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ORIGINAL ARTICLE

Three novel *IGF1R* mutations in microcephalic patients with prenatal and postnatal growth impairment

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Summary

Background *IGF1R* gene mutations have been associated with varying degrees of intrauterine and postnatal growth retardation, and microcephaly.

Objective To identify and characterize *IGF1R* gene variations in a cohort of 28 Argentinean children suspected of having IGF-1 insensitivity, who were selected on the basis of the association of pre/postnatal growth failure and microcephaly.

Methods The coding sequence and flanking intronic regions of *IGF1R* gene were amplified and directly sequenced. Functional characterization was performed by two *in vitro* assays: 1) [Methyl-^{3}H] thymidine incorporation into DNA in fibroblast cell primary cultures from patients and controls treated with IGF-1 for 16-24 h. 2) PI3K/Akt pathway was evaluated with phospho-Akt (Ser473) STAR ELISA Kit (Millipore) in fibroblast cultures from patients and controls stimulated with IGF-1 for 10 min. Prepubertal clinical and GH-IGF-1 axis evaluation was followed up.

Results We identified three novel heterozygous missense mutations in three unrelated patients, *de novo* p.Arg1256Ser, *de novo* p.Asn359Tyr and p.Tyr865Cys. In control cells, proliferation assay showed that IGF-1 significantly induced DNA synthesis at 20 h and Akt phosphorylation assay that it significantly stimulated phosphorylation after 10 min ($P < 0{\cdot}05$ by ANOVA and Bonferroni Tests). However, no significant increase was observed in any of the three patient fibroblasts in both functional studies. GH therapy growth response in two patients was inconsistent.

Conclusion These variations led to failure of the IGF1R function causing pre- and postnatal growth retardation and microcephaly. Microcephaly should be considered in the evaluation of SGA patients, because it seems to favour the frequency of detection of *IGF1R* mutations.

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Introduction

Components of the IGF system are ubiquitously expressed throughout pre- and postnatal life, regulating the development of most, if not all, tissues and organs. The mitogenic and metabolic effects of IGF-1 are mediated through type 1 IGF receptor (IGF1R) that is structurally related to the insulin receptor and is encoded by the *IGF1R* gene (15q26·3). The functional receptor is a tetramer $\alpha 2/\beta 2$ with the extracellular dimeric α subunits involved in ligand binding, whereas intrinsic tyrosine kinase activity is located within the β subunits.[1] Upon the IGF-1 binding to the receptor, which undergoes autophosphorylation, the MAPK/Erk and PI3K/Akt pathways are activated and mediate effects on cell proliferation and differentiation, cell survival, metabolic homoeostasis and neurodevelopment, among others.[2]

Animal models for defective *IGFs* or *IGF1R* genes have illustrated the role of the GH-independent IGF-1 production in foetal growth and development.[3] In humans, clinical data and *in vitro* studies have demonstrated that the copy number of the *IGF1R* gene has a profound effect on prenatal and early postnatal growth.[4]

Approximately 10–20% of children born small for gestational age (SGA) do not show spontaneous postnatal catch-up growth,[5] and the causes of pre- and postnatal growth deficit remain unclear in most cases. Since 2003, several *IGF1R* gene mutations have been described as a genetic cause of pre- and postnatal growth retardation due to IGF-1 insensitivity in humans.[6–19] These reports supported the important role of the IGF-IGF1R signalling in growth control and the causal relationship between *IGF1R* gene mutations and SGA. Even though most affected subjects were born SGA without catch-up growth in postnatal life, a great variability in the clinical and biochemical expression of the mutations has become apparent.

The aim of this study was to identify and characterize *IGF1R* gene variations in a cohort of 28 Argentinean patients suspected

of having IGF-1 insensitivity, who were selected on the basis of the association of growth failure and microcephaly.

Materials and methods

Study population

Of 74 SGA patients without catch-up growth followed in the Endocrinology Service from January 2010 to December 2013, we selected 28 unrelated Argentinean children suspected of having IGF-1 insensitivity according to the following criteria: (i) being born SGA, defined as a birth weight (W) and/or length (L) <−2 SDS for gestational age,[20] (ii) postnatal growth failure (defined as W and L lower than −2 SDS below the normal mean for age and sex (iii) normal karyotype, and (iv) microcephaly (defined as head circumference below −2 SDS for age and sex). Patients with major genetic abnormalities, severe chronic illnesses or endocrine disorders were excluded from the study.

For the functional studies, skin biopsies from an abdominal zone were obtained from selected patients. In the case of controls, samples from subjects in the same range of ages (C1: 3·57 and C2 4·88 years and similar ethnic origin were obtained during minor surgery.

This study was approved by the Ethics Committee of the Garrahan Pediatric Hospital. Written informed consent for the study was obtained from parents or tutors.

Clinical and biochemical measurements

Height, weight and head circumference were measured with standard equipment. Anthropometric SD score (SDS) was calculated on the basis of an Argentinean reference population.[21] Bone age was determined using the Greulich and Pyle method.[22]

Routine biochemical parameters (complete blood count, serum protein, serum electrolytes, liver, kidney and thyroid function, and screening for celiac disease) were measured by standard techniques.

Serum GH was measured using the Immulite assay and the GH standard 98/574 (cut off: 4·7 μg/l).[23] Serum IGF-1 and IGFPB3 were determined by automated chemiluminescent assay systems (Immulite®, Diagnostic Products Corp, Los Angeles, CA, USA) which use monoclonal murine anti-IGF-1 an anti-IGFBP3, respectively, and values were converted to SDSs based on normative data from our laboratory.[24]

Fasting serum insulin was determined by the Imx sys lens (ABBOTT Laboratories, Abbot Park, Chicago, IL, USA); assay sensitivity was 1·0 μU/ml; interassay coefficient of variation ranged from 3·8 to 4·5%. Serum glucose was determined by a Hitachi 911 autoanalyser. The homoeostatic model assessment for insulin resistance index (HOMA-IR = fasting glucose (mmol/l) x fasting insulin (mUI/ml)/22·5) was used for estimating insulin resistance. A standard oral glucose tolerance test (OGTT) performed after a 10-h overnight fast was carried out. Glucose and insulin were determined every 30 min for 2 h. Glucose tolerance was evaluated according to WHO criteria, derived from oral glucose tolerance test (OGTT).[25] Chromosome and G-banding analyses were carried out according to standard procedures.

Molecular analysis of the IGF1R *gene*

Genomic DNA was isolated from mononuclear cells of the affected subjects and relatives according to standard procedures. The coding sequence (exon 1-21) and flanking intronic regions of *IGF1R* gene were PCR amplified from genomic DNA, using specifics primers.[7] Each purified product (Qia Quick PCR purification kit, Qiagen, Buenos Aires, Argentina) was direct sequenced using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Buenos Aires, Argentina) and 3130 Genetic Analyzer capillary DNA sequencer (Applied Biosystems). The nucleotide sequence obtained was compared with those from GenBank accession number: NG_009492.

In silico *assays*

In silico tools were applied to identify the potential functional impact of newly found variants. *In silico* was assessed using online tools such as sequence homology-based tool, SIFT (Sorting Intolerant from Tolerant; http://sift.jcvi.org/) version 2·0·6, the structure-based tool, PolyPhen2 (Polymorphism Phenotyping; http://genetics.bwh.harvard.edu/pph2) and Mutation Taster (http://www.mutationtaster.org/).

Fibroblast cell primary culture

Fibroblast cultures were established from skin biopsies obtained from an abdominal zone from patients affected by the variations (P1, P2 and P3) and two control subjects (C1 and C2). Cell cultures were maintained in Dulbecco's modified Eagle medium and F12 (DMEM/F12, SIGMA, Buenos Aires, Argentina) containing 10 or 20% foetal bovine serum (FBS), at 37 °C in a humidified atmosphere with 5% CO2. Fibroblasts were subcultured for 3 passages and then stored in liquid N_2 until the time of performing the assay. Finally, all fibroblasts were subcultured once more to collect the necessary cell number for the study. All studies were performed at passage 4. Fibroblasts were stimulated with different concentrations of IGF-1, and the highest response was observed at 50 ng/ml.

IGF1-dependent DNA synthesis assay

Fibroblast cultures from all subjects were incubated in 24-well plates with DMEM/F12 containing 10% FBS. Subconfluent cells were washed with saline solution and starved for 24 h in DMEM/F12 containing 0·1% BSA. According to the protocol described by Kawashima *et al.*,[7] fibroblasts were stimulated with 50 ng/ml of IGF-1 for 16, 20 and 24 h. [Methyl-^{3}H] thymidine (1 mCi/ml) was added for 4 h prior to basal conditions and to 12, 16, 20 h of IGF-1 treatment, to give a final concentration of 1 μCi/ml. Cells were washed with PBS and fixed by 10% trichloric acid for 2 h at 4 °C. They were solubilized with 0·1 M NaOH and 0·2% SDS for over 2 h at 37 °C, and the solubilized

radioactivity was counted. All assays were performed sixfold, at the same time, in seven 24-well plates (one plate for each, patients and controls), at a concentration of $2{\cdot}5 \times 10^4$ cell/well.

AKT phosphorylation stimulation by IGF-1

Fibroblast cultures from all subjects were incubated in 24-well plates with DMEM/F12 containing 10% FBS. Subconfluent cells were washed with saline solution and starved for 24 h in DMEM/F12 containing 0·1% BSA. Cells were stimulated with 50 ng/ml of IGF-1 for 10 min and then were washed twice with PBS. The fibroblasts were lisyed, and protein extraction was performed by standard procedures. AKT phosphorylation was measured with phospho-Akt (Ser473) STAR ELISA Kit (Merk Millipore, Tecnolab, Buenos Aires, Argentina) following the manufacturer's instructions. Protein concentration for each well was measured by Bradford assay, and the results are expressed by mg of protein. The entire assay was performed by duplicated.

Results

We identified four novel heterozygous missense *IGF1R* gene variations by DNA sequencing in 4 of 28 unrelated patients, *de novo* p.Arg1256Ser (P1) and p.Asn359Tyr (P2), and inherited p.Tyr865Cys (P3) and p.Arg1337Cys (P4), as described in Fig. 1. In P1, P2 and P3 phenotypic characteristics, segregation with the growth impairment phenotype, molecular analyses and functional tests (described below) suggest that these variations affect IGF1R function and they are, therefore, considered as new gene mutations. An additional allelic variant p.Arg1337Cys (P4) was not found in 120 alleles from control subjects, and it did affect a highly conserved amino acid of the IGF1R protein. However, it was considered to be a rare benign allelic variation because it did not cosegregate with the growth impairment phenotype in the patient's family (Figure S3B) and abnormal activity was not confirmed by functional studies (Figure S4).

Clinical evaluation

Clinical description of subjects carrying *IGF1R* variations in heterozygosis are shown in Tables 1 and 2. As shown in Table 1, all patients were born at term. Severe prenatal weight and longitudinal growth restriction was evidenced in all patients and in 2 of 3 subjects (P1 and P3), respectively. Microcephaly was very marked in the one newborn in whom head circumference measurement was available (P3). P1 had mild phenotypic anomalies.

Table 2, Figure. S1 and S2 show patterns of body length and head circumference in patients and first-degree relatives carrying *IGF1R* gene mutations. At admission, all patients were 2·8 years old or younger and they were followed for several years during childhood. Initially, they had body lengths and/or weights below −2 SDS, but P1 and P3 underwent spontaneous body length catch-up growth until the age of 4·5–5 years. In the three patients, spontaneous head circumference catch-up growth was observed, but in all cases, it remained <−2 SDS. Mental development was normal in 2 subjects and mildly delayed in one.

P1 and P2 were treated with rhGH (47 μg per kilogram of body weight per day, given daily sc) for 2 years (Table 2). At start, P1 had already been in a process of spontaneous longitudinal catch-up growth, which was not modified by rhGH therapy. P2 showed a moderate improvement in HSDS (Δ +0·9 SDS) after 2 years of rhGH. Head circumference did not increased under rhGH therapy in P1 and showed a slight increase in P2.

P1 and P2 had *de novo* variations in the *IGF1R* gene, and no family history of SGA, short stature and/or microcephaly was reported in both. Pedigre of P3 is shown in Figure S3. P3's mother, who had microcephaly (HC −2·1 SDS) and shared the same variation as the respective index case.

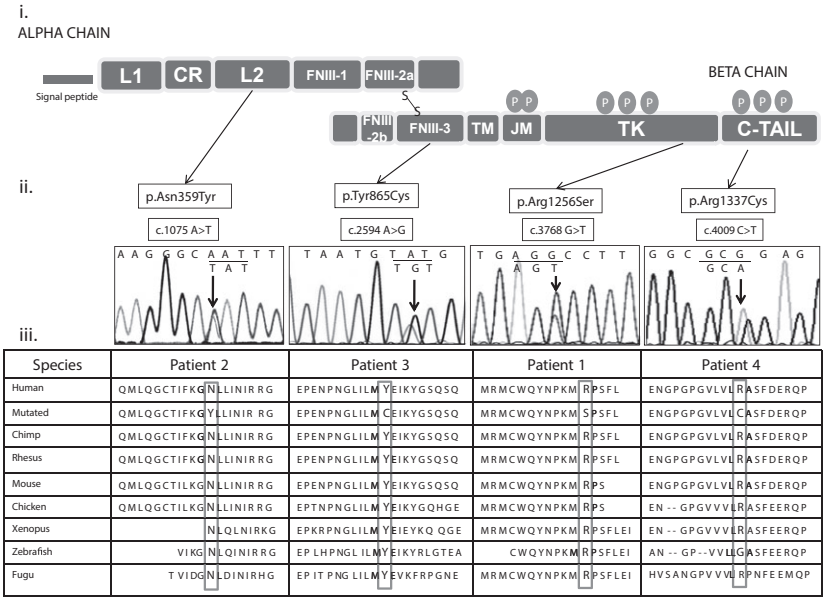

Fig. 1 Panel (i) Cartoon of the hemi-receptor protein: L1, L2, leucine-rich domains; CR, cysteine-rich domain; FNIII, type III fibronectin repeats; TM, transmembrane domain; JM, juxtamembrane region; TK, tyrosine kinase domain; C-tail, COOH-terminal tail. The variations affected the L2, FNIII type III, TK and C-tail domains. (ii) Chromatograms of *IGF1R* gene variations present in four unrelated patients. Electropherograms showing replacement of asparagine to tyrosine, at position 359 (c.1075 C>T) in P2; tyrosine to cysteine at position 865 (c.2594 A>G) in P3; arginine to serine at position 1256 (c.3768 G>T) in P1; and arginine to cysteine at position 1337 (c.4009 C>T) in P4. (iii) Conservation of the affected amino acids in different species. The pictures revealed that the four amino acid substitutions affect a highly conserved amino acid of the IGF1R protein.

Table 1. Clinical characteristics of neonates bearing IGF1R variations in heterozygosis

Patient, sex	GA weeks	Weight Kg, (SDS)	Length cm, (SDS)	HC cm, (SDS)	Karyotype	Molecular variation	Phenotype associations
P1, male	37	1·9, (−3·76)	42, (−4·39)	n.a.	46,XY	*de novo* p.Arg1256Ser	SGA, triangular face, small ears, clinodactily, long philtrum
P2, male	38	2·37, (−2·33)	48, (−1·1)	n.a.	46,XY	*de novo* p.Asn359Tyr	SGA
P3, female	38·5	2·23, (−2·49)	43, (−3·46)	31, (−2·6)	46,XX	Familial p.Tyr865Cys	SGA

GA, gestational age, SDS, standard deviation score, HC, head circumference, P, patient, SGA, small for gestational age, AGA, adequate for gestational age, n.a., not available.

Table 2. Postnatal auxology, GH/IGF-1 axis and mental development in subjects bearing IGF1R variations in heterozygosis

Subject, Follow-up	CA y	BA y	L/Height cm, (SDS)	Weight Kg, (SDS)	HC cm, (SDS)	Serum GH µg/l	IGF-1 ng/ml (SDS)	IGFBP3 mg/l (SDS)	Mental devel.
P1 (M)									
Adm.	1·5	–	74 (−3·0)	7·7 (−3·1)	42·5 (−4·0)	8·0	231 (+2·9)	5·2 (+3·4)	Mild delay
Exam.	2·8	1·4	86 (−2·1)	9·8 (−3·1)	44·8 (−3·5)				
rhGH Ons.	4·5	3·0	97 (−1·7)	12·6 (−2·7)	46·2 (−3·0)				
rhGH Res.	6·4	4·0	109 (−1·5)	16·1 (−2·2)	47·0 (−3·0)		261 (+1·7)	5·2 (+2·3)	
P2 (M)									
Adm.	2·8	–	81 (−3·6)	10·5 (−2·5)	43·8 (−4·6)	0·27	58 (+0·5)	2·6 (−0·2)	Normal
rhGH Ons.	5·0	3·0	94 (−3·0)	14·0 (−2·2)	47·0 (−2·5)				
rhGH Res.	7·0	6·0	109·5 (−2·1)	17·8 (−1·87)	48·8 (−2·1)		192 (+0·8)	4·5 (+0·7)	
P3 (F)									
Adm	1·9	1·5	78 (−2·4)	7·9 (−3·0)	43·0 (−4·0)	1·18	211 (+1·7)	4·7 (+1·9)	Normal
Last Exam.	5·0	5·5	99 (−1·6)	12·3 (−3·4)	45·6 (−3·0)	1·04	306 (+1·9)	4·8 (+1·6)	
P3 mother	35	–	161 (+0·0)		52·2 (−2·1)				

CA, chronological age, BA, bone age, P, patient, L/Height, length/height, HC, head circumference, Mental devel., mental development, Adm., at admission. Exam., follow-up clinical examination, rhGH Ons., onset of rhGH treatment, Res., response to rhGH after 2 years of treatment, Last exam, last clinical examination, M, male, and F, female.

Routine laboratory assessment and standard chromosome analysis were normal in all patients (including analysis of chromosome 15). In all subjects, OGTTs results, insulin levels and HOMA-IR calculations were normal. At admission (Table 2), all serum IGF-1 and IGFBP3 levels were above 0 SDS for age and sex, except for serum IGFBP3 in P2 (SDS = −0·2).

Molecular analysis

The nucleotide sequences of genomic DNA in P1 revealed a *de novo* heterozygous variation in exon 21, substituting G for T at cDNA nucleotide position 3768, changing arginine to serine at codon 1256 (p.Arg1256Ser) in the tyrosine kinase domain of the IGF1R. In P2, a *de novo* heterozygous variation in exon 4, substituting A for T at cDNA nucleotide position 1075, was found, changing asparagine to tyrosine at codon 359 (p.Asn359-Tyr) in the ligand-binding L2 domain of the receptor molecule. In P3, a heterozygous variation in exon 12, substituting A for G at cDNA nucleotide position 2594, was detected, changing tyrosine to cysteine at codon 865 (p.Tyr865Cys) in the fibronectin type III-3 domain of the IGF1 receptor. The mother was also found to be heterozygous for the same *IGF1R* gene variation.

To determine whether these variations are present in the general population, 60 control subjects (120 alleles) were screened by DNA sequencing. No allele carrying these variations was detected, suggesting that they probably are not common polymorphisms. These variations were not found in the databases of NCBI.

To analyse the evolutionary conservation of the amino acids affected by the novel variations, the sequence alignment of IGF1R proteins from different species was examined. This approach revealed that the amino acid substitutions affect highly conserved amino acids of the IGF1R protein (Fig. 1), suggesting that these novel variants might be deleterious for protein activity.

Other tools such as (i) PolyPhen2 which predicts that the variations found were probably damaging with a score of 1·000 (score 0 to 1·000) and (ii) Mutation Taster which predicts that the alterations might be disease causing suggest that variations did affect protein function. Additionally, (iii) SIFT tool showed that the p.Arg1256Ser and p.Tyr865Cys variations also affect protein function with a highly deleterious tolerance index score of 0·00, while the p.Asn359Tyr and p.Arg1337Cys variations were predicted to be tolerated.

Functional studies

IGF-1-*stimulated DNA synthesis in fibroblasts.* DNA synthesis (^{3}[H]-thymidine uptake) was determined after treating with IGF-1 (50 ng/ml) for 16, 20 and 24 h. We observed that IGF-1 significantly induced DNA synthesis in patients and controls *vs* basal in all conditions (16, 20 and 24 h, $P < 0{\cdot}05$ by ANOVA and Bonferroni tests). Moreover, we observed that IGF-1 significantly induced DNA synthesis in C1 and C2 at 20 h *vs* 16 and 24 h of treatment ($5{\cdot}15 \pm 0{\cdot}67$-fold and $6{\cdot}37 \pm 1{\cdot}00$-fold increase over basal, respectively, $P < 0{\cdot}05$ by ANOVA and Bonferroni tests). However, no significant increases in DNA synthesis were observed in fibroblasts from P1, P2 and P3 at 20 h *vs* 16 and 24 h of treatment. Results are shown in Fig. 2A.

Akt *phosphorylation stimulated by* IGF-1. Akt phophorylation was significantly stimulated in the control subjects by IGF-1 ($P < 0{\cdot}05$ by ANOVA and Bonferroni tests). However, no significant stimulation in PI3K/Akt pathway was observed in P1, P2 and P3. Results are shown in Fig. 2B.

Discussion

IGF1R gene defects are considered as an infrequent cause of pre- and postnatal growth retardation even though the true prevalence of *IGF1R* gene defects among patients with these growth characteristics is unknown. Since the first description by Abuzzahab M *et al.* in 2003,[6] several individuals from different families have been reported showing a great phenotypic and biochemical variability.[7–19] It therefore seemed clinically relevant to define more appropriate selection criteria to look for this molecular diagnosis among children with weight and longitudinal growth deficiencies.

In SGA patients, the estimated frequency of IGF1R mutations previously reported ranged between 2 and 4%.[6,7,17,18] However, subjects studied were selected according to different and strict criteria of growth restriction and biochemical parameters.[26] In this report, due to the fact that several reports point out to the high prevalence of microcephaly among subjects with IGF1R mutations,[26] we have also included low head circumference as additional criterion along with pre- and postnatal growth impairment to perform the molecular analysis of the *IGF1R* gene. Using this approach, we have found three missense mutations in the *IGF1R* gene in a cohort of 28 unrelated Argentinean patients (10·7 %). Our findings reinforce the importance of measuring head circumference in the evaluation of SGA babies and short-statured patients.

The IGF-1/IGF1R system plays an important role in brain development. Indeed, it has been shown that IGF-1 stimulates the proliferation of neural progenitors and possibly pluripotent neural stem cells, the survival of neurons and oligodendrocytes, as well as differentiation of neurons (neuritic outgrowth and synaptogenesis), and of oligodendrocytes (expression of myelin gene proteins and myelination).[27] Small head circumference, a clinical marker of small brain volume, has also been reported in GHR-deficient, low- serum IGF-1 Laron syndrome patients, as part of their growth retardation and acromicria.[28,29]

The *IGF1* gene would be another potential candidate to look for molecular defects in gene analysis because pre- and postnatal growth impairment, microcephaly along with several degrees of sensorineural deafness and intellectual deficit have also been reported in patients with *IGF1* gene defects.[3,30,31] The finding of serum levels of IGF-1, in immunoassays using monoclonal antibodies, above mean reference values in all of our patients' samples made these other diagnoses (Laron syndrome and *IGF1* gene mutations) improbable, even though heterozygous *IGF1*

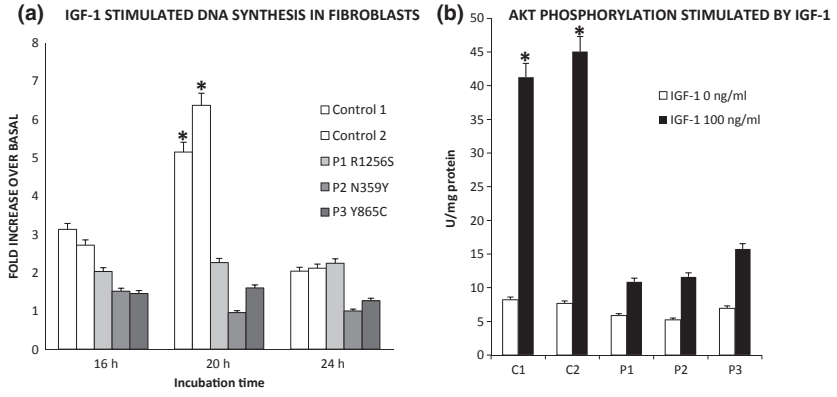

Fig. 2 (a) Effect of IGF-1 on DNA synthesis in fibroblast cell primary cultures. DNA synthesis in fibroblasts from all cases and two normal controls was determined using a ^{3}[H]-thymidine incorporation assay. See *Patients and Methods.* The assay was preformed after 16, 20 and 24 h of IGF-1 treatment (in six duplicates per time). A significant increase of ^{3}[H]-thymidine incorporation was observed after 20 h *vs* 16 and 24 h of IGF-1 treatment in controls with normal growth phenotype. ^{3}[H]-Thymidine incorporation in fibroblast from all patients was significantly lower than that in normal controls. Results are expressed as fold increase over basal condition (X ± SD) and were analysed by ANOVA and Bonferroni test. *$P < 0{\cdot}05$ 20 h *vs* 16 and 24 h. (b) Phosphorylated Akt stimulated by IGF-1. A total of 80·000 cells/wells were incubated in 24-well plates and stimulated with 100 ng/ml of IGF-1. The fibroblasts were lysed, and the protein concentration was measured by Bradford assay, and the phosphorylated Akt was determinated with phospho-Akt (Ser473) STAR ELISA Kit (Millipore). Bradford assay was read at 595 nm, and phophorylated Akt was read at 450 nm. Results are expressed as units per mg of protein (X ± SD) and were analysed by ANOVA and Bonferroni test. *$P < 0{\cdot}05$ *vs* basal conditions.

mutations might share similar clinical findings and elevated non-functional IGF-1 levels has been described by Walenkamp MJ *et al*[32] in one report.

Even though postnatal catch-up growth in body length and weight was observed in our patients (P1 and P3), microcephaly remained reinforcing the role of IGF1R signalling during brain development. In addition to the major manifestations (growth retardation and microcephaly), our patients also illustrated the phenotypic variability observed among individuals carrying heterozygous mutations of the *IGF1R* gene in whom genotype–phenotype correlations are not evident. Moreover, the mother bearing the p.Tyr865Cys mutation showed stature within the normal range but presented microcephaly. Normal final height was reported in 4 affected cases,[12,13,16,17] suggesting that there may be some recovery postnatal growth in some cases. However, all of these individuals had a history of low weight and/or length at birth and one patient also had microcephaly in adulthood.[17] The low expressivity or penetrance of mutant alleles might explain the variability in growth restriction and the borderline growth-retarded phenotype in some mutational carriers, suggesting that haploinsufficiency might not always result in clinically significant IGF1 resistance.[15,26]

The response to rhGH therapy was variable in the two treated patients. In P2, a slight increase in head circumference was also observed. In other conditions that do not involve IGF1R, such as Laron syndrome and isolated growth hormone deficiency, HC increase under IGF-1 and/or rhGH treatment has been reported.[28,33] The potential benefit of rhGH therapy on growth in our two patients was uncertain. Twelve children with *IGF1R* gene mutation have been treated with rhGH with inconsistent results.[6,9–11,13–18] However, the response to rhGH therapy among treated individuals is difficult to evaluate because of heterogeneity of the available data regarding age of onset, duration of therapy and dose regimes, as well as therapy goal. Apart from the age of onset, time of treatment and dose used, the specific impact of the *IGF1R* mutations on cell growth and proliferation and the genetic background might influence the final outcome of rhGH therapy among patients.

We investigated the presence of point mutations in the *IGF1R* gene by direct DNA sequencing and detected three novel heterozygous nonsynonymous exonic variants causing a change in the encoded amino acid (p.Arg1256Ser, p.Asn359Tyr and p.Tyr865Cys). The p.Arg1256Ser (P1) and p.Asn359Tyr (P2) were *de novo* variations, while p.Tyr865Cys (P3) was also identified in at least one first-degree relative, the mother of P3. *In silico* prediction models indicated that the amino acid substitutions, which were located at highly conserved amino acid residues, probably affect protein function. Functional analyses were performed by evaluating IGF-1-stimulated DNA synthesis and Akt phosphorylation in fibroblast cultures grown from abdominal skin biopsies. The assay showed a significant decrease in cell proliferation and phosphorylation of signal trasductor Akt after IGF-1 treatment in the three patients. These findings strongly suggest that the variations led to inhibition of IGF-1-dependent cell proliferation and affect signal transduction, and they also suggest that these alterations led to failure of the IGF1R function causing pre- and postnatal growth retardation and microcephaly.

The *de novo* p.Arg1256Ser mutation found in P1, the first variation described so far in exon 21, affects the tyrosine kinase domain of the IGF1 receptor, similar to the p.Gly1155Ala mutation described by Kruis *et al.*[12] The binding of the IGF-1 to the receptor leads to receptor autophosphorylation of several intracellular tyrosine residues and activation of the receptor intrinsic tyrosine kinase, resulting in recruitment of cytoplasmic components of a multiple downstream signalling cascade, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT/PKB) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways.[2] We propose that p.Arg1256Cys affects this downstream signalling cascade and leads to inhibition of cell proliferation. The p.Asn359Tyr mutation in P2 compromises the extracellular L2 domain of IGF1R, involved in ligand binding and receptor internalization. A mutation affecting the ligand-binding L2 domain has also been reported by Kawashima *et al*[16] in a Japanese girl who did not respond well to rhGH. It was postulated that the variation p.R413L described in the Japanese patient resulted in attenuation of IGF signalling and decreased internalization of IGF1R, possibly resulting in inhibition of cell proliferation induced by IGF-1. The mutation in P2 could have affected the signalling pathway and internalization of IGF1R, like in Kawashima's patient, and resulted in inhibition of cell proliferation explaining the pre- and early postnatal growth retardation observed in this patient. However, in contrast to Kawashima's patient, P2 showed different biochemical profile and growth response to rhGH, suggesting that the phenotypic consequences of the two variants affecting ligand-binding L2 domain might not be the same. The p.Tyr865Cys mutation found in P3 and in her mother is located in the extracellular fibronectin type III-3 domain of the receptor molecule. Labarta *et al*[17] described a family carrying the p.Tyr487Phe mutation affecting the fibronectin type III-2a domain, close to the first disulphide bond (Cys514) between the two IGF1R α-subunits. It was hypothesized that this change might disturb the dimerization process; however, disturbance of the type 1 IGF-1 receptor in the presence of p.Tyr865Cys mutation found in P3 remains to be elucidated. Alterations of signal transduction lead to inhibition of cell proliferation and cause pre- and postnatal growth retardation. Even though variable specific impacts of each heterozygous *IGF1R* mutation on cell growth might explain different responses among affected subjects, the genetic background of patients with the same mutation might also contribute to phenotypic variability.

In summary, this study reports 3 new heterozygous *IGF1R* mutations in children who were admitted because of pre- and postnatal growth failure and microcephaly. These clinical signs, particularly microcephaly, should be considered as important tools in the evaluation of SGA patients, because microcephaly allowed us to increase the frequency of detection of *IGF1R* mutation in our cohort. Long-term follow-up, as well as the study of individuals with less stringent selection criteria, is needed to evaluate the impact of the *IGF1R* mutations in humans.

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Nothing to declare.

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