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Isolated Growth Hormone Deficiency: Testing the *little* Mouse Hypothesis in Man and Exclusion of Mutations within the Extracellular Domain of the Growth Hormone-Releasing Hormone Receptor

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ABSTRACT

The phenotypic characteristics of isolated growth hormone deficiency (IGHD) type IB in humans, such as autosomal recessive inheritance, time of onset of growth retardation, diminished secretion of growth hormone (GH) and IGF-I, proportional reduction in weight and size, and delay in sexual maturation, has much in common with the phenotype of the homozygous *little/little* (*lit/lit*) mouse. Sequencing of the GH releasing hormone (GHRH) receptor in *lit/lit* mice has shown a single nucleotide substitution within the extracellular peptide binding domain at codon 60 that changed aspartic acid to glycine. Therefore, the GHRH receptor is a reasonable candidate gene for causing IGHD in humans. DNA from 65 unrelated healthy Caucasians of normal stature and 65 children with IGHD type IB of whom 12 did not respond to exogenous treatment with GHRH were studied. Restriction endonuclease analysis, linkage studies, and polymerase chain reaction amplification and sequencing of the whole extracellular domain including the first three membrane spanning domains of the GHRH receptor gene were performed. None of the analyses revealed any structural abnormalities in these patients with IGHD. This suggests that a *lit/lit* mouse equivalent is an unlikely explanation for the majority of children with IGHD. Although gross structural abnormalities in the whole gene have been ruled out in this study, mutations in the carboxyl terminus are still possible, and, therefore, the remaining part of the gene needs to be sequenced. ***(Pediatr Res* 38: 962–966, 1995)**

Abbreviations

IGHD, isolated GH deficiency
GH, growth hormone
r-hGH, recombinant hGH
GHRH, growth hormone releasing hormone
***lit/lit* mouse,** *little/little* mouse
PCR, polymerase chain reaction
RFLP, restriction fragment length polymorphism

In the United Kingdom, the prevalence of growth hormone deficiency is reported to be between 1 in 4 000 and 1 in 10 000 (1). Although most cases are sporadic and believed to result from exogenous cerebral insults or developmental abnormalities, 5–30% of GH-deficient patients have a GH-deficient relative; therefore, in these cases the disorder is suggested to be mainly familial (2). Four distinct familial types of IGHD have been defined on the basis of inheritance and other hormone deficiencies (3). This classification includes IGHD type IA, autosomal recessive with absent endogenous GH; type IB, autosomal recessive with diminished GH; type II, autosomal dominant with diminished GH; and type III, X-linked with diminished GH.

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Although most cases of IGHD type IA are due to homozygous deletions of the GH-1 gene encoding the pituitary-derived GH, kindreds with IGHD have been recently reported in whom two distinct point mutations caused a nonsense and splicing defect affecting the hGH-1 gene (4). The loci and mutations responsible for other Mendelian forms of IGHD still remain unknown (3, 5), but linkage studies have excluded the GH-1 locus in more than 50% of the autosomal IGHD (types IB and II) families in which the genetic defect causing the IGHD phenotype segregated independently of the GH gene (5, 6). The regulation of GH secretion is complex, and there are, therefore, multiple sites at which a genetic defect may perturb homeostasis, resulting in GH deficiency and short stature. Mutational changes in genes that are specifically related to GH would be expected to cause IGHD, and those that are shared by other pituitary cells or by other tissues would be expected to produce a more complex phenotype. Inasmuch as GHRH peptide does

not influence other cells, the receptor is probably restricted to somatotroph cells. An insufficiency/deficiency of GHRH has been suggested to explain many cases of IGHD, and its gene has been studied as a candidate gene without any abnormalities demonstrated (6, 7).

Since Mayo cloned the human pituitary-specific receptor for GHRH and a homozygous mutation within the GHRH binding domain of the receptor was described to be the molecular basis of the defect in the *lit/lit* mouse, an animal model of autosomal recessive isolated GH deficiency, the GHRH receptor gene has become a testable candidate gene for IGHD (8, 9) (Fig. 1). Thus, the aim of the present study was to screen the GHRH receptor gene for structural alterations in patients suffering from short stature classified as IGHD.

METHODS

Patients. DNA from 65 unrelated healthy Caucasians of normal stature (10) were studied as controls. In addition, 65 children with IGHD type IB attending the Growth Disorder Clinic either at the Middlesex Hospital in London or at the University Children's Hospital in Bern, Switzerland, were studied. The parents of the affected children studied were normal for height and sex (10). Children from Mediterranean, Asian, African, and Semitic descent were excluded from the study. The criteria for IGHD type IB included no demonstrable causes for IGHD including GH-gene cluster abnormalities, short stature ≤2.5 SDS, decreased growth rate, retarded bone age, normal karyotype, normal thyroid function, and peak GH levels below 4 μg/L after stimulation test (insulin-induced hypoglycemia). Details of the patients are given in Table 1. Twelve children were treated with exogenous GHRH for a 6-mo period, but did not respond at all. Either twice daily s.c. injections (25 μg/kg/d) of GHRH (1–29)-NH_2 or GHRH (1–40) s.c. by a pulsatile pump at 3-h intervals, over a 12-h period at night, at a dose of 1–2 μg/kg/pulse were administered. Data are presented in Table 2. Standard auxologic assessment was performed (11). No child had evidence of organic disease, psychosocial deprivation, or any eating disorder, and all had normal renal and hepatic function. All children showed a sustained response to r-hGH treatment over 1–10 y (median 4.8 y).

Figure 1. Schematic structure of the GHRH receptor showing the location of the aspartic acid (*Asp*) 60 to glycine (*Gly*) mutation causing the *lit/lit* mouse phenotype. The GHRH receptor is a 423-amino acid protein that contains the seven potential membrane-spanning domains characteristic of G protein-coupled receptors. The approximate sites of intron/exon boundaries are indicated as well as the PCR-amplified gene fragment (*).

Preparation of the DNA. Genomic DNA was isolated from peripheral leukocytes and from EBV-transformed lymphoblasts as previously described (6, 12). The concentration of each sample was determined by measuring the OD of the purified DNA at 260 and 280 nm.

Restriction endonuclease digestion and Southern blotting. Samples of DNA (5 μg) were digested to completion with *Bam*HI and the restriction enzymes shown in Table 3. After electrophoresis in 0.5–1.2% (wt/vol) agarose gel, the DNA fragment were transferred onto a nylon membrane (Hybond-N, Amersham, Rahn Zurich, Switzerland) and hybridized to the GHRH receptor cDNA probe. HPR3Z is a 1.6-kb human GHRH receptor cDNA clone described by Mayo (9) inserted into pGEM7Z as previously described (9, 13). The filters were then washed at 65°C and autoradiographed at −70°C using intensifying screens (6, 12, 13).

PCR amplification of the extracellular binding domain of the GHRH receptor. Gene fragments coding for the extracellular binding domain of the GHRH receptor were PCR-amplified in patients and controls (14). One microgram of genomic DNA was added to 100 μL of reaction mixture of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM of each deoxy-NTP, 0.1 mM of each primer, and 4 U of *Taq* DNA polymerase (Boehringer Mannheim, Rotkreuz, Switzerland). The up- and down-stream primers corresponded to nucleotides 188–206 (sense: 5′-GGAGATGCCCAACACCAC-3′) and 773–790 (antisense: 5′-GAACCAGCCACCAGAAGG-3′) covering approximately 600 bp (9). The PCR reaction mixture (without *Taq* DNA polymerase) was denatured for 6 min at 94°C and 4 U of *Taq* DNA polymerase were added at 80°C. Then the solution was cycled 35 times (94°C, 1.0 min; 63°C, 1.0 min; 72°C, 1.5 min), followed by a 10-min extension at 72°C.

Restriction endonuclease detection of mutations. Aliquots of PCR amplification products were digested with the enzymes shown in Table 4. The enzymes *Fok*I was chosen because the mutation in the *lit/lit* mouse on codon 60 is from GAT (Asp) to GGT (Gly) (8, 9, 15) which destroys the *Fok*I restriction site (GGATGN).

DNA sequencing. The PCR products were cleaned by filtration with Qiaquick PCR purification kit (Qiagen, Hilden, Germany), then digested with different enzymes and correspondingly cloned into a PCR 1000 vector (Invitrogen Corp. BV, NV Leek, The Netherlands). The cloned GHRH receptor PCR products were purified from an overnight culture by the alkaline lysis method (16), followed by digestion with 4 U of RNase (Sigma Chemical Co. Chemie, Buchs SG, Switzerland) and precipitation by addition of an equal volume of a 1.6 M NaCl 13% polyethylene glycol solution. The plasmid DNA was then sequenced by the dideoxy method (17) using dsDNA cycle sequencing system (Boehringer Mannheim; MWG-Bio Tech AG, Münchenstein, Switzerland).

Table 1. *Anthropometric details of the 65 prepubertal children with idiopathic growth hormone deficiency*

Sex (M/F)	Age (y)	Bone age (y)	hGH peak on ITT (μg/L)	Height SDS for chronologic age	Height velocity SDS
42/23	2.6–9.4 (5.9)	1.2–6.9 (4.0)	<0.3–1.5 (0.8)	−5.8 to −2.5 (3.1)	−4.9 to −1.0 (−2.5)

The median is given in parentheses. ITT, Insulin tolerance test; SDS, standard deviation score.

Table 2. *Clinical details of 12 prepubertal children unsuccessfully treated with exogenous GHRH*

Sex (M/F)	Age (y)	Bone age (y)	Height SDS for chronologic age	Pretreatment height vel. (cm)	GHRH height vel. (cm)	Pretreatment height vel. SDS	GHRH height vel. SDS
6/6	4.6–9.6 (6.2)	3.2–6.9 (5.9)	−4.6 to −2.5 (3.5)	2.5–4.9 (3.9)	3.3–5.2 (4.1)	−3.6 to −1.3 (−2.7)	−3.5 to −1.9 (−2.9)

The median is given in parentheses. Vel., velocity; SDS, standard deviation score.

Table 3. *Analysis of search for RFLPs recognized by probe for GHRH receptor*

Enzyme	Base pairs (no.)	Bands (no.)	Base pairs screened	Chromosomes screened	Total base pairs screened*
*Bcl*I	6	3	24	260	6240
*Bgl*I	6	5	36	260	9360
*Bgl*II	6	5	36	260	9360
*Eco*RI	6	3	24	260	6240
*Fok*I	5	3	20	260	5200
*Hinc*II	2 × 6	3	36	260	9360
*Hind*III	6	1	12	260	3120
*Msp*I	4	6	28	260	7280
*Pst*I	6	3	24	260	6240
*Pvu*II	6	4	30	260	7800
Totals			270	2600	91.26×10^6

* Total base pairs screened = (no. of base pairs in restriction site) × (no. of bands +1) × (no. of chromosomes screened).

Table 4. *Restriction endonuclease detection of mutations in children with IGHD*

Enzyme	Base pairs (no.)	Bands (no.)	Base pairs screened	Chromosomes screened	Total base pairs screened*
*Alu*I	4	6	20	130	2 600
*Fok*I	5	4	15	130	1 950
*Hae*III	4	8	28	130	3 640
*Pvu*II	6	5	24	130	3 120
Totals			87	130	11 310

* Total base pairs screened = (no. of base pairs in restriction site) × (no. of bands −1) × (no. of chromosomes screened).

RESULTS

Restriction endonuclease digestion and Southern blotting. The GHRH receptor cDNA probe was used to examine sets of chromosomes of healthy subjects and children with IGHD to check for gross gene abnormalities. No mutation was found (Table 3) having screened a total of 91.26×10^6 bp.

Linkage analysis. Linkage analysis was performed using the *Bam*HI RFLP for the GHRH receptor locus we reported previously in controls (13). The distribution of the alleles were identical in all three groups, controls, children with IGHD, and children with IGHD who did not respond to GHRH treatment (Table 5).

Table 5. *Frequencies of RFLPs identified in children with IGHD and controls*

	RFLP alleles (kb)	Controls	IGHD	GHRH nonresponding IGHD
*Bam*HI	2.6/2.4	0.71/0.29 (130)	0.7/0.3 (106)	0.72/0.28 (24)

Numbers of chromosomes analyzed are in parentheses.

PCR amplification of the extracellular binding domain of the GHRH receptor. Using the primers described, genomic PCR fragments were obtained and screened for gross alterations by restriction enzyme analysis (Table 4). However, no alteration could be found and the *Fok*I digestion pattern in controls and patients was identical suggesting that in our children with IGHD who did not respond to the GHRH treatment not the same mutation as in the *lit/lit* mouse found is present.

DNA sequencing. DNA from the children who did not respond to GHRH treatment spanning from exon 2 to 7 was sequenced. Neither a mutation nor a deletion was found when compared with sequences obtained from the control population (8, 9).

DISCUSSION

GHRH is a peptide hormone (42–44 amino acids in various species) that is synthesized in the hypothalamus and stimulates the synthesis and secretion of GH from pituitary somatotroph after its binding to the GHRH receptor (18–20). The recent

identification and cloning of a receptor for GHRH has allowed to study its role in the etiology of growth disorders to be examined (8, 9, 21). The GHRH receptor is a 423-amino acid protein that contains the seven membrane-spanning domains characteristic of G protein-coupled receptors (Fig. 1) (8).

The phenotype of IGHD type IB in humans has much in common with the phenotype of homozygous *lit/lit* mice including autosomal recessive inheritance, time of onset of growth retardation, diminished secretion of GH and IGF-I, proportional reduction in weight and skeletal size, and delay in sexual maturation (22–25). Sequencing of the GHRH receptor gene in *lit/lit* mice has shown a single nucleotide substitution in codon 60 that changed aspartic acid to glycine (8). This substitution in the extracellular binding domain prevents GHRH binding to the receptor (8). Therefore, GHRH receptor would appear to be a reasonable candidate gene for mutation in patients with IGHD. The aim of the study was to screen the extracellular domain for mutations which may, first, cause short stature in patients with IGHD and, second, explain the nonresponsiveness to GHRH treatment in a more specific group of IGHD patients.

As with any new candidate gene, Southern blot analysis was performed to screen for gross deletions and for informative RFLPs followed by linkage analysis. As shown in Table 3 and 5, neither restriction enzyme analysis nor the linkage analysis were successful in defining any GHRH receptor abnormality. Thereafter, PCR amplification of the extracellular domain of the GHRH receptor was performed. Following the known pattern of the mouse GHRH receptor we amplified from amino acid 47 to 240 including exon 3 which contains the *lit/lit* mutation, exon 4 to 6 and part of exon 2 and 7. These exons encode the extracellular domain and the first three membrane spanning loops (Fig. 1) (8). Restriction enzyme pattern and sequence analysis were identical in controls and patients with IGHD and, therefore, no structural abnormalities as described in the *lit/lit* mice were found in the extracellular binding domain which would explain first the phenotype of IGHD IB and second the nonresponse to GHRH treatment in some of these patients.

However, we did not sequence exons 8–13 which encode the remaining 4 membrane spanning domains and the intracellular domains. In humans, the exon and intron boundaries in that region are still unknown, and further sequencing of the genomic GHRH receptor in all these patients will be important as several recent examples underscore the potential involvement of G protein-coupled receptor in inherited diseases or phenotypic variations. In congenital nephrogenic diabetes insipidus for instance, several different mutations within the vasopressin V2 receptor have been described (26–28). In addition, recently thyroid dysfunctions have been reported by Sunthornthepvarakul *et al.* (29) caused by mutations within the extracellular domain of the TSH receptor gene resulting in TSH resistance.

Patients with a phenotype of IGHD type IB who do not respond to GHRH treatment but show a growth promoting effect on r-hGH therapy may have a defect in the GHRH receptor signaling pathway. Such a defect could theoretically occur anywhere in the signaling cascade, from the receptor to the molecules activating adenylate cyclase including G_s subtypes of G proteins. Additionally, in several cell types elevated cAMP leads to the phosporylation and activation of transcription factor CREB by protein kinase A (30, 31). One target for CREB action is the 5′ up-stream region of the hGH-1 gene which regulates expression of the GH gene in somatotroph cells, thus providing a pathway by which GH expression is regulated.

To summarize, we have studied the extracellular domain of the GHRH receptor (introns, exons 2–7) in 65 healthy controls and in 65 patients phenotypically classified as having IGHD type IB. Any GH gene cluster abnormalities were excluded. In addition, DNA from 12 patients growing well on r-hGH replacement therapy but who did not respond to GHRH treatment were studied. Neither restriction enzyme analysis, linkage analysis, nor sequencing data revealed any structural abnormalities in these patients excluding the possibility of a *lit/lit* mouse equivalent in humans. Although gross structural abnormalities in the whole gene have been ruled out in our study, mutations in the carboxyl terminus are still possible and, therefore, the whole gene including introns will need to be sequenced.

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REFERENCES

1. Vimpani GV, Vimpani AF, Lidgard GP, Cameron EHD, Farquhar JW 1977 Prevalence of severe growth hormone deficiency. BMJ 2:427–430
2. Rona RJ, Tanner JM 1977 Aetiology of idiopathic growth hormone deficiency in England and Wales. Arch Dis Child 52:197–208
3. Phillips III JA 1989 Inherited defects in growth hormone synthesis and action. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The Metabolic Basis of Inherited Disease, 6th Ed. McGraw-Hill, New York, pp 1965–1983
4. Cogan JD, Phillips III JA, Sakati N, Frisch H, Schober E, Milner RDG 1993 Heterogeneous growth hormone (GH) gene mutations in familial GH deficiency. J Clin Endocrinol Metab 76:1224–1228
5. Phillips III JA, Cogan JD, Raskin S, Parker RA 1992 Pedigree analysis of familial isolated growth hormone deficiency. Am J Hum Genet 51(Suppl):A1469
6. Mullis PE, Patel M, Brickell PM, Brook CGD 1990 Isolated growth hormone deficiency: analysis of the growth hormone (GH) releasing hormone gene and the GH gene cluster. J Clin Endocrinol Metab 70:187–190
7. Jurado LA, Phillips III JA, Francke U 1994 Exlusion of the growth hormone (GH)-releasing hormone gene mutations in familial isolated GH deficiency by linkage and single strand conformation analysis. J Clin Endocrinol Metab 78:622–628
8. Lin SC, Lin CR, Gukovsky I, Lusis AJ, Sawchaenko PE, Rosenfeld MG 1993 Molecular basis of the *little* mouse phenotype and implications for cell type-specific growth. Nature 364:208–213
9. Mayo KE 1992 Molecular cloning and expression of a pituitary-specific receptor for the growth hormone-releasing hormone. Mol Endocrinol 6:1733–1744
10. Tanner JM, Whitehouse RH, Takaishi M 1966 Standards from birth to maturity for height, weight, height velocity and weight velocity: British children, part II. Arch Dis Child 41:613–635
11. Brook CGD 1982 Growth Assessment in Childhood and Adolescence. Blackwell, Oxford
12. Mullis PE, Patel MS, Brickell PM, Brook CGD 1991 Constitutionally short stature: analysis of the insulin-like growth factor-I gene and the human growth hormone cluster. Pediatr Res 29:412–415
13. Cao Y, Wagner JK, Eblé A, Hindmarsh P, Mullis PE 1994 *Bam*HI RFLP for the GHRHR locus. Hum Mol Genet 3:682
14. Saiki RK, Gelfand DH, Stoffel S, Scharf SS, Higuchi R, Horn GT, Mullis KB, Erlich HA 1988 Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science 239:487–491
15. Godfrey P, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE 1993 GHRH receptor of *little* mice contains a missense mutation in the extracellular domain that disrupts receptor function. Nature Genet 4:227–231
16. Sambrook J, Fritsch EF, Maniatis T 1989 Small-scale preparations of plasmid DNA. In: Ford N, Nolan C, Ferguson M, Ockler M (eds) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 1.25–1.28
17. Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain-terminating

inhibitors. Proc Natl Acad Sci USA 74:5463–5467
18. Rivier J, Spiess J, Thorner M, Vale W 1982 Characterization of growth hormone-releasing factor from a human pancreatic islet tumor. Nature 300:276–278
19. Frohman L, Jansson J-O 1986 Growth hormone releasing hormone. Endocr Rev 7:223–253
20. Guillemin R, Brazeau P, Bohlen P, Esch F, Ling N, Wehrenberg WB 1982 Growth hormone releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585–587
21. Gaylinn BD, Harrison JK, Zysk, JR, Lyons CE, Lynch KR, Thorner MO 1993 Molecular cloning and expression of human anterior pituitary receptor for growth hormone-releasing hormone. Mol Endocrinol 7:77–84
22. Cheng TC, Beamer WG, Phillips III JA, Bartke A, Mallonee RL, Dowling C 1983 Etiology of growth hormone deficiency in Little, Ames, and Snell dwarf mice. Endocrinology 113:1669–1674
23. Eicher EM, Beamer WG 1976 Inherited ateliotic dwarfism in mice: characterization of the mutation, *little*, on chromosome 6. J Hered 67:87–91
24. Beamer WG, Eicher EM 1976 Stimulation of growth in *little* mouse. J Endocrinol 71:37–45
25. Underwood LE, Van Wyk JJ 1992 Normal and aberrant growth. In: Wilson JD, Foster DW (eds) William's Textbook of Endocrinology, 8th Ed., WB Saunders, Philadelphia, pp 1079–1138
26. Holtzman EJ, Harris HW, Kolakowski LF, Guay-Woodford LM, Botelho B, Ausiello DA 1993 Brief report: a molecular defect in the vasopressin V2-receptor gene causing nephrogenic diabetes insipidus. N Engl J Med 328:1534–1537
27. Yuasa H, Ito M, Oiso Y, Kurakawa M, Watanabe T, Oda Y, Ishizuka T, Tani N, Ito S, Shibata A, Saito H 1994 Novel mutations in the V2 vasopressin receptor gene in two pedigrees with congenital nephrogenic diabetes insipidus. J Clin Enodcrinol Metab 79:361–365
28. Merendino JJ, Spiegel AM, Crawford, O'Carroll AM, Brownstein MJ, Lolait SL 1993 Brief report: a mutation in the vasopressin V2-receptor gene in a kindred with X-linked nephrogenic diabetes insipidus. N Engl J Med 328:1538–1341
29. Sunthornthepvarakul T, Gottschalk ME, Hayashi Y, Refetoff S 1995 Brief report: Resistance to thyrotropin caused by mutations in the thyrotropin-receptor gene. N Engl J Med 332:155–160
30. Gonzalez GA, Montminy MR 1987 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–689
31. Sheng M, Thompson MA, Greenberg ME 1991 CREB: a Ca^2-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427–1430