GENETICS

GH-1 Gene Splicing Mutations: Molecular Basis of Hereditary Isolated Growth Hormone Deficiency in Children

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Children, residents of the Russian Federation, with congenital isolated growth hormone deficiency, were screened for mutations of *GH-1* gene, the main gene of this deficiency. Twenty-eight children from 26 families with total congenital isolated growth hormone deficiency were examined. Direct sequencing of *GH-1* detected five splicing mutations in intron 2, intron 3, and exon 4, two of them were never described previously. Three dominant negative mutations of *GH-1* splicing, the basis for autosomal dominant isolated growth hormone deficiency (type II), are presented: IVS2 -2A>T, IVS3 +2T>C, and IVS3 +1G<A. *GH-1* is the main gene of type II isolated growth hormone deficiency in patients living in the Russian Federation. All detected mutations of *GH-1* impair splicing processes, which distinguishes them from mutations in other forms of isolated growth hormone deficiency. The detected variety of *GH-1* splicing mutations attests to allele genetic heterogeneity of this pathology. The "hot spot" of mutations is 5'-donor splicing site of *GH-1* intron 3, while IVS3 +1G>A mutation can be regarded as the most incident in type II isolated growth hormone deficiency in the Russian population.

Key Words: *GH-1 gene; splicing mutations; growth hormone deficiency*

Genetic approaches based on concepts and methods of molecular genetics become priority in studies of the pathogenesis of endocrine diseases in children [11]. The spectrum of genes involved in the pituitary embryogenesis and regulation of the "growth hormone (GH) — insulin-like growth factors" system in humans is now known. Congenital isolated growth hormone deficiency (IGHD) is a heterogeneous disease uniting familial and sporadic forms with different types of inheritance. The main gene of congenital IGHD is *GH-1* gene. Mutations in this gene in children of different ethnic groups were described [13,14]. Mutation analysis of *GH-1* was not carried out in patients with IGHD in Russia before.

We studied *GH-1* gene mutations in Russian children with congenital IGHD and detected possible "hot spots" of mutations in *GH-1* gene responsible for this disease.

MATERIALS AND METHODS

Twenty-eight children (16 boys and 12 girls) from 26 families with congenital IGHD were examined.

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Ten children from 8 families (6 families with type II and 2 with type IB IGHD) presented with familial and 18 patients with sporadic form of the disease. The patients were included into group for molecular analysis on the basis of the following criteria: growth retardation since infancy (during the first year of life), height <-2 SDS (standard deviation coefficient for chronological age and sex), total GH deficiency (peak release <5 ng/ml) after stimulation with insulin (Actrapid HM, Novo Nordisk; 0.1 U/kg intravenously) and clonidine (Organika; 0.15 mg/m^2 body surface orally), normal basal blood levels of free T_4 (or total T_3/T_4), hydrocortisone, and prolactin. Chronological age (*M±SD)* of patients was 8.3±4.3 years, bone age 4.6±3.6 years. Height SDS (H SDS) at the age of 1 year corresponded to -3.2±1.2, at the age of 2 years -4.3±0.9. The majority (67.8%) of children had a large impending forehead, depressed bridge of the nose, small features, and bluish scleras. Maximum blood concentration of GH after stimulation was 1.39±1.80 ng/ml. The peak of GH did not surpass 2 ng/ml in 74% children. Body length at birth was 50.3±2.8 cm.

Genome DNA was isolated from venous blood leukocytes by the method of Lindblom and Holmund based on phenol-chloroform extraction. Four DNA fragments of *GH-1* gene ([2], GenBank accession N J03071) covering exons 2-5 and their flanking regions were amplified using DNA amplifier (Pushchino) and analyzed by single stranded DNA conformation polymorphism (SSCP) method and direct DNA sequencing. Exon 1 was excluded from analysis because of its small size (10 n. p.) and absence of data on mutations significant for the studied disease in this exon. The sequences of original primers synthesized in Litech Firm, specific for each primer, are presented in Table 1.

PCR was carried out in 30 μl amplified mixture of the following composition: 0.25 μM each oligonucleoide primer, 200 μM each deoxynucleotide triphosphate, 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Twin-20, 2-6 mM (depending on each pair of primers) $MgCl_2$, 1-1.5 U Taq DNA polymerase (Fermentas), and 0.1-0.5 μg genome DNA. The mixture was denatured for 7 min at 94°C and then subjected to PCR cycles (32 cycles for exons 2, 4, and 5; 34 cycles for exon 3): 45 sec denaturing at 94°C, 45 sec annealing at 57°C (exons 2 and 5), 65°C (exon 3), or 60°C (exon 4), 45 sec elongation at 72°C with subsequent 8-min incubation at 72°C. DNA samples were screened for mutations in *GH-1* gene by SSCP method. SSCP with alkaline denaturing was used: 7 μl PCR product was mixed with 0.5 μl 5 M NaOH, 0.5 μl 0.5 M EDTA, and 4.5 μl deionized water. The mixture was warmed to 42°C for 15 min and 3.0 μl formamide mixed with dyes (0.5% xylene cyanol, 0.5% bromophenol blue) was added. The sample was applied onto 10% PAAG with 5% glycerol and analyzed by electrophoresis (5 V/cm, 0.5× Tris-borate buffer, 18-20 h at room temperature). After electrophoresis the gel was fixed and stained using Silver Sequence DNA Staining Reagents kit (Promega Corp.). DNA samples with changed electrophoretic mobility (detected by SSCP) were analyzed by direct gene sequencing. DNA material after amplification, gel extraction, and purification served as the matrix for direct sequencing: 60 μl PCR product was mixed with 12 μl dye, electrophoresed in 1% low-melting agarose in 1× TAE buffer with ethidium bromide (1 μg/ml). DNA matrix was purified using Wizard PCR PREPS DNA Purification System columns (Promega Corp.). Direct automated sequencing (5' and 3') of purified PCR products was carried out using ABI Sequencing dye terminator kit (Perkin Elmer) on an ABI PRISM 373 DNA Sequencer (Perkin Elmer) at Laboratory for Automated Sequencing of DNA, Engelgardt Institute of Molecular Biology, Russian Academy of Sciences.

RESULTS

Mutations of *GH-1* gene were identified in 9 (32.1%) of 28 children. All detected *GH-1* mutations were splicing mutations in a heterozygotic state located in different splicing sites, including intron 2, intron 3, and exon 4 (Table 2). Two of the detected five mutations were never described previously.

Splicing mutations of *GH-1* were detected in all six families (in 7 children) with autosomal dominant type of IGHD inheritance (type II): IVS2 -2A>T (A>T substitution of second nucleotide of 3'-acceptor splicing site in intron 2), IVS3 +2T>C (T>C substitution of second nucleotide of 5'-donor splicing site in intron 3), IVS3 +1G>A (G>A sub-

TABLE 1. Sequences of Primers Used for PCR Amplification of *GH-1* Gene Exons 2-5 and Flanking 5' and 3' Regions

Exon	Primer	Sequence
2	F	5'-CGGCTCCCTCTGTTGCCCTCT-3'
	R	5'-CCCCTTCCTGCCACCCCTGAT-3'
3	F	5'-AATGGGAGCTGGTCTCCAGCG-3'
	R	5'-GGGGCTCTGACTACAGGTCTC-3'
4	F	5'-GTGGATGCCTTCTCCCCAGGC-3'
	R	5'-GGGGCTCCAGGATTGGGGAC-3'
5	F	5'-GAATGAGAAAGGGAGGGAACAGTA-3'
	R	5'-CTGGAGTGGCAACTTCCAGGG-3'

TABLE 2. *GH-1* Gene Splicing Mutations in Children with Familial and Sporadic IGHD

Location	Mutation type	Nucleotide substitution	Number of	
			children	families
Intron 3	5'-donor splicing site	IVS3 +1G→A	5	4[x]
Intron 3	5'-donor splicing site	IVS3 +2T→C	1	1[x]
Intron 2	3'-acceptor splicing site	IVS2 -2A→T*	1	1[x]
Intron 3	5'-donor splicing site	IVS3 +5G→A	1	1
Exon 4	3'-acceptor splicing site	456G→A*	1	1

Note. *Not described previously; [x]families with type II IGHD.

stitution of the first nucleotide in 5'-donor splicing site of intron 3). These mutations destroy highly conservative IVS2 acceptor or IVS3 donor slicing sites critical for normal splicing.

New splicing mutation IVS2 -2A>T located in the second nucleotide of conservative ag-dinucleotide of 3'-acceptor splicing site in *GH-1* intron 2 (tagGAA) (Fig. 1) was detected in two patients from one family with type II IGHD. Two generations of patients suffer from congenital IGHD in this family (Fig. 2).

The body height at the age of 1 year was -2.7 SDS. IGHD was diagnosed at the age of 5.2 years (H SDS -3.25), with the peak GH level in clonidine test 1.19 ng/ml. In the mother IGHD was diagnosed at the age of 33 years; her body height was 141.4 cm (H SDS -3.47), the peak GH level in insulin test being 1.8 ng/ml.

Splicing mutation IVS2 +2T>C, located in the second nucleotide of 5'-donor splicing site conservative gt-dinucleotide of *GH-1* intron 3 (TCCgtg) (Fig. 1), was detected in one family with type II

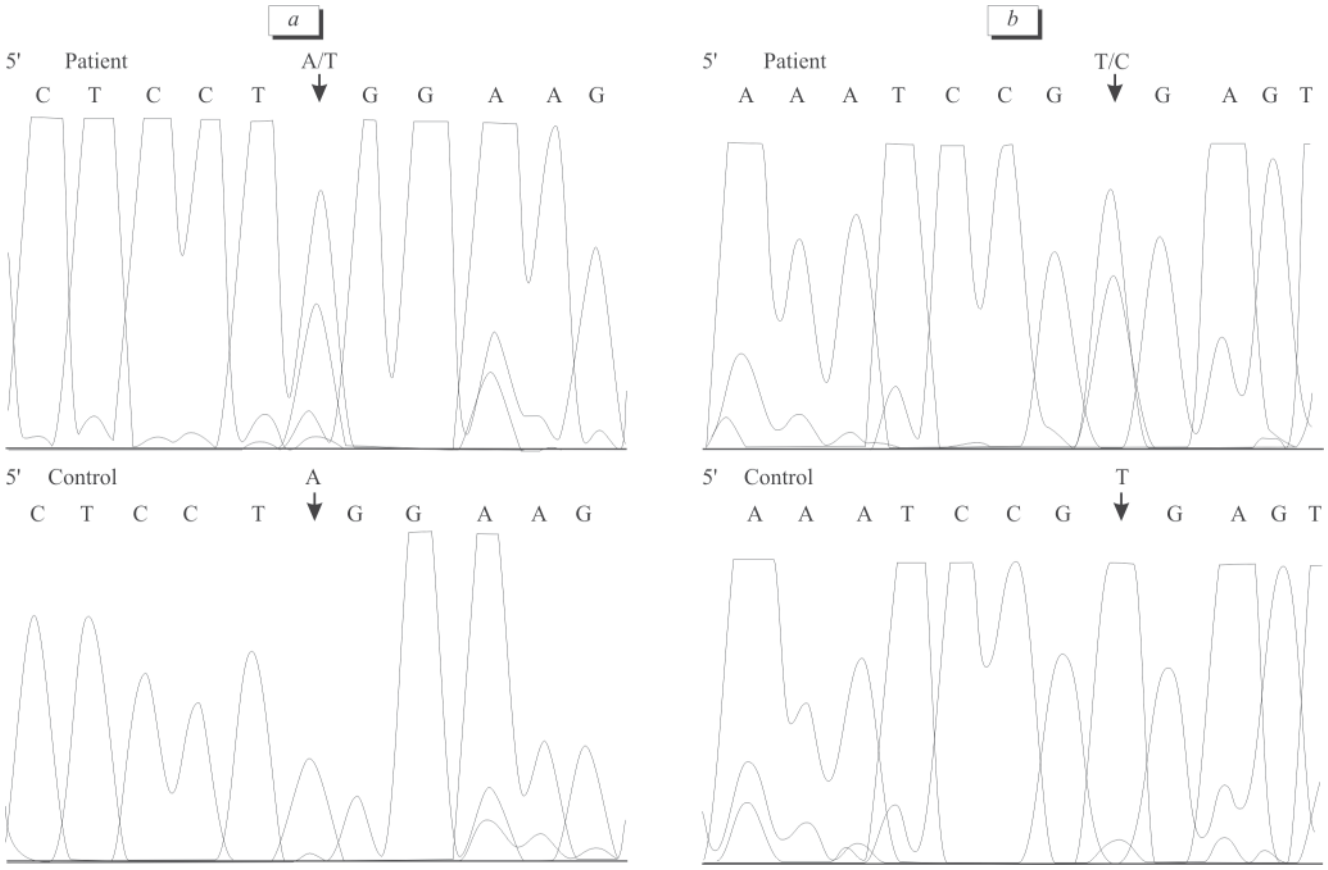

Fig. 1. Fragments of sequence of genome DNA of *GH-1* gene in families with type II isolated growth hormone deficiency (IGHD). Mutations in heterozygotic state. *a*) IVS -2A>T in second nucleotide of intron 2 3'-acceptor splicing site; *b*) IVS3 +2T>C in second nucleotide of intron 3 5'-donor splicing site.

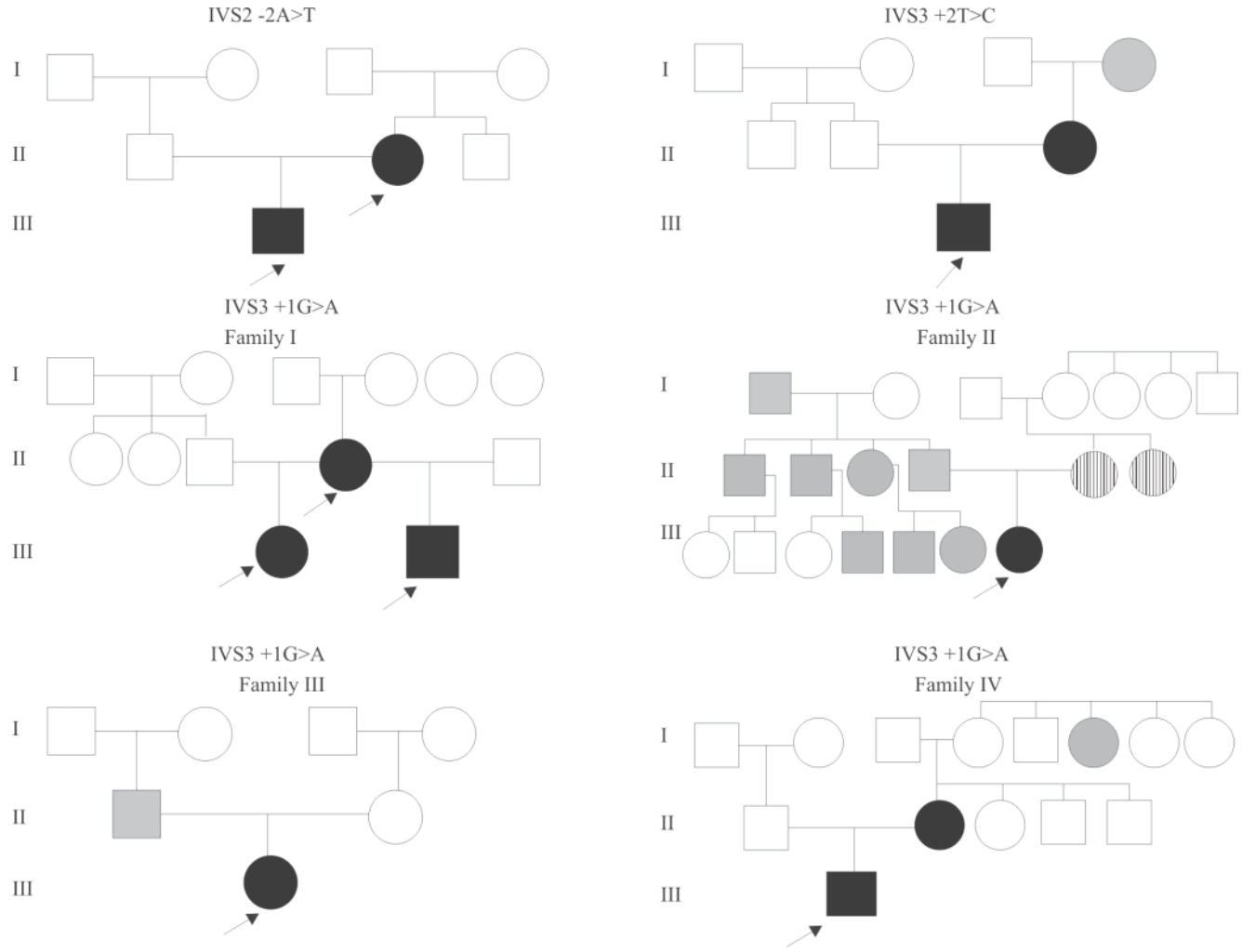

Fig. 2. Genealogy of families with congenital type II IGHD resultant from splicing mutations in *GH-1* gene. Black symbols with an arrow: patients with detected *GH-1* mutation; black symbols: patients with proven IGHD, no molecular analysis was carried out; gray symbols: family members with presumable IGHD (could not be examined); cross-hatched symbols: patients with Seckel syndrome.

IGHD. The family consisted of three generations of patients suffering from IGHD. The boy's height at the age of 1 year was -3.46 SDS; at the age of 7.8 years (H SDS -4.4) the diagnosis was confirmed by insulin and clonidine tests (GH peaks 0.6 and 1.8 ng/ml, respectively). The patients had concomitant coarctation of the aorta. In the mother (height 119.0 cm, H SDS -7.2) IGHD was diagnosed at the age of 27 years, with GH peak in insulin test <0.1 ng/ml. Grandmother's (maternal line) height was also 119.0 cm.

Splicing mutation IVS3 +1G>A, located in the first nucleotide of 5'-donor splicing site conservative gt-dinucleotide in *GH-1* intron 3 (Fig. 3) was detected in 5 children from 4 families with type II IGHD. In the first of these families sister and brother from different fathers inherited the pathological allele from the mother. The disease was diagnosed in the sister at the age of 13 years (H SDS -4.7, peak GH concentration after insulin and clonidine stimulation 0.6 ng/ml), in the brother at the age of 1 year (H SDS -3.1, blood level of IGF-I <2.5 ng/ml). IGHD was diagnosed in their mother at the age of 38 years, when her height was 126.5 cm (H SDS -5.9) and peak GH in insulin test was 0.05 ng/ml.

Genealogy of the second family included, in addition to the proband, short father (H SDS -6.7) and grandfather (paternal line; H SDS -4.5). IGHD was diagnosed in the girl at the age of 5.2 years (H SDS -3.9 years), peak GH level after clonidine stimulation was 1.4 hg/ml. Her father's family included short aunt (145 cm), two uncles (140 cm each), and their three children (130 cm). The girl's short mother (H SDS -5.7) suffered from Seckel syndrome. The aunt in mother's family (H SDS -5.0), also with the Seckel syndrome phenotype, died at the age of 18 years from bone sarcoma.

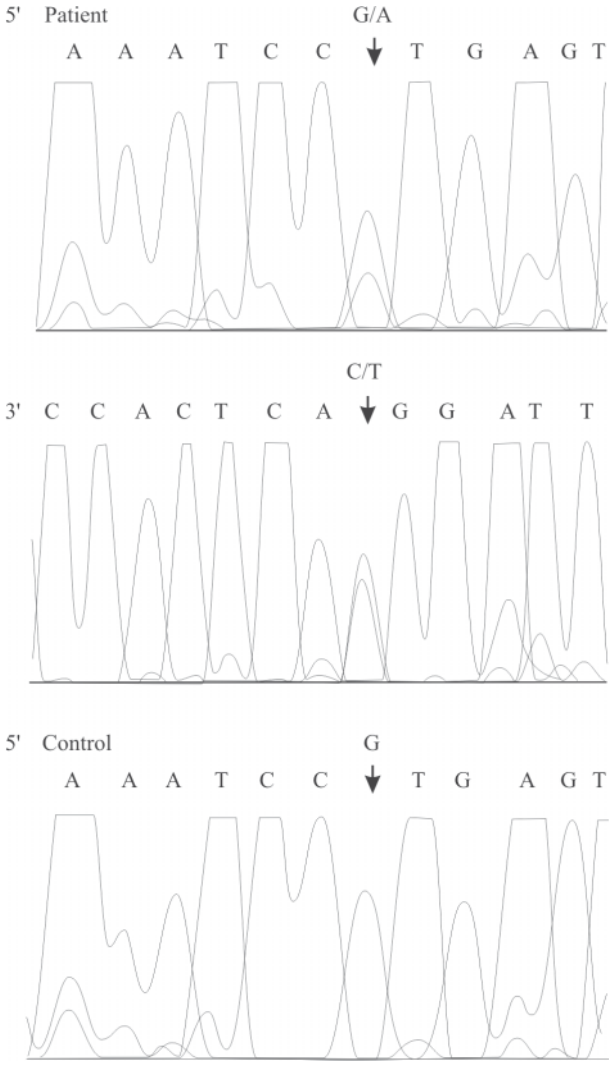

Fig. 3. Fragments of *GH-1* gene genome DNA sequence in families with type II isolated growth hormone deficiency (IGHD). Mutation in first nucleotide of intron 3 5'-donor splicing site in the heterozygote state.

In the third family IGHD was diagnosed in a girl at the age of 2.3 years (H SDS -3.8), peak GH level after clonidine stimulation 0.7 ng/ml. Short father (H SDS -3.7) could not be examined.

The fourth family consisted of two generations of patients suffering from congenital IGHD. Genetic analysis in the son was carried out by the group of scientists headed by J. A. Phillips III (Vanderbilt University, Nashville, USA). Final height of the patient at the age of 20 years (initial examination) was 144.8 cm (H SDS -4.5), pubertal stage IV according to Tanner, peak GH level after insulin stimulation <0.1 ng/ml. Height of patient's 59-year-old mother was 123.4 cm (H SDS -6.5), peak GH level in insulin test <0.15 ng/ml.

The spectrum of detected *GH-1* dominant negative splicing mutations [7] destroying invariant IVS2/exon 3-acceptor and exon 3/IVS3-donor splicing sites supplemented the list of known gene defects associated with IGHD [3,4,10]. We identified three different mutations causing autosomal dominant segregation of the disease, including one new mutation in intron 2 and one in exon 4 of *GH-1*. *GH-1* splicing mutations in type II IGHD were highly incident (100%, 6/6 families), in contrast to sporadic cases. Mutation IVS3 +1G>A was described in patients of different ethnic groups [5,8,12], in two cases these were *de novo* mutations [9]. These results rule out the "founder effect" as the explanation of high incidence of the mutation. It has been shown that IVS3 +1G>A mutation leads to omission of exon 3 with loss of amino acids 32-71 and shortening of the final protein to 151 amino acid residues [5]. As mutation IVS3 +1G>A involves the CpG sequence, characterized by increased incidence of mutations because of spontaneous deamination of methylated cytosine [1,6], this site can be regarded as a "hot spot" for mutations. This hypothesis is confirmed by IVS3 +1G>A mutation, detected in four of six unrelated families with type II IGHD. We present data on the greatest number of families with type II IGHD, confirmed by genetic findings, in one country (Russian Federation). The mechanism responsible for dominant negative effect of *GH-1* mutant allele remains not quite clear. It is considered that GH storage and secretion in the somatotroph secretory granules are impaired because of interactions between the mutant and nonmutant protein [1]. All mutations described in this paper most likely lead to omission of exon 3 in mature mRNA sequence. Hence, we suppose that the loss of *GH-1* exon 3 causes modification of GH molecule, leading to interaction of this mutant protein with normal protein molecules and thus inactivates them.

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