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Prevalence of GH-1 Gene Deletion in Patients with Isolated Growth Hormone Deficiency in Japan

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Abstract. A multicenter study was carried out to investigate the prevalence of growth hormone (GH-1) gene deletions among patients with isolated growth hormone deficiency (IGHD) and extremely short stature in Japan, using PCR method. Genomic DNA was extracted from the whole blood samples of 48 patients (34 males and 14 females) at 20 hospitals. All the patients fulfilled the inclusion criteria as follows: (1) IGHD patients whose every peak serum GH level in more than two tests <5 ng/m*l* and (2) pretreatment height <-3SD, regardless of family history and facial feature characteristic of GH-1 gene deletion. The subjects were screened for deletions in GH-1 gene, using a PCR method that could identify deletions of 6.7, 7.0 and 7.6 kbp. Three (6.25%) out of 48 subjects were found to have such deletion fragments. The first case was a boy homozygous for deletion of 6.7 kbp fragments. The second case was a girl heterozygous for 6.7 kbp deletion. A direct sequence analysis revealed a 2-bp deletion in exon 3 on the remaining allele that created a stop codon in exon 4. The third case was a boy also heterozygous for 6.7 kbp deletion. By direct sequencing analysis, three point mutations were detected in the promoter region on the opposite allele together with a four-base addition at base 250. One of the mutations was in the area of Pit-1 binding site (at base -123). The latter two cases apparently represent new types of compound heterozygote of GH-1 gene deletion. Our results suggest that GH-1 gene mutation is not so rare in extremely short IGHD children in Japan.

Key words: Isolated growth hormone deficiency (IGHD), IGHD type IA, GH-1 gene, Polymerase chain reaction (PCR), Deletion mutation

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HUMAN growth hormone (GH) is encoded by the GH-1 gene which resides on chromosome 17 (17q22–24) [1, 2]. Abnormalities in GH-1 gene cause familial types of GH deficiency, resulting in short stature. The familial isolated GH deficiency (IGHD) was classified into 3 types by Phillips [3] on the basis of the mode of inheritance. IGHD type IA and IB are autosomal recessive while type II is autosomal dominant and type III is X-linked recessive. IGHD type IA is caused by large deletions in the GH-1 gene. Mutations of GH-1, growth hormone releasing hormone (GHRH) or GHRH receptor gene is supposed to cause IGHD type IB, but GHRH gene mutations are unlikely to be a causative factor of IGHD type IB [4].

IGHD patients with extreme short stature have been studied for GH-1 gene deletions among Oriental Jews [5], Japanese and Chinese [6], British Caucasian

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[7], North European [8, 9], Mediterranean [8, 9], Turkish [8] and Asian [9] children. Examination of 10 IGHD patients from Nagoya University in Japan, failed to detect any GH-1 gene deletion [6]. However, we felt that we could detect GH-1 gene deletions in such patients if they were collected from different parts of Japan. We therefore organized a multicenter study group to investigate the prevalence of GH-1 gene deletion in Japan. Our study revealed 3 cases of GH-1 gene deletions including two new types of compound heterozygote.

Subjects and Methods

Forty-eight Japanese patients (34 males and 14 females) with IGHD from 20 hospitals were examined. The names of the participating doctors and their hospitals are listed in Table 1. The inclusion criteria were: 1) IGHD in whom every peak serum GH level in more than two standard pharmacological tests was <5 ng/m*l* and 2) pretreatment height SD scores were <-3, regardless of family history and facial feature characteristic of IGHD type IA. Informed consent was obtained from the parents of all the patients before participating in this study. The clinical and endocrinological characteristics of the patients are summarized in Table 2. There was only one patient with family history and facial features peculiar to IGHD type IA.

Genomic DNA was isolated from peripheral leukocytes and the concentration was determined as described before [10]. PCR amplifications were performed by the method of Vnencak-Jones *et al.* [13]. The reaction mixtures (total volume, 100 μl) contained 100 ng of genomic DNA, oligonucleotide primer, dNTPs buffer and Taq polymerase. The samples were amplified by 30 repeated cycles as follows:1) denaturation, 30 sec. at 94°C, 2) annealing, 30 sec. at 60°C and 3) extension, 2 min. at 72°C. Following PCR, the products (1900 bp) were digested with restriction enzyme Sma I and the resulting DNA fragments were subjected to electrophoresis on 2% SeaKem LE agarose gels to identify 6.7, 7.0 and 7.6 kbp deletions in GH-1 gene.

The subjects who were heterozygous for GH-1 gene deletions were further analyzed by direct sequencing to detect small abnormalities in the appar-

Table 1. Members of the GH Gene Study Group

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Table 2. Clinical and endocrinological characteristics of the subjects

	Number of Patients	Age (yrs) (Mean)	Height SDS	Peak GH (ng/ml)		IGF-I (U/ml)
				Arginine	L-DOPA	
Male	34	12.5	−4.84±3.26	2.62±2.68	1.88±4.32	0.34±0.41
Female	14	12.8	−5.76±2.02	2.77±3.93	2.20±1.60	0.43±0.26
Total	48	12.6	−5.09±3.01	2.66±4.04	1.96±4.24	0.36±0.39

ently normal GH-1 gene (on the opposite allele).

Results

In this study, we found deletions in GH-1 gene in 3 (6.3%) out of 48 patients. The auxological and endocrinological findings of the specific cases are summarized in Table 3.

In normal control, 4 bands (1900, 761, 712 and 448 bp) are expected to be seen after gel electrophoresis of the PCR products digested with Sma I. However, the above three cases demonstrated abnormal patterns after electrophoresis of the digested PCR products. In case 1 [11], only two bands (1470 and 448 bp) were detected. This finding suggested that the patient was homozygous for the 6.7 kbp deletion of the GH-1 gene. Interestingly, other members of the family (father, mother and two sisters) were heterozygous for the 6.7 kbp deletion. There was a history of consanguinity in this family. The patient had a vaulted forehead and his growth was suppressed by GH antibody produced during GH treatment.

In the second case [10], a total of five bands (4 normal as well as a rare 1470 bp band) were detected when the sample DNA was treated in an identical fashion as in case 1. The patient and her father were heterozygous for the 6.7 kbp deletion of GH-1 gene. In contrast, the sample from her mother demonstrated a restriction enzyme pattern identical to that of a healthy subject. After PCR amplification and direct sequencing, a deletion of 2 bp (AG) in exon 3 of the GH-1 gene on one allele was detected in the patient and her mother. This 2 bp deletion in the exon 3 resulted in a frameshift mutation followed by a stop codon in exon 4. Consequently, a small molecular GH with amino acid residues 1-105 was produced which might have been bioinactive. At present, work is under way to determine any abnormality of the polypeptide produced by the abnormal GH-1 gene. Family history revealed no consanguinity. This patient did not have the facial feature characteristic of IGHD type IA and grew well after GH administration without any antibody production.

Case 3 [12] had a similar electrophoretic pattern to that of case 2. The patient, therefore, was expected to have some abnormality in his apparently normal allele to cause severe short stature. The mother was heterozygous for the 6.7 kbp deletion, while the electrophoretic pattern of the Sma I digested material of the PCR products was apparently normal in his father. A direct sequencing analysis revealed three point mutations (T to C at base −123, A to G at base −6 and A to T at base −1) in the promoter region and a four-base (AGAA) addition at base 250 of intron 1 of the GH-1 gene (on the remaining allele)

Table 3. Auxological and endocrinological findings in cases with GH-1 gene deletion

	Age	Height SD Score	Peak Serum GH		Response to GH Treatment	GH Antibody
			L-DOPA (ng/ml)	Arginine (ng/ml)		
Case 1	4.4	−8.1	0.7	0.9	(−)	(+)
Case 2	5.6	−3.0	1.2	2.1	(+)	(−)
Case 3	9.2	−7.8	1.0	1.0	(+)	(−)

both in the patient and his father. The Pit-1 gene was normal in the patient. His facial appearance was normal and no consanguinity was noted in his family history. He responded well to GH therapy without any antibody production.

Discussion

Previously, GH-1 gene abnormalities were examined by Southern blot analysis after digestion of genomic DNA with various restriction endonucleases including BamH I, Bgl I, EcoR I, Hinc II and Msp I. However, this method is laborious and time consuming. Thus, as an alternative, we adopted a PCR amplification method which was simple, rapid and comparable for detection of GH-1 gene deletions. This method requires only a small amount of DNA and even a spot of blood on a filter paper is sufficient to analyze the sample.

The PCR amplification is carried out using 23 and 26 base oligoprimers for annealing to the portions that flank the GH-1 gene and are highly homologous [13]. The amplified products are 1,900 and 1,921 bp, from the 5′ and the 3′ portion, respectively, and are indistinguishable after electrophoresis. Digestion of the 1,921 bp product with Sma I yields three fragments, 762, 711 and 448 bp. In contrast, the 1,900 bp product has no cleavage site for Sma I and consequently four fragments are detected on agarose gel electrophoresis.

Three sizes of GH-1 gene deletion are distinguishable. The 6.7 kbp deletion has an unequal DNA sequence between the 3′ portion and the 5′ portion. This fusion fragment has only one cleavage site from the 3′ portion and yields a 1,472 bp fragment which is characteristic of 6.7 kbp deletion and distinguishable in homozygote as well as in heterozygote, and a 448 kbp fragment after Sma I digestion. Deletion of 7.0 kbp loses the 5′ portion with GH-1 gene. As a result, the 1,900 bp fragment is not recognized and the three fragments produced from 1,921 bp after Sma I digestion are seen on the agarose gel. Deletion of 7.6 kbp loses the 3′ portion together with the GH-1 gene. Thus, only the 1,900 bp product of PCR is recognizable on the agarose gel after digestion with Sma I.

The incidence of IGHD type IA has been reported in several countries (Table 4). In 1989, Parks *et al.* [4] identified GH-1 gene deletions in 6 (11%) out of 56 patients with severely short IGHD (height SD score < -4) in Israel using Southern blotting. All of the affected individuals were Oriental Jews whose prevalence was 38% (5 of 13 cases). However, Mullis *et al.* [7] could not detect any GH-1 gene deletions among 53 British Caucasian children with IGHD and height less than -3 SD.

In another study, utilizing PCR method, Kamijo *et*

Table 4. Prevalence of GH-1 gene deletion in different ethnic groups

Reference	Nationality	Height SDS	Peak GH in Provocative tests (ng/ml)	Affected/Total	Prevalence (%)	Screening Method
Parks *et al.*	Oriental Jewish	< -4.0	<4	5/13	38.5	Southern Blotting
Mullis *et al.* (1990)	British	< -3.0	<1.5	0/53	0	Southern Blotting
Mullis *et al.* (1992)	North European	< -4.5	<4	3/32	9.4	PCR
	Mediterranean	< -4.5		3/22	13.6	
	Turkish	< -4.5		4/24	16.7	
Kamijo *et al.* (1991)	Chinese	< -4.0	<5	3/26	11.5	PCR
	Japanese	< -4.0		0/10	0	
Wagner *et al.* (1998)	North European	< -4.5	<4	6/69	8.7	PCR
	Mediterranean	< -4.5		4/34	11.8	
	Asian	< -4.5		9/48	18.7	
Present Study (1997)	Japanese	< -3.0	<5	3/48	6.3	PCR
		< -4.0		2/39	5.1	
		< -4.5		2/30	6.7	

al. [6] detected the 6.7 kbp deletion in 3 (12%) out of 26 Chinese children with severe isolated GH deficiency and short stature (< -4 SD). However, there were no GH-1 gene deletions among 10 Japanese IGHD patients. In their second study, Mullis *et al* [8] found GH-1 gene deletions in IGHD children (with height SD score < -4.5) from northern Europe (9.4%), Mediterranean region (13.6%) and Turkey (16.6%) by PCR method. Recently, Wagner *et al.* [9] investigated GH-1 gene abnormalities (including IGHD type 1A, 1B and 2) and the occurrence rates of the abnormalities were 11.6%, 14.7% and 31.2% in Northern Europe, Mediterranean and Asian countries, respectively.

In this study, using PCR method we found 3 cases (6.25%) which were homozygous and compound heterozygous for the 6.7 kbp deletion of GH-1 gene. One of the compound heterozygotes had only a 2-base deletion in exon 3 on an apparently normal allele. As a result, a frame shift mutation occurred generating a stop codon (after the codon of amino acid residue 131) in exon 4 and raising the question whether this patient belonged to IGHD type IA or type IB. At present, the definitions of type 1A and 1B are not clear. In another heterozygous case, three point mutations were detected, among which T to C transition at base -123 that could affect GH-1 gene expression, because TAAAT (from base -123 to -118) is involved in Pit-1 binding to GH-1 gene. However, more experiments are needed to explain this hypothesis. Interestingly, we detected an antibody to GH in one case homozygous for the 6.7 kbp deletion. The difference between antibody producer and non-producer remains to be clarified.

In conclusion, our results indicate that IGHD type IA is not so rare in Japan as had been thought, if severe GH-deficient and extremely short patients are examined regardless of family history and facial features peculiar to patients with GH-1 gene deletion.

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