Clinical and molecular characterization of Brazilian patients with growth hormone gene deletions

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Abstract

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Genomic DNA from 23 patients with isolated growth hormone (GH) deficiency (12 males and 11 females: heights -4.9 ± 1.4 SDS) was screened for GH gene deletions by restriction endonuclease analysis of polymerase chain reaction amplification products. Three unrelated patients had typical features of severe GH deficiency and deletions (6.7 kb in two and 7.6 kb in one) of the GH gene. The two patients with 6.7-kb deletions developed growth-attenuating anti-GH antibodies whereas the patient with the 7.6-kb deletion continued to grow with GH replacement therapy. Our finding that 3/23 (~13%) Brazilian subjects had GH gene deletions agrees with previous studies of severe isolated GH deficiency subjects in other populations. Two of three subjects (67%) with deletions developed blocking antibodies despite administration of exogenous GH at low doses. Interestingly, only 1/10 of cases with affected relatives or parental consanguinity had GH-1 gene deletions.

Introduction

In 1971, Illig et al. (1) described familial type I-A isolated growth hormone (GH) deficiency with autosomal recessive inheritance, short length compared to weight at birth, typical facies and early growth failure leading to severe dwarfism. While these subjects initially responded to GH therapy with a strong anabolic response, within a few months they developed high titers of GH antibodies associated with growth attenuation. In 1981, deletions of 6.7 kilobases (kb) including the structural gene for GH-1 were documented in these patients by Southern blot analysis (2). More recently, GH gene deletions were diagnosed by polymerase chain reaction (PCR) techniques (3,4). In addition to deletions, frameshift and nonsense mutations of the GH gene have also been found in some subjects with this phenotype (for a review, see Ref. 5). To determine the type and frequency of GH gene deletions in Brazilians with severe isolated GH deficiency we screened DNAs from 23 subjects by PCR. We report here that 3/23 (~13%) patients had GH gene deletions as the cause of their clinical features and severe GH deficiency. Inter-

estingly, 2/3 (67%) of those with deletions developed blocking antibodies despite administration of exogenous GH at low doses.

Material and Methods

Informed consent was obtained from the parents before the study and the protocol was approved by the Hospital Ethics Committee.

Patients

The study was conducted on 23 patients (12 males and 11 females, height standard deviation score (SDS) range -2.3 to -7.9, mean ± SD -4.9 ± 1.4) with isolated GH deficiency from the Division of Endocrinology, University Hospital, São Paulo. The patients belonged to 19 families. Three siblings belonged to a family with consanguineous parents. Each of two families with unrelated parents had two siblings who were both studied. One patient had related parents and a known sibling with GH deficiency that was not available for study. In two families with related parents a single patient was affected. Thus, 10/23 (~43%) of our cases were familial or had consanguineous parents. Patients 1, 2 and 3 were not related. Isolated GH deficiency was diagnosed in children with short stature (height <-2.0 SD) by failure to achieve normal serum GH levels after at least two pharmacological stimulation tests, usually clonidine (0.1 mg/m², *po*) and insulin-induced hypoglycemia (0.1 U/kg, *iv*). Peak GH levels were considered to be normal when higher than 7 µg/l in a immunoradiometric assay (Wallac, Turku, Finland) and 3.0 µg/l in an immunofluorimetric assay (Delfia, Wallac). Combined pituitary hormone deficiency was ruled out by normal basal serum T3, T4, cortisol and prolactin levels and normal cortisol, prolactin, TSH, LH, and FSH responses to combined *iv* infusion of insulin (0.1 U/kg), thyrotropin releasing-hormone (200 µg) and gonadotropin releasing-hormone (100 µg).

Anthropometric measurements

Heights were measured with a stadiometer and height SDS were calculated using Brazilian reference standards (6).

Hormone assays

Serum GH levels were measured by an immunoradiometric assay up to 1994 (7) and by an immunofluorimetric assay thereafter. GH antibodies were detected by patient serum binding to a GH tracer (8). Cortisol, T3, T4, TSH and prolactin were measured with commercial RIA kits. IGF-I was measured by RIA after extraction using a kit from Nichols Diagnostic Institutes (San Juan Capistrano, CA). IGF-binding protein 3 was measured by IRMA using a kit from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA).

Molecular study

DNA isolation. Genomic DNA was isolated from peripheral blood leukocytes from all patients and from the parents of patients 1-3.

PCR amplification and restriction endonuclease analysis of PCR products. PCR amplification reactions were carried out in a total volume of 100 µl containing 400 ng of genomic DNA, 1 µM of each oligonucleotide primer, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 *Taq* polymerase units. The primer sequence was sense 5'G GATCCAGCCTCAAAGAGCTTAC3' and antisense 3'GAATTCCCAGAGCCTTGA GCAATGGA5' (9). The reaction mixtures were denatured at 94ºC for 6 min, and then subjected to 30 amplification cycles in a thermocycler (Perkin Elmer Cetus, Norwalk, CT). Each cycle consisted of a 30-s denaturation period at 94ºC, a second annealing

period at 60ºC and a 2-min extension period at 72ºC. After the 30 amplification cycles the reactions were allowed to anneal for 10 min at 72ºC and then cooled to 0ºC (3). Following PCR amplification, 25 µl of each reaction was digested with *Sma*I using conditions specified by the commercial supplier (New England Biolabs, Beverly, MA). PCR products of patient 3 and her parents were also digested with *Bgl*I and *Hae*II (9). The resulting DNA fragments were then subjected to electrophoresis on 5% polyacrylamide gels in TBE (90 mM Tris-HCl and 90 mM boric acid) and the fragments were visualized by ethidium bromide staining.

The 6.7- and 7.6-kb deletions that include the GH gene were detected using methods previously described (9). After PCR amplification of homologous repeats normally lying 5' and 3' (1900 and 1919 bp, respectively) from the GH gene, the products were digested with *Sma*I restriction endonuclease. This treatment yields digestion products of 1470 and 446, 1900, or 1900, 762, 711 and 446 bp when derived from genomic DNA templates of individuals with the 6.7-kb, 7.6-kb or no deletions, respectively (9) (Figure 1).

Results

The clinical features of the 3 patients with GH gene deletions are shown in Table 1 and Figure 2. The 3 patients had a large forehead, low nasal bridge, thin hair, increased subcutaneous fat, and a high-pitched voice and the boy had a micropenis. Developmental milestones were normal. The parents of patient 1 were first cousins and two sisters were of low-normal height (-0.6 and -2.0 SDS, respectively) but had normal responses to GH stimulation tests. Patients 2 and 3 were products of nonconsanguineous marriages; patient 2 had 2 brothers and patient 3 one brother, all of normal height. The three families were from different cities and no relationship among them was known (Table 1).
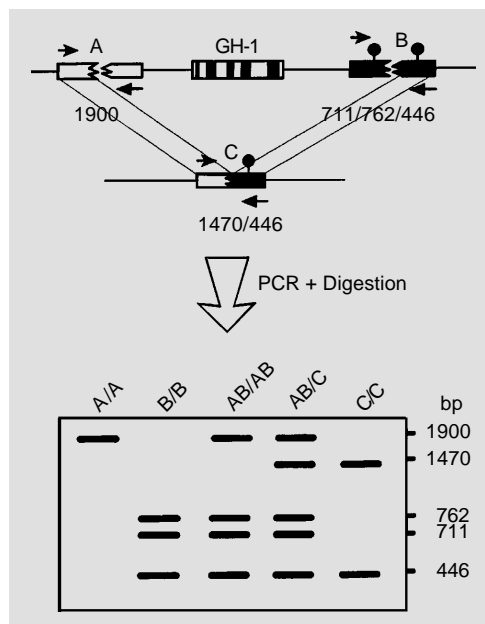

Figure 1 - Diagram of the results of unequal recombination that produces GH-1 gene deletions. Note that the normal PCR amplification products (A and B) generated by the primers indicated by arrows contain no or two *Sma*I recognition sites. In contrast, the fusion product (C) associated with 6.7-kb deletions including GH-1 contains a single *Sma*I recognition site. The corresponding sizes of DNA fragments obtained after digestion of products from normal controls (AB/AB), deletion heterozygotes (AB/C) and deletion homozygotes (C/C) with *Sma*I are shown below.

Table 1 - Clinical features of three Brazilian patients with GH gene deletions.

	Patient 1	Patient 2	Patient 3
Age (years)/Sex	1.75/F	3.25/M	2.91/F
Height (cm)/SDS	60.0/-7.9	74.8/-6.0	77.9/-4.8
Weight (kg)/span (cm)	5.8/56.5	8.8/76.5	11.5/75.0
Upper/lower segment (cm)	36.5/23.5	41.6/35.5	43.6/34.0
Head circumference (cm)	45.5	46.5	49.5
Birth weight (g)/length (cm)	2850/45	3340/42	2970/48
Father's height (cm)/SDS	159.1 (-1.9)	165.0 (-1.1)	172.0 (-0.1)
Mother's height (cm)/SDS	147.0 (-2.0)	153.0 (-1.1)	154.5 (-0.8)
Consanguinity	Yes	No	No
City/state of origin	São Vicente/SP	Cruzeiro/SP	Bragança/SP

Complete GH stimulation tests were difficult to perform in patient 2 because of small veins. Laboratory data are shown in Table 2. Serum cortisol, prolactin, TSH, T3 and T4 levels were all normal in the 3 patients. Magnetic resonance imaging of pituitary and stalk were normal in patients 1 and 2 and not available for patient 3.

A molecular basis for the GH deficiency was investigated by studying patient DNA. Digestion of the PCR products from patient 2 (Figure 3, lane 1) with *Sma*I yielded two fragments of 1470 and 446 bp which were identical to those obtained using DNA from

Figure 2 - Clinical photographs of patients 1 (A) and 2 (B) with 6.7-kb growth hormone gene deletions.

a known GH-1 gene deletion patient (lane 6) as template. In the family of patient 1, the patterns obtained from the DNA of the two sisters (lanes 3 and 4) were identical to that of a control (lane 7) which contains 1900-, 762-, 711- and 446-bp fragments. In contrast, their father (lane 2) has an additional 1470-bp band which indicates that he is heterozygous for the GH-1 gene deletion, and their affected sister (patient 1) has DNA fragments of 1470 and 446 bp (lane 5), indicating that she is homozygous for the GH-1 gene deletion (Figure 3). The mothers of patients 1 and 2 were also heterozygous for the 6.7-kb deletion. The PCR product obtained from genomic DNA of patient 3 yielded only 1900-bp digestion products after *Sma*I digestion. PCR products from patient 3, as well as from samples from her parents, were also digested with *Bgl*I and *Hae*II and yielded patterns consistent with homozygosity for a 7.6-kb deletion including the GH-1 gene in patient 3, and strongly suggested heterozygosity for the 7.6-kb deletion in her parents.

Anti-GH antibodies were absent before GH replacement therapy. In case 1, pituitary GH was started at 0.03 U x kg body weight per week, divided into daily subcutaneous injections, approximately 1/10 of the then usual standard replacement dose. After 3 months GH antibodies (1/100 titer) appeared and the growth response was absent. Replacement with standard doses in patients 1 and 2 resulted in an increased growth rate for 6 months. After 1 year, anti-GH antibody titer was 1/100000 and 1/1000, in patients 1 and 2, respectively, and there was no growth response to pituitary or recombinant GH therapy. In contrast, patient 3 continued to have a catch-up growth after 2 years of standard GH replacement therapy.

Discussion

The diagnosis of GH deficiency in patients with short stature is usually based on

failure to achieve an arbitrary threshold or cut-off levels of serum GH after at least two standard pharmacological GH stimulation tests, but these criteria are not absolute (10). The patients in this study presented with short stature, and low pre- and post-stimulation GH values, which excluded resistance to GH, as seen in Laron dwarfism (11). A genetic basis for GH deficiency was suggested by the presence of affected siblings or consanguineous parents in 6/19 families, suggesting an autosomal recessive mode of inheritance. Among the 3 patients with deletions, none had affected relatives and only patient 1 had consanguineous parents. The three patients with GH gene deletions had typical clinical features of profound growth hormone deficiency, including severe short stature, especially in patients 1 and 2. However, in other patients with a similar phenotype deletions were not detected.

The GH-1 gene is normally located in the 5' end of the 48-kb human GH gene cluster on the long arm of chromosome 17, that also includes 5' → 3', the chorionic somatomammotrophin (CSH) pseudogene-1, and the CSH-1, GH-2 and CSH-2 genes. These genes retain 92-98% homology in the immediate flanking, intervening and coding sequences. GH-1 gene deletions probably arise from homologous unequal recombination, resulting from chromosomal misalignment during meiosis, facilitated by the presence of highly homologous regions that flank the GH gene (12).

As shown in Figure 3, patients 1 and 2 are homozygous while the father of patient 1 is heterozygous for the gene deletion. Although the patterns obtained are the same for both patients, further studies are needed to determine if the deletions are identical or represent independent deletion events.

In our series, GH gene deletions were demonstrated in only one of the four consanguineous families but not in any of the 8 familial cases. Some of the remaining cases may possibly include some with small dele-

Table 2 - Laboratory data for three Brazilian patients with GH gene deletions.

*IRMA, Immunoradiometric assay. **IFMA, Immunofluorimetric assay.

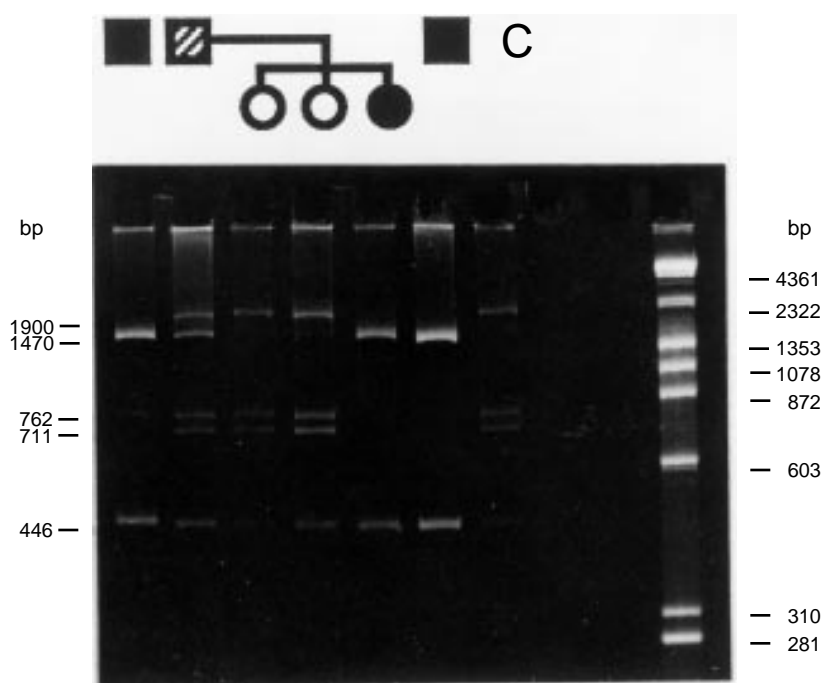|	Patient 1	Patient 2	Patient 3
Bone age at chronological age (year)	0.8 at 2.9	2.0 at 3.25	1.25 at 2.6
Peak GH (µg/l) clonidine/insulin	1.4/<0.6*	<0.25*/−	<0.1/0.1**
Insulin-like growth factor I (ng/ml)	22	22	8
IGF-binding protein 3 (mg/l)	0.44	0.42	−

Figure 3 - Detection of DNA fragments obtained by ethidium bromide staining after *Sma*I digestion and electrophoresis in patient 2, family of patient 1, a patient with known GH-1 gene deletion and a normal control (C). *Lanes 1* and *5*: Patients 2 and 1; *lanes 2-4*: father and normal sisters of patient 1, respectively; *lane 6*: known Chinese GH-1 deletion homozygote; *lane 7*: normal control.

tions or point mutations in exons, introns or promoter regions of the GH-1 gene (5).

The incidence of deletions of ~13% in this Brazilian series is similar to that reported in the literature (5,13). Mullis et al. (13) reported GH-1 gene deletions in 3/32 (~9%) Northern-European, 3/22 (~14%) Mediterranean and 4/24 (~17%) Turkish patients. Of these deletions, 8/10 (80%) involved 6.7 kb and the remaining 20%

7.6 kb. Only 1/10 (10%) of our cases with affected relatives or parental consanguinity had GH-1 gene deletions.

Interestingly, both patients with 6.7-kb deletions developed growth-attenuating anti-GH antibodies whereas the patient with 7.6-kb deletion continued to grow in response to GH replacement therapy. This variation in antibody responses has been observed in other GH-1 deletion cases (9). The formation of GH antibodies has been attributed to complete lack of endogenous GH during the period in which immune tolerance develops. One possible explanation for greater immune tolerance of patients with 7.6-kb deletion is that translocation of the promoter sequences, normally found 5' from GH-1, into the corresponding region of the CSH pseudogene-1 might cause partial expression of CSH, which could induce immune tolerance to exogenous GH due to its homology with GH (9). Along this line, it is interesting that patient 3 with the 7.6-kb deletion had a less severe growth deviation before therapy than the two patients with 6.7-kb deletions, suggesting a less complete absence of GH. However, since different responses to GH therapy have been observed within sibs with the same size deletion, factors other than molecular heterogeneity in the GH-1 gene deletions themselves must be involved (14).

A single Brazilian patient with a 6.7-kb GH gene deletion has been previously reported by Mullis et al. (15). The patient developed growth-attenuating anti-GH antibodies after replacement therapy with GH of pituitary origin and also suffered from cystic fibrosis. Since the gene for cystic fibrosis is located on chromosome 7 and the gene for GH on chromosome 17, this association was probably a coincidence.

We conclude that the incidence of GH-1 gene deletions is 3/23 (~13%) in Brazilian patients with severe isolated GH deficiency. Furthermore, we found that both subjects with the 6.7-kb deletion and 2/3 of all our deletion subjects developed blocking antibodies despite the administration of exogenous GH at low doses. Interestingly, gene deletions were found in only 1/10 patients and in 1/6 families with parental consanguinity or the presence of familial cases, suggesting a different genetic origin for their GH deficiency.

Acknowledgments

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