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Familial short stature and intrauterine growth retardation associated with a novel mutation in the IGF-I receptor gene

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Complete List of Authors:	Labarta-Aizpún, José I.; Hospital Infantil Miguel Servet, Pediatrics and Endocrinology Unit Barrio, Eva; Hospital Infantil Miguel Servet, Molecular Genetics Audi, Laura; HOSPITAL VALL D'HEBRON, PEDIATRIC ENDOCRINOLOGY Fernández-Cancio, Mónica; Hospital Vall d'Hebron, Paediatrics; HOSPITAL VALL D'HEBRON, PEDIATRIC ENDOCRINOLOGY Andaluz, Pilar; Hospital Vall d'Hebron, Paediatrics De Arriba, Antonio; Gobierno de Aragón, Fundación Andrea Prader Puga, Beatriz; Gobierno de Aragón, Fundación Andrea Prader Calvo, María T.; Hospital Infantil Miguel Servet, Molecular Genetics Unit Mayayo, Esteban; Hospital Infantil Miguel Servet, Pediatrics and Endocrinology Unit Carrascosa, A; Hospital Vall d'Hebron, paediatrics Ferrández-Longás, Angel; Hospital Infantil Miguel Servet, Pediatrics and Endocrinology Unit
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Authors: José I. Labarta [1], Eva Barrio [2,3], Laura Audí [4], Mónica Fernández-Cancio [4], Pilar Andaluz [4], Antonio de Arriba [1,3], Beatriz Puga [3], María T. Calvo [2], Esteban Mayayo [1], Antonio Carrascosa [4], Angel Ferrández-Longás [1].

[1] Endocrinology Unit. Department of Pediatrics. [2] Molecular Genetics Unit. Hospital Infantil Universitario "Miguel Servet". Zaragoza. Spain.

[3] "Andrea Prader" Center of Growth and Development. Government of Aragón, Zaragoza. Spain.

[4] Department of Pediatrics and Pediatric Endocrinology Research Unit. Vall d'Hebron Institut de Recerca (VHIR), Hospital Vall d'Hebron. Autonomous University of Barcelona. CIBERER (Center for Biomedical Research on Rare Diseases), Instituto de Salud Carlos III. Barcelona. Spain.

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Corresponding author:

Dr. José I. Labarta. Endocrinology Unit. Department of Pediatrics. Hospital Infantil Universitario "Miguel Servet". Zaragoza 50009. Spain. Phone: + 34 976 765500 (ext 3475). Fax: + 34 976 765633. e-mail: jilabarta@salud.aragon.es

José I. Labarta and Eva Barrio contributed equally to the manuscript.

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ABSTRACT

Context. IGF-I is essential for normal human growth and mediates its effects through the IGF-I receptor (IGF1R). IGF1R mutations have been associated with varying degrees of intrauterine and postnatal growth retardation.

Objective. To identify *IGF1R* gene mutations in a short-statured family with intrauterine growth retardation, microcephaly and normal IGF-I serum concentrations.

Methods. Direct DNA sequencing was used to identify *IGF1R* mutations. Multiplex ligation dependent probe amplification (MLPA) analyses were performed for deletions and duplications of all *IGF1R* exons. Functional studies were performed to assess its pathogenicity.

Results. A novel heterozygous *IGF1R* missense mutation in exon 7 (c.A1549T, p.Y487F) was identified in a short-statured girl with severe prenatal growth retardation and microcephaly. The same mutation was also identified in her mother, who presented prenatal and postnatal growth failure, and her short-statured maternal grandmother, both exhibiting microcephaly. The index case experienced partial response to rhGH. Functional studies performed in dermal fibroblasts from the index case and her mother showed normal binding of IGF-I but IGF-I activation of intracellular signalling measured as AKT and ERK phosphorylation were markedly reduced, with child values being lower than those of the mother. IGF-I stimulation of DNA synthesis was significantly reduced compared to controls.

Conclusion. Our results show a novel missense mutation in the *IGF1R* gene (c.A1549T, p.Y487F) associated with prenatal and postnatal growth failure and microcephaly in the context of familial short stature. The functional studies are in line with the inactivation of one copy of the *IGF1R* gene with variable expression within the same family.

INTRODUCTION

IGF-I is essential for prenatal and postnatal growth and development. The mitogenic effects of IGF-I are mediated through the IGF1R, a cell-surface tyrosine kinase receptor encoded by the *IGF1R* gene (15q26.3) (1). Ligand binding to IGF1R leads to receptor autophosphorylation on several intracellular tyrosine residues and activation of the receptor intrinsic tyrosine kinase, resulting in recruitment of cytoplasmic components of multiple downstream signaling cascade, including the phosphatidylinositol 3-kinase PI3K/Akt and MAPK/Erk pathways (2).

The role of IGF1R in human growth and development was first studied in mouse models (3,4). Knockout of the gene for IGF1R led to severe decrease in fetal size and death shortly after birth. In contrast, targeted partial invalidation of the IGF1R gene in mice causes mild pre- and post-natal growth retardation (5).

The underlying causes of intrauterine and postnatal growth retardation are not completely elucidated. Homozygous deletions or mutations in the *IGF-I* gene causes severe intrauterine and postnatal growth retardation and developmental delay (6,7). The possibility that abnormalities of *IGF1R* could result in some combination of intrauterine and postnatal growth failure was first supported by observations in patients with deletions of chromosome 15q and consequent haploinsufficiency for *IGF1R*. Patients suffering from heterozygous inactivating *IGF1R* mutations display varying degrees of intrauterine and postnatal growth retardation with a variable degree of mental retardation and dysmorphic features (8-19).

We report a novel heterozygous *IGF1R* gene point mutation (c.A1549T) predicting p.Y487F, identified in a family with severe short stature, microcephaly and intrauterine growth retardation. Functional studies argue for the pathogenicity of the mutation.

PATIENTS AND METHODS

Clinical and auxological evaluation

The Ethics Committee of the Children's University Hospital Miguel Servet approved all clinical studies and parents gave informed consent for the studies. Height was determined with Harpenden stadiometer and weight and head circumference were measured with standard equipment. Psychomotor development was evaluated with Brunet-Lezine (< 2 years) and Mc Carthy (3-6 years) tests. Results are expressed in SD score (SDS) based on Spanish Longitudinal Study of Growth and Development (20). Bone age was evaluated by the method of Greulich and Pyle (21).

Biochemical measurements

Plasma GH was measured by a solid-phase two-site chemiluminescent immunometric assay (Immulite, 2000 Siemens). Plasma IGF-I and IGFBP-3 were determined by a specific solid-phase enzyme-labeled chemiluminescent immunometric assay (Immulite 2000, Siemens). Serum glucose, hepatic, renal, thyroid function and insulin were measured by standard techniques in the hospital laboratory.

Genetic studies

Samples

We have screened 91 children with short stature. These children presented intrauterine growth restriction, defined as a birth weight and/or length less than mean -2.0 SDS for gestational age, familial short stature and normal IGF-I and IGFBP-3 serum levels. We also have studied 126 control samples.

Gene analysis

Genomic DNA was extracted from peripheral blood by standard procedures. The entire coding and intronic flanking sequences of *IGF1R* were amplified by PCR using total DNA from whole blood. The amplified products were purified and directly sequenced using an automated DNA sequencer (AbiPrism 310, Applied Biosystems, Foster City, CA). Exon 7 of the *IGF1R*

gene was amplified by PCR, purified and sequenced using genomic DNA as template in 126 controls and family samples. Sequences of oligonucleotides used for amplification and sequencing were designed using the programmes Primer3 and Xprimer.

MLPA analyses

MLPA analyses were performed using the IGF1R probe kit (SALSA MLPA P217) commercially available from MRC-Holland, Amsterdam, Netherlands. This set of probes allows screening for deletions and duplications of all IGF1R exons, in two PCR reactions. MLPA reactions were executed using the manufacturer´s protocol with minor modifications (22). Dosage ratio values of < 0.5 and > 1.5 were used as boundaries for deletions and duplications respectively (23).

Functional studies

Dermal fibroblast culture

A punch skin biopsy was obtained with informed consent from the child and her mother. Fibroblasts were grown from explants and cultured in MEM with 10% FBS. Dermal fibroblasts from normal controls of similar chronological age were obtained from patients undergoing minor surgery. All cells were subcultured up to the 8th passage when all experiments were performed. Mean doubling time of cells from the index patient and her mother was extremely long, thus 6 months were required to obtain sufficient cells at the 8th passage.

IGF-1 binding assay

Eighth-passage confluent fibroblasts from each of the 4 different cultures were trypsinized and plated in 12-microwell tissue culture plates at a density of 100,000 cells/well in 1 ml MEM with 10% FBS. After 72h, the medium was aspirated, fibroblasts washed twice with 1 ml of serum- and antibiotic-free medium and incubated for 16h at 4ºC with 1 ml of serum and antibiotic-free medium containing ^{125} I-IGF-1 (120,000 cpm; 0.06 x 10^{-9}M) with/without increasing concentrations of IGF-1 (0.13 to 130 x 10^{-9}M). Four different wells were prepared for

each condition. At the end of the incubation period, media were aspirated, cells washed twice with 1 ml of serum- and antibiotic-free medium and cells solubilized in 1 ml of NaOH 1N at 37ºC for 1 h; 0.5 ml were transferred to a gamma counter and the rest kept at -20ºC for protein content determination. Results were expressed as cpm specifically bound and as femtomoles bound per mg of protein; maximal binding capacity (Bmax) and affinity (Kd) were calculated according to Scatchard analysis.

IGF1R, AKT and ERK phosphorylation by IGF-1

IGF1R phosphorylation stimulation by IGF-1 was measured in cell extracts from 12-well plate cultures: 100,000 cells per well were plated, 6 wells per culture (child control, child Y487F, mother control and mother Y487F), with 1 ml MEM and 10% FBS; cells were incubated for 48h, then for a further 24h with 1 ml MEM and 0.5% FBS followed by stimulation with increasing amounts of IGF-1 (0-10-25-50-100 ng/ml) for 10 min at 37ºC. Cells were washed with ice-cold PBS, scraped in 1 ml of ice-cold RIPA lysis buffer (Millipore®) containing pepstatin A, aprotinin, leupeptin and PMSF, incubated in ice for 15 min, vortexed for 10 sec and centrifuged for 10 min at 12000 rpm at 4ºC: extract aliquots were frozen at -80ºC for total protein, total IGF1R and phospho-IGF1R measurement. Total IGF1R (beta subunit) and phospho(Tyr1135/1136)-IGF1R were measured by commercial ELISA kits (Millipore®) following the manufacturer's instructions. The ratio of phospho-IGF1R to total IGF1R indicated the relative proportions in each extract. AKT and ERK phosphorylation stimulation by IGF-1 was measured with two commercial, cell-based fluorogenic ELISAs (Millipore®): AKT (Ser473) Dual Detect CELISA assay kit and ERK 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) Dual Detect CELISA assay kit. Briefly, 20,000 cells/well were plated in the 96-microwell plate supplied in each kit. Four wells were plated for each condition and each culture [child control, child (Y487F), mother control and mother (Y487F)]. Cells were incubated at 37ºC with 0.1 ml MEM and 10% FBS for 16h followed by a further 16h with 0.1 ml MEM and 0.5% FBS followed by stimulation with increasing amounts of IGF-1 (0-10-25-50-100 ng/ml) in 0.1 ml of serum- and antibiotic-free MEM for 10 min for the AKT plate and 15 min for the ERK plate.

The procedures indicated by the manufacturer were followed and phosphorylated AKT (Ser473) and ERK 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) contents were read at 460 nm emission whereas total AKT and ERK 1/2 were read at 590 nm. Background without antibody was subtracted in each well for each culture. The 460/590 ratio in each well indicated the relative amount of phosphorylated protein with respect to the amount of total protein.

^{3}H-Thymidine incorporation into DNA

Eighth passage confluent fibroblasts were trypsinized and plated in 96-microwell tissue culture plates at a density of 20,000 cells/well in 0.3 ml of MEM serum- and antibiotic-free medium. After 48h, the medium was aspirated and fibroblasts were incubated for 48h with IGF-1 at increasing concentrations (10 to 100 ng/ml) or FBS (10%). Control wells were incubated with medium containing the same amount of BSA (0.1 %) as under IGF-1 conditions. [^{3}H]-thymidine (5 µCi/ml) was added for the last 24h of incubation. Four different wells were prepared for each condition. Incubations were terminated by medium aspiration, followed by extensive cell washing with 2.5% acetic acid in distilled water. Fibroblasts were then treated with 0.5% Triton X-100 (250 µl/well; 10 min) and collected in a multiple cell harvester on glass fiber filters. The filters were dried and counted in 5 ml Biodegradable Counting Scintillant (Amersham Biosciences, UK) in a Beta-Counter (BECKMAN LS650 Multi-purpose Scintillation Counter) for 5min. The result for each well was expressed in cpm and, for each condition, as a percentage of mean radioactivity in four wells compared with the control condition (100%).

Statistical analysis

Comparison of AKT and ERK phosphorylation and cell proliferation stimulation by IGF-1 among the heterozygous Y487F carriers and controls was performed by ANOVA (Bonferroni/Dunn test) using StatView 4.5 software (SAS, Cary, NC).

RESULTS

Case reports

Patient A is now a 7.5 year-old girl who was sent to our Unit when she was 1.5 years of age for evaluation of short stature. She was born at 37 ^{6} weeks of gestational age and her birth weight was 1960 grs (-3.46 SDS), length 43 cms (-4.91 SDS) and head circumference 28 cms (-5.7 SDS). Apgar score was normal. Her pregnancy was uneventful except for gestational diabetes type A1. Intrauterine growth retardation was diagnosed at third term of gestation. The placenta was reported to be small with low weight.

She was a proportionate short-statured girl, her length was 73.3 cms (-2.84 SDS), weight 7.070 kgrs (-3.4 SDS), head circumference 43 cms (-3.37 SDS) and bone age 9 months. Other possible causes of growth retardation were excluded. Poor appetite was detected along infancy. Psychomotor development was evaluated and her developmental quotient was in the normal range but slightly retarded in the 10th-25th centile. Postnatal growth retardation was detected and at 2 years of age her height was -3.44 SDS, weight -3.53 SDS, BMI -2.83 SDS and head circumference was -3.36 SDS. She showed normal random GH serum levels (7.6 ng/mL) with low IGF-I serum levels (16 ng/mL) and normal IGFBP-3 (1.62 μg/mL). At 3.2 years of age she had normal IGF-I and IGFBP-3 serum levels (62 ng/mL and 2.27 μg/mL, respectively). At 3.4 years old her height was 87.4 cms (-3.19 SDS), weight 9.6 kg (-3 SDS) and she initiated treatment with rhGH (0.035 mg/kg/day). Clinical characteristics and growth response of index case is presented in Table 1.

Patient B (mother of index case) was evaluated at 36 years of age and her height was 154 cms (-1.6 SDS), BMI was 16.6 kg/m^2 and her head circumference was 52 cms (-2.5 SDS). Her birth weight was 2.200 gr (-2.8 SDS) and birth length 46 cms (-2.6 SDS). Her menarche was at 13 years of age. Random analysis showed serum levels of GH of 14.9 ng/mL, IGF-I of 199 ng/mL, IGFBP-3 of 2.88 μg/mL, with normal thyroid function, insulin and glucose values. Lumbar DEXA scan showed a T value of -1.6 SDS (0.988 gr/cm2) and femoral neck DEXA

scan showed a T value of -1.8 SDS (0.784 gr/cm2). Father´s height of the index case was 171 cms (-0.83 SDS) and target height was 155.3 cms (-1.34 SDS). Short stature was noted on the maternal side of the family (Figure 1A). At 61 years of age maternal grandmother´s height (Patient C) was 148.3 cms (-2.6 SDS), BMI 25.4 kg/m^2 and head circumference 51.5 cms (-2.9 SDS). She had menarche at 14.5 years of age. Mother and maternal grandmother´s intelligence quotient were completely normal.

Genetic studies

Index case showed the missense point mutation in exon 7 (c.A1549T, p.Y487F) in heterozygosis (change of thyrosine for phenylalanine in codon 487); this mutation in exon 7 affects the extracellular domain of IGF1R (α-subunit). This mutation was also observed in heterozygosis in the mother and the maternal grandmother, both affected of short stature, and was not found in controls (n=126) nor in the father or the mother´s sister, both with normal stature (Figure 1B). We have analyzed the index case and mother, father, aunt, maternal grandmother and greatgrandaunt for large genomic rearrangements in *IGF1R* gene by MLPA and no deletions or duplications were observed in the cases studied.

Functional studies

IGF-1 binding assay

^{125}I-IGF-1 binding displacement by IGF-1 was similar in all four cultures (Figure 2). Maximal binding capacity (Bmax) and dissociation constant (Kd) were similar in child control and mutated index case (646 and 639 x 10^{-15} M/mg protein and 0.23 and 0.32 x 10^{-9} M/L, respectively) as they were similar in mother control and mutated mother (721 and 756 x 10^{-15} M/mg protein and 0.32 and 0.38 x 10^{-9} M/L, respectively).

IGF1R, AKT and ERK phosphorylation by IGF-1

Although both the heterozygous mutated cell cultures from the child and her mother increased in relative phospho(Tyr1135/1136)-IGF1R content with increasing IGF-1 concentrations, levels were lower than those in the respective controls (Figure 3, I). Relative amounts of baseline phosphorylated AKT and ERK were significantly reduced in the child

(Y487F) (B) compared with the child control (A) ($p<0.01$) and with the mother (Y487F) (D) ($p<0.01$) (Figure 3, II and III). AKT and ERK phosphorylation was significantly stimulated in each culture by IGF-1 from 10 ng/ml, reaching maximum values for ERK with IGF-1 at this concentration and at 25-100 ng/ml for AKT. However, values in both the child (Y487F) (B) and the mother (Y487F) (D) were significantly lower than those of the respective controls (A and C) for AKT ($p<0.05$), with the child values being lower than those of the mother ($p<0.05$). Although ERK phosphorylation stimulation by IGF-1 was more intense in the child (Y487F) (B) compared with the child control (A), relative values were significantly lower ($p<0.05$). The mother (Y487F) (D) values did not differ significantly compared with the mother control (C) for ERK phosphorylation stimulation by IGF-1 (Figure 3, III).

^{3}H-Thymidine incorporation into DNA

Statistically-significant differences were observed between conditions and among cultures. At baseline (control), "mother control" incorporated significantly less than the "child control" ($p=0.004$), and the "mother Y487F" significantly less than the "mother control". No significant increase was observed in any culture for IGF-1 at 10 ng/ml whereas increases were significant compared with the "control condition" in all cultures with IGF-1 at 50 and 100 ng/ml; however, statistically-significant differences were observed among the cultures: the child heterozygous mutated cell ("child Y487F") increases were significantly lower than those of the "child control" ($p=0.02$ for IGF-1 at 50 ng/ml and $p=0.0007$ for IGF-1 at 100 ng/ml; 1.76 vs. 2.96-fold for IGF-1 at 100 ng/ml) and similar differences were observed between the "mother Y487F" cells and those of the "mother control" (3.37 vs. 4.79-fold for IGF-1 at 100 ng/ml). Similar differences among the cultures were observed for the condition in which 10% FBS was added (3.89 vs. 6.90-fold and 4.83 vs. 6.43-fold for the mutated vs. the control, child and adult cultures, respectively) with all increases being significantly higher than with IGF-1 at 100 ng/ml (Figure 4).

DISCUSSION

In this study we present a novel heterozygous *IGF1R* mutation (p.Y487F) in three short-statured members of the same family with a variable clinical expression. The functional studies showed the pathogenic effect of the new mutation that resulted in partial inactivation of one allele resulting in haploinsufficiency and concomitant reduction of IGF1R signaling pathway.

The *IGF1R* mutation Y487F affects the extracellular domain owing to a change from tyrosine to phenylalanine, i.e. a polar to a non-polar aminoacid. Because of the charge change it seems likely that the mutant receptor cannot preserve its active conformation and consequently the substitution could result in IGF1R inactivation. This mutation is close to the first disulfide bond (Cys514) between the two IGF1R α-subunits and it may be hypothesized that the dimerization process may be disturbed.

The *in vitro* functional study was carried out in cultured dermal fibroblasts from the patient and her mother and results were compared with those of normal controls. No modification in IGF-I binding characteristics could be observed, thereby demonstrating that the heterozygous expression did not affect the process of IGF-1 binding by the receptor *in vitro*. However, IGF1R autophosphorylation and downstream signaling pathway protein phosphorylation stimulation by IGF-1 was significantly decreased in both heterozygous carriers. Although IGF1R autophosphorylation and AKT and ERK phosphorylation significantly increased under graded IGF-I concentrations, both baseline levels and those reached under stimulation were significantly decreased. In parallel with the phenotype, the lowering of response to IGF-I was more severe in the child. The functional study of an *IGF1R* mutation in the same domain as that found in our patients was conducted by Inagaki *et al* (12) by transfecting the wild-type and the R481Q mutated IGF1R allele into NIH3T3 fibroblasts and similar results were observed. Cell proliferation stimulation was also shown to be affected by the heterozygous expression of the mutated Y487F protein. This reduction in cell proliferation in response to IGF-I administration indicates that reduced activation of IGF1R signaling ultimately affects IGF-I-dependent cell growth as it has been shown in other mutations (11-13).

The patients we present have several manifestations associated with IGF-I resistance such as prenatal and postnatal growth failure, microcephaly, delayed bone maturation, low placental weight, normal IGF-I serum levels and marginal response to rhGH. The degree of growth failure in patients with *IGF1R* mutations is variable; it appears that patients with an affected mother are more severely prenatally growth retarded (11) than if the affected member is the father (16) or if there are no familial antecedents. It could be argued that maternal IGF-I resistance during pregnancy could affect placental size and as a consequence fetal growth (11) as it was observed in our case where the placenta was reported to be diminished. This hypothesis is supported by the finding that placentas from intrauterine growth retardation pregnancies are characterized by decreased expression of IGF-I and IGF1R and signal transduction proteins (24-25) as we have previously reported (26). The family we present showed variability regarding prenatal and postnatal growth failure with the index case being more severely affected than her mother. The mother and maternal grandmother showed a moderate delay in menarche as it has been found in other patients (11,14,17). Osteoporosis has not been reported in the cases studied and our observation is in agreement with this finding (11).

IGF-I deficiency caused by homozygous *IGF1* gene mutation has been associated with microcephaly and developmental delay (6,7,27). In contrast, the impact of IGF-I resistance on mental development is variable (9). The index case and the rest of the family members affected showed microcephaly without intellectual delay. This is in contrast with the presence of a below average psychomotor development and speech retardation in some patients with IGF1R mutation (8-10,15). Opposite to patients with genetic IGF-I deficiency, audition is not affected in patients with heterozygous *IGF1R* mutations reflecting that partial impairment of IGF-I signalling is sufficient for normal development of the inner ear.

We observed normal IGF-I serum levels that increased when treated with rhGH. Even in patients with IGF1R deletion, IGF-I has been reported to be low in early infancy (28). Nutritional disorders and poor food intake is frequent in patients with *IGF1R* mutations and this

may explain the low or normal levels of IGF-I observed in some cases (11,12) which normalizes after realimentation.

The functional studies of each of the heterozygous *IGF1R* mutation reported, including the novel mutation presented in this study, indicate that higher amounts of IGF-I are needed to achieve levels of Tyr-phosphorilated IGF1R equivalent to those of the control and that increased concentrations of IGF-I may lead to normalizing activation of the IGF1R pathway. This observation indicates that rhGH and/or rhIGF-I treatments may confer a potential of benefit to these patients. In the reported cases treatment with rhGH have showed inconsistent results. While some of them have not result in significant catch up growth (8,12,13,15) other have shown modest short-term response (10,16) or satisfactory long-term response as is the case of a patient with IGF1R haploinsufficiency due to terminal 15q26.2 deletion (29). The index case was treated with rhGH showing partial response. The final outcome of rhGH therapy may depend not only on the dose used, as pharmacological doses are needed to overcome IGF-I resistance, and duration of therapy but also on the specific impact that each heterozygous *IGF1R* mutation may have on cell growth and on the genetic background of the patient (30).

In summary, in this study we describe a novel *IGF1R* mutation associated with partial IGF-I resistance in the context of familial short stature associated with intrauterine growth retardation. *In vitro* experiments argue for its functional impact by impairing IGF-I signal transduction suggesting that the mutation results in inactivation of one copy of the *IGF1R* gene. We support the concept of analyzing *IGF1R* gene when facing short statured-children associated with intrauterine growth retardation and microcepahly, especially if familial antecedents are present in the maternal side, with normal or elevated levels of IGF-I and less than expected response to rhGH treatment.

REFERENCES

1. Adams, T.E., Epa, V.C., Garret, T.P., *et al.* (1990) Structure and function of the type 1 insulin-like growth factor receptor. *Cellular Molecular Life Sciences*, **57,** 1050-1093.
2. Le Roith, D., Werner, H., Beitner-Johnson, D., *et al*. (1995) Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocrine Reviews*, **16,** 143-163.
3. Walenkamp, M.J. & Wit, J.M. (2007) Genetic disorders in the GH-IGF-I axis in mouse and man. *European Journal of Endocrinology*, **157,** suppl 1, 15-26.
4. Barker, J., Liu, J.P., Robertson, E.J., *et al*. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, **75,** 73-82.
5. Holzenberger, M., Leneuve, P., Hamard, G., *et al*. (2000) A targeted partial invalidation of the insulin-like growth factor I receptor gene in mice causes a postnatal growth deficit. *Endocrinology*, **141,** 2557-2566.
6. Woods, K.A., Camacho-Hubner, C., Savage, M.O., *et al*. (1996) Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth fator-I gene. *New England Journal of Medicince*, **335,** 1363-1367.
7. Walenkamp, M.J., Karperien, M., Pereira, A.M., *et al*. (2005) Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. *Journal of Clinical Endocrinology and Metabolism*, **90,** 2855-2864.
8. Abuzzahab, M.J., Schneider. A., Goddard, A., *et al*. (2003) IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. *New England Journal of Medicince*, **349,** 2211-22.
9. Kawashima, Y., Kanzaki, S., Yang, F., *et al*. (2005) Mutation at cleavage site of insulin-like growth factor receptor in a short stature child born with intrauterine growth retardation. *Journal of Clinical Endocrinology and Metabolism*, **90,** 4679-4687.
10. Raile, K., Klammt, J., Schneider, A., *et al*. (2006) Clinical and functional characteristics of the human Arg59Ter insulin-like growth factor I receptor (IGF1R) mutation:

implications for a gene dosage effect of the human IGF1R. *Journal of Clinical Endocrinology and Metabolism*, **91,** 2264-2271.

11. Walenkamp, M.J., Van der Kamp, H.J., Pereira, A.M., *et al*. (2006) A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the insulin-like growth factor I receptor. *Journal of Clinical Endocrinology and Metabolism*, **91,** 3062-3070.
12. Inagaki, K., Tiulpakov, A., Rubtsov, P., *et al*. (2007) A familial insulin-like growth factor-I receptor mutant leads to short stature: clinical and biochemical characterization. *Journal of Clinical Endocrinology and Metabolism*, **92,** 1542-1548.
13. Fang, P., Schwartz, I.D., Johnson, B.D., *et al*. (2009) Familial short stature caused by haploinsufficiency of the insulin-like growth factor I receptor due to nonsense-mediated messenger ribonucleic acid decay. *Journal of Clinical Endocrinology and Metabolism*, **94,** 1740-1747.
14. Kruis, T., Klammt, J., Galli-Tsinopoulou, A., *et al*. (2010) Heterozygous mutation within a kinase-conserved motif of the insulin-like growth factor I receptor causes intrauterine and postnatal growth retardation. *Journal of Clinical Endocrinology and Metabolism*, **95,** 1137-1142.
15. Wallborn, T., Wüller, S., Klammt, J., *et al*. (2010) A heterozygous mutation of the insulin-like growth factor-I receptor causes retention of the nascent protein in the endoplasmic reticulum and results in intrauterine and postnatal growth retardation. *Journal of Clinical Endocrinology and Metabolism*, **95,** 2316-2324.
16. Choi, J.H., Kang, M., Kim, G.H., *et al*. (2011) Clinical and functional characteristics of a novel heterozygous mutation of the IGF1R gene and IGF1R haploinsufficiency due to terminal 15q26.2 >qter deletion in patients with intrauterine growth retardation and postnatal catch-up growth failure. *Journal of Clinical Endocrinology and Metabolism*, **96**, E130-E134.

17. Fang, P., Cho, Y.H., Derr, M.A., *et al*. (2012). Severe short stature caused by novel compound heterozygous mutations of the insulin-like growth factor-I receptor (IGF1R). *Journal of Clinical Endocrinology and Metabolism*, **97**, E243-E247.

18. Mohn, A., Marcovecchio, M.L., De Giorgis, T., *et al*. (2011) An insulin-like growth factor-I receptor defect associated with short stature and impaired carbohydrate homeostasis in an Italian pedigree. *Hormone Research in Paediatrics*, **76,** 136-143.

19. Kawashima, Y., Higaki, K., Fukushima, T., *et al*. (2012) Novel missense mutation in the IGF-I receptor L2 domain results in intrauterine and postnatal growth retardation. Clinical Endocrinology, "accepted article", doi: 10.1111/j.1365-2265.2012.04357.x

20. Ferrández-Longás, A., Baguer, L., Labarta, J.I., *et al*. (2005) Longitudinal study of normal spanish children from birth to adulthood: anthropometric, puberty, radiological and intellectual data. *Pediatric Endocrinology Reviews*, **2,** suppl 4, 423-462.

21. Greulich, W.W. & Pyle, S.I. (1959) *Radiographic atlas of skeletal development of the hand and wrist*. In 2nd Edition, Stanford University Press, California.

22. Schouten, J.P., McElgunn, C.J., Waaijer, R., *et al*. (2002) Relative quantification of 40 nucleic acids sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res*, **30**, e57.

23. Taylor, C.F., Charlton, R.S., Burn, J., *et al*. (2003) Genomic deletions in MSH2 or MLH1 are a frequent cause of hereditary non-polyposis colorectal cancer: identification of novel and recurrent deletions by MLPA. *Human Mutation*, **6**, 428-433.

24. Laviola, L., Perrini, S., Belsanti, G., *et al*. (2005) Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. *Endocrinology,* **146,** 1498-1505.

25. Iñiguez, G., González, C.A., Argandoña, F., *et al*. (2010) Expression and protein content of IGF-I and IGF-I receptors in placentas from small, adequate and large for gestational age. *Hormone Research in Paediatrics*, **73,** 320-327.

26. Calvo, M.T., Romo, A., Gutiérrez, J.J., *et al*. (2004) Study of genetic expression of intrauterine growth factors IGF-I and EGFR in placental tissue from pregnancies with

intrauterine growth retardation. *Journal of Pediatric Endocrinology and Metabolism*, **17,** suppl 3, 445-450.

27. Netchine, I., Azzi, S., Houang, M., *et al*. (2009) Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. *Journal of Clinical Endocrinology and Metabolism*, **94,** 3913-3921.
28. Ester, W.A., Van Duyvenvoorde, H.A., De Wit, C.C., *et al*. (2009) Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype. *Journal of Clinical Endocrinology and Metabolism*, **94,** 4717-4727.
29. Walenkamp, M.J., De Muinck Keizer-Schrama, S.M.P.F., De Mos, M., *et al*. (2008) Successful long term growth hormone therapy in a girl with haploinsufficiency of the insulin-like growth factor-I receptor due to terminal 15q26.2 >qter deletion detected by multiplex ligation probe amplification. *Journal of Clinical Endocrinology and Metabolism*, **93,** 2421-2425.
30. Hammer, E., Kutsche, K., Haag, F., *et al*. (2004) Mono-allelic expression of the IGF-I receptor does not affect IGF responses in human fibroblasts. *European Journal of Endocrinology*, **151,** 521-529.

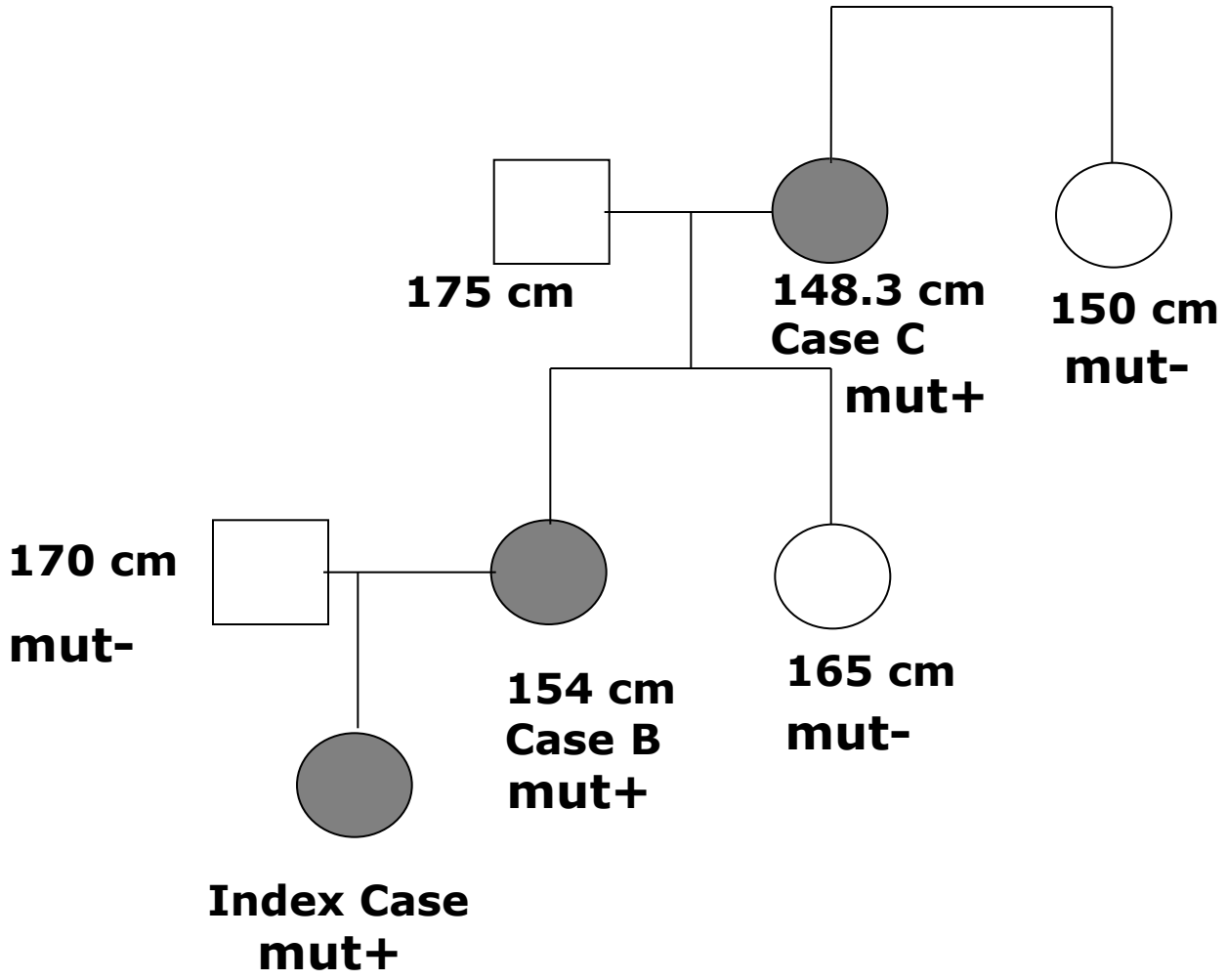
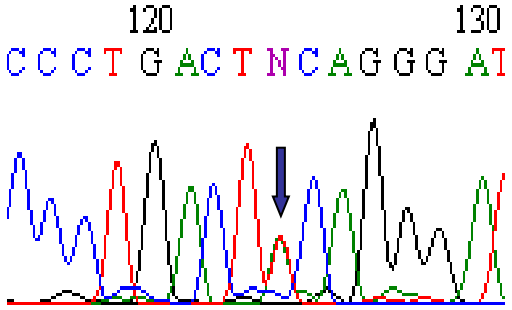
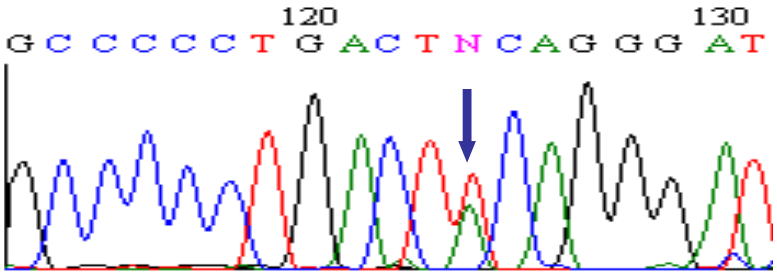

175 cm
148.3 cm
Case C
mut+
150 cm
mut-
170 cm
mut-
154 cm
Case B
mut+
165 cm
mut-
Index Case
mut+

Figure 1A

Case A: index case



Case B: mother



Case C: maternal grandmother

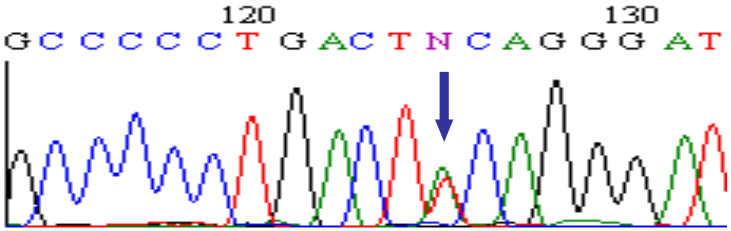


Figure 1B

^{125}I-IGF-1 binding displacement curve by increasing concentrations of IGF-1

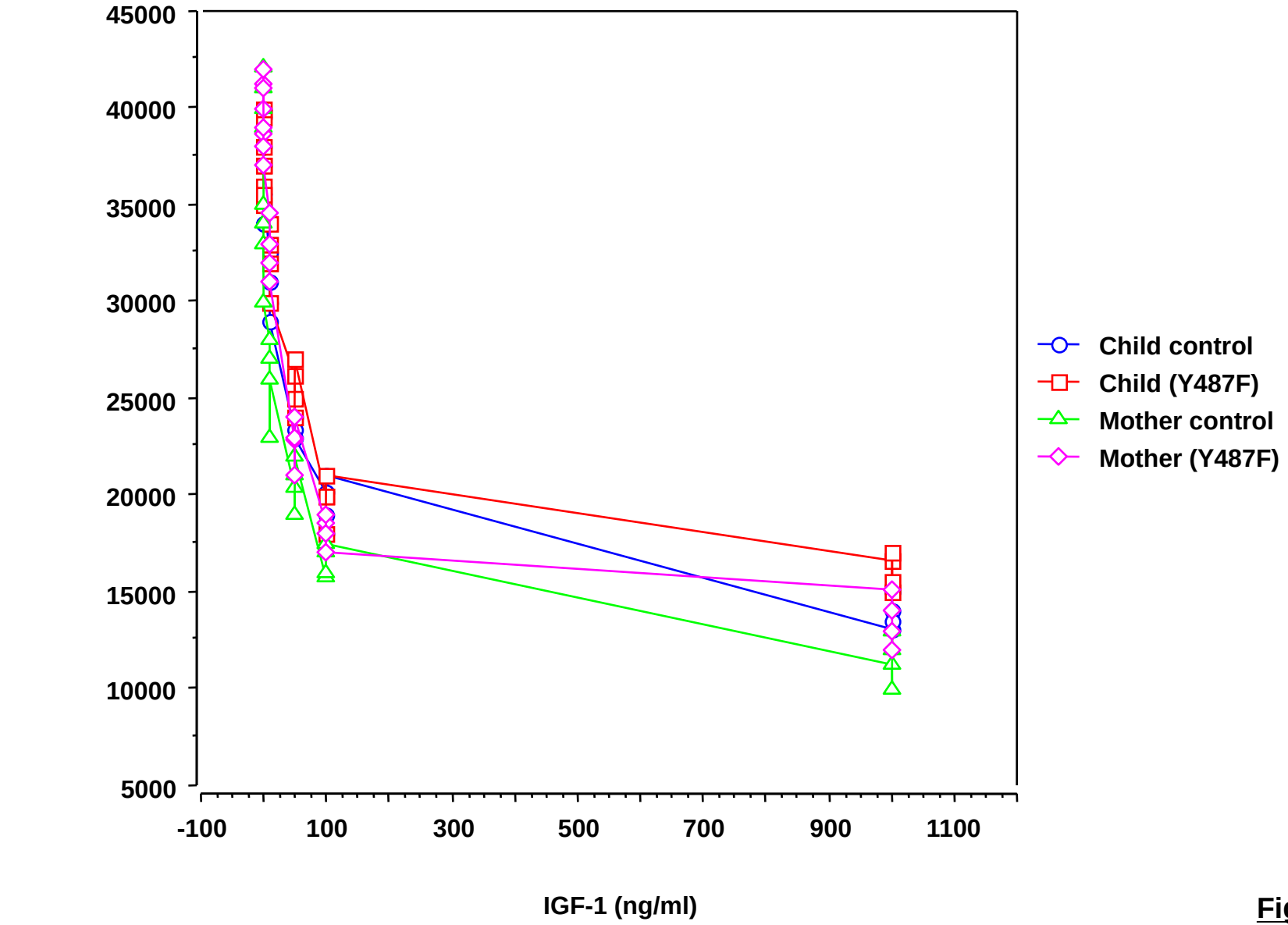
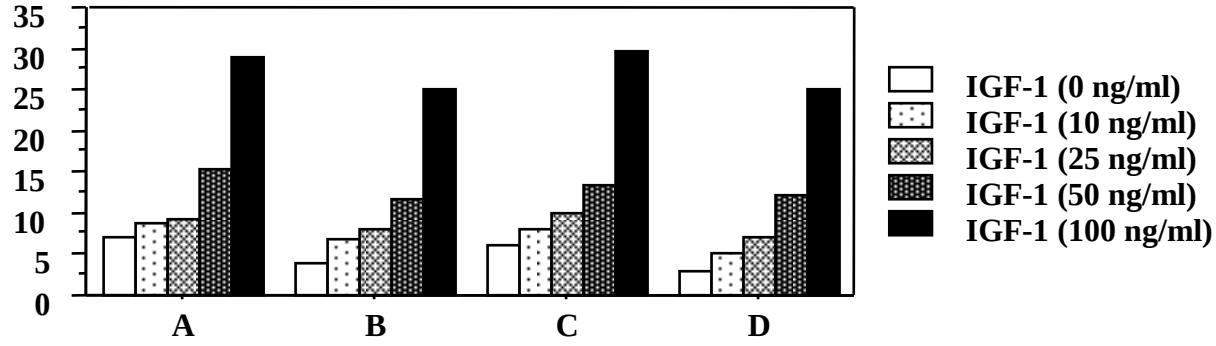
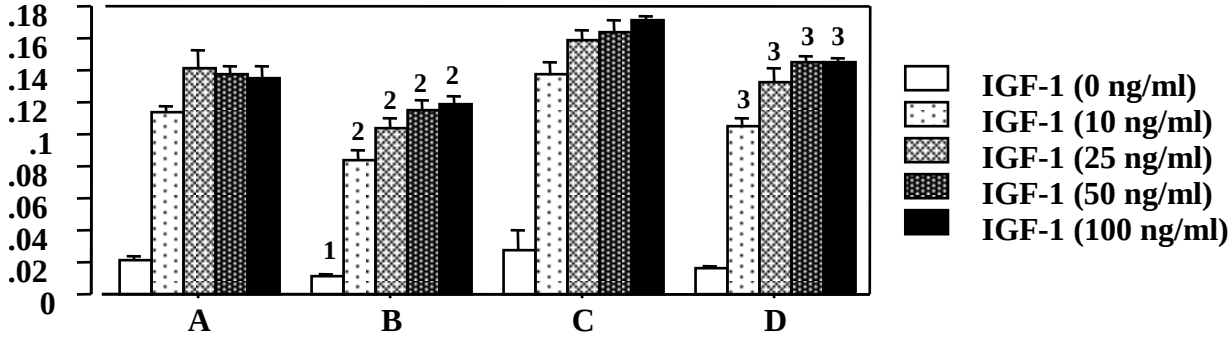


Figure 2

I) Phosphorylated IGF1R / Total IGF1R



II) Phosphorylated AKT / Total AKT



III) Phosphorylated ERK / Total ERK

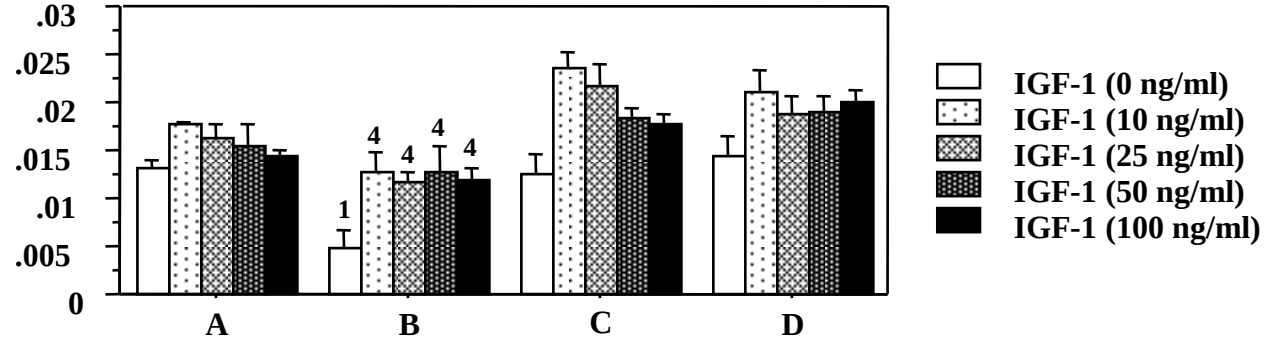


Figure 3

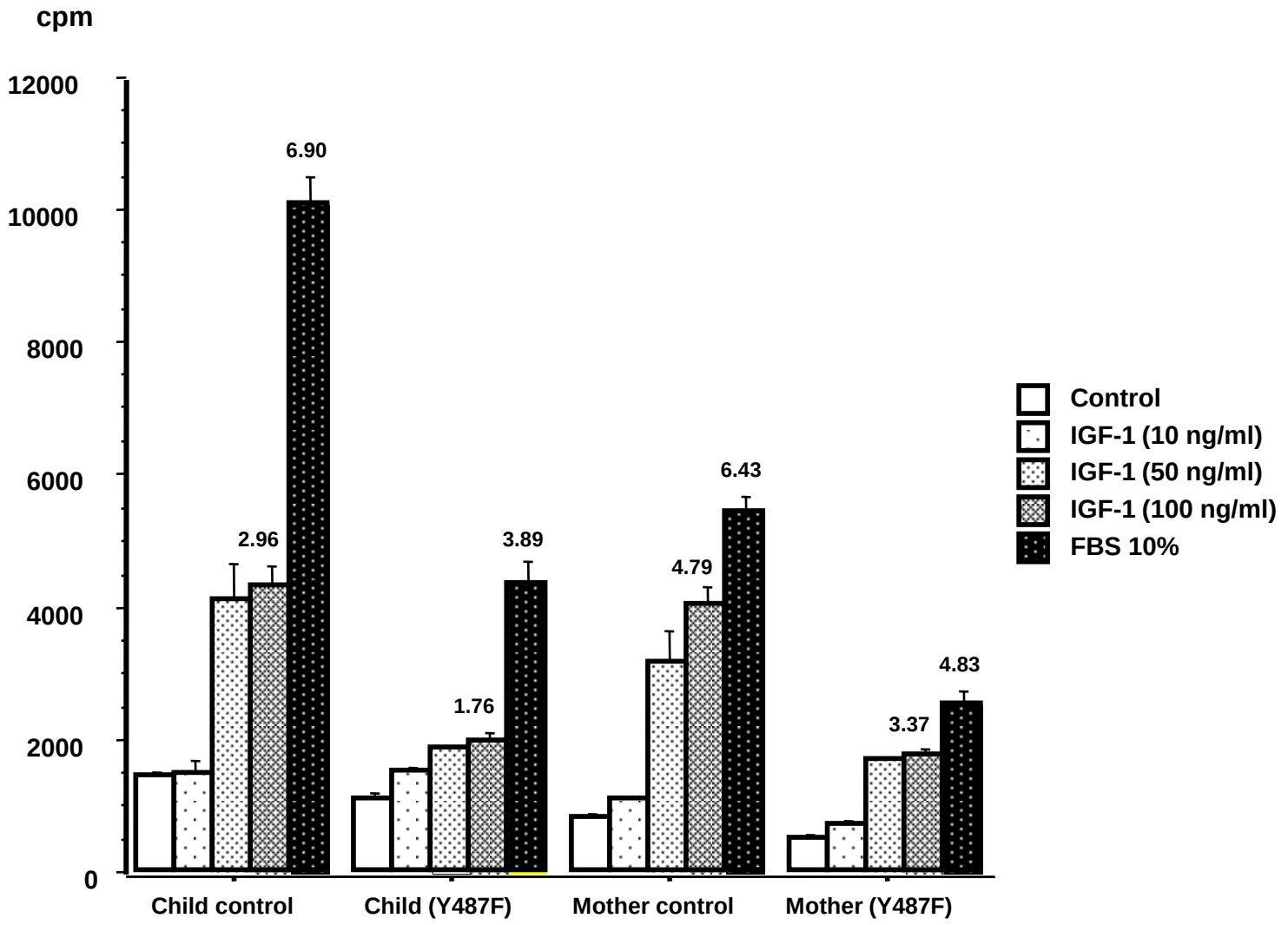

3H-Thymidine incorporation into DNA in skin fibroblasts
cpm
12000
10000
8000
6000
4000
2000
0
6.90
2.96
3.89
1.76
6.43
4.79
4.83
3.37
Child control
Child (Y487F)
Mother control
Mother (Y487F)
Control
IGF-1 (10 ng/ml)
IGF-1 (50 ng/ml)
IGF-1 (100 ng/ml)
FBS 10%

Figure 4

Figure 1

A: Pedigree of the proband case and heights of the family members. Shaded circles represent the IGF1R mutation cases (mut+). Mut-: absence of mutation.

B: Chromatogram of IGF1R DNA sequence of exon 7 in the index case (case A), the mother (case B) and the grandmother (case C) obtained by direct sequencing of PCR products revealed a heterozygous missense mutation corresponding to Y487F.

Figure 2

Ninth-passage confluent fibroblasts from each of the 4 different cultures [(child control, child (Y487F), mother control and mother (Y487F)] were plated in 12-well tissue culture plates at a density of 100,000 cells/well in 1 ml MEM with 10% FBS. After 72h, the medium was aspirated, fibroblasts washed and incubated for 16h at 4ºC with 1 ml of serum and antibiotic-free medium containing ^{125} I-IGF-1 (120,000 cpm; 0.06 x 10^{-9}M) with/without increasing concentrations of IGF-1 (0-1-10-50-100-1000 ng/ml; 0.13 to 130 x 10^{-9}M). Four different wells were prepared for each condition. At the end of the incubation period, media were aspirated, cells washed twice with 1 ml of serum- and antibiotic-free medium and cells solubilized in 1 ml of NaOH 1N at 37ºC for 1 h; 0.5 ml were transferred to a gamma counter and the rest kept at -20ºC for protein content determination. Results were expressed as cpm bound per mg of protein. The curves represent the mean of quadruplicates. No differences were observed between patient and control curves.

Figure 3

I) IGF1R phosphorylation stimulation by IGF-1 was measured in cell extracts from 12-well plate cultures: 100,000 cells per well were plated, 6 wells per culture (A = child control, B = child Y487F, C = mother control and D = mother Y487F), with 1 ml MEM and 10% FBS; cells were incubated for 48h, then for a further 24h with 1 ml MEM and 0.5% FBS followed by stimulation with increasing amounts of IGF-1 (0-10-25-50-100 ng/ml) for 10 min at 37ºC. Cells were washed with ice-cold PBS, scraped in 1 ml of ice-cold RIPA lysis buffer (Millipore®) containing pepstatin A, aprotinin, leupeptin and PMSF, incubated in ice for 15 min, vortexed for 10 sec and centrifuged for 10 min at 12000 rpm at 4ºC: extract aliquots were frozen at -80ºC for total protein, total IGF1R and phospho-IGF1R measurement. Total IGF1R (beta subunit) and phospho(Tyr1135/1136)-IGF1R were measured by ELISA. The ratio of phospho-IGF1R to total IGF1R (IU/ng) indicated the relative proportions in each extract.

II-III) AKT and ERK phosphorylation stimulation by IGF-1 was measured with cell-based fluorogenic ELISAs. 20,000 cells/well were plated in the 96-microwell plate: four wells for each condition and each culture [A = child control, B = child (Y487F), C = mother control and D = mother (Y487F)]. Cells were incubated at 37ºC with 0.1 ml MEM and 10% FBS for 16h followed by a further 16h with 0.1 ml MEM and 0.5% FBS followed by stimulation with increasing amounts of IGF-1 (0-10-25-50-100 ng/ml) in 0.1 ml of serum- and antibiotic-free MEM for 10 min for the AKT plate and 15 min for the ERK plate. The phosphorylated AKT (Ser473) and ERK 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) contents were read at 460 nm emission whereas total AKT and ERK 1/2 were read at 590 nm. Background without antibody was subtracted in each well for each culture. The 460/590 ratio in each well indicated the relative amount of phosphorylated to total AKT (II) or ERK (III). Results for each culture and IGF-1 concentration are represented as means and standard deviations of quadruplicates.

[1] $p<0.01$ compared with A and D; [2] $p<0.05$ compared with A and D; [3] $p<0.05$ compared with C; [4] $p<0.05$ compared with A.

Figure 4

Eighth passage confluent fibroblasts from four cultures ["child control", "child (Y487F)", "mother control" and "mother (Y487F)"] were plated in 96-microwell tissue culture plates at a density of 20,000 cells/well in 0.3 ml of MEM serum- and antibiotic-free medium. After 48h, the medium was aspirated and fibroblasts were incubated for 48h with IGF-1 at increasing concentrations (10 to 100 ng/ml) or FBS (10%). Control wells were incubated with medium containing the same amount of BSA (0.1 %) as under IGF-1 conditions. [^{3}H]-thymidine (5 mCi/ml) was added for the last 24h of incubation. Four different wells were prepared for each condition. Incubations were terminated by medium aspiration, followed by extensive cell washing with 2.5% acetic acid in distilled water. Fibroblasts were then treated with 0.5% Triton X-100 (250 ml/well; 10 min) and collected in a multiple cell harvester on glass fiber filters. The filters were dried and counted in 5 ml Biodegradable Counting Scintillant (Amersham Biosciences, UK) in a Beta-Counter (BECKMAN LS650 Multi-purpose Scintillation Counter) for 5min. The result for each condition in each culture is expressed as mean cpm and standard deviation of quadruplicates.

Table 1. Auxological, clinical and biochemical characteristics of case A (index patient) before and during rhGH treatment

Chronological age yrs	1.5	3.4	4.3	5.4	7.5
Height SDS	-2.81	-3.19	-2.88	-2.37	-2.39
Weight SDS	-3.39	-3.0	-2.78	-2.41	-2.45
BMI SDS	-2.57	-2.48	-2.58	-2.18	-2.29
Tricipital skinfold SDS		1.05	-1.8	-2.13	-1.84
Subscapular skinfold SDS		-1.0	-1.8	-1.31	-1.46
Head Circumference SDS	-3.36	-4.07	-3.81	-3.91	
Bone Age yrs	0.75	2	3.12	3.75	6
IGF-I (ng/mL)		39	171	245	
IGFBP-3 (mcgr/mL)		1.78	2.85	3.38	
Psychomotor development	Centile 10-25	Centile 50	Centile 50		
rhGH (µg/kg/day)		31 *	36	38	31

* Onset of rhGH treatment.

Results for height, weight, BMI, head circumference and bone age are expressed in SDS and psychomotor development in centiles according to the Spanish Longitudinal Growth Study (20).