Type IA isolated growth hormone deficiency (IGHD) consistent with compound heterozygous deletions of 6.7 and 7.6 Kb at the *GH1* gene locus

Deficiência isolada de hormônio do crescimento tipo IA (DIGH) consistente com deleções heterozigotas compostas de 6,7 e 7,6 Kb no locus *gênico* GH1

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SUMMARY

Isolated growth hormone deficiency (IGHD) may result from deletions/mutations in either *GH1* or *GHRHR* genes. The objective of this study was to characterize the molecular defect in a girl presenting IGHD. The patient was born at 41 weeks of gestation from non-consanguineous parents. Clinical and biochemical evaluation included anthropometric measurements, evaluation of pituitary function, IGF-I and IGFBP-3 levels. Molecular characterization was performed by PCR amplification of *GH1* gene and *SmaI* digestion of two homologous fragments flanking the gene, using genomic DNA from the patient and her parents as templates. At 1.8 years of age the patient presented severe growth retardation (height 61.2 cm, -7.4 SDS), truncal obesity, frontal bossing, doll face, and acromicria. MRI showed pituitary hypoplasia. Laboratory findings confirmed IGHD. *GH1* gene could not be amplified in samples from the patient while her parents yielded one fragment of the expected size. *SmaI* digestion was consistent with the patient being compound heterozygous for 6.7 and 7.6 Kb deletions, while her parents appear to be heterozygous carriers for either the 6.7 or the 7.6 Kb deletions. We have characterized type IA IGHD caused by two different *GH1* gene deletions, suggesting that this condition should be considered in severe IGHD, even in non-consanguineous families. Arq Bras Endocrinol Metab. 2012;56(8):558-63

SUMÁRIO

A deficiência isolada do hormônio do crescimento (DIGH) pode ser resultado de deleções/mutações no gene *GH1* ou no gene *GHRHR*. O objetivo deste estudo foi caracterizar o defeito molecular em uma menina que apresenta DIGH. A paciente nasceu às 41 semanas de gestação de pais não consanguíneos. As avaliações clínica e bioquímica incluíram medidas antropométricas, avaliação da função pituitária e concentrações de IGF-I e IGFBP-3. A caracterização molecular foi feita por meio de amplificação do *GH1* por PCR e digestão com *SmaI* de dois fragmentos homólogos flanqueando o gene, usando-se DNA genômico da paciente e de seus pais como padrões. Com 1,8 ano de idade, a paciente apresentou atraso grave no crescimento (altura 61,2 cm, -7.4 DP), obesidade central, protuberância frontal, face de boneca e acromicria. A RM mostrou hipoplasia pituitária. Os achados laboratoriais confirmaram a DIGH. O gene *GH1* não pôde ser amplificado nas amostras da paciente, enquanto as amostras de seus pais produziram um fragmento do tamanho esperado. A digestão com *SmaI* foi consistente com a paciente ser heterozigota composta para deleções para 6,7 e 7,6 Kb, enquanto seus pais parecem ser carreadores heterozigotos para deleções de 6,7 ou 7,6 Kb. Caracterizamos a DIGH tipo IA causada por duas deleções diferentes no gene G*H1*, sugerindo que essa condição pode ser considerada na DIGH grave, mesmo em famílias não consanguíneas. Arq Bras Endocrinol Metab. 2012;56(8):558-63

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INTRODUCTION

Growth hormone deficiency (GHD) is a relatively common disorder, occurring in 1 out of 4,000 to 10,000 live births (1). Most frequently, it occurs as a sporadic condition of unknown aetiology (2) but severe forms of isolated GHD (IGHD) may have a genetic basis (3). In patients with severe growth retardation (height less than -4.5 SDS) presenting IGHD, the prevalence of *GH1* or *GHRHR* gene defects, either mutations or deletions, could be as high as 20%, depending on the population (4,5). More recently, Alatzoglou and cols. have reported an 11.1% prevalence of *GH1* or *GHRHR* molecular defects in IGHD pedigrees, which increased to 38.6% in familial cases (6). Familial IGHD has been associated with four Mendelian disorders (7,8), including two autosomal recessive (Type IA and IB), one autosomal dominant (Type II) and one X-linked (Type III) form. Type IA IGHD was first described by Ruth Illig and cols. (9) in 1970 in three Swiss siblings with severe short stature, early growth retardation, extreme dwarfism in adulthood, and a particular phenotype. These patients developed high titers of anti-GH antibodies, which arrested their growth response to pituitary-extracted GH treatment. Using Southern blot analysis in these patients, Phillips III and cols. (10) later characterized a homozygous 7.5 Kb deletion that included *GH1* gene.

The aim of this report was to characterize the molecular defect in a female patient who fulfilled the criteria for severe IGHD.

SUBJECT AND METHODS

Case report

We report a small-for-gestational-age female patient born from non-consanguineous parents by vaginal delivery after 41 weeks of gestation. Birth weight and length were 2,460 g (-2.0 SDS) and 44 cm (-3.7 SDS), respectively. Both parents presented normal height: father 165.5 cm (-1.07 SDS) and mother 154.5 cm (-1.01 SDS). The patient's target height was 153.7 ± 8.5 cm. The first evaluation, at 10 months of age, showed severe growth retardation (height 57 cm, -5.9 SDS) (Table 1). Auxological parameters were expressed in cm and SDS according to Argentinean references (11). Her physical examination showed truncal obesity, frontal bossing, doll face, and acromicria (Figure 1A). She had normal psychomotor development. Laboratory findings confirmed severe growth hormone deficiency with no GH response to an arginine test, low IGF-I and IGFBP-3, and normal GHBP serum levels (Table 2). Brain MRI showed severe anterior pituitary hypoplasia (Figure 1B). She started rhGH replacement therapy (0.33 mg/kg.week) at 2.4 years of age. Although her growth rate improved during the first 6 months of treatment (12.8 cm/year), afterwards she developed anti-GH antibodies, and her growth velocity decreased to 2.8 cm/year (Figure 1C). Levels of IGF-I and IGFBP-3 remained very low on rhGH treatment.

Table 1. Auxological evaluation

Chronological age (years)		Birth	0.8	2.4	2.8	3.3
Height	(cm)	44.0	57.0	67.5	73.0	74.4
	(SDS)	-3.7	-5.9	-6.3	-5.2	-5.2
Weight	(g)	2,480	5,100	7,700	8,600	8,980
	(SDS)	-2.0	-3.6	-3.7	-3.4	-3.6
Head circumference	(cm)	34.1	42.1	43.5		47
	(SDS)	0.0	-2.0	-2.0		-2.0
Body proportions	(cm)		37.0	43.5	45.0	46.6
	(pc)		25	25	10	25
Bone age (years)				1.6		2.0
Height velocity (cm/year)			16.2	6.6	13.7	2.8
rhGH treatment (months)				0	5	11

Table 2. Endocrinological evaluation

Chronological age (years)		1.8	2.4	2.8	3.3	Reference values
Prolactin (ng/mL)		21	38.6			3-15
Cortisol (μg/dL)		43	18.3	16.1	31	6-21
ACTH (pg/mL)		40		77	75	10-50
TSH (mIU/mL)		2.81	8.10	5.04	8.13	0.50-6.50
FT4 (ng/mL)		1.28	1.70	1.53	1.75	0.80-2.00
GH (ng/mL) maximal response to arginine test		< 0.05				> 6.00
IGF–I (ng/mL)	ICMA	< 25		< 25	< 25	56-140
	RIA	< 11		< 11	< 11	25-158
IGFBP-3 (μg/mL)		< 0.5		< 0.5	< 0.5	2.0-4.4
GHBP (nmol/L)		1.62				1.04-6.17
Anti-GH antibodies titers				1/1,000	1/1,000	Negative
rhGH treatment (months)			0	5	11	

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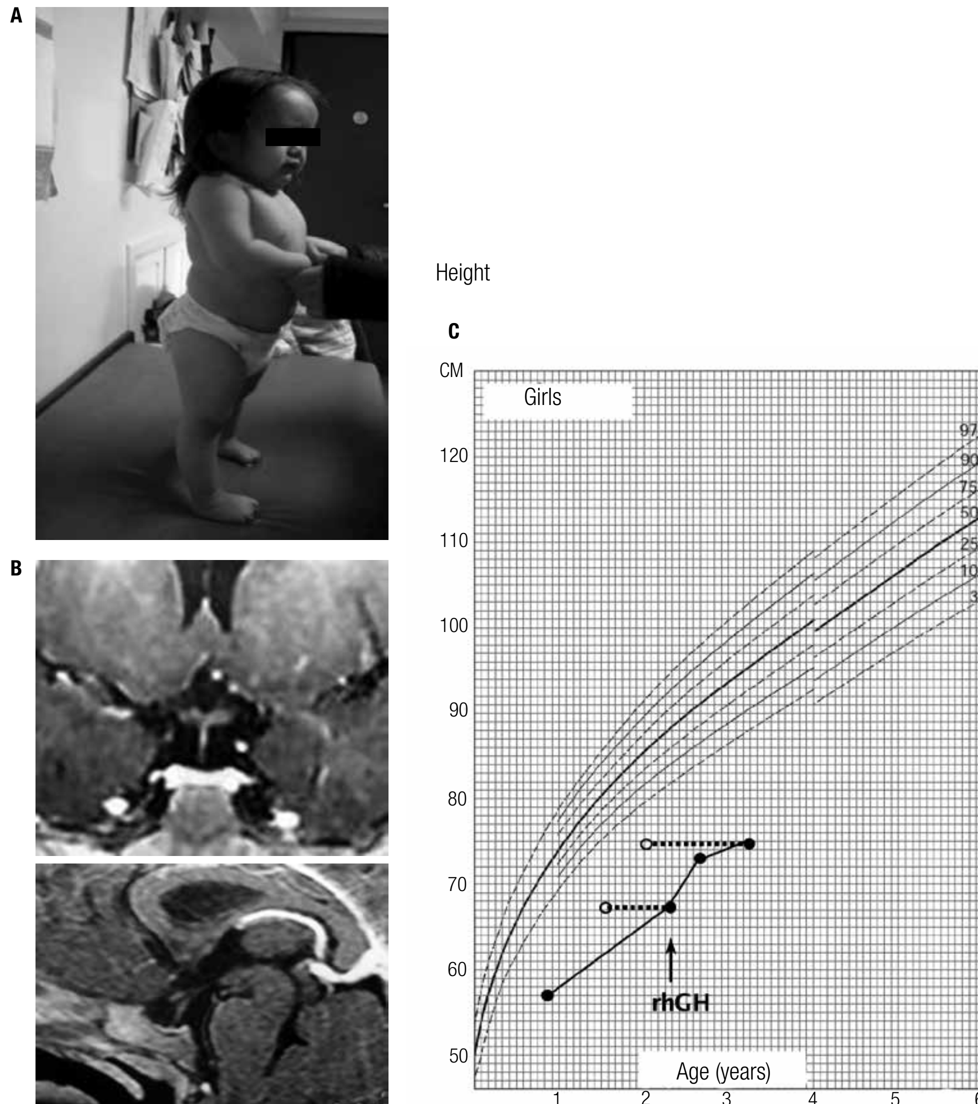

Figure 1. A. Patient at 1.8 years of chronological age (written consent for publication was obtained from the parents). **B.** Brain MRI showing pituitary hypoplasia. **C.** Growth chart. Closed circles: height for chronological age. Open circles: bone age.

Hormone assays

Serum growth hormone (GH) secretion was evaluated by an arginine provocative test (0.5 g/kg body weight). GH, IGF-I, IGFBP-3, and ACTH serum levels were measured by chemiluminiscent immunometric assays (ICMA, IMMULITE® 2000 system, Siemens Healthcare Diagnostics Products Ltd, Gwynedd, UK); cortisol, prolactin, TSH, and free T4 (FT4), by electrochemiluminescence assays (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) using a Cobas e411 analyzer. IGF-I levels were also measured by an *in house* RIA after serum extraction by the acid-ethanol method followed by cryoprecipitation (12), and GHBP serum concentration was determined by an *in house* time-resolved fluorometric immunofunctional assay modified from Fisker and cols. (13,14). Anti-GH antibodies were determined by an *in house* ELISA as follows: A 96-well plate was coated with rhGH and non-specific sites on the coated wells were blocked with phosphate buffer (PBS; pH = 7.4) containing BSA (2 g/L). After removal of the blocking solution, the serum sample (diluted 1/10 in PBS) was added and incubated overnight at 4°C. The plate was washed and incubated for two hours with rabbit polyclonal horseradish peroxidase-labeled total anti-human IgG (DakoCytomation, Glostrup, Denmark). The plate was washed and the substrate (tetramethyl benzidine) was added. After 5 minutes, the reaction was stopped by addition of sulfuric acid, and absorbance was determined at 450 nm.

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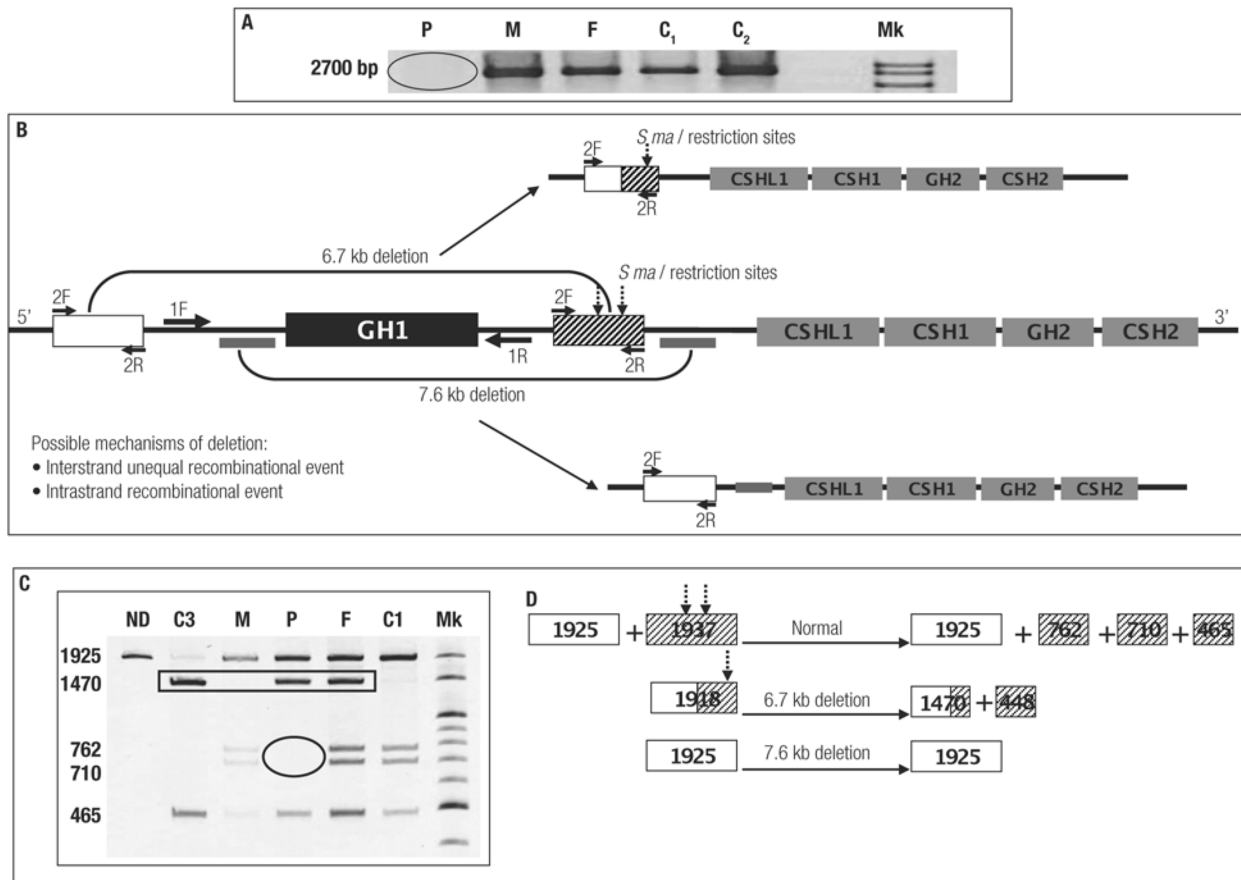

Figure 2. A. *GH1* gene amplification (1.5% agarose gel electrophoresis, ethidium bromide staining). *GH1* gene PCR amplification yielded no product using the proband's genomic DNA as template (P), while her parents (M, F) showed one band of the expected size (2,700 bp), similar to normal controls (C_1, C_2). Mk: 100 bp DNA ladder. **B.** Schematic representation of *GH1* gene in the cluster context, and genomic organization resulting from 6.7 and 7.6 Kb deletions. 1F, 1R: oligonucleotide primers for *GH1* gene PCR amplification. 2F, 2R: oligonucleotide primers for simultaneous PCR amplification of 5' and 3' homologous flanking sequences (white and shaded boxes, respectively) that give rise to the 6.7 Kb deletion. Grey boxes: homologous regions that give rise to 7.6 Kb deletion upon unequal recombination. Dotted arrows indicate *SmaI* restriction enzyme sites present in 3' homologous flanking region. **C.** *SmaI* digestion (5% polyacrylamide gel electrophoresis, ethidium bromide staining). Fragment pattern was consistent with the father (F) being heterozygous carrier for 6.7 Kb deletion, and the patient (P), compound heterozygous for 6.7 and 7.6 Kb deletions. Unfortunately, the band pattern for the mother (M), presumably carrier of a 7.6 Kb deletion, could not be distinguished from a normal control (C1). C3: Patient known to be homozygous for 6.7 Kb deletion. Mk: 100 bp DNA ladder. ND: non-digested PCR product. **D.** Diagram showing the expected size of the DNA fragments obtained after *SmaI* digestion.

Magnetic resonance imaging (MRI): MRI examination was carried out in sagittal and coronal T1 images of the brain, sellar and suprasellar structures, with and without gadolinium contrast.

Molecular characterization

Genomic DNA was isolated from peripheral venous blood by cetyltrimethylammonium bromide (CTAB) lysis buffer and chloroform-isoamyl alcohol extraction (15). Written informed consent for molecular studies was obtained from the parents.

PCR amplification of the whole *GH1* gene (Gene ID 2688, RefSeqGene: NG_011676.1) was performed using GoTaq® DNA polymerase (Promega Corporation, Madison, USA) and oligonucleotide primers GH1F (5'-ccagcaatgctcagggaaag-3') and GH1R (5'-tgtcccaccggttgggcatggcaggtagcc-3') (16). PCR mixtures were denatured for 2 min at 94°C and submitted to 30 cycles at 92°C for 1 min; 61°C for 45 sec; and 68°C for 3 min, followed by final extension at 68°C for 10 minutes. The resulting PCR product (2700 bp) was visualized by agarose gel electrophoresis and ethidium bromide staining.

Characterization of *GH1* gene deletion was performed according to the method by Vnencak-Jones and cols. (17) modified by Mone and cols. (18). Briefly, two homologous sequences flanking *GH1* gene, and the fusion fragments resulting from different *GH1* gene dele-

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tions, were simultaneously amplified by PCR with the following primers: 5'-tccagcctcaaagagcttacagtc-3' (GH2F) and 5'-cgttttctctagtctagatcttcccagag-3' (GH2R). PCR mixtures were denatured at 94°C for 3 min and submitted to 30 cycles at 94°C for 1 min; 64°C for 45 sec; and 72°C for 3 min, followed by a 10-min final extension at 72°C. The resulting PCR fragments were digested overnight at 37°C with *SmaI* restriction endonuclease (RO141S, New England Biolabs, MA, USA) according to the manufacturer's protocol, and the digested products were visualized by ethidium bromide staining after electrophoresis on a 5% polyacrylamide gel.

RESULTS

Biochemical evaluation: IGHD was confirmed by lack of response of GH to an arginine test, undetectable levels of IGF-I and IGFBP-3, normal thyroid function (normal to slightly elevated TSH with normal FT4 levels), normal ACTH, and elevated cortisol levels. Prolactin levels were slightly above the upper normal range (Table 2).

PCR amplification of *GH1* gene: *GH1* gene PCR amplification yielded no product using two different genomic DNA samples of the proband as template, while her parents showed one amplicon of the expected size, similar to DNA from normal controls (Figure 2A). This result was suggestive of *GH1* gene deletion in the patient.

Characterization of *GH1* gene deletion: following PCR amplification of two homologous sequences flanking *GH1* gene, the *SmaI* restriction enzyme digestion band pattern obtained was consistent with the patient being compound heterozygous for 6.7 and 7.6 Kb deletions, while her father displayed a pattern consistent with a heterozygous carrier of the 6.7 Kb deletion. The pattern obtained in the mother could not be distinguished from that of a normal control, and was compatible with both the mother having two normal alleles or being a heterozygous carrier of the 7.6 Kb deletion (Figure 2C). Unfortunately, only those heterozygous carriers for the 6.7 Kb deletion can be unambiguously detected by this assay, which does not enable the differentiation between normal homozygous individuals and 7.6 Kb deletion heterozygous carriers (19,20). As a consequence, we were not able to confirm whether the proband inherited the 7.6 Kb deletion from her mother, or if this deletion arose as a *de novo* event.

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DISCUSSION

The characteristic phenotype of severe GH deficiency or resistance includes craniofacial disproportion, frontal bossing, truncal obesity, doll face, and acromicria. The absence of basal or stimulated GH together with normal secretion of other pituitary hormones, support the diagnosis of IGHD, suggesting a molecular defect in the *GH1* gene. This gene is located in the long arm of chromosome 17 (17q24.2) as part of a cluster of 5 homologous genes, arranged from 5' to 3' as follows: *GH1*, *CSHL1* (chorionic somatommamotropin pseudogene), *CSH1* (chorionic somatommamotropin gene 1, or placental lactogen), *GH2* and *CSH2*. The cluster genes share a high degree of identity not only in coding, but also in intervening and flanking sequences. The three pairs of homologous sequences present upstream and downstream of *GH1* gene provide a basis for the high susceptibility of this gene to suffer unequal recombination events due to misalignment that give rise to the most common gene deletions (20,21).

To date, several different length deletions within the GH-gene cluster (6.7, 7.0, 7.6, 45, double deletions) have been characterized as molecular defects in IGHD (20-24), with the 6.7 Kb deletion as the most frequent one (80%). These patients show severe growth retardation early in infancy (first 6 months of age), undetectable GH levels and, in most of the cases, they develop anti-GH antibodies that impair growth response to exogenous GH treatment. However, in spite of having the same genetic defect and developing similar anti-GH antibodies titers, growth response to GH treatment may be quite heterogeneous depending on the neutralizing effects of these antibodies (25,26). In our patient, an initial 6-month good response to rhGH therapy slowed down when high titers of anti-GH antibodies developed. Therefore, it appears that rhIGF-I remains as the only alternative therapeutic approach.

Most of the cases reported to date arose in consanguineous families. However, the occurrence of compound heterozygous cases with one deleted and one mutated allele (20,27,28), or different *GH1* gene deletions (29), as the present case, suggests that this diagnosis should be considered in patients with severe growth retardation, particularly in early infancy, even in non-consanguineous families. Since most type-IA IGHD patients develop anti-GH antibodies after rhGH treatment, molecular diagnosis has clinical implications for the management of patients with this condition.

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