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Insulin-Like Growth Factor-I Gene Analysis in Subjects with Constitutionally Variant Stature

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ABSTRACT. The IGF-I gene from leukocyte DNA of a control population of normal stature was studied using Southern blotting. Restriction fragment lengths for 21 enzymes were determined and three restriction fragment length polymorphisms (RFLP) were found (*Eco*RV, *Hin*dIII, and *Pvu*II). In addition, the IGF-I gene of 64 constitutionally short subjects, five Pygmies, and 10 constitutionally tall subjects was analyzed. No IGF-I gene alterations were detectable by Southern blot in any of these conditions. Linkage analysis using genetic markers (RFLP) yielded results that were uninformative for five constitutionally short families investigated, owing to the limited number of RFLP and their low incidence (17% for the 5.2-kb *Hin*dIII, 5-kb *Pvu*II RFLP alleles, and 13% for the 13-kb *Eco*RV RFLP allele). The *Eco*RV RFLP was found to map near Exon 1. The incidence of the 13-kb polymorphic allele with *Eco*RV proved to be lower (4%) in the group with constitutionally short stature than in controls. These results could suggest that modifications in the region of the IGF-I gene may be involved in constitutionally short subjects. (*Pediatr Res* 27:488–491, 1990)

Abbreviations

GH, growth hormone
RFLP, restriction fragment length polymorphism

The well-established role of IGF-I in postnatal growth is reflected clinically by variations in serum levels that are related to GH status in particular (1). Pygmies, who have normal GH secretion, exhibit a primary IGF-I deficiency (2). In children and adolescents with constitutionally variant stature, short subjects on average have lower, and tall subjects have higher levels of IGF-I than normals (3–6). Although these statural deviations represent etiologically heterogenous groups, the frequently familial nature of the deviations suggests that genetic alterations may be involved. We therefore set out to analyze the IGF-I gene using different restriction endonucleases, first in a control population, then in subjects with constitutionally variant stature and in Pygmies.

MATERIALS AND METHODS

Control subjects for gene analysis were French adults ($n = 56$) of normal stature (normal range ± 1 SD) (7). Subjects with constitutionally variant stature were selected on the basis of criteria defined previously (3, 4). Sixty-four short subjects were studied (55 of whom were unrelated). Fifty-eight were children and adolescents, and six were fully grown. Ht was below −2 SD for the French population (7), the average being −2.7 SD. Birth size was 49 cm ± 1.2 (mean ± SD). The mean body mass index (wt/ht^2) was 15.8 ± 1.6, which is within the normal range. Five families were also investigated, comprising 24 subjects of whom 13 were constitutionally short. All short subjects had GH levels exceeding 14 ng/mL after ornithine infusion and the mean of the GH peaks (22.7 ± 7.3 ng/mL, mean ± SD) was no different from that of normals (8). Ten constitutionally tall subjects (nine children and adolescents and one adult) were studied (ht above +2 SD, the mean being +3.4 SD). In all cases, wt was within the normal range (mean ± 1 SD for ht). Thyroid hormone levels were normal in all subjects (free T4: 14.2 ± 2.9 pg/mL, TSH: 1.38 ± 0.5 μU/mL). Serum IGF levels were determined after separation by acidic gel filtration using methods reported earlier (9). IGF-I was measured by RIA and IGF-II by protein-binding assay using a cerebrospinal fluid binding protein with a selective affinity for IGF-II (10).

IGF-I levels were within the normal range (mean ± 2 SD) in the 58 constitutionally short children and adolescents, but two-thirds of them (37 of 58) had values below the normal age mean (Fig. 1). Although there was no statistically significant difference between this group and controls, an earlier study covering a much larger number of subjects did reveal significantly lower levels (4). The mean value for the six fully-grown subjects was 378 ± 79 ng/mL (mean ± SD), which is similar to that for normal adults (300 ± 56 ng/mL). The constitutionally tall adult in this study had a normal IGF-I level (326 ng/mL). All but two of the tall children and adolescents, however, had IGF-I levels above the normal age mean (Fig. 1). Here, too, an earlier study on larger numbers of subjects had shown significantly higher IGF-I levels in constitutionally tall subjects than in controls (3, 6). IGF-II levels were normal in all subjects with constitutionally variant stature.

Samples were obtained from five adult Pygmies (four men and one woman) of the Aka tribe (Lobaye, Central African Republic). The mean ht of the men in the tribe was 154.2 ± 6.3 cm (mean ± SD) and of the women, 144.2 ± 6.4 cm (11). These ht are below −3 SD of the normal French population.

Preparation of genomic DNA. The procedure used was that of Miller *et al.* (12) with minor modifications. Leukocyte DNA was isolated from 10–20 mL blood (or buffy coats of nucleated cells) collected in anticoagulant (EDTA). Blood samples were stored at −80°C until extraction of the DNA. Samples were rapidly thawed at 37°C, washed twice with 40 mL 0.02 M Tris-HCl, pH 7.5, 0.005 M $MgCl_2$. The lysed cells were resuspended in 3.5 mL 0.02 M Tris-HCl, pH 7.5, 0.4 M NaCl, 0.002 M EDTA and incubated for at least 4 h (or overnight) at 56°C with 0.2 mL 10% SDS and 80 μL proteinase K (10 mg/mL). Proteins were

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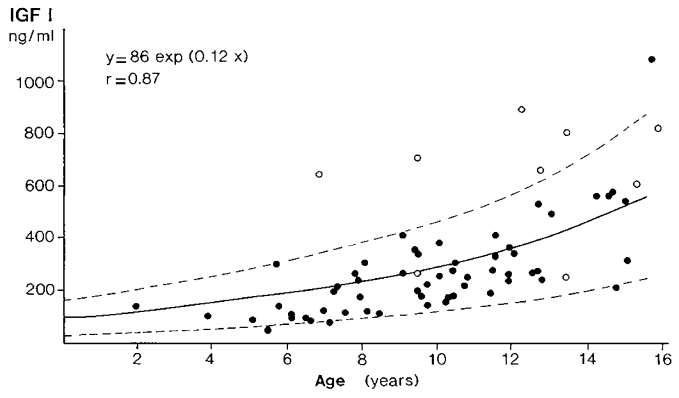

Fig. 1. Age-related variations in IGF-I levels during the growth period in constitutionally short (●) and tall (○) children and adolescents. The corresponding regression lines (mean ± 2 SD) for control children and adolescents have been reproduced for purposes of comparison.

precipitated by adding 1 mL saturated NaCl, shaken vigorously, and centrifuged. Two vol of absolute ethanol were added to the supernatant at room temperature and the precipitated DNA strands removed with a pipette before being transferred to a microcentrifuge tube and resuspended in 0.2 mL 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA.

DNA probes. The human liver IGF IA cDNA insert (1073 bp) from phage λ-TG03 (13) was subcloned in plasmid PGEM4 (pTG 3906). The 660-bp *Eco*RI-*Bam*HI fragment, which contains the 5′-untranslated region, the coding region, and part of the 3′-untranslated region corresponding to the IGF IA mRNA (Exons 1, 2, 3, and 5) was used, as was the 152-bp 5′-*Eco*RI-*Rsa*I fragment corresponding to Exon 1.

Southern blot analysis. Ten μg of purified leukocyte DNA were digested with 80 IU of restriction endonuclease as recommended by the manufacturers (Appligene, Illkirch, France, or New England Biolabs, Inc., Boston, MA) and the digested DNA submitted to electrophoresis in 0.7 to 1.2% agarose gels with 1 mg/L ethidium bromide. Thereafter the DNA was denatured and transferred to a nylon membrane (Gene Screen Plus, New England Nuclear, Boston, MA) according to the alkaline method (14). Transfer was carried out for 3 h using a solution of 0.4 M NaOH, 0.6 M NaCl as transfer solution. The membrane was then neutralized with 0.5 M Tris, pH 7.5, twice for 15 min and twice for 10 min with 2 × SSPE (1 × SSPE = 0.01 M phosphate buffer, pH 6.8, 0.001 M EDTA, 0.15 M NaCl) and baked for 45 min at 80°C.

Hybridization with IGF-specific probe was achieved as follows. First, the DNA blot was incubated for 2 h at 42°C with prehybridization buffer comprising 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 5 × Denhardt's Solution (1 × Denhardt's = 0.02 g/L Ficoll, 0.02 g/L polyvinyl-pyrrolidone, 0.02 g/L BSA), 0.05 M sodium phosphate buffer, pH 6.5, 1% SDS, and 100 mg/L sonicated, denatured salmon sperm DNA. Then, hybridization was continued for 20 h at 42°C using the same buffer without the salmon sperm DNA and with 5% dextran sulphate and approximately 10^7 cpm/mL of the probe (sp act: $1 - 2 \times 10^9$ cpm/μg DNA). The 660-bp probe was labeled with α-^{32}P dATP (Amersham, Little Chalford, England) by random priming (kit, Amersham) according to the manufacturer's instructions. The blot was washed twice for 15 min at room temperature in a solution containing 2 × SSC, 0.1% SDS and twice for 30 min at 65°C with 0.1 × SSC, 0.1% SDS and then exposed to x-ray film (Curix RPl, Agfa-Gevaert, Rueil-Malmaison, France) with two intensifying screens (Hi-Plus-Du Pont, Cronex, Du Pont, Wilmington, DE) for 2 to 4 d at −80°C. The 152-bp 5′-*Eco*RI-*Rsa*I fragment was also used as a probe, but labeled by nick-translation (kit, Amersham) (sp act: 4×10^8 cpm/μg DNA). Hybridizations with each of the two probes were done sequentially. After washing at 45°C with 0.4 M NaOH for 30 min and then with 0.1 × SSC, 0.1% SDS, 0.2 M Tris, pH 7.5, for 30 min (according to the membrane manufacturer's instructions) to remove the previously hybridized probe, the blot was rehybridized with the second labeled probe.

RESULTS

Restriction Fragment Length Analysis in Control Population. Twenty-one restriction enzymes were used to analyze human IGF-I DNA. The fragment lengths determined are shown in Table 1. Only three of these enzymes, (*Eco*RV, *Hin*dIII, and *Pvu*II) revealed RFLP (Table 1; Figs. 2 and 3*a*) (15). The polymorphic sites for *Hin*dIII and *Pvu*II are linked. The incidence of the *Hin*dIII and *Eco*RV RFLP in the control French population are shown in Table 2. When the 152-bp 5′ *Eco*RI-

Table 1. *Restriction fragment lengths of human IGF-I gene in control population*

Restriction endonuclease	Controls (*n*)	Fragment lengths (kb)				
*Apa*I	24	25.0	22.0	6.0		
*Ase*I	14	5.4	3.8	3.4	2.1	
*Ava*II	51	2.0	1.7	1.3	1.2	
*Bam*HI	20	18.0	9.2	7.0	1.0	
*Bcl*I	18	7.4	6.0	2.5	1.8	
*Bgl*I	19	22.0	19.0	12.0		
*Bgl*II	20	11.5	8.0	3.5	2.1	
*Eco*RI	16	7.7	7.0	4.5	1.4	
*Hae*III	15	2.0	0.6	0.3		
*Hin*fI	20	0.8	0.7	0.6	0.4	
*Hph*I	24	2.2	1.3	0.3		
*Kpn*I	21	9.6				
*Mbo*I	19	1.0	0.6			
*Msp*I	19	5.5	3.5	2.0	1.5	0.7
*Rsa*I	17	0.9	0.7	0.4		
*Sac*I	23	6.1	3.6	2.3		
*Taq*I	25	13.4	4.7	3.3	2.4	
*Xba*I	20	5.0	3.5	2.3		
*Hin*dIII	56	7.6	6.6	5.2/4.9*	3.0	
*Pvu*II	56	7.4	5.0/ 4.7*	2.7	1.4	
*Eco*RV	56	20.0	13.0/11.5*	8.7	5.0	

The lengths of the fragments were determined by Southern blot analysis with 21 enzymes, three of which yield polymorphism (*RFLP).

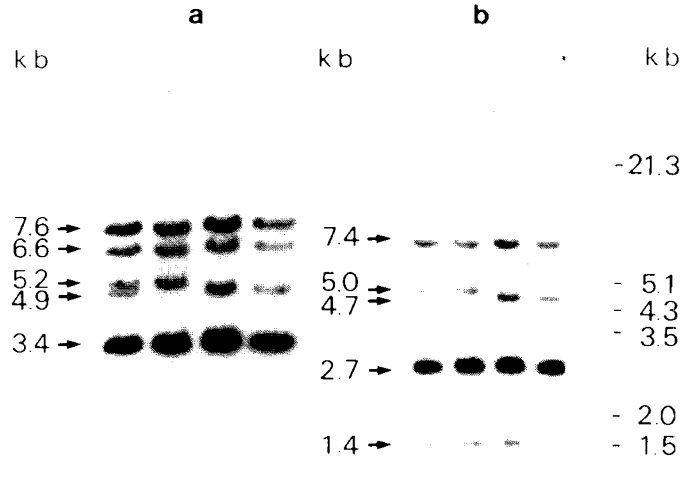

Fig. 2. Southern blot analysis of human DNA from normal subjects, using the 660-bp IGF-I cDNA probe. Genomic DNA digested with *Hin*dIII (*a*) and *Pvu*II (*b*) revealed polymorphism. The DNA fragment lengths determined using size markers (*Hin*dIII-*Eco*RI-digested phage λ-DNA) are shown in each panel. The size of the mol wt marker, *Hin*dIII-*Eco*RI-digested phage λ-DNA, is shown on the *right*.

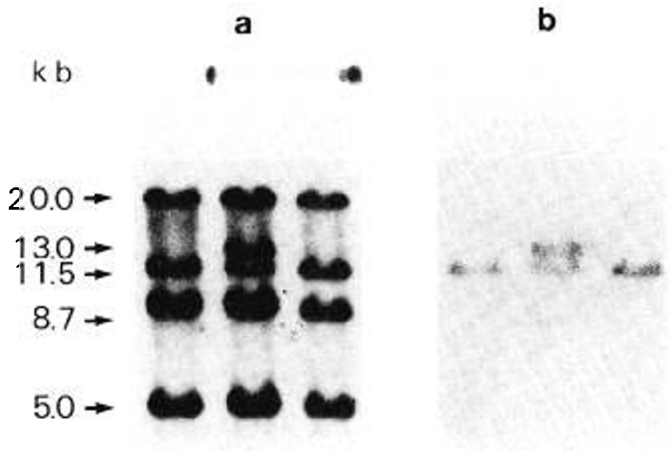

Fig. 3. Southern blot analysis showing the *Eco*RV restriction pattern of the IGF-I gene. (*a*), Hybridization with IGF-I cDNA probe (660 bp: Exons 1, 2, 3, and 5); (*b*), Same blot hybridized with the 5′ *Eco*RI-*Rsa*I fragment (152 bp: part of Exon 1) of the same IGF-I cDNA insert. The DNA fragment lengths determined using size markers (*Hin*dIII-*Eco*RI-digested phage λ-DNA) are shown as in Figure 2.

Table 2. *Allele frequencies of HindIII and EcoRV polymorphisms at human IGF-I locus in control subjects and in unrelated constitutionally short subjects (CSS)*

		Controls $n = 56$	CSS $n = 55$
*Hin*dIII*	5.2-kb allele	17%	17%
	4.9-kb allele	83%	83%
*Eco*RV	13-kb allele	13%	4%†
	11.5-kb allele	87%	96%

* *Hin*dIII and *Pvu*II RFLP were linked and yielded the same percentages.

† The difference in incidence of the 13-kb allele between constitutionally short subjects and controls was significant ($p < 0.02$) (χ^2 test).

*Rsa*I fragment of the IGF-I cDNA (which maps to Exon 1) was used as probe, only the 11.5- and 13-kb polymorphic fragments were observed with the *Eco*RV-digested DNA (Fig. 3*b*).

Human IGF DNA in Subjects with Growth Abnormalities. Constitutionally short subjects. The patterns obtained from Southern blotting using various enzymes, *Ava*II, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RV, *Hin*dIII, *Hin*fI, *Pvu*II, and *Taq*I, were identical to those in controls. Neither IGF-I gene deletions nor abnormalities in the genomes of these subjects were detectable by the method used. *Eco*RV and *Hin*dIII were used as genetic markers to investigate the incidence of RFLP in this group (55 unrelated individuals). The incidence of RFLP for *Hin*dIII was similar to that in the control population. That of the polymorphic 13-kb *Eco*RV was, however, significantly lower (as evaluated by χ^2 test, $p < 0.02$) (Table 2). Analysis of five families using *Hin*dIII and *Eco*RV was uninformative for the IGF-I gene.

Constitutionally tall subjects. The IGF-I gene of 10 such subjects was analyzed using the enzymes *Hae*III, *Bgl*I, *Eco*RV, *Hin*dIII, *Pvu*II, and *Taq*I, and no abnormalities were detected in the patterns of the blots.

Pygmies. Five Pygmy samples were analyzed using *Eco*RV, *Hin*dIII, *Pvu*II, and *Taq*I, yielding results identical to those of controls (Fig. 4).

DISCUSSION

Analysis of the IGF-I gene in a control population of normal ht enabled us to characterize the restriction profiles and determine fragment lengths for 21 restriction enzymes, as well as to detect RFLP. Of the enzymes tested, only three (*Eco*RV, *Hin*dIII,

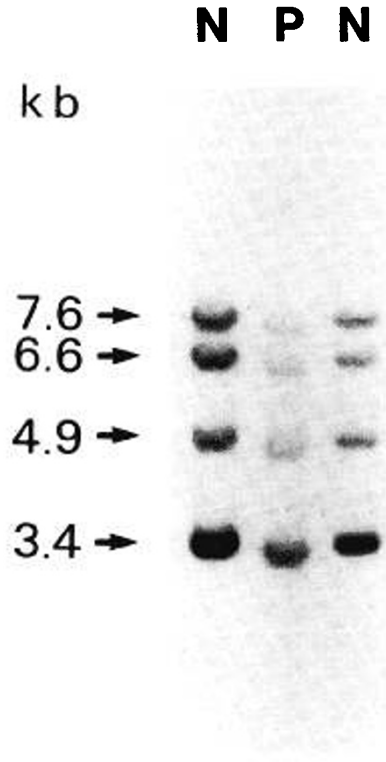

Fig. 4. Southern blot analysis of Pygmy (*P*) and control (*N*) DNA digested with *Hin*dIII hybridized with the 660-bp IGF-I cDNA probe. The DNA fragment lengths determined using size markers (*Hin*dIII-*Eco*RI-digested phage λ-DNA) are shown as in Figure 2.

and *Pvu*II) revealed polymorphism (15–18), indicating that there is high conservation of the IGF-I gene. *Hin*dIII and *Pvu*II RFLP were found to be linked. Variation of restriction fragment length between the two alleles was 300 bp for the two enzymes, which suggests that an insertion-deletion mechanism would account for the variation (19). The incidence of *Hin*dIII and *Pvu*II polymorphism in the French population studied was 17% for one allele and 83% for the other. Allele polymorphisms for *Eco*RV were 13 and 87%. The RFLP with *Eco*RV and *Hin*dIII were not linked. Site polymorphisms for *Hin*dIII and *Pvu*II mapped to the region around Exon 5 (18), whereas that for *Eco*RV was found to map around Exon 1.

With these results for the IGF-I gene in a normal population, it was possible to make comparisons with those for subjects with constitutionally variant stature. In short subjects where the etiology of the condition is probably heterogeneous, differences in IGF-I levels may well reflect differences in GH secretion not detected by pharmacological tests (20–22). However, Zadik *et al.* (22), studying 24-h integrated GH concentrations, found more than half of the values within the normal range. A possible hypothesis would therefore be that individual variations exist in the expression of the IGF-I gene which accompany normal levels of GH (6). In our studies, Southern blotting revealed no significant deletions or other anomalies in the IGF-I gene of any of these subjects, even in short children with markedly reduced IGF-I levels. The short stature of Pygmies has been attributed to a primary IGF-I deficiency (2), although a recent report suggests a possible defect at the GH receptor (23). In our study, the restriction profile obtained with leukocyte DNA indicated no detectable anomaly of the IGF-I gene.

With a view to determining whether finer modifications of the IGF-I gene structure may be responsible for the variations in the levels of this factor, we used the RFLP as genetic markers to trace transmission of the different alleles of the IGF-I gene within families. The limited number of RFLP, *i.e.* three, of which two were linked, and their low incidence (17 and 13%) gave insuffi-

cient information for linkage analysis of the five families tested. Nonetheless, the proportion of the 13-kb polymorphic allele obtained with *Eco*RV proved to be lower (4%) in the 55 unrelated constitutionally short subjects than in controls (13%). This means that constitutionally short individuals may comprise a group that is genetically different from normals. More refined techniques (such as genome amplification by the polymerase chain reaction) will need to be applied to establish whether or not anomalies of the IGF-I gene exist in these subjects.

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