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Detection of Growth Hormone Gene Defects by Dideoxy Fingerprinting (ddF)

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Abstract. We carried out screening for mutations in the GH-1 gene in 29 sporadic Japanese subjects with severe Isolated Growth Hormone Deficiency (IGHD) by dideoxy fingerprinting (ddF). Three of 29 (~ 10%) were heterozygous for each of the following GH-1 gene mutations including: 1) an G→A transition in the third codon of the GH-1 signal peptide of exon 1 resulting in a Threonine to Alanine substitution, 2) a G→A transition in the first base of the donor splice site of IVS 3 (+1G→A) and 3) a G→A transition in the 183rd codon of the GH-1 mature peptide of exon 5 resulting in an Arginine to Histidine substitution. One of three was heterozygous for both mutations of 1) and 2). The IVS 3 (+1G→A) mutation has been previously reported in affected individuals from three unrelated families with IGHD type II (autosomal dominant form). This mutation destroys the GH IVS 3 donor splice site, causing skipping of exon 3 and loss of the codons for amino acids 32–71 of the mature GH peptide. Our findings indicate that 1) ddF screening of genomic DNAs provides a practical tool to detect GH gene mutations and 2) some sporadic cases of IGHD may be caused by GH gene alternations.

Key words: Isolated Growth Hormone Deficiency (IGHD), GH-1 gene, Dideoxy fingerprinting, Splicing
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THE FREQUENCY of GH deficiency is estimated to range from 1/4,000 ~ 1/10,000 in various studies [1–4]. While most cases are sporadic, from 3–30% of individuals with isolated GH deficiency (IGHD) have an affected first degree relative, suggesting many cases may be familial. Familial IGHD is associated with several modes of inheritance. These include autosomal recessive (IGHD IA and IB), autosomal dominant (IGHD II) and X-linked (IGHD III) forms [5]. Of subjects having the severe autosomal recessive phenotype (IGHD IA), about 13% have complete deletions of the GH-1 gene [6], and frameshift and nonsense mutations are reported in some of the others [7]. The less severe autosomal recessive form (IGHD IB) is due to less severe mutations including a donor splice site mutation of IVS 4 [8]. In some with an autosomal dominant mode of inheritance (IGHD II), donor splice site mutations of IVS 3 have been detected, which have a dominant negative effect [9, 10]. These findings suggest that a large variety of molecular defects may cause familial IGHD. To facilitate detection of these heterogeneous mutations, dideoxy fingerprinting (ddF) has been used to examine the GH genes of GHD subjects in several previous studies [11–14]. In these studies of consecutive GHD cases of varying severity, from 10–44% of samples had changes detected by ddF. To determine the frequency and types of GH-1 mutations occurring in sporadic cases of severe IGHD, we screened DNAs from 29 Japanese subjects utilizing ddF analysis of the entire GH-1 gene and sequenced those having variant patterns.

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Subjects and Methods

Subjects

We studied DNAs from 29 Japanese individuals affected with severe IGHD, all having heights before GH treatment < −4 SD. None had a family history of relatives with IGHD or short stature. Peak serum GH levels by l-dopa, arginine and insulin stimulation were all < 7 ng/m*l*. Levels of thyroxine were normal and bone ages were delayed. In all cases, GH-1 gene deletions were excluded by polymerase chain reaction (PCR) amplification and digestion of the product with Sma I restriction endonuclease [15].

Methods

DNA isolation: Genomic DNA was isolated as previously described from leukocytes of peripheral blood samples, and the concentration of each sample was determined by measuring the optical density of the purified DNA at 260 nm [16].

PCR amplification of genomic DNA: 2.7 kb PCR products including the entire region of the GH-1 gene were amplified. 200 ng of genomic DNA was added to a 100 μl reaction mixture of 50 mmol/L Tris-HCl (pH 9.2), 16 mmol/L $(NH_4)_2SO_4$, 1.75 mmol/L $MgCl_2$, 350 μmol/L of each deoxy-NTP, and 10 μmol/L of each primer. To minimize Taq polymerase errors during PCR amplification, 2.5 U Taq & Pwo DNA polymerase (BOEHRINGER MANNHEIM) were used because of the proofreading properties of the latter. The forward and reverse primers corresponded to nucleotides 4556–4575 (Primer 11: 5′-CCAGCAATGCTCAGGGAAAG-3′) and the complement of 7226–7255 (Primer 2a: 5′-TGTCCCACCGGTTGGGCATGGCAGGTAGCC-3′) of the GH-1 gene cluster (Fig. 1). These primers are specific for the GH-1 gene and do not amplify any of the other components of the GH gene cluster. The PCR reaction mixture was denatured for 2 min at 92 °C and cycled 10 times (92 °C, 10 sec; 65 °C, 30 sec; and 68 °C, 3 min) and cycled 20 times (92 °C, 10 sec; 65 °C, 30 sec; and 68 °C, 3 min + cycle elongation of 20 sec for each cycle), followed by a 7-min elongation at 68°C [14].

PCR of 2.6 kb nested fragments: 2 μl of 2.7 kb PCR product was added to a 100 μl reaction mixture of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 200 μmol/L of each deoxy-NTP, 10 μmol/L of each primer, and 2.5 U Taq DNA polymerase. The forward primer of the second round of PCR was 4599–4618 (Primer 33: 5′- TTCTCTCTAGTGGTCAGTGT-3′); the reverse primer was 7187–7206 (Primer 32: 5′-CTCCTGCTGGTATAGTTATT-3′)

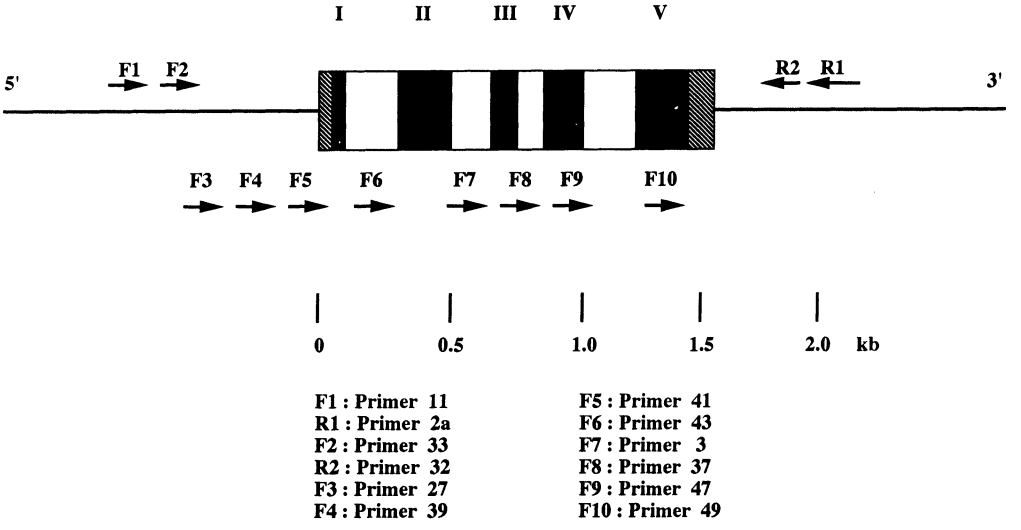

Fig. 1. The structure of the GH-1 gene and the location of primers used for long PCR, nested PCR and ddF are schematically shown. Exons, introns and nontranslated sequences are depicted by solid, open and shaded rectangles.

(Fig. 1). The PCR reaction mixture was denatured for 6 min at 94 °C and cycled 32 times (94 °C, 1 min; 52 °C, 45 sec; 72 °C, 3 min), followed by 10 min at 72 °C. The resulting PCR products were cleaned by filtration with a Microcon-30 (Amicon, Beverly, MA, U.S.A.) and used as templates for dideoxy fingerprinting and sequencing [14].

Dideoxy Fingerprinting (ddF): We used 8 primers to complete ddF screening of the GH-1 gene (see Table 1). These 8 primers are spaced at interval of 218 bp to 360 bp spanning the 2.6 kb nested PCR fragments that contain the GH-1 gene. The ddF reaction was done using the dsDNA Cycle Sequencing System (GIBCO BRL, Gaitherburg, MD, U.S.A.). At first, 1 μl of H_2O, 1 μl of 5 X Kinase Buffer, 1 μl of [γ-^{32}P] ATP (1 μCi) and 1 μl of T4 Polynucleotide Kinase (1 U) were added to 1 μl of each primer (1 μmol/L). The mixture was incubated at 37 °C for 30 min, then at 55 °C for 10 min. To all 5 μl of each end-labeled primer, 24 μl of H_2O, 4.5 μl of 10 X Taq sequencing buffer, 2 μl of template DNA and 0.5 μl Taq DNA polymerase (2.5 U) were added. Then 8 μl of the prereaction mixture and 2 μl of a mixture containing 2 mM ddTTP were added. The sequencing reaction mixture was denatured at 95 °C for 5 min, cycled 20 times (95 °C, 30 sec ; 55 °C, 30 sec ; 70 °C, 1 min) and then cycled 10 times (95 °C, 30 sec ; 70 °C, 1 min), followed by a 10-min elongation at 72 °C. After cycle sequencing reaction, 1 U of Terminal deoxynucleotidyl Transferase (TdT) was added to each sequencing reaction mixture. After incubation at 37 °C for 30 min, 5 μl of Stop Solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added. The mixture was then heated at 95 °C for 5 min and chilled on ice, and 3 μl of each reaction was loaded per lane on a 5% non-denaturing polyacrylamide gel. The samples were electrophoresed at 25 W at 4 °C for ~ 3 h with 50 mM Tris-borate, pH 8.3 and 1 mM EDTA. The gel was transferred to gel blot papers, dried and exposed to BioMax film (Eastman Kodak, Rochester, NY, U.S.A.).

Sequencing: For samples in which changes were found by ddF analysis, sequencing was performed by the cycle sequencing method using the dsDNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, MD, U.S.A.).

Results

Dideoxy Fingerprinting (ddF)

Autoradiogram of ddF analysis are shown in Fig. 2. Four heterozygous changes were found in three subjects (Subject 5, 22, 26).

Sequencing

The sequences of the GH-1 gene of these three subjects with ddF changes are shown in Fig. 3. Subject 5 was heterozygous for a G→A transition in the first base of the donor splice site of IVS 3 and an A→G transition in the third codon of exon 1 which results in a Threonine to Alanine substitution in the third amino acid residue of the GH-1 signal peptide. Subject 26 also was heterozygous for the same exon 1 mutation as subject 5. Subject 22 was heterozygous for a G→A transition in the 183rd codon of the GH-1 mature peptide which changes an Arginine to Histidine in exon 5.

Table 1. Primers used in ddF screening of the GH-1 gene

Primer No.	Sequence	Location
27	5′-CAGGACTGAATCGTGCTCAC-3′	4676–4695
39	5′-TATCTCTGGCTGACACTCTGTGC-3′	4934–4956
41	5′-GAACCACTCAGGGTCCTGTG-3′	5186–5205
43	5′-CCCTCTGTTGCCCTCTGGTT-3′	5466–5485
3	5′-CTGGGAAATAAGAGGAGGAGACT-3′	5715–5737
37	5′-CTCAGAGTCATTCCGACACCCT-3′	5933–5955
47	5′-GCCTCTGACAGCAACGTCTA-3′	6176–6195
49	5′-GCAGACCTACAGCAAGTTCG-3′	6536–6555

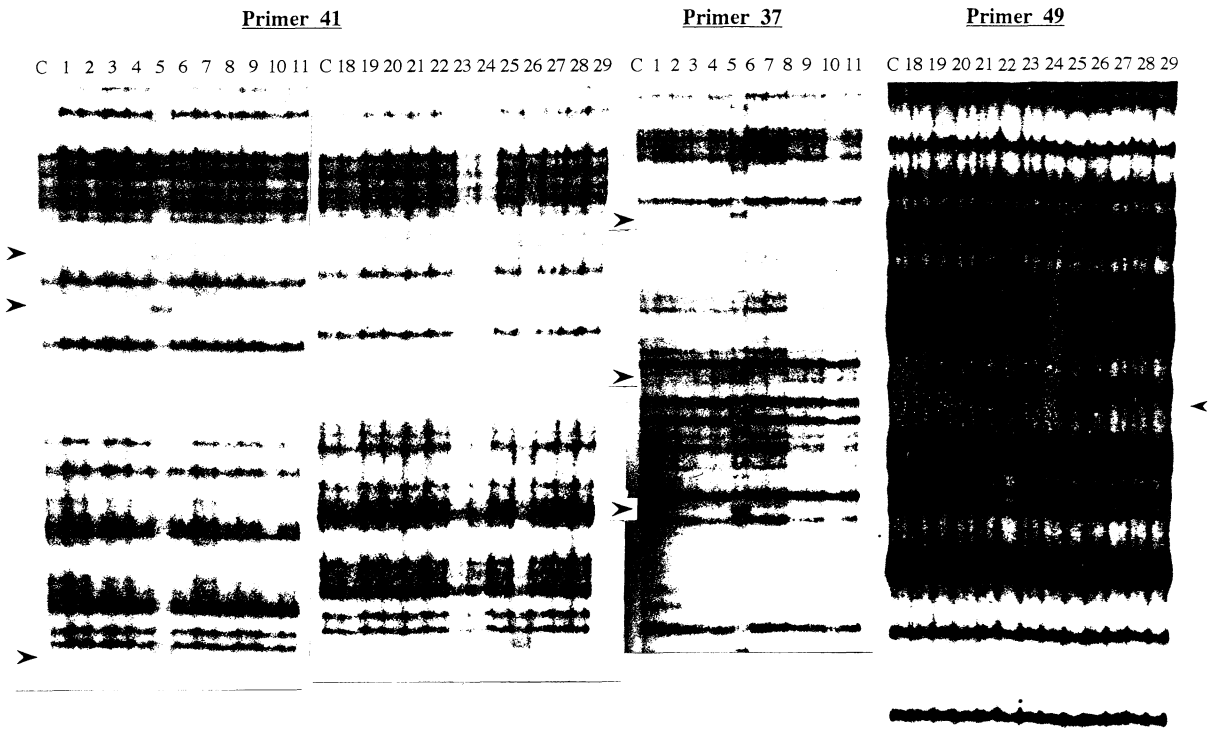

Fig. 2. Autoradiogram of ddF analysis. Lanes 1–29 contain products from corresponding Japanese subjects, while lane C was from a control. Heterozygous changes were found in lane 5 and 26 by primer 41, in lane 5 by primer 37 and in lane 22 by primer 49.

Discussion

Dideoxy fingerprinting is a hybrid method between dideoxy sequencing and single-strand conformation polymorphism (SSCP) analysis that can detect the presence of single base and other sequence changes in PCR-amplified segments [17]. It involves a Sanger sequencing reaction with one dideoxynucleotide followed by non-denaturing gel electrophoresis. Blaszyk *et al.* used ddF to screen for mutations in the p53 tumor suppressor gene in primary breast cancers and detected 100% of p53 mutations in their samples with few false positives [18]. The ddF method provides advantages over SSCP in that it yield information about the location of the sequence change within the PCR product and increases the sensitivity of detection to almost 100% [6, 17, 18].

Using ddF analysis, we found that 3/29 (10%) of sporadic Japanese subjects with severe IGHD were heterozygous for mutations of the GH-1 gene. One of them was heterozygous for both a G→A transition in the first base of the donor splice site of IVS 3 (+1G→A) and an A→G transition in exon 1 affecting the third codon of the GH-1 signal peptide. The IVS 3 (+1G→A) mutation has been previously reported in affected individuals from three unrelated families with IGHD type II [10]. This mutation destroys the GH IVS 3 donor splice site and causes skipping of exon 3 and loss of the codons for amino acids 32–71 of the mature GH peptide, which correspond to the 17.5 kilodalton isoform of GH protein. The *de novo* origin of the mutation was documented in one of three families studied. Recently, a G→C transversion in the same donor splice site was reported in a sporadic German case with severe IGHD [19]. This mutation had a dominant negative effect and also arose *de novo*. These and our findings constitute 5 unrelated cases of donor splice mutations of IVS 3. It is interesting that 3/5 (60%) arose *de novo*, suggesting that sporadic occurrence of severe IGHD does not exclude a genetic basis of the disorder.

The A→G transition detected in exon 1 of subject 5 and 26 converts a Threonine codon (ACA) to an

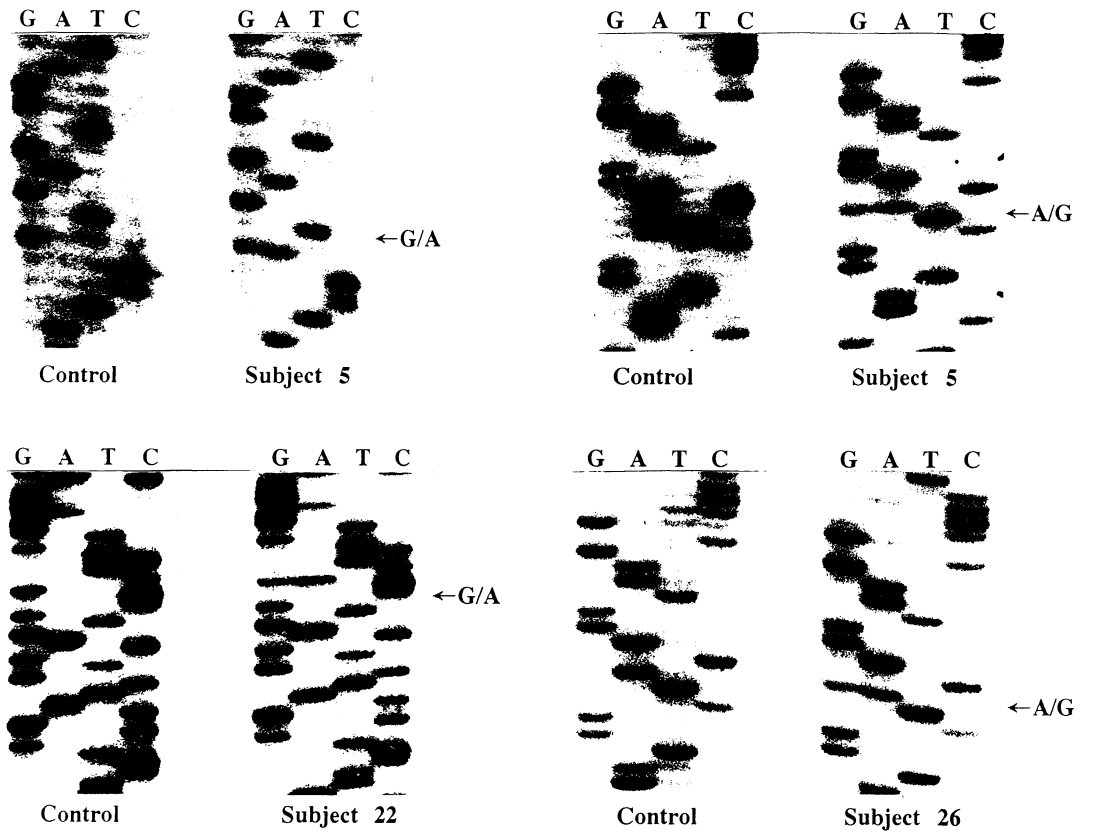

Fig. 3. Sequencing analysis of three affected individuals and a normal control. Subject 5 was heterozygous for a G→A transition in the first base of IVS 3 and an A→G transition in exon 1 (upper left and right, respectively). Subject 22 was heterozygous for a G→A transition in exon 5 (lower left). Subject 26 also has the same mutation (lower right) in exon 1 as Subject 5 (upper right).

Alanine codon (GCA). While this mutation has been previously detected in three Brazilian and one Thai GHD subjects, all of whom were heterozygous [11, 13], its significance is unclear. But since no segregation or functional studies have proven that it affects GH expression or function, it may be a silent polymorphism.

We also found that subject 22 was heterozygous for a G→A transition in exon 5 that affects the 183rd codon of the GH-1 mature peptide. This G →A substitution converts an Arginine codon (CGC) to a Histidine codon (CAC) at nucleotide 6661. This mutation has also been detected in a Swiss subject with GHD (Primus Mullis and Joy D. Cogan, personal communication), but again no effect on GH expression or function is proven.

In conclusion, ddF is an efficient method for screening the entire GH-1 gene. From previous studies of Japanese and Chinese subjects with severe IGHD, Kamijo *et al.* [20] found that ~ 12% had GH-1 gene deletions. In this study, we found that ~ 10% of sporadic Japanese subjects with severe IGHD had GH-1 gene alternations. The frequency of these ddF alternations appears to be less than that reported for consecutive Czecho and Slovakian (44%) versus Thai subjects (24%) with GHD [12, 13], but is similar to that of Brazilian subjects (10%). Further studies are needed to determine the proportion of those changes that are functionally important.

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