Genetic disorders in the growth hormone-IGF-I axis

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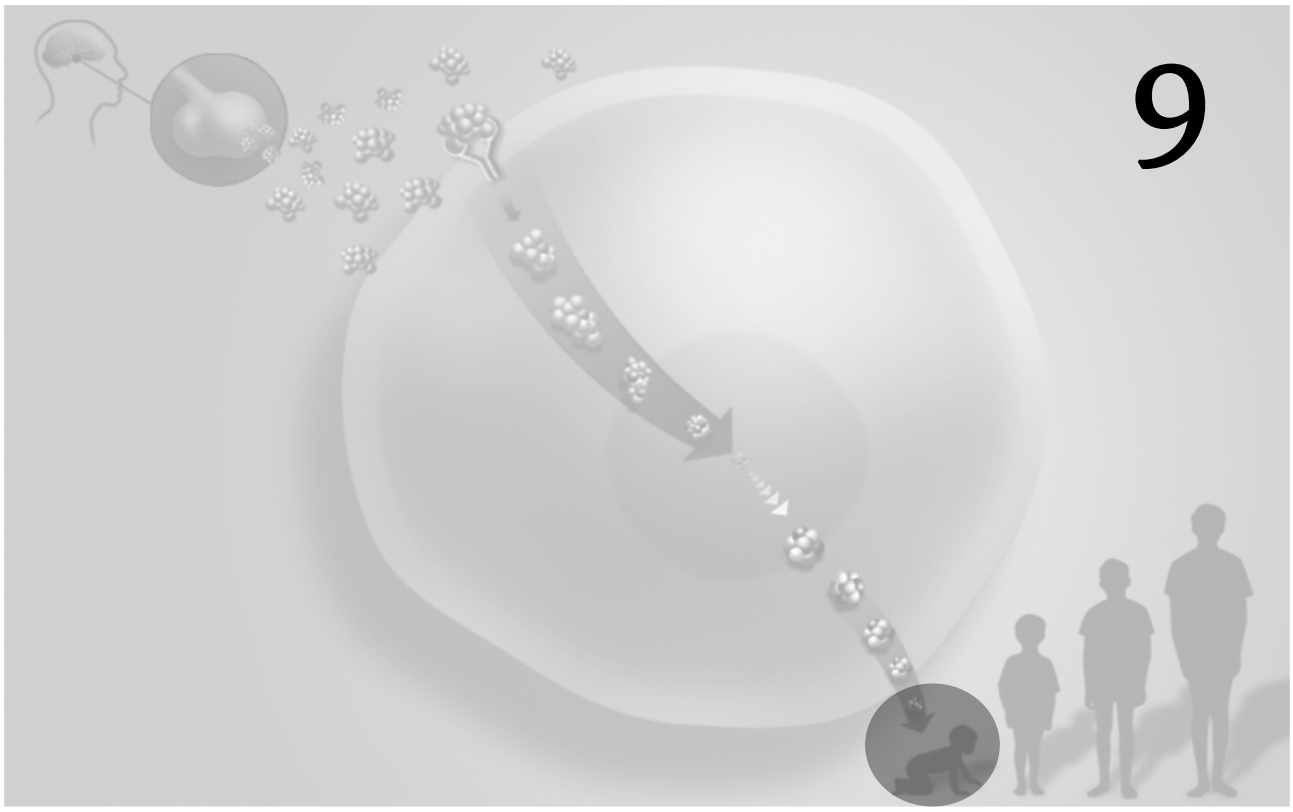
Succesful long-term growth hormone therapy in a girl with haploinsufficiency of the IGF-I receptor due to a terminal 15q26.2 → qter deletion



9

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Abstract

Context: Microscopically visible heterozygous terminal 15q deletions encompassing the IGF1R gene are rare and invariably associated with intrauterine growth retardation and short stature. The incidence of submicroscopic deletions is unknown, as well as the effect of growth hormone therapy in this condition.

Objective: To describe the use of a novel genetic technique [multiplex ligation probe amplification (MLPA)] to detect haploinsufficiency of the IGF1R gene in a patient suspected of an IGF1R gene defect and evaluate the effect of long-term GH therapy.

Patient: We describe a 15 yr old adolescent, born small for gestational age, who showed persistent postnatal growth retardation, mild developmental delay and elevated IGF-I levels. She had been treated with GH since the age of 5 yr.

Methods: Mutation analysis of the IGF1R gene was performed by DNA sequencing followed by MLPA and array Comparative Genomic Hybridization (aCGH) to examine gene copy number changes. Dermal fibroblast cultures were used for functional analysis.

Results: Sequence analysis revealed no abnormalities in the IGF1R gene. With MLPA, a deletion of one copy of the IGF1R gene was detected, which was defined by aCGH to a loss of 15q26.2→qter. In concordance with IGF1R haploinsufficiency, IGF1R mRNA expression was decreased and activation of IGF1R and PKB/Akt after a challenge with IGF-I was decreased in the patient's fibroblasts, though to a lesser extent. IGF-I binding assays showed normal binding affinity and maximal binding of the IGF1R. GH treatment resulted in a good growth response and final height within the normal range.

Conclusions: The phenotype of a heterozygous terminal 15q deletion resembles that of a heterozygous inactivating IGF1R mutation with respect to intrauterine growth retardation, microcephaly, short stature, and elevated IGF-I levels. Long term growth hormone therapy is well tolerated and causes growth acceleration in childhood resulting in a normal adult height. MLPA and aCGH are useful tools to detect submicroscopic deletions of the IGF1R gene in patients born small for gestational age with persistent growth failure.

Introduction

Insulin-like growth factor I (IGF-I) is required for normal intrauterine and postnatal growth. The biological functions of IGF-I are mediated through the type 1 IGF receptor (IGF1R). The IGF1R gene is located on the distal long arm of chromosome 15 (15q26.3). Heterozygous inactivating mutations of the IGF1R gene result in a phenotype of intrauterine and postnatal growth failure and microcephaly, with a variable degree of psychomotor retardation (1-4). Mild dysmorphic features have been described in some cases (1, 3). An isolated homozygous or heterozygous deletion of the IGF1R gene has not yet been described.

Heterozygous terminal deletions of the distal long arm of chromosome 15, including the IGF1R, have been reported in only a few cases: 6 patients with a15q26.1 → 15qter deletion (5-9) and 2 patients with a 15q26.2→15qter deletion (10-12). Intrauterine growth retardation (IUGR) is present in almost all cases, with a birth weight varying between -1.8 SDS and –5.6 SDS and a birth length between -1.3 SDS and -5.5 SDS. In all cases, except one, the karyotype was abnormal, showing the terminal deletion of 15q.

We report a female patient with pre- and postnatal growth failure and elevated plasma IGF-I levels. The karyotype was normal. This clinical picture resembles the presentation of patients with a heterozygous mutation in the IGF1R gene (1-4). However, sequencing of the IGF1R gene did not reveal any abnormalities. Since the clinical picture may also be caused by haploinsufficiency of the IGF1R gene, we performed multiplex ligation-dependent probe amplification (MLPA) and found loss of one copy of the IGF1R gene. The borders of the deletion were mapped using array-comparative genomic hybridization (aCGH). 15q26.2→qter was deleted, including IGF1R gene. We show that long term growth hormone therapy in a patient with IGF1R haploinsufficiency improves growth considerably and leads to a normal adult height without notable side-effects.

Methods

The patient and her parents provided written informed consent

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 (13). Sitting height and sitting height/height ratio were also expressed as SDS for the Dutch population (14).

Bone Mineral Density (BMD)
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 (15). BMD and BMAD results were compared with age- and sex-matched reference values and expressed as SDS (16, 17).

Biochemical measurements
Plasma GH was measured with Plasma GH was measured with Immulite 2000 a solid-phase, two-site chemiluminescent immunometric assay (DPC, Los Angeles, CA) using the WHO NIBSC 1st international standard 80/505 (1mg=2.6 IU). An arginine stimulation test was performed with 0.5 g/kg arginine iv over 30 min and blood samples at 0 and 30 min. A clonidine stimulation test was performed with 0.15 mg/m^2 clonidine orally, collecting blood samples every 30 minutes until 150 min.

Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1 and IGFBP-3 were determined by specific RIAs (18, 19). 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 (20). Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of 6 healthy adult controls. An IGF-I generation test was performed with 1 mg/m^2 body surface GH (Humatrope, Lilly) during 4 days, followed by 2 mg/m^2 during 3 days.

Genetic analysis

Fluorescence In Situ Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was performed on cultured dermal fibroblasts according to standard procedures. The probes used were the 15q subtelomeric PAC clone 154P1 (GS-154P1, (21) and the BAC clone 342L10 (RP11-342L10, "Cancer_1E9", located in 15q26.3, BACPAC Resource Center (http://bacpac.chori.org/order.php).

Sequence analysis

Total RNA was isolated from cultured fibroblasts and reverse transcribed into cDNA. The coding regions of the IGF1R were amplified by PCR using overlapping primer combinations and subjected to direct sequencing as described previously (22). Genomic DNA was isolated from whole blood according to the salting out procedure described by Miller et al (23). All coding exons of the IGF1R were PCR amplified and subjected to direct sequencing as described previously (3).

Multiplex ligation-dependent probe amplification (MLPA)

MLPA probes for the IGF1R were designed according to the criteria described by White et al (24) and were directed to exons 2, 8 and 18 of the IGF1R gene, which are conserved exons in different species (Table 1). The oligonucleotide probes were ordered from Illumina Inc. (San Diego, CA) and used without purification.

Table 1. Sequence of the MLPA probes.

Gene	exon	Upstream (U) / downstream (D)	Sequence (5'-3')	Primer length (bp)	Fragment length (bp)	label
IGF1R	2	U	GATGTGTGAGAAGACCACCATCAACA	44	92	HEX
		D	ATGAGTACAACTACCGCTGCTGGACCACAA	48		
	8	U	CTACATGGGCTGAAGCCCTGGACTCAG	45	94	HEX
		D	TACGCCGTTTACGTCAAGGCTGTGACCCTCA	49		
	18	U	CAGTCCTAGCACCTCCAAGCCTGAGCA	45	90	HEX
		D	AGATGATTCAGATGGCCGGAGAGATTG	45		
Labeled primer			GGGTTCCCTAAGGGTTGGA			
Unlabeled primer			GTGCCAGCAAGATCCAATCTAGA			

All reagents for the MLPA were obtained from MRC Holland (Amsterdam, the Netherlands). Reactions were performed according to White *et al*. (24) and the manufacturer's instructions. In short, 50 ng of genomic DNA in a final volume of 1 µl was heated at 98°C for 5 min and subsequently cooled at 25°C. 0.375 µl probe mix (4 fmol/µl), 0.25 µl H_2O and 0.375 µl SALSA MLPA buffer were added to each sample, heat denatured at 95°C for 2 minutes and followed by hybridization at 60°C for 2.5-3 hours. Samples were kept at 54°C and 8 µl Ligase-65 mix (0.75 µl buffer A, 0.75 µl buffer B, 0.25 µl Ligase and 6.25 µl H_2O) was added to each sample. After 10-15 min, the reaction was stopped by heat inactivation at 98°C for 5 min and cooled at 4°C. The MLPA product (10 µl) was added to 20 µl PCR mix at 60°C and 33 cycles of 20 sec. 95°C, 30 sec at 62°C and 60 sec. at 72°C were carried out. From each PCR reaction 2 µl of product was mixed with 15 µl deionized formamide and 0.5 µl ROX-500 Genescan. Product separation was performed using capillary electrophoresis on an ABI 310 (Applied Biosystems). The data analysis was performed as described by White *et al*. (24).

Array comparative genomic hybridization (aCGH)

To determine the boundaries of the deletion we performed array-based comparative genomic hybridization (aCGH) using the 44B Human Genome CGH Microarrays (Agilent, Santa Clara, CA). These arrays contain $43x10^3$ 60-mer oligonucleotide probes (mostly exonic) that span the human genome with an average spacing of 35 Kb. aCGH analysis was performed according to the manufacturer's protocols. After hybridization and washing, slides were dried and scanned using a microarray scanner (Agilent, Santa Clara, CA). Images were analyzed with Agilent's CGH Analytics software.

Functional analysis

Real Time PCR was performed with the Biorad iQ5 multicolor real-time PCR detection system using Hs_IGF1R_SG Quantitect Primer Assay primers (Qiagen, Valencia, CA). Fibroblast cultures of the patient and of two healthy donors were used for Western blotting. Cells were stimulated for 10 min with or without 10 ng/ml IGF-I. Blots were probed with an anti-phospho-PKB/Akt, total PKB/Akt (Cell Signaling Technology, Beverly, MA), anti-phospho-IGF1R (Biosource International, Camarillo, CA) and total IGF1R (Cell Signaling Technology, Beverly, MA) antibodies as described previously (22). Binding studies were performed using iodinated

IGF-I in the presence of an excess of an IGF-I analog that is bound by IGFBPs but not by the IGF1R ($Ala^{31}Leu^{60}$-IGF-I, GroPep, Adelaide, Australia) (23). 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 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 (23). After 18 h, cells were washed and solubilized in 1 M NaOH. Radioactivity was determined using a γ-counter.

Results

Case report

A 4.5 year old girl presented to the growth clinic of the Sophia Children's Hospital with severe growth retardation. She was born after 39 wk gestation as the third child of healthy unrelated parents. The pregnancy was uneventful. Her birth weight was 2.1 kg (-3.0 SDS), birth length was 47 cm (-1.3 SDS), and head circumference 33 cm (-2 SDS)(25). Father's height was 182 cm (-0.3 SDS) and the height of her mother was 165.5 cm (-0.8 SDS). Her target height corrected for secular trend (4.5 cm per generation) was 171.8 (0.2 SDS) (13). Her sister's height was 0 SDS, her brother's height was +1.3 SDS. Motor development was normal: she started sitting at the age of 10 months and walking at 17 months. Speech development was slow.

At the age of 4.5 yr her height was 93.5 cm (-3.5 SDS) (Fig. 1), weight 14.8 kg (weight for height +0.7 SDS), BMI 17 kg/m^2 (1.0 SDS), sitting height 51.6 cm (sitting height/ height ratio 0.55 = 0.1 SDS). At physical examination she had a puppet face and a high-pitched voice. Her upper legs were strikingly muscular and there was increased abdominal fat distributed in a lobular pattern. She wore strong glasses because of extreme myopia (-10 and -7.5). There were no dysmorphic features and no abnormalities at further physical examination.

IgG and IgA anti-gliadin antibodies were negative, virtually excluding celiac disease. Thyroid function was normal. The karyogram showed a normal female pattern

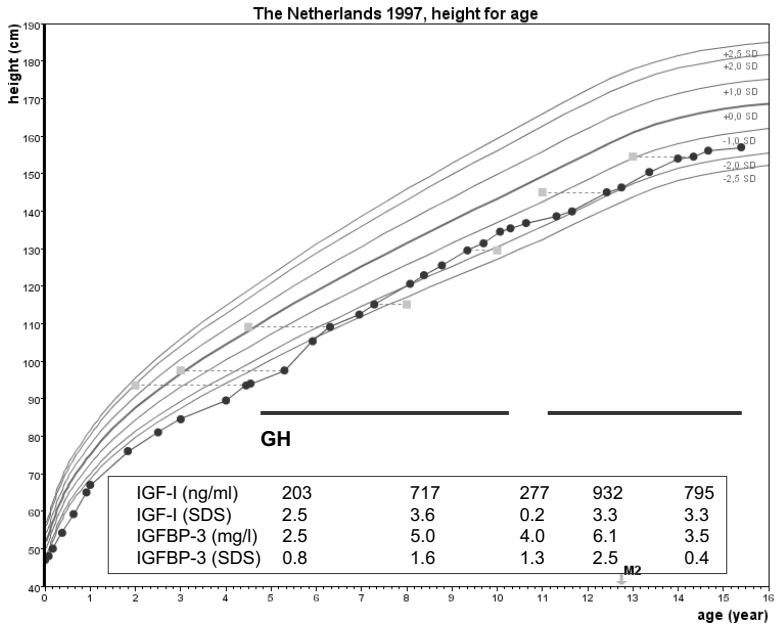

Figure 1. Growth curve
The black dots represent height measurements, the gray squares the bone age, M2 indicates Tanner breast stage 2. The black horizontal lines indicate the periods of GH treatment.

(46,XX). IGF-I was 203 ng/ml (+2.5 SDS), IGFBP-3 2.49 mg/l (+0.8 SDS), IGFBP-1 46.6 ng/ml (normal). GH stimulation tests were performed with clonidine and arginine on separate days, resulting in peak GH concentrations of 8.4 and 25 IU/liter, respectively. At the start of the IGF-I generation test IGF-I was 224 ng/ml, after 4 days of GH injections (1 mg/m^2 body surface) IGF-I was 375 ng/ml, after another 3 days of GH (2 mg/m^2) IGF-I was 480 ng/ml (normal range 115-329 ng/ml).
Bone age was delayed by 2.5 years. BMD was 0.56 g/cm^2 (-1.80 SDS), BMAD was 0.25 g/cm^3 (-1.09 SDS) and total body BMD was 0.77 g/cm^2 (-1.39 SD). Lean body mass SDS was –2.45 and percentage body fat SDS was 0.75.

At the age of 5.3 years GH therapy was started in a dosage of 1 mg/m^2.day sc (Humatrope, Lilly) (equivalent to 0.26 mg/kg body weight/week). A rapid catch-up growth occurred, followed by a stabilisation at -2 SDS (Fig. 1). During GH

therapy IGF-I was elevated (Fig. 1). BMD and lean body mass SDS increased. After two years of GH-treatment lumbar spine BMD SDS was –1.55, BMAD SDS –0.62, total body BMD SDS –0.95, and lean body mass SDS –0.96. GH therapy was interrupted at the age of 10.8 years for 6 months, resulting in a decrease in height velocity and IGF-I level (Fig. 1). Puberty started at the age of 12.8 years and final height was reached at 15 years (157 cm, -1.6 SDS), 1.8 SD lower than target height SDS. Head circumference was 53 cm (-1.1 SDS). She had a regular menstrual cycle. She has recently completed high school.

Genetic and functional analysis

Sequence analysis revealed no mutation in the coding exons of the IGF1R gene. MLPA showed a heterozygous deletion of the three exons (2, 8 and 18) of the IGF1R gene that were included in the MLPA assay (Fig. 2). This deletion was not present in the DNA of the parents. FISH confirmed the IGF1R deletion, together with a deletion of the 15q telomere. aCGH revealed that the deletion comprises 5.2 Mb of the terminal part of the long arm of chromosome 15 (15q26.2 → 15qter), starting from the SPATA8 gene, spanning 34 genes.

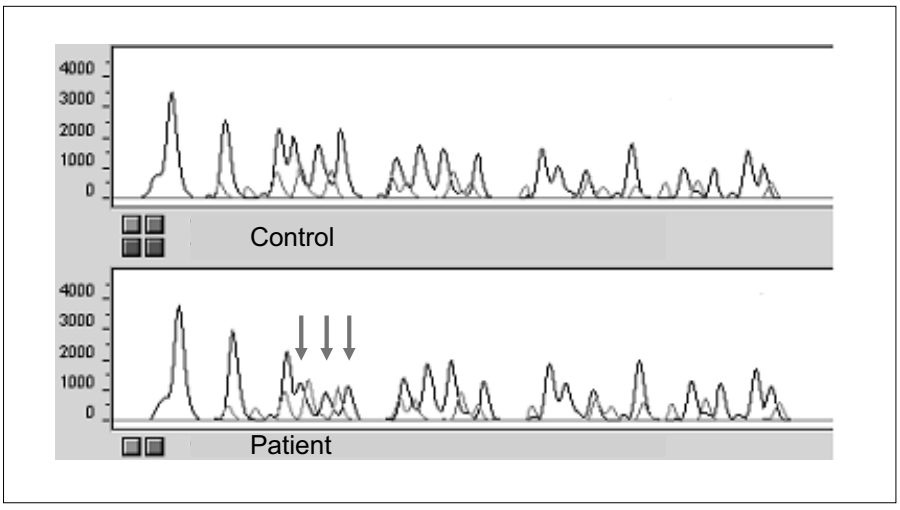

Figure 2. Genescan of the MLPA analysis of the IGF1Rgene.
The arrows in the *lower* panel show the lower peaks of the 3 IGF1R probes in the patient compared with the control in the *upper* panel, indicating a deletion of IGF1R.

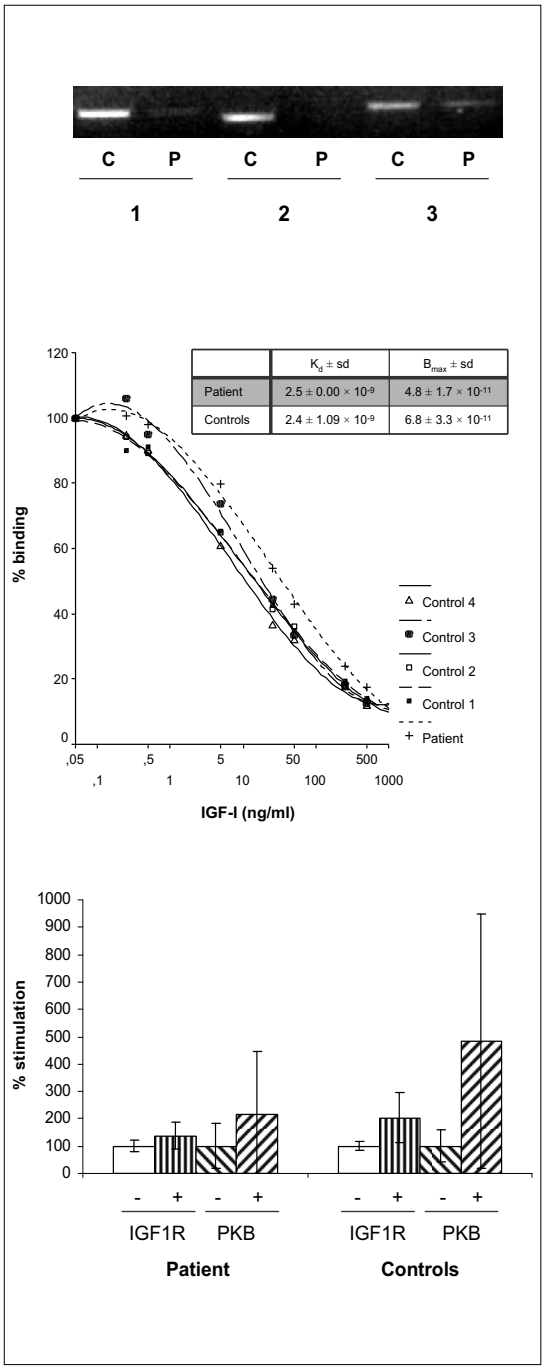

Figure 3. Functional analysis

A. Expression of three IGF1R mRNA fragments (P = patient, C = control). Cultured dermal fibroblasts showed decreased expression of IGF1R mRNA in the patient *vs* a random control.

B. Equal amounts of cells of the patient and four controls were seeded in 24-wells plates. At confluency, cells were incubated with [^{125}I]IGF-I in the presence of 250 ng/ml Ala^{31}Leu^{60}-IGF-I and increasing amounts of unlabeled native IGF-I. After 18 h, cells were washed and binding of [^{125}I]IGF-I was determined. Data represent the mean of two quadruplicate experiments and are expressed as percentage of total binding in the presence of competition with the lowest concentration IGF-I (0.05 ng/ml), which was set to 100% after correction for non-specific binding. The displacement curve of the patient's cells was indistinguishable from controls.
Scatchard analysis was performed for the calculation of the binding affinity (K_d) and binding capacity (B_{max}) of the patient's cells and controls. The K_d and B_{max} of our patient showed no significant differences compared with various controls. Values represent the mean of two quadruplicate experiments ± SD.

C. Dermal fibroblasts of the patient 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 (picture not shown). Densitometric quantification of the Western blots was performed. Data are expressed as a ratio of phosphor specific IGF1R or PKB/Akt and total IGF1R or PKB/Akt, respectively. The ratio of the unstimulated lysates was set at 100%. The activation of the IGF1R and PKB/Akt tended to be lower in the patient, but this did not reach significance.

RT-PCR analysis using various fragments of the IGF1R gene showed decreased expression of IGF1R mRNA in fibroblasts of the patient versus a normal control (Fig. 3A). This was confirmed by quantitative PCR (qPCR) showing an approximately 5 times reduction in IGF1R mRNA expression compared to a panel of normal controls (data not shown). Binding studies showed normal binding affinity and a trend towards decreased total binding (not significant) of iodinated IGF-I to patient's cells in comparison with a panel of control cell lines (Fig. 3B). Western blot demonstrated a comparable level of total IGF1R protein expression in the patient compared with controls (data not shown); however, autophosphorylation of the IGF1R and activation of PKB/Akt upon a challenge with 10 ng/ml IGF-I for 10 min were reduced (Fig. 3C), although this did not reach significance.

Discussion

Partial IGF-I resistance due to heterozygous deletions or inactivating mutations of the IGF1R gene is a rare cause of short stature. We present a girl with a heterozygous terminal deletion of the long arm of chromosome 15, including the IGF1R gene.

The IGF1R is a tyrosine kinase receptor, consisting of a heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein. Binding of IGF-I to the receptor results in autophosphorylation of three intracellular tyrosine residues and activation of the receptor's intrinsic tyrosine kinase. Subsequently, distinct intracellular signaling pathways are activated, which induce amongst others protein synthesis and glucose transport and regulate cell proliferation, differentiation and apoptosis (26). A deletion of one copy of the IGF1R may result in haploinsufficiency due to lower expression of IGF1R mRNA and protein. Indeed, in dermal fibroblasts of the patient IGF1R mRNA expression was decreased compared with a panel of controls. However, this resulted not in reduced protein expression, diminished IGF-I binding or decreased activation of downstream signaling upon a challenge with IGF-I. Maximal binding and phosphorylation of IGF1R and PKB/AKT tended to be lower but this did not reach significance. Also others have reported inconsistent results regarding IGF1R haploinsufficiency due to a 15q deletion in dermal fibroblasts and it was suggested that dermal fibroblasts were less suited to study the functional consequences of IGF1R haploinsufficiency (6, 10). This contrasts findings in a family with a missense

mutation in the intracellular kinase domain of the IGF1R, which resulted in strongly decreased activation of downstream signaling in dermal fibroblasts (3). It may well be that the consequences of haploinsufficiency are cell type dependend, with little effect in dermal fibroblasts but strong effects in growth plate chondrocytes that are responsible for longitudinal growth.

A pure terminal deletion of 15q, without the presence of a ring chromosome, has only been described in a few cases (5-11). In all cases, except one, the deletion was detected by regular karyotyping. Pinson et al. diagnosed a patient with a terminal 15q deletion unexpectedly: looking for a maternal 15q11-q13 deletion to exclude Angelman syndrome, they accidently observed that the specific telomeric control probe on one chromosome 15 was missing. In our case the deletion was not diagnosed with regular karyotyping, but detected through the MLPA technique. We have developed a MLPA probe-mixture directed against 3 different exons of the IGF1R and several other genes for the evaluation of children with unexplained short stature. In our patient MLPA proved successful in detecting the IGF1R deletion, which was further characterized with aCGH. Since the MLPA assay can be easily extended to include as many as at least 40 different probe sets, this technique is ideally suited for the evaluation of copy number changes of multiple genomic regions simultaneously. We, therefore, expect that these techniques will significantly contribute to the elucidation of genetic causes of unexplained short stature in the future.

Some of the phenotypical features can be attributed to the loss of one copy of the IGF1R gene, while other features are probably the result of the absence of other genes in the deleted region.
In all cases of terminal 15q deletion, except in one child with a diabetic mother (9), IUGR was present. IUGR is also a common feature in patients with inactivating IGF1R mutations (1-4), indicating that IGF1R haploinsufficiency is responsible for the poor prenatal growth. Also, the postnatal growth pattern in our patient and in other cases with this condition is comparable with the growth in patients with a heterozygous inactivating mutation in the IGF1R gene, suggesting that the growth retardation in patients with terminal 15q deletion is the result of partial IGF-I resistance.

Another common feature in patients with partial IGF-I resistance is microcephaly with or without delayed psychomotor development. The normal development in our patient, our earlier observations of a normal intellectual development in an adult woman with a heterozygous missense mutation (3), in combination with the normal intelligence reported for three other cases with IGF1R missense mutations (1, 2, 4) suggest that the degree of developmental delay is predominantly determined by the deletion of other genes on 15q26.2→qter.

The dysmorphic features described in the patients with terminal 15q deletion, including our own patient, mainly include craniofacial characteristics and anomalies of hand and feet. These features are uncommon in patients with IGF1R mutations and probably result from the loss of other genes in the gene-rich 15q subtelomeric region. Our patient had extreme myopia, which has not been reported in other patients with IGF-I resistance. Although the IGF1R is expressed in the lens (27), it is unlikely that myopia is the result of IGF-I resistance, as one would expect to find this more frequently in other patients with IGF-I resistance. We have now shown that the absence of major dysmorphic features does not exclude a terminal 15q deletion.

We have shown that the growth retardation caused by IGF1R haploinsufficiency can successfully be treated with GH: height at start of therapy was -3.5 SDS and final height was -1.6 SDS, within the population range, but 1.8 SD below target height. This observation is partially consistent with the effects of GH therapy in two patients with IGF1R haploinsuffiency (6, 10). One of them (6) was treated from 3.5 years onwards during three periods with different dose regimes. During the first two periods catch-up growth was observed, but this was not sustained and at the age of 12 years height was -5.5 SDS, similar to height SDS at start. Okubo et al. (10) treated a patient with 1.7 U/day from the age of 22 months resulting in improved growth (-4.9 SDS to -3.2 SDS at the age of 9 years). The result of two years of GH treatment (30 µg/kg/day) administered to a patient with an inactivating mutation of the IGF1R gene was reported by Raile et al (28). Height SDS increased from -2.5 to -1.5. Thus, we conclude that patients with partial IGF-I resistance due to heterozygous IGF1R deletions or mutations may benefit from GH therapy in terms of longitudinal growth, possibly due to the direct effect of increased concentrations of GH in combination with strongly elevated plasma IGF-I levels that may partially overcome the decreased IGF-I sensitivity.

In conclusion, the characteristics of a heterozygous terminal 15q deletion are dominated by partial IGF-I resistance, due to IGF1R haploinsufficiency, while the dysmorphic features and the developmental delay are probably the result of haploinsufficiency of other genes in this chromosomal region. GH treatment considerably improves growth during childhood and leads to a normal adult height, though still below the genetic target. A normal karyogram in patients with features suggestive for IGF-I resistance does not exclude small deletions. MLPA followed by aCGH is a powerful diagnostic strategy to detect submicroscopic deletions in children with short stature.

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