

## HETEROGENEOUS PHENOTYPES OF JAPANESE CASES WITH A GROWTH HORMONE GENE DELETION

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**Summary** We studied four Japanese patients with isolated growth hormone (hGH) deficiency from three different families. There was consanguinity in two of the three families, and three patients were second cousins. Each patient was homozygous for a deletion of approximately 7.5 kilobases, which included the hGH-N gene. The deletions in three patients belonging to two different families were associated with the same restriction fragment length polymorphism haplotype, while the deletion of the other patient was associated with a different haplotype. All patients were treated with injections of pituitary hGH. The response to the therapy differed among them, that is, two patients belonging to different families showed a poor response together with the presence of anti-hGH antibodies, while the other two patients belonging to the same family showed a rather good response together with the absence of such antibodies. It is suggested that the production of anti-hGH antibodies is not solely due to the gene deletion, but that an unknown immunological disposition might also be involved.

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## INTRODUCTION

Single gene disorders of isolated human growth hormone (hGH) deficiency are classified into three different types: autosomal recessive (type I), autosomal dominant (type II) and X-linked recessive (type III) (Phillips III, 1983). Illig (1970) and Illig *et al.* (1971) distinguished a subgroup among type I patients, referred to type I-A, on clinical grounds. Later, Phillips III *et al.* (1981) clarified, based on restriction endonuclease analysis, that the Swiss patients described by Illig *et al.* (1970) were homozygous for a deletion of approximately 7.5 kilobases (kb) of DNA which contained the normal hGH gene (hGH-N). Subsequent studies disclosed the disease in children in Austria (Phillips III, 1983), Argentina (Phillips III, 1983; Rivarola *et al.*, 1984), Japan (Phillips III, 1983; Nishi *et al.*, 1984), Italy (Braga *et al.*, 1985), Israel (Laron *et al.*, 1985) and France (Goossens *et al.*, 1986). The phenotypes of the affected subjects differed in several respects. We describe four additional Japanese patients from three different families with a hGH-N gene deletion.

## SUBJECTS AND METHODS

*Subjects.* Four Japanese patients with type I-A isolated hGH deficiency from three different families were studied with informed consent. Their clinical and laboratory data are presented in Table 1. Cases 1 and 2 were siblings. The clinical record of case 4 was reported elsewhere (Mori *et al.*, 1981). Both the parents of cases 1 and 2 and the parents of case 3 were second cousins. The parents of case 4 were unrelated. All four patients received hGH (Crescormone®; AB Kabi, Stockholm, Sweden), for periods of three months to over 10 years, depending on the response. Plasma hGH, TSH, FSH, ACTH and prolactin were measured with commercial radioimmunoassay kits. Plasma from the patients was repeatedly examined for the presence of anti-hGH antibodies by the method of Roth *et al.* (1964). Clinical and laboratory data for the first patient described in Japan (Nishi *et al.*, 1984) are included in Table 1 for a reference.

*DNA probe.* cDNA of hGH, a kind gift from Dr. P.H. Seeburg, was prepared by the cleared lysis procedure and purified on CsCl gradients. The internal 490 base pair *Pvu*II fragment was labeled by nick translation, with a final specific activity of  $1 \times 10^8$  cpm/ $\mu$ g.

*Preparation of DNA and blot hybridization.* Total genomic DNAs were extracted from lymphoid cell lines established with the Epstein-Barr virus (Matsuda *et al.*, 1977) or whole blood by the method of Kunkel *et al.* (1977). DNA samples were completely digested using restriction enzymes *Bam*HI, *Hind*III, *Bgl*II, *Hinc*II and *Msp*I, and then fractionated by electrophoresis on 0.8% agarose gels. Blotting onto nitrocellulose filters was carried out by the method of Southern (1975). The

Table 1. Clinical and laboratory observations in patients with a hGH gene deletion.

	Case 1	Case 2	Case 3	Case 4	Case 5 <sup>b</sup>
<b>Parents</b>					
Consanguinity	+, second cousins		+, second cousins	—	+, second cousins
Father's height (cm)	167 (−0.3 SD)		163 (−1.1 SD)	158 (−1.9 SD)	162 (−0.7 SD)
Mother's height (cm)	149 (−1.6 SD)		150 (−1.4 SD)	152 (−0.9 SD)	149 (−1.0 SD)
Affected siblings	+(cases 1 and 2 were siblings)		—	—	—
<b>Patients</b>					
Sex	male	male	male	female	male
Delivery	normal	normal	normal	normal	normal
Birth weight (g)	2,600	2,740	2,495	2,900	3,050
At admission	5y8m	1	2y	7y9m	2y4m
Height (cm)	79.6 (−7.2 SD)	58.5 (−11.4 SD)	62.8 (−6.8 SD)	83.6 (−7.1 SD)	65.3 (−6.6 SD)
Weight (g)	1,000 (−3.4 SD)	5,410 (−4.3 SD)	6,310 (−3.8 SD)	11,700 (−3.5 SD)	6,190 (−4.7 SD)
Appearance					
Large vaulted forehead	+	+	+	+	+
Small nose with a retracted bridge	+	—	+	+	+
Cryptorchidism	—	—	—	/	+
Congenital dislocation of the hips	—	—	—	—	+
Bone age	4y5m	1y	1y6m	6y5m	1y
Blood sugar level on fasting (mg/dl)	45	45	40	75	42
hGH at rest (ng/ml) <sup>a</sup>	0.9	0.5	0.6>	1.0	0.5>
Maximum hGH level after stimulation (ng/ml) <sup>a</sup>	1.3	1.2	1.1	1.7	0.5
Other endocrine functions	normal	normal	normal	normal	normal
Antibodies for hGH after therapy	—	—	++ (1 : 600, 000)	++ (1 : 1,000)	++ (1 : 600, 000)
Karyotype	46,XY	46,XY	46,XY	46,XX	46,XY

<sup>a</sup> Levels below 1.5 ng/ml are considered to be indistinguishable from 0 values. <sup>b</sup> This was the first case in Japan and the data are cited from Nishi *et al.* (1984).

hybridization buffer contained 50% formamide, 5×SSC, 10×Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulphate (SDS) and 200 µg/ml of denatured salmon sperm DNA. Hybridization was performed at 42°C for 24 hr. Each filter was washed three times for 5 min with excess 1×SSC (0.3 M NaCl, 0.03 M Trisodium citrate)/0.1% SDS at room temperature and then washed for 1 hr with 0.1×SSC/0.1% SDS at 56°C. The filter was then exposed to an X-ray film, with a Dupont Lightning-plus intensifying screen, at -80°C for 24 hr.

*Enzymes and chemicals.* The restriction enzymes were purchased from Takara Shuzo Co. and Toyobo Co., and *E. coli* DNA polymerase I was from New England Biolabs. [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) was obtained from Amersham. The nitro-cellulose filters were from Schleicher and Schuell.

## RESULTS

### *Clinical course*

For case 1, treatment with hGH injections was initiated at the age of 5. The initial dose of hGH was 4 U/week, and later the dose was increased gradually to 6, 8, 12 and 16 U/week at the age of 10, 13 and 14, respectively. At the age of 13 when his height was 124.7 cm which corresponds to the height at the age of 8, he developed clinical signs of puberty and so cyproterone acetate (an anti-androgenic drug) was administered for a while in order to delay pubertal maturation. At the age of 14, his serum testosterone level was within normal adult limits and the development of his genitalia corresponded to stage III of Tanner's classification. Repeated examinations for anti-hGH antibodies gave negative results consistently. Generally speaking, he showed a rather good response to the treatment (Fig. 1A). Case 2 was the younger brother of case 1. He also showed a good response to 4 U/week hGH injections, and anti-hGH antibodies were never detected throughout the repeated examinations (Fig. 1A).

For case 3, we started giving injections of 4 U/week of hGH at the age of 5, and his height increment was 1 cm per 1 month during 3 months of the therapy (Fig. 1A). After that, his height increment became almost unmeasurable and subcutaneous fat tissue atrophy was observed on the buttocks at the site of injections. His serum anti-hGH antibodies titer was increased to 1 : 600,000. The patient and his parents refused the continuation of the treatment.

Case 4, a female patient, was treated with 6 U/week hGH injections at the age of 8. Her height increment was 4.3 cm during the first 6 months of the therapy, while during the next 6 months of the therapy her height increment was only 0.6 cm (Fig. 1B). Similar to in case 3, she developed subcutaneous fat tissue atrophy around the buttocks after the repeated injections. Her anti-hGH antibodies titer was positive, being 1 : 1,000, and remained highly positive even 3 years after discontinuation of the therapy. Soon after her treatment was discontinued when she

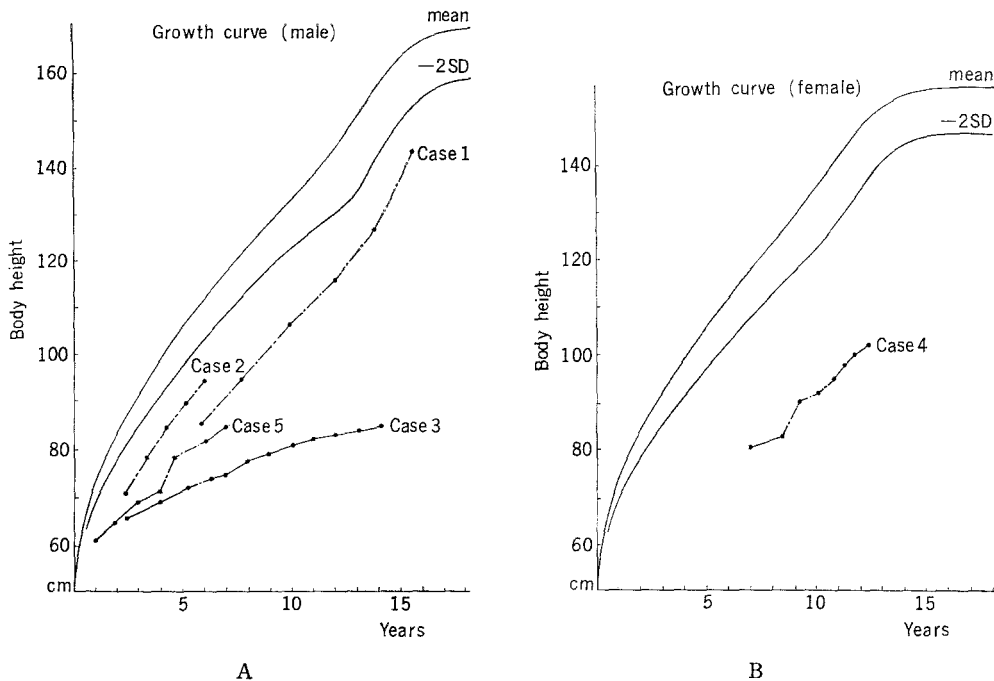


Fig. 1. A: Growth curve of case 1, 2, 3 and 5 (males). B: Growth curve of case 4 (female).

was 10 years and 8 months old, pubertal signs including stage IV breasts and the menarche were observed.

#### *Restriction endonuclease analysis*

The autoradiogram patterns of DNAs after *Bam*HI digestion of cultured lymphoid cells from each patient showed the absence of the 3.8 kb fragment that contains the hGH-N gene. A representative pattern for cases 1, 2, 3 and 4, and a reference case with a hGH gene deletion (Nishi *et al.*, 1984) is shown in Fig. 2. All tested parents appeared to have approximately half of the 3.8 kb DNA fragment, indicating a heterozygous state. Other DNA fragments of 8.5, 6.7, 5.3, and 3.0 plus 1.2 kb, which correspond to the human chorionic somatomammotropin-like (hCS-L), hChS-B, hCS-A and hGH-V genes (Seeburg, 1982), were all observed in each patient. The results of *Hinc*II digestion confirmed the absence of the hGH-N gene (Fig. 3). None of the affected patients showed the hybridizing 4.5 or 6.7 kb fragment, which is found in normal samples, depending on the presence or absence of a polymorphic restriction site immediately 5' of the first exon of the gene (Laron *et al.*, 1985).

Digestion with *Hind*III yielded three hybridizing fragments in normal samples; 26, 23 and 16 kb in length. The largest one, the 26 kb fragment, contains the hGH-N and hCS-L genes. The 23 and 16 kb fragments contain the hCS and hGH-V

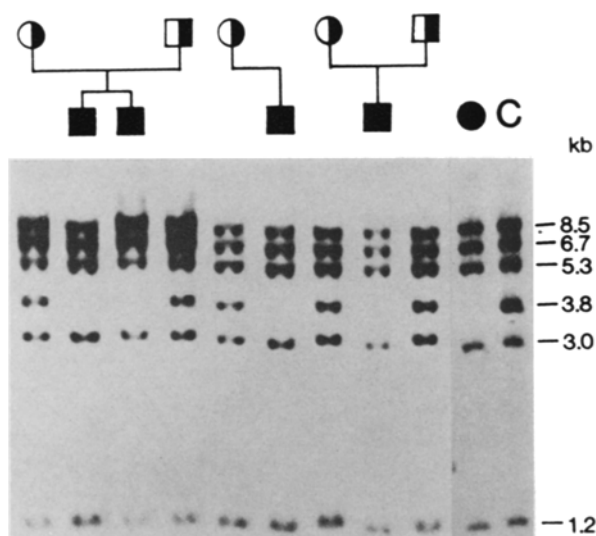


Fig. 2. Restriction endonuclease *Bam*HI "signatures" from members of four Japanese families with isolated growth hormone deficiency type 1A (case 1, 2, 3, 5 and 4; at far right is a normal control). All affected children lack the 3.8 kb fragment which contained hGH-N gene.

genes, respectively (Phillips III, 1983). All patients showed the absence of the 26 kb gene but the presence of a fragment of 18.5 kb in length (Fig. 3). This result indicated that the deletion in each patient was 7.5 kb in length and that it contained the hGH-N gene. The hCS-L gene appeared to be intact, when the observation on *Bam*HI digestion was taken into account.

Digestion with *Bgl*III and *Msp*I permitted assignment of restriction fragment length polymorphism (RFLP) haplotypes for individual growth hormone gene clusters. As shown in Fig. 3 and Table 2, cases 1, 2 and 4 had the same haplotype as that of the reference case (Nishi *et al.*, 1984), but case 3 had a different haplotype.

#### DISCUSSION

The hGH-N gene was analyzed in our four patients by the methods described previously (Phillips *et al.*, 1981; Rivarola *et al.*, 1984; Nishi *et al.*, 1984; Laron *et al.*, 1985). The deletion size and RFLP haplotype for the hGH gene clusters in cases 1, 2 and 4, and a reference case were similar to those of an oriental Jewish family (Family IV in Laron *et al.*, 1985), while the deletion and haplotype of case 3 was similar to those of Swiss, Argentinian (Phillips, 1983) and other oriental Jewish families (Families I, II and III in Laron *et al.*, 1985). In the present study, only a rough estimation of the deletion length of the gene was made. Further precise analysis is required to determine whether the gene defect with the observed similar

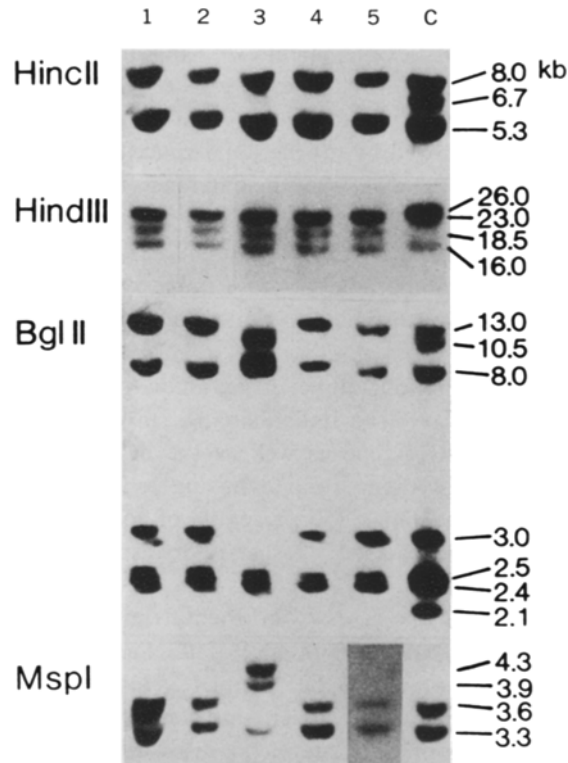


Fig. 3. Signatures of five children with isolated growth hormone deficiency type 1A after digestion with *HincII*, *HindIII*, *BglII*, and *MspI* (case 1, 2, 3, 4 and 5; at far right is a normal control). All affected children lack 6.7 kb fragment by *HincII* digestion. They showed presence of 18.5 kb fragment and absence of 26 kb fragment by *HindIII* digestion. Two different RFLP haplotypes are seen in the children by the *BglII* and *MspI* digestion.

Table 2. Restriction fragment length polymorphism haplotypes of the patients with a growth hormone gene deletion.

	<i>Bam</i> HI	<i>Hinc</i> II	<i>Hind</i> III	<i>Bgl</i> IIA	<i>Bgl</i> IIIB	<i>Msp</i> IA	<i>Msp</i> IB
Case 1	Del <sup>b</sup>	Del	18.5	13.0	3.0	3.6	3.3
Case 2	Del	Del	18.5	13.0	3.0	3.6	3.3
Case 3	Del	Del	18.5	10.5	8.0	4.3	3.9
Case 4	Del	Del	18.5	13.0	3.0	3.6	3.3
Case 5 <sup>a</sup>	Del	Del	18.5	13.0	3.0	3.6	3.3

<sup>a</sup> The data presented by Phillips III (1983) were confirmed in the present study. <sup>b</sup> Del, deletion.

deletions in cases from different families are identical. Illig *et al.* (1970, 1971) reported the clinical characteristics of patients with the disease: (1) short height, particularly in relation to weight at birth, (2) early growth retardation leading to extreme dwarfism in adulthood, (3) typical facies, and (4) a high antibody titer with an initial course of hGH, rapidly followed by (5) the appearance of hGH antibodies with the arrest of growth. Cases 3 and 4, and the reference case had all these features, while cases 1 and 2 from the same family showed a better response to hGH, compared to the other cases, together with the absence of anti-hGH antibodies. The former observations were comparable to those for a Swiss family (Illig, 1970; Illig *et al.*, 1971) and the latter were similar to those for oriental Jewish families (Laron *et al.*, 1985).

Despite antibody formation, three Argentinian siblings continued to grow slowly (Rivarola *et al.*, 1984). Two Italian siblings, in whom the gene deletion was 8.5 kb, containing the hGH-N gene as well as the hCS-L gene, showed different responses to hGH. One of them, despite having a higher anti-hGH antibodies titer, showed a fairly good response (50 percentile of control), while the other one, with a lower antibodies titer, showed a rather poor response (less than 3 percentile of control) (Braga *et al.*, 1985).

As far as our cases and the cases from oriental Jewish families are concerned, the proposed hypothesis, *i.e.*, that the presence of hGH antibodies might be the cause of the slow growth rate, seemed to be feasible. However, when the reports of Argentinian and Italian families are taken into account, it is likely that another factor may also affected the growth rate in the disease. For further discussion the nature of the anti-hGH antibodies should be clarified in each case.

Since the gene deletion was similar and the hGH preparation used was identical in each affected patient in our study, the heterogenous responses to hGH, as to antibody production, might be due to not only the gene deletion, but also to other factors including their immunological dispositions. The absence of anti-hGH antibodies or a good response to exogenous hGH cannot be used to rule out isolated growth hormone deficiency in cases with a hGH gene deletion on clinical grounds.

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