22) was subcloned into pShuttle transfer plasmid (AdenoVator Vector system; QBiogen, Carlsbad, CA). Final construction of the adenovirus (Ad-F-STAT5b) followed the manufacturer's protocol.

Transfection experiments

HEK293 cells were transfected with vector or vector carrying cDNA encoding GHR variants as described previously (23). After 24 h transfection, the cells were starved in DMEM supplemented with 0.1% BSA for 16 h before treatments with hGH (a generous gift from Genentech, Inc., South San Francisco, CA) as indicated.

Adenovirus infections of primary dermal fibroblasts

Fibroblasts were infected with adenovirus Flag-tagged STAT5b at a multiplicity of infection of 1000 as described previously (23). After 48 h of infection, the cells were starved 16 h in DMEM supplemented with 0.1% BSA before GH treatments as indicated, and cell lysates were collected for Western immunoblot analysis.

GH binding assay by monolayer cells

HEK293 cells transfected with GHR variants were starved overnight and washed with Hank's balanced salt solution saline buffer (Hyclone, Logan, UT) supplemented with 0.1% BSA. The cells were then incubated with I^{125}-GH (NEX-100; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) in the presence (specific binding) or absence (total binding) of unlabeled GH for 3 h at room temperature. The cells were washed three times with 0.1% BSA-Hank's balanced salt solution buffer, solubilized with cell culture lysis buffer (Promega, Madison WI) and the activity of I^{125}-GH in the lysates counted with a 1470 WIZARD γ-counter (PerkinElmer Life Sciences, Inc., Wallac Oy, Finland).

Western immunoblot analysis and immunoprecipitation

Preparation of cell lysates and subsequent Western blot analyses were performed as described previously (24). Immunoprecipitations of the fibroblast lysates with anti-FLAG-M2-agarose beads (Sigma, St. Louis, MO) were performed following the manufacturer's protocol. The antibodies used in this study were: rabbit polyclonal IgG against phospho-Tyr694-STAT5 (dilution 1:1000) from Cell Signaling Technology (Beverly, MA); rabbit polyclonal IgG against hGHR (GHRcyt-AL47) (dilution 1:2000) (25), a generous gift from Dr. Stuart J. Frank (University of Alabama, Birmingham, AL); mouse monoclonal IgG against STAT5b (G-2) (dilution 1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies (antimouse IgG and antirabbit IgG) were obtained from Amersham-Pharmacia Biotech (Uppsala, Sweden). All immunoblot data shown are representative of at least two independent experiments.

Luciferase reporter assays

Cell lysates from transfected HEK293 cells were analyzed for reporter activity from an 8 × GHRE(Spi2.1)-pGL2 firefly luciferase construct (p8 × GHRE-LUC) as previously described (23). Luciferase activities were measured with a luminometer (Turner Designs, Sunnyvale, CA) and normalized to total protein concentration.

Results

The GHR gene in the two patients is a C94S/H150Q compound heterozygote

Sequencing of the GHR gene from genomic DNA obtained from either whole blood or cultured fibroblasts revealed that patient 1 carries two novel compound heterozygous missense mutations: a G to C transversion in exon 5 on one allele resulted in the change of codon C94 (TGT) into S94 (TCT); a T to G transversion in exon 6 on the other allele resulted in a H150 (CAT) to Q150 (CAG) substitution (Fig. 1B). These mutations were also confirmed at the cDNA level (data not shown). The same mutations were identified in the genomic DNA of patient 2 (Fig. 1B). Analysis of the genomic DNA from the other family members revealed that the father and one of the siblings (sister 2) were heterozygous for C94S, and the mother and another sibling (sister 1) were heterozygous for H150Q (Fig. 1B and Table 2), thus confirming the paternal origin of the C94S mutation and the maternal origin of the H150Q mutation.

In addition to the identified missense mutations, we determined whether GHR exon 3 was retained (GHRfl) or ex-

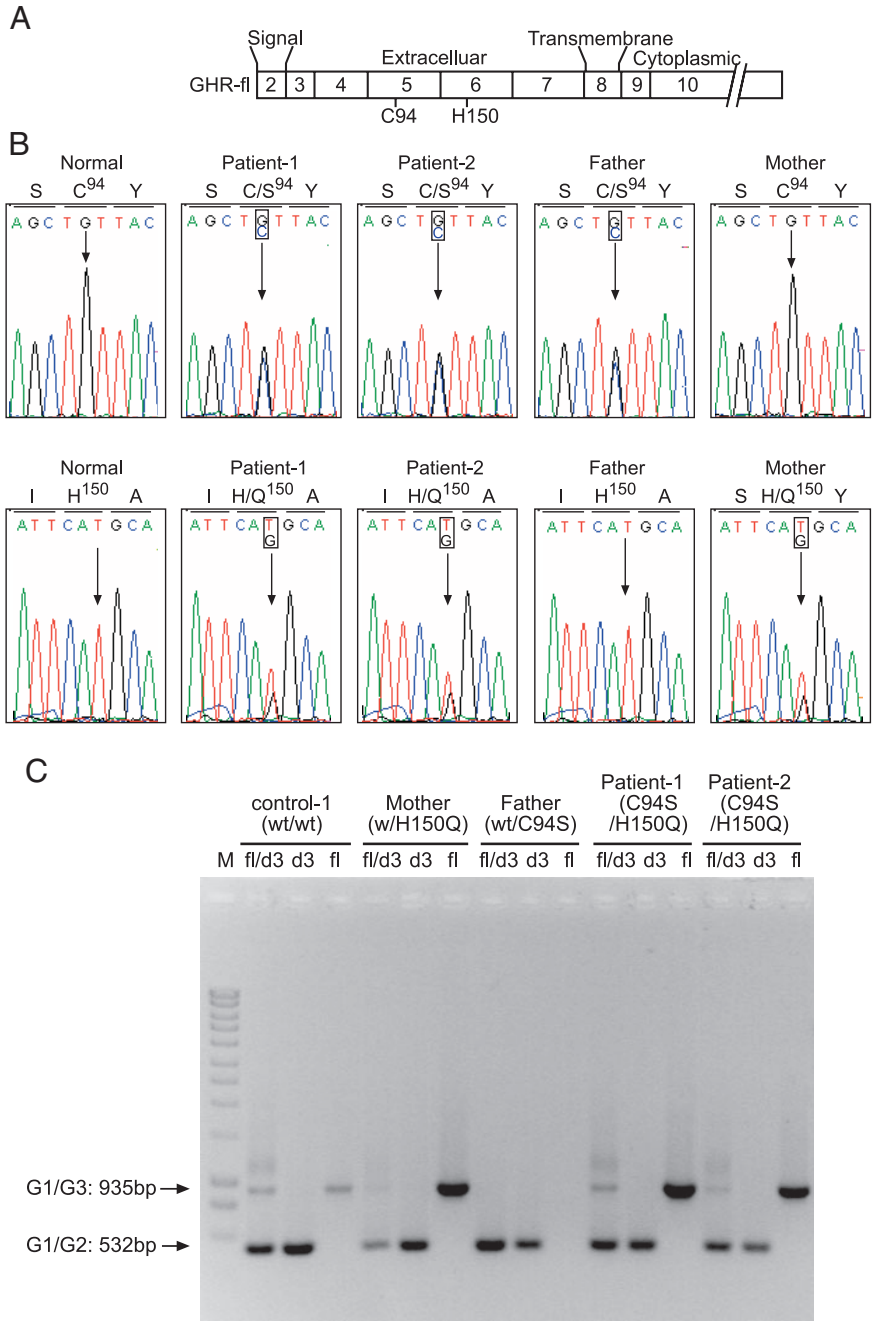

FIG. 1. Family-based genetic analyses of the GHR gene. A, Schematic depiction of the human GHR gene. B, Electropherogram of genomic DNA sequence (sense strand) from two patients, their parents, and a normal control. The *arrow* indicates the normal sequence, the heterozygous mutations at codons C94 of exon 5 (G to C transversion), or the heterozygous mutations at codons H150 of exon 6 (T to G transversion). C, Genotyping the exon 3 loci of the GHR gene. As described previously (20), the 935-bp product amplified with primer G_1/G_3, indicates the presence of exon 3 on the GHR gene, whereas the 532-bp product amplified with primer G_1/G_2, indicates the absence of exon 3 on the GHR gene. M, Marker; fl, full-length, exon 3-retained GHR; d3, exon 3-excluded GHR; fl/d3, heterozygote of GHRfl and GHd3.

cluded (GHRd3) because GH responsiveness has been reported to be affected by the GHRd3 isoform (26). Genotyping the exon 3 loci of the GHR gene indicated that the two patients and their mother are heterozygous for GHRfl and GHRd3, whereas the father is homozygous for GHRd3 (Fig. 1C). We therefore conclude that C94S mutation was carried on the GHRd3 variant [GHRd3(C94S)], whereas the H150Q mutation is on GHRfl [GHRfl(H150Q)].

GHRd3(C94S) cannot bind GH

To assess the ability of each GHR variant to bind GH, GHR variants were constructed and transfected into HEK293 cells. As indicated by Western immunoblot analyses, both GHRd3(C94S) and GHRfl(H150Q) were stably expressed at levels comparable with the wild-type GHR (Fig. 2A). It was of note that GHRd3(C94S), compared with the wild type,

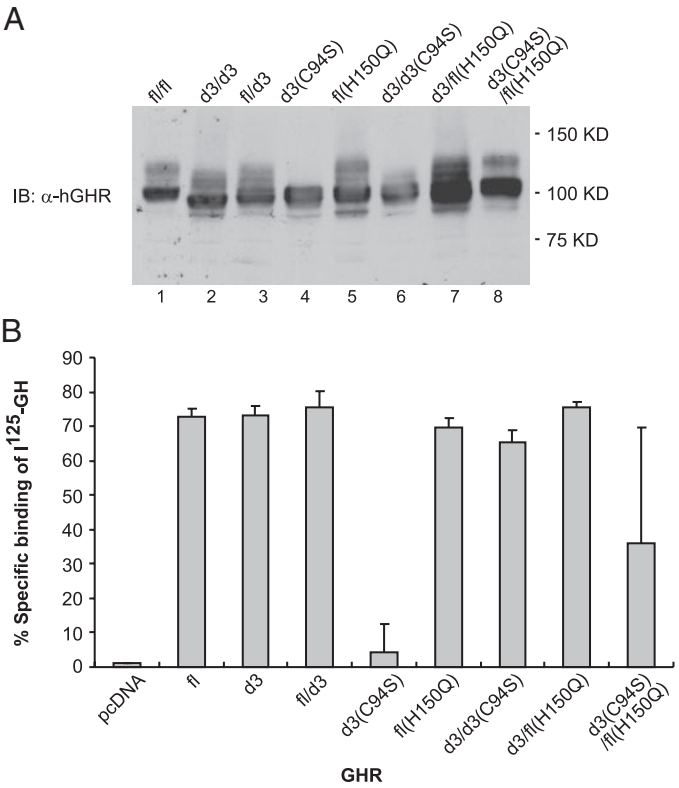

FIG. 2. I^{125}-GH binding to the surface of monolayer HEK293 cells expressing GHR variants. A, Expression of GHR variants in HEK293 cells. The cell lysates were collected from HEK293 cells transfected with either 1 μg vector plus either 1 μg vector or 1 μg GHR variants (0.5 μg each of two cotransfected variants) as described in *Subjects and Methods*. The expression of each GHR variant was analyzed by Western immunoblot (IB), using an antibody against the cytoplasmic domain of the hGHR protein. The molecular markers are indicated to the right of the blot. B, I^{125}-GH binding. HEK293 cells on 12-well plate were transfected with 0.4 μg vector, 0.4 μg vector carrying GHR variants, or 0.2 μg each of two cotransfected variants. Then 10^5 cpm/well (~1 ng/ml) of I^{125}-GH were added to determine the total binding or specific binding (in the presence of 10 μg/ml unlabeled GH). The data are presented as percentage of specific binding (mean ± SE) from four independent experiments performed in duplicates.

consistently lacks the higher molecular form (115–130 kDa) corresponding to mature, normally glycosylated GHR (Fig. 2A, lane 4) (27, 28).

The monolayer GH binding assay indicated that the total specific binding by homozygous GHRd3(C94S) was only 6% of that observed with wild-type GHR, whereas the binding of GH by homozygous GHRfl(H150Q) was indistinguishable from wild-type GHR (Fig. 2B). Furthermore, when GHRd3(C94S) was cotransfected with wild-type GHRd3 or GHRfl(H150Q) (1:1), the resultant GHRd3/GHRd3(C94S) heterozygote exhibited similar GH binding as did wild-type GHR, whereas the compound heterozygote had total specific binding of GH only 50% of that observed with wild-type GHR (Fig. 2B).

The compound heterozygote showed a dose-dependent defect on GH-induced STAT5b activation

Endogenous STAT5b activation induced by GH (1–100 ng/ml) was examined in HEK293 cells transfected with vector or the GHR variants. In HEK293 cells transfected with vector, minimal tyrosine-phosphorylated STAT5b (pSTAT5b) was observed upon GH treatment (Fig. 3A, lanes 1, 5, 9, and 13), reflecting the modest amount of endogenous GHR in this cell type. Overexpression of wild-type GHRfl, GHRd3, or combination GHRfl/GHRd3 activated STAT5b similarly, with robust pSTAT5b observed in response to 10 and 100 ng/ml GH (Fig. 3A, lanes 10–12 and 14–16).

Strikingly, both mutants in their homozygous state showed significant reduction on GH-induced STAT5b activation. In the cells expressing the GHRd3(C94S), minimal pSTAT5b was observed upon treatment with GH (1–100 ng/ml) (Fig 3B, lanes 11 and 15), consistent with the inability of the C94S mutant to bind GH (Fig. 2B), thus suggesting that this mutant is essentially incapable of mediating GH action *in vivo*. The GHRfl(H150Q), compared with wild-type GHR, demonstrated modestly reduced ability to mediate GH-induced pSTAT5b (Fig. 3B, lanes 12 and 16), suggesting that this mutant might show a significant defect in transducing GH-induced signal in response to physiological concentrations of GH *in vivo*.

When the two GHR mutants were coexpressed at 1:1 ratio with wild-type GHR in HEK293 cells, activation of STAT5b was similar to the wild-type GHR alone (Fig. 3C, lanes 9–11 and 13–15). In contrast, coexpression of GHRd3(C94S) and GHRfl(H150Q), mimicking the compound heterozygote state of the two patients, elicited significantly reduced pSTAT5b at 10 ng/ml GH (Fig. 3C, lane 12). At 100 ng/ml GH, pSTAT5b was detected but at low levels (Fig. 3C, lane 16).

In addition to reconstitution systems, the effects of C94S or H150Q mutations on GH-induced signal transduction were also examined in primary dermal fibroblasts established from patient 1 (compound heterozygote), her mother (H150Q heterozygote), and her father (C94S heterozygote). To better visualize STAT5b activation in the fibroblasts, STAT5b was overexpressed by infecting cells with adenovirus carrying N-terminal Flag-tagged human STAT5b (F-STAT5b) cDNA. Consistent with observations in the reconstituted HEK293 cells, the GH-induced activation of F-STAT5b was largely abolished in patient 1 cells, in response to 10 and 100 ng/ml of GH (Fig. 3D, lanes 8 and 12), thus indicating the detrimental effects of the compound heterozygote on GH-induced signal transduction *in vivo*. Interestingly, unlike observations in HEK293 cells, F-STAT5b activation in primary fibroblasts from the mother [GHRd3/GHRfl(H150Q] heterozygote) and father [GHRd3/GHRd3(C94S) heterozygote] were also significantly impaired (Fig. 3D, compare lane 5 with lanes 6 or 7 and lane 9 with lanes 10 and 11). Thus, in this assay system, activation of F-STAT5b was severely impaired in the compound heterozygote and partially impaired in the simple heterozygotes at the concentrations of GH used in the experiment.

The compound heterozygote causes a reduction of GH-induced gene transcription

The effects of C94S or H150Q mutation on GH-induced gene transcription were determined in reconstituted HEK293 cells, using luciferase reporter assays. No significant GH-induced Luc activity was observed in the cells expressing

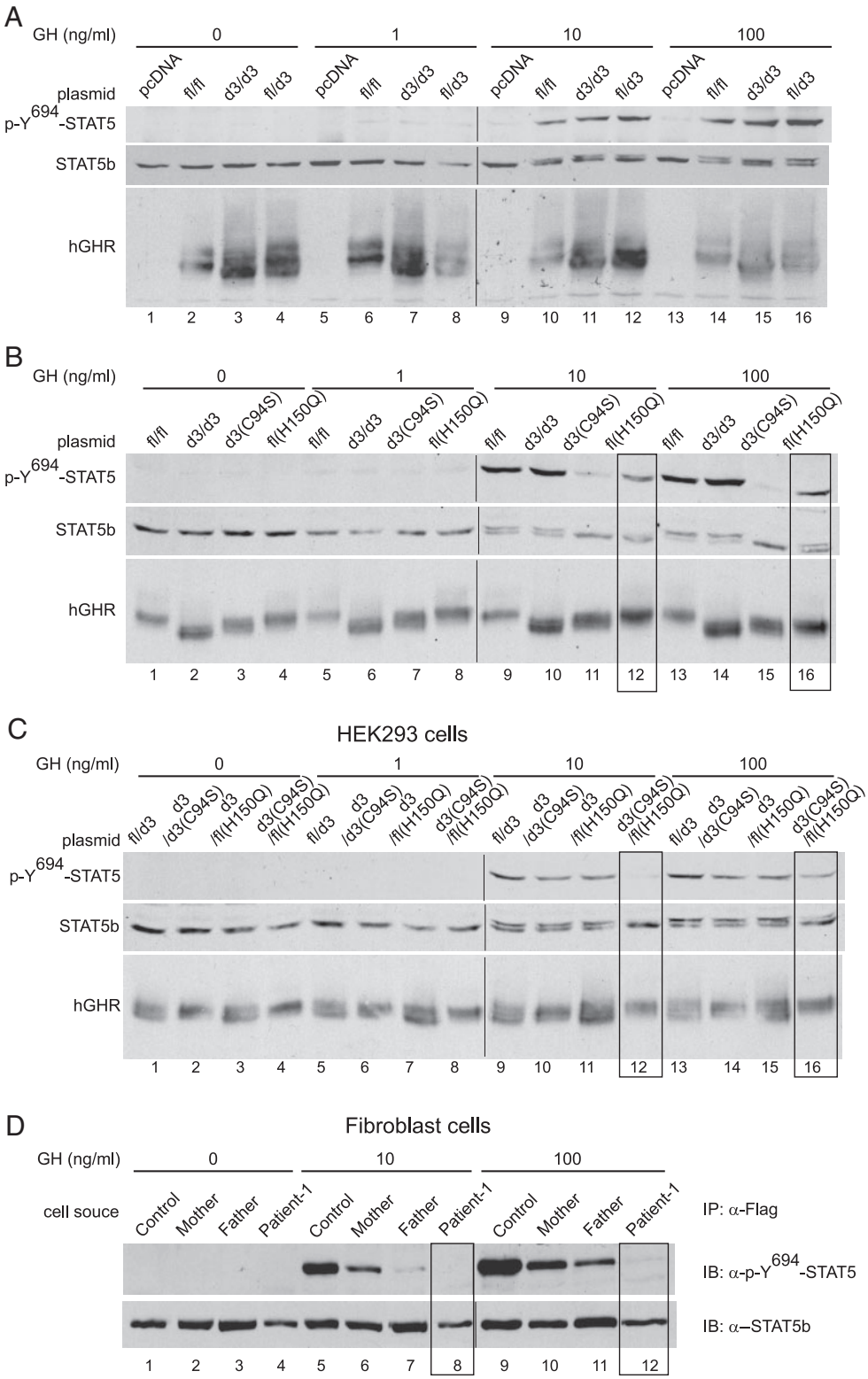

FIG. 3. GH-induced STAT5b activation in HEK293 cells expressing GHR variants. A–C, HEK293 cells were transfected with cDNAs carrying GHR variants as described in Fig. 2 and were treated with or without 1, 10, or 100 ng/ml GH as indicated. The cell lysates were collected 20 min after treatment and subjected to Western immunoblot analyses (~60 μg cell lysates per lane) with specific antibodies against proteins indicated at the *left* of each blot. Western immunoblots for phospho-STAT5 protein were stripped and reprobed with anti-STAT5b antibody or anti-hGHR antibody as indicated. D, Fibroblasts infected with adenovirus carrying cDNA encoding F-STAT5b were treated with or without 10 or 100 ng/ml GH as indicated. The cell lysates were collected 20 min after treatment, immunoprecipitated (IP) with anti-FLAG-M2-agarose beads (~800 μg cell lysates per immunoprecipitation), and probed for phospho-STAT5. The immunoblot (IB) for phospho-STAT5 was subsequently stripped and reprobed with anti-STAT5b antibody.

endogenous GHR (transfected with pcDNA) (Fig. 4A), consistent with the minimal STAT5b activation observed in these cells (Fig. 3A).

In the cells expressing homozygous GHRd3(C94S), Luc activity was not induced at any dose of GH used (Fig. 4A). On the other hand, homozygous GHRfl(H150Q) did not appear to impact luciferase activity at these GH concentrations. In cells expressing both GHR mutations, GHRd3(C94S)/GHRfl(H150Q), luciferase activity was markedly reduced at GH concentrations of 1 and 10 ng/ml, with partial recovery at GH concentrations of 50–100 ng/ml.

Discussion

In this study, we report two GHI patients from a nonconsanguineous Austrian family, who presented with severe growth retardation, elevated circulating levels of GH, but low levels of serum IGF-I and IGFBP-3. The older patient failed to respond to GH treatment but modestly responded to IGF-I therapy. The analyses of the GHR gene led to identification of two novel GHR mutations, one in each allele, with the mutations recessively inherited. Our *in vitro* functional analyses demonstrated the potential detrimental effects of the compound heterozygous mutations on GHR signal transduction and gene regulation, thus supporting the mutant GHR as the etiology for the growth failure and IGF deficiency observed in the patients.

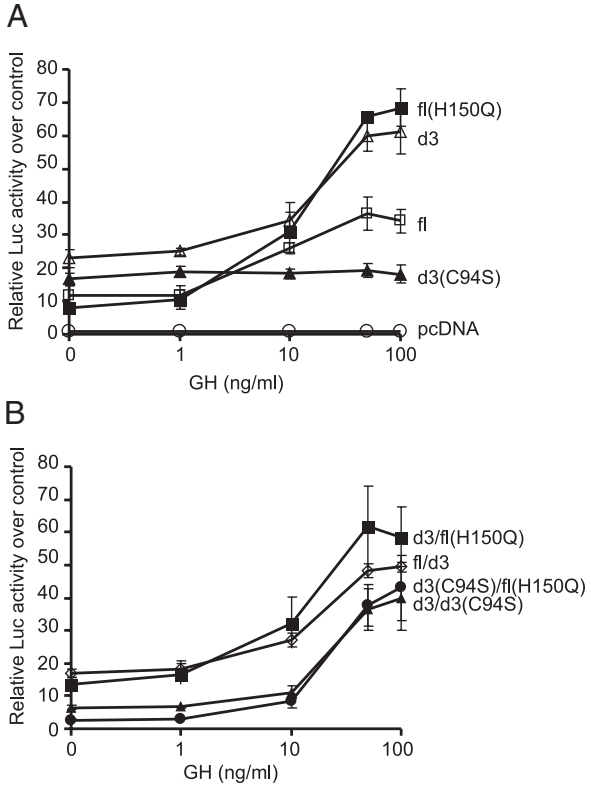

FIG. 4. GH induced luciferase activity in HEK293 cells expressing GHR variants. HEK293 cells were transfected with 1 μg luciferase reporter plasmid (p8xGHRE-LUC) plus either 1 μg vector or 1 μg GHR variants (0.5 μg each of two cotransfected variants) as indicated in *Subjects and Methods*. The Luc activities were measured with the luciferase assay system (Promega), following the manufacturer's protocol, and were normalized to total protein concentration. A and B, Relative Luc activity over control (no exogenous GHR expressed), which compares the levels of STAT5b-dependent gene expression activated by different GHR variants upon GH treatment. The results presented (mean ± SE) are from three independent experiments performed in duplicate.

The two mutations identified, C94S and H150Q, are both located in the extracellular domain of the GHR. The extracellular domain of GHR contains seven cysteine residues, with residues C38 and C48, C83 and C94, and C108 and C122 forming three intracellular disulfide bonds (29). The remaining unpaired cysteine 241 is believed to form a disulfide bond upon GH-induced GHR dimerization (30). A mutation of C94S would thus be predicted to disrupt the formation of the C83-C94 disulfide bond and lead to further alterations in GHR structure. Interestingly, *in vitro* mutagenesis studies had suggested that of the three disulfide bonds, the C83-C94 disulfide bond was the most critical for GHR to bind GH (27). Consistent with these inferences, our *in vitro* studies demonstrated that GH cannot bind to the surface of cells expressing homozygous GHRd3(C94S) (Fig. 2B), and the C94 mutation would, in homozygous form, thus render a nonfunctional GHR *in vivo* (Figs. 3 and 4).

The significance of these extracellular cysteine residues in normal GHR function was also suggested by the previous identification of a homozygous missense mutation C38S in a patient with GHI syndrome, although no functional studies were provided for this GHR mutant (31).

Unlike residue C94, our results indicate that the homozygous GHRfl(H150Q) has normal GH binding (Fig. 2B) but is aberrant for GH-induced signal transduction and gene expression in response to physiological concentrations of GH (1–10 ng/ml) (Figs. 3B and 4A). At higher doses of GH (50–100 ng/ml), the GH-induced STAT5b activation and Luc activity in the cells expressing GHRfl(H150Q) reached a level comparable with that seen in the cells expressing wild-type GHR (Figs. 3B and 4A). These results are in contrast to a previous *in vitro* mutagenesis study, in which generation of an H150D mutation caused an 89% reduction in GH-induced gene expression, even in response to 200 ng/ml GH (32). Such dramatically reduced response to GH was likely due to a severe disruption in GHR dimerization and GH-induced receptor orientation, although binding of GH was reportedly unaffected (32). This suggests that replacing H150 with a noncharged residue (Gln, Q), as in our patients, has a milder effect on GHR structure and function than replacement of H150 with a charged residue (Asp, D).

When the GHRfl(H150Q) was coexpressed with GHRd3(C94S) in HEK293 cells, both GH-induced STAT5b activation and STAT5b-dependent gene expression were significantly reduced, compared with cells expressing GHRfl(H150Q) or wild-type GHR (Figs. 3C and 4B). Because GHRd3(C94S) is a nonfunctional GHR, these observed GH responses, particularly the increase in STAT5b activation and gene expression in response to higher doses of GH (50–100 ng/ml) (Figs. 3C and 4B) are presumably attributed to the contributions of the GHRfl(H150Q). This also suggests that if expressed at 1:1 ratio, the GHRfl(H150Q) can potentially compensate for the defective GHRd3(C94S) at higher doses of GH treatment. However, the compound heterozygous GHR mutations abolished GH-induced STAT5b activation in the fibro-

blasts from patient 1 (Fig. 3D), who also failed to respond to a trial of GH treatment, suggesting that, under *in vivo* conditions: 1) GHRd3(C94S) may be preferentially expressed; 2) GHRd3(C94S) has a dominant-negative effect; 3) GHRd3(C94S) cannot form stable dimers with GHRfl(H150Q); or 4) therapeutic levels of GH cannot adequately compensate for aberrant GHR function (*in vivo*). Indeed, the somewhat milder and atypical features of the two patients, compared with classical GHI syndrome, undoubtedly reflects the incomplete nature of the receptor defects.

It has been difficult, to date, to demonstrate convincingly that heterozygous mutations in GHR can result in clinically significant GH insensitivity, except in rare dominant-negative situations. In the present case, through genetic and functional analyses, our results indicate that the clinical phenotype of the patients is reasonably attributable to the additive effects of each heterozygous mutation. Furthermore, our functional analyses suggest that the C94S heterozygous state could cause partial GHI (Figs. 3D and 4B), although impact on growth appeared modest, with statures of family members carrying the heterozygous C94S GHR within the normal range (−1.0 and −0.6 SDS). It was of note that the one child who carries two normal GHR alleles has a height of +1.5 SDS, which was 1.5–2 SDS above those of heterozygote family members, suggesting the intriguing possibility that the heterozygous state may, in fact, have some biological consequence. Further studies of the extended family would be necessary to determine the consistency of this observation.

We hypothesize that in patients who are heterozygous for GHR mutations that, in an homozygous or compound heterozygous state would result in clinical GHI, stature within the normal range may be achieved by either compensatory GH secretion or compensatory GH signaling downstream of the GHR. In situations in which such compensatory mechanisms prove inadequate, short stature may be the result. Indeed, the same may well be true for heterozygosity for genes controlling other aspects of the GH-IGF axis, such as GH signaling, IGF-I gene expression, or IGF-I receptor function, in which varying ability of the individual to compensate may explain, at least in part, deviations from mean stature.

Acknowledgments

We thank Tercica, Inc. (Brisbane, CA) for supplying recombinant human IGF-I for the patient who is still on treatment.

Received November 29, 2006. Accepted March 28, 2007.

Address all correspondence and requests for reprints to: Dr. Stefan Riedl, Department of Pediatrics, University of Vienna, 1090 Vienna, Austria. E-mail: stefan.riedl@meduniwien.ac.at; or Dr. Vivian Hwa, Department of Pediatrics, NRC5, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 9723-3098. E-mail: hwav@ohsu.edu.

This work was supported by Pfizer Corp. Austria (to H.F. and S.R.).

Disclosure Statement: P.F., S.R., S.A., K.L.P., B.M.L., G.H., V.H., and H.F. have nothing to disclose. R.G.R. consults and received lecture fees from Tercica.

References

1. **Savage MO, Attie KM, David A, Metherell LA, Clark AJ, Camacho-Hubner C** 2006 Endocrine assessment, molecular characterization and treatment of growth hormone insensitivity disorders. Nat Clin Pract Endocrinol Metab 2:395–407
2. **Laron Z** 2004 Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958–2003. J Clin Endocrinol Metab 89:1031–1044
3. **Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, Tsubaki J, Pratt KL, Bezrodnik L, Jasper H, Tepper A, Heinrich JJ, Rosenfeld RG** 2003 Growth hormone insensitivity associated with a STAT5b mutation. N Engl J Med 349:1139–1147
4. **Hwa V, Little B, Adiyaman P, Kofoed EM, Pratt KL, Ocal G, Berberoglu M, Rosenfeld RG** 2005 Severe growth hormone insensitivity resulting from total absence of signal transducer and activator of transcription 5b. J Clin Endocrinol Metab 90:4260–4266
5. **Woods KA, Camacho-Hubner C, Savage MO, Clark AJ** 1996 Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 335:1363–1367
6. **Behncken SN, Waters MJ** 1999 Molecular recognition events involved in the activation of the growth hormone receptor by growth hormone. J Mol Recognit 12:355–362
7. **Brown RJ, Adams JJ, Pelekanos RA, Wan Y, McKinstry WJ, Palethorpe K, Seeber RM, Monks TA, Eidne KA, Parker MW, Waters MJ** 2005 Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. Nat Struct Mol Biol 12:814–821
8. **Laron Z, Pertzelan A, Mannheimer S** 1966 Genetic pituitary dwarfism with high serum concentration of growth hormone—a new inborn error of metabolism? Isr J Med Sci 2:152–155
9. **Godowski PJ, Leung DW, Meacham LR, Galgani JP, Hellmiss R, Keret R, Rotwein PS, Parks JS, Laron Z, Wood WI** 1989 Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. Proc Natl Acad Sci USA 86:8083–8087
10. **Rosenfeld R, Rosenbloom A, Guevara-Aguirre J** 1994 Growth hormone (GH) insensitivity due to primary GH receptor deficiency. Endocr Rev 15:369–390
11. **Fielder PJ, Guevara-Aguirre J, Rosenbloom AL, Carlsson L, Hintz RL, Rosenfeld RG** 1992 Expression of serum insulin-like growth factors, insulin-like growth factor-binding proteins, and the growth hormone-binding protein in heterozygote relatives of Ecuadorian growth hormone receptor deficient patients. J Clin Endocrinol Metab 74:743–750
12. **Goddard AD, Covello R, Luoh SM, Clackson T, Attie KM, Gesundheit N, Rundle AC, Wells JA, Carlsson LM** 1995 Mutations of the growth hormone receptor in children with idiopathic short stature. The Growth Hormone Insensitivity Study Group. N Engl J Med 333:1093–1098
13. **Woods KA, Dastot F, Preece MA, Clark AJL, Postel-Vinay M-C, Chatelain PG, Ranke MB, Rosenfeld RG, Amselem S, Savage MO** 1997 Phenotype: genotype relationships in growth hormone insensitivity syndrome. J Clin Endocrinol Metab 82:3529–3535
14. **Ayling RM, Ross R, Towner P, Von Laue S, Finidori J, Moutoussamy S, Buchanan CR, Clayton PE, Norman MR** 1997 A dominant-negative mutation of the growth hormone receptor causes familial short stature. Nat Genet 16:13–14
15. **Pantel J, Grulich-Henn J, Bettendorf M, Strasburger CJ, Heinrich U, Amselem S** 2003 Heterozygous nonsense mutation in exon 3 of the growth hormone receptor (GHR) in severe GH insensitivity (Laron syndrome) and the issue of the origin and function of the GHRd3 isoform. J Clin Endocrinol Metab 88:1705–1710
16. **Kaji H, Nose O, Tajiri H, Takahashi Y, Iida K, Takahashi T, Okimura Y, Abe H, Chihara K** 1997 Novel compound heterozygous mutations of growth hormone (GH) receptor gene in a patient with GH insensitivity syndrome. J Clin Endocrinol Metab 82:3705–3709
17. **Enberg B, Luthman H, Segnestam K, Ritzen EM, Sundstrom M, Norstedt G** 2000 Characterisation of novel missense mutations in the GH receptor gene causing severe growth retardation. Eur J Endocrinol 143:71–76
18. **Tiulpakov A, Rubtsov P, Dedov I, Peterkova V, Bezlepkina O, Chrousos GP, Hochberg Z** 2005 A novel C-terminal growth hormone receptor (GHR) mutation results in impaired GHR-STAT5 but normal STAT-3 signaling. J Clin Endocrinol Metab 90:542–547
19. **Riedl S, Frisch H** 2006 Effects of growth hormone (GH) and insulin-like growth factor-I therapy in patients with gene defects in the GH axis. J Pediatr Endocrinol Metab 19:229–236
20. **Pantel J, Machinis K, Sobrier ML, Duquesnoy P, Goossens M, Amselem S** 2000 Species-specific alternative splice mimicry at the growth hormone receptor locus revealed by the lineage of retroelements during primate evolution. J Biol Chem 275:18664–18669
21. **Ross RJ, Esposito N, Shen XY, Von Laue S, Chew SL, Dobson PR, Postel-Vinay MC, Finidori J** 1997 A short isoform of the human growth hormone receptor functions as a dominant negative inhibitor of the full-length receptor and generates large amounts of binding protein. Mol Endocrinol 11:265–273
22. **Hwa V, Little B, Kofoed EM, Rosenfeld RG** 2004 Transcriptional regulation of insulin-like growth factor-I by interferon-γ requires STAT-5b. J Biol Chem 279:2728–2736
23. **Fang P, Kofoed EM, Little BM, Wang X, Ross RJ, Frank SJ, Hwa V, Rosenfeld RG** 2006 A mutant signal transducer and activator of transcription 5b, asso-

ciated with growth hormone insensitivity and insulin-like growth factor-I deficiency, cannot function as a signal transducer or transcription factor. J Clin Endocrinol Metab 91:1526–1534
24. **Fang P, Hwa V, Rosenfeld RG** 2006 Interferon-γ-induced dephosphorylation of STAT3 and apoptosis are dependent on the mTOR pathway. Exp Cell Res 312:1229–1239
25. **Zhang Y, Guan R, Jiang J, Kopchick JJ, Black RA, Baumann G, Frank SJ** 2001 Growth hormone (GH)-induced dimerization inhibits phorbol ester-stimulated GH receptor proteolysis. J Biol Chem 276:24565–24573
26. **Dos Santos C, Essioux L, Teinturier C, Tauber M, Goffin V, Bougneres P** 2004 A common polymorphism of the growth hormone receptor is associated with increased responsiveness to growth hormone. Nat Genet 36:720–724
27. **van den Eijnden MJ, Lahaye LL, Strous GJ** 2006 Disulfide bonds determine growth hormone receptor folding, dimerisation and ligand binding. J Cell Sci 119:3078–3086
28. **Harding PA, Wang XZ, Kelder B, Souza S, Okada S, Kopchick JJ** 1994 *In vitro* mutagenesis of growth hormone receptor Asn-linked glycosylation sites. Mol Cell Endocrinol 106:171–180
29. **Fuh G, Mulkerrin MG, Bass S, McFarland N, Brochier M, Bourell JH, Light DR, Wells JA** 1990 The human growth hormone receptor. Secretion from *Escherichia coli* and disulfide bonding pattern of the extracellular binding domain. J Biol Chem 265:3111–3115
30. **Zhang Y, Jiang J, Kopchick JJ, Frank SJ** 1999 Disulfide linkage of growth hormone (GH) receptors (GHR) reflects GH-induced GHR dimerization. Association of JAK2 with the GHR is enhanced by receptor dimerization. J Biol Chem 274:33072–33084
31. **Sobrier ML, Dastot F, Duquesnoy P, Kandemir N, Yordam N, Goossens M, Amselem S** 1997 Nine novel growth hormone receptor gene mutations in patients with Laron syndrome. J Clin Endocrinol Metab 82:435–437
32. **Chen C, Brinkworth R, Waters MJ** 1997 The role of receptor dimerization domain residues in growth hormone signaling. J Biol Chem 272:5133–5140

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.