Case report

A novel splicing mutation in exon 4 (456G>A) of the GH1 gene in a patient with congenital isolated growth hormone deficiency

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ABSTRACT

Isolated Growth Hormone Deficiency (IGHD) due to GH1 gene defects has a variable inheritance pattern: autosomal recessive, autosomal dominant, and X-linked. The autosomal dominantly inherited form, IGHD II, is mainly caused by heterozygous mutations of splicing around the exon 3/IVS3 boundary region of the GH1 gene resulting in exon 3 skipping of transcripts. We have previously reported findings on GH1 gene mutations in 28 Russian patients with severe congenital IGHD (-3.22±1.2 height SDS at the age of 1yr); five heterozygous dominant negative splice site mutations in intron 2, intron 3, and exon 4 of the GH1 gene were identified in 32.1% of the cohort. In the present report we describe a novel 456G>A heterozygous mutation of splicing of the last base of the 3'-acceptor splice site of exon 4 within the GH1 in a 4.2-year old, extremely short (–5.32 height SDS) girl with congenital IGHD. The mutation involves a highly conserved GGGgtg sequence of the exon 4/IVS4 boundary region of the GH1 gene. The predicted effect of the 456 G>A mutation is perturbed splicing with possible skipping of exon 4 of the GH1 gene. The novel heterozygous 456 G>A mutation in exon 4 expands the spectrum of dominant negative splicing defects within the GH1 gene, responsible for congenital IGHD.

Key words: GH1 gene, Isolated growth hormone deficiency, Splice site mutation

INTRODUCTION

Hereditary Isolated Growth Hormone Deficiency (IGHD) is a heterogeneous disorder with different modes of inheritance. GH1 gene is the main candidate gene for congenital IGHD in children. Familial IGHD due to GH1 gene defects is associated with four hereditary forms. Two forms are inherited as autosomal recessive traits (IGHD IA, IGHD IB), one form is inherited as an autosomal dominant trait (IGHD II), and one form is inherited as an X-linked disorder (IGHD III).[1-3]

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Received 20-06-06, Revised 02-08-06, Accepted 15-08-06

Splicing mutations of the GH1 gene represent an invariant type of mutation responsible for familial autosomal dominant (type II) isolated growth hormone deficiency (IGHD II). The majority of reported dominant negative mutations are located around the exon 3/IVS3 boundary region of the GH1 gene and cause IGHD II by perturbing GH mRNA splicing.[4-6] De novo GH1 splicing mutations in IVS3 have also been reported in some patients.[7] No splicing mutation located within exon 4 of the GH1 gene has thus far been described in congenital IGHD.

We have recently reported allelic genetic heterogeneity of IGHD II in the Russian population.[6] A high incidence of the GH1 splicing mutations in families with IGHD II was shown. We identified five heterozygous mutations of splicing in intron 2, intron 3, and exon 4 of the GH1 gene and described in detail families with IGHD II due to mutations within intron 2 and intron 3. According to our data, the 5'-donor splice site of intron 3 of the GH1 gene is the mutational "hot spot" and IVS3 +1G>A mutation is a common molecular defect in IGHD II in Russian patients.

We herein report one more mutation* of splicing within GH1 gene in a patient with IGHD, i.e. a heterozygous transition from guanine to adenine in the last base of the 3'-acceptor splice site of exon 4 (456 G>A), which has not previously been reported.

The study was approved by the Ethical Clinical Research Committee of the Endocrinology Research Center.

PATIENT AND METHODS

1. Patient

The girl herein described was born at term with no perinatal complications. Birth length was 47 cm (-1.5SDS) and birth weight was 2.95kg (-1.1SDS). Growth retardation became apparent in the first few months of life and at age 1yr her height was –4.53SDS (Table 1). Isolated GH deficiency was diagnosed at the Endocrinology Research Center at the chronological age of 4.2yr. The patient was extremely short with a height of 78.4cm (–5.32SDS) and a phenotype of congenital GHD, including prominent forehead, mid-facial hypoplasia, and saddle nose. The bone age was 1.3yr, "bone age: chronological age" ratio was 0.31. Peak GH level was undetectable (<0.1μg/liter) on a clonidine test. Serum free T_4, TSH and cortisol levels were normal. She was started on recombinant GH therapy and the height velocity increased to 12.0 cm/yr.

Table 1. Clinical and hormonal data of the patient with isolated GH deficiency and a heterozygous 456G>A splicing mutation in exon 4 of the GH1 gene

	Patient
Chronological age (yr)	4.2
Bone age (yr)	1.3
Height (cm)	78.4
Height SDS	
present examination/age 1yr	-5.32/-4.53
BMI SDS/BMI SDS (HA)	-0.03/-1.28
GH level in clonidine test (μg/liter)	
Basal/stimulated	<0.1/<0.1
Free T4 (pmol/l)	14.8
TSH (mU/l)	1.2
Cortisol (nmol/l)	322.0

HA - height age, BMI - body mass index, SDS - standard deviation score, yr - years
Normal ranges: peak GH, above 10 μg/liter; free T_4, 10-25pmol/liter; TSH, 0.3-3.5mU/liter; cortisol, 150-650nmol/liter.

The family tree consisted of three generations of short-statured individuals (Figure 1). Apart from the proband, the pedigree included a short (144.3 cm; -3.03SDS) but non-GH-deficient mother with stimulated peak GH level of 12.8μg/liter during an insulin tolerance test. The molecular defect, a 456G>A mutation in the GH1 gene, was not detected in the mother. A short (138cm; -5.78SDS) grandfather on the maternal line was not available for study. The patient's father's height was 165 cm (-1.46HSDS).

2. Mutation analysis of the GH1 gene

Genomic DNA was extracted from leukocytes using the phenol/chloroform method (Lindblom, Holmund, 1988). Four DNA fragments of the GH1

*This work was presented in part at the 41st Annual Meeting of the European Society for Paediatric Endocrinology, (Madrid, Spain, 25-28 September, 2002).

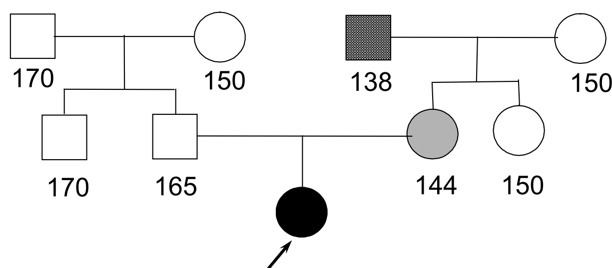

Figure 1. Pedigree of the patient with congenital isolated GH deficiency due to a heterozygous 456G>A splicing mutation in exon 4 of the GH1 gene. *Black symbol with arrow:* patient with the reported GH1 mutation; *grey symbol:* mother with short stature, non-GH deficient; *stippling symbol:* grandfather with short stature, not available for study.

(Genbank Accession No J03071)[8] covering exons 2, 3, 4, 5 and their boundary regions were amplified on thermal cycler (DNA Technology, Russia) and analyzed by SSCP for possible DNA alterations. Exon 1 was not included in the study because of its small size and an absence of data regarding functional GH1 mutations in this exon. The 30-μl reaction mixture consisted of 0.25 μM of each primer, 200 μM of each d-NTP, 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH4)_2SO_4$, 0.01% Twin-20, 2 mM - 6 mM (depending on each pair of primers) $MgCl_2$, and 1-1.5 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Sequences of primers are shown in Table 2.

The PCR reaction mixture was denaturated for 7 min at 94°C and cycled 32 times (exons 2, 4, 5) or 34 times (exon 3): 94°, 45 sec; 57° (exons 2, 5), or 65° (exon 3), or 60° (exon 4), 45 sec; and 72°, 45 sec, followed by an 8-min extension at 72°C. The resulting GH1 PCR products were electrophoresed on a 6% polyacrylamide gel (15V/cm, 1h, 1xTBE buffer) with 10-min pre-electrophoresis. After electrophoresis, DNA was visualized for the detection of single-strand conformation polymorphism (SSCP) by silver staining. 7μl of PCR product was mixed with 0,5μl of 5M NaOH, 0,5μl of 0,5 M EDTA, and 4,5μl dH2O. The mixture was heated for 15min at 42°C. After 3μl of formamide buffer (95% formamide, 0,5% xylol cyanol, 0,5% bromphenol blue) was added PCR product was electrophoresed on a 10% polyacrylamide gel (acrylamide/bisacrylamide ratio was 29/1) with 5% glycerin (5V/cm, 0,5xTBE, 18-20h, room t°). The gel was then developed using Silver Sequence DNA Staining Reagents kit (Promega, USA) according to the manufacturer's protocol. To confirm the SSC-polymorphism, direct sequencing was performed. PCR products were extracted from 1% low-melting agarose and purified by Wizard PCR PREPS DNA Purification System (Promega, USA) and then used for direct sequencing as templates. Both forward and reverse strands were sequenced on DNA Sequencer Pharmacia Biotech according to the protocol recommended by the company.

RESULTS

One novel mutation of splicing in exon 4 of the GH1 gene is described. Figure 2 illustrates the finding of a heterozygous transition of guanine to adenine in the last base of the 3'-acceptor splice site of exon 4 of the GH1 gene. The mutation shown occurs at base position 456 (456 G>A) and involves a highly conserved GGGgtg sequence of the exon 4/IVS4 boundary region of the GH1 gene (Figure 3). The predicted effect of the 456 G>A mutation

Table 2. Primers used for amplification of exons 2-5 of the GH1 gene and flanking 5'- and 3'-intronic sequences

Region	Primer	Sequence
Exon 2	Forward	5'- CGGCTCCCTCTGTTGCCCTCT -3'
	Reverse	5'- CCCCTTCCTGCCACCCCTGAT -3'
Exon 3	Forward	5'- AATGGGAGCTGGTCTCCAGCG -3'
	Reverse	5'- GGGGCTCTGACTACAGGTCTC -3'
Exon 4	Forward	5'- GTGGATGCCTTCTCCCCAGGC -3'
	Reverse	5'- GGGGCTCCAGGATTGGGGAC -3'
Exon 5	Forward	5'- GAATGAGAAAGGGAGGGAACAGTA -3'
	Reverse	5'- CTGGAGTGGCAACTTCCAGGG -3'

is perturbed splicing with possible skipping of exon 4 of the GH1 gene.

DISCUSSION

The autosomal dominant form of isolated GH deficiency is a subject of special interest for pediatric endocrinologists in terms of genetic and phenotypic variability. To date, the reported dominant negative mutations in IGHD II or de novo splicing mutations are located in intron 2, intron 3, intron 4, and exon 3 of the GH1 gene. No splicing mutation in exon 4 of the GH1 gene has so far been described in congenital IGHD.

Figure 2. Genomic DNA sequence analysis (3´) of exon 4/IVS4 GH1 boundary region. Heterozygous transition of G to A in the last base of the 3´-acceptor splice site of exon 4 (456G>A) in a patient with isolated GH deficiency.

IGHD II is mainly caused by heterozygous splice site mutations in intron 3 of the GH1 gene around the exon 3/IVS3 boundary region. These mutations affect the normal GH mRNA splicing, leading to the complete skipping of exon 3 and the loss of amino acids 32-71. The resultant alternative spliced product ($^{\Delta exon3}$hGH) represents the short-length 17.5-kDa isoform of GH that lacks the loop connecting the first two helices of GH with ^{53}Cys of the internal disulfide bridge. The dominant negative effect of splicing mutations involves different alterations within the secretory pathway through interactions between del32-71-GH and wild-type protein in secretory granules of somatotrophs. The 17.5-kDa isoform of GH decreases intracellular stability of the 22-kDa isoform by suppressing its intracellular accumulation and secretion in tissue culture cells; it moreover destroys somatotrophs, disrupts the Golgi apparatus, and impairs some protein trafficking in transgenic mice.[9-15]

To date, seven dominant negative mutations of splicing have been described in intron 3 of the GH1 gene in humans. The first one, IVS3 +1G>A mutation located in the first nucleotide of the invariant gt dinucleotide of the 5'-donor splice site of intron 3 (TCCgtg) of the GH1 gene, is a mutational "hot spot". IVS3 +1G>A mutation has been identified

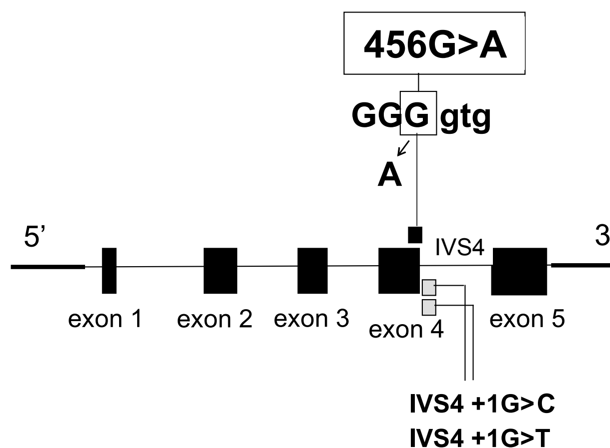

Figure 3. Schematic presentation of splicing mutations within exon 4/IVS4 boundary region in the GH1 gene in children with congenital isolated GH deficiency. Black box; the reported mutation, grey boxes; data of the literature (Cogan JD et al, 1993; Phillips JA 3rd, Cogan JD, 1994; Wagner JK et al, 1998)

in IGHD II families from Sweden, North America, South Africa, India,[16] Chile,[17] Japan,[18] the Russian Federation,[6] and other ethnic groups;[20] also as a de novo mutation in cases from Japan.[18,19] The second one, a de novo IVS3 +1G>C mutation, was described in a patient from Germany[21] as well as in a Dutch family.[22] The third one, IVS3 +2T>C mutation located in the second nucleotide of the invariant gt dinucleotide of the 5'-donor splice site of intron 3, was first reported in a IGHD II family from the Black Sea region of the Russian Federation.[6,23] Subsequently, IVS3 +2T>C mutation has been described in one family from Germany in which two siblings and the father were affected.[11] IVS3 +5G>A mutation was reported in an IGHD II Chilean family[17] and in a Japanese family from Kyoto.[24] IVS3 +5G>A mutation was also included in the spectrum of five GH1 splicing defects identified in the Russian population.[6] IVS3 +6T>C mutation[20,25,26] was first identified in a Turkish family with IGHD II.[25] Finally, the last two reported splicing mutations in intron 3 of the GH1 gene were located apart from the canonical IVS3 5'-donor splice sites; IVS3 +28G>A (ISEm1) mutation, first reported in a Thai family with three affected children, and IVS3Δ28-45 (ISEm2) mutation disrupt intron splicing enhancer (ISE) elements.[16,27-29] The overall size of intron 3 of the GH1 gene has been shown to be crucial for exon 3 inclusion.[30]

Two dominant negative splicing mutations have been described in intron 2 of the GH1 gene, causing complete skipping of exon 3 from the GH1 mRNA transcript. IVS2 –2A>T mutation, first revealed in a IGHD II family from the region of central Russia, affects the highly conserved tagGAA sequence of the invariant agent of the 3'-acceptor splice site in intron 2.[6,31] IVS2 -1G>A is the second intron 2 acceptor splice site mutation detected in a IGHD II family.[32]

Two dominant negative splicing mutations were reported in intron 4 of the GH1 gene. Both IVS4 +1G>C[1] and IVS4 +1G>T[33] mutations have been described in Saudi Arabian families with IGHD, type IB.

Finally, two exon splice enhancer (ESE) mutations that disrupt splicing regulatory sequences and weak exon 3 recognition have been reported within exon 3 of the GH1 gene. Exon splice enhancer element (ESE1) includes the first seven nucleotides of exon 3.[30] A heterozygous G-to-T transversion at the first nucleotide of the exon 3 (E3 +1G>T) deleted exon 3 in mature mRNA and resulted in IGHD II in a Japanese family.[34] E3 +5A>G mutation has also been described.[35]

Exon 4 of the GH1 has not previously been recognized as a site for splicing defects responsible for IGHD. As mentioned above, we have identified five dominant-negative splicing mutations in the GH1 gene in children with IGHD from the Russian Federation.[6,36] We described in detail IGHD II families that harbored both two novel (IVS2 –2A>T; IVS3 +2T>C) mutations and a "hot spot" (IVS3 +1G>A) mutation. We herein describe the third novel mutation of splicing in the GH1 gene localized within exon 4.

A heterozygous transition from guanine to adenine in the last base of the 3'-acceptor splice site of exon 4 (456G>A) of the GH1 gene was identified in one Russian patient with congenital IGHD. The 456G>A mutation spans the highly conserved GGGgtg sequence of the exon 4/IVS4 boundary region of the GH1 gene. The predicted effect of this mutation is perturbed splicing with possible skipping of exon 4 of the GH1 gene. The 456G>A mutation in exon 4 of the GH1 gene is close to the homozygous IVS4 +1G>C mutation in intron 4, which has been reported in three brothers with IGHD, type IB,[1] and the IVS4 +1G>T mutation reported in a family with IGHD, type IB.[33]

The reported girl had dramatic postnatal growth retardation with height SDS of -4.53 at 1yr of age. This degree of growth delay in the early months of life is the highest among the group of Russian children harboring mutations in other splicing sites of the GH1 gene.[6] As a result, the height SDS of the girl decreased to -5.32 SDS at 4.2yr of age. It is worth pointing out that the patient had the lowest birth length (-1.5 SDS, gestational age 40 weeks) among the above- mentioned cohort of patients with GH1 splicing defects. Clinical features of congenital GHD, including prominent forehead, mid-facial hypoplasia, and saddle nose, were also present. Bone age chronological age ratio (0.31 at CA 4.2yr) was also the lowest among Russian children with other GH1

splicing mutations of the GH1 gene.

On the basis of our results, it can be assumed that a phenomenon of splicing defects in the GH1 gene is well defined in GH deficiency, but the list of splicing mutations is far from being complete. Our results demonstrate for the first time that, in addition to intron 2, 3, 4, and exon 3, one more novel site of splicing defect in congenital IGHD is located on exon 4 of the *GH1* gene. We speculate that 456G>A transition in the highly conserved GGGgtg sequence of the exon 4/IVS4 boundary of GH1 results in perturbed splicing and altered protein product. A novel heterozygous 456G>A dominant negative mutation in exon 4 expands the spectrum of splicing defects within the GH1 gene responsible for congenital isolated GHD so far described.

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