6

Genetic analysis of short children with apparent growth hormone insensitivity

J.M. Wit[1], H.A. van Duyvenvoorde[1,2,3], S.A. Scheltinga[3], S. de Bruin[3], L. Hafkenscheid[3], S.G. Kant[3], C.A.L. Ruivenkamp[3], A.C.J. Gijsbers[3], J. van Doorn[4], E. Feigerlova[5], C. Noordam[6], M.J. Walenkamp[7], H. Claahsen-van de Grinten[6], P. Stouthart[8], I.E. Bonapart[9], A.M. Pereira[2], J. Gosen[10], H.A. Delemarre-van de Waal[1], V. Hwa[5], M.H. Breuning[3], H.M. Domené[11], W. Oostdijk[1], M. Losekoot[3]

[1] Department of Pediatrics, [2] Department of Endocrinology and Metabolic Diseases, [3] Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands
[4] Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, The Netherlands
[5] Department of Pediatrics, Oregon Health & Science University, Portland, United States
[6] Department of Pediatrics, University Medical Center Nijmegen St. Radboud, Nijmegen, The Netherlands
[7] Department of Pediatrics, VU University Medical Center, Amsterdam, the Netherlands
[8] Department of Pediatrics, Orbis Hospital, Sittard, The Netherlands
[9] Department of Endocrinology, Venizeleio General Hospital, Heraklion, Crete, Greece
[10] Department of Pediatrics, Rijnland Hospital, Leiderdorp, The Netherlands
[11] Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Hospital de Niños R. Gutiérrez, Buenos Aires, Argentina

Hormone Research in Paediatrics 2012;77(5):320–333

Abstract

Background/Aims: In short children, a low IGF-I and normal GH secretion may be associated with various monogenic causes, but their prevalence is unknown. We aimed at testing *GH1*, *GHR*, *STAT5B*, *IGF1*, and *IGFALS* in children with GH insensitivity.

Subjects and Methods: Patients were divided into three groups: group 1 (height SDS <–2.5, IGF-I <–2 SDS, n = 9), group 2 (height SDS –2.5 to –1.9, IGF-I <–2 SDS, n = 6) and group 3 (height SDS <–1.9, IGF-I –2 to 0 SDS, n = 21). An IGF-I generation test was performed in 11 patients. Genomic DNA was used for direct sequencing, multiplex ligation-dependent probe amplification and whole-genome SNP array analysis.

Results: Three patients in group 1 had two novel heterozygous *STAT5B* mutations, in two combined with novel *IGFALS* variants. In groups 2 and 3 the association between genetic variants and short stature was uncertain. The IGF-I generation test was not predictive for the growth response to GH treatment.

Conclusion: In severely short children with IGF-I deficiency, genetic assessment is advised. Heterozygous *STAT5B* mutations, with or without heterozygous *IGFALS* defects, may be associated with GH insensitivity. In children with less severe short stature or IGF-I deficiency, functional variants are rare.

Introduction

In approximately 80% of children who are referred to a pediatric clinic because of short stature, no definite cause can be established, even after a thorough diagnostic workup including extensive biochemical screening and radiologic investigations [1–4]. Such children are usually classified as idiopathic short stature (ISS) [1]. Within the ISS group, especially those children with a low circulating IGF-I level, in the face of a normal or even elevated GH secretion (also labeled 'primary IGF-I deficiency'), pose a diagnostic challenge.

At present, the known monogenic causes of short stature which are associated with a low serum IGF-I level and normal or elevated GH secretion include defects of *GH1* (bioinactive GH) [5], *GHSR* (ghrelin receptor) [6], *GHR* (GH receptor) [7], *STAT5B* [8], *IGF1* [9] and *IGFALS* [10; for reviews, see 11, 12]. With increasing numbers of reports on these gene defects it has become clear that the phenotype is more variable than suggested by the first cases. For example, the clinical features of homozygous *GHR* mutations can vary substantially depending on the location of the mutation. Furthermore, heterozygosity for the same gene defect may be associated with a mild negative effect on growth (approx. 1 SD), as observed for mutations in *IGF1* [9, 13, 14] and *IGFALS* [15–17]. On the other hand, some cases of heterozygosity for a *GHR* mutation are associated with pronounced growth failure [12, 18].

So far, only monogenic causes of primary IGF-I deficiency have been reported. However, it is conceivable that primary IGF-I deficiency can also be associated with the cumulative effect of digenic or oligogenic defects, in a similar fashion as recently shown for hypogonadism [19]. This view is supported by the results from genome-wide association studies that have shown that height is determined by more than 180 genes [20].

Based on the clinical and biochemical phenotype of children with monogenetic disorders of the GH-IGF-I axis, we and others have proposed algorithms for the diagnostic approach of children with short stature [11, 12, 21]. As a first step, IGF-I generation tests theoretically should allow one to discriminate between a normal GH sensitivity (*GH1* and *GHSR* defects) and GH insensitivity (*GHR*, *STAT5B*, *IGF1*, *IGFALS* defects) [11, 21]. However, the diagnostic accuracy of the various regimens for an IGF-I generation test is uncertain [reviewed in 22]. The second step would be genetic testing of the most likely candidate gene(s). Finally, as a third step, we have suggested a whole-genome single nucleotide polymorphism (SNP) array analysis [11].

In this paper we present the results of a genetic analysis for *GH1*, *GHR*, *STAT5B*, *IGF1*, and *IGFALS*, followed by whole-genome SNP array analysis, in short children with various degrees of IGF-I deficiency and a normal or increased GH secretion pattern.

Subjects and Methods

Subjects

DNA from patients with short stature was sent to us for genetic analysis. For this study we included patients with short stature (height standard deviation score (SDS) <–1.9, according to Dutch references [23]) and no abnormalities with respect to medical history, physical examination, radiologic, and biochemical investigations, that could point to a diagnosis. GH secretion was either normal (6.7–26.6 µg/l) or elevated (arbitrarily defined as ≥ 26.7 µg/l), as assessed by a standard GH provocation test (clonidine or arginine test) (except for 2 cases where it was not tested), and body proportions (sitting height/height ratio) were normal (between –2.5 and +2.5 SDS for age) [24].

Subjects were divided into three groups, based on height SDS and serum level of IGF-I (assessed at least twice). Group 1 consisted of severely short children (height SDS <–2.5) with a low serum level of IGF-I (<–2 SDS). According to the recently proposed algorithm [11], these children would be suitable candidates for genetic testing. Children in group 2 were less short (height SDS between –2.5 and –1.9), but IGF-I deficient (serum IGF-I <–2 SDS). Group 3 consisted of children with a wide range of short stature (height SDS <–1.9) and a serum IGF-I in the lower normal range (i.e. between –2 and 0 SDS).

Auxological data and clinical characteristics were collected from case records. Birth weight, length and head circumference were expressed as SDS according to Swedish reference data [25]. In children of whom no birth length or head circumference were available, length and head circumference SDS in the first 3 months of life were used as proxy estimates. Height at presentation as well as parental heights were expressed as SDS according to Dutch nation-wide reference charts [23]. Conditional target height (cTH) SDS was calculated as 0.72 × mean parental height SDS [26], and the distance between height SDS and cTH SDS was recorded. The growth response to GH treatment is expressed as the change in height SDS [23] during the first year. Body mass index (BMI) was calculated as height (m)/weight (kg)^2 and expressed as SDS for the Dutch 1980 references, which were collected before the start of the obesity epidemic [27]. The serum IGF-I data presented in the tables were obtained at clinical presentation.

Biochemical Tests

Serum levels of IGF-I were measured either in Leiden University Medical Center or University Medical Center Utrecht (UMCU) on the Immulite 2500 or Immulite 1000 (Siemens, Munich, Germany), respectively. Both methods were correlated to the original in-house IGF-I RIA for which age references had been determined [28]. Serum IGFBP-3 was determined in Utrecht as described previously [28] and in Leiden on the Immulite 2500. Results were expressed as SDS for age and gender, after correction for inter-assay differences, based on previously

described age references [28]. GH was measured in local laboratories, but a nation-wide quality control system assured that the results of different assays were comparable [29]. Results are expressed as µg/l (1 µg/l = 3 mU/l according to the most recent standard (IS 98/574)).

For subjects suspected for ALS deficiency, serum ALS levels were determined using the ELISA kit of Mediagnost (Reutlingen, Germany). Intra-assay variations were 6.6 and 6.8% at mean levels of 911 and 1,338 mU/ml, respectively. Inter-assay variations were 9, 8 and 8% at mean levels of 931, 1,061, and 1,926 mU/ml, respectively. Since ALS levels in the circulation depend on age and gender (although less than serum levels of IGF-I and IGFBP-3), values were transformed to SDS using reference values. In order to establish normative ranges for ALS in the circulation, non-fasting blood samples were collected from 159 children (81 girls, 78 boys) ranging in age from 10 months to 18 years. These samples were derived either from healthy children from several primary schools in the Netherlands, selected populations of patients (e.g. with minor ear, nose or throat conditions) or their healthy siblings visiting the UMCU. In addition, serum or EDTA plasma samples of adolescents and adults (176 females, 181 males) ranging from 18 to 78 years were obtained from the Red Cross Blood Bank Utrecht (Utrecht, the Netherlands), and from healthy volunteers working in the endocrine department of UMCU. None of these subjects were suffering from malnutrition or showed signs of acute disease or endocrine abnormalities. All samples were obtained after informed consent and stored at –20°C until analysis.

The different molecular-size classes of endogenous IGF-IGFBP complexes in plasma were determined by neutral gel filtration through a 1.6 × 60 cm HiLoad Superdex-200 column, as described previously [15]. In order to investigate if the abnormal profiles could be normalized by spiking the samples with excess rhIGFBP-3, we used glycosylated hIGFBP-3, isolated from pooled normal human plasma using the purification procedure as modified by Martin and Baxter [30].

IGF-I Generation Test

The GH dose-escalation IGF-I generation test was performed as previously described [21]. In brief, GH was administered once a day subcutaneously for 1 week with a dosage of 0.7 mg/m^2/day. In case of an insufficient response (defined as a change of <1 SDS), the dose was doubled (1.4 mg/m^2/day), and if the response was still too low, a third series of GH injections (2.8 mg/m^2/day) was administered. Serum IGF-I was measured before the first injection and after the seventh one. Washout periods between subsequent doses of GH lasted at least 4 weeks.

Genetic Tests

Genomic DNA was isolated from peripheral blood samples using the Autopure LS Instrument (Gentra Systems). Direct sequencing was carried out according to standard procedures (primer sequences are available upon request).

Variants were classified as polymorphisms on the basis of their presence in dbSNP (build 132) or as polymorphisms in gene-specific mutation databases (HGMD). Missense mutations were classified as pathogenic or unclassified variant (UV) based on their presence in gene-specific mutation databases and in silico prediction programs (Polyphen, SIFT). Intronic and neutral amino acid substitution variants were analyzed with various in silico splice predict software programs (Human Splicing Finder, SpliceSiteFinder-like, MaxEntScan, and NNSPLICE) to determine the potential effect on splicing. Different Multiplex Ligation-dependent Probe Amplification (MLPA) kits (MRC Holland, The Netherlands) were used to detect deletions and duplications in various genes: P262 for *GHR*, *IGF1* and *STAT5b*, P216 for *LHX4*, *POU1F1*, *HESX1*, *PROP1*, *GHRHR*, *LHX3* and *GH1*, P18D1 for *SHOX*, and P217 for *IGF1R*, *IGFBP3* and *IGFALS*, according to the manufacturer's instructions. The Affymetrix GeneChip Human Mapping 262K *NspI* array was used according to the instruction provided in the Affymetrix GeneChip Human Mapping 500K Manual (http://www.affymetrix.com) to detect copy number variants (CNVs). SNP copy number was assessed in the patient using CNAG (Copy Number Analyzer for GeneChip®) Version 2.0 [31].

Functional Studies

Antibodies

The following antibodies were used: anti-phospho-STAT5 (Tyr694) from Cell Signaling Technology (Beverly, Mass., USA), anti-STAT5b (G2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif., USA), anti-FLAG M2 antibody from Sigma (St. Louis, Mo., USA), and anti-mouse IgG and anti-rabbit IgG from Amersham-Pharmacia Biotech (Uppsala, Sweden).

Generation of Recombinant Mutant N-FLAG-STAT5b Plasmids

N-terminally FLAG-tagged wild-type STAT5b (F-STAT5b) was described previously [32]. FLAG-STAT5b-V498M (F-V498M) was generated by site-specific mutagenesis (QuickChange II Site-Directed Mutagenesis Kit; Stratagene, La Jolla, Calif., USA), using F-STAT5b as template. The primers used were forward 5'-gggtgccatttgcc**a**tgcctgacaaagtg-3' and reverse 5'-cactttgtcaggca**t**ggcaaatggcaccc-3'. The nucleotide substitutions (in bold) are underlined. The resulting F-V498M variant was confirmed by DNA sequencing.

Cell Culture and Transfection Experiments

HEK293 cells stably transfected with the full-length human *GHR* cDNA, HEK293(hGHR)

[33], were maintained as recommended. For reconstitution studies, HEK293(hGHR) cells were seeded at 2×10^5/well, grown to ~60% confluence in 6-well tissue culture plates, and transiently transfected with 1 µg of vector, pcDNA3.1, or 1 µg of vector carrying F-STAT5b, or F-V498M, using TransIT-LT1 (Mirus, Madison, Wisc., USA). After 24 h of transfection, cells were starved for 9 h prior to a 20-min treatment with 100 ng/ml rhGH (generous gift from Serono), as described previously [32]. Transfection experiments were performed in duplicates, at least three independent times.

Luciferase Reporter Assay

Luciferase (pGHRE-LUC)-transfected HEK293(hGHR) cells were analyzed for reporter activity using the luciferase assay system (Promega Corp., Madison, Wisc., USA). A total input of DNA was 2 µg/well: 1 µg of pGHRE-LUC plus 1 µg of pcDNA3.1, or relevant F-STAT5b variant (F-STAT5b, or F-V498M). After treatment with GH for 24 h, collected cell lysates (total amount is as indicated) were analyzed for reporter activity using a luminometer (BioTek Instruments Inc., Winooski, Vt., USA). The results (from at least two independent experiments, performed in duplicate) are reported as relative fold induction ± SD, compared to activities detected in 20 µg of total protein of untreated, pcDNA3.1 transfected cell lysates, which was given an arbitrary unit of 1.

Results

Auxology and Biochemistry

Clinical and biochemical characteristics of the subjects in groups 1–3 are shown in tables 1–3, respectively. Three out of 9 children in group 3 were born small for gestational age (SGA). The average GH peak after provocation was clearly elevated (112 mU/l), and in 7 out of 9 cases the GH peak was ≥26.7 µg/l. In group 2, none of the 6 children was born SGA, and only 1 had a GH peak ≥26.7 µg/l. In group 3, 10 out of 21 children were born SGA, and 10 out of 19 had a GH peak ≥26.7 µg/l. Two participants (DD and JK) did not undergo a provocation test because both their serum IGF-I levels were within the normal range and GH treatment was approved because of SGA. Most patients were treated with biosynthetic GH and all except 2 cases in group 3 (NV, Δ height SDS 0.1; DD, Δ height SDS 0.3) showed a normal growth response to GH during the first year of treatment (Δ height SDS 0.4–1.1).

IGF-I Generation Test

In 11 of the 36 subjects, GH sensitivity was studied using an IGF-I generation test (table 4). A normal response was defined as an increase of the serum IGF-I level with >1 SDS on a dosage of 0.7 mg/m^2/day (equivalent to 25 µg/kg/day at a body surface area of 1 m^2). This was

Table 1 Clinical and biochemical features of group 1 (height SDS < -2.5, IGF-I < -2.0 SDS), ranked according to height SDS

Patient	Sex	Birth weight[a]	Birth length[a]	Birth HC[a]	Age years	Height[a]	HC[a]	BMI[a]	Paternal height[a]	Maternal height[a]	Height cTH[a]	GHmax µg/l	IGF-I[a]	IGFBP-3[a]	Clinical features	ΔHSDS (GHdose)
IR	M	–1.8	–2.1	0.3	12.47	–4.7	–1.8	–2.3	–3.6	–2.0	–2.7	34.7	–2.9	0.2	–	0.5 (1.4 +GnRHa)
RZ[b]	M	0.2	–2.3	–1.3	3.24	–4.5	–2.5	–0.3	–3.0	–1.6	–2.9	95.3	–3.2	–5.2	high-pitched voice, obesity	No R
BV	F	–0.9	–0.1	–	38.34	–4.5	–2.6	–1.1	–3.0	–1.9	–2.7	8	–2.8	–0.2	–	No R
ER	F	0.4	0.6	1.3	8.41	–3.9	–0.2	0.3	–3.4	–1.9	–2.0	26.7	–2.1	1.1	–	0.5 (0.8)
IZ[b]	M	0.2	–	–	6.98	–3.6	–1.2	1.3	–3.0	–1.6	–2.0	68.7	–3.6	–2.6	high-pitched voice, obesity	No R
CH	M	–1.7	–1.1	–	4.88	–3.5	–	–0.7	–1.3	–1.2	–2.6	35.3	–2.5	–2.9	eczema	0.4/0.6 years (2.8)
TW	M	–2.5	–5.3	–0.7	3.72	–3.3	–2.2	–1.9	1.7	0.0	–4.0	29.3	–2.3	–1.8	–	0.8 (1.0)
AL	F	0.8	–1.0	–2.3	5.82	–3.2	–0.8	–0.7	0.8	–2.1	–2.8	10.7	–3.1	–1.6	–	1.2 (1.0)
CG	F	–1.1	–	–	61.51	–3.0	–2.0	1.7	–0.6	–1.6	–2.2	27.7	–2.0	–1.2	low BMD, Graves	No R
Mean	5M/4F	–0.7	–1.6	–0.5	16.2	–3.8	–1.7	–0.4	–1.7	–1.6	–2.6	37.3	–2.7	–1.7	–	–

HC = head circumference; cTH = conditional target height; GHmax = maximum GH peak after provocation; ΔHSDS (GHdose) = response to growth hormone treatment, expressed as change in height SDS; GH dose = GH dose (mg) per m^2 body surface per day; No R = no treatment; BMD = bone mineral density.

[a] All auxological data and serum levels of IGF-I and IGFBP-3 are expressed as standard deviation score (SDS). [b] Brothers.

Table 2 Clinical and biochemical features of group 2 (height SDS -2.5 to -1.9, IGF-I < -2.0 SDS), ranked according to height SDS

Patient	Sex	Birth weight[a]	Birth length[a]	Birth HC[a]	Age years	Height[a]	HC[a]	BMI[a]	Paternal height[a]	Maternal height[a]	Height cTH[a]	GHmax µg/l	IGF-I[a]	IGFBP-3[a]	Clinical features	ΔHSDS (GH dose)
JH	M	–0.9	–1.7	–1.4	5.43	–2.4	–1.3	–1.1	–0.1	–0.2	–2.3	14.7	–3.4	–3.0	–	0.9 (1.0)
JB	M	–0.1	–0.1	–0.4	5.25	–2.4	1.6	–0.1	0.5	–1.5	–2.0	14	–3.0	–2.5	–	1.0 (1.3)
KB	M	0.3	0.7	0.3	3.72	–2.4	–0.3	0.2	–2.6	0.4	–1.6	33.7	–3.3	–0.5	dysmorphic	0.5 (1.0[b])
WW	M	0.1	1.0	0.4	11.26	–2.2	–1.3	–2.1	–0.9	0.1	–1.9	12.3	–2.8	–1.7	–	No R
MH	F	–0.1	0.9	1.3	4.25	–2.0	–0.1	–0.6	0.5	–0.1	–2.1	12.3	–3.7	–4.7	dysmorphic	No R
JT	F	0.7	–0.2	–0.4	4.09	–1.9	–1.5	–0.3	0.7	0.0	–2.2	21.7	–2.5	–1.8	dysmorphic	1.0 (1.3)
Mean	4M/2F	0.0	0.1	0.0	5.7	–2.2	–0.5	–0.7	–0.3	–0.2	–2.0	18	–3.1	–2.4	–	–

HC = head circumference; cTH = conditional target height; GHmax = maximum GH peak after provocation; ΔHSDS (GH dose) = response to growth hormone treatment, expressed as change in height SDS; GH dose = GH dose (mg) per m^2 body surface per day; No R = no treatment.

[a] all auxological data and serum levels of IGF-I and IGFBP-3 are expressed as standard deviation score (SDS). [b] Age at start 7.2 years. The dose was increased from 0.7 to 1.0 mg/m^2 after 3 months.

Table 3 Clinical and biochemical features of group 3 (height SDS < -1.9, IGF-I -2 to 0 SDS), ranked according to height SDS

Patient	Sex	Birth weight[a]	Birth length[a]	Birth HC[a]	Age years	Height[a]	HC[a]	BMI[a]	Paternal height[a]	Maternal height[a]	Height cTH[a]	GHmax µg/l	IGF-I[a]	IGFBP-3[a]	Clinical features	ΔHSDS (GH dose)
DD	M	–2.2	–2.6	–	5.16	–5.2	–1.6	–1.1	–2.2	–3.6	–3.1	–	–1.3	–0.4	–	0.3 (1.3)
BrKa	F	–1.7	–2.7	–1.1	0.87	–4.0	–1.6	–1.0	–1.5	–0.4	–3.3	12	–0.1	–2.0	–	1.1 (1.0)
LR	F	–1.1	–1.1	2.3	3.38	–3.7	–0.8	0.4	–2.4	–1.7	–2.2	7.3	–1.5	0.4	–	No R
JK	F	–1.8	–2.4	–	6.43	–3.4	–1.8	–0.4	–0.5	–1.7	–2.6	–	–1.0	–1.5	–	0.7 (1.0)
MV[b]	M	–0.9	–0.5	–0.3	4.07	–3.3	–0.7	–0.1	–2.6	–3.5	–1.1	24	–1.6	–2.6	SH/H +2.4 SDS	1.0 (1.0)
BrKo	M	–0.3	–1.3	–0.6	8.14	–3.2	–0.6	–0.5	–0.3	–0.9	–2.8	13.7	–1.0	0.1	–	No R
GA	M	0.0	0.5	–0.2	6.60	–3.1	–0.1	–1.4	–1.7	–1.2	–2.1	23.7	–1.9	–0.7	–	1.1 (1.1)
NK	M	–0.5	–1.2	0.3	4.83	–3.0	–1.0	–0.1	–1.8	0.0	–2.4	48.3	0.1	0.9	dev delay	No R
MH	M	–1.2	–2.6	–	8.19	–2.8	–0.3	–1.1	–0.2	–2.3	–1.9	18.7	–1.7	–0.7	–	1.1 (1.0)
SH	M	1.6	0.1	0.2	4.97	–2.7	0.6	–0.8	–1.4	0.1	–2.2	28	–1.9	–1.5	dev delay	No R
RK	M	–1.0	0.7	_	6.80	–2.6	–0.5	–0.3	–2.8	–1.0	–1.3	16.7	–1.6	–2.0	–	No R
VO[c]	M	–1.6	–2.0	–0.4	13.38	–2.6	–1.2	–2.1	–0.1	–2.3	–1.7	48.7	–1.2	–0.3	–	0.5 (1.5)
NV[b]	F	–1.3	–1.7	–1.2	3.45	–2.5	–0.4	–0.2	–2.6	–3.5	–0.3	40.3	–0.7	–2.1	–	0.1 (0.7)
GY	F	–2.3	–1.4	–0.1	4.41	–2.5	0.0	–0.2	–0.6	–2.9	–1.2	48.3	–1.1	–0.4	frontal bossing	No R
MM	M	–3.2	–4.1	–1.8	5.92	–2.4	–2.0	–1.1	–0.5	–0.8	–1.9	50	–0.4	0.8	hypospadia	No R
TO[c]	M	–2.9	–2.0	–2.1	13.38	–2.4	–1.7	–1.9	–0.1	–2.3	–1.5	41.7	–1.5	–0.7	–	0.4 (1.5)
WD	M	0.1	–0.8	0.4	4.11	–2.4	–0.3	–0.5	–1.5	–4.3	–0.3	14.7	–1.9	–1.1	–	No R
JO	M	0.0	–0.5	–0.9	9.42	–2.2	–0.2	–0.2	–1.6	–1.2	–1.2	11	–0.7	–1.4	–	No R
JR	M	–0.9	–2.4	–	4.84	–2.2	–1.4	–1.4	–0.3	–1.6	–1.5	29.3	–0.8	1.9	–	No R?
BaKo	M	0.4	0.5	0.2	4.12	–1.9	–1.7	–1.2	0.4	–0.6	–1.8	40.7	–0.8	–0.9	–	No R
KBr	M	–2.5	–2.2	–1.2	4.98	–1.9	–0.7	–2.1	–0.5	–1.5	–1.2	27.7	–0.9	–0.5	–	No R
mean	16M/5F	–1.1	–.0	–0.4	6.1	–2.9	–0.9	–0.8	–1.2	–1.8	–1.8	28.7	–1.1	–0.7	–	–

HC = head circumference; cTH = conditional target height; GHmax = maximum GH peak after provocation; ΔHSDS (GH dose) = response to growth hormone treatment, expressed as change in height SDS; GH dose = GH dose (mg) per m^2 body surface per day; No R = no treatment; SH/H = sitting height/height ratio; dev delay = developmental delay.
[a] all auxological data and serum levels of IGF-I and IGFBP-3 are expressed as standard deviation score (SDS). [b] Siblings. [c] Twin brothers.

observed for 3 subjects, i.e. JH, KB (group 2) and (borderline response) NV (group 3). A low response (a serum IGF-I increase <1 SDS) to the lowest dose of GH, but a sufficient response to the intermediate dosage (1.4 mg/m^2/day) of GH, suggesting partial GH resistance, was observed for 5 patients (group 1: ER and CH; group 2: JB and JT; group 3: MV). One patient (IR, group 1) only showed an IGF-I response to the highest GH dose, suggesting a severe GH insensitivity. Finally, 2 patients (brothers RZ and IZ) were virtually insensitive to GH, and were not treated with GH.

The short-term serum IGF-I responses to GH in the IGF-I generation test did not correlate well with the growth response to long-term GH treatment (tables 1–4), although the variety of doses makes a comparison difficult. On average, the growth response of the 3 children with a normal IGF-I generation test was 0.5 SDS (range 0.1–0.9) in the first year, and of the 5 children with partial insensitivity it was 0.8 SDS (range 0.5–1.0). One of the two poor responders in terms of growth response (NV) had a borderline normal IGF-I response. The patient with severe GH insensitivity (IR) was treated with both GH and GnRH analogue from 13.8 till 15.7 years of age, and GH was continued until 17.0 years. Her height SDS at start of GH treatment was -4.6 SDS and increased to –3.4 SDS at 17 years.

Genetic Findings

Group 1

Five out of 9 patients exhibited one or more variants in direct sequencing of genes known to be involved in the regulation of growth or in the whole-genome SNP array analysis (table 5). These findings are described in detail below.

The 2 brothers (RZ and IZ) both showed abdominal obesity, a high-pitched voice, mid-face hypoplasia, and frontal bossing, and the biochemical picture of complete GH insensitivity (tables 1, 4). They shared a heterozygous unclassified variant (UV) in *STAT5B* (p.Glu315Ala) which was not inherited from their mother (unfortunately, no DNA was available from their father). RZ was 0.9 SD shorter than his brother, and has two additional genetic variants. His shorter stature may be caused by the UV in *IGFALS* (p.Arg548Trp) rather than by the interstitial deletion (maximally 272.5 kb) in Xq25 containing one gene (*WDR40C*). This is supported by both the lower 150 kDa ternary complex peak (fig. 1b) and serum ALS level (–1.2 SDS) in RZ when compared with the results for IZ (serum ALS: 1.1 SDS) (fig. 1c).

Table 4 Results of the GH dose escalation IGF-I generation test (according to Walenkamp and Wit [21])

		Low dose (0.7 mg/m²/day × 7)				Intermediate dose (1.4 mg/m²/day × 7)				High dose (2.8 mg/m²/day × 7)			
		IGF-I SDS		IGFBP-3 SDS		IGF-I SDS		IGFBP-3 SDS		IGF-I SDS		IGFBP-3 SDS	
Patient	Age years	0	7	0	7	0	7	0	7	0	7	0	7
IR	12.2	–3.4	–2.6	–4.3	–3.0	–3.0	–2.7	–3.8	–3.1	–2.7	–1.4	–3.3	–3.1
RZ	4.6	<–3.4	<–3.4	–3.1	–2.6	<–3.4	<–3.4	–1.8	–1.8	<–3.5	<–3.5	–2.2	–2.6
ER	7.3	–1.7	–1.7	–0.1	–0.1	–2.0	+0.5	+0.3	+0.9	–	–	–	–
IZ	7.4	–4.2	–4.3	–2.9	–2.9	–3.9	–4.5	–2.4	–2.7	–3.8	–3.4	–2.4	–2.7
CH	5.2	–3.0	–2.3	–3.0	–2.3	–3.4	–1.7	–3.2	–2.4	–2.7	–1.6	–2.3	–1.7
JH	7.3	–3.9	–2.5	–2.6	–0.1	–	–	–	–	–	–	–	–
JB	5.4	<–3.0	–2.4	–2.5	–1.5	<–3.0	–2.1	–3.5	–1.2	<–3.1	–1.6	–2.6	–0.7
KB	7.0	–3.3	–0.9	–0.5	+1.2	–	–	–	–	–	–	–	–
JT	4.5	–2.5	–2.9	–3.0	–2.5	–3.1	–1.6	–4.3	–1.1	–	–	–	–
MV	4.9	–2.1	–2.4	+0.8	–3.4	–3.0	–0.4	–1.5	–2.5	–	–	–	–
NV	11.3	–1.4	–0.4	–	–	_	–	–	–	–	–	–	–

0 = Measurement at start (day 0); 7 = Measurement after 7 injections (day 7).

Table 5 Genetic findings in group 1

Patient	*GH1*	*GHR*	*STAT5B*	*IGFALS*	SNP array
RZ[a]			het UV c.944A>C p.Glu315Ala	het UV c.1642C>T p.Arg548Trp	arr Xq25 (SNP_A-2190162→SNP_A-2154195)×0 mat, containing 1 gene (*WDR40C*)
BV		het POL c.266+22G>T		het UV c.860C>T p.Pro287Leu	
IZ[a]			het UV c.944A>C p.Glu315Ala		
CH			het UV c.1492G>A p.Val498Met	het UV c.1133C>T p.Pro378Leu	
AL					arr 2q24.3q31.1 (SNP_A-1903408→SNP_A-1965788)×3 mat, 2q31.1 (SNP_A-2147815→SNP_A-2193545)×3 mat, containing 6 and 2 genes, respectively[b]

het = Heterozygous; UV = unclassified variant; mat = maternal; POL = polymorphism.
[a] Brothers. [b] The most proximal duplication contains 6 protein coding genes: *NOSTRIN*, *SPC25*, *G6PC2*, *ABCB11*, *DHRS9* and *LRP2*. The most distal duplication contains two protein coding genes: *DYNC1I2* and *SLC25A12*.

Addition of purified hIGFBP-3 (2.0 µg) per ml serum of RZ restored 150 kDa complex formation efficiently, i.e. it became comparable to the pattern found when the same amount of IGFBP-3 was added to 1 ml serum from IZ (fig. 1c) or a normal control (fig. 1a). This result would suggest that patient's ALS should be capable of effective 150 kDa complex formation at a higher level of IGFBP-3. Possibly, RZ's variant *IGFALS* has a reduced affinity for IGF-IGFBP-3 complexes, which only becomes manifest at his relatively low endogenous level of serum IGFBP-3.

A boy with clinical features suggestive for a *STAT5B* defect (CH), including severe constitutional eczema from infancy, borderline elevated serum prolactin, low serum immunoglobulins, and a very low IgG antibody response to hemophilus B vaccination at 6 months of age, showed heterozygous UVs in *STAT5B* (p.Val498Met, maternally transmitted) and *IGFALS* (p.Pro378Leu, paternally transmitted). The *STAT5B* missense mutation is located in exon 13 and affects a highly conserved nucleotide and amino acid. In reconstitution studies, homozygous expression of the mutant *STAT5B* was lower than the wild-type gene (fig. 2). However, immunologically equivalent amounts of the mutant STAT5b protein could still be phosphorylated by the GH-GHR signaling pathway (fig. 2a) and was able to induce transcriptional activities comparable to that of wild-type STAT5b (fig. 2b).

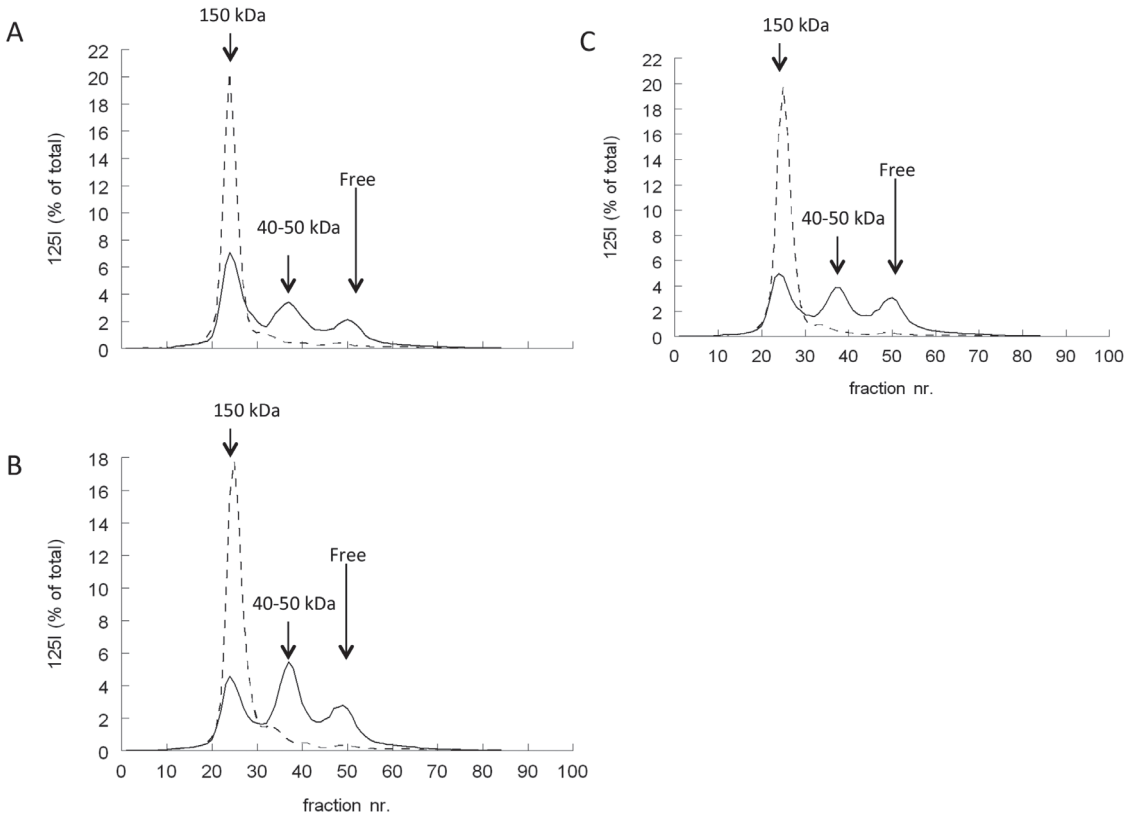

Figure 1 S200 gel filtration column chromatography. **a** Representative column profile for normal adult serum. **b** Serum RZ. **c** Serum IZ. Solid line: without addition; dotted line: addition of purified hIGFBP-3 (2.0 µg/ml serum).

The *IGFALS* variant appears to lead to a partial ALS deficiency, since serum ALS was –1.8 SDS and column chromatography showed a pattern consistent with a heterozygous *IGFALS* defect (fig. 3). This variant has not been found in previous studies, nor in controls. PolyPhen 2 [34] predicts that the *IGFALS* variant could be pathogenic (HumDiv 0.999 score; HumVar 0.985 score). However, addition of purified hIGFBP-3 (2.0 µg/ml) to patient's serum led to increased 150 kDa complex formation (data not shown), and became similar to that observed in normal serum (fig. 1a). It remains to be determined whether the heterozygous *STAT5B* p.Val498Met mutation by itself, or synergistically with the heterozygous *IGFALS* mutation, is the cause of patient's severe short stature and his low serum IGF-I level.

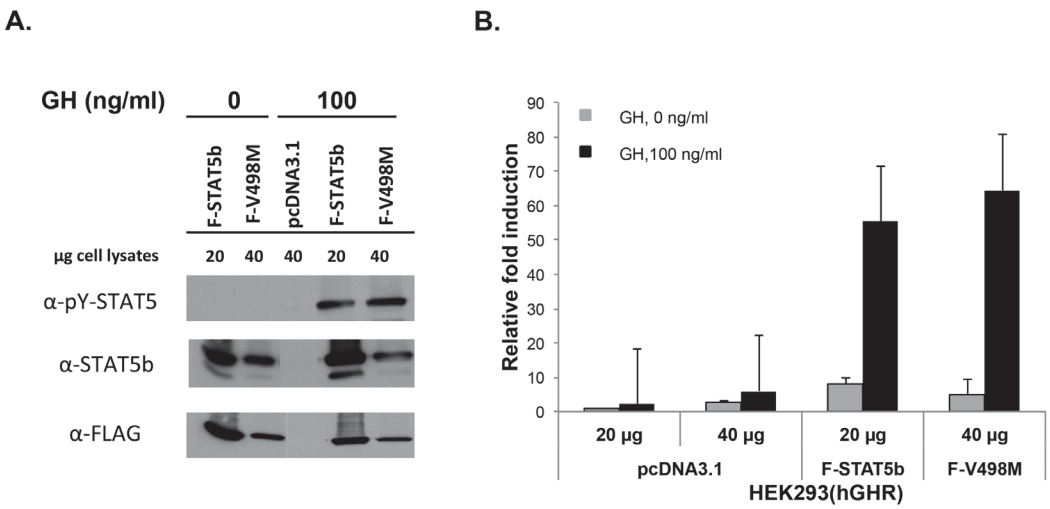

Figure 2 Reconstitution studies of the heterozygous *STAT5B* p.V498M mutation identified in CH. Reconstitution studies were performed in HEK293(hGHR). To compare immunologically equivalent amounts of FLAG-tagged STAT5b protein, loading of cell lysates from cells transfected with F-V498M was twice that of cells transfected with wild-type F-STAT5b or with vector, pcDNA3.1. **a** Western immunoblot analyses of GH-induced STAT5b tyrosine phosphorylation (pY-STAT5). Primary antibodies are indicated on the left side of panels. **b** GH-induced luciferase reporter activities.

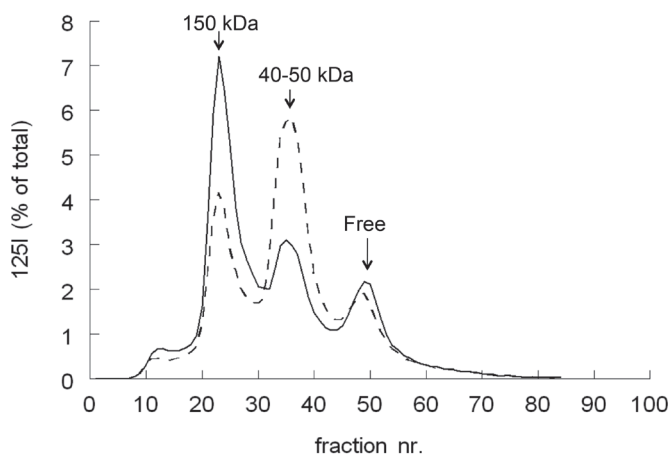

Figure 3 S200 gel filtration column chromatography. Column profile for serum of CH (dotted line) compared with normal adult serum (solid line).

Table 6 Genetic findings in groups 2 and 3

Patient	*GH1*	*GHR*	*STAT5B*	*IGFALS*	SNP array
Group 2					
JB				het POL c.1386C>T, p.Tyr462Tyr (pat)	
KB	het POL c.10+52A>G, c.10+56A>T (mat)				
Group 3					
DD					arr3p12.3(SNP_A-2129422→SNP_A-2233269)×3 Not of mat origin. Pat DNA n.a. containing part of *ROBO2*
BrKa				het UV in 5'UTR c.56-30A>T (mat)	
LR		het UV c.1319G>T, p.Cys440Phe (mat)			
JK					arr15q24.2q24.3(SNP_A-4204149→SNP_A-4240707)×3 mat containing *C15orf27*, *ETFA*, *ISL2* and *SCAPER*
MV[a]		het POL c.558A>G, p.Gly186Gly			
NV[a]					arr16q12.1 (SNP_A-2104022→SNP_A-1828829)×3 mat containing *CBLN1*, *AC0076.14.7*, *C16orf78* and *ZNF423*
MM			het POL c.682-117C>T		arr1q25.1(SNP_A-1800278→SNP_A-4225405)×1 mat containing *ZBTB37*, *SERPINC1*, *RC3H1* and *RABGAPIL*
JO				het UV c.860C>T, p.Pro287Leu (mat) and het POL c.1386C>T, p.Tyr462Tyr	

het = Heterozygous; UV = unclassified variant; mat = maternal; pat = paternal; 5'UTR = 5'untranslated region.
[a] Siblings.

AL had two different interstitial duplications in the long arm of chromosome 2, which have not been described in the normal population. No association with height is known for any of these genes. However, her mother, who was also short (table 1) also carried both duplications, suggesting a functional role of one or both of these duplications. AL responded very well to a regular dose of GH (table 1).

It is uncertain to which extent the heterozygous UV in *IGFALS* (p.Pro287Leu) and the polymorphism in the *GHR* contributed to the severely short stature and low serum IGF-I level of BV. This *IGFALS* variant has been considered to represent a SNP with low abundance (MAF = 0.0053), in silico analysis predicted it to be benign, and serum IGFBP-3 level was within the normal range, all suggestive for a neutral variant. However, this UV has been found previously in patients with ISS [35], and in vitro expression of the mutant p.Pro287Leu ALS protein resulted in increased trans-Golgi co-localization, suggesting impaired trafficking [36]. BV's brother who has comparable short stature (height SDS –5.5) carries the same *GHR* and *IGFALS* variants, in addition to two *IGFALS* polymorphisms (c.1566G>A, p.Thr522Thr and c.1386C>T, p.Tyr462Tyr).

Group 2

In 2 out of 6 patients, DNA variants could be detected (table 6), described in detail below.

In JB, the low serum levels of IGF-I and IGFBP-3 would suggest ALS deficiency. Indeed, the concentration of ALS in serum was low (–2.4 SDS) and 150 kDa ternary complex formation reduced, as assessed by column chromatography, being consistent with partial *IGFALS* deficiency (fig. 4a). As in cases RZ and CH, the profile normalized after adding excess purified hIGFBP-3 (2.0 µg/ml) (data not shown). Also on GH treatment the column profile normalized (fig. 4a) as well as serum ALS (0.8 SDS), and the growth response was excellent (table 2). However, direct sequencing of *IGFALS* only showed a c.1386C>T, p.Tyr462Tyr polymorphism transmitted from his normal statured father, that has been encountered in 18.0% of ISS subjects and 14.8% of normal children [35]. Serum levels of IGF-I, IGFBP-3 and ALS in carriers for this SNP do not differ from those in non-carriers, both in ISS and normal children [H.M. Domené, pers. commun.]. We then performed column chromatography in both parents (fig. 4b) and measured serum ALS. Serum of the relatively short mother (height –1.5 SDS) showed a reduced 150 kDa ternary complex, although serum ALS was normal (0 SDS). The father (with a height of 0.5 SDS) had a normal profile and a high serum ALS (+2.6 SDS). Although we have no good explanation for these findings, they are suggestive for the presence of an *ALS* abnormality that cannot be discovered by sequencing of the coding domains of the gene. Both for the patient and his mother, the addition of hIGFBP-3 to their serum led to a substantial increase in 150 kDa complex formation, to the same extent as encountered for

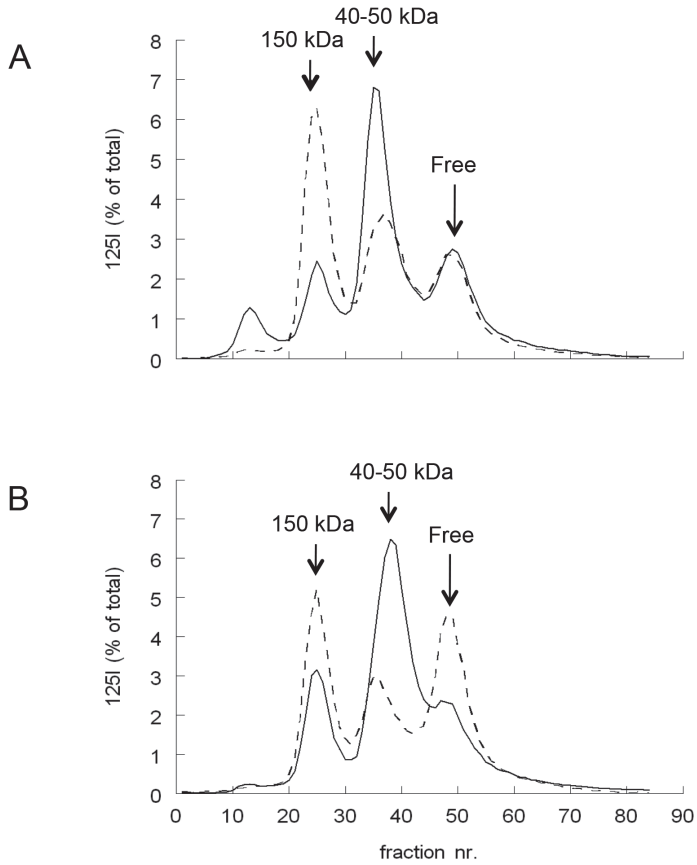

Figure 4 S200 gel filtration column chromatography. **a** Column profile of JB before GH treatment (solid line) and after 4 years of GH treatment (dotted line). **b** Column profile of mother (solid line) and father (dotted line).

normal serum (fig. 1a), suggesting only a partial decrease of IGFALS affinity for IGF-IGFBP-3 binary complexes.

KB had several characteristics of a bioinactive GH molecule, such as short stature, a very low serum IGF-I level that increased sufficiently after a low dose of GH in an IGF-I generation test, and a very high GH peak after provocation. However, curiously the *GH1* variant found in intron 1 originated from KB's mother, who had a normal height (+0.4 SDS).

His short father (height SDS –2.6) had a normal serum IGF-I and IGFBP-3. KB's growth rate increased moderately on GH treatment (1 mg/m^2, started at 7.2 years) and serum IGF-I normalized as well (+0.6 SDS after 1 year), supporting the possibility that his endogenous GH is not biologically active.

Group 3

In 8 out of 21 patients, DNA variants were detected (table 6), but in all cases the pathogenic relevance remains uncertain, as outlined below.

Out of the 4 copy number variations (CNVs) found with SNP array analysis, the 1.4-Mb duplication detected in DD, containing part of *ROBO2*, a non-coding RNA (ncRNA) and a microRNA (miRNA), may suggest an association with short stature. DD was born SGA, remained extremely short and microcephalic, and has short parents (table 3). His mother does not carry the variant, but his younger brother with a similar growth curve (most recent height –4.3 SDS) carries the same duplication. Unfortunately, no DNA from his father is available. The duplication has not been described as a genomic variation in the available databases, however overlapping duplications have been found in two other families investigated in our laboratory. The index patients of these families had short stature, but further analysis of the family showed that the duplication was inherited from a parent with normal stature.

MM had normal serum levels of IGF-I and IGFBP-3, but a very high serum GH peak level after provocation, suggesting either a defect of the IGF-I receptor (*IGF1R*), a post-receptor defect, or an *IGF1* defect. No abnormalities could be found in the *IGF1R* nor *IGF1*, but a small interstitial deletion was detected in chromosome 1q, containing 4 genes (table 6). His mother with a normal height (–0.8 SDS) carried the same deletion. The CNVs detected in MM and the other 2 cases (JK and NV) have not been associated previously with short stature, and, as far as known, no clear candidate genes are present in the deleted or duplicated regions.

Two patients showed UVs in *IGFALS* (BrKa and JO), but in both cases it is unlikely that the clinical and biochemical features can be explained due to these mutations. In BrKa the UV in the 5' UTR of *IGFALS* was inherited from her normal statured mother (height SDS –0.4). During GH treatment there was an excellent growth response (table 3), an elevated level of serum ALS (+2.7 SDS), and normal 150 kDa ternary complex formation. In JO, two variants were found in *IGFALS*, although serum ALS was 0.2 SDS and most of ^{125}I-IGF-I migrated in the 150 kDa ternary complex peak, after column chromatography. Moreover, as explained in the case of BV, it is uncertain whether the p.Pro287Leu variant leads to pathogenic effects. The p.Tyr462Tyr is considered to represent a polymorphism.

RK (table 3) had biochemical characteristics of partial ALS deficiency (a lower serum

IGFBP-3 level than serum IGF-I, a column chromatography pattern typical for partial ALS deficiency, and a serum ALS value of –1.1 SDS), but mutation screening and MLPA did not show any *IGFALS* variant.

LR inherited an UV of the *GHR* from her mother with a height of –1.7 SDS, which makes it unlikely that this variant by itself had caused severe GH insensitivity in this patient. The *GHR* variant in MV represents a synonymous polymorphism.

Polymorphisms

Within the whole cohort of ISS subjects investigated, the frequency of the known polymorphism in *GHR* (a deletion of exon 3, d3) was 50% full length, 34% heterozygous for d3, and 16% homozygous d3/d3. Two known polymorphisms of *IGF1* were found in 3 cases (exon 2: c.64–23A>C in 1 case, exon 3: c.221–164G>A in the other 2 cases).

Discussion

In our group 1 (9 patients with severe short stature (height SDS <–2.5) and decreased serum IGF-I), 5 were found to carry a gene variant. In 3 of them (RZ, IZ and CH), heterozygous mutations of *STAT5B* appeared to be involved in the observed GH insensitivity. In 2 other patients the associations between the respective genetic variants in *IGFALS* and short stature remains uncertain. Thus, according to the previously proposed clinical algorithm [11], the yield in terms of established diagnoses was 3/9, i.e. 33%. By contrast, the association between the genetic variants observed in groups 2 and 3 with the clinical and biochemical features remained uncertain.

With respect to the two novel heterozygous *STAT5B* variants, for which in silico analysis suggested pathogenicity, we first speculated that these mutations could exert dominant negative effects. However, so far we have not been able to confirm this. Alternatively, mutations may exist in non-coding regions of the *STAT5B* gene (e.g. the promoter) in the other allele, resulting in a compound heterozygous defect, or in unidentified genes involved in its regulation of expression. Another interesting novel observation is that several patients show a combination of variants in two genes that are known to be involved in the regulation of growth, for example, the *STAT5B* and *IGFALS* variants in CH and RZ. We hypothesize that, similar to the finding of a high percentage of abnormalities of oligogenic origin in hypogonadotropic hypogonadism [19], GH insensitivity may be of digenic, oligogenic or polygenic origin.

Although we found three *GHR* variants, the only one for which the association with GH resistance cannot be excluded is the c.1319G>T, p.Cys440Phe mutation. However,

the maternal transmission (maternal height –1.7 SDS) makes its pathogenicity doubtful, which is in line with previous publications on this variant (in older nomenclature termed p.Cys422Phe), showing no difference in signaling in vitro, when compared with controls [reviewed in 37].

It is also unlikely that the variant in *GH1* exerts a pathogenic effect in KB, since the *GH1* variant was inherited from the normal statured mother, and the response to long-term GH treatment was only moderate. We have not been able to test for *GHSR* variants, but the prevalence of abnormalities in this gene appears to be low [6, 38–40].

The biological significance of the heterozygous variants in *IGFALS* observed in 6 patients within the whole cohort of ISS subjects remains uncertain. In previous studies, we showed that heterozygosity for dysfunctional *IGFALS* mutations may lead to approximately a 1 SD height loss [15–17]. In contrast to his brother IZ, RZ had an additional p.Arg548Trp *IGFALS* variant besides the *STAT5B* variant which both brothers carried. The differences between the 2 brothers with respect to height SDS (i.e. 0.9 SD), serum ALS levels, and the relative size of the 150 kDa peak after column chromatography, is consistent with an additional effect of the heterozygous *IGFALS* variant in RZ. Possibly, this variant decreases the affinity of IGFALS, since in the presence of a high concentration of IGFBP-3, 150 kDa complex formation increased substantially. The p.Arg548Trp *IGFALS* variant is described as a SNP in several databases, and has been encountered previously in 2 ISS children as well as in 2 normal control children [H. R. Domené, pers. commun.]. However, in silico analysis by PolyPhen predicts a damaging effect, and preliminary in vitro studies on the expression of the p.Arg548Trp ALS mutant protein indicate an increased trans-Golgi co-localization and a reduction in ALS secretion [36]. The 2 ISS children carrying this heterozygous variant had serum levels of IGF-I, IGFBP-3 and ALS all below –2.0 SDS. On the other hand, 2 normally statured children carrying this same heterozygous variant showed circulating levels of IGF-I, IGFBP-3 and ALS within the respective normal ranges [H. R. Domené, pers. commun.]. The p.Pro378Leu *IGFALS* variant in combination with the *STAT5B* variant in CH may have contributed to his severe short stature and GH insensitivity. The role of the p.Pro287Leu *IGFALS* variant that we found in 2 other patients is less clear. In BV it is unlikely that this is the cause of the severe short stature, whereas in JO the column profile did not point to ALS deficiency.

An interesting observation was a low or low-normal serum ALS level and reduced 150 kDa ternary complex formation in 2 children (JB and RK) with a phenotype and biochemical features suggestive for a heterozygous *IGFALS* defect. Nonetheless, *IGFALS* sequencing only showed a common (neutral) synonymous SNP and the WT sequence of nucleotides, respectively. This suggests that there may be abnormalities of ALS secretion that are not caused by exonic variants. In 1 of them (JB), serum ALS and the 150 kDa ternary complex

formation normalized on GH treatment, as well as in vitro after addition of exogenous hIGFBP-3. With respect to the genetic finding in patient BrKa, there is no reported *cis*-element in the 5'-UTR promoter region of the *IGFALS* gene affected by the variant (c.56–30A>T) [41, 42].

Our data show that the GH dose-escalating IGF-I generation test has some diagnostic value by allowing discrimination between severe GH insensitivity, as in the cases with *STAT5B* variants, and reduced bioactivity of endogenous GH. However, the predictive value of this test for the efficacy of GH treatment is low, since almost all patients who were treated with GH showed an adequate growth response, independent of the results of the IGF-I generation test. It must be emphasized, however, that some of them received supraphysiologic doses of GH. Further studies on alternative regimens of the test including a larger number of patients are needed before definitive conclusions about its value in the diagnostic workup of patients with IGF-I deficiency can be reached.

Besides the variants in known genes involved in growth disorders, we found novel CNVs in 6 short children. It is possible that in some of these patients the CNVs are associated with the phenotype, but in none of these CNVs clear candidate genes were involved. This finding is concurrent with a recent report on an increased burden of lower-frequency deletions in children with short stature [43]. With the accumulation of CNV data on many more patients into the databases, novel gene defects may be discovered that play a role in IGF-I generation and growth.

In conclusion, the diagnostic yield of genetic testing in children with severe short stature (height <–2.5) and low serum IGF-I is approximately 30%, which appears sufficient to advise genetic assessment of the genes that are currently known to be associated with GH insensitivity. We have presented evidence that heterozygous *STAT5B* mutations may be associated with GH insensitivity. In at least two cases we found evidence for a digenic origin of short stature. In children with less severe short stature and/or modestly decreased serum IGF-I levels, the likelihood of finding variants in these genes is much lower, suggesting that other, as yet unknown, genes play a role.

Acknowledgements

Thanks are due to all members of the Leiden Growth Genetics Working Group for useful discussions, to Ms. A. Autar (Leiden University Medical Center, LUMC) for technical assistance, to Dr. B. Ballieux (LUMC) for measuring serum IGF-I and IGFBP-3, and to the following clinicians for providing clinical information about the patients from whom DNA samples were obtained: Dr. N. Biermasz (LUMC); Dr. A.J.M. van den Broek (Diaconessenhuis, Leiden); Dr. A. Clement-Boers (Hagaziekenhuis, The Hague); Dr. O.M. Dekkers (LUMC); A.C. den Dulk (LUMC); Dr. S. Elkerbout (Rijnland Hospital, Leiderdorp); Dr. A.C. Engelberts

(Diaconessenhuis, Leiden); Dr. M.N. Gerding (Deventer Ziekenhuis, Deventer); Dr. S.E. Hannema (LUMC); Dr. D.A.J.P. Haring (Diaconessenhuis, Leiden); Dr. H. Havers (Rijnland Hospital, Leiderdorp); Dr. I. de Kruijff (Hofpoort Hospital, Woerden); Dr. P. Mourad-Baars (LUMC); Dr. D. Mul (Hagaziekenhuis, The Hague); Dr. P.H.M. Ooijevaar (Diaconessenhuis, Leiden); Dr. H.M. Reeser (Hagaziekenhuis, The Hague); Dr. J. Rehbock (Langeland Hospital, Zoetermeer); Dr. P.A.W.A. Renardel de la Valette (Antonius Hospital, Nieuwegein); Dr. H. Roggeveen (LUMC); Prof. C. Schrander-Stumpel (Maastricht University Medical Center, Maastricht); Dr. E. Stam (Langeland Hospital, Zoetermeer); Dr. W.H. Stokvis-Brantsma (LUMC); Dr. J. de Vos (Maastricht University Medical Center, Maastricht); Dr. M. Wagenvoort (Hagaziekenhuis, The Hague); Dr. J.G.C.M. van Zoest (Diaconessenhuis, Leiden). We thank the patients and their parents for their willingness to participate.

Reference List

1 Wit JM, Clayton PE, Rogol AD, Savage MO, Saenger PH, Cohen P: Idiopathic short stature: definition, epidemiology, and diagnostic evaluation. Growth Horm IGF Res 2008;18:89-110.

2 Oostdijk W, Grote FK, De Muinck Keizer-Schrama SM, Wit JM: Diagnostic approach in children with short stature. Horm Res 2009;72:206-217.

3 Kant SG, Grote F, de Ru MH, Oostdijk W, Zonderland HM, Breuning MH, Wit JM: Radiographic evaluation of children with growth disorders. Horm Res 2007;68:310-315.

4 Grote FK, Oostdijk W, De Muinck Keizer-Schrama SM, van Dommelen P, van Buuren S, Dekker FW, Ketel AG, Moll HA, Wit JM: The diagnostic work up of growth failure in secondary health care; an evaluation of consensus guidelines. BMC Pediatr 2008;8:21.

5 Takahashi Y, Kaji H, Okimura Y, Goji K, Abe H, Chihara K: Brief report: short stature caused by a mutant growth hormone [see comments]. N Engl J Med 1996;334:432-436.

6 Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S: Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. J Clin Invest 2006;116:760-768.

7 Godowski PJ, Leung DW, Meacham LR, Galgani JP, Hellmiss R, Keret R, Rotwein PS, Parks JS, Laron Z, Wood WI: Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. Proc Natl Acad Sci U S A 1989;86:8083-8087.

8 Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, Tsubaki J, Pratt KL, Bezrodnik L, Jasper H, Tepper A, Heinrich JJ, Rosenfeld RG: Growth hormone insensitivity associated with a STAT5b mutation. N Engl J Med 2003;349:1139-1147.

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

10 Domene HM, Bengolea SV, Martinez AS, Ropelato MS, Pennisi P, Scaglia P, Heinrich JJ,

Jasper HG: Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene. N Engl J Med 2004;350:570-577.

11 Wit JM, Kiess W, Mullis P: Genetic evaluation of short stature. Best Pract Res Clin Endocrinol Metab 2011;25:1-17.

12 David A, Hwa V, Metherell LA, Netchine I, Camacho-Hubner C, Clark AJ, Rosenfeld RG, Savage MO: Evidence for a continuum of genetic, phenotypic, and biochemical abnormalities in children with growth hormone insensitivity. Endocr Rev 2011;32:472-497.

13 Walenkamp MJ, Karperien M, Pereira AM, Hilhorst-Hofstee Y, van Doorn J, Chen JW, Mohan S, Denley A, Forbes B, van Duyvenvoorde HA, van Thiel SW, Sluimers CA, Bax JJ, de Laat JA, Breuning MB, Romijn JA, Wit JM: Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 2005;90:2855-2864.

14 van Duyvenvoorde HA, van Setten PA, Walenkamp MJ, van DJ, Koenig J, Gauguin L, Oostdijk W, Ruivenkamp CA, Losekoot M, Wade JD, De MP, Karperien M, Noordam C, Wit JM: Short stature associated with a novel heterozygous mutation in the insulin-like growth factor 1 gene. J Clin Endocrinol Metab 2010;95:E363-E367.

15 van Duyvenvoorde HA, Kempers MJ, Twickler TB, van Doorn J, Gerver WJ, Noordam C, Losekoot M, Karperien M, Wit JM, Hermus AR: Homozygous and heterozygous expression of a novel mutation of the acid-labile subunit. Eur J Endocrinol 2008;159:113-120.

16 Domene HM, Hwa V, Argente J, Wit JM, Camacho-Hubner C, Jasper HG, Pozo J, van Duyvenvoorde HA, Yakar S, Fofanova-Gambetti OV, Rosenfeld RG: Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res 2009;72:129-141.

17 Fofanova-Gambetti OV, Hwa V, Wit JM, Domene HM, Argente J, Bang P, Hogler W, Kirsch S, Pihoker C, Chiu HK, Cohen L, Jacobsen C, Jasper HG, Haeusler G, Campos-Barros A, Gallego-Gomez E, Gracia-Bouthelier R, van Duyvenvoorde HA, Pozo J, Rosenfeld RG: Impact of heterozygosity for acid-labile subunit (IGFALS) gene mutations on stature: results from the international acid-labile subunit consortium. J Clin Endocrinol Metab 2010;95:4184-4191.

18 Rosenfeld RG, Belgorosky A, Camacho-Hubner C, Savage MO, Wit JM, Hwa V: Defects in growth hormone receptor signaling. Trends Endocrinol Metab 2007;18:134-141.

19 Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, Dwyer AA, Quinton R, Hall JE, Gusella JF, Seminara SB, Crowley WF, Jr., Pitteloud N: Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci U S A 2010;107:15140-15144.

20 Lango AH, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F, Willer CJ, Jackson AU, Vedantam S, Raychaudhuri S, Ferreira T, Wood AR, Weyant RJ, Segre AV, Speliotes EK, Wheeler E, Soranzo N, Park JH, Yang J, Gudbjartsson D, Heard-Costa NL, Randall JC, Qi L, Vernon SA, Magi R, Pastinen T, Liang L, Heid IM, Luan J, Thorleifsson G, Winkler TW, Goddard ME, Sin LK, Palmer C, Workalemahu T, Aulchenko YS, Johansson A, Zillikens MC, Feitosa MF, Esko T, Johnson T, Ketkar S, Kraft P, Mangino M, Prokopenko I, Absher D, Albrecht E, Ernst F, Glazer NL, Hayward C, Hottenga JJ, Jacobs KB, Knowles JW, Kutalik Z, Monda KL, Polasek O, Preuss M, Rayner NW, Robertson NR, Steinthorsdottir V, Tyrer JP, Voight BF, Wiklund F, Xu J, Zhao JH, Nyholt DR, Pellikka N, Perola M, Perry JR, Surakka I, Tammesoo ML, Altmaier EL, Amin N, Aspelund T, Bhangale T, Boucher G, Chasman DI, Chen C, Coin L, Cooper MN, Dixon AL, Gibson Q, Grundberg E, Hao K, Juhani JM, Kaplan LM, Kettunen J, Konig IR, Kwan T, Lawrence RW, Levinson DF, Lorentzon M, McKnight B, Morris AP, Muller M, Suh NJ, Purcell S, Rafelt S, Salem RM, Salvi E, Sanna S, Shi J, Sovio U, Thompson JR, Turchin MC, Vandenput L, Verlaan DJ, Vitart V, White CC, Ziegler A, Almgren P, Balmforth AJ, Campbell H, Citterio L, De GA, Dominiczak A, Duan J, Elliott P, Elosua R, Eriksson JG, Freimer NB, Geus EJ, Glorioso N, Haiqing S, Hartikainen AL, Havulinna AS, Hicks AA, Hui J, Igl W, Illig T, Jula A, Kajantie E, Kilpelainen TO, Koiranen M, Kolcic I, Koskinen S, Kovacs P, Laitinen J, Liu J, Lokki ML, Marusic A, Maschio A, Meitinger T, Mulas A, Pare G, Parker AN, Peden JF, Petersmann A, Pichler I, Pietilainen KH, Pouta A, Ridderstrale M, Rotter JI, Sambrook JG, Sanders AR, Schmidt CO, Sinisalo J, Smit JH, Stringham HM, Bragi WG, Widen E, Wild SH, Willemsen G, Zagato L, Zgaga L, Zitting P, Alavere H, Farrall M, McArdle WL, Nelis M, Peters MJ, Ripatti S, van Meurs JB, Aben KK, Ardlie KG, Beckmann JS, Beilby JP, Bergman RN, Bergmann S, Collins FS, Cusi D, den HM, Eiriksdottir G, Gejman PV, Hall AS, Hamsten A, Huikuri HV, Iribarren C, Kahonen M, Kaprio J, Kathiresan S, Kiemeney L, Kocher T, Launer LJ, Lehtimaki T, Melander O, Mosley TH, Jr., Musk AW, Nieminen MS, O'Donnell CJ, Ohlsson C, Oostra B, Palmer LJ, Raitakari O, Ridker PM, Rioux JD, Rissanen A, Rivolta C, Schunkert H, Shuldiner AR, Siscovick DS, Stumvoll M, Tonjes A, Tuomilehto J, van Ommen GJ, Viikari J, Heath AC, Martin NG, Montgomery GW, Province MA, Kayser M, Arnold AM, Atwood LD, Boerwinkle E, Chanock SJ, Deloukas P, Gieger C, Gronberg H, Hall P, Hattersley AT, Hengstenberg C, Hoffman W, Lathrop GM, Salomaa V, Schreiber S, Uda M, Waterworth D, Wright AF, Assimes TL, Barroso I, Hofman A, Mohlke KL, Boomsma DI, Caulfield MJ, Cupples LA, Erdmann J, Fox CS, Gudnason V, Gyllensten U, Harris TB, Hayes RB, Jarvelin MR, Mooser V, Munroe PB, Ouwehand WH: Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 2010;467:832-838.

21 Walenkamp MJE, Wit JM: Genetic disorders in the growth hormone-IGF-I axis. Horm Res 2006;66:221-230.

22 Wit JM, Bang P: European perspective on treatment approaches for growth failure. Pediatr Endocrinol Rev 2008;5 Suppl 3:862-868.

23 Fredriks AM, Van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, Roede MJ, Verloove-Vanhorick SP, Wit JM: Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res 2000;47:316-323.

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

25 Niklasson A, Ericson A, Fryer JG, Karlberg J, Larwence C, Karlberg P: An Update of the Swedish Reference Standards for Weight, Length and Head Circumeference at Birth for Given Gestational Age (1977- 1981). Acta Pæd Scand 1991;80:756-762.

26 Hermanussen M, Cole J: The calculation of target height reconsidered. Horm Res 2003;59:180-183.

27 Cole TJ, Roede MJ: Centiles of body mass index for Dutch children aged 0-20 years in 1980--a baseline to assess recent trends in obesity. Ann Hum Biol 1999;26:303-308.

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

29 Ross HA, Lentjes E, Menheere PP: The Consensus Statement on the Standardization and Evaluation of Growth Hormone and Insulin-like Growth Factor Assays Lacks a Recommendation to Attempt Efficacious Harmonization. Clin Chem 2011;57:1463–1464.

30 Martin JL, Baxter RC: Insulin-like growth factor-binding protein from human plasma. Purification and characterization. J Biol Chem 1986;261:8754-8760.

31 Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S,

Bailey DK, Kennedy GC, Ogawa S: A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 2005;65:6071-6079.

32 Hwa V, Little B, Kofoed EM, Rosenfeld RG: Transcriptional regulation of insulin-like growth factor-I by interferon-gamma requires STAT-5b. J Biol Chem 2004;279:2728-2736.

33 Maamra M, Finidori J, Von LS, Simon S, Justice S, Webster J, Dower S, Ross R: Studies with a growth hormone antagonist and dual-fluorescent confocal microscopy demonstrate that the full-length human growth hormone receptor, but not the truncated isoform, is very rapidly internalized independent of Jak2-Stat5 signaling. J Biol Chem 1999;274:14791-14798.

34 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR: A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248-249.

35 Scaglia P, Domene HM, Martinez A, Keselman A, Pipman P, Bengolea SV, Karabatas L, Ropelato G, Lescano E, Ballerini G, Heinrich J, Jasper H: Heterozygous IGFALS gene mutations are present in both idiopathic short stature (ISS) and normal children: Impact on height, and acid-labile subunit (ALS) levels; 2009, p 246.

36 Guida MC, Nistchke R, Domene HM, Karabatas L, Scaglia P, Martinez A, Keselman A, Bergada I, Cassinelli H, Bengolea SV, Heinrich JJ, Pipman V, Ropelato MG, Ballerini MG, Rey RA, Jasper HG: Intracellular trafficking effects of insulin-like growth factor acid labile subunit gene (IGFALSD) mutations identified in idiopathic short stature (ISS) children; 2010, p 4.

37 Savage MO, Camacho-Hubner C, David A, Metherell LA, Hwa V, Rosenfeld RG, Clark AJ: Idiopathic short stature: will genetics influence the choice between GH and IGF-I therapy? Eur J Endocrinol 2007;157 Suppl 1:S33-S37.

38 Pantel J, Legendre M, Nivot S, Morisset S, Vie-Luton MP, Le Bouc Y, Epelbaum J, Amselem S: Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. J Clin Endocrinol Metab 2009;94:4334-4341.

39 Inoue H, Kangawa N, Kinouchi A, Sakamoto Y, Kimura C, Horikawa R, Shigematsu Y, Itakura M, Ogata T, Fujieda K: Identification and functional analysis of novel human growth hormone secretagogue receptor (GHSR) gene mutations in Japanese subjects with short stature. J Clin Endocrinol Metab 2011;96:E373-E378.

40 Pugliese-Pires PN, Fortin JP, Arthur T, Latronico AC, Mendonca BB, Villares SM, Arnhold IJ, Kopin AS, Jorge AA: Novel inactivating mutations in the GH secretagogue receptor gene in patients with constitutional delay of growth and puberty. Eur J Endocrinol 2011;165:233-241.

41 Rhoads RP, Greenwood PL, Bell AW, Boisclair YR: Organization and regulation of the gene encoding the sheep acid-labile subunit of the 150-kilodalton insulin-like growth factor-binding protein complex. Endocrinology 2000;141:1425-1433.

42 Suwanichkul A, Boisclair YR, Olney RC, Durham SK, Powell DR: Conservation of a growth hormone-responsive promoter element in the human and mouse acid-labile subunit genes. Endocrinology 2000;141:833-838.

43 Dauber A, Yu Y, Turchin MC, Chiang CW, Meng YA, Demerath EW, Patel SR, Rich SS, Rotter JI, Schreiner PJ, Wilson JG, Shen Y, Wu BL, Hirschhorn JN: Genome-wide association of copy-number variation reveals an association between short stature and the presence of low-frequency genomic deletions. Am J Hum Genet 2011;89:751-759.