Genetic disorders in the growth hormone-IGF-I axis

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Citation

Walenkamp, M. J. E. (2007, November 8). *Genetic disorders in the growth hormone-IGF-I axis*. Retrieved from https://hdl.handle.net/1887/12422

A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the IGF-I receptor

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Abstract

Context: The type 1 IGF-I receptor (IGF1R) mediates the biological functions of IGF-I. Binding of IGF-I to the IGF1R results in autophosphorylation of the intracellular β-subunit and activation of intracellular signaling.

Objective: The objective of this study was to evaluate the functional characteristics of a novel IGF1R mutation and describe the phenotypic features of two patients with this mutation.

Design: The study was performed in a university hospital.

Patients: We describe a 35-yr-old female with mild intrauterine growth failure, progressive postnatal growth retardation, severe failure to thrive, and microcephaly. Her daughter was born with severe intrauterine growth retardation and also showed postnatal failure to thrive and microcephaly.

Results: We found a heterozygous G3148→A nucleotide substitution in the IGF1R-gene, changing a negatively charged glutamic acid at position 1050 into a positively charged lysine residue (E1050K). E1050 is a conserved residue in the intracellular kinase domain. Dermal fibroblasts of the mother showed normal binding of iodinated IGF-I, but autophosphorylation and activation of downstream signaling cascades upon challenging with IGF-I was markedly reduced. Consequently, the maximal [^{3}H]thymidine incorporation upon a challenge with a dose range of IGF-I was reduced compared with a panel of control cells (3.65 ± 1.79 *vs.* 6.75 ± 4.7-fold stimulation ($P < 0.01$)). These data suggest that the mutation result in the inactivation of one copy of the IGF1R gene.

Conclusions: These two patients support the key role for IGF-I in intrauterine and postnatal growth. The different phenotypes of these and earlier described patients may be associated with variability in IGF-I signaling. The degree of intrauterine growth retardation may be partially determined by the presence or absence of maternal IGF-I resistance.

Introduction

IGF-I plays a key role in intrauterine development and postnatal growth and metabolism. IGF-I deficiency due to a homozygous deletion or mutation of the IGF-I gene results in severe intrauterine and postnatal growth failure, mental retardation and deafness (1, 2). The biological functions of IGF-I are primarily mediated through the type 1 IGF receptor (IGF1R). The IGF1R gene is located on the distal long arm of chromosome 15 (15q26.3) and has a similar organization compared with the insulin receptor (IR) gene, with sequence homology varying from 41-84% depending on the domain (3). Both are heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoproteins, synthesized as a single chain prereceptor and consisting of an α-subunit that is mainly involved in ligand binding and a β-subunit containing the tyrosine kinase domain. Ligand binding to the tyrosine kinase receptor results in receptor autophosphorylation on intracellular tyrosine residues and activation of the receptor's intrinsic tyrosine kinase, initiating distinct intracellular signaling pathways (4).

Although the IR gene and the IGF1R gene are homologous, genetic disturbances lead to a different phenotypical spectrum. Mutations of the IR gene in humans present with a heterogeneous phenotype, ranging from mild insulin resistance to leprechaunism (5). The clinical features of patients with IGF1R mutations are less well defined. So far, no cases have been found with a homozygous IGF1R mutation, and observations in mice suggest that such defect is lethal: IGF1R knock out mice die within min after birth due to respiratory failure (6). Heterozygous IGF1R mutations presenting with intrauterine and postnatal growth retardation have been observed in three families (7, 8). Heterozygous mutant mice are phenotypically normal (6), but targeted partial invalidation of the IGF1R-gene in mice causes postnatal growth deficiency (9).

Abuzzahab *et al.*(7) described a girl and a boy with mutations in the IGF1R. The girl was compound heterozygous for two missense mutations in exon 2, resulting in reduced ligand binding and decreased receptor phosphorylation on IGF-I stimulation. The boy had a nonsense mutation in exon 2, resulting in reduced expression of IGF1R. Both children had severe intra-uterine growth retardation and postnatal growth failure. Recently, a heterozygous mutation in the cleavage site of the proreceptor of IGF1R was reported in a 6 year old Japanese girl and her mother, present-

ing with mild intrauterine growth retardation and postnatal short stature (8). We now describe a mother and her daughter with the first missense mutation in the intracellular kinase domain of the IGF1R.

Methods

Patient A provided written informed consent for herself and her daughter (Patient B) for all investigations. The Medical Ethics Committee of the Leiden University Medical Center approved the protocol.

Clinical measurements and auxology
Height and sitting height were determined with a Harpenden stadiometer, and head circumference was assessed with a tape measure. Height and head circumference were expressed as standard deviation score (SDS) based on Dutch references (10). Sitting height and sitting height/height ratio were also expressed as SDS for the Dutch population (11).

Radiological and sonographic measurements
BMD (g/cm^2) of the lumbar spine and total body was measured by dual-energy x-ray absorptiometry (DXA) (Lunar, DPXL/PED, Lunar Radiation Corporation, Madison, WI). Ancillary DXA-derived data were used to calculate lumbar spine volumetric BMD [bone mineral apparent density (BMAD)] with the model BMAD = BMD x [4/(x width)], as validated before (12). BMD and BMAD results were compared with age- and sex-matched reference values and expressed as SDS.
Cardiac ultrasound in patient A was performed (GE System 7 – Vingmed, Milwaukee, WI, USA) and routine images (for assessment of left ventricular systolic function) and color Doppler data (to detect valvular abnormalities) were obtained.

Biochemical measurements
Plasma GH in both patients was measured with time resolved IFMA (Wallac/PE, Turku, Finland), using the WHO 80/505 as a standard (1 mg = 2.6 IU). Spontaneous GH secretion was assessed in case A after an overnight fast by sampling every 20 min from 0900-1200 (3-hr GH profile). An arginine stimulation test (0.5 g/kg iv over 30 min, from 0-30 min) and a combined GH-Releasing-Hormone (GHRH) (1

µg/kg iv at 0 min) and arginine test was performed in case A. An arginine stimulation test (0.5 g/kg iv over 30 min) was performed in case B.

Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1 and IGFBP-3, were determined by specific RIAs (13, 14) With the exception of IGFBP-1 smoothed references based on the LMS method were available for all parameters allowing conversion of patients data to SDS values (15). Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of six healthy adult controls.

A 75-g oral glucose tolerance test performed after a 10-h overnight fast was used to classify the patient as having normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes on the basis of the American Diabetes Association 1997 criteria (16). Glucose was assayed by an automated glucose oxidase method. Plasma insulin and C-peptide levels were measured by specific RIAs. The insulin secretion ratio or insulinogenic index was calculated as (30- to 0-min plasma insulin)/30-min glucose, which correlates well with direct measures of stimulated insulin secretion (17, 18). The homeostatic model assessment for insulin resistance index (HOMA-IR = fasting glucose (mmol/l) × Fasting insulin (mUI/ml)/22.5) was used for estimating insulin action. High HOMA-IR index denotes low insulin sensitivity and thus insulin resistance (19).

Molecular and functional studies
A skin biopsy from patient A was taken, and a culture of dermal fibroblasts was established. Total RNA was isolated and reverse transcribed into cDNA. The coding regions of the IGF1R were amplified by PCR using overlapping primer combinations (Table 1) and subjected to direct sequencing as described previously (2). Genomic DNA was isolated from whole blood of the patients and of 87 unrelated individuals with proportionate growth retardation, of whom 19 also had intrauterine growth retardation. All coding exons of the IGF1R were PCR amplified and subjected to direct sequencing. Primer combinations are indicated in Table 2. Fibroblast cultures of the patient and of eight healthy donors were used for a thymidine incorporation assay in response to IGF-I as described previously (20). Each of these cultures was used in at least two independent thymidine incorporation assays performed in quadruplicate. The interexperimental variance for each dose of IGF-I varied between 10 and 25%. For Western blotting, cells were stimulated for 10 min with a dose

Table 1. Overlapping primersets used for PCR amplification and sequencing of the IGF1R cDNA.

Primer	Sequence (5'-3')	Nucleotide[a]
hIGF1R 1 F	5'- TTTGAGAAAGGGAATTTCATCC - 3'	12 - 33
hIGF1R 2 R	5'- AGACACCGGCATAGTAGTAGTGG - 3'	823 - 845
hIGF1R 3 F	5'- TTGCCGCCACTACTACTATGC - 3'	795 - 815
hIGF1R 3 R	5'- GTTATGATGATGCGATTCTTCG - 3'	1568 - 1589
hIGF1R 4 F	5'- GACATAAACACCAGGAACAACG - 3'	1471 - 1492
hIGF1R 4 R	5'- CTTCTCGGCTTCAGTTTTGG - 3'	2172 - 2191
hIGF1R 5 F	5'- ATTGAGGAGGTCACAGAGAACC - 3'	2083 - 2104
hIGF1R 5 R	5'- GGAAGACGTACAGCATAATCACC - 3'	2920 - 2942
hIGF1R 6 F	5'- CACATCTCTCTCTGGGAATGG - 3'	2766 - 2786
hIGF1R 6 R	5'- CCGAGTAAGTGGTGAAGACTCC - 3'	3625 - 3646
hIGF1R 7 F	5'- TGGAGTCTTCACCACTTACTCG - 3'	3603 - 3624
hIGF1R 7 R	5'- TAAAGGCCCATGTCAGTTAAGG - 3'	4390 - 4411

F, Forward; R, Reverse
[a] The numbers correspond to nucleotide position in the IGF1R mRNA with accession number
 NM_000875

range of IGF-I, 1 x 10^{-6} M insulin or 10 ng/ml epidermal growth factor (EGF). Blots were probed with an anti-phospho-PKB/Akt, total PKB/Akt, anti-phospho-ERK-1 and -2, anti-phospho-IGF1R (Biosource International, Camarillo, CA), and a total IGF1R β (Cell Signaling technology, Beverly, MA) antibody as described previously (21). Binding studies were performed using iodinated IGF-I in the presence of an IGF-I analogue that is bound by IGFBPs but not by the IGF1R (Ala ^{31}Leu^{60}-IGF-I, GroPep) (22). In short, fibroblasts of the patient and controls were incubated at 4°C with 30,000 cpm [^{125}I]IGF-I, 250 ng/mL Ala^{31}Leu^{60}-IGF-I and graded amounts of unlabeled native IGF-I in 250 µL of HEPES binding buffer (100 mM HEPES (pH 7.8), 0.5% fatty-acid-free BSA, 120 mM sodium chloride, 1.2 mM magnesium sulfate, 5 mM potassium chloride, 15 mM sodium acetate, and 10 mM dextrose) as previously described (7, 22). After 18 h, cells were washed and solubilized in 1 M NaOH. Radioactivity was determined using a γ-counter.

Table 2. Primer sets for the amplification and sequencing of the coding exons of the IGF1R.

exon	name	primer sequence	exon	name	primer sequence
1	ex01-F	aaggggaatttcatcccaaa	11	ex11-F	aagtcatagaaaagacaaaagagg
	ex01-R	aggaaaagttcccgcagtg		ex11-R	ccactaggttgtgaggaagg
2	ex02a-F	tccttctaactgagacgtttacc	12	ex12-F	gaacccaaatccaactttgt
	ex02a-R	gcatttttctcaatcctgatg		ex12-R	catatgctgtcaatggatgg
2	ex02b-F	ttctacaactacgccctggt	13	ex13-F	ctgcattcatgggaaattg
	ex02b-R	cagaagagaagggaggtcaa		ex13-R	accctgctttcagtttttacc
3	ex03-F	tcatctccgtctctcctctc	14	ex14-F	tgtgaagaaatgaaatgagca
	ex03-R	gggcgggtagtgaccaca		ex14-R	accactcagccacagaaagt
4	ex04-F	cagactcaattatgtgtgtttttg	15	ex15-F	atgtatggaggtggggtttt
	ex04-R	aagcccatattttagtggtga		ex15-R	cttcctttcccaaattagca
5	ex05-F	agcacacagtgacacaatcc	16	ex16-F	cgttctgtctaagggcttgt
	ex05-R	gcacgctgcttattttgtaa		ex16-R	caaaggcaagacaccaaac
6	ex06-F	gcaggtgcgctaacatcg	17	ex17-F	caacgaagcttctgtgatga
	ex06-R	gctgttatcatgatggttgg		ex17-R	tttccttgcagggagattat
7	ex07-F	aagcaagacaggtgcttttc	18	ex18-F	aagaaattggcatggaaaaa
	ex07-R	cgtagccctgtcaacagaat		ex18-R	taatgccaacaaagtcctca
8	ex08-F	tgagggttttgatgtcagag	19	ex19-F	gctccagcgtgtgactct
	ex08-R	caggcatagctcactgctaa		ex19-R	agactgagctggtggaaagt
9	ex09-F	ctgttggcttgccagagtat	20	ex20-F	ttgttcagtccatccctttc
	ex09-R	acaggaatgaacggtcaca		ex20-R	cccaagaaaacaggtatttga
10	ex10-F	gctttcattcccactcttgt	21	ex21-F	agggctgtgttcagtgct
	ex10-R	agtgggttttgccaactg		ex21-R	aggcttgtgaatggattgtt

To facilitate sequence analysis, oligonucleotides included an M13 (tgt-aaa-acg-acg-gcc-agt) or M13 rev (cag-gaa-aca-gct-atg-acc) sequence primer.
F, Forward and R, Reverse

Results

Case reports

Patient A was born after 40 wk gestation as the second daughter of a nonconsanguineous marriage. This pregnancy was complicated by hyperemesis gravidarum. Her birth weight was 2.6 kg (-2.1 SDS), and birth length was 49 cm (-0.34 SDS) (23). The height of her father was 171.5 cm (-1.0 SDS) and of her mother 158.0 cm (-1.3 SDS). Her target height was 162.8 cm (-1.2 SDS) (10). Postnatally severe failure to thrive and poor appetite were noticed. She needed nasogastric tube feeding during the first year of life. Psychomotor development was normal. At 3.3 yr of age her height was 88 cm (-2.9 SDS) and her weight was 9.9 kg (weight for height, – 3.3 SDS). Additional investigations excluded hypothyroidism and renal failure. A normal female karyotype (46 XX) was found. In 1975, an arginine stimulation test was performed with a maximum GH response of 62 mU/liter. In 1981, an exercise test showed a peak GH concentration of 109 mU/liter. Bone age was 10 yr at the chronological age of 12.1 yr.

Recently, she came again to our attention through her daughter, who had similar problems (patient B). At present, patient A is 35 yr of age. At physical examination her height was 144.6 cm (-4.0 SDS), weight 47 kg (-1.7 SDS), sitting height/height ratio 0.52 (-0.1 SDS), body mass index 22.5 kg/m^2 and head circumference 50.2 cm (-3.0 SDS) (10, 11). Blood pressure was 135/85 mm Hg. There were no dysmorphic features (Fig. 1). Cardiovascular, respiratory and abdominal examinations were all normal. Menarche had occurred at 18 yr. She has worked as administrative assistant after completing high school. Neuropsychological tests showed an above average intelligence. Biochemical characteristics are summarized in Table 3. Plasma IGF-I was in the upper normal range and IGFBP-1 was low. Ingestion of 75 g glucose suppressed GH secretion (GH nadir, 0.2 mU/liter). Glucose tolerance was slightly impaired: fasting glucose, 4.8 mmol/liter; 2-h glucose, 8.5 mmol/liter; fasting insulin, 14 mU/liter. Stimulated insulin secretion, as reflected by the insulinogenic index, was normal: 12.3 (mU/liter x mmol/liter^{-1}). HOMA-IR index indicated mild insulin resistance: index: 3.1 (mU/liter x mmol/liter^{-1})

BMD at the lumbar spine (L2-L4) was 1.07 gr/cm^2 (-0.5 SDS*),* BMAD (L3) was 0.43 gr/cm^3 (+1.3 SDS). Abdominal ultrasound revealed no abnormalities of liver,

Table 3. Biochemical characteristics of case A at 35 yr of age and case B at 15 months of age.

Variable	Case A		Case B	
	Value	SDS	Value	SDS
GH max. (mU/liter) after arginine stimulation	62[a]		222	
GH max. (mU/liter) in 3-h GH profile	3.4			
GH max. (mU/liter) after GHRH/arginine	131			
Total IGF-I (ng/ml)	239	1.6	145[b]	2.9
IGF-II (ng/ml)	656	-0.5	430	0.9
IGFBP-1 (ng/ml)	< 5 [c]		21[c]	
IGFBP-3 (µg/ml)	2.63	0.1	1.9	1.0

[a] performed at the age of 5.7 yr

[b] Before placing the gastrostoma, IGF-I was 68 ng/ml (-0.1 SDS)

[c] Normal range for nonfasting subjects is 24-58 ng IGFBP-1/ml. After overnight fasting, there is an average 5-fold rise in normal individuals.

spleen, kidneys, pancreas, uterus, and ovaries. Cardiac ultrasound was normal.

Patient B, the daughter of patient A, was born after 39 + 1/7 wk gestation. Her father was from Hindoestani descent. This pregnancy was complicated by hyperemesis gravidarum and oligohydramnion. Birth weight was 2100 g (-3.3 SDS), length 42 cm (-4.2 SDS) (23), and placental weight was 290 gr. (-2.4 SDS) (24). Head circumference was 33.3 cm at 2 months of age (-5.6 SDS). At physical examination, she had a triangular face, brachycephaly, mild hypotelorism, a small mouth with thin lips, and prominent ears (Fig. 1). Because of extremely poor appetite a percutaneous gastrostoma was positioned at the age of 10 months. Failure to thrive persisted (Fig. 2). Biochemical characteristics at 16 months of age are summarized in Table 3. Most noteworthy is the elevated plasma IGF-I after realimentation and the low IGFBP-1. An arginine stimulation test was performed at 14 months of age. Bone age was 1.5 yr at the chronological age of 1.05 yr and 1.75 yr at the age of 1.6 yr . Psychomotor development was determined with the Bayley test at 9 months and showed a motor delay of 3 months. After feeding through the gastrostoma and with physical therapy, motor skills progressed rapidly. At the age of 15 months a normal mental and slightly delayed motor development was found.
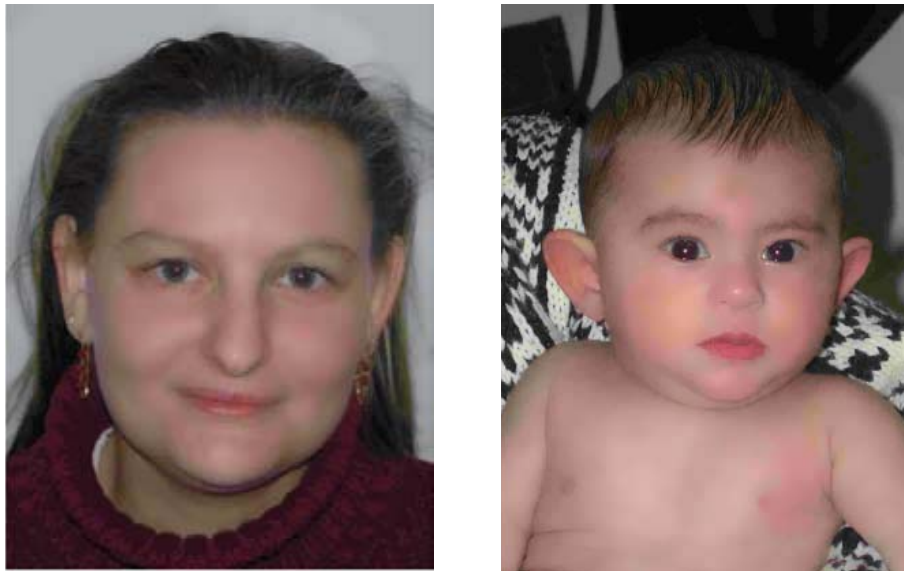
Figure 1. Patient A at the age of 35 yr and her daughter at the age of 4.5 months (patient B).
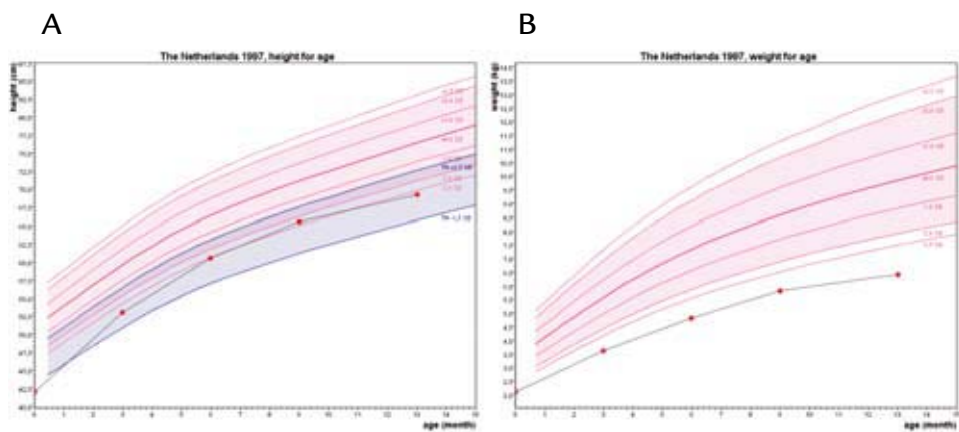

Figure 2. A, Length for age of patient B, with the pink area showing the normal distribution (-2 to +2 SDS) and the blue area showing the target range; B, weight for age.

Mutational analysis

Considering the increased IGF-I levels in both patients, we hypothesized that the clinical symptoms could be caused by a deletion or an inactivating mutation in the IGF1R-gene. IGF1R cDNA was isolated by RT-PCR from fibroblasts of patient A. Sequence analysis showed, besides a known polymorphism, a heterozygous G >A nucleotide substitution at position 3148, changing glutamic acid to lysine at position 1050 of the mature IGFIR protein (E1050K) (Fig. 3). No other mutations were found after sequence analysis of all coding exons of the IGF1R-gene. In genomic DNA of patient B, the same mutation was found. Patient B did not carry the polymorphism. In addition, we sequenced all coding exons of a panel of 87 growth-retarded patients of whom 19 also had intrauterine growth retardation. In none of these patients was the G3148A substitution or another mutation in the IGF1R present. This excluded that the G3148A substitution was a common polymorfism and provided further support for its pathogenicity.
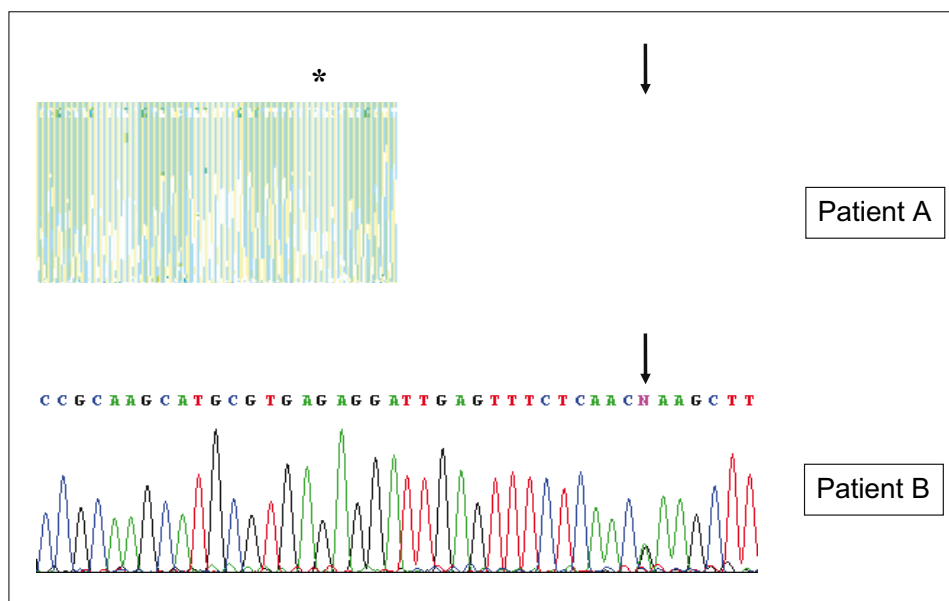

Figure 3. Sequence analysis of IGF1R cDNA of patient A (*top*) and genomic DNA of patient B (*bottom*). The *asterisk* indicates the polymorphism. The *arrow* indicates the heterozygous G→A nucleotide alteration. The wild-type and mutant alleles are both expressed in fibroblasts of patient A.
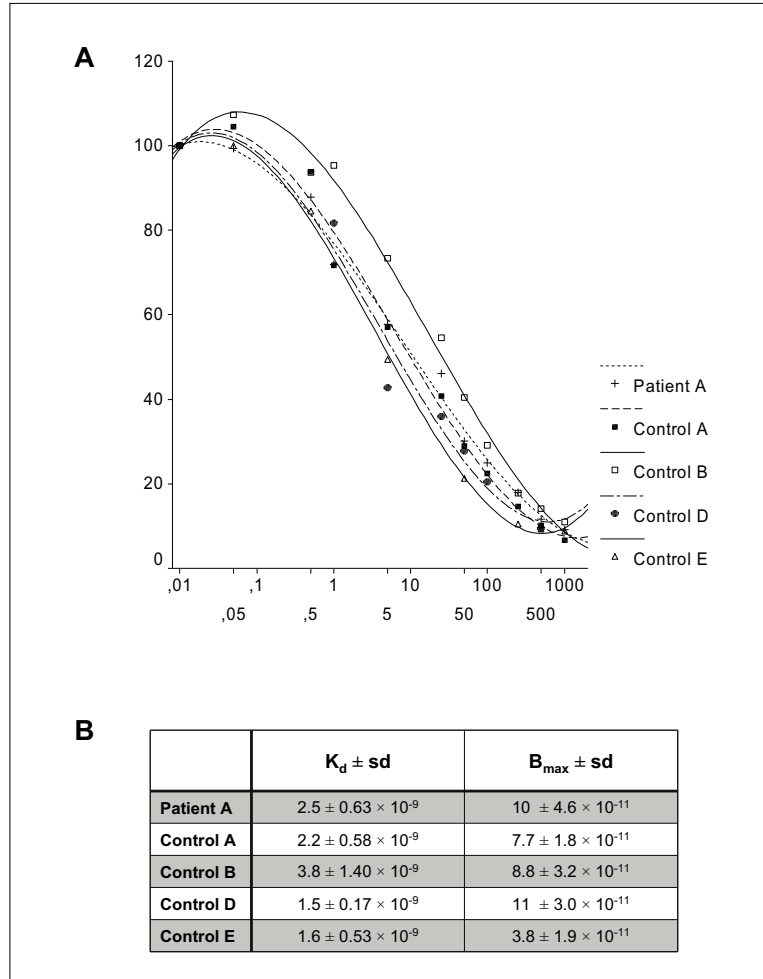
Figure 4. IGF-I binding studies.

A. Equal amounts of cells of patient A and four controls were seeded in 24-well plates. At confluency, cells were incubated with $[^{125}I]$IGF-I in the presence of 250 ng Ala^{31}Leu^{60} IGF-I and increasing amounts of unlabeled IGF-I. After 18 h, cells were washed and binding of $[^{125}I]$IGF-I was determined. Data represent the mean of four quadruplicate experiments and are expressed as percentage of total binding in the absence of competition with unlabeled IGF-I, which was set to 100% after correction for nonspecific binding. The displacement curve of the patient's cells was indistinguishable from controls.

B. Scatchard analysis was performed, with GraphPad for Windows, for the calculation of the binding affinity (K_d) and binding capacity (B_{max}) of the patient's cells and controls. Both values were in the normal range. Values represent the mean of four quadruplicate experiments ± SD.

Functional analysis

E1050 is a highly conserved amino acid residue located in the intracellular tyrosine kinase domain. The charge change induced by the amino acid substitution is presumed to result in inactivation of the IGF1R. Based on PCR and sequence analysis, both mutant and wild-type alleles were equally well expressed in fibroblasts of patient A (Fig. 3). In addition, we did not observe a difference in the expression of the IGF1R mRNA in the patient's *vs.* control cells (data not shown). Binding studies showed that the mutation did not affect binding affinity or total binding of iodinated IGF-I to patient's cells in comparison with a panel of control cell lines (Fig. 4). Western blot demonstrated a comparable level of total IGF1R protein expression in the patient compared with controls; however, autophosphorylation of the IGF1R and activation of PKB/Akt upon a challenge with IGF-I for 10 min were markedly reduced (Fig. 5, A and B). Stimulation with a dose range of IGF-I also showed that autophosphorylation of the IGF1R and activation of the downstream targets PKB/Akt and to a lesser extent Erk1/2 were reduced in cells of the patient compared with an age- and sex-matched healthy control (Fig. 5C). This reduction in activation of intracellular signal transducers was specific for IGF-I, because it was not seen when EGF or insulin were used as stimuli (Fig. 5D), excluding global unresponsiveness of the cells of the patient. Finally we determined the incorporation of [^{3}H]thymidine after a challenge with a dose range of IGF-I and compared the response in the patient's cells with the average response in a panel of fibroblast cultures of eight non-growth-retarded individuals. As shown in Fig. 5E, the maximal response was almost 50% reduced compared with controls (3.65 ± 1.79 *vs.* 6.75 ± 4.7-fold stimulation ($P < 0.01$). These results are in line with the inactivation of one copy of the IGF1R caused by the E1050K mutation.

Table 4. Clinical features of the four families with heterozygous IGF1R mutations.

nr	Subject	Mutation	Origin of mutation	Birth weight	Birth length	Head circ.	Last reported height	Dysmorphic features	Development
1A	Index case	R108Q K115N	father and mother	-3.5			-4.8	no	Verbal IQ 134 Perf. IQ 89 Psychiatric anomaly
1B	Mother	K115N		-2.0			-1.6		
1C	Father	R108Q		-2.0			-2.8		
2A	Index case	R59stop	mother	-3.5	-5.8	- 4.6 at birth	-2.6	yes	Mild motor and speech retardaton
2B	Brother	R59stop	mother	-2.7	-2.1				
2C	Mother	R59stop	father or de novo	-2.4	-1.6		-2.6		
3A	Index case	R709Q	mother	-1.5	-1.0		-2.1		IQ 60
3B	Mother	R709Q		-1.6			-2.9		Normal
4A	Mother	E1050K	father or de novo	-2.1	-0.3	- 3.0 at 35 yr	-4.0	no	Verbal IQ 110 Perf. IQ 112
4B	Daughter	E1050K	mother	-3.3	-4.2	- 5.6 at 2 months	-2.3	yes	normal

Families 1 and 2 are described by Abuzzahab *et al.* (7). Family 3 is described by Kawashima *et al.* (8). Family 4 is described in this article. Data are expressed as SDS.
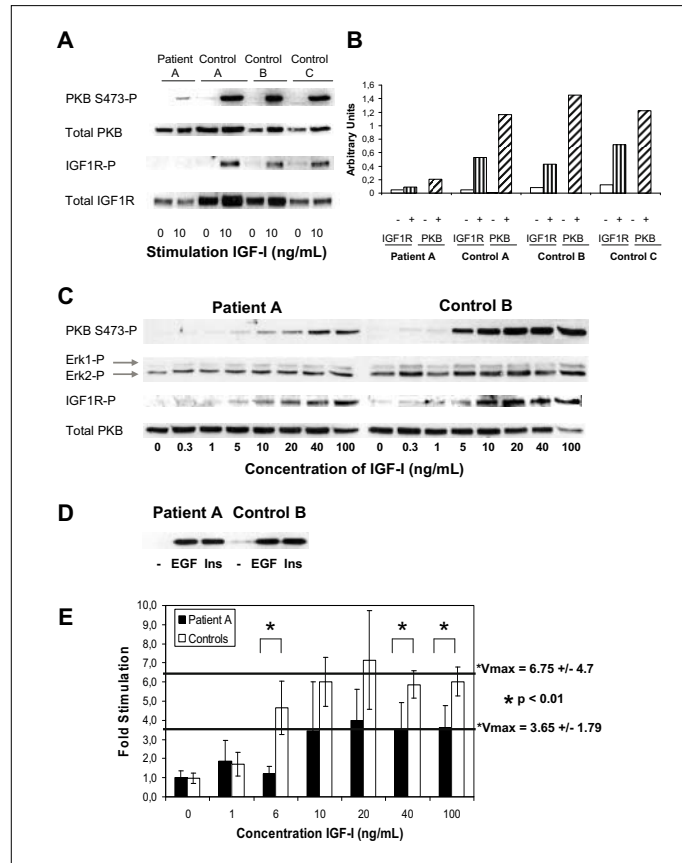
Figure 5. The E1050K missense mutation results in partial IGF-I resistance.

A. Dermal fibroblasts of patient A and controls were stimulated with 10 ng/ml IGF-I for 10 min. Protein lysates were collected and 25 μg of protein was used for Western blotting using phosphospecific IGF1R and PKB/Akt (Ser473) antibodies and total IGF1R and PKB/Akt antibodies. A representative picture of a triplicate experiment is shown.

B. Densitometric quantification of the Western blot shown in A. Data are expressed as a ratio of phosphospecific IGF1R or PKB/Akt and total IGF1R or PKB/Akt, respectively.

C. Activation of PKB/Akt by phosphorylation on Ser473 and of phosphor-Erk-1 and −2 as well as autophosphorylation of the IGF1R in fibroblasts of patient A and an age- and sex-matched control was determined by Western blot after a challenge with a dose range of IGF-I. Protein lysates were collected after 10-min stimulation. Total PKB/Akt was used to check for equal loading.

D. To exclude global unresponsiveness, fibroblasts of patient A and the age- and sex-matched control were challenged with 10 ng/ml EGF and 1 x 10^{-6} M Insulin (Ins). Protein lysates were collected and equal amounts were loaded on a gel and subjected to Western blot to determine phosphorylated PKB/Akt. Both stimuli were equally potent in activating PKB/Akt in cells of the patient and the control.

E. Fibroblasts of patient A and fibroblasts of eight healthy donors were stimulated with a dose range of IGF-I and the incorporation of [³H]-thymidine was determined. The maximal response expressed as fold stimulation (Vmax) was calculated by averaging the responses observed at concentrations of IGF-I of 10, 20, 40, and 100 ng/ml at which the plateau phase was reached. Data are expressed as fold stimulation ± SEM. *, $P < 0.05$).

Discussion

In this study, we describe two cases, a mother and daughter, with the first hetero-zygous missense mutation in the intracellular tyrosine kinase domain of the IGF1R. This IGF1R mutation results in partial resistance to IGF-I, which presents with intrauterine and postnatal growth retardation and failure to thrive. These cases, together with the three previously described families with IGF1R mutations (7, 8), and two cases with homozygous IGF-I defects (1, 2) have provided a unique oppor-tunity to study the role of the IGF-I/IGF1R system in human physiology.

Our patients have a mutation in exon 16, coding for the intracellular tyrosine kinase domain of the receptor. The tyrosine kinase (catalytic) domain is part of the cytoplasmatic portion of the β-chain of the IGF1R. Binding of IGF-I to the extra-cellular α-chain induces a conformational change in the structure of the receptor leading to autophosphorylation of three tyrosines in the activation loop of the catalytic domain of the β chain. Phosphorylation of the tyrosine residues results in a dramatic conformational change (25, 26). Glutamic acid at position 1050 (E1050) is extremely well conserved in all tyrosine kinase domains available in the database of the National Center for Biotechnology Information (NCBI) and is believed to play an important role in stabilizing the active conformational state.

The equivalent of E1050 in the IR is E1047. Upon binding insulin, the intracellular kinase domain is activated and conformational changes are induced. The change in three-dimensional structure places E1047 in close proximity of K1030 (K1033 in the IGF1R). This lysin residue is also highly conserved in tyrosine kinase domains and is located in the ATP-binding site. Hydrogen bonding between the negatively charged E1047 and the positively charged K1030 is believed to stabilize the structure of the activated receptor upon binding of insulin (25). Similarly, hydrogen bonding between E1050 and K1033 is believed to stabilize the active conformation of the IGF1R upon IGF-I binding. Due to the charge change induced by the E1050K substi-tution, it seems likely that the mutant receptor cannot preserve its active conforma-tion. Consequently, the substitution should result in inactivation. This was confirmed by functional studies showing a dramatic reduction in autophosphorylation of IGF1R and in activation of downstream signaling pathways in cells of patient A compared with a panel of controls upon a challenge with IGF-I. The mutation did not have an

effect on IGF-I binding or on IGF1R mRNA and protein expression. Compared with the PKB/Akt pathway, the reduction in activation of the Erk1/2 pathway was less pronounced. This can be explained by assuming that less kinase activity is required to saturate activation of the Erk1/2 pathway compared with the PKB/Akt pathway. Finally, the maximal response determined in a [^{3}H]thymidine incorporation assay was almost 50% reduced compared with a panel of fibroblasts derived from healthy controls. Taken together, these data are fully in line with the inactivation of one copy of the IGF1R as a result of the E1050K substitution and suggests that the IGF-I resistance in our patients is caused by IGF1R haploinsufficiency. They furthermore confirm the important function of E1050 in stabilizing the active conformation of the tyrosine kinase domain as predicted by structural models (25, 26).

Although we believe that the best explanation of the phenotype observed in our two patients is the heterozygous IGF1R mutation leading to partial IGF-I resistance, we acknowledge that the results cannot be viewed as definitive proof. Even if one accepts that the mutated IGF1R must be dysfunctional based on its structure and that in *in vitro* studies of cultured fibroblasts stimulation of the the PKB/Akt pathway is reduced, one still has to assume that one functional allele is not sufficient for a full biological activity, at least with respect to growth of the skull, trunk, and extremities. Clinical observations on more patients, combined with detailed *in vitro* studies, are needed before it can be concluded that IGF1R haploinsufficiency indeed causes a clinical syndrome of pre- and postnatal growth failure.

The most striking clinical characteristics of patients with primary IGF-I resistance are intrauterine and postnatal growth retardation. The growth data of the reported patients with a mutation of the IGF1R gene are summarized in Table 4. Most patients have intrauterine growth retardation, but the degree of growth failure is variable. The patients with an affected mother (patients 2A, 2B, and 4B, and possibly 1A) seem to be more severely growth retarded than the patients with an apparently unaffected mother (patients 2C and 4A). One can hypothesize that maternal IGF-I resistance during pregnancy may affect placental size and, as a consequence, fetal growth. This hypothesis is supported by a strongly positive correlation between the rate of IGF-I increase during pregnancy and placental weight (27, 28) and by the finding that placentas from IUGR pregnancies are characterized by decreased expression of IGF1R and signal transduction proteins (29).

Postnatally, we saw a stimulated GH response to arginine in patient B, reflecting the state of IGF-I resistance. The direct IGF-I independent effects of GH could account for the better postnatal than prenatal growth in patient B. Another explanation could be that later in life the body compensates for the lack of IGF-I effect by producing more IGF-I receptor per cell. In the adult patient, GH secretion was normal, while it had been high in childhood and adolescence. This suggests that the GH-IGF-I feedback loop may change with age.

Concerning puberty, our patient A had a late onset of puberty. In the earlier reported patients, timing of puberty was not described in detail, except for the compound heterozygous patient in the report of Abuzzahab *et al*. (7) who had a normal pubertal development. The patients with IGF-I deletion and mutation had delayed puberty (1, 2). In Laron syndrome puberty is delayed, more so in boys than in girls (30). In patients with ALS deficiency puberty is extremely delayed (31). Taking all data together, it appears that IGF-I plays a role in pubertal onset.

So far, all patients with heterozygous IGF1R mutations with a reported head circumference have been microcephalic (Table 4). This is in line with the severe microcephaly found in both patients with primary IGF-I deficiency (-8 SDS in the case of IGF-I mutation and – 5.3 SDS in the case of IGF-I deletion) (1, 2). Carriers of the IGF-I mutation had a lower head circumference than noncarriers (-1.0 SDS *vs*. – 0.4 SDS) (2). This confirms the important role of IGF-I in intrauterine brain development as is known from IGF-I knockout mice (32). In contrast, the findings regarding intellectual and emotional development seem to vary substantially (Table 4).

Both our patients needed tube feeding due to poor appetite and failure to thrive, so it is tempting to believe that the IGF1R mutation is associated with this phenomenon. Observations in rats lend some support to this speculation: in rats, IGF-I receptors are localized in the hypothalamic arcuate nucleus (33), whereas the arcuate nucleus integrates signals regulating appetite (34). Insulin is known to penetrate the blood-brain barrier, where it acts as an anorexigenic signal, decreasing intake and body weight (34). Although data on the role of IGF-I on appetite are limited, we hypothesize that IGF-I has a similar effect. In our case B we observed that plasma IGF-I levels can be within the normal range in a state of poor nutrition. After restoring the nutritional state by a gastrostoma, IGF-I increased from -0.1 SDS to 2.9 SDS.

In our adult patient, an increased HOMA-IR was found, reflecting a moderate degree of insulin resistance, which could not be explained by an increased fat mass, since body mass index as well as stimulated insulin secretion were normal. Primary IGF-I deficiency is associated with insulin resistance (35) and treatment with recombinant human IGF-I improved insulin sensitivity in a patient with homozygous IGF-I mutation (36). Administration of recombinant human IGF-I to patients with severe insulin resistance and to patients with type 2 diabetes improves insulin sensitivity (37). These observations support the hypothesis that IGF-I is necessary for normal insulin sensitivity.

Comparing the phenotypes of the affected individuals in the four families with an IGF1R mutation, the degree of mental performance, dysmorphic features, and failure to thrive varies substantially (Table 4). This may reflect a spectrum of remaining IGF-I signaling activity. This is supported by our finding in patient A, that [^{3}H]thymidine can be incorporated in fibroblasts that are stimulated with high doses of IGF-I, although the maximal response is significantly reduced compared with controls. With regard to therapy, this *in vitro* response to high-dose IGF-I is an important argument for the potential benefits of GH or IGF-I treatment. Our patient B has just started GH treatment (1.4 mg/m2/day).

In conclusion, the novel heterozygous mutation in the intracellular tyrosine kinase domain of the IGF1R leads to intrauterine and postnatal growth retardation to a similar extent as the previously described mutations in the extracellular ligand binding part of the receptor. The degree of remaining IGF-I signaling may explain the different phenotypes seen in patients with IGF1R mutations. Maternal IGF-I resistance may affect placental size and explain part of the variance in birth weight of carriers of an IGF1R mutation. Heterozygous IGF1R mutations can be expected in patients born small for gestational age with a small head circumference in the presence of relatively high levels of IGF-I. Genetic analysis in such patients may provide important information on genotype-phenotype relations and the role of the IGF-I system in intrauterine and postnatal growth and development in human.

References

1. Woods KA, Camacho-Hubner C, Savage MO, Clark AJ. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 1996;335(18):1363-1367.

2. Walenkamp MJ, Karperien M, Pereira AM, Hilhorst-Hofstee Y, Van Doorn J, Chen JW et al. Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 2005;90(5):2855-2864.

3. Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 2000;57(7):1050-1093.

4. LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 1995;16(2):143-163.

5. Nakae J, Kido Y, Accili D. Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev 2001;22(6):818-835.

6. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (IGF-1) and type 1 IGF receptor (IGF1R). Cell 1993;75(1):59-72.

7. Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E et al. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 2003;349(23):2211-2222.

8. Kawashima Y, Kanzaki S, Yang F, Kinoshita T, Hanaki K, Nagaishi Ji et al. Mutation at cleavage site of insulin-like growth factor receptor in a short-stature child born with intrauterine growth retardation. J Clin Endocrinol Metab 2005;90(8):4679-4687.

9. Holzenberger M, Leneuve P, Hamard G, Ducos B, Perin L, Binoux M et al. A targeted partial invalidation of the insulin-like growth factor I receptor gene in mice causes a postnatal growth deficit. Endocrinology 2000;141(7):2557-2566.

10. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E et al. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res 2000;47(3):316-323.

11. Fredriks AM, van Buuren S, van Heel WJM, Dijkman-Neerincx RHM, Verloove-Vanhorick SP, Wit JM. Nationwide age references for sitting height, leg length, and sitting height/height ratio, and their diagnostic value for disproportionate growth disorders. Arch Dis Child 2005;90(8):807-812.

12. Kroger H, Vainio P, Nieminen J, Kotaniemi A. Comparison of different models for interpreting bone mineral density measurements using DXA and MRI technology. Bone 1995;17(2):157-159.

13. Rikken B, Van Doorn J, Ringeling A, Van den Brande JL, Massa G, Wit JM. Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. Horm Res 1998;50(3):166-176.

14. Buul-Offers SC, van Kleffens M, Koster JG, Lindenbergh-Kortleve DJ, Gresnigt MG, Drop SL et al. Human insulin-like growth factor (IGF) binding protein-1 inhibits IGF-I-stimulated body growth but stimulates growth of the kidney in snell dwarf mice. Endocrinology 2000;141(4):1493-1499.

15. Cole TJ. The LMS method for constructing normalized growth standards. Eur J Clin Nutr 1990;44(1):45-60.

16. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20(7):1183-1197.

17. Phillips DI, Clark PM, Hales CN, Osmond C. Understanding oral glucose tolerance: comparison of glucose or insulin measurements during the oral glucose tolerance test with specific measurements of insulin resistance and insulin secretion. Diabet Med 1994;11(3):286-292.

18. Wareham NJ, Phillips DI, Byrne CD, Hales CN. The 30 minute insulin incremental response in an oral glucose tolerance test as a measure of insulin secretion. Diabet Med 1995;12(10):931.

19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28(7):412-419.

20. Kamp GA, Ouwens DM, Hoogerbrugge CM, Zwinderman AH, Maassen JA, Wit JM. Skin fibroblasts of children with idiopathic short stature show an increased mitogenic response to IGF-I and secrete more IGFBP-3. Clin Endocrinol (Oxf) 2002;56(4):439-447.

21. Denley A, Wang CC, McNeil KA, Walenkamp MJ, van Duyvenvoorde H, Wit JM et al. Structural and functional characteristics of the val44met insulin-like growth factor I missense mutation: correlation with effects on growth and development. Mol Endocrinol 2005;19(3):711-721.

22. Siebler T, Lopaczynski W, Terry CL, Casella SJ, Munson P, De Leon DD et al. Insulin-like growth factor I receptor expression and function in fibroblasts from two patients with deletion of the distal long arm of chromosome 15. J Clin Endocrinol Metab 1995;80(12):3447-3457.

23. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta Paediatr Scand 1991;80(8-9):756-762.

24. Kloosterman GJ. [Intrauterine growth and intrauterine growth curves]. Maandschr Kindergeneeskd 1969;37(7):209-225.

25. Hubbard SR. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J 1997;16(18):5572-5581.

26. Favelyukis S, Till JH, Hubbard SR, Miller WT. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat Struct Biol 2001;8(12):1058-1063.

27. Clapp III, Schmidt S, Paranjape A, Lopez B. Maternal insulin-like growth factor-I levels (IGF-I) reflect placental mass and neonatal fat mass. American Journal of Obstetrics and Gynecology 2004;190(3):730-736.

28. Chellakooty M, Vangsgaard K, Larsen T, Scheike T, Falck-Larsen J, Legarth J et al. A longitudinal study of intrauterine growth and the placental growth hormone (GH)-insulin-like growth factor I axis in maternal circulation: association between placental GH and fetal growth. J Clin Endocrinol Metab 2004;89(1):384-391.

29. Laviola L, Perrini S, Belsanti G, Natalicchio A, Montrone C, Leonardini A et al. Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. Endocrinology 2005;146(3):1498-1505.

30. Laron Z. Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. J Clin Endocrinol Metab 2004;89(3):1031-1044.

31. Domene HM, Bengolea SV, Martinez AS, Ropelato MG, Pennisi P, Scaglia P et al. Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene. N Engl J Med 2004;350(6):570-577.

32. D'Ercole AJ, Ye P, O'Kusky JR. Mutant mouse models of insulin-like growth factor actions in the central nervous system. Neuropeptides 2002;36(2-3):209-220.

33. Garcia-Segura LM, Rodriguez JR, Torres-Aleman I. Localization of the insulin-like growth factor I receptor in the cerebellum and hypothalamus of adult rats: an electron microscopic study. J Neurocytol 1997;26(7):479-490.

34. Wynne K, Stanley S, McGowan B, Bloom S. Appetite control. J Endocrinol 2005;184(2):291-318.

35. Camacho-Hubner C, Savage M. Insulin-like growth factor -I deficiency. Horm Res 2001;55 Suppl:117-20.

36. Camacho-Hubner C, Woods KA, Miraki-Moud F, Hindmarsh PC, Clark AJ, Hansson Y et al. Effects of recombinant human insulin-like growth factor I (IGF-I) therapy on the growth hormone-IGF system of a patient with a partial IGF-I gene deletion. J Clin Endocrinol Metab 1999;84(5):1611-1616.

37. Moses AC, Young SC, Morrow LA, O'Brien M, Clemmons DR. Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. Diabetes 1996;45(1):91-100.