Successful long-term growth hormone therapy in a girl with haploinsufficiency of the IGF-I receptor due to a terminal 15q26.2 -> qter deletion detected by multiplex ligation probe amplification

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Abstract

Context: Microscopically visible heterozygous terminal 15q deletions encompassing the IGF1R gene are rare and usually associated with intrauterine growth retardation and short stature. The incidence of submicroscopic deletions is unknown, as well as the effect of growth hormone (GH) 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: A 15 yr old adolescent, born small for gestational age (SGA) showed persistent postnatal growth retardation, microcephaly, and elevated IGF-I levels. She had been treated with GH since the age of 5 yr.

Methods: MLPA and array Comparative Genomic Hybridization (aCGH) were performed to examine gene copy number changes. Dermal fibroblast cultures were used for functional analysis.

Results: With MLPA, a deletion of one copy of the IGF1R gene was detected, defined by aCGH as a loss of 15q26.2->qter. IGF1R mRNA expression was decreased in fibroblasts. IGF-I binding and IGF1R protein expression, as well as activation of IGF1R autophosphorylation and PKB/Akt by IGF-I tended to be lower but this did not reach statistical significance. GH treatment resulted in a good growth response and a normal adult height.

Conclusions: MLPA and aCGH are useful tools to detect submicroscopic deletions of the IGF1R gene in patients born SGA with persistent growth failure. The phenotype resembles that of a heterozygous inactivating IGF1R mutation. Long term GH therapy causes growth acceleration in childhood and a normal adult height.
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), 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) and dysmorphic features (1, 3).

Microscopically visible heterozygous terminal 15q deletions encompassing the IGF1R gene have been reported in only a few cases: 6 patients with a 15q26.1 -> 15qter deletion (5-9) and 3 patients with a 15q26.2 -> 15qter deletion (10-12). A low birth size was present in almost all cases, birth weights varying between -1.8 SDS and –5.6 SDS and birth lengths between -1.3 SDS and -5.5 SDS.

We report a female patient with pre- and postnatal growth failure and elevated plasma IGF-I levels with a microscopically normal karyotype, in whom multiplex ligation-dependent probe amplification (MLPA) and array-comparative genomic hybridization (aCGH) showed that 15q26.2->qter was deleted, including the IGF1R gene.

Methods

The patient and her parents provided written informed consent for clinical and genetic studies. Height, sitting height, weight and head circumference were measured with standard equipment and expressed as standard deviation score (SDS) (13). GH reserve was assessed with an arginine test and clonidine test and GH was measured with Immulite 2000 (DPC, Los Angeles, CA) using the WHO NIBSC 1st international
standard 80/505 (1mg=2.6 IU). Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1 and IGFBP-3 were determined by specific RIAs, as reported earlier (3).

**Genetic analysis**

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, (14) and the BAC clone 342L10 (RP11-342L10, "Cancer_1E9", located in 15q26.3, BACPAC Resource Center (http://bacpac.chori.org/order.php).

Total RNA was isolated from cultured fibroblasts and reverse transcribed into cDNA. Genomic DNA was isolated from whole blood and 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 White *et al* (15) and were directed to exons 2, 8 and 18 of the IGF1R gene, which are conserved exons in different species (detailed information on the probes can be obtained from the authors). The oligonucleotide probes were ordered from Illumina Inc. (San Diego, CA) and used without purification. All reagents for the MLPA were obtained from MRC Holland (Amsterdam, the Netherlands). Reactions and data analysis were performed according to White *et al*. (15) and the manufacturer’s instructions.

**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
These arrays contain $43 \times 10^3$ 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 2 healthy donors (a 29 year old female and 48 year old male) were used for Western blotting (we have not found evidence that IGF1R expression and responsiveness are dependent on age, passage number and sex). Cells were stimulated for 10 min with or without 10 ng/ml IGF-I. Blots were probed with an anti-phospho-Protein kinase B (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 (16). 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) (17). In short, fibroblasts of the patient and 6 controls (between 5 and 59 years old) were incubated at 4°C with 30,000 cpm $[^{125}\text{I}]$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, as previously described (17). After 18 h, cells were washed and solubilized in 1 M NaOH. Radioactivity was determined using a $\gamma$-counter.
Results

Case report

A 4.5 year old girl presented with severe growth retardation (height -3.5 SDS). She was born after 39 wk gestation as the third child of healthy unrelated parents after an uneventful pregnancy, with a weight of 2.1 kg (-3.0 SDS), a length of 47 cm (-1.3 SDS), and head circumference of 33 cm (-2 SDS)(18). Target height corrected for secular trend (4.5 cm per generation) was 171.8 cm (0.2 SDS) (13) and 2 sibs had a normal height (0 and +1.3 SDS). Motor development was normal (she started sitting at the age of 10 months and walking at 17 months), but speech development was slow.

Body proportions were normal and body mass index was 17 kg/m$^2$ (1.0 SDS). She had a high-pitched voice, 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.

Celiac disease, hypothyroidism and other possible causes of growth retardation were excluded and the karyogram was normal (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). Two growth hormone (GH) stimulation tests (clonidine and arginine) resulted in peaks of 8.4 and 25 mU/L. After 4 daily GH injections (1 mg/m$^2$ body surface, Humatrope, Lilly, Indianapolis, IN) IGF-I rose from 224 to 375 ng/ml, after another 3 days of GH (2 mg/m$^2$) IGF-I was 480 ng/ml. Bone age was delayed by 2.5 years.
At 5.3 years GH therapy was started (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 a height of -2 SDS, while IGF-I was approximately +3.5 SDS. Lean body mass SDS increased to –0.96. Puberty started at the age of 12.8 years and at 15 years she reached her adult height of 157 cm (-1.6 SDS, 1.8 SD lower than her target height SDS). Head circumference was 53 cm (-1.1 SDS). She had a regular menstrual cycle and 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 all 3 IGF1R exons (2, 8 and 18) included in the MLPA assay (Fig. 1), which was not present in parental DNA. FISH confirmed the IGF1R deletion and 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.

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. 2A). 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 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. 2B). Western blot demonstrated that total IGF1R protein was comparable to controls (data not shown), and autophosphorylation of the IGF1R and activation of protein kinase B (PKB/Akt) upon a challenge with 10 ng/ml IGF-I for 10
min tended to be lower than controls, but this did not reach statistical significance (Fig. 2C).

**Discussion**

This case report confirms that the phenotype of a heterozygous IGF1R deletion is similar to that of a heterozygous IGF1R mutation and shows that MLPA is an efficient technique to detect IGF1R haploinsufficiency. We demonstrate that short children with this condition respond well to GH treatment.

The clinical presentation of this patient (SGA, persistent short stature, microcephaly and elevated IGF-I) is similar to that of the rare cases with heterozygous IGF1R mutations (1-4). Children with a pure terminal deletion of 15q, without the presence of a ring chromosome (5-12) have a similar growth phenotype. Because deletions of the IGF1R have not been observed in healthy controls, we believe that this phenotype is caused by IGF1R haploinsufficiency. Children with a terminal deletion of IGF1R often have additional abnormalities and we propose that these are due to the defects in other genes in the deleted region. For example, the normal development in our patient, in the adult woman with a heterozygous missense mutation (3), and 3 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 15q26.1->qter. Similarly, the dysmorphic features described in the patients with terminal 15q deletion, such as craniofacial characteristics and anomalies of hand and feet, are uncommon in patients with IGF1R mutations. Our patient had extreme myopia, which have not been reported in other patients with IGF-I resistance.
In all reported cases with 15qter deletion, except one (11), the deletion was detected by regular karyotyping. In our case the deletion was not diagnosed with regular karyotyping, but it was detected through MLPA, and further characterized with aCGH. Since the MLPA assay can be easily extended to include as many as 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 this technique will significantly contribute to the elucidation of genetic causes of unexplained short stature in the future, and propose that children presenting with the phenotype of IGF-I resistance should be tested with MLPA, specific for IGF1R, followed by sequencing of the gene.

In vitro, a deletion of 1 copy of the IGF1R would be expected to result in 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, we did not observe reduced protein expression, and the tendency towards a diminished IGF-I binding and decreased activation of downstream signaling upon a challenge with IGF-I did not reach statistical significance. This is in line with the inconsistent results reported earlier, suggesting that dermal fibroblasts may be a suboptimal model to study the functional consequences of IGF1R haploinsufficiency (6, 10). In contrast, in the family with a missense mutation in the intracellular kinase domain of the IGF1R we observed a strongly decreased activation of downstream signaling (3). A possible explanation of this discrepancy is a dominant-negative effect of the mutation, resulting in a more pronounced effect in vitro; alternatively the consequences of haploinsufficiency may be cell type dependent, with possibly a
relatively strong effect in growth plate chondrocytes, or the fibroblast model may be too insensitive and variable to pick up statistically significant differences.

We have shown that the growth retardation caused by IGF1R haploinsufficiency can successfully be treated with GH, although full catch-up was not reached. This partially confirms earlier reports (6, 10, 19) and we suggest that this positive effect may be due to direct GH effects 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. GH treatment considerably improves growth. A normal karyogram in patients with features suggestive for IGF-I resistance does not exclude small deletions, and MLPA is a powerful diagnostic strategy to detect submicroscopic deletions.
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novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90:2855-2864


Figure legends

Figure 1 Genescan of the MLPA analysis of the IGF1R gene

The arrows in the lower panel show the lower peaks of the three IGF1R probes in the patient compared to the control in the upper panel, indicating a deletion of IGF1R.

Figure 2: 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 4 controls (aged 29-59 years).

Scatchard analysis was performed for the calculation of the binding affinity ($K_d$) and binding capacity ($B_{max}$) of the patient’s cells and 6 controls (5-59 years). The $K_d$ and $B_{max}$ of our patient showed no significant differences compared with controls. Values represent the mean of two quadruplicate experiments ± SD.

C. Dermal fibroblasts of the patient and 2 controls (29 and 48 years) 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.
Figure 1
Figure 2

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