Study of GH Sensitivity in Chilean Patients with Idiopathic Short Stature

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We hypothesized that some children with idiopathic short stature in Chile might bear heterozygous mutations of the GH receptor. We selected 26 patients (3 females, 23 males) from 112 patients who consulted for idiopathic short stature at the University of Chile. Their chronological age was 8.3 ± 1.9, and bone age was 6.1 ± 1.0 yr. Their height was -3.0 ± 0.7 SDS; IGF-I, -1.2 ± 1.1 SD; IGF binding protein 3, -0.7 ± 2.0 SDS; and GH binding protein, 0.4 ± 0.8 SDS. Patients were admitted, and blood samples were obtained every 20 min to determine GH concentrations overnight. Coding sequences and intron-exon boundaries of exons 2–10 of GH receptor gene were amplified by PCR and subsequently analyzed through single-strand conformational analysis. Mean serum GH concentration, over 12-h, was 0.20 ± 0.08 nM; pulse amplitude, 0.40 ± 0.15 nM; number of peaks, 5.8 ± 1.5 peaks/12 h; peak value of GH during the 12-h sampling, 1.03 ± 0.53 nM; and area under the curve, 151.4 ± 56.1 nM/12 h. There were positive correlations between mean GH *vs.* area under the curve ($P < 0.001$) and GH peak ($P < 0.01$).

The single-strand conformational analysis of the GH receptor gene showed abnormal migration for exon 6 in 9 patients and for exon 10 in 9 patients, which (by sequence analysis) corresponded to 2 polymorphisms of the GH receptor gene: an A-to-G transition in third position of codon 168 in exon 6 and a C-to-A transversion in the first position of codon 526 in exon 10. We further sequenced all coding exons and intron-exon boundaries in the most affected patients (nos. 6, 9, 11, 14, 15, 16, and 23). This analysis revealed a C-to-T transition in codon 161 of exon 6 in patient 23, which results in an amino acid change (Arg to Cys) in an heterozygous form in the patient and his father. In conclusion, the results of our study suggest that, in Chilean patients with idiopathic short stature, GH receptor gene mutations are uncommon, although we cannot exclude mutations that were missed by single-strand conformational analysis or mutations within introns or in the promoter regions of the GH receptor gene. (*J Clin Endocrinol Metab* 86: 4375–4381, 2001)

G ROWTH FAILURE CAN be the result of primary growth abnormalities such as bone dysplasias, chromosomal abnormalities, and intrauterine growth retardation, as well as genetic short stature. Secondary growth disorders include systemic illnesses and endocrine disorders that may affect the GH-IGF axis. Once known causes of short stature have been excluded, these patients are defined with various terms, including idiopathic short stature (ISS), which indicates that the cause of their growth failure has not yet been explained. Some of these children may show biochemical features suggestive of GH insensitivity (GHIS) such as low IGF-I, low IGF binding protein 3 (IGFBP-3), and high spontaneous or poststimulated GH plasmatic levels.

The term GHIS describes a group of inherited disorders characterized by a reduction in the biological effects of GH in the presence of normal or elevated circulating levels of GH (1). These disorders are characterized by poor postnatal growth and short stature and low circulating levels of IGF-I and IGFBP-3 (2). The original description of this syndrome, known as Laron syndrome, was first recognized 3 decades ago. The affected children have variable growth retardation,

with heights between -2.2 to -10.4 SD, hypoglycemic episodes, and acromicria, among other dysmorphic features (2, 3). The etiology of the disorder is caused by GH receptor (GHr) defects. Over 30 mutations in the GHr gene, either in homozygous or heterozygous form, have been reported (4). The human GHr gene consists of 9 coding exons, spanning at least 87 kb of chromosome 5. Exon 2 encodes the signal peptide; exons 3–7, the extracellular domain; exon 8, the transmembrane domain; and exon 9 and part of exon 10, the intracellular domain (5). The majority of the mutations in GHr gene are located in the portion involving exons 3–7, which also codifies for the GH binding protein (GHBP), generated by limited proteolysis of the GHr (6). For this reason, most GHIS patients show GHBP levels below the normal range (7)

Recently, the presence of partial GHr insensitivity has been reported in patients with ISS (8, 9). These patients have mean 12-h GH levels significantly higher than the subset of patients with ISS without GH partial insensitivity (10). Abnormalities in the GHr gene may cause deficiencies in signaling and, as a consequence, reduced expression of IGF-I and elevated GH levels.

We are aware of only two molecular genetic studies regarding the prevalence of partial GHIS in ISS patients. Goddard and co-workers (8, 11) found eight patients carrying a mutation in the GHr gene leading to an heterozygous ge-

notype, among 100 patients with ISS. Sánchez *et al.* (9) found no definitive mutation within the GHr gene among 17 children with ISS. We hypothesized that some patients with well-characterized ISS in Chile might bear heterozygous mutations in the GHr gene, which would cause growth retardation.

Materials and Methods

Patients

The study was approved by the Institutional Review Board of the San Borja Arriarán Hospital in Santiago, Chile. Informed consent was obtained from at least 1 parent of each patient. Candidates for the study were selected from a total of 112 patients who consulted for ISS (height, -2.5 SDS), in the pediatric endocrine clinic at the Institute of Maternal and Child Research of the University of Chile. Selection criteria included lack of pubertal development, short stature (with a height of less or equal to -2.5 SD), slow growth velocity (defined as less than 10th percentile for bone age), normal birth weight, delayed bone age, GH levels above 0.45 nM (10 ng/ml) after stimulation with clonidine or hypoglycemia, low IGF-I, and/or low IGFBP-3. We excluded other identifiable endocrine, genetic, skeletal dysplasias, or psychosocial causes of short stature. We did not specifically seek patients with a Laron phenotype, because patients with partial GHIS do not necessarily show this phenotype. Specifically, the children had normal body proportions and normal cell blood count, blood chemistries, plasma electrolytes, renal and liver function tests, urinalysis, stool parasites, thyroid function, and karyotype (girls).

Among the 112 patients, we selected 26 patients (23 males and 3 females) who met all the inclusion criteria. Clinical characteristics of the patients and their parents are depicted in Table 1. Patients were admitted 4 h before the assessment of spontaneous GH secretion, to become acclimatized to the hospital environment. Height was measured 10 times with the use of a Harpenden stadiometer. We used National Center for Health Statistics growth curves, which have been found to be applicable

to Chilean children (12, 13). Bone age was determined by the method of Greulich and Pyle. Baseline concentrations of GH were assessed by obtaining blood samples, through a short iv catheter placed in the forearm vein. Blood samples were obtained every 20 min, from 2000 h to 0800 h, to determine GH concentrations. Nine patients were submitted to an IGF-I generation test (14). All the selected patients were from Chile and had at least 2 Chilean surnames from Spanish origin. Ethnically, the Chilean population is considered to be a mixture of 30% Amerindian and 70% European (mainly Spanish) origin (15).

Hormone assays

Serum GH was measured by a double-antibody RIA with a sensitivity of 0.8 ng/ml and inter- and intraassay coefficients of variation (CV) of 10% and 6.5%, respectively. GH was labeled with the lactoperoxidase method (16). All reagents for GH RIA were donated by the National Hormone and Pituitary Program. (human GH-I-3, antihuman GH-2 antisera, human GH-reference preparation). Serum insulin-like growth factor I was measured by RIA, after acid-ethanol extraction, using a reference standard purchased from Bachem (Torrance, CA) and an antiserum (NIH UB2–495) donated by the National Hormone and Pituitary Program (Rockville, MD). This assay has an intraassay CV of 7.5% and an interassay CV of 11.1% (17, 18). Serum IGFBP-3 was measured by immunoradiometric analysis with a commercial kit (Diagnostic Systems Laboratories, Inc., Webster, TX) with a interassay CV of 1.8%, and a intraassay CV of 1.1%. The IGF-I and IGFBP-3 detection limits were 10 ng/ml and 0.05 mg/liter, respectively. GHBP was determined by monoclonal RIA [monoclonal antibody (Mab) 263] with inter- and intraassay CVs of 11.4% and 5.2%, respectively (19). This assay incubates the patient's serum with I^{125}-labeled GH plus Mab 263 against the GHr in the presence or absence of nonlabeled GH. The soluble form of GHr (GHBP) binds to Mab 263 plus nonlabeled GH to form the trimolecular complex anti-GHR<>GHBP<>^{125}I-hGH. Separation of bound/free form of I^{125}-hGH was performed using a second antibody (antimouse IgG, whole molecule, Calbiochem 401210, San Diego, CA) and polyethylene glycol. The percent binding of GHBP from each patient is in reference to 100% binding of GHBP obtained from a pool of adult serum.

TABLE 1. Clinical and laboratory data of patients at study entry

Patient no.	Sex F/M	CA (yr)	Height (SDS)	P/T (SDS)	BA del (yr)	IGF-I (SDS)	IGFBP3 (SDS)	GHBP (SDS)	GHBP (%)	Peak GH (nM)
1	F	4.7	−3.1	0.6	2.0	−1.4	−2.0	0.3	55.0	3.09
2	F	7.6	−2.6	−0.6	2.6	−0.9	−1.0	0.5	60.0	0.91
3	M	9.8	−3.0	0.8	2.0	−1.9	2.3	−0.1	55.0	0.91
4	M	8.7	−2.5	1.9	1.8	−1.2	0.3	−1.4	34.0	1.83
5	M	9.9	−3.2	−0.9	4.0	0.4	−1.5	−0.5	48.0	0.81
6	M	8.3	−4.3	0.3	3.0	−3.4	−3.8	0.0	56.0	0.65
7	M	9.5	−2.7	1.1	2.0	−2.1	0.0	0.0	56.6	0.57
8	M	9.8	−2.5	1.3	3.8	−1.2	−1.5	0.1	58.0	1.90
9	M	10.5	−2.5	−0.6	2.2	−3.1	−2.5	1.2	75.3	2.60
10	M	10.5	−2.7	−0.2	3.9	0.2	−1.0	1.2	75.3	0.94
11	M	9.2	−2.5	0.8	3.1	−2.9	−1.5	0.4	63.1	1.27
12	M	9.7	−2.5	−0.3	2.5	−1.1	0.5	−0.2	52.8	0.70
13	M	7.7	−3.1	1.0	3.5	−1.2	−1.3	0.6	52.8	1.40
14	M	7.9	−4.2	1.4	2.7	−3.3	−3.2	1.4	63.7	0.78
15	M	5.4	−2.5	−0.6	1.1	−2.0	−2.3	0.1	45.4	0.99
16	M	6.7	−4.0	−0.5	1.3	−2.0	−3.2	0.1	45.5	1.48
17	M	8.7	−2.5	0.9	2.7	−0.8	−1.5	0.9	69.8	0.72
18	M	4.8	−2.5	1.5	1.0	−1.1	−3.8	0.5	54.2	0.54
19	M	5.8	−2.5	0.3	1.8	−0.7	−2.0	1.6	75.7	0.66
20	M	8.8	−2.7	1.4	1.0	−0.5	0.5	0.0	56.5	0.52
21	M	10.4	−2.5	1.0	1.3	−0.1	0.3	−0.3	51.9	1.51
22	M	9.2	−5.2	−0.3	1.5	−1.0	1.0	−0.4	49.2	0.74
23	M	6.3	−2.7	−0.7	2.0	−0.1	3.3	0.2	47.2	1.69
24	M	5.3	−3.5	−1.2	2.5	−1.4	1.0	1.7	77.2	1.75
25	F	10.2	−2.8	1.5	2.2	1.0	2.8	0.0	63.8	1.45
26	M	10.5	−3.0	5.0	1.0	−0.5	1.3	2.1	89.0	0.98
Mean		8.3	−3.0	0.6	2.2	−1.2	−0.7	0.4	58.9	1.21
SD		1.9	0.7	1.3	0.9	1.1	2.0	0.8	12.3	0.65

The percent binding of GHBP from each patient is in reference to 100% binding of GHBP obtained from a pool of adult serum.
CA, Chronological age; BA del, bone age delay; Peak GH, peak of stimulated GH (clonidine or insulin).

Analysis of the pulsatile characteristics of GH secretion was performed using the computer program Pulsar (20).

The IGF-I generation test included a baseline (d 1) blood sample for measurement of serum IGF-I level. From d 1–4, GH was administered at a dose of 0.1 U = 0.03 mg GH sc at night. On d 5, at 0900 h, we obtained a blood sample for the final serum IGF-I level determination (14).

Statistical analysis

The statistical analysis of the data was performed by ANOVA. Data are expressed as the mean ± SEM.

Molecular genetic study

DNA was isolated from peripheral leukocytes of each patient (21). PCR amplification, covering coding sequences and intron-exon boundaries of exons 2–9, was performed. Only the coding region of exon 10 was PCR-amplified using three overlapping pairs of primers. PCR primers are shown in Fig. 1. In exons 2, 4, 5, 6, 7, 8, and 9, PCR amplification involved an initial denaturation for 5 min at 95 C, followed by 28 cycles consisting of 1 min at 95 C, 1 min at 48 C, and 1 min at 72 C. Exon 3 was amplified with an initial denaturation of 2 min at 94 C, followed by 28 cycles involving a denaturation step of 30 sec at 94 C, an annealing of 30 sec at 55 C, and an extension period of 30 sec at 72 C. The three overlapping fragments of the coding region of exon 10 were amplified with a touchdown PCR program including an initial denaturation of 2 min at 94 C; followed by 3 cycles consisting of 94 C for 30 sec, 59 C for 30 sec, and 72 C for 30 sec; another 3 cycles consisting of 94 C for 30 sec, 57 C for 30 sec, and 72 C for 30 sec; and finally, 25 cycles including a denaturation of 30 sec at 94 C, annealing of 30 sec at 55 C, and extension of 30 sec at 72 C. All PCR amplification reactions ended with a final extension period of 10 min at 72 C. After PCR amplification, all DNA fragments were purified from agarose gels using Geneclean II (Bio 101, Inc., Vista, CA).

For the single-strand conformational analysis (SSCA), PCR products were subjected to electrophoresis on MDE (BioWhittaker, Inc., Rockland, ME) 0.5× in the presence of glycerol, at 18 C, 6 W for 14 h; or, in the absence of glycerol, at 18 C, 3 W for 13 h. The gels were silver-stained after electrophoresis. Sequence analysis of all exons was performed by direct sequencing using the dsDNA Cycle Sequencing System (Life Technologies, Inc., Gaithersburg, MD), and γ-^{32} P-ATP, in the cases indicated in *Results*. Electrophoresis was carried out into 6% acrylamide/bisacrylamide (19:1) gels, in the presence of 8 M urea. Gels were dried and subjected to autoradiography for 24–48 h.

Results

Twenty-six children (3 females and 23 males) met our inclusion criteria for ISS. Their clinical data are summarized in Table 1. Their mean chronological age was 8.3 ± 1.9 yr (range, 10.5–4.7 yr), mean bone age was 6.1 ± 1.0 yr (9.5–2.7 yr), and mean bone age delay was 2.2 ± 0.9 yr. The mean height of these patients was −3.0 ± 0.7 SDS, and mean weight was 0.6 ± 1.3 SDS for chronological age. Therefore, these children had an increased weight for height. Mean plasma IGF-I level was −1.2 ± 1.1 SD (14.5 ± 5.7 nM), and the mean IGFBP-3 level was −0.7 ± 2.0 SD (70 ± 28 nM). GHBP levels (0.4 ± 0.8 SDS) were within the normal levels for our Chilean age- and sex-matched population. None of our patients had a GHBP below −2.0 SD, and only 1 patient (no. 26) had a GHBP level slightly above the normal range (2.1 SD).

In the assessment of nocturnal GH secretion, the mean 12-h serum GH concentration was 0.20 ± 0.08 nM, (range, 0.1–0.44 nM), well above the range used as a criteria to define GH deficiency (22, 23) and above the mean normal values reported for normal North American prepubertal children (24). Mean pulse amplitude was 0.4 ± 0.15 nM, and mean number of peaks was 5.8 ±1.5 peaks during 12 h. The mean peak value of GH during the 12-h sampling was 1.03 ± 0.53 nM (range, 3.1–0.45 nM). The mean area under the curve (AUC) of GH was 151.4 ± 56.1 nM/12 h. There were significant positive correlations between mean GH during spontaneous secretion *vs.* AUC (r = 0.99, $P < 0.001$) and between mean GH during spontaneous secretion *vs.* GH peak during spontaneous secretion (r = 0.89, $P < 0.01$). IGFBP-3 and IGF-I did not correlate with mean GH levels during spontaneous secretion (r = 0.14 and r = −0.05, respectively). We also analyzed whether there were any correlations between patient height SDS and the following factors: midparental height SDS, mother's height SDS, father's height SDS, mean GH, GH peak during spontaneous and stimulated secretion, and GH AUC. All these correlations were not significant. In addition, there were no correlations between IGF-I SDS *vs.* spontaneous GH peak and *vs.* stimulated GH peak, and IGFBP-3 SDS *vs.* spontaneous GH peak and *vs.* stimulated GH peak. There was no correlation between GHBP SD and each of the following parameters: mean GH levels, peak-stimulated and peak-spontaneous GH, IGF-I, IGFBP-3, stimulated IGF-I, and percent increase of IGF-I in the patients who underwent an IGF-I generation test. The results of GH sampling for each patient are summarized in Table 2. Mean IGF-I percent in-

EXON	PRIMER SEQUENCE
2	Forward: TCTGCTTTTAATTGCTGGGCTTT Reverse: GAATACAGTTCAGTGTTGTTTCAA
3	Forward: TACACAGGGTCATATCAGATTG Reverse: CTATTCCAGTTACTACCATCCC
4	Forward: ATATGACTCACCTGATTTCATGC Reverse: TAGGTACATCCATGGAGAGGAA
5	Forward: GCTACAACATGATTTTTGGAACAA Reverse: CATTTATTTAGTCTAAAACTATGTCA
6	Forward: AATATTAAATTGTGTCTGTCTGTGT Reverse: GAAAGAAAAGTCAAAGTGTAAGGT
7	Forward: TTGAGTTGTTGACTCTTTGGCC Reverse: AACTGTTATATTGACAAAAGCC
8	Forward: GAAACTGTGCTTCAACTAGTC Reverse: AACGGTACAAAATACCAA
9	Forward: AGAATATGTAGCTTTTAAGATGTCA Reverse: GACAGGAGTCTTCAGGTGTTAA
10a	Forward: GAGTTTCTTTTCATAGATCTTC Reverse: TTAACCTCTGTGGCTGAG
10b	Forward: ACATGAGGGTACCTCAGA Reverse: CAGAAGTAGGCATTGTCC
10c	Forward: GGAAATGGTCTCACTCTG Reverse: CAAAAGAAAGGCTAAGGC

FIG. 1. PCR primers used for amplification of coding sequences and intron-exon boundaries of exons 2–9.

crease during the IGF-I generation test was 27.57 ± 42.3%. Data are summarized in Table 3.

The SSCA of the nine coding exons of the GHr gene showed bands with an abnormal migration for exon 6 in nine

TABLE 2. Results of overnight GH sampling and pulsatile characteristics analysis by Pulsar

Patient no.	Mean GH (nM)	Peak GH (nM)	Peak amplitude (nM)	Peak number	AUC (nM)
1	0.20	0.80	0.32	8.0	148
2	0.25	1.40	0.53	5.0	187.2
3	0.20	0.86	0.42	4.0	151
4	0.10	0.86	0.27	3.0	77
5	0.15	0.71	0.28	6.0	107.3
6	0.15	0.92	0.49	6.0	111.4
7	0.29	1.60	0.62	5.0	211.4
8	0.20	0.79	0.26	9.0	148.4
10	0.12	0.62	0.35	5.0	88
11	0.26	1.29	0.57	4.0	194.7
12	0.28	1.33	0.65	4.0	209
13	0.44	3.11	0.75	5.0	325.3
14	0.12	0.75	0.27	5.0	90.9
15	0.18	0.79	0.32	7.0	128.9
16	0.19	1.15	0.26	7.0	136.1
17	0.18	0.89	0.40	6.0	128.8
18	0.14	0.45	0.17	8.0	97.5
19	0.24	1.42	0.42	6.0	177.7
20	0.16	0.67	0.33	6.0	117.2
21	0.19	0.53	0.26	7.0	139
22	0.21	0.80	0.42	7.0	153.6
23	0.32	1.37	0.65	4.0	245.2
24	0.24	1.00	0.42	5.0	173.8
25	0.19	1.07	0.26	7.0	141.8
26	0.13	0.54	0.23	7.0	96.6
Mean	0.21	1.03	0.40	5.8	151.4
SD	0.07	0.53	0.16	1.5	56.1
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Mean	0.20	NA	0.50	11.1	NA
SD	0.11	NA	0.73	16.2	NA
GH def		NA			NA
Mean	0.05	NA	0.15	3.4	NA
SD	0.03	NA	0.11	2.4	NA
Normal		NA			NA
Mean	0.18	NA	0.41	9.1	NA
SD	0.09	NA	0.21	4.6	NA

GH secretion analysis was not performed in patient 9. NA, Not applicable; GH def, GH deficiency; Normal, normal statured children. Data are obtained from *N Engl J Med* 1988, 319:201–207 (Ref. 20).

TABLE 3. Results of the IGF-I generation test in nmol/liter and SDS of IGF-I at baseline and d 5

Patient no.	Baseline IGF-I nM	Baseline IGF-I SDS	d 5 IGF-I nM	d 5 IGF-I SDS	% Increase from baseline
9	50.2	−0.50	44.5	−0.75	−11.4
12	51.6	0.57	64.0	1.26	23.9
15	28.4	−0.05	31.1	0.35	9.6
17	25.0	−0.30	54.9	2.90	119.2
18	33.2	0.58	36.6	0.94	10.2
19	25.6	−0.25	29.3	0.15	14.7
22	30.6	−0.60	29.1	−0.70	−5.1
23	20.4	−0.16	35.7	1.70	75.0
26	32.3	−1.28	36.2	−1.12	12.1
Mean	33.04	−0.14	40.16	0.53	27.6
SD	10.9	0.64	12.12	1.31	42.3

Percent increase of IGF-I from d 1–5 in nmol/liter (divide by 7.7 for ng/ml).

patients, and for exon 10 in nine patients. The sequence of the aberrant band in exon 6 revealed an A-to-G transition in the third position of codon 168, with no amino acid change. This nucleotide change has been previously described in normal subjects (8, 9). In exon 10, we found a C-to-A transversion in the first position of codon 526, deriving into an amino acid change, Ile to Leu. No other nucleotide changes were found by SSCA. Both nucleotide changes were found in some patients as well as in two control DNAs, concordant with polymorphic changes (Table 4). Because some patients exhibited more severe clinical and biochemical features, we selected patients 6, 9, 11, 14, 15, 16, and 23 for sequencing of all coding exons. The complete sequence of the exons, including their exon-intron boundaries, revealed a C–T point mutation on codon 161 of exon 6 in patient 23. This mutation, which results in an amino acid change, Arg to Cys, was present in an heterozygous form in the patient (number 23 in tables) and in his father and was absent in the mother (Fig. 2A). The C–T transition results in a gain of a NlaIII restriction site as shown in Fig. 2B. A fragment of 268 bp, corresponding to exon 6, was amplified by PCR and digested with NlaIII restriction enzyme. As shown in Fig. 2B, the patient and his father are heterozygous for the mutation.

We performed a screening assay for this mutation in 50 Chilean control individuals (100 chromosomes) and found that this mutation was not present, excluding the possibility of a polymorphism (data not shown). The heights of the index case and his father are −2.7 and −0.2 SDS, respectively. Neither the father nor the patient have a specific phenotype. IGF-I plasma values in the affected child and in the father were 20.4 nM and 33.6 nM, respectively. IGFBP-3 plasma values in the affected child and in the father were 84 nM and 164.5 nM, respectively. There-

TABLE 4. Polymorphic changes detected in the ISS patients studied in codon 168 of exon 6 and in codon 526 in exon 10

| Patient no.	G168	I526L
1	W	Heterozygous
2	W	Heterozygous
3	W	Heterozygous
4	W	Heterozygous
5	W	W
6	Homozygous	W
7	W	W
8	W	W
9	Homozygous	Heterozygous
10	Homozygous	Heterozygous
11	Homozygous	Heterozygous
12	Homozygous	W
13	Homozygous	W
14	W	Heterozygous
15	Homozygous	W
16	W	Heterozygous
17	W	W
18	Homozygous	W
19	Homozygous	W
20	W	W
21	W	W
22	W	W
23	W	W
24	W	W
25	W	W
26	W	W

W, Wild-type.
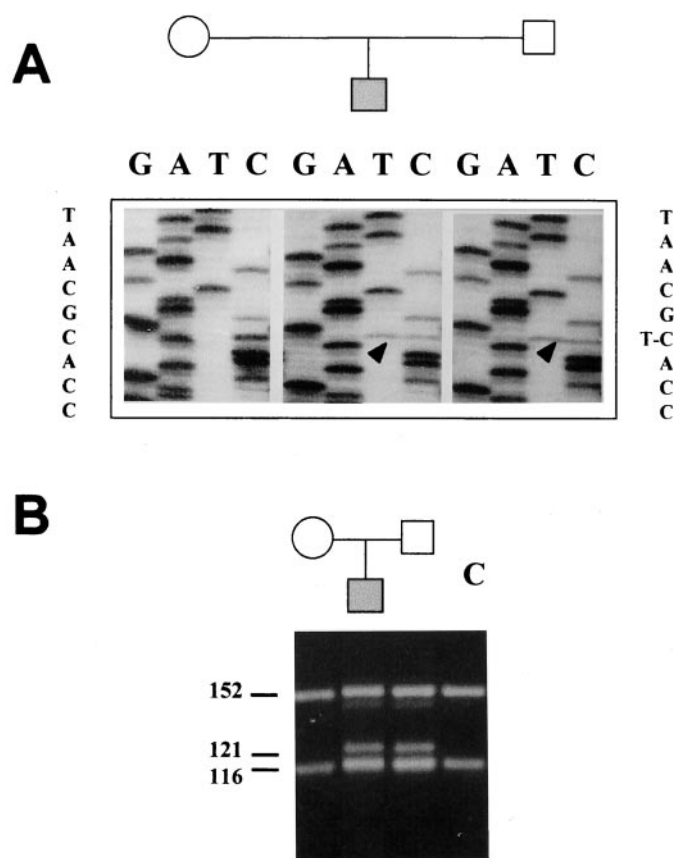
FIG. 2. A, Partial autoradiogram of the mutated and normal sequences from family members. Exon 6 from the GHR gene was amplified by PCR in each family member, and the products were purified and sequenced directly. An *arrow* indicates the mutations in each case. The normal sequences are indicated *at the left*, and the respective mutations are indicated *at the right*. B, Restriction analysis with NlaIII in family members. A fragment of 268 bp, corresponding to exon 6, was amplified by PCR, digested with NlaIII, and subjected to electrophoresis on Nusieve-agarose gels (2%–1%). NlaIII-digested fragments from exon 6 are indicated *at the left*. Normal fragments: 152 bp, 116 bp. Mutated fragments: 116 bp, 121 bp, (and 31 bp, not shown).

after, we performed an IGF generation test in the affected child. His stimulated values of IGF-I and IGFBP-3 were 35.7 nM and 129.5 nM, respectively, showing modest increases from baseline (Table 3). The patient exhibited a GH secretory pattern that might be attributable to diminished GH sensitivity. These features were a robust GH response to a GH stimulation test (1.69 nM), which is one of the greatest GH responses within the ISS group tested. In addition, during spontaneous secretion, the patient showed a mean GH concentration of 0.32 nM (which is 1.6 sD above the normal range reported), an AUC of 245.2 nM (which is 1.7 SDS above the mean GH AUC for the ISS group tested), and an increased peak amplitude of 0.65 nM (mean normal, 0.41 ± 0.21).

In addition, we analyzed whether there were any clinical or biochemical differences in the patients with heterozygous or homozygous polymorphisms. We analyzed the parents' height, peak GH after GH stimulation test, IGF-I, IGFBP-3 levels, GH analysis of 12-h spontaneous secretion (peak, number of peaks, amplitude of peaks, and AUC), and did not

find any relation between these parameters among the patients bearing the same polymorphism.

Discussion

We studied Chilean children with ISS, attempting to document the presence of mutations in the GHr. There are more heterozygous carriers of mutant GHrs than there are homozygous subjects, but it is unclear how often heterozygosity results in the phenotype of short stature. In the study from Goddard *et al.* in children with ISS, the authors found 3 patients bearing heterozygous mutations and 1 compound heterozygote, among 14 patients with ISS and low GHBP and IGF-I (11). In a population of ISS selected by less stringent criteria, 4 out of 86 patients were found to bear GHr mutations (8); and in another study, 1 of 17 ISS patients was probably a heterozygous mutant (9). Thus, the expression of both the complete GHIS phenotype in the homozygous patients and the partial GHIS phenotype in the heterozygous carriers is highly variable.

It has been shown, by several studies, that mutations of the GHr gene, in the heterozygous state, have different effects on carriers. In some cases, the mutation in only one allele acts in a dominant negative fashion, leading to severe growth failure (25–27). In other cases, heterozygous relatives of GH-insensitive children exhibit a wide variation of height sD scores, as shown by Woods *et al.* (3) in a cohort of families from different countries, and by Laron *et al.* (28). In addition, Rosenbloom *et al.* (29), in an Ecuadorian cohort in which several members are affected by the same recessive splice mutation in exon 6 (E180splice), showed that heterozygous relatives were not significantly short. Therefore, it seems that the site of the mutation within the gene, and the specific modification at the protein level, influence the observed phenotype of heterozygous individuals.

The distribution and general range of mean GH levels and of peak amplitude of GH secretory bursts in these ISS children were not significantly different from those of North American normal prepubertal children (24). However, the number of peaks showed by our patients was significantly higher than the reference population (24). This could be a consequence of a diminished inhibition of GHRH pulse regulator, with a higher probability of being insensitive to GH, as an explanation for their short stature and decreased growth velocity. Among our 26 patients, 4 children had mean night GH levels below 0.14 nM (3.0 ng/ml) during the spontaneous secretion, the cut-off value used by some authors as a criteria for GH neurosecretory dysfunction. Three of these 4 patients had higher BMIs than our cohort of ISS children. Three of these patients had GHBP levels within the normal range, and 1 had a GHBP level slightly above the normal range. These results clearly differ from those of other researchers, who have observed a higher incidence of approximately 45% of low night GH levels in short children (23). In addition, the GHBP levels in our patients also differ from other studies that demonstrate that around 20% of ISS patients have GHBP levels of −2 SDS (8, 10).

Therefore, we question the usefulness of GHBP levels as a selection criteria for GHIS. The first cases of partial insensitivity, described by Goddard *et al.* (11), had extremely low

GHBP levels; but subsequent cases had normal (30) and even increased levels (31), depending on how the mutation affects the structure of the GHr gene. Because the mechanism of GHBP excision from the whole receptor is not fully understood, we did not rule out a patient with GHBP levels within the normal range. In addition, it is important to point out that GHBP values show remarkable variation in individuals of the same age range. In this regard, we tried to identify individuals with mild disorders, which might not show a significant reduction in GHBP levels.

The change in IGF-I concentrations, after GH administration, showed high variability among the subjects tested, as has been reported before (14, 32). Although this test can add some information to support the diagnosis of GHIS, we question its usefulness as a primary screening test for partial GHIS.

The molecular analysis of the GHr gene from these patients showed one heterozygous mutation on codon 161 from exon 6, resulting in an amino acid change, Arg to Cys. We cannot conclude yet that this mutation has an effect on GHr function. The GHr extracellular domain contains seven cysteines, two of them codified in exon 4, four in exon 5, and one in exon 7 (5). Six of these cysteines (1–2, 3–4, 5–6) form disulfide bonds stabilizing the extracellular domain structure (33). It is possible that the new cysteine at codon 161 in exon 6 disrupts one of the three disulfide bridges or creates a new bond binding to cysteine 141, thus affecting the extracellular domain structure. This mutation, R161C, has been described in one Laron patient in a homozygous form (34), and in a heterozygous form in three ISS patients, one of them a compound heterozygote (8). The fact that this mutation in a homozygous form is responsible for complete GHIS strongly suggests that GHr function may be affected in this case. The two heterozygous patients with ISS, described by Goddard *et al.*, showed height SDS values between −2.0 and −3.1, similar to the patient reported in the present study.

It is noteworthy that all the patients reported to date who bear this mutation in an heterozygous form have normal or mildly reduced levels of IGF-I and GHBP. The difference in the phenotypes that are associated with a single mutation is highlighted by comparing the heterozygous R161C (8), homozygous (34), and compound heterozygous patients (11) reported. This finding implies that the phenotypic consequences of these mutations may depend on the patient's genetic makeup. Additional alterations in the GHr gene or in other loci could contribute to the phenotypic diversity of short stature. The molecular analysis performed on the rest of the patients revealed only two polymorphisms, already described in the normal population. However, it is important to point out that SSCA can detect approximately 50–90% of mutations, depending on the sequence in which the nucleotide changes are located. Therefore, we cannot rule out the possibility that the children we studied may have mutations that were missed by SSCA. In addition, there could be mutations in the promoter region of the GHr gene or within intronic regions that were not analyzed in this study that do not affect GHBP levels.

The fact that we were not able to find more than one mutation in the GHr gene, among the patients selected for this study, may reflect the variety of causes responsible for ISS. The six patients selected for sequencing of all coding exons showed height SDS values between −2.5 and −4.3, IGF-I levels between −2 and −3.4 sd, and IGFBP-3 levels between −1.5 and −3.8 sd. Because IGF-I and IGFBP-3 synthesis and secretion are dependent on GHr function, and the gene seems to be free of mutations, we hypothesize that mutations in genes that codify for related proteins that participate in the signal transduction pathway are possible.

Although the clinical and biochemical features of our patients are compatible with partial GHIS, short stature may be caused by neurosecretory dysfunction (23), an increased proportion of circulating non-22-kDa GH isoforms, (20/22 K) (35), low bioactive GH (36), pseudoautosomic deletions of the homeobox gene SHOX (37), or minimal bone dysplasias and partial GHIS (38). These causes may help explain the absence of correlation found between the patients statures and their mean GH, mean GHBP, IGF-I, IGFBP-3, and peak GH.

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