0021-972X/99/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 84, No. 3
Printed in U.S.A.

Familial Dwarfism due to a Novel Mutation of the Growth Hormone-Releasing Hormone Receptor Gene*

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ABSTRACT

Isolated growth hormone (GH) deficiency (IGHD) is a rare cause of short stature. The same mutation of the gene encoding the growth hormone-releasing hormone receptor (GHRHR) has been identified as the basis for IGHD in three families from the Indian subcontinent. The prevalence and heterogeneity of defects in the GHRHR gene are not known.

Twenty-two dwarf members of a large, extended kindred containing at least 105 affected members with autosomal recessive short stature underwent extensive endocrine evaluation, which confirmed markedly reduced or undetectable serum concentrations of GH that did not increase in response to different stimuli.

DNA sequences of the 13 exons and intron-exon boundaries of the GHRHR gene were determined in an index patient. A novel homozygous 5′ splice site mutation (G→A at position +1) in IVS1 was found. Thirty of the affected subjects tested were homozygous for this mutation, and 64 clinically unaffected patients were either heterozygous for the mutation (n = 41, including 9 obligate carriers) or homozygous for the wild-type sequence (n = 23).

We describe a novel mutation in the GHRHR gene as cause of dwarfism in the largest kindred with familial IGHD described to date. (*J Clin Endocrinol Metab* **84:** 917–923, 1999)

THE incidence of short stature due to isolated deficiency of growth hormone (IGHD) is estimated to be 1/3,480–1/10,000 live births (1–4). While the majority of these cases are sporadic and thought to be caused by a variety of hypothalamic or pituitary insults, anatomic abnormalities are found in only 12% of such patients examined by magnetic resonance imaging (5). This suggests that genetic rather than structural defects may account for growth hormone (GH) deficiency in a significant proportion of cases. The incidence of familial IGHD, defined as the occurrence of IGHD in two first-degree relatives, is controversial and has been estimated to represent between 5 and 30% of cases (6). Both autosomal dominant and recessive as well as X-linked modes of inheritance have been observed in familial IGHD, suggesting that several different molecular mechanisms must account for GH deficiency (6). The gene encoding GH (GH1), located at 17q23, has been the most extensively studied candidate for IGHD. A variety of GH1 defects, including deletions, frameshifts, splice site, and nonsense mutations, which lead to decreased GH expression or action have been described (6, 7). However, as IGHD is not associated with mutations in the GH1 gene in most families (7), defects in other genes must cause some forms of IGHD.

Recent studies of the development and function of the pituitary somatotroph have provided additional candidate genes for IGHD. Proliferation of pituitary somatotrophs and secretion of GH are under dual hypothalamic regulation, stimulated by GHRH and inhibited by somatostatin (8). These hormones bind to specific cell surface receptors that interact with the heterotrimeric G proteins, G_s (GHRH) or G_i (somatostatin), to stimulate or inhibit the production of cAMP by adenylyl cyclase, respectively. The ability of GHRH to stimulate GH secretion and to enhance somatotroph proliferation was first recognized clinically with the discovery of pituitary hyperplasia in patients with ectopic GHRH-secreting tumors (9). These actions were later confirmed experimentally by the overexpression of GHRH in transgenic mice (10). Other studies have more directly demonstrated the role of the cAMP-dependent signal transduction pathway in the regulation of GH secretion. Natural or experimentally induced activating mutations in the α subunit of G_s ($G_s\alpha$) that lead to constitutive stimulation of adenylyl cyclase are associated with excessive secretion of GH as well as soma-

Received October 5, 1998. Revision received November 13, 1998. Accepted November 25, 1998.

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*This work was supported in part by NIH Grants DK34281 (M.A.L.), DK35592 (J.A.P.), and NIH-NCRR GCRC Grant 5 M01RR 00052, and two grants from the Genentech Foundation for Growth and Development (R.S. and G.B.). R.S. was supported in part by NIDDK 1 T32 DK07751 Training Grant. The University of São Paulo group was supported in part by a grant from Pharmacia & Upjohn, Inc., São Paulo Brazil and by a grant from São Paulo State Research Foundation (FAPESP). The University of Sergipe group was supported in part by a grant from Conselho Nacional de Pesquiza e Tecnologia CNPQ, Brazil.

totroph proliferation (11, 12). By contrast, inhibition in transgenic mice of the transcription factor CREB, downstream effector of cAMP action, leads to somatotroph depletion and dwarfism (13). These observations suggest that familial IGHD could result from genetic defects that impair GHRH synthesis, secretion, or action, as well as gene defects that affect development of pituitary somatotrophs or synthesis of GH. Although no muations have been identified in the gene for GHRH (14), recent studies have disclosed inactivating mutations of the GHRH receptor (GHRHR) gene that account for complete GH deficiency and profound dwarfism in the *little* mouse (15–17), a naturally occurring murine model for human IGHD, and in three families originating from the Indian subcontinent (18–20). The GHRHR gene encodes a 423-amino acid receptor protein that is expressed primarily in the pituitary somatotroph and is essential for GHRH-stimulated secretion of GH (21). In the present study, we analyzed the GHRHR gene for mutations in GH-deficient patients who are part of a large Brazilian kindred with at least 105 members with familial dwarfism.

Subjects and Methods

Subjects

We studied members of a large extended pedigree with familial dwarfism who have been identified in adjacent cities in Itabaianinha, a rural county in the state of Sergipe, located in northeastern Brazil. Inhabitants of this region are thought to be of Portuguese descent. They have high frequency of consanguineous marriages (21.7% of 322 unions examined). Marriages between first degree cousins, second degree cousins, and uncle and niece were 53%, 45.6%, and 1.4%, respectively. The diagnosis of dwarfism was based on early growth failure, proportionate short stature, and radiologic evidence of delayed bone age as determined using the Greulich and Pyle atlas (22). Affected subjects are very short and attain an adult stature that ranges between 105 and 135 cm (4.5–8.6 SD below the mean for a normal population). In addition, patients have high-pitched voices and increased abdominal fat accumulation. Except for a somewhat delayed onset of puberty, which does not affect their fertility, they do not manifest any signs or symptoms that suggest deficiency of other pituitary hormones. Clinical information about a subgroup of these patients (10–20 yr old) is summarized in Table 1. Although we are aware that the unique genetic background and the delayed puberty of this population can complicate the evaluation of bone age, the values are dramatically lower than respective chronological ages, consistent with the diagnosis of GH deficiency.

For many years women were advised against pregnancy because of concerns regarding cephalo-pelvic disproportion if carrying a normal fetus, but recently several of them had successful deliveries of normal infants via cesarean section.

The number of affected individuals is estimated to be at least 105 in a total population of 32,000, corresponding to a calculated prevalence of 1 case of dwarfism per 304 inhabitants. Pedigree analysis demonstrates an autosomal recessive mode of transmission of dwarfism (Fig. 1).

Ten patients have been treated with recombinant human GH for 1 yr, and each showed a brisk increase in growth velocity without reduced responsiveness over time (Hayashida C., R. Gondo, C. Ferrari, S. Toledo, R. Salvatori, M. Levine, M. Ezabella, N. Abelin, D. Gianella Neto, B. Wajchenberg; manuscript in preparation).

These studies were approved by the appropriate institutional review committees, and all subjects gave informed consent.

Hormonal studies

Comprehensive evaluation of GH secretion was performed in affected subjects, whose clinical characteristics are shown in Table 1. They all underwent clonidine stimulation test. Nine were also tested with insulin tolerance test and four with acute GHRH stimulation test.

After an overnight fast, an indwelling iv catheter was placed in each subject for the purpose of repeated blood sampling. For the clonidine test, the clonidine was given orally. Its dose was calculated either according to body surface (0.15 mg/m^2 body surface) or according to weight (0.075 mg for children below 15 kg of body weight, 0.1 mg for weight between 15.1 and 25 kg, and 0.15 mg for weight above 25 kg). Blood samples were collected at −30, 0, 60, 90, and 120 min from its administration. For the insulin tolerance test, insulin (0.1 U/kg body weight) was administered iv as a single bolus injection. Blood samples were collected at −15, 0, 15, 30, 45, 60, and 90 min. For the GHRH stimulation test, two basal (*i.e.* pretreatment) blood samples were obtained at −30 and 0 min for measurement of GH. After a single injection of GHRH (1 μg/kg, Geref, Serono, Norwell, MA) as an iv bolus and blood samples were obtained 15, 30, 45, 60, and 90 min. Two of the patients (nos. 9 and 12) were subjected to a prolonged GHRH stimulation test: GHRH (5 μg/kg) was injected sc, in the evening, at 2000 h for 6 consecutive days, and a standard GHRH stimulation test (above) was performed on the morning of the 7th day.

TABLE 1. Anthropometric measurements and bone ages in selected patients with familial dwarfism from the Itabaianinha county

Patient	Sex	Age (yr)	Height (cm)	Height SD	Weight (kg)	Bone age (yr)
1	M	5.2	86.0	−4.5	13.0	3.0
2	M	8.5	102.5	−4.3	15.0	5.5
3	M	9.2	105.0	−4.7	14.5	5.0
4	F	10.0	108.1	−5.0	20.2	5.7
5	F	10.5	108.4	−4.9	18.0	8.5
6	F	11.2	102.8	−6.0	16.4	6.9
7	F	11.2	105.7	−5.6	16.2	7.8
8	F	11.5	117.0	−4.4	21.0	5.5
9	M	11.7	107.4	−5.9	14.4	8.0
10	F	12.0	127.0	−2.7	36.0	8.5
11	M	12.5	106.2	−6.9	16.1	2.7
12	M	13.0	104.3	−6.6	14.4	6.0
13	M	13.1	104.0	−6.7	16.0	8.5
14	F	13.7	107.5	−7.0	19.0	11.0
15	M	15.3	107.0	−8.4	15.2	4.0
16	F	17.0	122.0	−5.9	28.0	13.0
17	F	18.0	116.5	−7.3	26.0	15.0
18	F	19.2	133.0	−4.9	42.0	16.0
19	F	19.4	120.0	−7.0	26.5	16.0
20	F	19.6	119.0	−7.2	23.5	15.5
21	F	20.1	129.9	−4.7	40.0	16.0
22	M	20.1	116.4	−7.6	20.8	13.0

Bone age was calculated with Greulich and Pyle atlas.

Quantitation of GH, LH, and FSH was performed using commercial fluoroimmunoassay (Auto Delfia, Wallac, Turku, Finland). Serum cortisol (INCSTAR Corp., Stillwater, MN) and thyroxine (Diagnostic Products Corporation, Los Angeles, CA) were measured using commercial RIA. Serum concentration of thyrotropin (DPC, Los Angeles, CA), insulin-like growth factor 1 (IGF-1) (Active IGFI DSL 6600, Diagnostic System Laboratories, Webster, TX), and IGF-binding protein 3 (IGFBP3) (Active IGFBP-3 DSL 5600) were determined using commercial immunoradiometric assays. The GH assay had a detection limit of 0.1 ng/mL, with intra- and interassay variabilities of 3.7 and 5.0%, respectively.

PCR amplification and analysis of the GHRHR gene

We isolated genomic DNA from 10–20 mL of peripheral blood with published methods (23, 24). The 13 exons and the flanking intronic sequences of the GHRHR gene were amplified by polymerase chain reaction (PCR) from genomic DNA. Primers are listed in Table 2 and correspond to intronic sequences situated 9–41 bases from intron-exon junctions, based on published sequences (25, 26). With the exception of exon 2 and 3 (separated by a short intron), each exon was amplified individually. The exon 1 sense primer annealed to the promoter region, and the exon 13 antisense primer annealed to the untranslated region of exon 13. Genomic DNA (250–500 ng) was amplified via PCR in a 25 μL reaction mixture containing 25 pmol of each primer, 50 μM potassium

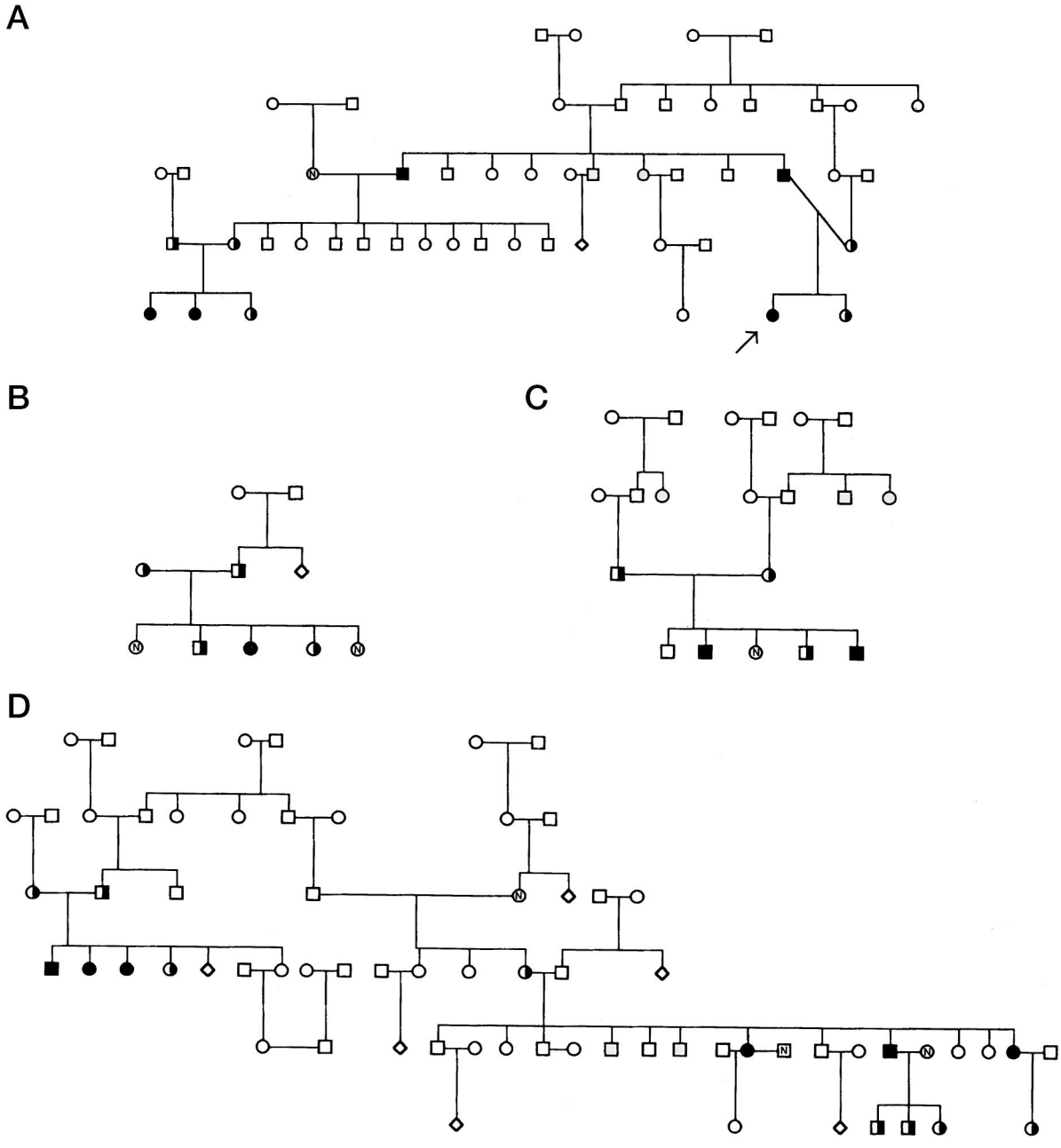

FIG. 1. Six representative subpedigrees (labeled A through F) of the Itabaininha kindred with isolated growth hormone deficiency. *Circles* indicate females, *squares* indicate males, and *diamonds* indicate miscarriages. *Solid black symbols* are GHRH receptor IVS1 + 1 G→A genotype-confirmed affected subjects, and *half-black symbols* are genotype-confirmed heterozygotes; *gray shading* indicates dwarf subjects whose DNA was not available. "N" indicates genotype-confirmed IVS1 + 1 G (normal) sequence. The *arrow* indicates the index patient. The pedigrees and genotypes are consistent with autosomal recessive inheritance.

chloride, 1.5 mM magnesium chloride, 5 mM TRIS (pH 8.3), 200 μM of each deosynucleotide triphosphate, and 1.25 U of *Taq* polymerase. After an initial denaturation at 94 C for 4 min, amplification was performed for 40 cycles at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec, and a final 10-min period of elongation at 72 C. The PCR products were purified and sequenced directly using the Amersham Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Cleveland, OH).

To optimize our ability to distinguish PCR products derived from the wild-type and mutant GHRHR allele (see below), we amplified exon 1 and part of IVS1 from genomic DNA with a primer pair ("DGGE" primers in Table 2) in which the sense oligonucleotide had been synthesized with a 5′ GC-rich sequence 45 bases long (27, 28). The amplification products were separated using denaturing gradient gel electrophoresis (DGGE) as previoiusly published (28). This technique is able to detect single-base substitutions in 100–1000-bp long DNA fragments (27). Optimal resolution was empirically obtained by electrophoresis at constant voltage (160 V) for 6 h at 60 C through 7.5% polyacrylamide gels containing a denaturing gradient of 50–90% (100% denaturant = 7 M urea and 40% formamide).

Haploptype analysis

We refined the localization of the human GHRHR gene by PCR of genomic DNA from the Stanford G3 Radiation Hybrid panel (29, 30)

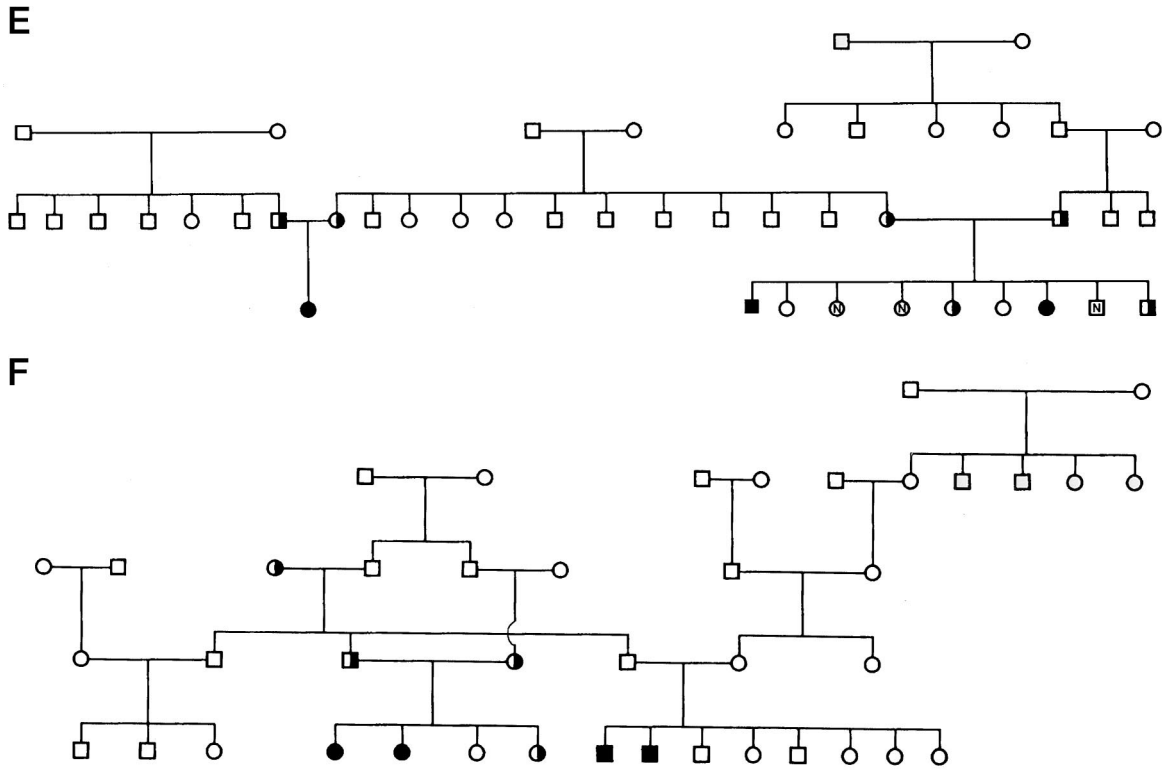

FIG. 1.—Continued.

using the "mapping" and "D7S526" primers listed in Table 2. This localization permitted us to identify polymorphic DNA markers that would enable assignment of GHRHR gene haplotypes.

Results

Hormonal studies

All of the 22 affected individuals who were evaluated showed a flat response to clonidine or insulin-induced hypoglycemia or GHRH (peak GH = 0.63 ± 0.61 ng/mL). In addition, serum levels of IGF-1 (6.7 ± 2.8 ng/mL *vs.* normal 65–724 ng/mL) and IGFBP3 (0.43 ± 0.17 μg/mL *vs.* normal 1.8–6.4 μg/mL) were markedly low. Two patients who were treated with daily injections of GHRH for six days before a GHRH stimulation test showed no response to the acute bolus of GHRH (peak GH < 0.25 ng/mL), confirming complete resistance to exogenous GHRH.

All affected patients tested showed normal cortisol responses to insulin-induced hypoglycemia. Thyroid function was normal in all patients except patient 22, who had mild (subclinical) primary hypothyroidism. Serum concentrations of gonadotropins were appropriate for age in all affected male and female patients.

Identification of the mutation

The nucleotide sequences of the entire coding region and of the flanking intronic sequences of the GHRHR gene in the index case were normal except for a homozygous G→A transition of the first base of the 5′ splice site at the beginning of IVS1 (IVSI+1G→A) (Fig. 2). The G→A transition created an additional *Nla* III restriction site in the amplicon, which enabled direct confirmation of the base change by endonuclease digestion of PCR-amplified genomic DNA (Fig. 3).

To avoid the possibility of misclassification due to incomplete digestion of PCR products with *Nla III*, we optimized conditions for DGGE that would allow us to distinguish individuals who were homozygous for the wild-type gene from patients who were homozygous or heterozygous for the mutation (Fig. 4).

Using DGGE we screened 29 other affected patients and 64 clinically unaffected sibs and parents of affected patients in this kindred. All affected patients were homozygous for the mutation. In addition, all clinically unaffected individuals were either heterozygous for the mutation (n = 41, including 9 obligate carriers), or homozygous for the wild-type genotype (n = 23). Two unaffected subjects, apparently unrelated, showed a heterozygous pattern on DGGE that differed from that of subjects with the IVS1 + 1 A mutation. Direct nucleotide sequencing showed that these subjects were heterozygous for a novel C→T polymorphism in the untranslated region of exon 1 that is predicted to be of no biological significance. One of these subjects had married a clinically affected patient who was homozygous for the splice site mutation, and all three of their children are heterozygous for both the IVS1 + 1 A mutation and the exon 1 polymorphism and are phenotypically normal.

Because the IVS1 + 1 G→A sequence variant was homozygous in all clinically affected patients, but was heterozygous or absent in 64 unaffected subjects, and absent in 96 normal chromosomes from the Centre Etude Polymor-

TABLE 2. Primers used to amplify the 13 exons and intron-exon boundaries of the GHRH receptor gene, for gene mapping ("Mapping" and "D7S526") and for amplification of the exon 1-intron 1 junction for Denaturing Gradient Gel Electrophoresis ("DGGE")

	Sense primers	Anti-sense primers
Exon 1	5′AACGGCTGTGTCAGGGGACAG3′	5′CAGAGGCCAGAGGGTCTCAG3′
Exons 2–3	5′GACACCCAAATGGCTTGGCTCAT3′	5′GCCACTTCCAGATGAAAGCACCTC3′
Exon 4	5′AGAGGGAAGGAGTTGTGGCTAGAG3′	5′GTCCATGGCAGTCCTTCCTCCAA3′
Exon 5	5′TCCAAAGGCCCAGAAGCTGA3′	5′TGGGAAATAAGGAGCCAA3′
Exon 6	5′CAGAATCCCCTCTCCCTGCTT3′	5′CTGCCCAGCCCCTTCACCCATG3′
Exon 7	5′TGGGTGTCCCAGCTCTGAAGCAC3′	5′TTTACCTCCCCATGGTGCCCAA3′
Exon 8	5′AGATCTCAGAGTCAAGGATGCAGA3′	5′TCCACTCCACACCCCATGTAGGA3′
Exon 9	5′AGGTGCCTGCGTCACCACAGTGA3′	5′CAGTAGTTCCAGGGAAGTTGACT3′
Exon 10	5′GCCACCCAAGGCTACCCCCTGAC3′	5′TGGATGAGACAGACACATTGAAT3′
Exon 11	5′TGAAGTGCACACGACAGTTTCTA3′	5′GCAACAGCACCTCCCTCCAGCAC3′
Exon 12	5′AGGCCAAAGGGTTCTGATGGG3′	5′TTAGGTCTGGTGGGAGGGGGA3′
Exon 13	5′GACCTTCCTAACGTCCTCTTC3′	5′CAGCTGGGGTGGGGATGTGGC3′
Mapping	5′CTGCCTCATCACGCCACT3′	5′TACAGGTTTATTGGCTCCTCTG3′
D7S526	5′AACAAGGGCTTCTGCTGAG3′	5′CCATCTTGGTGTTGAGGGC3′
DGGE	5′GC_{45}-AACGGCTGTGTCAGGGGACAG3′	5′CAGGCTCCAGTTGGCCCTGT3′

phism Humain (CEPH) panel (data not shown), we concluded that this mutation caused IGHD in this family. To determine the frequency of the IVS1 + 1 G→A mutation as a cause of dwarfism, we screened DNA samples from nine additional unrelated patients with IGHD by DGGE. In all cases, affected subjects were found to have the wild-type, IVS1 + 1 G sequence.

Haploptype analysis

Radiation hybrid mapping placed the human GHRHR locus very close to the D7S632/D7S2041E loci (logarithm of odds (LOD) = 1000 at 0.00 cR) and between D7S2492 (45 cRs 64 1.35 Mb) and D7S1834 (39 cRs 64 1.2 Mb). We also mapped D7S526 and found it to colocalize with GHRHR and D7S632 (LOD 1000 at 0.00 cR).

Discussion

Four naturally occurring murine models of congenital, autosomal recessive GH deficiency exist: the *Snell* (*dw*), the *Ames* (*df*), the *Spontaneous Dwarf Rat* (*SDR*), and the *little* (*lt*) mouse (15–17, 31–33). In the first two models, pituitary glands are hypoplastic and depleted of somatotrophs, thyrotrophs, and lactotrophs, causing a multihormonal pituitary failure, while in the *SDR* rat and in the *little* mouse the hypoplasia is limited to somatotrophs, and only GH is deficient. The *Snell* mouse results from a point mutation in the gene encoding for the pituitary transcription factor Pit-1, which is required for the development of all three pituitary cell lines (31). The *Ames* mouse results from a mutation of another transcription factor (Prop-1), which acts as activating factor of the Pit-1 gene (32). The *SDR* rat has a point mutation in the GH gene (33). The *little* mouse has a missense mutation in the extracellular domain of the GHRHR gene, which does not allow proper binding of GHRH (16, 17). Analogous human diseases have been described for all 4 murine models: multiple cases of familial panhypopituitary dwarfism, resembling the *Snell* and *Ames* phenotype, have been proven to be caused by PIT-1 mutations or PROP-1 mutations (6, 34). About 12.5% of patients with IGHD have mutations in the GH1 gene (7). Recently, Cao *et al.* analyzed GHRHR sequences corresponding to the coding region of part of the extracellular domain and portions of the transmembrane regions of the GHRHR in 12 children with IGHD who did not respond to treatment with GHRH and found no genetic abnormalities (35). Subsequently, 3 families of similar ethnic backgrounds were found with identical nonsense mutations (Glu72X) in exon 3 (18–20). In the present study, we analyzed the entire GHRHR gene in an unusually large IGHD family that contained at least 105 dwarf members. We found a novel mutation in the consensus GT of the 5′ splice site of IVS1, which is required for correct splicing of IVS1 from RNA transcript to form the messenger RNA (mRNA). As the GHRHR gene is expressed only in the pituitary (21), there is no easily accessible tissue from which one can directly analyze GHRHR mRNA processing in patients carrying this mutation. Moreover, several attempts to amplify GHRHR mRNA from fresh and transformed lymphocytes via nested reverse transcriptase PCR were unsuccessful (data not shown). However, as this guanine is conserved in 100% of mammalian genes (36), the G→A transition is predicted to prevent the normal removal of IVS1 during processing of the nascent GHRHR transcript. Similar IVS1 + 1 G→A transitions have been described in at least 9 other human genes (37–46). In each case, the mutation was shown to be the cause of the disease phenotype. In those cases that have been analyzed, the IVS1 + 1 G→A mutation was associated with retention of intron sequences in the mature mRNA and utilization of a downstream cryptic splice donor site. Failure to normally excise IVS1 was predicted to result in production of a truncated protein, due to either the presence of a terminator codon in the retained intronic sequence or to a shift in the reading frame of downstream exonic sequences (37, 44, 45). By analogy to these examples, we predict that the IVS1 + 1 G→A mutation of the GHRHR gene leads to retention of IVS1 sequences. Although no potential cryptic splice site has been identified downstream of the normal donor splice site, an in-frame TAG stop codon is located 213 nucleotides from the end of the exon 1 coding region. Thus, retention of even a portion of IVS1 would lead to generation of a markedly truncated protein, if one is produced at all, that would likely lack any receptor activity.

Given the large number of affected patients and the high

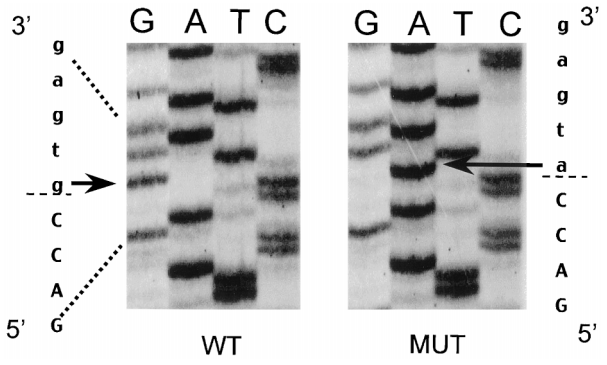

FIG. 2. Sequence analysis of the exon 1-IVS1 junction of the GHRHR gene from genomic DNA of the index patient (MUT). Normal sequence (WT) is from an unrelated normal subject. The exonic sequence is indicated in *capital letters*, and the IVS1 sequence is indicated in *small case letters*. The G→A transition is pointed by the *arrow*.

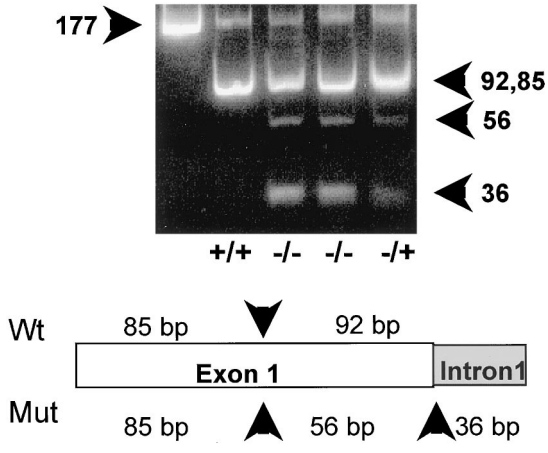

FIG. 3. Restriction analysis of the amplified exon1-IVS1 fragment of the GHRHR gene. Genomic DNA was amplified with the exon1 primer pair described in Table 2 to yield a 177 bp fragment (left lane). *Nla III* digestion of the amplified fragment from a normal individual (+/+) yields 92 and 85 bp fragments that cannot be separated by electrophoresis through an 18% polyacrylamide gel. A band reflecting incomplete digestion is present in all lanes. Longer incubation time or increased enzyme concentration did not result in complete digestion. The G→A transition at the exon 1-IVS1 junction creates an additional *Nla III* restriction site so that digestion of the amplicon from 2 affected individuals (−/−) yields three bands (85, 56, and 36 bp) (plus the 177 bp band due to incomplete digestion). The Xylene cyanol dye front runs with the 56-bp band, reducing the intensity of the ethidium bromide fluorescence. DNA from an obligate heterozygous individual (−/+) generates four bands when digested with *Nla III* (92 and 85 bp from the normal allele, 85, and 56, and 36 bp from the mutant allele, with reduced intensity of the lower bands relative to the −/− subjects).

degree of consanguinity of this population, it is most likely that the IVS1 + 1 G→A mutation arose in a single founder several generations ago. However, as the mutation occurs at a CpG dinucleotide, a sequence with increased mutagenicity due to the spontaneous deamination of a methyl cytosine (47, 48), we performed haplotype analysis to determine whether the mutation may have arisen in several different GHRHR genes. Radiation hybrid mapping placed the human GHRHR locus very close to D7S632/D7S2041E and between D7S2492 and D7S1834. The D7S2492 locus, which was estimated to be 1–2 million bp from the GHRHR gene, contains a DNA

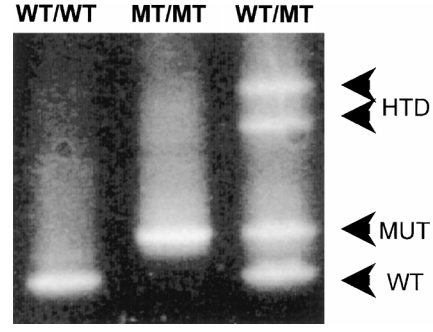

FIG. 4. Denaturing gradient gel electrophoresis of the amplified exon1-IVS1 fragment of the GHRHR gene. Leukocyte genomic DNA was amplified with the DGGE primer pair described in Table 2. DNA from an unrelated normal subject (WT/WT) has a single homoduplex band (WT *arrow*). DNA from an affected subject (MT/MT) yields a single homoduplex band (MUT *arrow*) that migrates more slowly in the gel. DNA from an obligate heterozygous subject (WT/MT) has four bands: two homoduplex bands (one from the wild-type and one from the mutant allele) and two more slowly migrating bands (HTD *arrow*), representing heteroduplexes generated by annealing of the forward and reverse strands of the G and A alleles during PCR amplification.

polymorphism. As this physical distance is estimated to correspond to 1–2% recombination, the D7S2492 and GHRHR loci should exhibit genetic disequilibrium and, in most cases, should be inherited together. The haplotypes of the mutant GHRHR alleles were very similar when 76 mutant alleles (IVS1 + 1A) were examined (mutant alleles were represented by two haplotypes, A-1 and A-2, which accounted for 85% and 15% of alleles, respectively). In contrast, normal GHRHR alleles (IVS1 + 1G) were represented by five different haplotypes, G-1 (29%), G-2 (14%), G-3 (29%), G-4 (21%), and G-5 (7%). Our finding, that 85% of mutant alleles share a common (A-1) haplotype that is present in only 29% (G-1) of normal subjects, suggests that the mutation arose only once and that its widespread distribution represents inheritance from a common ancestor (*i.e.*, a founder effect). Therefore, propagation of this mutation within the gene pool and the subsequent generation of large numbers of affected subjects and carriers was facilitated by the unusually high incidence of consanguineous marriages among inhabitants of a geographically isolated region.

We also evaluated the frequency of this mutation in other unrelated patients with GHRH-resistant IGHD (34). None of the nine probands showed the IVS1 + 1 G→A mutation, despite this being a potential mutational "hot spot".

The profound clinical and biochemical consequences of GHRHR gene mutations further define the molecular pathophysiology of IGHD. The possibility of a partial clinical phenotype in patients heterozygous for the exon 3 Glu72X mutation was suggested (19). The uniquely large size of our kindred will allow us to establish phenotype-genotype correlation for patients who are heterozygous for this GHRHR mutation.

The prevalence of mutations in the GHRHR gene remains to be determined. Ours is the fourth report of a mutation in the GHRHR gene; the other three reports (18–20) described identical mutations in apparently unrelated families. Our finding of a novel mutation suggests that defects in the GHRHR may be a more common cause of IGHD than previously suspected.

Acknowledgments

We wish to thank Dr. Primus E. Mullis (University of Bern, Bern Switzerland) for providing DNA samples from patients with IGHD. The University of São Paulo group wishes to thank Dr. Bernardo Leo Wajchenberg for suggestions and advice, Dr. Carmela Ferrari, Dr. Suely Kazue N. Marie, Neusa Abelin, Daniel Giannella-Netto, and Monica de Souza Rustilli for their technical help. The University of Sergipe group wishes to thank Dr. Berenice B. Mendonca and Dr. Antonio F.V. Souza for technical help and Mario C. Pereira Oliveira for technical and secretarial assistance.

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