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General introduction

General introduction

Normal body growth can be defined as the progression of height, weight, and head circumference in line with established standards for a given population. It starts at conception and proceeds through various developmental stages, controlled by genetic, environmental, psychosocial and nutritional factors (1;2).

Growth can be divided into four stages: fetal life, infancy, childhood and puberty. Fetal growth is mainly regulated by insulin-like growth factors (IGFs; IGF-I and IGF-II) and insulin, and environmental factors (nutrition, maternal factors, and placental function) have a larger impact than genetic factors. In the second trimester of fetal life growth velocity is at its maximum. In infancy (up to ~3 years) nutritional factors, the growth hormone (GH)-IGF-I system, as well as genetic factors play a role. In the first two to three years the child seeks its own growth channel, which is highly correlated with target height (gender-corrected mid-parental height). In childhood (from 3 years to puberty) growth continues at a lower and gradually diminishing rate, with a small growth spurt during mid-childhood. In this phase growth is predominantly under the influence of GH and thyroid hormone. Puberty is characterized by a growth spurt followed by a rapid decrease of growth velocity followed by fusion of the epiphyseal plate (growth plate), thereby terminating growth. Besides GH and IGF-I, estrogen is the main determinant of pubertal growth and epiphyseal fusion in boys and girls. Boys have a later onset of pubertal growth than girls, which gives them an additional two years of prepubertal growth. This, together with the greater amplitude of pubertal growth in boys, leads to a difference of 12-13 cm in adult height between the sexes. There is a wide variation in pubertal timing within each sex, but the net result is a comparable adult height in early and late maturers (2).

Height is a highly heritable and classic polygenic trait, and to a lesser degree influenced by environmental factors. It is estimated that about 80% of the variation in adult height among individuals is due to genetic factors (3). The GH-IGF-I axis is an important regulator of longitudinal growth. Genetic defects in this axis have been shown to be responsible for abnormal growth, and can have a large influence on adult height. These mutations, however, are rare and do not explain the 'normal' variation in height among people. Due to new techniques, such as genome-wide association studies (GWAS), 180 genes associated with height have been identified, and studies on even larger cohorts have suggested that there are at least 700 genes associated with height (Visscher, personal communication). While the individual contribution of each of these genes on height is small, the cumulative effect of all these genes is estimated to determine ~10% of the variance. This thesis focuses on the detection of genetic defects in the GH-IGF-I axis and on the results of whole genome SNP-arrays and next-generation sequencing to identify novel genes influencing height.

Genetic defects in the GH-IGF-I axis in short stature

The GH-IGF-I axis is the most important system regulating longitudinal growth. The first somatomedin hypothesis was formulated in 1957 and since then it has been updated due to new insights. The term somatomedin is derived from the hypothesis that GH (also known as somatotropin) does not exert its effects directly on tissues but only through an intermediary substance, first named sulfation factor, later somatomedin, and finally IGF. This hypothesis was later shown to be only partially correct. The latest version of the somatomedin hypothesis will be explained here.

GH is produced in and secreted by the pituitary. GH secretion is regulated by the hypothalamic factors GH releasing hormone (GHRH) and somatostatin. The pulsatile fashion of GH secretion is regulated by an interaction between these hormones. Another potent stimulator of GH secretion is ghrelin. This is an appetite stimulating peptide produced mainly by the stomach; plasma levels increase before meals and decrease thereafter (4). Ghrelin binds to its receptor, the growth hormone secretagogue receptor (GHSR1a), which is highly expressed in the brain and in the pituitary, resulting in GH secretion. Binding of GH to the transmembrane receptor (GHR, a cytokine receptor that lacks intrinsic kinase activity), which exists as a pre-assembled non-functional dimer (5), is followed by conformational changes leading to stabilization of the dimer and induction of signal transduction through the recruitment and activation of Janus kinase 2 (JAK2) (6;7). An important intermediary of the complex activated signaling pathways is Signal Transducer and Activator of Transcription 5b (STAT5b). STAT5b is recruited to the intracellular domain of the activated GHR and subsequently phosphorylated (activated) by JAK2. Phosphorylated STAT5b forms a homodimer and translocates to the nucleus, where it binds to GH-responsive elements (GHRE) in DNA and activates transcription of target genes, including *IGF1*, *IGFBP3* and *IGFALS*.

IGF-I can be synthesized by almost any tissue in the body, in an autocrine and paracrine fashion, but circulating (endocrine) IGF-I is mainly produced in the liver (8). Approximately 80–90% of circulating IGFs form a 150 kDa ternary complex with IGFBP-3 or -5 and the acid-labile subunit (ALS) (9), 10–15% are bound in a 40–50 kDa binary complex to IGFBPs, whereas less than 1% is found in the free form (10). The 150 kDa complexes cannot cross the capillary endothelial barrier, which prolongs the half-life of IGFs, IGFBP-3, and IGFBP-5 in the circulation (9;11;12). This seems to play an important role in the regulation of the bioavailability of IGFs to the tissue compartments. When carried in ternary complexes, the half-life of IGF-I is more than 12 h, much longer than that of binary bound IGF-I (30–90 min) and free IGF-I (~ 10 min) (13). IGF-I binds to the extracellular domain (α-subunit) of the tetrameric ($\alpha_2\beta_2$) transmembrane IGF-I receptor (IGF1R, a tyrosine kinase receptor), leading

to autophosphorylation of the intracellular tyrosine kinase domain (β-subunit). This results in recruitment of cytoplasmic components of downstream signaling pathways, including the PI3K/Akt and MAPK/Erk pathways, ultimately leading to cell proliferation, survival and growth (6;14).

The original somatomedin hypothesis proposed that GH binding to its receptor stimulated IGF-I production by the liver, which independently affected growth (15). Since then new insights made clear that: 1) IGFs and the GHR are expressed in most tissues; 2) GH also has local (direct) effects independent of those mediated by the increase in circulating IGF-I; 3) IGF-I cannot be the sole mediator of GH action because IGF-I repletion does not fully restore the deficits found in GH insensitivity; 4) some of the IGF effects oppose the known actions of GH; and 5) both locally produced and circulating IGF-I are important growth mediators. This led to the latest somatomedin hypothesis (2007); "the augmentative/counteractive hypothesis", which implies that IGFs enhance (augment) the anabolic actions of GH while reducing (countering) its potentially undesirable effects of hyperglycemia (gluconeogenesis) and depletion of lipid stores (lipolysis) (16).
In the following paragraphs the genetic defects in various components of the GH-IGF-I axis causing growth disorders are summarized.

GHRH
Although it is considered to be a candidate gene, to date no GH releasing hormone *(GHRH)* gene mutations or deletions causing isolated GH deficiency (IGHD) have been described (17;18).

GHRHR
The GH releasing hormone receptor (GHRHR) belongs to the G protein-coupled receptor (GPCR) superfamily characterized by a seven transmembrane domain structure. Mutations in the *GHRHR* have been estimated to account for 10% of the patients with autosomal recessive familial IGHD. The phenotype of these patients include proportionate short stature, normal intelligence, variable anterior pituitary hypoplasia, normal GHRH and GHBP levels, undetectable or very low GH levels, low IGF-I and IGFBP-3 levels, absent or subnormal GH response to stimulation tests and a good response to exogenous GH. The presence of an intermediate phenotype has been hypothesized in heterozygous carriers, although they do not show signs of IGHD (19-21).

GHSR1a
The GHSR is a member of the GPCR superfamily. There are two isoforms of GHSR: GHSR1a which is active and GHSR1b which is truncated and has no known biological activity. The

GHSR1a is a constitutive active receptor which stimulates GH secretion after binding to Ghrelin. Homozygous and heterozygous mutations in the *GHSR1a*, causing a decrease in the constitutive activity, have been identified in patients with idiopathic short stature (ISS), IGHD, and constitutional delay of growth and puberty. Familial analysis suggests a dominant mode of inheritance with incomplete penetrance (22-25).

GH1

IGHD can also be caused by a mutation or deletion of the *GH1* gene. Heterozygous missense mutations in the *GH1* gene can lead to the secretion of biologically inactive GH, resulting in GH insensitivity (GHI). Clinical characteristics include severe short stature, normal or slightly increased GH levels, low IGF-I levels, and a good response in terms of growth and circulating IGF-I to exogenous GH. However, normal statured family members carrying the same mutation have been described, suggesting a variable penetrance (6;20;26). In one case a homozygous missense mutation of the *GH1* gene was described, leading to the absence of the disulfide bridge Cys-53 to Cys-165, resulting in reduced binding affinity for the GH receptor (GHR) and subsequently activation of the JAK2/STAT5 signaling pathway. Similar to the cases with heterozygous missense mutations, both growth and circulating IGF-I responded well to exogenous GH. The parents of the patient, who were heterozygous carriers of the mutation, had normal height (27).

Homozygous deletions of the *GH1* gene have also been described in patients with IGHD. These patients usually have birth weight and length in the normal range but experience severe postnatal growth retardation with a height SDS less than -4.5. The sizes of the deletions vary, with 6.7 kb occurring most frequently. The remaining deletions include 7.6, 7.0 and 45 kb. The deletions arise at meiosis via unequal recombination and crossing over within the *GH*-gene cluster. Patients with homozygous *GH1* deletions and frameshift mutations frequently develop anti-GH antibodies during exogenous GH treatment. However, this is an inconsistent finding, even within families (6;20).

The genetic defects in the *GHRHR*, *GHSR1a*, and *GH1* gene causing IGHD, as described above, are relatively rare causes of short stature.

GHR

Defects in the *GHR* gene lead to Laron syndrome, also called GH insensitivity syndrome (GHIS) or classical GH insensitivity. Since 1966, more than 250 patients have been described worldwide. Clinical characteristics include a mean birth length of -1 SDS or lower (indicating some GH dependency during late pregnancy), severe postnatal growth retardation, normal head circumference and very low circulating IGF-I, IGFBP-3 and ALS levels despite increased GH secretion. In most families this disorder is inherited in an autosomal recessive

fashion, including homozygous or compound heterozygous mutations. Deletions, missense, nonsense and splice site mutations have been reported. Milder phenotypes of GH insensitivity caused by *GHR* mutations (including splice site, compound heterozygous, and heterozygous dominant negative mutations) have also been described, illustrating the heterogeneous phenotype in this disorder (6;20;28).

STAT5B

Molecular defects in GH signal transduction pathways appear to be very rare. To date, 7 different homozygous *STAT5B* gene mutations have been described, including insertions, deletions, and missense and nonsense mutations. The phenotype of these patients include autosomal recessive inheritance, normal birth weight and length, followed by postnatal growth failure and severe GH resistance (also against exogenous GH), normal head circumference, delayed puberty in most cases, and in all cases except one a severe immune disorder. Biochemical profiles show normal or elevated GH secretion, very low circulating IGF-I, IGFBP-3 and ALS levels (also after GH stimulation), and in all cases elevated serum prolactin. A heterozygous defect may lead to a mild decrease in height SDS, but this has still to be confirmed by data from additional heterozygous carriers (6;7;28).

IGF1

Only 6 mutations in the *IGF1* gene have been described so far. The first reported patient had a homozygous deletion of exon 4 and 5 of the *IGF1* gene, resulting in severe intrauterine and postnatal growth failure, microcephaly, mental retardation and sensorineural deafness. Circulating IGF-I was undetectable and IGFBP-3 and ALS were normal, but GH was elevated (29). In the second patient with the same phenotype, a homozygous nucleotide substitution located in the upstream core polyadenylation signal at the 3' untranslated region in exon 6 of *IGF1* was reported (30), but the same variant was subsequently found in unaffected children, thus probably is a polymorphism (31). The third and fourth reported cases both showed homozygous missense mutations that reduced the affinity of *IGF1* for its receptor (32;33). The third case had a similar clinical phenotype as the first reported case, but the biochemical profile differed to some extent: circulating IGF-I was elevated, as well as GH and ALS, and IGFBP-3 was normal (32). Remarkably, the fourth case had a similar but milder phenotype compared to the previous reported patients, including milder growth deficiency, microcephaly, and intellectual impairment, and normal hearing. Circulating IGF-I concentrations differed according to the immunoassay used, and IGFBP-3 and ALS levels were in the upper normal range or above (33). The difference between these two cases consists of a difference in reduced binding affinity for the IGF1R; case 3 had a 90-fold decreased affinity compared to a 2- to 3-fold decreased affinity in case 4. This partially

diminished IGF-I activity had marked consequences for growth and development (6;32-35). Family members who were heterozygous carriers of the *IGF1* defect have a modest decrease of height and head circumference (29;32;36). The fifth and sixth reports concerned families with a heterozygous mutation in the *IGF1* gene associated with severe short stature (37-39). In none of the 3 index cases in the two reports deafness was observed. Microcephaly, intellectual impairment and intrauterine growth failure was observed at a variable degree, but not present in all cases. Circulating IGF-I was low and IGFBP-3 was normal in all index cases (37-39). In the first report, two children with severe short stature and a maternally derived novel heterozygous frameshift mutation in exon 3 of the *IGF1* gene and their relatives were described, and a dominant negative effect of the putative truncated protein was hypothesized. This was however not supported by subsequent functional studies. The cause of the growth failure was speculated to be a combination of partial IGF-I deficiency, placental IGF-I insufficiency, and other genetic factors (38;39). The most recent report described a large kindred with severe short stature, in which a novel heterozygous splicing mutation in intron 4 of the *IGF1* gen was found to segregate with short stature. There were however other family members who were short but wild-type for *IGF1*, thus it remains unclear whether the growth retardation in this family is due to more than the *IGF1* mutation alone (37).

IGFALS

The *IGFALS* gene encodes for the soluble protein ALS, a member of the leucine-rich repeat superfamily. The proteins belonging to this family are characterized by their ability to participate in protein-protein interactions (12). ALS is synthesized primarily in the liver and regulated by GH (40). Molecular defects in the *IGFALS* gene appear to be more frequent compared to the other candidate genes for primary IGF deficiency, besides the *GHR* gene. Since the first report of a homozygous *IGFALS* mutation (41), a total of 16 unique homozygous or compound heterozygous mutations in 21 patients with ALS deficiency have been described (42;43). The different types of *IGFALS* mutations include missense and nonsense mutations, frameshift mutations with premature stop codons, and in-frame duplications resulting in the insertion of extra amino acids (6;44). The phenotype of these patients includes autosomal recessive inheritance, extremely low levels of circulating ALS with inability to form the ternary complex with IGFBP-3 and IGF-I, severely reduced levels of IGF-I together with even more profoundly reduced IGFBP-3 levels, and a mild growth retardation apparently out of proportion to the degree of IGF-I and IGFBP-3 deficits, with the possibility of reaching a normal adult height. Pubertal delay in boys and insulin insensitivity are common findings, but not present in all cases. Studies in family members who were either heterozygous carriers of the *IGFALS* mutation or had a homozygous wild-

type *IGFALS* gene showed that heterozygosity resulted in approximately 1.0 SDS height loss in comparison with wild-type, whereas homozygosity or compound heterozygosity for *IGFALS* mutations resulted in a further height loss of 1.0 to 1.5 SDS, suggestive of a gene-dosage effect (43). An important lesson learned from these families is that local IGF-I appears to be more important for growth than circulating IGF-I.

IGF1R

Heterozygous and compound heterozygous defects (mutations or deletions) of the *IGF1R* gene, causing IGF-I resistance, have been described several times since the first report of 2 children with short stature and unexplained intrauterine growth retardation with a compound heterozygous or heterozygous *IGF1R* mutation (45). Clinical characteristics of patients with a heterozygous or compound heterozygous *IGF1R* mutation include small birth size, short stature, small head circumference, and relatively high circulating IGF-I levels (>0 SDS) (6;28;46-48). Until recently, homozygous mutations in the *IGF1R* gene had not been described. First, these were thought to be lethal, because *igf1r -/-* mice die shortly after birth (46;47), but homozygosity for a hypomorphic *IGF1R* mutation has recently be associated with a similar phenotype as observed in (compound) heterozygous damaging mutations (49). In patients with a heterozygous deletion of the *IGF1R* and surrounding genes (most times a 15q terminal deletion) additional clinical manifestations can be seen, including developmental delay, and facial, cardiac and limb abnormalities, probably caused by haploinsufficiency of one of the surrounding genes (6;28;46-48;50).

Genetic defects in the GH-IGF-I axis in tall stature

Tall stature can be caused by excessive GH secretion in childhood. In most of these children the excessive GH secretion results from a pituitary adenoma or hyperplasia. This is extraordinarily rare in early childhood, and its frequency only slightly increases in adolescence. A pituitary adenoma can be part of a genetic condition predisposing to pituitary and other tumors. Multiple endocrine neoplasia type 1 (MEN1), Carney complex type 1 (CNC1), familial isolated pituitary adenomas (FIPA) and McCune-Albright syndrome (MAS) are genetically determined endocrine-related tumor syndromes resulting in excessive GH secretion (51-53). MEN1 and CNC1 are autosomal dominant disorders caused by germline mutations in the *MEN1* tumor suppressor gene and the *PRKAR1A* gene, respectively. Mutations in the *CDKN1B* gene (alias p27/KIP1) are associated with a MEN-1- like syndrome. FIPA can be caused by inactivating mutations in the *AIP* gene, but the remaining predisposition genes are largely unknown. MAS is caused by a somatic mutation in the *GNAS1* gene, thus this is a genetic, but not inherited, disorder (51-53).

Sotos syndrome which is characterized by tall stature, craniofacial features and mental

retardation is caused by haploinsufficiency of the *NSD1* gene, either caused by a mutation or deletion (54-57). In a recent study a significant association was demonstrated with the Mitogen-Activated Protein Kinase (MAPK) pathway, which is part of the downstream signaling pathways of the GH and IGF-I receptors. Phosphorylation studies in skin fibroblasts demonstrated a possible diminished activity state of the MAPK/ERK pathway and the hypothesis was proposed that deregulation of the MAPK/ERK pathway in Sotos syndrome results in altered hypertrophic differentiation of NSD1 expressing chondrocytes and may be a determining factor in statural overgrowth and accelerated skeletal maturation (58). The MAPK/ERK signaling pathway is an important regulator of cell differentiation, proliferation and apoptosis and has been implicated in many human diseases. Activating mutations in this pathway have been identified as the causative factor in a number of short stature syndromes, such as Noonan, Costello and LEOPARD syndrome (59).

Duplication of the *IGF1R* caused by a trisomy of 15q26-qter is frequently associated with tall stature, mental retardation and often macrocephaly (60;61). Cell proliferation studies on skin fibroblasts of a patient with three copies of the *IGF1R* gene showed accelerated growth, and IGF1R phosphorylation and binding were increased, while cells of a patient with only one copy of the *IGF1R* gene showed slower growth and decreased phosphorylation and binding, compared with controls (62). There seems to be a gene dosage effect of the *IGF1R* gene, causing tall stature when increased and short stature when decreased.

Strategies to identify genetic defects

There are different strategies and techniques that can be used to search for a genetic defect causing a growth disorder, or any other congenital syndrome. One of these is the candidate gene approach. By thoroughly investigating the clinical and biochemical phenotype of the patient and compare this to the medical literature, one or more candidate genes can be selected. Sanger sequencing and/or multiplex ligation-dependent probe amplification (MLPA) analyses to identify mutations and/or copy number variations of these genes can be performed to investigate the presence of a genetic defect.

If the candidate gene approach does not result in a causative genetic defect or if there is no candidate gene, another approach is a whole genome single-nucleotide polymorphism (SNP) array to identify copy number variations or uniparental disomy. Microdeletions and -duplications, with a size >10 kb, can be identified with this technique and can result in the identification of novel genes involved in the regulation of longitudinal growth.

The most recent available technique in identifying genetic defects is next-generation sequencing. With this technique the whole genome or whole exome (only the part of the genome coding for genes) can be sequenced. With this high-resolution technique mutations of single nucleotides can be detected. Instead of sequencing one candidate

gene, all genes in the human genome are sequenced and analyzed for the presence of a mutation, inversing the search strategy and increasing the probability to identify new genes involved in longitudinal growth. However, this approach also brings new challenges, such as how to establish the pathogenicity of the detected genetic defect.

Outline of this thesis

In this thesis, we aimed to identify genetic causes of growth disorders, using different search strategies. We further aimed at studying genotype-phenotype correlations, in order to increase the understanding of the regulation of longitudinal growth.

Chapter 1 presents a general introduction and the outline of this thesis.

A. Candidate gene approach

The first family with a heterozygous mutation in the *IGF1* gene associated with severe short stature is described in *chapter 2*, followed by functional analysis of the mutant IGF-I in *chapter 3*.

Three brothers with short stature, microcephaly and ALS deficiency due to a homozygous mutation in the *IGFALS* gene and their family members are described in *chapter 4*. In this report we propose that there might be a gene-dosage effect, resulting in some negative effect on height and head circumference in heterozygous carriers.

B. Combined candidate gene and whole genome approach

In *chapter 5* we describe the results of a study in 100 children born small for gestational age (SGA) with persistent short stature in which we examined 18 growth-related genes with MLPA for possible aberrations. This resulted in 2 patients with a heterozygous 15q terminal deletion including the *IGF1R* gene. A subsequent review of the literature shows that small birth size, short stature, small head size, relatively high IGF-I levels, developmental delay, and micrognathia are the main predictors for an *IGF1R* deletion.

Chapter 6 describes the genetic analysis of short children with apparent GH insensitivity, combining two different search strategies; the candidate gene and whole genome SNP array approach. Patients were divided into three groups based on their height and circulating IGF-I levels. The group including children with severe short stature and IGF-I deficiency (lowest height and circulating IGF-I levels) revealed three patients with two novel heterozygous *STAT5B* mutations, in two of them combined with novel heterozygous *IGFALS* variants.

C. Whole genome approach

In *chapter 7* the use of the whole genome SNP array approach in patients with ISS is described, in an effort to identify rare variants that may cause short stature. In 22.1% of the included families potentially pathogenic CNVs which may be associated with short stature were identified, although their precise role remains unclear.

D. Combined whole genome approach and functional studies

In *chapter 8* the use of whole exome sequencing in a patient with extreme tall stature is described, identifying a heterozygous mutation in the *NPR2* gene. Functional studies using the synthetic derived mutant NPR2 protein and skin fibroblast of the patient revealed increased basal and stimulated NPR2 activity, indicative for an activating mutation which results in extremely tall stature.

Chapter 9 provides a general discussion on the findings described in this thesis, including future perspectives, followed by a summary in English and in Dutch in *chapter 10.*

Reference List

1. Ergun-Longmire B, Wajnrajch M. Growth and growth disorders. In: New M, editor. www. endotext.org, Pediatric Endocrinology. 2010.

2. Clayton PE, Gill MS. Normal growth and its endocrine control. In: Brook CGD, Hindmarsh PC, editors. Clinical Pediatric Endocrinology. 2001: 95-114.

3. Lango Allen H, Estrada K, Lettre G et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 2010; 467(7317):832-838.

4. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402(6762):656-660.

5. Gonzalez L, Curto LM, Miquet JG, Bartke A, Turyn D, Sotelo AI. Differential regulation of membrane associated-growth hormone binding protein (MA-GHBP) and growth hormone receptor (GHR) expression by growth hormone (GH) in mouse liver. Growth Horm IGF Res 2007; 17(2):104-112.

6. David A, Hwa V, Metherell LA et al. Evidence for a continuum of genetic, phenotypic, and biochemical abnormalities in children with growth hormone insensitivity. Endocr Rev 2011; 32(4):472-497.

7. Rosenfeld RG, Belgorosky A, Camacho-Hubner C, Savage MO, Wit JM, Hwa V. Defects in growth hormone receptor signaling. Trends Endocrinol Metab 2007; 18(4):134-141.

8. Rosen CJ. Serum insulin-like growth factors and insulin-like growth factor-binding proteins: clinical implications. Clin Chem 1999; 45(8 Pt 2):1384-1390.

9. Boisclair YR, Rhoads RP, Ueki I, Wang J, Ooi GT. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J Endocrinol 2001; 170(1):63-70.

10. Domene HM, Bengolea SV, Jasper HG, Boisclair YR. Acid-labile subunit deficiency: phenotypic similarities and differences between human and mouse. J Endocrinol Invest 2005; 28(5 Suppl):43-46.

11. Hwa V, Haeusler G, Pratt KL et al. Total absence of functional acid labile subunit, resulting in severe insulin-like growth factor deficiency and moderate growth failure. J Clin Endocrinol Metab 2006; 91(5):1826-1831.

12. Janosi JB, Ramsland PA, Mott MR, Firth SM, Baxter RC, Delhanty PJ. The acid-labile subunit of the serum insulin-like growth factor-binding protein complexes. Structural determination by molecular modeling and electron microscopy. J Biol Chem 1999; 274(33):23328-23332.

13. Domene HM, Martinez AS, Frystyk J et al. Normal growth spurt and final height despite low levels of all forms of circulating insulin-like growth factor-I in a patient with acid-labile subunit deficiency. Horm Res 2007; 67(5):243-249.

14. LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 1995; 16(2):143-163.

15. Salmon WD, Jr., Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J Lab Clin Med 1957; 49(6):825-836.

16. Kaplan SA, Cohen P. The somatomedin hypothesis 2007: 50 years later. J Clin Endocrinol Metab 2007; 92(12):4529-4535.

17. Mullis P, Patel M, Brickell PM, Brook CG. Isolated growth hormone deficiency: analysis of the growth hormone (GH)-releasing hormone gene and the GH gene cluster. J Clin Endocrinol Metab 1990; 70(1):187-191.

18. Perez Jurado LA, Phillips JA, III, Francke U. Exclusion of growth hormone (GH)-releasing hormone gene mutations in familial isolated GH deficiency by linkage and single strand conformation analysis. J Clin Endocrinol Metab 1994; 78(3):622-628.

19. Martari M, Salvatori R. Diseases associated with growth hormone-releasing hormone receptor (GHRHR) mutations. Prog Mol Biol Transl Sci 2009; 88:57-84.

20. Mullis PE. Genetics of GHRH, GHRH-receptor, GH and GH-receptor: its impact on pharmacogenetics. Best Pract Res Clin Endocrinol Metab 2011; 25(1):25-41.

21. Walenkamp MJ, Pereira AM, Oostdijk W et al. Height gain with combined growthhormone and gonadotropin-releasing hormone analog therapy in two pubertal siblings with a

growth hormone-releasing hormone receptor mutation. J Clin Endocrinol Metab 2008; 93(1):204-207.

22. Inoue H, Kangawa N, Kinouchi A et al. 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(2):E373-E378.

23. Pantel J, Legendre M, Nivot S et al. Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. J Clin Endocrinol Metab 2009; 94(11):4334-4341.

24. Pantel J, Legendre M, Cabrol S et al. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. J Clin Invest 2006; 116(3):760-768.

25. Pugliese-Pires PN, Fortin JP, Arthur T et al. Novel inactivating mutations in the GH secretagogue receptor gene in patients with constitutional delay of growth and puberty. Eur J Endocrinol 2011; 165(2):233-241.

26. Walenkamp MJ, Wit JM. Genetic disorders in the growth hormone - insulin-like growth factor-I axis. Horm Res 2006; 66(5):221-230.

27. Besson A, Salemi S, Deladoey J et al. Short stature caused by a biologically inactive mutant growth hormone (GH-C53S). J Clin Endocrinol Metab 2005; 90(5):2493-2499.

28. Wit JM, Camacho-Hubner C. Endocrine regulation of longitudinal bone growth. Endocr Dev 2011; 21:30-41.

29. 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(18):1363-1367.

30. Bonapace G, Concolino D, Formicola S, Strisciuglio P. A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 2003; 40(12):913-917.

31. Coutinho DC, Coletta RR, Costa EM et al. Polymorphisms identified in the upstream core polyadenylation signal of IGF1 gene exon 6 do not cause pre- and postnatal growth impairment. J Clin Endocrinol Metab 2007; 92(12):4889-4892.

32. Walenkamp MJ, Karperien M, Pereira AM et al. Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 2005; 90(5):2855-2864.

33. Netchine I, Azzi S, Houang M et al. Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. J Clin Endocrinol Metab 2009; 94(10):3913-3921.

34. Denley A, Wang CC, McNeil KA et al. Structural and functional characteristics of the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development. Mol Endocrinol 2005; 19(3):711-721.

35. Forbes BE. Molecular mechanisms underlying insulin-like growth factor action: How mutations in the GH: IGF axis lead to short stature. Pediatr Endocrinol Rev 2011; 8(4):374-381.

36. Woods KA. Molecular Mechanisms in Growth Hormone Insensitivity. London: Paediatric Endocrinology Section, Department of Endocrinology, St Bartholomew's Hospital London, 1998.

37. Fuqua JS, Derr M, Rosenfeld RG, Hwa V. Identification of a Novel Heterozygous IGF1 Splicing Mutation in a Large Kindred with Familial Short Stature. Horm Res Paediatr 2012; 78(1):59-66.

38. van Duyvenvoorde HA, van Setten PA, Walenkamp MJ et al. Short stature associated with a novel heterozygous mutation in the insulin-like growth factor 1 gene. J Clin Endocrinol Metab 2010; 95(11):E363-E367.

39. van Duyvenvoorde HA, van Doorn J, Koenig J et al. The severe short stature in two siblings with a heterozygous IGF1 mutation is not caused by a dominant negative effect of the putative truncated protein. Growth Horm IGF Res 2011; 21(1):44-50.

40. Ooi GT, Cohen FJ, Tseng LY, Rechler MM, Boisclair YR. Growth hormone stimulates transcription of the gene encoding the acid-labile subunit (ALS) of the circulating insulin-like growth factor-binding protein complex and ALS promoter activity in rat liver. Mol Endocrinol 1997; 11(7):997-1007.

41. Domene HM, Bengolea SV, Martinez AS et al. Deficiency of the circulating insulin-like

growth factor system associated with inactivation of the acid-labile subunit gene. N Engl J Med 2004; 350(6):570-577.

42. Domene HM, Hwa V, Jasper HG, Rosenfeld RG. Acid-labile subunit (ALS) deficiency. Best Pract Res Clin Endocrinol Metab 2011; 25(1):101-113.

43. Fofanova-Gambetti OV, Hwa V, Wit JM et al. 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(9):4184-4191.

44. Domene HM, Hwa V, Argente J et al. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res 2009; 72(3):129-141.

45. Abuzzahab MJ, Schneider A, Goddard A et al. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 2003; 349(23):2211-2222.

46. Klammt J, Kiess W, Pfaffle R. IGF1R mutations as cause of SGA. Best Pract Res Clin Endocrinol Metab 2011; 25(1):191-206.

47. Kawashima Y, Takahashi S, Kanzaki S. Familial short stature with IGF-I receptor gene anomaly. Endocr J 2012; 59(3):179-185.

48. Ester WA, van Duyvenvoorde HA, de Wit CC et al. Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype. J Clin Endocrinol Metab 2009; 94(12):4717-4727.

49. Gannage-Yared MH, Klammt J, Chouery E et al. Homozygous mutation of the IGF1 receptor gene in a patient with severe pre- and postnatal growth failure and congenital malformations. Eur J Endocrinol 2013; 168(1):K1-K7.

50. Rudaks LI, Nicholl JK, Bratkovic D, Barnett CP. Short stature due to 15q26 microdeletion involving IGF1R: report of an additional case and review of the literature. Am J Med Genet A 2011; 155A(12):3139-3143.

51. Horvath A, Stratakis CA. Clinical and molecular genetics of acromegaly: MEN1, Carney complex, McCune-Albright syndrome, familial acromegaly and genetic defects in sporadic tumors. Rev Endocr Metab Disord 2008; 9(1):1-11.

52. Kant SG, Wit JM, Breuning MH. Genetic analysis of tall stature. Horm Res 2005; 64(3):149-156.

53. Keil MF, Stratakis CA. Pituitary tumors in childhood: update of diagnosis, treatment and molecular genetics. Expert Rev Neurother 2008; 8(4):563-574.

54. Kurotaki N, Imaizumi K, Harada N et al. Haploinsufficiency of NSD1 causes Sotos syndrome. Nat Genet 2002; 30(4):365-366.

55. Tatton-Brown K, Douglas J, Coleman K et al. Genotype-phenotype associations in Sotos syndrome: an analysis of 266 individuals with NSD1 aberrations. Am J Hum Genet 2005; 77(2):193-204.

56. De Boer L, Kant SG, Karperien M et al. Genotype-phenotype correlation in patients suspected of having Sotos syndrome. Horm Res 2004; 62(4):197-207.

57. De Boer L, van Duyvenvoorde HA, Willemstein-Van Hove EC et al. Mutations in the NSD1 gene in patients with Sotos syndrome associate with endocrine and paracrine alterations in the IGF system. Eur J Endocrinol 2004; 151(3):333-341.

58. Visser R, Landman EB, Goeman J, Wit JM, Karperien M. Sotos Syndrome Is Associated with Deregulation of the MAPK/ERK-Signaling Pathway. PLoS ONE 2012; 7(11):e49229.

59. Bentires-Alj M, Kontaridis MI, Neel BG. Stops along the RAS pathway in human genetic disease. Nat Med 2006; 12(3):283-285.

60. Chen CP, Lin YH, Au HK et al. Chromosome 15q overgrowth syndrome: prenatal diagnosis, molecular cytogenetic characterization, and perinatal findings in fetus with dup(15)(q26.2q26.3). Taiwan J Obstet Gynecol 2011; 50(3):359-365.

61. Kant SG, Kriek M, Walenkamp MJ et al. Tall stature and duplication of the insulin-like growth factor I receptor gene. Eur J Med Genet 2007; 50(1):1-10.

62. Okubo Y, Siddle K, Firth H et al. Cell proliferation activities on skin fibroblasts from a short child with absence of one copy of the type 1 insulin-like growth factor receptor (IGF1R) gene and a tall child with three copies of the IGF1R gene. J Clin Endocrinol Metab 2003; 88(12):5981-5988.

Part A

Candidate gene approach

2

Short stature associated with a novel heterozygous mutation in the *insulin-like growth factor 1* gene

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Abstract

Context: Homozygous *IGF1* deletions or mutations lead to severe short stature, deafness, microcephaly and mental retardation. Heterozygosity for an IGF-I defect may modestly decrease height and head circumference.

Objective: The objective of the study was to investigate the clinical features of heterozygous carriers of a novel mutation in the *IGF1* gene in comparison with noncarriers in a short family and to establish the effect of human GH treatment.

Subjects: Two children, their mother, and their maternal grandfather carried the mutation and were compared with two relatives who were noncarriers.

Results: The two index cases had severe short stature (height SD Score –4.1 and –4.6), microcephaly, and low IGF-I levels. Sequencing of *IGF1* revealed a heterozygous duplication of four nucleotides, resulting in a frame shift and a premature termination codon. The mother and maternal grandfather had the same *IGF1* mutation. Adult height (corrected for shrinking and secular trend) and head circumference SD Score of carriers of the paternally transmitted mutation was –2.5 and –1.8, in comparison with –1.6 and 0.3 in noncarriers, respectively. After 2 yr of GH treatment, both index cases exhibited increased growth.

Conclusions: Heterozygosity for this novel *IGF1* mutation in children born from a mother with the same mutation, presumably in combination with other genetic factors for short stature, leads to severe short stature, which can be successfully treated with GH.

Introduction

Insulin-like growth factor-I (IGF-I) plays a critical role in the regulation of pre- and postnatal growth and development in humans and rodents (1;2). Prenatal IGF-I production is largely growth hormone (GH) independent, and is mainly affected by placental sufficiency, fetal nutrition, and insulin (3). Postnatally IGF-I production and secretion is predominantly regulated by GH and nutrition (1). IGF-I can be synthesized by almost any tissue in the body, but circulating IGF-I is mainly produced in the liver (4).

A total IGF-I deficiency caused by a homozygous *IGF1* deletion or missense mutation, leads to severe intrauterine and postnatal growth failure, microcephaly, mental retardation, and sensorineural deafness (5;6). In another patient with the same phenotype, a homozygous nucleotide substitution located in the upstream core polyadenylation signal at the 3' untranslated region in exon 6 of *IGF1* was reported (7), later shown to be a polymorphism (8). Recently a fourth patient was described with a homozygous missense *IGF1* mutation (9), exhibiting intrauterine and postnatal growth failure, microcephaly, mild intellectual impairment, but normal hearing.

There is some evidence that heterozygosity for an *IGF1* defect is associated with a modest decrease of height. The eight heterozygous carriers of the *IGF1* deletion had a height between –0.6 and –2.1 SD Score (SDS), and serum IGF-I was below the normal range in five (5;10). The nine heterozygous carriers of the missense mutation showed a subtle, but statistically significant, inhibition of intrauterine and postnatal statural and cranial growth (6).

In this report we describe two children with severe short stature and a maternally derived novel heterozygous mutation in exon 3 of the *IGF1* gene and their relatives. All subjects provided written informed consent.

Materials and Methods
Clinical, radiological, and audiometric measurements
A full physical examination was performed as described previously (11). Bone mineral density was measured by dual-energy x-ray absorptiometry using the Hologic Scanner (QDR 4500; Hologic, Waltham, MA) at the lumbar spine and femoral neck. Z-scores were calculated as a function of age, sex, and ethnicity for a matched population using the Hologic reference database. Hearing ability at different sound frequencies (500–4000 Hz) was measured with an AC40 clinical audiometer (Interacoustics, Eden Prairie, MN).

Biochemical measurements

Plasma acid-labile subunit (ALS), IGF-I, IGF-II, IGF binding protein (IGFBP)-1, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-6 were determined as described previously (6). Serum insulin levels, after an overnight fast, were determined by a semiautomatic luminescence method (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). GH levels are expressed as micrograms per liter (conversion rate 1 µg/liter = 3.0 IU, standard World Health Organization international standard 98/574).

Molecular studies with genomic DNA of the family members

Genomic DNA was isolated from whole blood (12). All exons of *IGF1* (GenBank accession number NM_000618) were sequenced as described previously (6).

For the analysis of single-nucleotide polymorphisms, the Affymetrix GeneChip human mapping 262K *NspI* array (Affymetrix, Santa Clara, CA) was used. Detection of copy number changes was performed using Copy Number Analyser for GeneChip (CNAG) version 2.0 (www.genome.umin.jp/) (13;14).

Results

Subjects

Clinical and biochemical features are shown in Table 1. Adult height SDS is corrected for shrinking and secular trend (15).

Index case III-1 was born after an uncomplicated 40-wk pregnancy. There were feeding difficulties in infancy, slow motor development, poor growth, and delayed bone age (2.5 yr at 5.9 yr) (Supplemental Fig. 1A). She had a prominent forehead, thin lips, and fine eyebrows. IGF-I was low, but GH maximum during two provocation tests was normal (10.7 µg/liter, normal >6.7 µg/liter). At an IGF-I generation test (1), baseline plasma IGF-I was –2.7 SDS and IGFBP-3 was 0.3 SDS. Only on the highest GH dose IGF-I normalized (–0.6 SDS).

Her younger brother (index case III-2) was born after an uncomplicated 42-wk pregnancy. Besides cow milk allergy and asthma, there were no medical problems, except poor growth and delayed bone maturation (Supplemental Fig. 1B). He had a prominent forehead. Plasma IGF-I was low and the peak GH response to arginine borderline (5.6 µg/liter).

Case II-3, the father of the index cases, had a height of –1.3 SDS, a head circumference (HC) of –1.0 SDS and normal IGF-I and IGFBP-3.

Their mother (case II-2) had feeding problems in infancy and poor growth. At 8.5 yr, height SDS was –3.0 (16), and the GH peak after exercise was 11.5 µg/liter. Breast development started at 11.3 yr at a height of 131.0 cm, and pubertal height gain was only 16.2 cm.

The mother's sister (case II-1) was also short as a child (height SDS –2.0) (16). Her pubertal growth spurt started at 9.75 yr at a height of 128.0 cm, and pubertal height gain was

28.5 cm. The GH peak after exercise was 6.3 µg/liter.

Their maternal grandfather (case I-1), born in 1941, was admitted to the hospital in the first 6 months for failure to thrive. Since 32 yr of age, he is known with poor bone quality, overweight, early dementia, chronic diarrhea, headache, reflux esophagitis due to a hernia diaphragmatica, gout, and a cataract. Reportedly his father, as well a several members of his father's family, was short.

Case I-2, the probands' maternal grandmother, had always been healthy without any medication.

Molecular studies with genomic DNA of the family members

Sequence analysis of *IGF1* of cases I-1, II-2, III-1, and III-2 revealed a heterozygous duplication, indicated as +/–, of four nucleotides, resulting in a frame shift at position 35 of the mature IGF-I protein and a premature stopcodon (c.243_246dupCAGC, p.Ser83GlnfsX13) (http://www.hgvs.org/mutnomen/recs-prot.html). The other family members did not carry the mutation (+/+) (Fig. 1).

Sequence analysis of *FGFR3* in cases II-2, III-1 and III-2 and *SHOX* in cases II-2 and III-1 showed no abnormalities. Case III-2 showed a 47,XYY karyotype. His sister (case III-1) and father (case II-3) had a normal karyotype.

Single-nucleotide polymorphism array analysis showed an interstitial deletion (rs13073496 to rs1119180) of about 0.4 Mb on chromosome 3q26.1 in cases I-1, II-1, II-2, III-1 and III-2. All these subjects, except case II-1, are also heterozygous carriers of the *IGF1* mutation (Fig. 1). The deleted area does not contain any genes or noncoding RNAs. None of the known variations in the Database of Genomic Variants in this region (http://projects.tcag.ca/variation/) had 100% overlap with this deletion.

Comparison of clinical and biochemical features between carriers and noncarriers of the IGF-I mutation

Carriers of the *IGF1* mutation tended to have a lower height SDS (–3.4 *vs.* –1.6, P = 0.172) and had a significantly lower HC (–1.9 *vs.* 0.3, P = 0.002). In case II-2, bone mineral density was normal. Tone audiometry in the two probands was normal. In carriers plasma IGF-I was lower than in noncarriers (P = 0.004).

Response to GH treatment

Both index cases received GH (1.4 mg/m²/day) (Supplemental Fig. 1), which was well tolerated. After 2 yr, height SDS in case III-1 increased by +1.0, she remained prepubertal, and plasma IGF-I rose from –2.6 to +0.6 SDS. IGFBP-3 increased from –0.4 to +0.5 SDS
In case III-2 height SDS increased by +1.5 SD, IGF-I from –2.4 to +0.3 SDS, and IGFBP-3 from –1.0 to +0.3 SDS.

Table 1 Clinical features and biochemical characteristics of the heterozygous and noncarriers

	I-1	II-2	III-1	III-2	I-2	II-1
IGF1 gene	+/−	+/−	+/−	+/−	+/+	+/+
Gender	M	F	F	M	F	F
Age (yr)	65.2	35.5	8.2	6.2	64.5	37.2
Birth weight (kg) [SDS]	n.a.	2.8 [−1.6]	2.3 [−2.9]	3.3 [−1.2]	4.0–5.0	3.0 [−1.1]
Birth length (cm) [SDS]	n.a.	47 [−1.6]	44 [−3.8]	50 [−1.0]	n.a.	48 [−1.1]
Height (cm) [SDS][a]	164 [−1.4]	147.2 [−3.5]	108.9 [−4.1]	98.7 [−4.6]	153.2 [−1.2]	156.5 [−2.0]
HC (cm) [SDS]*	54.7 [−1.8]	52.5 [−1.7]	47.8 [−2.4]	49.0 [−1.6]	55.5 [0.1]	56.0 [0.4]
BMI (kg/m^2) [SDS]	28.6 [3.5]	31.3 [4.7]	14.1 [−1.3]	13.5 [−1.6]	27.0 [2.7]	40.0 [8.8]
Sitting height: height [SDS]	0.54 [1.7]	0.55 [2.2]	0.55 [1.6]	0.57 [2.5]	0.53 [0.7]	0.55 [1.7]
IGF-I (ng/ml) [SDS]	66 [−2.0]	87 [−1.8]	76 [−2.3]	35 [−2.6]	126 [0.6]	131 [−0.4]
IGF-II (ng/ml) [SDS]	529 [1.6]	599 [1.7]	640 [2.0]	542 [1.4]	665 [3.0]	719 [2.8]
IGFBP-1 (ng/ml) [SDS][b]	23	5	143 [0.3]	378 [1.3]	62	7
IGFBP-2 (ng/ml) [SDS]	143 [−0.6]	83 [−1.7]	155 [−1.3]	288 [0.2]	244 [0.5]	81 [−1.7]
IGFBP-3 (mg/liter) [SDS]	2.0 [0.1]	2.8 [0.8]	3.6 [1.2]	2.1 [0.1]	3.3 [2.0]	2.7 [0.7]
IGFBP-4 (ng/ml) [SDS]	242 [1.1]	212 [0.6]	109 [−1.4]	117 [−1.1]	184 [−0.1]	133 [−0.9]
IGFBP-6 (ng/ml) [SDS]	252 [1.0]	148 [0.2]	83 [−1.1]	89 [−0.3]	131 [−0.9]	141 [0.0]
ALS (mg/liter) [SDS]	16.6 [−0.1]	22.8 [0.3]	20.1 [1.1]	11.5 [−0.4]	20.7 [1.2]	24.8 [0.7]
Insulin (IU/liter)[c]	8.5	22.2	< 2.0	< 2.0	< 2.0	9.6

To convert IGF-I to nanomoles per liter, multiply by 0.131; to convert IGF-II to nanomoles per liter, multiply by 0.134; to convert ALS to nanomoles per liter, multiply by 15.8; to convert IGFBP-1 to nanomoles per liter, multiply by 0.033; to convert IGFBP-2 to nanomoles per liter, multiply by 0.032; to convert IGFBP-3 to nanomoles per liter, multiply by 33.3; to convert IGFBP-4 to nanomoles per liter, multiply by 0.038; to convert IGFBP-6 to nanomoles per liter, multiply by 0.034. n.a., Not available; BMI, body mass index; ALS, acid-labile subunit.

[a] Adult height SDS is corrected for shrinking and secular trend by the equations of Niewenweg *et al.* (15).

[b] Normal range for nonfasting subjects is 24–57 ng IGFBP-1 per milliliter. After overnight fasting there is an average 5-fold rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 yr.

[c] Normal range for healthy subjects after overnight fasting is less than 25 IU/liter.

* Statistical difference between heterozygous and wild-type family members.
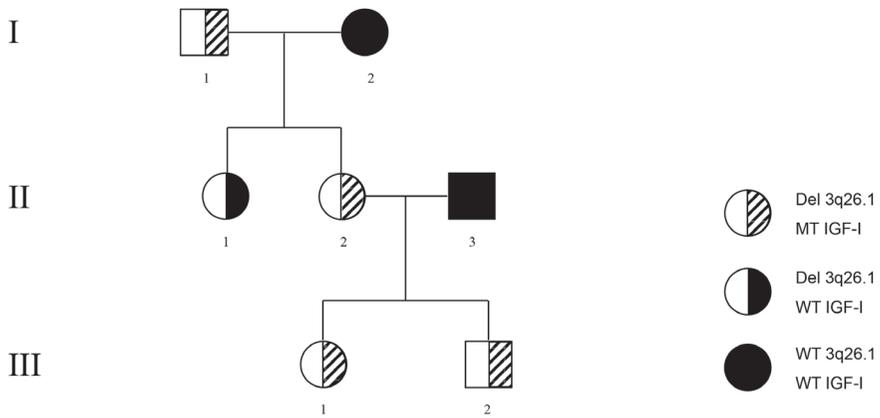
Figure 1

Pedigree of the family. The heterozygous carriers of the *IGF1* mutation are indicated with a *diagonally striped right half of the symbol* and the noncarriers are indicated with a *filled black right half*. The carriers of the 3q26.1 deletion are indicated with a *white left half of the symbol* and the noncarriers are indicated with a *filled black left half*. On the right, the different combinations of wild-type (WT) or mutant (MT) *IGF1* and deleted or normal 3q26.1 is explained. On the left the generation number is indicated and the family number is indicated below each individual.

Discussion

We have shown that heterozygosity for a maternally derived *IGF1* frameshift mutation is associated with severe short stature and microcephaly. The probands' mother, who inherited the same mutation from her father, was less short than the two probands. However, she was still shorter and had a smaller HC and lower plasma IGF-I than her sister, who is not carrying the mutation. This suggests that the combination of maternal and fetal IGF-I deficiency may have had a larger effect on growth than fetal IGF-I deficiency alone, similar to previous observations in a family with an *IGF1R* defect (11). Most carriers had feeding difficulties in infancy, comparable with that observed in patients with homozygous *IGF1* and heterozygous *IGF1R* defects (5;6;11;17). Biochemically, GH secretion was not elevated, similarly to observations in children with IGF-I receptor defects (17) and mice with brain-targeted inactivation of *igf1r* (18). Long-term GH treatment of the probands resulted in a good growth response. Mutant IGF-I was synthesized and used for several *in vitro* experiments. It could not bind to the IGF-I receptor or antagonize the growth-promoting effect of IGF-I. It could bind to IGFBPs but was not incorporated into 150-kDa complexes (data not shown). Details will be reported elsewhere.

We hypothesize that the severe short stature in the two probands might be caused by a cumulative effect of three factors: 1) *IGF1* haploinsufficiency; 2) placental dysfunction due to maternal *IGF1* haploinsufficiency; and 3) other genetic factors associated with (mild) short stature.

The first part of our hypothesis is supported by the observation that case II-2 was in childhood 1 SD and in adulthood 1.5 SD shorter than her wild-type sister (case II-1). Indirect support comes from our previous study showing that heterozygosity for a missense *IGF1* mutation leads to a 0.6 SD lower height, as well as an inhibition of intrauterine and cranial growth (6;19). The heterozygous carriers of an *IGF1* deletion also exhibited reduced height (5;10). The difference between pubertal height gain in cases II-2 and II-1 suggests that IGF-I deficiency may have a strong impact on pubertal growth.

The second part is based on the observation that birth length (−3.8 SDS) and childhood height (−4.1 SDS) of case III-1 is 1–2 SD lower than of her mother (−1.6 and −3.0 SDS, respectively). It is further supported by our previous observations in a family with an *IGF1R* mutation (11), in which the proband's length was much more affected than in her mother, who did not inherit the mutation maternally. Some more indirect evidence for such effect of maternal IGF-I deficiency on placental and fetal growth includes the strong correlation between the rate of IGF-I increase during pregnancy and placental weight (11). Furthermore, placentas from intrauterine growth retardation pregnancies are characterized by decreased expression of IGF-I receptor and associated signal transduction proteins (11).

With respect to the third part (other genetic factors), we have found no other gene defects in this family, except for an interstitial deletion on chromosome 3q26.1 in most cases. There is no evidence linking this region to short stature. Interestingly, the male proband has a 47,XYY karyotype, known to be associated with tall stature. This may have mitigated the negative effect of IGF-I deficiency on intrauterine growth and may have increased his growth response to GH.

In conclusion, our observations suggest that maternally and paternally derived *IGF1* haploinsufficiency leads to a height loss of 2 and 1 SD, respectively, and to a smaller HC, in comparison with wild-type relatives. In combination with a familial predisposition for short stature, this can lead to a height far below the normal range, which can be treated successfully with GH.

Reference List

1. Walenkamp MJ, Wit JM 2006 Genetic disorders in the growth hormone - insulin-like growth factor-I axis. Horm Res 66:221-230

2. Walenkamp MJ, Wit JM 2007 Genetic disorders in the GH IGF-I axis in mouse and man. Eur J Endocrinol 157 Suppl 1:S15-S26

3. Rosenfeld RG 2003 Insulin-like growth factors and the basis of growth. N Engl J Med 349:2184-2186

4. Rosen CJ 1999 Serum insulin-like growth factors and insulin-like growth factor-binding proteins: clinical implications. Clin Chem 45:1384-1390

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

6. 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 2005 Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90:2855-2864

7. Bonapace G, Concolino D, Formicola S, Strisciuglio P 2003 A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 40:913-917

8. Coutinho DC, Coletta RR, Costa EM, Pachi PR, Boguszewski MC, Damiani D, Mendonca BB, Arnhold IJ, Jorge AA 2007 Polymorphisms identified in the upstream core polyadenylation signal of IGF1 gene exon 6 do not cause pre- and postnatal growth impairment. J Clin Endocrinol Metab 92:4889-4892

9. Netchine I, Azzi S, Houang M, Seurin D, Perin L, Ricort JM, Daubas C, Legay C, Mester J, Herich R, Godeau F, Le Bouc Y 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. J Clin Endocrinol Metab 94:3913-3921

10. Woods KA 1998 Molecular Mechanisms in Growth Hormone Insensitivity. London: Paediatric

Endocrinology Section, Department of Endocrinology, St Bartholomew's Hospital London.

11. Walenkamp MJ, van der Kamp HJ, Pereira AM, Kant SG, van Duyvenvoorde HA, Kruithof MF, Breuning MH, Romijn JA, Karperien M, Wit JM 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. J Clin Endocrinol Metab 91:3062-3070

12. Miller SA, Dykes DD, Polesky HF 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

13. Gijsbers AC, Bijlsma EK, Weiss MM, Bakker E, Breuning MH, Hoffer MJ, Ruivenkamp CA 2008 A 400kb duplication, 2.4Mb triplication and 130kb duplication of 9q34.3 in a patient with severe mental retardation. Eur J Med Genet 51:479-487

14. Kulharya AS, Flannery DB, Norris K, Lovell C, Levy B, Velagaleti GV 2008 Fine mapping of breakpoints in two unrelated patients with rare overlapping interstitial deletions of 9q with mild dysmorphic features. Am J Med Genet A 146A:2234-2241

15. Niewenweg R, Smit ML, Walenkamp MJ, Wit JM 2003 Adult height corrected for shrinking and secular trend. Ann Hum Biol 30:563-569

16. van Wieringen JC, Wafelbakker F, Verbrugge HP, de Haas JH 1971 Growth diagrams 1965 the Netherlands. Leiden/Groningen, The Netherlands: Nederlands Instituut voor Praeventieve Geneeskunde/Wolters Noordhoff.

17. Ester WA, van Duyvenvoorde HA, de Wit CC, Broekman AJ, Ruivenkamp CA, Govaerts LC, Wit JM, Hokken-Koelega AC, Losekoot M 2009 Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype. J Clin Endocrinol Metab 94:4717-4727

18. Kappeler L, De Magalhaes FC, Dupont J, Leneuve P, Cervera P, Perin L, Loudes C, Blaise A, Klein R, Epelbaum J, Le BY, Holzenberger M 2008 Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism. PLoS Biol 6:e254

19. Denley A, Wang CC, McNeil KA, Walenkamp MJ, van DH, Wit JM, Wallace JC, Norton RS, Karperien M, Forbes BE 2005 Structural and functional characteristics of the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development. Mol Endocrinol 19:711-721

Supplemental Data

Methods
Biochemical measurements
Plasma levels of IGF-I, IGF-II, IGFBP-1, -2, -3, -4 and -6 were determined as described previously (1). ALS was measured by a commercially available ELISA (Diagnostics Systems Laboratories, Inc., Webster, TX). With the exception of IGFBP-1 for adults older than 24 years, smoothed references based on the LMS method were available for all parameters allowing conversion of patient data to SDS values (1). For the adult members of the family plasma IGFBP-1 concentrations after an overnight fast were compared with a reference group of 6 healthy adult controls. Levels of insulin were determined in serum, obtained after an overnight fast, by a semi-automatic luminescence method (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA).

Molecular studies with genomic DNA of the family members
Genomic DNA was isolated from whole blood according to the salting out procedure described by Miller et al (2). All exons of *IGF1* (GenBank accession number NM_000618) were PCR amplified and sequenced as described previously (1). The various primer sequences are available upon request.

For the analysis of single-nucleotide polymorphisms (SNPs)**,** the Affymetrix GeneChip human mapping 262K *NspI* array (Affymetrix, Santa Clara, CA) was used. This SNP array is capable of genotyping approximately 262,000 SNPs, with a ~12 kb resolution. The assay was performed according to the manufacturer's protocol, beginning with 250 ng DNA. Detection of copy number changes was performed using Copy Number Analyser for GeneChip (CNAG) version 2.0 (www.genome.umin.jp/), using a reference set of minimal 5 and maximal 10 control DNAs (3;4).

Supplemental Figure 1
Growth curves of the index cases. The dots represent height measurements, the squares represent bone age measurements, and GH treatment is indicated below the curves. The growth curves of index cases III-1 and III-2 are shown in panels A and B, respectively.

chapter
2 S

A

B
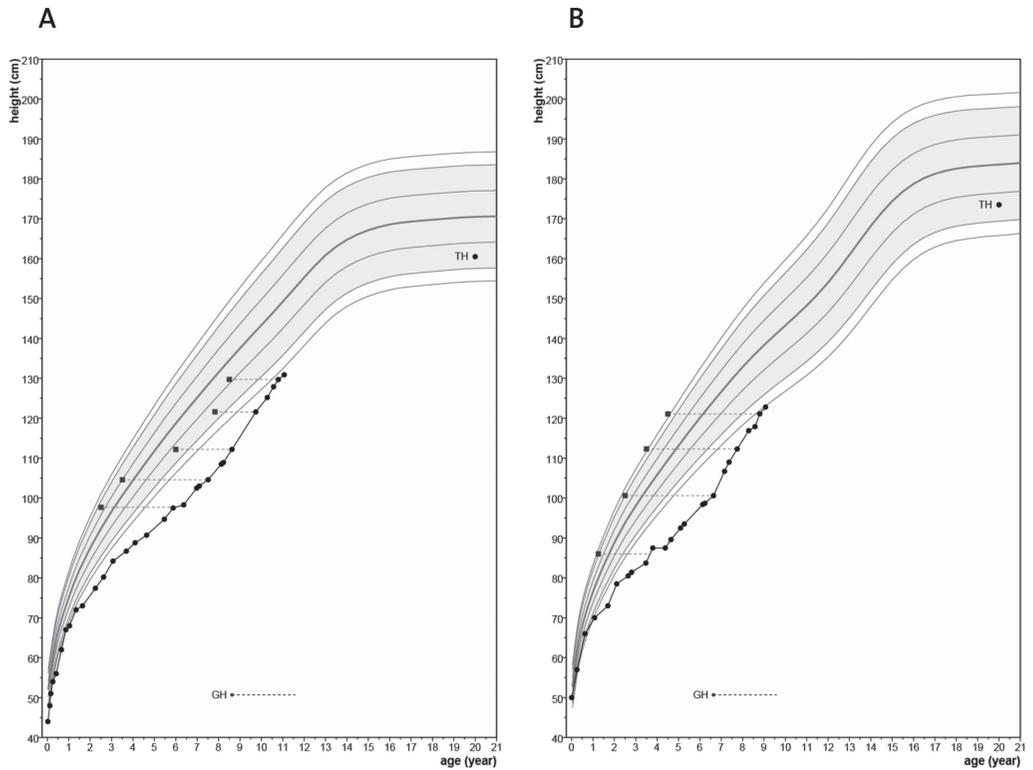
Reference List

1. 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 2005 Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90:2855-2864

2. Miller SA, Dykes DD, Polesky HF 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

3. Gijsbers AC, Bijlsma EK, Weiss MM, Bakker E, Breuning MH, Hoffer MJ, Ruivenkamp CA 2008 A 400kb duplication, 2.4Mb triplication and 130kb duplication of 9q34.3 in a patient with severe mental retardation. Eur J Med Genet 51:479-487

4. Kulharya AS, Flannery DB, Norris K, Lovell C, Levy B, Velagaleti GV 2008 Fine mapping of breakpoints in two unrelated patients with rare overlapping interstitial deletions of 9q with mild dysmorphic features. Am J Med Genet A 146A:2234-2241

chapter

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The severe short stature in two siblings with a heterozygous *IGF1* mutation is not caused by a dominant negative effect of the putative truncated protein

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Abstract

Objective: While in previous studies heterozygosity for an *Insulin-Like Growth Factor 1 (IGF1)* defect only modestly decreased height and head circumference, we recently reported on two siblings with severe short stature with a maternally transmitted heterozygous duplication of 4 nucleotides, resulting in a frame shift and a premature termination codon in the *IGF1* gene. In this paper we describe the structural and functional characteristics of the putative truncated IGF-I protein.

Design: Two children, their mother and maternal grandfather carried the mutation. In addition, two family members who were not affected were included in the study. Mutant (MT) IGF-I was synthesized in oxidized and reduced form using two methods. Neutral gel filtration studies were carried out with wild-type (WT) and synthetic MT IGF-I. Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptors were performed with EBNA-293 cells, stably transfected with the IGF-I receptor, and IM9 cells. L6 cells were used to examine the mitogenic potency and the potential antagonizing effect of synthetic MT IGF-I by [^{3}H]-thymidine incorporation assays.

Results: In the sera of both the carriers and non-carriers the proportion of ^{125}I-IGF-I that was associated with the 150 kDa complex was somewhat less (varying between ~37 and ~52%) than in normal pooled serum (~53– ~ 63%) and, instead, slightly increased amounts of radioactivity were eluted in the 40–50 kDa fraction (consisting of binary IGF–IGFBP complexes) or remained unbound. Synthetic MT IGF-I did not bind to the IGF-I receptor, nor antagonize the growth-promoting effect of IGF-I. It did bind to IGFBPs, but was barely incorporated into 150 kDa complexes. Because in all cases WT IGF-I immunoreactivity was recovered in one peak, corresponding to the MW of WT IGF-I, i.e. ~7.6 kDa, an interaction of circulating truncated mutant peptide with WT IGF- I is very unlikely.

Conclusions: There is no evidence that the severe short stature associated with heterozygosity for this novel *IGF1* mutation in children born from a mother with the same mutation is caused by a dominant negative effect of the truncated protein. We speculate that the growth failure is caused by a combination of partial IGF-I deficiency, placental IGF-I insufficiency, and other genetic factors.

Introduction

IGF-I is a highly conserved, single-chain polypeptide consisting of 70 amino acids, organized into four peptide domains: A, B, C and D. The B-domain and the first A domain helix are mainly responsible for binding to IGFBPs. The A-, B- and C- and, to a lesser extent, the

D-domain contribute substantially to IGF binding to its receptor (IGF1R) (1-8). The A domain also contains residues involved in low affinity binding to the IGF2R (4-9). The IGF-I pro-hormone contains an additional carboxy-terminal E peptide that is cleaved in the Golgi apparatus before secretion (10).

Approximately 80–90% of circulating IGFs form a 150 kDa ternary complex with IGFBP-3 or -5 and the acid-labile subunit (ALS) (11), 10–15% are bound in a 40–50 kDa binary complex to IGFBPs, whereas less than 1% is found in the free form (12). The 150 kDa complexes cannot cross the capillary endothelial barrier, which prolongs the half-life of IGFs, IGFBP-3, and -5 in the circulation (11, 13, 14). This seems to play an important role in the regulation of the bioavailability of IGFs to the tissue compartments. When carried in ternary complexes, the half-life of IGF-I is more than 12 h, much longer than that of binary bound IGF-I (30–90 min) and free IGF-I (~10 min) (15).

In mice IGF-I plays a major role in pre- and postnatal growth (16-17). Recent studies revealed that autocrine and paracrine IGF-I appears crucial for early postnatal and pubertal development of cortical bone, while circulating IGF-I, responsible for the endocrine effect, is mainly important for growth after puberty. In autocrine and paracrine IGF-I deficient mice high levels of serum IGF-I can permit postpubertal "catch-up" growth (18-20).

IGF-I is also needed for normal pre- and postnatal growth in humans, as shown by severe dwarfism in three patients with well documented homozygous *IGF1* defects, caused by the deletion of exons 4 and 5 (21), a missense mutation (22) and a missense mutation with a milder phenotype (23). There may be a fourth case (24), but although the patient's phenotype was very similar, the presumed genetic aberration in the polyadenylation site at the 3'UTR in exon 6 was later found to occur also in healthy controls (25). Patients with a homozygous *IGF1* defect exhibit severely affected intrauterine growth (about –4 SDS), whereas postnatally height SDS varies between –6.9 and –8.5, and head circumference between –4.0 and –8.0 SDS. Heterozygosity for an *IGF1* mutation or deletion may be associated with a mild height loss (in the order of 1 SD), but can present as short stature if occurring in a family with genetic predisposition for decreased height (22).

We recently reported on two siblings with severe short stature carrying a heterozygous duplication of four nucleotides in *IGF1*, resulting in a frame shift at position 35 of the mature IGF-I protein and a premature stop codon (c.243_246dupCAGC, p.Ser83GlnfsX13) (26). In the present paper we describe the laboratory studies that we performed to investigate whether this heterozygous mutation could cause such severe phenotype, either directly or by interfering with the biological action of WT IGF-I, in particular whether the truncated mutant IGF-I may exert a dominant negative or antagonistic effect on the function of wild-type IGF-I.

Subjects and Methods

Subjects

Clinical features, growth curves and plasma levels of IGFs, various IGFBPs and ALS of the 4
carriers of the *IGF1* mutation, as well as the pedigree, have been reported previously (26). In
short, the first index case was a 8.2 year old girl (III-1) born small for gestational age (birth
weight –2.9 and birth length –3.8 SDS) who presented with severe postnatal short stature
(height –4.1 SDS) and microcephaly (head circumference –2.4 SDS). Her 6.2 year old brother
(III-2) had a birth weight and length of –1.2 and –1.0 SDS, respectively, a height of –4.6 SDS,
and a head circumference of –1.6 SDS. Their mother (II-2) carried the same mutation and
had a height SDS of –3.5 and a head circumference of –1.7 SDS, and the affected maternal
grandfather (I-1) had a height of –1.4 SDS (corrected for age and secular trend) (27) and a
head circumference of –1.8 SDS. The unaffected maternal grandmother and aunt were less
short (–1.2 and –2.0 SDS) and had a normal head circumference (0.1 and 0.4 SDS). Serum
IGF-I SDS of the 4 carriers was approximately –2 SDS, significantly lower than in non-
carriers (*P* = 0.004). For both carriers and non-carriers of the *IGF1* mutation serum levels of
IGFBP-1, -2,-3, -4 and -6, as well as those of ALS, were within the respective normal ranges,
i.e. between –2 and +2 SDS (26). Case III-2 showed a 47,XYY karyotype.

Synthesis of mutant IGF-I

The biological properties of mutant (MT) IGF-I were tested with synthesized peptides.
Since the B-domain of MT IGF-I contains two unpaired cysteine residues, and it is unknown
whether the cysteines remain reduced or become oxidized (in case the peptide is stably
expressed *in vivo*), both oxidized and reduced forms were prepared, and used in the various
experiments.

One batch of the oxidized mutant peptide was prepared (Dr. L. Schäffer, Novo Nordisk
A/S, Maaloev, Denmark) by solid phase peptide synthesis on Tentagel S RAM resin using
Fmoc chemistry on a Liberty microwave synthesizer according to the manufacturer's
instruction. The formation of disulfide bridges was accomplished by treatment of the
peptide with 2% iodine in N-methylpyrrolidone for 1 hour prior to cleavage. The peptide was
cleaved from the resin with trifluoroacetic acid/water/triisopropylsilane (95%:2.5%:2.5%)
and purified by standard RP-HPLC.

Another batch of oxidized peptide, as well as a batch of reduced peptide, was prepared
in the laboratory of Prof. J. D. Wade (Howard Florey Institute, Melbourne, Australia). Region-
selectively S-protected A- and B-chains were synthesized separately as their C-terminal
amides using the continuous flow Fmoc-SPPS method as previously reported (28), using an
automatic PerSeptive Biosystems Pioneer peptide synthesizer (Massachusetts, USA) (29).
The identities of the crude peptides were confirmed by MALDI-TOF MS. Subsequently, the

mutant IGF-I peptide was prepared by sequential region-selective disulfide bond formation between the two chains also as previously described (28,29). Following a final RP-HPLC purification step, overall yield of mutant IGF-I was 5.01 mgs (9.5% relative to crude starting B-chain) which was satisfactory given the multiple steps involved in its preparation.

We show the results obtained with the peptides prepared by Prof. J.D. Wade but identical results were obtained with the one prepared by Dr. L. Schäffer.

Neutral gel filtration studies with WT and synthetic MT IGF-I

The different molecular-size classes of endogenous IGF-I (i.e. 150 kDa, 40–50 kDa, and unbound, free form) in plasma were determined by neutral gel filtration through a 1.6×60 cm Superdex 200 Hiload column, as described previously (30). Prior to column chromatography, each serum sample (250 μl) was incubated with 100 μl of ~100,000 cpm of either [125]I -WT hIGF-I or [125]I- MT IGF-I being dissolved in 50 mM sodium phosphate buffer pH 7.4, containing 0.2% BSA, 10 mM EDTA and 0.05% (w/v) Tween-20, for 17 h at 4°C. The various molecular size classes of complexes were eluted from the column at a rate of 1.2 ml/min using 0.05 M NH_4HCO_3 buffer pH 7.4. The ^{125}I content of each 1.2 ml fraction was counted in a gamma counter.

In order to investigate the ability of synthesized MT IGF-I to compete for incorporation into ternary and binary complexes, 250 μl aliquots of pooled normal human serum were incubated with 100 μl of the same amount of ^{125}I radiolabeled WT recombinant IGF-I in the presence of increasing concentrations of either recombinant wild-type IGF-I or synthetic MT IGF-I, as described above. The distribution of ^{125}I-IGF-I among the various molecular-size classes in each aliquot of serum was determined after separation by S200 gel filtration.

The possibility that WT IGF-I and eventually naturally existing MT IGF-I in serum interacted, for example by forming higher MW dimers or multimers, was investigated by Sephadex G-50 exclusion chromatography of Sep-Pak C18 extracts of sera from carriers and non-carriers. The 1.5×90-cm column was calibrated with lactalbumin (14.5 kDa), hIGF-II (7.5 kDa), and insulin-B (3.5 kDa). Acetic acid (0.1 M) was passed in a descending direction at a rate of 4.8 mL/h, and ~3 mL fractions were collected, dried and reconstituted in 50 mM sodium phosphate buffer pH 7.4, containing 0.2% BSA, 10 mM EDTA and 0.05% (w/v) Tween-20. In each column fraction the IGF-I immunoreactivity was determined by RIA (31).

Cell lines

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptors was performed with EBNA-293 stably expressing the IGF-I receptor (gift from Dr. J. Whittaker, Case Western Reserve University, Cleveland, Ohio) and IM9 human lymphocytes, respectively. The rat myoblast cell line (L6) used for thymidine incorporation was kindly provided by Bo

Falck Hansen, Novo Nordisk, Denmark. These cells express ~175,000 IGF-I receptors per cell and no insulin receptors (Sajeed, W. and De Meyts, P., unpublished data).

EBNA 293 cells transfected with the IGF-1 receptor

The EBNA-293 cells are human embryonic kidney (HEK) cells expressing Epstein-Barr nuclear antigen (EBNA-1) which are stably transfected with the IGF-I receptor (EBNA293t IGF-IR), kindly provided by Dr. J. Whittaker (Case Western Reserve University, Cleveland, Ohio) (32). The cells were cultured in DMEM high glucose with Glutamax-1 supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin (all from Gibco, Invitrogen, USA). 250 µg/ml Hygromycin B (Sigma-Aldrich, USA) and 400 µg/ml Geneticin (Gibco, Invitrogen, USA) were added to the media for selecting the stably transfected cells. The cells were grown at 37°C in 5% CO_2 humidified atmosphere. Cells were sub-cultured three times a week by washing the cells in 10 ml PBS w/o calcium and magnesium (Gibco, Invitrogen, USA), detaching the cells from the culture flask with 2 ml 0.05% Trypsin-EDTA (Gibco, Invitrogen, USA), followed by resuspending in 8 ml fresh media.

IM9 cells

The IM9 human lymphoblast cell line (American Type Culture Collection (ATCC), USA) is derived from a patient with multiple myeloma. The cell line carries the Epstein-Barr virus. The IM9 cells express about 20,000 insulin receptors (isoform A) per cell and have been used for several decades in insulin receptor binding kinetics studies (33). The cells, which grow in suspension, were cultured and used as described previously (33).

Rat L6 myoblasts

The L6 WT cells were cultured in 75 cm² flasks with DMEM (1000 mg/l glucose, Glutamax-1, Pyruvate) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. The cells were grown at 37°C in 5% CO_2 humidified atmosphere. The cells were sub-cultured three times a week, by washing the cells in 10 ml PBS w/o calcium and magnesium, and detaching them by adding 2 ml 0.05% Trypsin-EDTA. Cells were resuspended in 10 ml fresh media, and seeded with a split ratio of 1:6 for two days and 1:12 for three days of maintenance.

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptor

Binding competition between synthetic mutant peptides and ^{125}I-IGF-I (on EBNA293t IGF-IR cells) and ^{125}I-insulin (on IM9 cells), respectively, as well as accelerated dissociation of labeled ligand by cold ligand (negative cooperativity) were studied according to established protocols (33, 34).

[³H]-Thymidine incorporation assay

[³H]-Thymidine incorporation was used to examine the mitogenic potency of synthetic MT IGF-I using the L6 WT rat myoblast cell line. We used a recently developed optimized assay on cells synchronized in G0/G1 that gives a robust and highly reproducible response to growth factors, as described in detail previously (35).

Results

Mutation analysis

In the two index cases, their mother and maternal grandfather, we found a heterozygous duplication of four nucleotides of the *IGF1* gene, resulting in a frame shift at position 35 of the mature IGF-I protein and a premature stop codon (c.243_246dupCAGC, p.Ser83GlnfsX13) (Fig. 1A). If the peptide would be stably expressed *in vivo*, the mutation should result in a truncated C-domain, followed by a tail of 12 residues with an altered amino acid sequence showing no homology with the wild-type A- and D-domains (Fig. 1B). Therefore, the mutant peptide and wild-type IGF-I protein would have only the B-domain in common. Single-nucleotide polymorphism array analysis showed an interstitial deletion not containing any gene or noncoding RNAs (26) in all carriers and mother's sister (II-1).

A

T A T G G C T C C A G C N N N C N G A N G G A G G N N N N

c.243_246dupCAGC, p.Ser35GlnfsX13

B

	1			30	42
IGF-I:	GPETLCGAEL	VDALQFVCGD	RGFYFNKPT	GYGSSSRRAPQT	GIVDECCF ~

B domain ←————————————————————————→ C domain ←———→ A domain ←---→

	1			30	
MT IGF-I:	GPETLCGAEL	VDALQFVCGD	RGFYFNKPT	GYGSS QQSEGA	SDRHRG

B domain ←————————————————————————→ C domain ←------------------→

Figure 1

A. DNA sequence chromatogram of the four family members with the heterozygous *IGF1* mutation. The arrow indicates the duplication of the 4 nucleotides.

B. Sequence alignment of wild-type human IGF-I and the mutant IGF-I (MT IGF-I).

The MT *IGF1* has a heterozygous duplication of 4 nucleotides at position 247. The duplication leads to an IGF-I molecule truncated in its C-domain, which therefore only contains 5 of the 12 residues (Gly^{30}–Ser^{34}) originally seen in the C-domain in a wild-type IGF-I molecule. The truncation is followed by a tail of 12 residues with an altered amino acid sequence (red colour), that show no homology with the following A- or D-domain observed in wild-type IGF-I. Consequently, the MT IGF-I has no A- and D-domain though it still has an intact B-domain. The arrows mark the location of the different domains. The dotted arrow marks the altered C-domain, whereas the red colour marks the twelve altered residues in the truncated C domain.

Neutral gel filtration studies with WT and synthetic MT IGF-I

After equilibration with ^{125}I-IGF-I, in aliquots (n = 4) of pooled normal control serum a substantial part of the radioactivity (~53 – ~63%) was incorporated into the 150 kDa complex (Fig. 2A). In the sera of both the carriers and non-carriers the proportion of ^{125}I-IGF-I that was associated with the 150 kDa complex was somewhat less (varying between ~37 and ~52%) and, instead, slightly increased amounts of radioactivity were eluted in the 40–50 kDa fraction (consisting of binary IGF-IGFBP complexes) or remained unbound (data not shown).

Superdex 200 gel filtration column chromatography of normal serum after incubation with either ^{125}I-labeled WT hIGF-I or MT IGF-I (reduced form) revealed that the radiolabeled mutant peptide is able to bind to IGFBPs in serum but, in contrast to WT IGF-I, is hardly incorporated into 150 kDa complexes (Fig. 2A). Similar results were obtained for the oxidized form of MT IGF-I (data not shown).

In addition, competition gel filtration experiments were performed in which ^{125}I-WT hIGF-I was equilibrated with different aliquots of pooled normal serum in the presence of increasing amounts of either unlabeled WT-hIGF-I or MT IGF-I. As shown in figure 2B, in contrast to WT hIGF-I, MT IGF-I (reduced form) did not compete significantly with radiolabeled ligand for 150 kDa complex formation and only modestly for incorporation into 40–50 kDa complexes. The same results were encountered for the oxidized form of MT IGF-I (data not shown).

The results of acid G50 column chromatography of Sep-Pak C18 extracted sera from carriers of the *IGF1* mutation did not support the possibility that the putative circulating truncated mutant peptide forms heteromultimers with WT IGF- I, because in all cases WT IGF-I immunoreactivity was recovered in one peak corresponding to the MW of WT IGF-I, i.e. ~7.6 kDa (data not shown).

MT IGF-I (both reduced and oxidized) did not show any cross-reactivity in the semi-automated chemiluminescence IGF-I assay employed (30) nor in an in house RIA (31).

Binding analysis of synthetic MT IGF-I to the IGF1R and insulin receptor

We tested the ability of the reduced and two oxidized preparations of MT IGF-I to compete for ^{125}I-WT IGF-I binding to EBNA293t IGF-IR cells, as well as for ^{125}I-insulin binding to IM9 cells (oxidized peptide only). No competition was observed in either system at concentrations as high as 10 µM, indicating that the peptides do not bind to IGF-I receptors (Fig. 3) or insulin receptors (data not shown). Likewise, the peptides did not accelerate the dissociation of ^{125}I-IGF-I (negative cooperativity) and did not antagonize the negative cooperativity of cold IGF-I (data not shown).
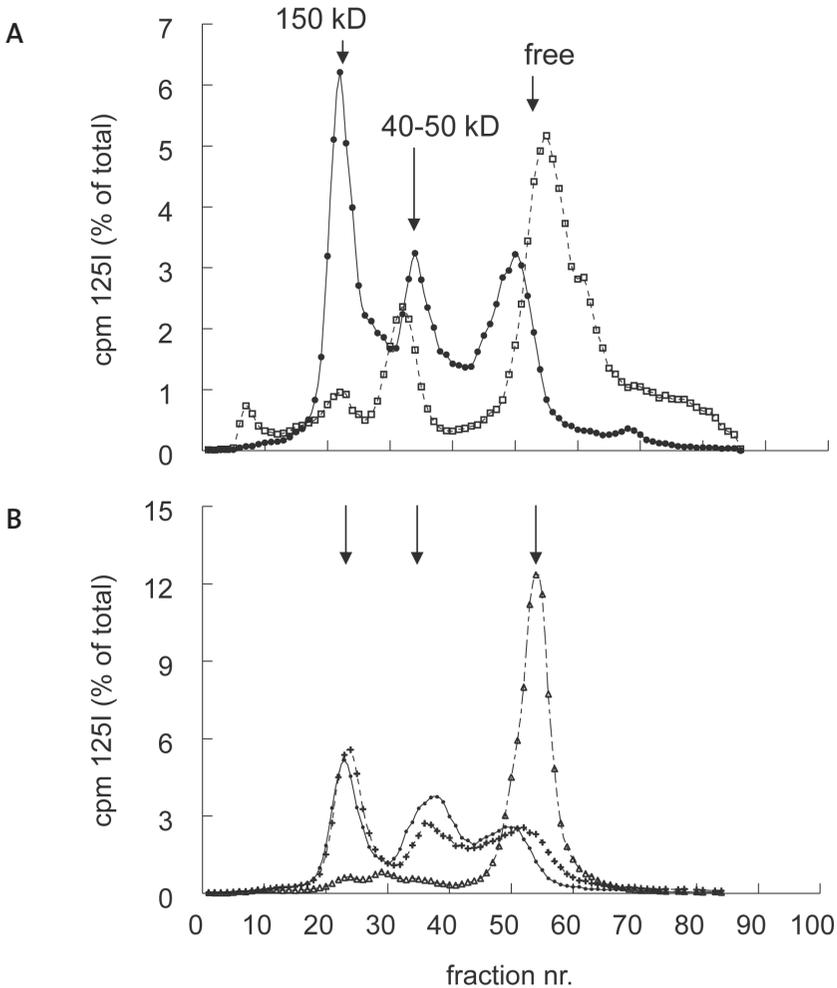
Figure 2

A. Aliquots of pooled normal serum were equilibrated with either ^{125}I radiolabeled WT hIGF-I (•——•) or synthetic MT IGF-I (reduced form)(□- - -□) , followed by Superdex 200 gel filtration column chromatography.

B. Distribution of radioactivity among the various molecular weight classes, as determined by Superdex 200 gel filtration in aliquots of pooled normal serum that had been equilibrated with ^{125}I radiolabeled WT in the absence (•——•) or presence (Δ- - - -Δ) of increasing amounts of unlabeled WT hIGF-I or reduced synthetic MT IGF-I (+ - - - +). Only the highest amounts of unlabeled WT hIGF-I and MT IGF-I employed are included in the figure, i.e. 26 pmol and 64 pmol, respectively.
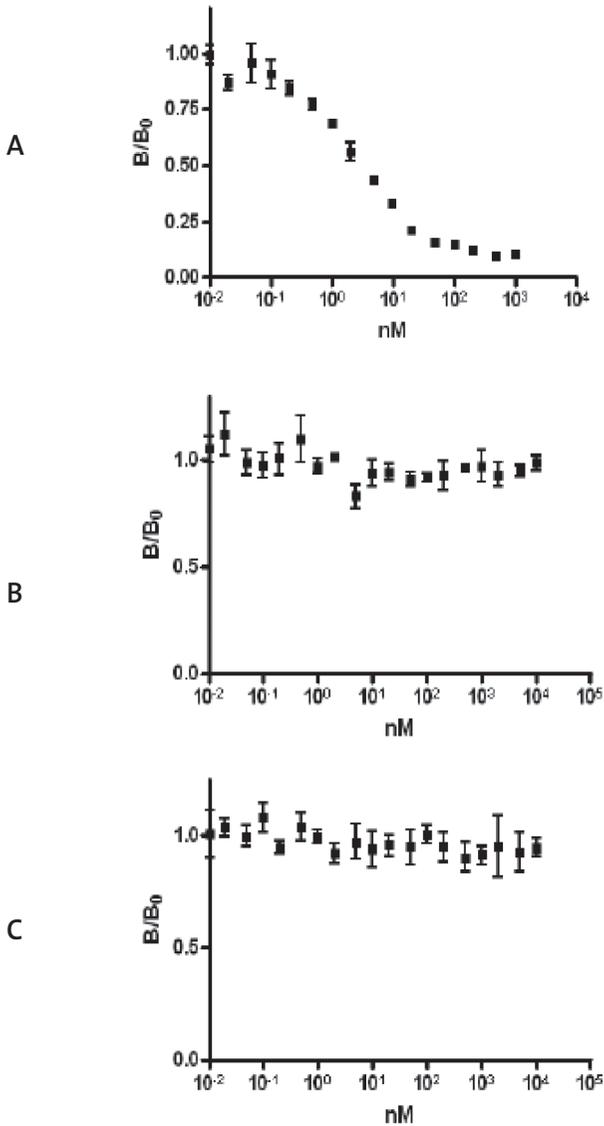
Figure 3

Competition of unlabeled IGF-I (A) and reduced (B) and oxidized (C) MT IGF-I for [125]I -IGF-I binding to EBNA-293 cells stably transfected with the IGF-I receptor. The data are plotted as bound tracer (as a fraction of tracer bound without unlabeled ligand), as a function of the concentration of competing ligand. IGF-I competes for tracer binding with a high affinity Kd of 1.3 nM, while both forms of the MT IGF-I are unable to compete at concentrations as high as 10 µM.

[³H]-Thymidine incorporation assay

The MT IGF-I peptides were tested for their ability to stimulate thymidine incorporation in L6 myoblasts. The cells showed a robust response to WT IGF-I, while the MT peptides showed no effect in the physiological molar range of WT IGF-I (0.13–13 nmol/L) (36). Only a very small increase in thymidine incorporation was observed at very high non-physiological concentrations of MT IGF-I, which is probably an artificial effect since at these concentrations there was no binding to the IGF-I receptor. Figure 4 shows data for the reduced mutant peptide; similar data were obtained with the oxidized peptides. The mutant oxidized and reduced peptides did not antagonize the response to WT IGF-I (data not shown).
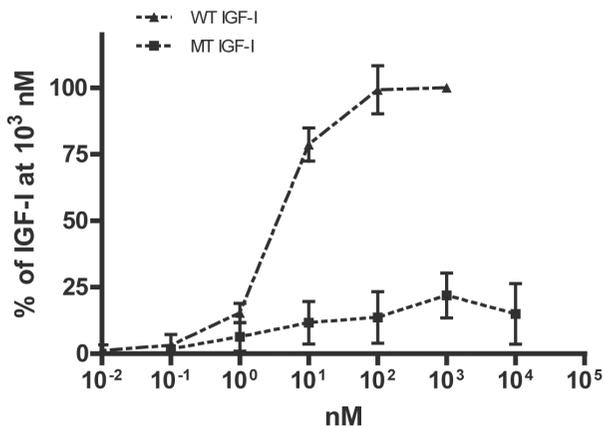
Figure 4

[³H]-Thymidine incorporation assay. Dilution series of MT IGF-I (0–10,000 nM) were used to stimulate L6 cell proliferation. The data were plotted as MT IGF-I response compared to WT IGF-I response at 10^3 nM in percent, as a function of the MT IGF-I concentration. WT IGF-I was included in each assay as a control and data were plotted as percent of WT IGF-I response at 10^3 nM. Data presented display three independent experiments performed in triplicates ± SD. MT IGF-I is not able to stimulate mitogenesis in normal physiological concentrations.

Discussion

In a previous study on a patient with a homozygous missense mutation in the *IGF1* gene, presenting with severe intrauterine and postnatal growth retardation, microcephaly, and sensorineural deafness**,** we also investigated family members who were heterozygous for this *IGF1* mutation. These subjects exhibited SDS values for height, head circumference, and birth weight that were lower than those for non-carriers from the same family, but still within the normal range (22). Hence, it was surprising that the severity of the short stature of the two siblings with the heterozygous *IGF1* mutation described in the present study was, in fact, of a similar order of magnitude as observed in one of the three subjects with a homozygous *IGF1* mutation (23), though less severe than the patients with a complete loss of function of IGF-I (21,22). This finding prompted us to investigate the possible functional effects of the putative truncated IGF-I peptide as encoded by the mutated *IGF1* gene.

Although we could not establish whether any MT IGF-I was present in the circulation, we first hypothesized that in case it would, this truncated peptide may exert a dominant negative effect on the biological action of the WT IGF-I. Insulin and IGF-I bind to their respective dimeric receptors with a complex mechanism that involves cross-linking of two partial binding sites (sites 1 and 2) on each receptor half. Two distinct sites of the ligand are involved, leading to high affinity binding (37). We reasoned that while the truncated IGF-I clearly lacks the whole set of determinants needed to cross-link the two receptor halves, it may still have sufficient affinity to bind to one of the two receptor binding sites, which would thus enable it to prevent the cross-linking by native IGF-I, resulting in a dominant negative or antagonistic effect.

However, we demonstrated that synthetic MT IGF-I could not bind to the IGF1R or the insulin receptor at all and lacked significant mitogenic potency. Moreover, MT IGF-I did not show any antagonistic effects on either the binding of WT IGF-I to the IGF1R and the insulin receptor or its mitogenic capacity. This result could be explained by the fact that MT IGF-I lacks critical A- and C-domain residues involved in receptor binding. Furthermore, it likely has a disordered structure with the potential for an abnormal disulfide bond as in the oxidized peptide. Thus, the short stature of the carriers of the heterozygous *IGF1* mutation cannot be attributed to a blockade of WT IGF-I action at the receptor level.

On the other hand, MT IGF-I still does contain various domains important for binding to IGFBPs. Therefore, we investigated the possibility that MT IGF-I might tightly bind to IGFBPs (including 150 kDa complexes with ALS) in such a way that it would not be readily exchangeable with WT IGF-I. In theory, this would lead to a decrease in the half-life of circulating WT IGF-I (as observed in ALS deficient patients), and, as a consequence, deregulation of its bioavailability. However, the results of our gel filtration experiments

with both synthesized forms of MT IGF-I did not provide any evidence for this possibility, probably due to a disordered structure as discussed above. In addition, there was no indication that MT IGF-I directly interacted with WT IGF-I.

The observation that the incorporation of WT ^{125}I-IGF-I into 150 kDa complexes in sera from both carriers and non-carriers of the *IGF1* mutation appeared to be somewhat less than in normal sera is difficult to explain. Subtle individual differences between the serum concentrations of the various IGFBPs or ALS may account for this finding, although for all the subjects investigated the levels of these proteins were within their normal ranges (26). In all members of this family, the serum levels of IGF-II appeared to be either in the upper normal range or even elevated (26). A relatively higher endogenous serum IGF-II pool may have competed with the WT ^{125}I-IGF-I for 150 kDa complex formation.

Circulating endogenous levels of WT IGF-I (MT IGF-I could not be detected by the IGF-I assays employed) in the four heterozygous carriers of the *IGF1* mutation were found to be significantly reduced, i.e. varying between -1.8 and -2.6 SDS (26). In each case, this appears to represent about half of that of the corresponding normal mean serum IGF-I level when adjusted for age and gender.

We therefore conclude that the severe short stature of the two index cases is not caused by a dominant negative or antagonistic effect on the function of WT IGF-I. We speculate that the severe short stature might be caused by a cumulative effect of three factors: 1) WT *IGF1* haploinsufficiency; 2) placental dysfunction due to maternal WT *IGF1* haploinsufficiency; and 3) other genetic factors associated with (mild) short stature (26). Our studies showed that MT IGF-I is not able to bind to the IGF1R, therefore we speculate that reduced height can be caused by WT *IGF1* haploinsuffiency; it seems that the WT copy of *IGF1* does not produce enough IGF-I to reach normal height. Another factor that may contribute to the short stature observed is placental dysfunction due to maternal WT *IGF1* haploinsufficiency, as discussed in our earlier paper (26). There is a strong correlation between the rate of maternal IGF-I increase during pregnancy and placental weight (38). Various studies have been performed to investigate IGF-I in intrauterine growth retardation pregnancies. Contradicting results have been observed in studies concerning placenta IGF-I and IGF1R mRNA and protein expression. Some studies reported increased expression of IGF-I and IGF1R in placentas from small for gestational age (SGA) newborns compared with appropriate for gestational age (AGA) newborns (39, 41), while other studies showed decreased or equal expression. Inconsistencies could be due to differences in the selection criteria for IUGR, length of gestation and the methodology used (40, 42). Based on the observation that birth length and childhood height of case III-1 is 1–2 SD lower than of her mother and our previous observations in a family with an *IGF1R* mutation (38), in which the proband's length was much more affected than in her mother, who did not inherit the

mutation maternally, we hypothesize that IGF-I and IGF1R play an important role in normal placental function and consequently in intrauterine growth.

Interestingly, we observed that GH therapy was efficacious in increasing height in the first two years of treatment, without adverse events, which may be explained by the normalisation of IGF-I availability.

chapter

3

Reference List

1. A.M. Brzozowski, E.J. Dodson, G.G. Dodson, G.N. Murshudov, C. Verma, J.P. Turkenburg, F.M. de Bree, Z. Dauter, Structural origins of the functional divergence of human insulin-like growth factor-I and insulin, Biochemistry 41 (2002) 9389-9397.

2. A. Denley, E.R. Bonython, G.W. Booker, L.J. Cosgrove, B.E. Forbes, C.W. Ward, J.C. Wallace, Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR, Mol. Endocrinol. 18 (2004) 2502-2512.

3. J. Dupont, M. Holzenberger, Biology of insulin-like growth factors in development, Birth Defects Res. C. Embryo. Today 69 (2003) 257-271.

4. B.E. Forbes, P.J. Hartfield, K.A. McNeil, K.H. Surinya, S.J. Milner, L.J. Cosgrove, J.C. Wallace, Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis, Eur. J. Biochem. 269 (2002) 961-968.

5. L. Gauguin, C. Delaine, C.L. Alvino, K.A. McNeil, J.C. Wallace, B.E. Forbes, P. de Meyts, Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I, J. Biol. Chem. 283 (2008) 20821-20829.

6. B.A. Magee, G.K. Shooter, J.C. Wallace, G.L. Francis, Insulin-like growth factor I and its binding proteins: a study of the binding interface using B-domain analogues, Biochemistry 38 (1999) 15863-15870.

7. P.J. Smith, E.L. Spurrell, J. Coakley, C.J. Hinds, R.J. Ross, A.R. Krainer, S.L. Chew, An exonic splicing enhancer in human IGF-I pre-mRNA mediates recognition of alternative exon 5 by the serine-arginine protein splicing factor-2/alternative splicing factor, Endocrinology 143 (2002) 146-154.

8. H. Vashisth, C.F. Abrams, All-atom structural models for complexes of insulin-like growth factors IGF1 and IGF2 with their cognate receptor, J. Mol. Biol. 400 (2010) 645-658.

9. T.E. Adams, V.C. Epa, T.P. Garrett, C.W. Ward, Structure and function of the type 1 insulin-like growth factor receptor, Cell Mol. Life Sci. 57 (2000) 1050-1093.

10. W.H. Daughaday, P. Rotwein, Insulin-like growth factors I and II. Peptide, messenger

ribonucleic acid and gene structures, serum, and tissue concentrations, Endocr. Rev. 10 (1989) 68-91.

11. Y.R. Boisclair, R.P. Rhoads, I. Ueki, J. Wang, G.T. Ooi, The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system, J. Endocrinol. 170 (2001) 63-70.

12. H.M. Domene, S.V. Bengolea, H.G. Jasper, Y.R. Boisclair, Acid-labile subunit deficiency: phenotypic similarities and differences between human and mouse, J. Endocrinol. Invest 28 (2005) 43-46.

13. V. Hwa, G. Haeusler, K.L. Pratt, B.M. Little, H. Frisch, D. Koller, R.G. Rosenfeld, Total absence of functional acid labile subunit, resulting in severe insulin-like growth factor deficiency and moderate growth failure, J. Clin. Endocrinol. Metab. 91 (2006) 1826-1831.

14. J.B. Janosi, P.A. Ramsland, M.R. Mott, S.M. Firth, R.C. Baxter, P.J. Delhanty, The acid-labile subunit of the serum insulin-like growth factor-binding protein complexes. Structural determination by molecular modeling and electron microscopy, J. Biol. Chem. 274 (1999) 23328-23332.

15. H.M. Domene, A.S. Martinez, J. Frystyk, S.V. Bengolea, M.G. Ropelato, P.A. Scaglia, J.W. Chen, C. Heuck, O.D. Wolthers, J.J. Heinrich, H.G. Jasper, Normal growth spurt and final height despite low levels of all forms of circulating insulin-like growth factor-I in a patient with acid-labile subunit deficiency, Horm. Res. 67 (2007) 243-249.

16. J. Baker, J.P. Liu, E.J. Robertson, A. Efstratiadis, Role of insulin-like growth factors in embryonic and postnatal growth, Cell 75 (1993) 73-82.

17. J.P. Liu, J. Baker, A.S. Perkins, E.J. Robertson, A. Efstratiadis, Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r), Cell 75 (1993) 59-72.

18. S. Elis, H.W. Courtland, Y. Wu, C.J. Rosen, H. Sun, K.J. Jepsen, R.J. Majeska, S. Yakar, Elevated serum levels of IGF-1 are sufficient to establish normal body size and skeletal properties even in the absence of tissue IGF-1, J. Bone Miner. Res. 25 (2010) 1257-1266.

19. Y. Wu, H. Sun, S. Yakar, D. LeRoith, Elevated levels of insulin-like growth factor (IGF)-I in

serum rescue the severe growth retardation of IGF-I null mice, Endocrinology 150 (2009) 4395-4403.

20. S. Yakar, H.W. Courtland, D. Clemmons, IGF-1 and bone: New discoveries from mouse models, J. Bone Miner. Res. 25 (2010) 2267-2276.

21. K.A. Woods, C. Camacho-Hubner, M.O. Savage, A.J. Clark, Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene, N. Engl. J. Med. 335 (1996) 1363-1367.

22. M.J. Walenkamp, M. Karperien, A.M. Pereira, Y. Hilhorst-Hofstee, J. van Doorn, J.W. Chen, S. Mohan, A. Denley, B. Forbes, H.A. van Duyvenvoorde, S.W. van Thiel, C.A. Sluimers, J.J. Bax, J.A. de Laat, M.B. Breuning, J.A. Romijn, J.M. Wit, Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation, J. Clin. Endocrinol. Metab. 90 (2005) 2855-2864.

23. I. Netchine, S. Azzi, M. Houang, D. Seurin, L. Perin, J.M. Ricort, C. Daubas, C. Legay, J. Mester, R. Herich, F. Godeau, Y. Le Bouc, Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development, J. Clin. Endocrinol. Metab. 94 (2009) 3913-3921.

24. G. Bonapace, D. Concolino, S. Formicola, P. Strisciuglio, A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency, J. Med. Genet. 40 (2003) 913-917.

25. D.C. Coutinho, R.R. Coletta, E.M. Costa, P.R. Pachi, M.C. Boguszewski, D. Damiani, B.B. Mendonca, I.J. Arnhold, A.A. Jorge, Polymorphisms identified in the upstream core polyadenylation signal of IGF1 gene exon 6 do not cause pre- and postnatal growth impairment, J. Clin. Endocrinol. Metab. 92 (2007) 4889-4892.

26. H.A. van Duyvenvoorde, P.A. van Setten, M.J. Walenkamp, J. van Doorn, J. Koenig, L. Gauguin, W. Oostdijk, C.A. Ruivenkamp, M. Losekoot, J.D. Wade, P. de Meyts, M. Karperien, C. Noordam, J.M. Wit, Short Stature Associated with a Novel Heterozygous Mutation in the Insulin-Like Growth Factor 1 Gene, J. Clin. Endocrinol. Metab. (2010) E363-E367.

27. R. Niewenweg, M.L. Smit, M.J. Walenkamp, J.M. Wit, Adult height corrected for shrinking and secular trend, Ann. Hum. Biol. 30 (2003) 563-569.

28. R.A. Bathgate, F. Lin, N.F. Hanson, L. Otvos Jr., A. Guidolin, C. Giannakis, S. Bastiras, S.L. Layfield, T. Ferraro, S. Ma, C. Zhao, A.L. Gundlach, C.S. Samuel, G.W. Tregear, J.D. Wade, Relaxin-3: improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo, Biochemistry 45 (2006) 1043-1053.

29. M.A. Hossain, R.A. Bathgate, C.K. Kong, F. Shabanpoor, S. Zhang, L.M. Haugaard-Jonsson, K.J. Rosengren, G.W. Tregear, J.D. Wade, Synthesis, conformation, and activity of human insulin-like peptide 5 (INSL5), Chembiochem. 9 (2008) 1816-1822.

30. H.A. van Duyvenvoorde, M.J. Kempers, T.B. Twickler, J. van Doorn, W.J. Gerver, C. Noordam, M. Losekoot, M. Karperien, J.M. Wit, A.R. Hermus, Homozygous and heterozygous expression of a novel mutation of the acid-labile subunit, Eur. J. Endocrinol. 159 (2008) 113-120.

31. B. Rikken, J. van Doorn, A. Ringeling, J.L. Van den Brande, G. Massa, J.M. Wit, 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. 50 (1998) 166-176.

32. J.W. Chen, T. Ledet, H. Orskov, N. Jessen, S. Lund, J. Whittaker, P. de Meyts, M.B. Larsen, J.S. Christiansen, J. Frystyk, A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum, Am. J. Physiol Endocrinol. Metab, 284 (2003) E1149-E1155.

33. P. de Meyts, J. Roth, D.M. Neville Jr., J.R. Gavin III, M.A. Lesniak, Insulin interactions with its receptors: experimental evidence for negative cooperativity, Biochem. Biophys. Res. Commun. 55 (1973) 154-161.

34. C.T. Christoffersen, K.E. Bornfeldt, C.M. Rotella, N. Gonzales, H. Vissing, R.M. Shymko, J. ten Hoeve, J. Groffen, N. Heisterkamp, P. de Meyts, Negative cooperativity in the insulin-like growth factor-I receptor and a chimeric IGF-I/insulin receptor, Endocrinology 135 (1994) 472-475.

35. C. Bonnesen, G.M. Nelander, B.F. Hansen, P. Jensen, J.S. Krabbe, M.B. Jensen, A.C. Hegelund, J.E. Svendsen, M.B. Oleksiewicz, Synchronization in G0/G1 enhances the mitogenic response of cells overexpressing the human insulin receptor A isoform to insulin, Cell Biol. Toxicol. 26 (2010) 293-307.

36. A. Denley, C.C. Wang, K.A. McNeil, M.J. Walenkamp, H. van Duyvenvoorde, J.M. Wit, J.C. Wallace, R.S. Norton, M. Karperien, B.E. Forbes, Structural and functional characteristics of

the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development, Mol. Endocrinol. 19 (2005) 711-721.

37. V.V. Kiselyov, S. Versteyhe, L. Gauguin, P. de Meyts, Harmonic oscillator model of the insulin and IGF1 receptors' allosteric binding and activation, Mol. Syst. Biol. 5 (2009) 243.

38. M.J. Walenkamp, H.J. van der Kamp, A.M. Pereira, S.G. Kant, H.A. van Duyvenvoorde, M.F. Kruithof, M.H. Breuning, J.A. Romijn, M. Karperien, J.M. Wit, A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the insulin-like growth factor I receptor, J. Clin. Endocrinol. Metab. 91 (2006) 3062-3070.

39. H. Dalcik, M. Yardimoglu, B. Vural, C. Dalcik, S. Filiz, S. Gonca, S. Kokturk, S. Ceylan, Expression of insulin-like growth factor in the placenta of intrauterine growth-retarded human fetuses, Acta Histochem. 103 (2001) 195-207.

40. G. Iniguez, C.A. Gonzalez, F. Argandona, E. Kakarieka, M.C. Johnson, F. Cassorla, Expression and protein content of IGF-I and IGF-I receptor in placentas from small, adequate and large for gestational age newborns, Horm. Res. Paediatr. 73 (2010) 320-327.

41. S. Sheikh, P. Satoskar, D. Bhartiya, Expression of insulin-like growth factor-I and placental growth hormone mRNA in placentae: a comparison between normal and intrauterine growth retardation pregnancies, Mol. Hum. Reprod. 7 (2001) 287-292.

42. M.H. Lee, Y.J. Jeon, S.M. Lee, M.H. Park, S.C. Jung, Y.J. Kim, Placental gene expression is related to glucose metabolism and fetal cord blood levels of insulin and insulin-like growth factors in intrauterine growth restriction, Early Hum. Dev. 86 (2010) 45-50.

4

Homozygous and heterozygous expression of a novel mutation of the acid-labile subunit

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Abstract

Context: Acid-labile subunit (ALS) deficiency due to homozygous inactivation of the *ALS* gene (*IGFALS*) is associated with moderate short stature, and in few cases pubertal delay. The clinical expression of heterozygosity is unknown.

Objective: To investigate the clinical, laboratory, and radiological features of homozygous and heterozygous carriers of a novel mutation in the *ALS* gene in comparison with non-carriers.

Subjects: Three short Kurdish brothers and their relatives.

Results: The index cases presented with short stature, microcephaly, and low circulating IGF-I and IGF-binding protein-3 (IGFBP-3), and undetectable ALS levels. Two were known with a low bone mineral density and one of them had suffered from two fractures. We found a novel homozygous *ALS* gene mutation resulting in a premature stopcodon (c.1490dupT, p.Leu497PhefsX40). The IGF-I, IGFBP-3, and ALS 150 kDa ternary complex was absent, and ALS proteins in serum were not detected with western blot. IGFPB-1 and IGFBP-2 were low and there was a mild insulin resistance. Five heterozygous carriers tended to have a lower height and head circumference than five non-carriers, and had low plasma ALS and IGFBP-3 levels. Bone mineral (apparent) density was low in two out of three homozygous carriers, but also in four out of nine relatives.

Conclusions: The clinical presentation of homozygous *ALS* mutations may, besides short stature, include microcephaly. Heterozygous carriers may have less statural and head growth, suggestive for a gene dosage effect.

Introduction

For both humans and rodents, it has been well established that insulin-like growth factor-I (IGF-I) plays a critical role in the regulation of intrauterine growth and development and postnatal growth and metabolism (1, 2). Total IGF-I deficiency leads to severe intrauterine and postnatal growth failure, microcephaly, mental retardation, and deafness (3, 4). Partial IGF-I resistance due to heterozygous mutations in the IGF-I receptor (*IGF1R*) gene results in moderate intrauterine and postnatal growth retardation and microcephaly (5-8).

A third defect that is associated with a reduced bioavailability of IGF-I is caused by a mutation in the acid-labile subunit gene (*ALS*). ALS can form, together with IGF-I (or IGF-II) and IGF-binding protein-3 (IGFBP-3, or IGFBP-5 for only 10%), a 150 kDa ternary complex. Under normal circumstances, 80–85% of circulating IGF-I is retained in this complex (9). The 150 kDa complexes cannot cross the capillary endothelial barrier, which prolongs the half-life of IGFs, IGFBP-3 and IGFBP-5, in the circulation (9-11). This seems to play an important role in the regulation of the bioavailability of IGFs to the tissue compartments.

ALS belongs to the superfamily of leucine-rich repeat (LRR) proteins, characterized by their ability to participate in protein–protein interactions. About 75% of the mature protein consists of the consensus motif for the LRR superfamily of proteins. These leucine-rich domains organize ALS into a doughnut-shaped structure (11, 12). Whereas ALS readily binds to binary complexes of IGFs and IGFBP-3, it does not interact directly with free IGFs and shows only a very low affinity for unliganded IGFBP-3 (13, 14).

So far, eight patients have been described with mutations in the *ALS* gene (10, 15-18). All cases described were characterized by moderate or mild growth failure, undetectable or very low circulating levels of ALS, and markedly reduced plasma concentrations of IGFBP-3 and IGF-I. In some cases, pubertal delay was noted, and a low bone mineral density (BMD) was found in one case (19).

In this report, we describe three brothers of Kurdish origin with short stature and microcephaly associated with a novel homozygous mutation in the *ALS* gene. In addition, we have evaluated the clinical and laboratory findings in ten relatives, five of whom were heterozygous for the mutation and five non-carriers.

Methods

All subjects described in this paper belong to a consanguineous family of Kurdish origin (Fig. 1). After mutation analysis, the subjects were divided into 'patients' (homozygous carriers), heterozygous carriers, and non-carriers. For each homozygous carrier, the medical history

as well as the results of previous investigations was retrieved. In 2007, all the subjects were again thoroughly investigated. All the subjects provided written informed consent.

Molecular studies

Genomic DNA was isolated from whole blood or buffy coats according to the salting out procedure described by Miller *et al.* (20). All exons of *ALS* (GenBank accession number AF192554) were PCR amplified and sequenced as described previously (4). The various primer sequences employed are available upon request.
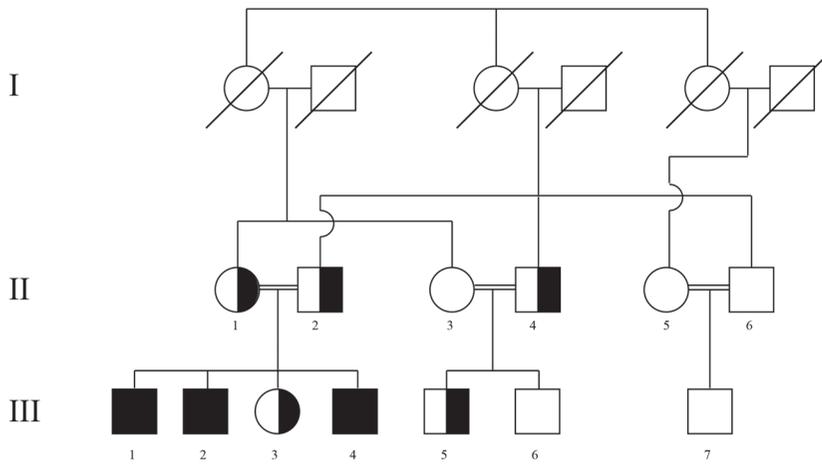
Figure 1

Pedigree of the family. The patients are indicated in black and the heterozygous carriers are indicated in white/black. On the left, the generation number is indicated and the family number is indicated below each individual.

Clinical measurements and auxology

Physical examination was performed including measurements of height and sitting height with a Harpenden stadiometer and head circumference with a tape measure. Height was expressed as standard deviation score (SDS) based on references for Turkish children living in the Netherlands (21). Head circumference and sitting height/height ratio were expressed as SDS for the Dutch population (22, 23). Differences between homozygous, heterozygous, and non-carriers were calculated with ANOVA.

Radiological measurements

BMD was measured by dual energy X-ray absorptiometry using the Hologic Scanner (QDR 4500W) at the lumbar spine, femoral neck, and total body. The volumetric bone mineral apparent density (BMAD) was calculated using the formula: BMAD (g/cm³) = BMD×(4/π×width of measurement area in lumbar spine) (24). An estimate of the mean width was derived from the scanned area (cm²) divided by the height of L2–L4. z-Scores were calculated using the reference data as reported by Fournier *et al.* (25). Subjects above the age of 20 years were compared with the reference data of subjects aged 18–20 years. The z-score of the subjects below the age of 9 years could not be calculated because there were no appropriate references.

Biochemical measurements

Plasma levels of IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-6 were determined as described previously (4), and ALS was measured by a commercially available ELISA kit (DSL, Inc., Webster, TX, USA) (26). All measurements were expressed as SDS, except for IGFBP-1 in adults older than 24 years, which was compared with a reference group of six healthy adult controls. 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. Prior to column chromatography, each serum sample (250 µl) was incubated with 100 µl of either ~80 000 c.p.m. ^{125}I-hIGF-I or ^{125}I-IGF-II, being dissolved in 50 mM sodium phosphate buffer (pH 7.4), containing 0.2% BSA, 10 mM EDTA, and 0.05% (w/v) Tween-20, for 17 h at 4°C. The various molecular size classes of complexes were eluted from the column at a rate of 1.2 ml/min using 0.05 M NH_4HCO_3 buffer (pH 7.4). The ^{125}I content of each 1.2 ml fraction was counted in a γ-counter. The hIGFBP-3 used in several of these experiments was isolated from human plasma according to Martin and Baxter (27). Western blotting on serum samples was performed as described previously (28), using an antibody against human amino-terminal ALS_{1-34} (DSL, Inc).

Results

Medical history of the ALS-deficient patients

Patient III-1 was born as the first of four children of consanguineous parents. In the first three years, he was short, frequently ill, had many episodes of diarrhea, and showed poor appetite and night sweating. At the age of 11.6 years, he moved with his family to the Netherlands. At the age of 12 and 14 years, he suffered fractures of the left wrist and arm, after minor trauma, and at radiographs, bone density appeared low. Reported pubertal onset was at about 14 years of age (mean age at Tanner stage 2 in Turkish adolescents living

in the Netherlands is 12.2 years) (21). He had a somewhat dysmorphic face with mandibular hypoplasia and a prominent forehead. The growth curve is shown in Fig. 2A.

At the age of 16.1 years, dietary calcium and vitamin D intake appeared low, and serum vitamin D25 was slightly decreased, but serum calcium was in the upper normal range and parathyroid hormone (PTH) was normal. Vitamin D p.o. for 6 months resulted in normalization of D25 levels. A grade I mitral and tricuspid insufficiency was detected at cardiologic investigation. The growth hormone (GH) peak after exercise was high (63 mU/l), IGF-I was very low (–6.9 to –5.0 SDS), and IGFBP-3 extremely low (–12.0 SDS). There was a poor response to GH in an IGF-I generation test with two different doses (0.8 and 1.6 mg/m^2 per day for 4 days); only on the higher dose IGF-I increased from 16 to 60 ng/ml. IGFBP-3 did not change.

At the age of 17.8 years, his bone age was adult, and he had reached a final height of 149.7 cm (–4.2 SDS) (Fig. 2A), indicating a fast progression through puberty, which probably led to the poor pubertal height gain (13.3 cm). At the age of 16, 17, and 19 years, total BMD and z-scores were 0.81 g/cm^2 (–3.3 SDS), 0.89 g/cm^2 (–3.9 SDS), and 0.91 g/cm^2 (–2.5 SDS) respectively, and femoral neck BMD was 0.827 (–1.5 SDS) at 19 years of age.

Patient III-2 moved with his family to the Netherlands at the age of 5 years. At 7 years of age, he was seen by an ophthalmologist because of strabismus. His growth chart is shown in Fig. 2B. Data on reported pubertal onset and progression are inconsistent.

Patient III-4 had a reported birth weight of 2.0 kg at an unknown gestational age. He remained short in childhood and adolescence (Fig. 2C), but bone maturation was not retarded. During infancy, he experienced poor appetite, frequent episodes of diarrhea, and night sweating. His left leg was ~5 cm shorter than the right leg, probably due to congenital coxa vara by congenital femoral hypoplasia. After an osteotomy, the difference was reduced to 3 cm. He had a similar dysmorphic face as patient III-1. Puberty onset was reported to have occurred between 12 and 14 years. Total BMD at 6, 10, and 12 years of age was 0.68 (–1.3 SDS), 0.76 (–1.2 SDS), and 0.62 (–2.0 SDS) respectively. The GH peak after exercise was high (118 mU/l), IGF-I was very low (–4.2 to –3.8 SDS), and IGFBP-3 extremely low (< –17 SDS). During IGF-I generation tests, employing GH doses 0.8 and 1.6 mg/m^2 per day for 4 days, IGF-I levels increased only marginally, from 38 to 46 ng/ml and 44 to 52 ng/ml respectively. DNA analysis for GH and GHR was normal.
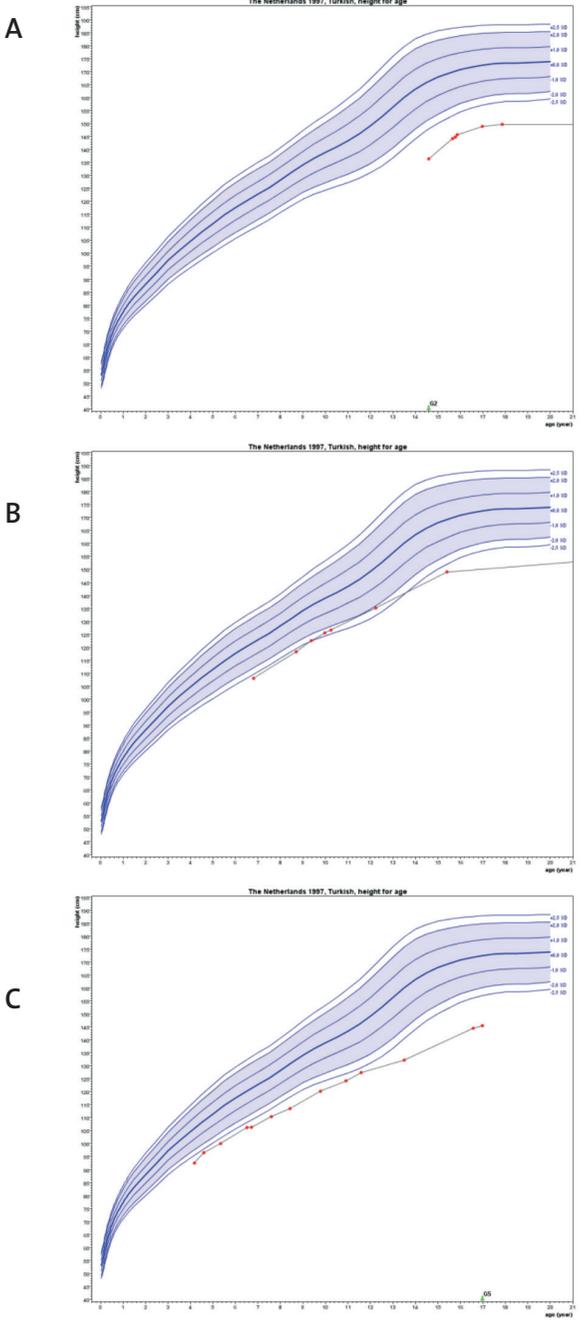
Figure 2
(A–C) Growth curves of patients III-1, III-2, and III-4 (index cases). Target height is 162.0
cm (–2.1 SDS). Dots represent height measurements, and G2 and G5 indicate Tanner
stages 2 and 5.

Molecular studies

Sequence analysis of the *ALS* gene of patients III-1, III-2, and III-4 revealed a homozygous duplication of a T nucleotide at position 1490 of the coding DNA, resulting in a frameshift and a premature stopcodon (c.1490dupT, p.Leu497PhefsX40; further indicated as −/−) (Supplemental Fig. 1). Five relatives were heterozygous carriers of the mutation (+/−) and the other five non-carriers (+/+) (Fig. 1).

Clinical, radiological, and biochemical features at recall

Clinical, radiological, and laboratory findings are summarized in Tables 1–3. Mean height and head circumference SDS of heterozygotes were 1 and 0.7 SD lower respectively than those of non-carriers, but the differences with non-carriers did not reach statistical significance (P = 0.25 and 0.13 respectively). The sitting height/height ratio was in the upper normal range in most cases, without apparent association with the *ALS* defect. Two homozygous patients exhibited a low BMD and one of them also a low BMAD, but also in four out of nine relatives low values were observed. Tone audiometry did not show a clinically relevant perceptive hearing loss in the patients or their relatives (data not shown).

In the three patients, ALS was not detected by ELISA, which was confirmed by western blotting of patient's sera (Supplemental Fig. 2). No ALS bands with aberrant molecular weights were detected. Fasting glucose levels were normal (5.3, 4.5, and 4.6 mmol/l), but insulin was slightly elevated (12, 17, and 14 mU/l), indicating mild insulin resistance. Plasma levels of IGFBP-3 were extremely low, and of IGF-I, IGF-II, IGFBP-1, and IGFBP-2 markedly reduced. Plasma IGFBP-4 and IGFBP-6, and serum testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and free thyroxine (FT4) levels were normal. In heterozygous carriers, plasma ALS and IGFBP-3 were low. In non-carriers, all parameters were normal.

Neutral gel filtration

While in normal control serum, most IGF-I (90%) is associated with the 150 kDa complex (Fig. 3A); in the sera of the three ALS-deficient patients there was almost no 150 kDa complex present. The 40–50 kDa fraction (consisting of binary IGF–IGFBP complexes) was markedly elevated and the free ^{125}I-IGF-I peak was reduced (Fig. 3B). Addition of purified hIGFBP-3 to these sera did not induce 150 kDa complex formation, while a slightly increased 150 kDa peak was observed upon addition to normal serum (data not shown). Similar results were obtained with ^{125}I-IGF-II (data not shown). In the sera of heterozygous carriers, the amount of the 150 kDa complexes was reduced and that of the 40–50 kDa complexes markedly increased (Fig. 3C). Column profiles of sera of the non-carriers were normal.

Table 1 Clinical features and biochemical characteristics of the homozygous carriers

	III-1	III-2	III-4	Mean
IGFALS gene	–/–	–/–	–/–	
Gender	M	M	M	
Age (year)	27.2	21.5	17.0	21.9
Height (cm) [SDS]	149.7 [–4.2]	153.2 [–3.6]	145.5 [–4.4]	149.5 [–4.1]
Head circumference (cm) [SDS]	52.5 [–3.0]	53.3 [–2.6]	51.2 [–3.2]	52.3 [–2.9]
BMI (kg/m²)	21.2	23.4	21.6	22.1
Sitting height: Height [SDS]	0.54 [2.2]	0.54 [2.0]	0.53 [1.1]	0.54 [1.8]
Total BMD (g/cm²) [z-score]	0.865	1.058	0.793 [–3.3]	0.905
BMD femoral neck [z-score]	0.591 [–2.4]	0.943 [0.1]	0.640 [–2.4]	0.725 [–1.6]
BMD L1–L4 [z-score]	0.625 [–4.2]	0.902 [–1.7]	0.590 [–3.1]	0.706 [–3.0]
BMAD L2–L4 [z-score]	0.209 [–2.8]	0.284 [–0.2]	0.230 [–1.7]	0.241 [–1.6]
IGF-I (ng/ml) [SDS]	<12 [< –7.2]	13 [–7.4]	29 [–5.6]	18 [–6.7]
IGF-II (ng/ml) [SDS]	66 [–6.6]	55 [–6.8]	49 [–6.7]	56.7 [–6.7]
IGFBP-1 (ng/ml) [SDS] ᵅ	27	12 [–1.5]	9 [–2.2]	16
IGFBP-2 (ng/ml) [SDS]	67 [–2.5]	95 [–1.8]	69 [–3.0]	77 [–2.4]
IGFBP-3 (mg/l) [SDS]	0.09 [–18.2]	0.09 [–18.5]	0.10 [–17.6]	0.10 [–18.1]
IGFBP-4 (ng/ml) [SDS]	172 [–0.1]	136 [–0.7]	157 [–0.3]	155 [–0.4]
IGFBP-6 (ng/ml) [SDS]	180 [0.4]	138 [–0.3]	148 [0.2]	155.3 [0.1]
ALS (mg/l) [SDS]	<0.07 [< –7.1]	<0.07 [< –5.7]	<0.07 [< –4.5]	<0.07 [< –4.5]

ᵅ Normal range for non-fasting subjects: 24–57 ng IGFBP-1 per ml. After overnight fasting, there is an average fivefold rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 years.

Table 2 Clinical features and biochemical characteristics of the heterozygous carriers

	II-1	II-2	II-4	III-3	III-5	Mean
IGFALS gene	+/−	+/−	+/−	+/−	+/−	
Gender	F	M	M	F	M	
Age (year)	46.1	52.2	41.6	20.1	7.4	33.5
Height (cm) [SDS]	141.7 [−3.4]	155.5 [−3.2]	154.8 [−3.3]	149.8 [−2.0]	126.3 [0.3]	145.6 [−2.3]
Head circumference (cm) [SDS]	53.8 [−0.9]	54.0 [−2.2]	55.0 [−1.6]	52.5 [−1.7]	51.6 [−0.4]	53.4 [−1.4]
BMI (kg/m^2)	27.2	24.3	27.0	24.4	14.7	23.5
Sitting height: Height [SDS]	0.53 [0.4]	0.54 [2.1]	0.53 [1.2]	0.53 [0.6]	0.53 [−0.4]	0.53 [0.8]
Total BMD (g/cm^2) [z-score]	0.974 [−0.9]	0.869	0.986	0.911 [−2.2]	0.751 [0.4]	0.898 [−0.9]
BMD femoral neck [z-score]	0.721 [−0.7]	0.648 [−1.3]	0.855 [0.0]	0.811 [−0.3]	0.732 [1.4]	0.753 [−0.2]
BMD L1–L4 [z-score]	0.805 [−1.7]	0.618 [−3.9]	0.692 [−3.5]	0.865 [−1.4]	0.577 [0.7]	0.711 [−2.0]
BMAD L2–L4 [z-score]	0.307 [−0.4]	0.164 [−4.4]	0.184 [−3.7]	0.308 [−0.4]	0.169	0.226 [−2.2]
IGF-I (ng/ml) [SDS]	99 [−1.1]	73 [−1.5]	86 [−1.6]	141 [−1.8]	73 [−1.4]	94 [−1.5]
IGF-II (ng/ml) [SDS]	352 [−1.0]	305 [−1.8]	305 [−1.7]	308 [−1.5]	296 [−1.1]	313.2 [−1.4]
IGFBP-1 (ng/ml) SDS] [a]	11	31	5	19 [−1.7]	164 [0.1]	46
IGFBP-2 (ng/ml) [SDS]	184 [0.1]	196 [0.3]	255 [0.9]	278 [1.5]	360 [1.1]	255 [0.8]
IGFBP-3 (mg/l) [SDS]	1.54 [−1.2]	0.93 [−2.9]	0.94 [−3.3]	1.19 [−3.2]	1.05 [−2.9]	1.13 [−2.7]
IGFBP-4 (ng/ml) [SDS]	196 [0.3]	117 [−1.1]	139 [−0.7]	139 [−0.8]	113 [−1.2]	141 [−0.7]
IGFBP-6 (ng/ml) [SDS]	185 [0.8]	176 [−0.3]	159 [−0.3]	147 [0.1]	112 [0.3]	156 [0.1]
ALS (mg/l) [SDS]	9.9 [−2.6]	10.5 [−1.9]	6.0 [−3.6]	9.0 [−3.0]	6.9 [−2.0]	8.5 [−2.6]

[a] Normal range for non-fasting subjects: 24–57 ng IGFBP-1 per ml. After overnight fasting, there is an average fivefold
 rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 years.

Table 3 Clinical features and biochemical characteristics of the non-carriers

	II-3	II-5	II-6	III-6	III-7	Mean
IGFALS gene	+/+	+/+	+/+	+/+	+/+	
Gender	F	F	M	M	M	
Age (year)	34.3	32.7	37.6	5.6	5.6	23.2
Height (cm) [SDS]	151.3 [−1.7]	160.6 [0.0]	156.6 [−3.0]	114.5 [−0.1]	107.0 [−1.8]	138.0 [−1.3]
Head circumference (cm) [SDS]	53.0 [−1.4]	54.0 [−0.8]	56.0 [−1.0]	52.0 [0.2]	50.5 [−0.7]	53.1 [−0.7]
BMI (kg/m²)	24.4	25.4	27.1	14.6	13.8	21.1
Sitting height: Height [SDS]	0.53 [0.4]	0.55 [1.5]	0.55 [2.5]	0.54 [−0.2]	0.54 [−0.3]	0.54 [0.8]
Total BMD (g/cm²) [z-score]	1.037 [−0.5]	0.905 [−2.0]	1.013	0.668 [−0.1]	–	0.906 [−0.9]
BMD femoral neck [z-score]	0.700 [−1.2]	0.683 [−1.4]	0.787 [−0.6]	0.659 [1.6]	–	0.707 [−0.4]
BMD L1–L4 [z-score]	0.903 [−1.3]	0.778 [−2.4]	0.754 [−3.0]	0.434 [−1.3]	–	0.717 [−2.0]
BMAD L2–L4 [z-score]	0.277 [−1.3]	0.239 [−2.4]	0.202 [−3.1]	0.141	–	0.215 [−2.3]
IGF-I (ng/ml) [SDS]	199 [0.8]	164 [0.0]	81 [−1.9]	144 [1.1]	77 [−0.5]	133 [−0.1]
IGF-II (ng/ml) [SDS]	436 [0.1]	543 [1.2]	441 [0.1]	546 [1.5]	302 [−0.9]	454 [0.4]
IGFBP-1 (ng/ml) [SDS] [α]	<5	20	78	125 [−1.0]	81 [−2.0]	62
IGFBP-2 (ng/ml) [SDS]	237 [0.7]	156 [−0.2]	336 [1.5]	242 [−0.5]	426 [1.5]	279 [0.6]
IGFBP-3 (mg/l) [SDS]	1.94 [−0.6]	2.0 [−0.6]	1.6 [−1.2]	2.52 [0.9]	1.66 [−0.7]	1.9 [−0.4]
IGFBP-4 (ng/ml) [SDS]	79 [−2.0]	91 [−1.8]	133 [−0.8]	126 [−0.9]	105 [−1.3]	107 [−1.4]
IGFBP-6 (ng/ml) [SDS]	125 [−0.3]	159 [0.5]	187 [0.4]	130 [1.2]	75.2 [−0.7]	135.2 [0.2]
ALS (mg/l) [SDS]	14.5 [−1.5]	–	–	16.1 [1.7]	_	15.3 [0.1]

To convert IGF-I to nanomoles per Liter (nmol/l), multiply by 0.131; to convert IGF-II to nmol/l, multiply by 0.134; to convert ALS to nmol/l, multiply by 15.8; to convert IGFBP-1 to nmol/l, multiply by 0.033; to convert IGFBP-2 to nmol/l, multiply by 0.032; to convert IGFBP-3 to nmol/l, multiply by 33.3; to convert IGFBP-4 to nmol/l, multiply by 0.038; to convert IGFBP-6 to nmol/l, multiply by 0.034.
[α] Normal range for non-fasting subjects: 24–57 ng IGFBP-1 per ml. After overnight fasting, there is an average fivefold rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 years.
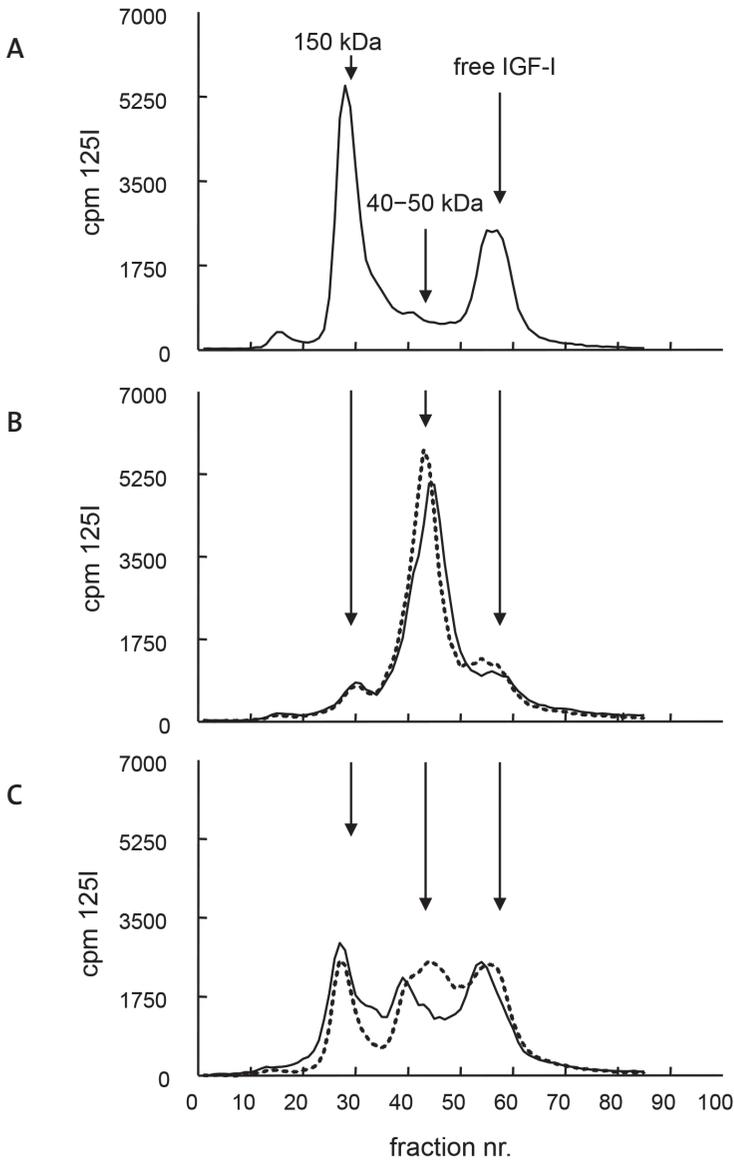
Figure 3
S200 gel filtration column chromatography. (A) Representative column profile for normal adult serum. (B) Serum of patient III-1 (solid line) and patient III-2 (dotted line), both exhibiting a homozygous mutation in the *ALS* gene. (C) Serum of the father II-2 (solid line) and uncle (II-4) (dotted line) of the index cases, both being heterozygous for the mutation.

Discussion

We report three brothers of Kurdish origin, born from a consanguineous family, with a novel homozygous frameshift mutation in the *ALS* gene, presenting with short stature and microcephaly. In two cases a low BMD was observed, in one of them twice resulting in a fracture; but further studies of BMD in their relatives suggest that other genetic factors may be involved. Biochemically, serum ALS was undetectable; IGFBP-3 extremely low; IGFs, IGFBP-2, and IGFBP-1 decreased; and the ternary 150 kDa complex was virtually absent. Most of the circulating IGFs were either sequestered by IGFBPs forming binary complexes or remained unbound. The western blotting experiments indicate that there is no truncated form of ALS present. Even if this were the case, a truncated ALS would not be able to form the stable 3D doughnut-shaped structure, which is essential for its function.

The growth pattern of all previously reported cases of ALS deficiency (10, 15, 16, 18) is characterized by moderate or mild short stature during childhood and adolescence and a normal or slightly decreased adult stature. Our ALS-deficient patients are much shorter in comparison with the reference population, but this may be due to other genetic factors in this highly consanguineous family. The difference between their mean (near) adult height SDS (–4.1 SDS) and that of the five heterozygous carriers (–2.3 SDS) is 1.7 SD, comparable with earlier reports. Data on head circumference have not been presented in earlier reports; thus it remains to be established whether microcephaly represents a clinical feature of homozygous ALS deficiency.

The growth pattern in humans with ALS deficiency is comparable with the mild growth failure observed in *Als* gene knock-out (ALS-KO) mice, and is in contrast with the severe growth failure as observed in patients with a total IGF-I deficiency (2). This is in line with the hypothesis that the contribution of locally produced IGF-I to longitudinal growth is more important than that of IGF-I derived from the circulation (10, 17). As speculated previously, local production of IGF-I may be even higher than normal due to increased GH secretion, and might thereby compensate, at least in part, for any deficiency of circulating IGF-I (10). Another possible explanation of the near-normal growth, in spite of reduced levels of circulating IGF-I, may include an increase in the IGF-I flux toward the peripheral tissue compartments at the expense of the circulating IGF-I pool that cannot be maintained at normal levels without ALS (17, 29). It is uncertain whether ALS deficiency affects fetal growth.

At present, it is uncertain if an *ALS* defect is associated with pubertal delay. Three patients described previously seem to have experienced late onset of puberty (17, 18), but in the other cases puberty was normal. In our index patient III-1, pubertal onset was late, in III-2 unknown, and in III-4 normal. The low BMD in two of our patients and in the first

published case (19), in combination with the low BMD in ALS-KO mice (30-32) and previous studies showing that circulating IGF-I levels are related to sufficient bone growth and acquisition of peak bone mass (33, 34), suggested that ALS deficiency might be associated with a low BMD. However, further observations in the patients' relatives did not support this.

Biochemically, in all cases reported so far, undetectable or very low levels of circulating ALS, extremely low IGFBP-3, and low IGF-I and IGF-II have been reported. The lack of 150 kDa complex formation is expected to shorten the half-life of IGF-I in the circulation (31), and possibly also that of IGFBP-3 (10). The low IGFBP-1 and IGFBP-2 plasma levels observed in two earlier papers (16, 18) and in our three patients suggest that this is part of the syndrome. A consequence of the low plasma IGFBP-1 (an inhibitor of IGF-I effects) may be that growth is less compromised than would be expected from the low circulating IGF-I levels alone.

In the cases tested, we found an increased GH response to provocative stimuli, confirming earlier observations (10, 16, 18). The increased GH secretion can be explained by the reduction in free IGF-I as observed in previous studies (10, 35, 36), but is in contrast to the normal free IGF-I and GH levels in ALS-KO mice (19, 31). An IGF generation test was performed in three previous cases (10, 16, 18), and showed virtually no response of IGF-I, similar to the two cases tested. Our patients had a mild insulin resistance, confirming earlier reports (16, 18).

Heterozygosity for an *ALS* defect may have some effect on stature and head circumference compared with non-carriers, but these differences did not reach statistical significance. Within the heterozygous and wild-type individuals, height and head circumference varied considerably, which may be due to other genes that negatively affect growth in this highly consanguineous family. As in most previous reports, parents of affected patients were relatively short (10, 18); a meta-analysis of all cases may shed more light on this issue. Biochemically, carriers had low plasma ALS and IGFBP-3, and IGF-I in the low normal range, as reported previously (10).

In conclusion, these three cases with a novel frameshift mutation of *ALS* resulting in undetectable circulating ALS levels show that besides short stature and possible delayed onset of puberty, microcephaly may also be part of the syndrome. Biochemically, the absence of circulating ALS leads to extremely low levels of IGFBP-3, very low levels of IGF-I and IGF-II, low plasma IGFBP-1 and IGFBP-2, and mild insulin resistance. Heterozygosity for an ALS defect may have a small effect on height and head circumference.

Reference List

1 Walenkamp MJ & Wit JM. Genetic disorders in the growth hormone - insulin-like growth factor-I axis. *Hormone Research* 2006 **66** 221-230.

2 Walenkamp MJ & Wit JM. Genetic disorders in the GH IGF-I axis in mouse and man. *European Journal of Endocrinology* 2007 **157** S15-S26.

3 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. *New England Journal of Medicine* 1996 **335** 1363-1367.

4 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. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 2855-2864.

5 Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, Kiess W, Klammt J, Kratzsch J, Osgood D, Pfaffle R, Raile K, Seidel B, Smith RJ & Chernausek SD. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. *New England Journal of Medicine* 2003 **349** 2211-2222.

6 Kawashima Y, Kanzaki S, Yang F, Kinoshita T, Hanaki K, Nagaishi J, Ohtsuka Y, Hisatome I, Ninomoya H, Nanba E, Fukushima T & Takahashi S. 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* 2005 **90** 4679-4687.

7 Walenkamp MJ, van der Kamp HJ, Pereira AM, Kant SG, van Duyvenvoorde HA, Kruithof MF, Breuning MH, Romijn JA, Karperien M & Wit JM. 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* 2006 **91** 3062-3070.

8 Inagaki K, Tiulpakov A, Rubtsov P, Sverdlova P, Peterkova V, Yakar S, Terekhov S & LeRoith D. A familial insulin-like growth factor-I receptor mutant leads to short stature: clinical and biochemical characterization. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 1542-1548.

9   Boisclair YR, Rhoads RP, Ueki I, Wang J & Ooi GT. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. *Journal of Endocrinology* 2001 **170** 63-70.

10 Hwa V, Haeusler G, Pratt KL, Little BM, Frisch H, Koller D & Rosenfeld RG. Total absence of functional acid labile subunit, resulting in severe insulin-like growth factor deficiency and moderate growth failure. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 1826-1831.

11 Janosi JB, Ramsland PA, Mott MR, Firth SM, Baxter RC & Delhanty PJ. The acid-labile subunit of the serum insulin-like growth factor-binding protein complexes. Structural determination by molecular modeling and electron microscopy. *Journal of Biological Chemistry* 1999 **274** 23328-23332.

12 Janosi JB, Firth SM, Bond JJ, Baxter RC & Delhanty PJ. N-Linked glycosylation and sialylation of the acid-labile subunit. Role in complex formation with insulin-like growth factor (IGF)-binding protein-3 and the IGFs. *Journal of Biological Chemistry* 1999 **274** 5292-5298.

13 Baxter RC & Martin JL. Structure of the Mr 140 000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. *PNAS* 1989 **86** 6898-6902.

14 Twigg SM & Baxter RC. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *Journal of Biological Chemistry* 1998 **273** 6074-6079.

15 Campos-Barros A, Heath KE, Diaz-González F, Barrios V, Martos GA, Pozo J, Caimari M, Gracia R, Argente J. **IGFALS** mutations define **a new form of impaired postnatal growth associated to low circulating IGF-I, IGFBP-3, and acid-labile subunit (ALS) levels**. *Hormone Research* 2007 **68** 33.

16 Domene HM, Bengolea SV, Martinez AS, Ropelato MG, 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. *New England Journal of Medicine* 2004 **350** 570-577.

17   Domene HM, Martinez AS, Frystyk J, Bengolea SV, Ropelato MG, Scaglia PA, Chen JW, Heuck C, Wolthers OD, Heinrich JJ & Jasper HG. Normal growth spurt and final height despite low levels of all forms of circulating insulin-like growth factor-I in a patient with acid-labile subunit deficiency. *Hormone Research* 2007 **67** 243-249.

18   Domene HM, Scaglia PA, Lteif A, Mahmud FH, Kirmani S, Frystyk J, Bedecarras P, Gutierrez M & Jasper HG. Phenotypic effects of null and haploinsufficiency of acid-labile subunit in a family with two novel IGFALS gene mutations. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 4444-4450.

19   Domene HM, Bengolea SV, Jasper HG & Boisclair YR. Acid-labile subunit deficiency: phenotypic similarities and differences between human and mouse. *Journal of Endocrinological Investigation* 2005 **28** 43-46.

20   Miller SA, Dykes DD & Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 1988 **16** 1215.

21   Fredriks AM, van Buuren S, Jeurissen SE, Dekker FW, Verloove-Vanhorick SP & Wit JM. Height, weight, body mass index and pubertal development reference values for children of Turkish origin in the Netherlands. *European Journal of Pediatrics* 2003 **162** 788-793.

22   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. *Pediatric Research* 2000 **47** 316-323.

23   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. *Archives of Disease in Childhood* 2005 **90** 807-812.

24   Kroger H, Kotaniemi A, Vainio P & Alhava E. Bone densitometry of the spine and femur in children by dual-energy x-ray absorptiometry. *Bone and Mineral* 1992 **17** 75-85.

25   Fournier PE, Rizzoli R, Slosman DO, Theintz G & Bonjour JP. Asynchrony between the rates of standing height gain and bone mass accumulation during puberty. *Osteoporosis International* 1997 **7** 525-532.

26 Yu H, Mistry J, Nicar MJ, Khosravi MJ, Diamandis A, van Doorn J & Juul A. Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *Journal of Clinical Laboratory Analysis* 1999 **13** 166-172.

27 Martin JL & Baxter RC. Insulin-like growth factor-binding protein from human plasma. Purification and characterization. *Journal of Biological Chemistry* 1986 **261** 8754-8760.

28 Ouwens DM, van der Zon GC, Pronk GJ, Bos JL, Moller W, Cheatham B, Kahn CR & Maassen JA. A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and p21ras-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. Evidence for IRS1-independent p21ras-GTP formation. *Journal of Biological Chemistry* 1994 **269** 33116-33122.

29 Payet LD, Firth SM & Baxter RC. The role of the acid-labile subunit in regulating insulin-like growth factor transport across human umbilical vein endothelial cell monolayers. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 2382-2389.

30 Beamer WG, Donahue LR, Rosen CJ & Baylink DJ. Genetic variability in adult bone density among inbred strains of mice. *Bone* 1996 **18** 397-403.

31 Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL, Ooi GT, Setser J, Frystyk J, Boisclair YR & LeRoith D. Circulating levels of IGF-1 directly regulate bone growth and density. *Journal of Clinical Investigation* 2002 **110** 771-781.

32 Hildebrand T, Laib A, Muller R, Dequeker J & Ruegsegger P. Direct three-dimensional morphometric analysis of human cancellous bone: microstructural data from spine, femur, iliac crest, and calcaneus. *Journal of Bone and Mineral Research* 1999 **14** 1167-1174.

33 Langlois JA, Rosen CJ, Visser M, Hannan MT, Harris T, Wilson PW & Kiel DP. Association between insulin-like growth factor I and bone mineral density in older women and men: the Framingham Heart Study. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 4257-4262.

34 Rosen CJ, Dimai HP, Vereault D, Donahue LR, Beamer WG, Farley J, Linkhart S, Linkhart T, Mohan S & Baylink DJ. Circulating and skeletal insulin-like growth factor-I (IGF-I) concentrations in two inbred strains of mice with different bone mineral densities. *Bone* 1997 **21** 217-223.

35 Daughaday WH. Circulating IGF-I deficiency and inactivation of the acid-labile subunit gene. *New England Journal of Medicine* 2004 **350** 1906.

36 Chapman IM, Hartman ML, Pieper KS, Skiles EH, Pezzoli SS, Hintz RL & Thorner MO. Recovery of growth hormone release from suppression by exogenous insulin-like growth factor I (IGF-I): evidence for a suppressive action of free rather than bound IGF-I. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2836-2842.

Supplemental Figures

Normal

Heterozygous

Homozygous
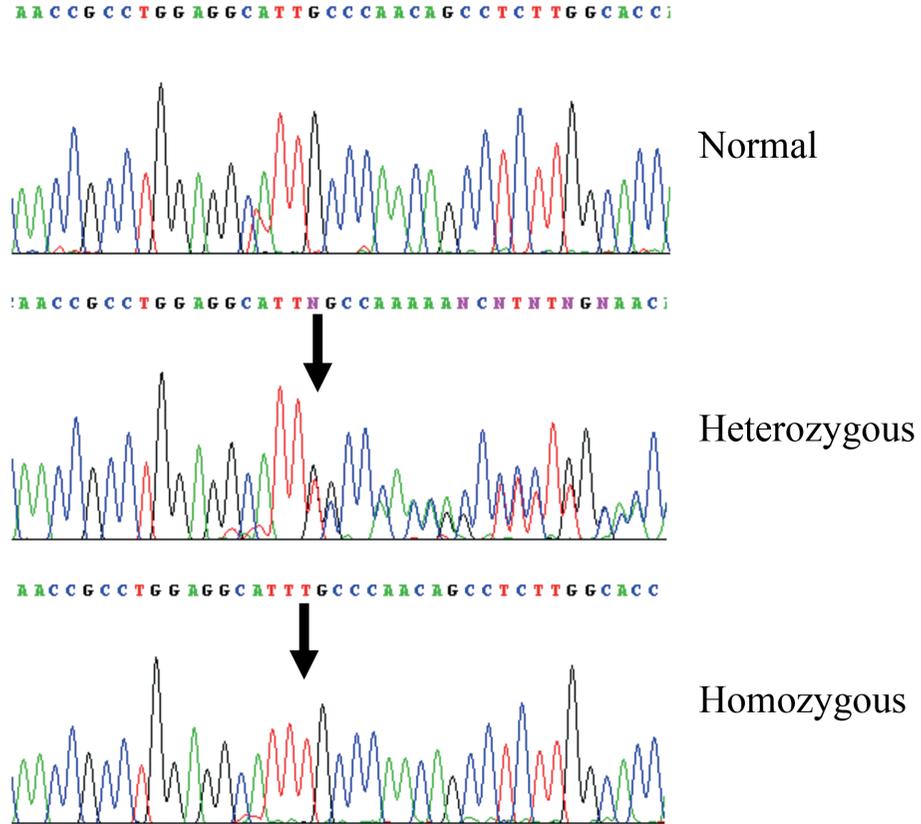
c.1490dupT, p.Leu497PhefsX40

Supplemental Figure 1
DNA sequence chromatogram of genomic *ALS* DNA. The upper panel represents the wild-type *ALS* sequence, the middle panel represents the sequence of heterozygous carriers of the mutation and the lower panel represents the sequence of the three brothers with the homozygous *ALS* mutation. The arrow indicates the duplication of a T nucleotide.

II-2	II-1	III-1	III-2	III-3	III-4	II-4	II-3	III-5	III-6	II-6	II-5	III-7	Co	Co

ALS

| +/− | +/− | −/− | −/− | +/− | −/− | +/− | +/+ | +/− | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ |
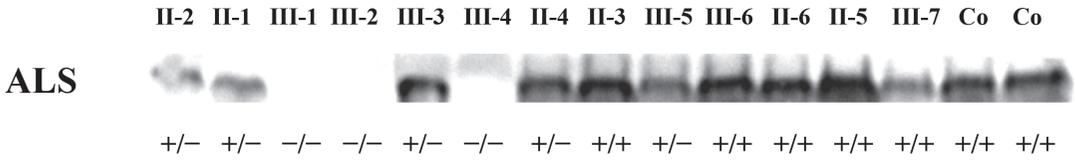
Supplemental Figure 2

Western blot analysis of ALS with an antibody against human amino-terminal ALS_{1-34} in serum samples of the family members and controls. Indicated above the blot is the family number. Below the blot is indicated if the serum sample is from a homozygous (−/−), heterozygous (+/−), or non-carrier (+/+) of the mutation.

chapter
4 S

Part B

**Combined candidate gene
and whole genome approach**

5

Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype

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Abstract

Context: Small for gestational age (SGA)-born children comprise a heterogeneous group in which only few genetic causes have been identified.

Objective: To determine copy number variations in 18 growth-related genes in 100 SGA children with persistent short stature.

Methods: Copy number variations in 18 growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2,* and *SOCS3*) were determined by an "in house" multiplex ligation-dependent probe amplification kit. The deletions were further characterized by single-nucleotide polymorphism array analysis.

Results: Two heterozygous *de novo* insulin-like growth factor 1 receptor (IGF1R) deletions were found: a deletion of the complete *IGF1R* gene (15q26.3, exons 1-21), including distally flanking sequences, and a deletion comprising exons 3-21, extending further into the telomeric region. In one case, serum IGF-I was low (−2.78 SD Score), probably because of a coexisting growth hormone (GH) deficiency. Both children increased their height during GH treatment (1 mg/m^2 per day). Functional studies in skin fibroblast cultures demonstrated similar levels of IGF1R autophosphorylation and a reduced activation of protein kinase B/ Akt upon a challenge with IGF-I in comparison with controls.

Conclusions: *IGF1R* haploinsufficiency was present in 2 of 100 short SGA children. GH therapy resulted in moderate catch-up growth in our patients. A review of the literature shows that small birth size, short stature, small head size, relatively high IGF-I levels, developmental delay, and micrognathia are the main predictors for an *IGF1R* deletion.

Introduction

Children born with a low birth weight and/or birth length corrected for gestational age (small for gestational age, SGA) comprise a heterogeneous group with a broad spectrum of clinical characteristics. Reduced size at birth may result from fetal, maternal, placental, and/or genetic factors. Although many children born SGA achieve sufficient growth to normalize their stature by 2 yr of age, approximately 15% maintain a height below –2 SD score and continue to be short throughout adolescence and adulthood (1). Short SGA children have a reduced lean body mass, fat mass, skinfolds, and body mass index (BMI) (2-4), as well as a lower caloric, fat, and carbohydrate intake (5). SGA children with a persistent short stature and/or a small head size have a higher risk of subnormal intellectual and psychological performance (6-9).

Genetic causes have only been found in a small proportion of short SGA children, including point mutations and deletions in the *IGF1* (10-12) and insulin-like growth factor 1 receptor (*IGF1R*) genes (13-26). The availability of the complete sequence of the human genome and the introduction of high-throughput DNA-scanning techniques provide novel tools to investigate the genetic basis of short stature. In this study, we used multiplex ligation-dependent probe amplification (MLPA) to investigate rapidly whether copy number variations in growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2,* and *SOCS3*) were present in a group of 100 children born SGA with persistent short stature. The extent of the two deletions that were found was determined with single-nucleotide polymorphism (SNP) array analysis. Functional studies on dermal fibroblasts were performed to investigate the IGF1R signal transduction pathway in the two patients and age- and sex-matched healthy controls.

chapter

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Subjects and Methods

Study population

The first hundred short SGA children participating in four prospective cohort trials evaluating the effect of GH treatment (3, 27) were included in the study. SGA was defined as a birth length and/or weight ≤–2 SD score for their gestational age (28), and only children were included who remained short in postnatal life [at age 3: height ≤–2.00 SD score: short SGA (29)]. All children were Caucasian and had an uncomplicated postnatal period. Severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities and psychosocial dwarfism were reasons to exclude children from the study. The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center (Rotterdam), and written informed consent was obtained.

Clinical and biochemical measurements

Birth and growth data before the start of GH treatment were retrieved from records of hospitals, community health services, and general practitioners. Children were systematically measured at the start of and during the GH trials (3, 27). Height and head circumference were expressed as SD scores (29). Body mass index was calculated (weight in kg/height in meters^2) and expressed as SD scores for age and sex (30). Body proportion was assessed by the sitting height/height ratio and expressed in SD scores (31). GH production was assessed by arginine and clonidine GH provocation tests, and GH was measured by AutoDelphia (Perkin-Elmer) and standardized according to World Health Organization 80/505 guidelines. Serum IGF-I and IGFBP-3 levels were measured in the SGA subjects as described previously (32, 33), and values were transformed to SD scores by adjusting for sex and age (33). Bone age was determined according to Greulich and Pyle (34). Dysmorphological examination was performed by an experienced clinical geneticist (L.C.P.G.).

Genetic analysis

Genomic DNA was extracted from peripheral blood samples (35). DNA from control samples was isolated from leukocytes using Puregene nucleic acid purification chemistries for the Autopure LS Instrument (Gentra Systems, Minneapolis, MN).

The "in house" probe kit was designed according to the criteria described in White *et al.* (36). The kit contained 34 probes (supplementary Table 1) in 18 different growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2,* and *SOCS3*). Reactions were performed as described by Walenkamp *et al.* (25). MLPA of all 21 *IGF1R* exons was performed using the MRC Holland P217 MLPA kit according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands).

All MLPA kits that were used were validated with DNA from patients with a deletion in that particular gene, that have been diagnosed with other molecular techniques. As a positive control, a patient that was previously published (25) was used. A number of normal individuals, including their parents, and a blank (no DNA) were used as negative controls.

The Affymetrix GeneChip Human Mapping 262K *NspI* array was used according to the instructions provided in the Affymetrix GeneChip Human Mapping 500K Manual (http://www.affymetrix.com). SNP copy number was assessed using CNAG (Copy Number Analyser for GeneChip) version 2.0 (37).

IGF1 gene sequencing of all four exons and flanking intron-exon boundaries was performed according to standard procedures (primers and conditions available upon request).

Functional studies

Skin biopsies were taken from patients A and B, and a culture of dermal fibroblasts was established (38). For Western blotting, fibroblasts were stimulated for 10 minutes with 5, 10, and 20 ng/ml IGF-I (PeproTech, Inc., Rocky Hill, NJ). Blots were probed with an antiphosphoprotein kinase B (PKB)/Akt (Ser[473]), total PKB/Akt, total IGF1Rβ (Cell Signaling Technology, Beverly, MA), and an antiphospho-IGF1R (Biosource International, Camarillo, CA) antibody as described previously (39).

Results

All 100 short SGA children were investigated with the "in house" MLPA growth kit, and two patients were identified with a deletion of the *IGF1R* gene. No copy number variants (CNVs) in the other growth-related genes were detected.

Patient A

Clinical description

Patient A was a girl who was born spontaneously after 40 weeks of gestation as the third child of nonconsanguineous parents. Because maternal age was 36 yr, her mother chose for amniocentesis showing a 46,XX karyotype. The pregnancy was complicated by vaginal bleeding and limited fetal movements. Her birth weight was 2890 g (–1.28 SD score) and birth length 47 cm (–2.21 SD score). The height of her father was 184.7 cm (0.40 SD score) and of her mother 176.6 cm (1.34 SD score), resulting in a (secular trend-corrected) target height of 178.8 cm (1.25 SD score). She had bilateral hip dysplasia and clubfeet, for which she had hip casting from 4 to 12 months of age. Her bilateral hearing loss improved by tympanostomy tubes, which were implanted at 3 yr of age. Psychomotor development was delayed. At 2.3 yr of age, her height was 78.9 cm (–3.46 SD score), weight 10.3 kg (–2.13 SD score weight for height), and head circumference 47.2 cm (–0.82 SD score). Arginine and clonidine stimulation tests were performed at age 2 with a maximal GH response of 19.1 mU/liter and 14.1 mU/liter, respectively. She had a delayed dentition, starting at age 2. At age 3 her bone age was 2 yr. Her IGF-I level was 46 ng/ml (–1.61 SD score) and IGFBP-3 level 1.17 mg/liter (–1.63 SD score). Cardiovascular, respiratory, and abdominal examinations were all normal. On magnetic resonance imaging of the hypothalamic and pituitary region, no abnormalities were seen.

From age 4 onward, GH treatment was initiated at a dose of 1 mg/m² per day (Fig. 1). At start of GH treatment, her height was 90.6 cm (–3.42 SD score), BMI 14.3 kg/m² (–1.02 SD

score), sitting height/height ratio 0.60 (0.00 SD score), and head circumference 48.6 cm (−0.95 SD score). Her serum total IGF-I level was 34 ng/ml (−2.78 SD score), IGFBP-3 level 1.35 mg/liter (−1.33 SD score), and her bone age was 1 yr behind. After 1 yr of GH treatment, height had increased by 1.02 SD and head circumference by 0.58 SD. Her serum total IGF-I level was 197 ng/ml (1.51 SD score), and IGFBP-3 level was 2.62 mg/liter (0.92 SD score). After 4 yr of GH treatment, she had an increase in height of +1.65 SD score and an increase in IGF-I level of +5.75 SD. Currently, at age 8.3, her height is 123.3 cm (−1.68 SD score). Cardiac ultrasound showed an undulating shape of the left ventricular wall which could not be further specified.

Dysmorphological examination showed hypertelorism, hypocanthal folds, medial flaring of the eyebrows, broad nasal bridge, and thick hair (Fig. 2). She had a triangular face, large mouth, short and pigmented upper lip, and low-placed, posterior rotated ears. Her abdomen was protruded. She had bilateral clinodactyly, short fingers, and proximally placed, broad thumbs. She had a bilateral sandal gap and broad forefeet with pes planus. There was hyperlaxity of the joints, especially of the elbows and knees (Beighton score 7/9). Dimples were present at the right flank and in the lumbar region. These dysmorphic features were absent in her parents. She goes to a regular primary school. Testing of her verbal and performance intelligence quotient showed an average intellectual level, with a score of 93 and 121 points, respectively. These scores indicate a disharmonic intellectual profile although being in the normal range. Her two brothers were born after 40 weeks gestation, had a normal birth size, and grew normally (data not shown).

Genetic analysis

Patient A had a deletion of all three probes in the *IGF1R* gene on the MLPA growth kit. Confirmation of this result with the MRC Holland P217 MLPA kit showed a heterozygous deletion of all probes in the *IGF1R* gene (exon 1-21), including the two telomeric control probes which were located at 2.0 and 2.8 Mb downstream of the *IGF1R* gene. Her parents and both brothers did not carry this deletion. SNP array analysis showed a terminal deletion from location rs12912857, the first deleted SNP probe located at 95.883.282 bp until rs7169385, the last deleted SNP probe located at 100.192.115 bp (Ensembl release 49), comprising a 4.5 Mb region on chromosome 15 (Fig. 3). No other pathogenic CNV was observed with SNP array analysis. Additional sequence analysis of the coding region of the *IGF1* gene, to exclude a mutation as a cause of the low IGF-I level revealed no mutation.
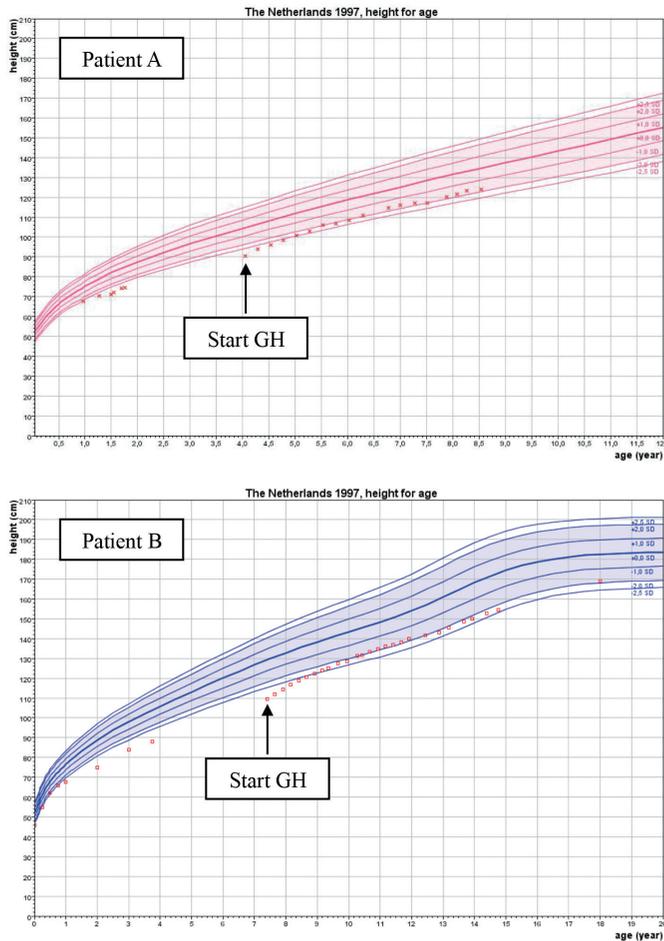
Figure 1 Growth charts of the two children with a *IGF1R* deletion.

Patient B

Clinical description

Patient B was a boy born spontaneously after 40 weeks of gestation as the first child of nonconsanguineous parents. A decrease in fetal growth was observed during the last trimester by ultrasound. His birth weight was 2600 g (–1.91 SD score) and birth length 47 cm (–2.21 SD score). The height of his father and mother was 187.6 cm (0.84 SD score) and 165.2 cm (–0.50 SD score), respectively. His secular trend-corrected target height was 187.4 cm (0.49 SD score). Bone age was 2 yr delayed at the chronological age of 4 yr. At age 2,

a rotation deformity of both tibial bones was observed, and tympanostomy tubes were implanted because of recurrent ear infections. At 3.0 yr of age, his height was 84.0 cm (−3.84 SD score), and his weight was 11.2 kg (−2.65 SD score). A clonidine stimulation test was performed at age 5 with a maximal GH response of 69.7 mU/liter. Cardiovascular, respiratory, and abdominal examinations were all normal.

From age 7 onward, he started GH treatment in a dose of 1 mg/m² per day (Fig. 1). At start of GH treatment, his height was 109.4 cm (−3.57 SD score), BMI 16.5 kg/m² (0.49 SD score), sitting height/height ratio of 0.55 (−1.86 SD score), and head circumference 51.3 cm (−0.53 SD score). IGF-I was 208 ng/ml (1.25 SD score) and IGFBP-3 level 2.99 mg/liter (1.24 SD score). After 1 yr of GH treatment, his height had increased by 0.83 SD and head circumference by 0.29 SD. His serum total IGF-I level was 356 ng/ml (2.28 SD score), and IGFBP-3 level was 1.99 mg/liter (−0.40 SD score). After 4 yr of GH treatment, he had an increase in height of +1.69 SD and an increase in IGF-I of +0.39 SD. Currently, at age 17, his height is 168.9 cm (−1.89 SD score).

From age 11 onward, he used methylfenidate (10/5/5 mg) because of attention-deficit hyperactivity disorder. After primary school, he started a secondary school for children with hearing and speech difficulties. Currently, he receives training for becoming a baker. Cardiac evaluation showed no abnormalities.

Dysmorphological examination showed hypertelorism, upward slant, thin upper lip, bilateral extra nipple, proximal implanted thumbs, and broad feet (Fig. 2). These dysmorphic features were absent in his parents and brother. His brother has a height within the normal range.

Genetic analysis

MLPA analysis showed a deletion of two of three *IGF1R* probes (exons 8 and 18). The MRC Holland P217 MLPA kit showed a heterozygous deletion of exons 3-21 of the *IGF1R* gene comprising also both telomeric control probes located at 2.0 and 2.8 Mb downstream of the *IGF1R* gene. His parents and brother did not carry the deletion. SNP array analysis showed a terminal deletion of 3.1 Mb on chromosome 15, ranging from rs11857366, first deleted SNP probe at 97.081.324 bp until rs7169385, last deleted SNP probe at 100.192.115 bp, containing 282 SNP probes (Fig. 3). No other pathogenic CNVs were observed in the SNP array analysis.

Patient A   **Patient B**

Figure 2 Physical characteristics of Patient A, at age 9, and of Patient B at 18 yr of age.

Functional analysis

In previous reports it was shown that a patient with a heterozygous *IGF1R* mutation (E1050K) demonstrated a decreased activation of IGF1R intracellular signaling upon challenging with IGF-I (26), and in a patient with a heterozygous *IGF1R* deletion the activation of the IGF1R and PKB/Akt tended to be lower, but this did not reach significance (25).

In our two patients Western blots from cultured skin fibroblasts demonstrated a slightly lower autophosphorylation of the IGF1R in patient B in comparison with controls (Fig. 4A), but on average autophosphorylation of the IGF1R in the two patients was similar to that in controls (Fig. 4B). There was a significantly reduced activation of PKB/Akt upon a challenge with 10 ng/ml IGF-I for 10 minutes compared with healthy controls. The levels of total PKB/Akt and IGF1R protein expression were similar to controls, although total IGF1R protein expression appeared lower in patient B (Fig. 4A). Stimulation of the fibroblasts with a dose range of IGF-I for 10 minutes (Fig. 4, C and D) demonstrated reduced phosphorylation of PKB/Akt in the patients compared with controls. Total PKB/Akt and IGF1R autophosporylation were similar in the patients and controls. Total IGF1R protein expression tended to be lower in the patients compared with controls.

Figure 3 Array analysis of chromosome 15 of Patient A showed a heterozygous deletion of 15q26.2 to 15qter, from rs12912857 to rs7169385, comprising the whole *IGF1R* gene. Patient B showed a heterozygous deletion of 15q26.3 to 15qter, from rs11857366 to rs7169385. This deletion starts within intron 2 of the *IGF1R* gene.
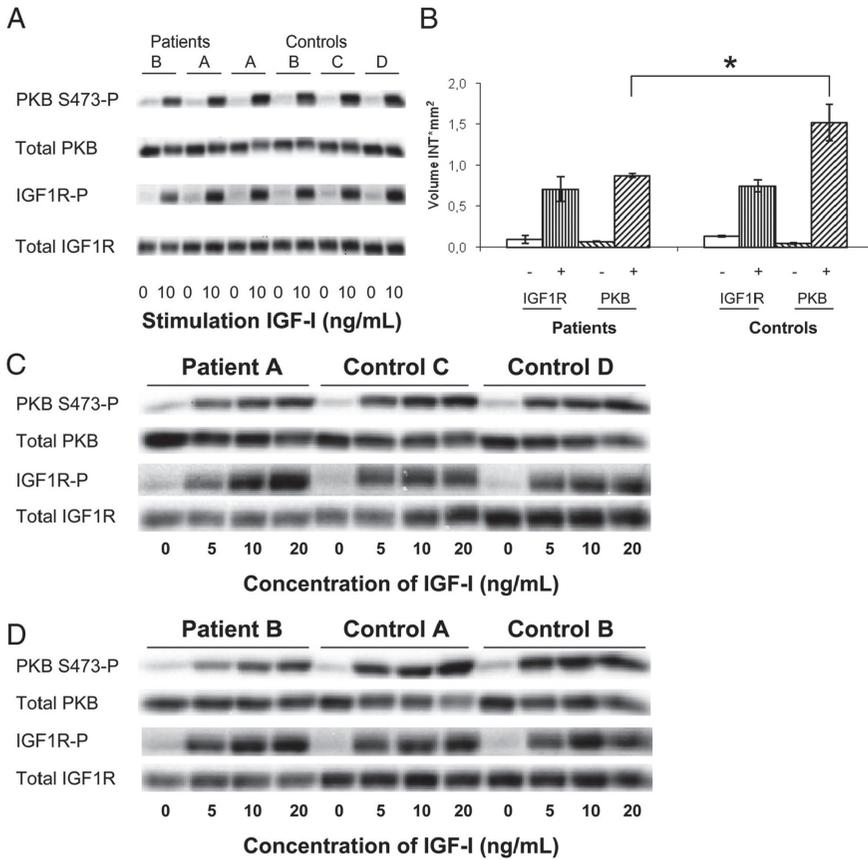
Figure 4 Functional analysis. A, Dermal fibroblasts of the patients and four age- and sex-matched controls were stimulated with 10 ng/ml IGF-I for 10 minutes. Protein lysates were collected, and 25 μg of protein was used for Western blotting with phosphospecific IGF1R and PKB/Akt (Ser[473]) antibodies and total IGF1R and PKB/Akt antibodies. B, Densitometric quantification of the Western blot shown in A. Data are expressed as a ratio of phosphospecific IGF1R or PKB/Akt and total IGF1R or PKB/Akt, respectively. The activation of PKB/Akt was significantly lower in the patients compared with the controls. Autophosporylation and total protein expression of the IGF1R tended to be lower in patient B, although this did not reach significance. C and D, Activation of PKB/Akt by phosphorylation on Ser[473] as well as autophosphorylation of the IGF1R in fibroblasts of Patients A and B and four age- and sex-matched controls were determined by Western blotting after a challenge with a dose range of IGF-I. Protein lysates were collected after a 10-minutes stimulation. Total PKB/Akt was used to check for equal loading.

Discussion

This study shows that *IGF1R* haploinsufficiency was detected in 2 of 100 short SGA children and that no other copy number variants were found in 18 other growth-associated genes. Because dominant *de novo* deletions are a well-recognized sign of pathogenicity, it is very likely that the observed deletions explain the short stature of both patients. In addition, according to the database of genomic variants (http://projects.tcag.ca/variation/) no deletion of the *IGF1R* gene region has been reported yet in the general population. This is further supported by previous clinical studies reporting subjects with *IGF1R* point mutations and deletions (13-15, 17-24, 26), including functional analysis of patients with a missense mutation and one with a complete deletion of the *IGF1R* gene (16, 25, 26). *In vitro* functional analysis of our patients showed almost similar IGF1R autophosporylation, a tendency toward reduced total IGF1R protein expression, and reduced intracellular PKB/Akt activation compared with healthy controls. Our results are in line with the findings we reported earlier on another patient with *IGF1R* haploinsufficiency (25) and suggest that this condition is characterized by a lower number of IGF1 receptors on the cell surface, resulting in less signal transduction. In the family with a missense mutation in the intracellular kinase domain of the *IGF1R*, we observed a stronger decrease in activation of downstream signaling (26). Previously we have hypothesized (25) that the discrepancy between the results in *IGF1R* deletions and mutations *in vitro* may be explained by a dominant-negative effect of the mutation, which would decrease the number of fully functional receptors to 25%, whereas haploinsufficiency would theoretically lead to a reduction of 50%.

In our fibroblast model, the expression of the IGF1R tended to be lower in the patient's cells but was not reduced with 50%. Downstream signaling as assessed by measuring phosphorylation of PKB/Akt was, however, reduced in the patient's fibroblasts. These observations are in line with previously reported results obtained in fibroblasts of a patient with a terminal 15q deletion, which also demonstrated a nonsignificant reduction in IGF1R expression but a much stronger and significant reduction in activation of downstream signaling (25). However, the observation that the effect on growth is similar in patients with *IGF1R* mutations and deletions suggests a similar biological effect on growth. Thus, the consequences of *IGF1R* haploinsufficiency may be cell type-dependent, with possibly a relatively strong effect in growth plate chondrocytes, which are responsible for longitudinal growth, whereas the differences in the fibroblast model are less pronounced.

Both children responded to GH treatment, increasing their height by 1.02 SD and 0.83 SD, respectively, after 1 yr of GH treatment. This growth response was comparable with the mean 1-yr growth response in short SGA children (approximately 0.8 SD in prepubertal short SGA children on the same GH dosage) (40).

This positive growth response can be explained by a combination of the direct effect of GH on the epiphyseal chondrocytes which is independent of the biological actions of serum IGF (41), and of elevated serum IGF-I levels, which may partially overcome the diminished sensitivity.

When previous case reports of children with an *IGF1R* deletion or mutation were reviewed, only six other children had received GH treatment (Table 1). The children who received a GH dose of 1 mg/m² per day increased their height with ~1.0 SD per year of GH treatment. Their serum IGF-I level increased with ~1.0 SD except for Patient A (discussed in the next paragraph). The other children received a higher GH dose but showed a similar variable response: a similar response in two of them, and no apparent response in two other patients (13, 16). Thus, six of eight children showed a beneficial effect of GH treatment.

An unexpected finding in Patient A was the low serum IGF-I level, in contrast to a high or normal serum IGF-I level usually observed in patients with an *IGF1R* mutation or deletion. Because no abnormalities in the *IGF1* gene were found, we hypothesize that this may be explained by a partial GH deficiency. The marginal response of GH in the provocation test and the observation that IGF-I and IGFBP-3 strongly rose by +4.3 SD and +2.2 SD after 1 yr of GH treatment support our hypothesis. In the child with an *IGF1R* missense mutation who presented with severe failure to thrive, serum IGF-I was initially not elevated (+1.3 SD score; van der Kamp, H.J., personal communication) (26), but after realimentation by a gastrostoma her IGF-I levels increased up to +2.9 SD score.

The children we identified had a relatively mild phenotype compared with the other patients with a terminal *IGF1R* deletion who had lung hypoplasia, atrial and/or ventricular septal defects, hypoplastic left atrial or ventricular heart, dextrocardia, and diaphragmatic hernia (Table 2) (19-25). We believe that these clinical signs are primarily linked to other genes in the area, as in children with an *IGF1R* mutation a much smaller number of additional characteristics has been observed. Poot *et al.* indicated *IGF1R* flanking genes that might be responsible for several characteristics of the variation in the phenotypes of children with an *IGF1R* deletion (20).

Both of our patients had hearing problems, showed mild developmental delay, and had proximal implanted thumbs. Hearing problems and developmental delay have been described in previous case reports of mutations in the *IGF1* and *IGF1R* gene and might also be caused by their tissue-specific expression in the auditory and central nervous system (42, 43).

To our knowledge, there are no diagnostic criteria for children with an *IGF1R* mutation or deletion. In Table 3 we have summarized clinical features of children with an *IGF1R* mutation or deletion according to their organ system. We identified major criteria that are predominantly related to growth restriction and minor criteria that are based on dysmorphic

features and signs of joint hypermobility. Based on the information summarized in Table 2, we propose the combinations of major and minor criteria which indicate a high likelihood of an *IGF1R* mutation or deletion. Interestingly, joint hypermobility is related to the fibrillin (*FBN1*) gene, which is located upstream of the *IGF1R* gene on 15q21. *FBN1* gene mutations are responsible for Marfan's syndrome, which is associated with tall stature. Because both clinical syndromes display joint hypermobility, we speculate that the *IGF1R* and *FBN1* genes together might play a role in variations in height and hypermobility in short SGA patients.

The remaining 98 short SGA children had no CNVs in the 18 growth-associated genes we selected, but we did not exclude mutations in these genes. Future studies are needed to investigate whether deletions or mutations in other genes, or combinations of several gene defects, are associated with the short SGA phenotype.

In summary, this study has shown that *IGF1R* haploinsufficiency was present in 2 of 100 short SGA children. This study illustrates that the combination of a small birth size, short stature, small head size, relatively high IGF-I level, developmental delay, and micrognathia is suggestive for children with an *IGF1R* deletion. Because GH therapy leads to a moderate catch-up growth of ~1.0 SD in the first year, we recommend that the SGA children with a persistent short stature are tested for an *IGF1R* deletion, particularly if some of the major or minor criteria are present. The MLPA has shown to be a valuable tool in rapidly identifying these relatively large deletions in short SGA children.

Table 1 Literature overview of short stature and/or SGA patients with a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion who received GH treatment

Molecular characterization	Patient A del(15)(q26.2)	Patient B del(15)(q26.3)	Abuzzahab (13) *IGF1R* R108Q/K115N	Abuzzahab (13, 44) *IGF1R* R59X	Inagaki (16) *IGF1R* R481Q	Walenkamp (26, 45) *IGF1R* E1050K	Siebler (23) del(15)(q26.1)	Walenkamp (25) del(15)(q26.2)
Birth								
GA (wk)	40	40	38	At term	41	39	39	39
Length (SD score)	−2.21	−2.74	NA	−5.89	−5.63	−2.94	NA	−1.95
Weight (SD score)	−1.28	−1.91	−4.33	−3.22	−3.06	−6.13	−3.05	−2.93
At start GH								
Age (yr)	4.0	7.0	4.5	6.4	13.6	1.6	3.5	5.3
Height (SD score)	−3.42	−3.57	~ −4.0[a]	−2.51	−5.0	−2.3	~ −5.0[a]	−3.5
IGF-I (SD score)	−2.78	1.25	"Normal"[b]	+1.20	⇑⇑[c]	2.1	NA[b]	2.5
GH dose (mg/m² per day)	1.0	1.0	1.6	0.9	2.1	1.4	1.6[d]	1.0
After 1 yr GH								
Height (SD score)	−2.40	−2.74	~ −4.0[a]	−1.96	−5.0[e]	−1.5[e]	~ −4.0[a]	−2.0[f]
IGF-I (SD score)	1.51	2.28	⇑⇑[b]	+1.77	NA	3.6	NA	3.5[f]
Change ht (SD)	*1.02*	*0.83*	*0*	*0.55*	*0*	*0.8*	*~1.0*	*1.5*
Change IGF-I (SD)	*3.53*	*1.03*	*⇑⇑[b]*	*0.57*	*NA*	*1.5*	*NA*	*1.0*

All birth weight and birth lengths are presented as standard deviation scores according to the Usher growth charts. NA, Not available.

[a] Estimated from the growth chart.

[b] No SD score is provided.

[c] IGF-I SD score not available, but level was above the normal range and unchanged after GH treatment.

[d] Three different GH doses were used, of which this dose was maintained for the longest period (age 8–10 yr).

[e] Measured after 6 months of GH treatment.

[f] Measurements are provided when height and IGF-I level stabilized.

Table 2 Overview of phenotypic characteristics in short SGA patients with a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion

Genotype		Phenotype										
Mutation or deletion	Study	Birth weight ≤ −2.0 SDS	Birth length ≤ −2.0 SDS	Birth HC ≤ −2.0 SDS and/or microcephaly	Postnatal height ≤ −2.0 SDS	IGF-I level > +1.0 SDS	Developmental delay and/or MR	Micrognathia/ triangular facies	Proximal placed dig I	Cardiac disorders	Total	Extra diagnoses
15q26.2→qter	Patient A	−	+	−	+	−	+	+	+	±	6	Clubfeet, hip dysplasia, aberrant left ventricular wall
15q26.3→qter	Patient B	−	+	−	+	+	+	−	+	−	5	
IGF1R exon 2	R108Q/ K115N (13)	+	NA	−	+	+	+	−	−	−	4	
IGF1R exon 2	R59X (13)	+	+	+	+	+	+	−	−	−	6	
IGF1R exon 7	R481Q (16)	+	+	NA	+	+	−	+	−	−	5	
IGF1R exon 11	R709Q (17)	−	−	NA	+	+	+	−	−	−	3	
IGF1R exon 11	R709Q (17)	+	NA	NA	+	−	NA	NA	NA	−	2	
IGF1R exon 16	E1050K (26)	+	−	+	+	+	−	−	−	−	4	
IGF1R exon 16	E1050K (26)	+	+	+	+	+	−	+	−	−	6	Oligohydramnios
15q26.1→qter	(14)	+	+	+	NA	NA	NA	−	−	+	4	Right-sided aorta, VSD, tethered spinal cord, death at 5 months

Feature	n	15q26.1→qter (15)	15q26.1→qter (23)	15q26.1→qter (23)	15q26.1→qter (21)	15q26.1 (24)	15q26.2→qter (19)	15q26.2→qter (20)	15q26.2→qter (25)	15q26.2 (22)	15q26.2 (18)
Diaphragmatic hernia, bicuspid aortic valve, aortic coarctation	2	+	+	NA	NA	NA	NA	NA	NA	–	+
Lung hypoplasia, diaphragmatic hernia	4	+	–	a	a	a	+	+	+	–	+
Cubitus valgus	5	+	+	+	+	NA	+	+	NA	NA	+
Oligohydramnios, hypoplastic lungs	6	+	–	+	+	+	+	+	NA	+	+
Gastroesophagal reflux	7	+	–	+	+	+	+	NA	+	+	+
Seizures, gastroesophagal reflux	8	+	+	+	+	+	+	+	+	NA	+
Oculocutaneous albinism, subluxation radial heads	7	+	+	NA	+	+	+	–	+	+	–
Severe myopia	4	+	–	–	+	+	–	+	–	NA	–
Clubfeet, genu recurvatum	5	+	–	–	+	NA	+	+	NA	NA	–
	4	+	–	–	+	NA	+	–	NA	NA	–
Total (number)		16	11	10	17	9	11	11	5	7	
Total (%)		80	79	71	100	75	69	61	26	35	

IGF1R is located at 15q25-26 (OMIM *147370). All birth weight and lengths were calculated as standard deviation scores (28).
HC, Head circumference ; MR, mental retardation; NA, not available.
a Died directly postpartum.

Table 3 Suggested clinical indicators for a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion based on published cases

Anatomical system	Major	Minor
1. Skeletal	Height ≤ –2.00[a] Birth length ≤ –2.00 Birth weight ≤ –2.00 Head circumference ≤ –2.00 IGF-I level SDS > 1.00	Triangular face/micrognathia Proximal placed thumb
2. Nervous system	Developmental delay Mental retardation	Tethered spinal cord Seizures
3. Cardiac	Right sided aorta, bicuspid aortic valve, aortic coarctation VSD Aberrant left ventricular wall	
4. Gastro-intestinal		Diaphragmatic hernia Gastroesophagal reflux
5. Connective tissue		Clubfeet Hip dysplasia Cubitus valgus Genu recurvatum Subluxation radial heads Severe myopia
6. Skin		Oculocutaneous albinism

[a] SD score. VSD, Ventricular septal defect.

Reference List

1. **Hokken-Koelega AC, De Ridder MA, Lemmen RJ, Den Hartog H, De Muinck Keizer-Schrama SM, Drop SL** 1995 Children born small for gestational age: do they catch up? Pediatr Res 38:267-271

2. **Arends NJ, Boonstra VH, Duivenvoorden HJ, Hofman PL, Cutfield WS, Hokken-Koelega AC** 2005 Reduced insulin sensitivity and the presence of cardiovascular risk factors in short prepubertal children born small for gestational age (SGA). Clin Endocrinol (Oxf) 62:44-50

3. **Arends NJ, Boonstra VH, Hokken-Koelega AC** 2004 Head circumference and body proportions before and during growth hormone treatment in short children who were born small for gestational age. Pediatrics 114:683-690

4. **Leger J, Garel C, Fjellestad-Paulsen A, Hassan M, Czernichow P** 1998 Human growth hormone treatment of short-stature children born small for gestational age: effect on muscle and adipose tissue mass during a 3-year treatment period and after 1 year's withdrawal. J Clin Endocrinol Metab 83:3512-3516

5. **Boonstra VH, Arends NJ, Stijnen T, Blum WF, Akkerman O, Hokken-Koelega AC** 2006 Food intake of children with short stature born small for gestational age before and during a randomized GH trial. Horm Res 65:23-30

6. **Lundgren EM, Cnattingius S, Jonsson B, Tuvemo T** 2001 Intellectual and psychological performance in males born small for gestational age with and without catch-up growth. Pediatr Res 50:91-96

7. **Brandt I, Sticker EJ, Lentze MJ** 2003 Catch-up growth of head circumference of very low birth weight, small for gestational age preterm infants and mental development to adulthood. J Pediatr 142:463-468

8. **Gale CR, O'Callaghan FJ, Godfrey KM, Law CM, Martyn CN** 2004 Critical periods of brain growth and cognitive function in children. Brain 127:321-329

9. **van Pareren YK, Duivenvoorden HJ, Slijper FS, Koot HM, Hokken-Koelega AC** 2004 Intelligence and psychosocial functioning during long-term growth hormone therapy in children born small for gestational age. J Clin Endocrinol Metab 89:5295-5302

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

11. **Bonapace G, Concolino D, Formicola S, Strisciuglio P** 2003 A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 40:913-917

12. **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** 2005 Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90:2855-2864

13. **Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, Kiess W, Klammt J, Kratzsch J, Osgood D, Pfaffle R, Raile K, Seidel B, Smith RJ, Chernausek SD** 2003 IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 349:2211-2222

14. **Bhakta KY, Marlin SJ, Shen JJ, Fernandes CJ** 2005 Terminal deletion of chromosome 15q26.1: case report and brief literature review. J Perinatol 25:429-432

15. **Biggio Jr JR, Descartes MD, Carroll AJ, Holt RL** 2004 Congenital diaphragmatic hernia: is 15q26.1-26.2 a candidate locus? Am J Med Genet A 126A:183-185

16. **Inagaki K, Tiulpakov A, Rubtsov P, Sverdlova P, Peterkova V, Yakar S, Terekhov S, LeRoith D** 2007 A familial insulin-like growth factor-I receptor mutant leads to short stature: clinical and biochemical characterization. J Clin Endocrinol Metab 92:1542-1548

17. **Kawashima Y, Kanzaki S, Yang F, Kinoshita T, Hanaki K, Nagaishi J, Ohtsuka Y, Hisatome I, Ninomoya H, Nanba E, Fukushima T, Takahashi S** 2005 Mutation at cleavage site of insulin-like growth factor receptor in a short-stature child born with intrauterine growth retardation. J Clin Endocrinol Metab 90:4679-4687

18. **Okubo Y, Siddle K, Firth H, O'Rahilly S, Wilson LC, Willatt L, Fukushima T, Takahashi S, Petry CJ, Saukkonen T, Stanhope R, Dunger DB** 2003 Cell proliferation activities on skin fibroblasts from a short child with absence of one copy of the type 1 insulin-like growth factor receptor (IGF1R) gene and a tall child with three copies of the IGF1R gene. J Clin Endocrinol Metab 88:5981-5988

19. **Pinson L, Perrin A, Plouzennec C, Parent P, Metz C, Collet M, Le Bris MJ, Douet-Guilbert N, Morel F, De Braekeleer M** 2005 Detection of an unexpected subtelomeric 15q26.2 --> qter deletion in a little girl: clinical and cytogenetic studies. Am J Med Genet A 138:160-165

20. **Poot M, Eleveld MJ, van 't Slot R, van Genderen MM, Verrijn Stuart AA, Hochstenbach R, Beemer FA** 2007 Proportional growth failure and oculocutaneous albinism in a girl with a 6.87 Mb deletion of region 15q26.2-->qter. Eur J Med Genet 50:432-440

21. **Roback EW, Barakat AJ, Dev VG, Mbikay M, Chretien M, Butler MG** 1991 An infant with deletion of the distal long arm of chromosome 15 (q26.1----qter) and loss of insulin-like growth factor 1 receptor gene. Am J Med Genet 38:74-79

22. **Rujirabanjerd S, Suwannarat W, Sripo T, Dissaneevate P, Permsirivanich W, Limprasert P** 2007 De novo subtelomeric deletion of 15q associated with satellite translocation in a child with developmental delay and severe growth retardation. Am J Med Genet A 143:271-276

23. **Siebler T, Lopaczynski W, Terry CL, Casella SJ, Munson P, De Leon DD, Phang L, Blakemore KJ, McEvoy RC, Kelley RI,** 1995 Insulin-like growth factor I receptor expression and function in fibroblasts from two patients with deletion of the distal long arm of chromosome 15. J Clin Endocrinol Metab 80:3447-3457

24. **Tonnies H, Schulze I, Hennies H, Neumann LM, Keitzer R, Neitzel H** 2001 De novo terminal deletion of chromosome 15q26.1 characterised by comparative genomic hybridisation and FISH with locus specific probes. J Med Genet 38:617-621

25. **Walenkamp MJ, de Muinck Keizer-Schrama SM, de Mos M, Kalf ME, van Duyvenvoorde HA, Boot AM, Kant SG, White SJ, Losekoot M, Den Dunnen JT, Karperien M, Wit JM** 2008 Successful Long-Term Growth Hormone Therapy in a Girl with Haploinsufficiency of the Insulin-Like Growth Factor-I Receptor due to a Terminal 15q26.2->qter Deletion Detected by Multiplex Ligation Probe Amplification. J Clin Endocrinol Metab 93:2421-2425

26. **Walenkamp MJ, van der Kamp HJ, Pereira AM, Kant SG, van Duyvenvoorde HA, Kruithof MF, Breuning MH, Romijn JA, Karperien M, Wit JM** 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. J Clin Endocrinol Metab 91:3062-3070

27. **Sas TC, Gerver WJ, De Bruin R, Mulder PG, Cole TJ, De Waal W, Hokken-Koelega AC** 2000 Body proportions during 6 years of GH treatment in children with short stature born small for gestational age participating in a randomised, double-blind, dose-response trial. Clin Endocrinol (Oxf) 53:675-681

28. **Usher R, McLean F** 1969 Intrauterine growth of live-born Caucasian infants at sea level: standards obtained from measurements in 7 dimensions of infants born between 25 and 44 weeks of gestation. J Pediatr 74:901-910

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

30. **Fredriks AM, van Buuren S, Wit JM, Verloove-Vanhorick SP** 2000 Body index measurements in 1996-7 compared with 1980. Arch Dis Child 82:107-112

31. **Gerver WJ, de Bruin R** 2001 Paediatric morphometrics, a reference manual. 2nd ed. Maastricht: University press Maastricht

32. **Hokken-Koelega AC, Hackeng WH, Stijnen T, Wit JM, de Muinck Keizer-Schrama SM, Drop SL** 1990 Twenty-four-hour plasma growth hormone (GH) profiles, urinary GH excretion, and plasma insulin-like growth factor-I and -II levels in prepubertal children with chronic renal insufficiency and severe growth retardation. J Clin Endocrinol Metab 71:688-695

33. **Rikken B, van Doorn J, Ringeling A, Van den Brande JL, Massa G, Wit JM** 1998 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 50:166-176

34. **Greulich WW, Pyle SI** 1959 Radiographic Atlas of Skeletal Development of the Hand and Wrist. 2nd ed. Stanford, CA: Stanford University Press

35. **Miller SA, Dykes DD, Polesky HF** 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

36. **White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, Breuning MH, den Dunnen JT** 2004 Two-color multiplex ligation-dependent probe amplification: detecting genomic rearrangements in hereditary multiple exostoses. Hum Mutat 24:86-92

37. **Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S** 2005 A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 65:6071-6079

38. **Kamp GA, Ouwens DM, Hoogerbrugge CM, Zwinderman AH, Maassen JA, Wit JM** 2002 Skin fibroblasts of children with idiopathic short stature show an increased mitogenic response to IGF-I and secrete more IGFBP-3. Clin Endocrinol (Oxf) 56:439-447

39. **Ouwens DM, van der Zon GC, Pronk GJ, Bos JL, Moller W, Cheatham B, Kahn CR, Maassen JA** 1994 A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and p21ras-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. Evidence for IRS1-independent p21ras-GTP formation. J Biol Chem 269:33116-33122

40. **Sas T, de Waal W, Mulder P, Houdijk M, Jansen M, Reeser M, Hokken-Koelega A** 1999 Growth hormone treatment in children with short stature born small for gestational age: 5-year results of a randomized, double-blind, dose-response trial. J Clin Endocrinol Metab 84:3064-3070

41. **van der Eerden BC, Karperien M, Wit JM** 2003 Systemic and local regulation of the growth plate. Endocr Rev 24:782-801

42. **Beck KD, Powell-Braxton L, Widmer HR, Valverde J, Hefti F** 1995 Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. Neuron 14:717-730

43. **Hawkins RD, Bashiardes S, Powder KE, Sajan SA, Bhonagiri V, Alvarado DM, Speck J, Warchol ME, Lovett M** 2007 Large scale gene expression profiles of regenerating inner ear sensory epithelia. PLoS ONE 2:e525

44. **Raile K, Klammt J, Schneider A, Keller A, Laue S, Smith R, Pfaffle R, Kratzsch J, Keller E, Kiess W** 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. J Clin Endocrinol Metab 91:2264-2271

45. **van der Kamp HJ, Walenkamp MJE, Wit JM** 2007 Growth hormone treatment is efficacious in short stature due to a hetrozygous missense mutation in the tyrosine kinase domain of the IGF-I receptor. Hormone Research 68 (suppl 1):170

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Supplemental Data

Table 1 Probe sequences of the "in house" MLPA kit.

Gene name	exon	Upstream (U)/ Downstream (D)*	Sequence	Total fragment length (bp)	Label
IGF1R	2	U	GATGTGTGAGAAGACCACCATCAACA	92	HEX
		D	ATGAGTACAACTACCGCTGCTGGACCACAA		
	8	U	CTACATGGGCTGAAGCCCTGGACTCAG	94	HEX
		D	TACGCCGTTTACGTCAAGGCTGTGACCCTCA		
	18	U	CAGTCCTAGCACCTCCAAGCCTGAGCA	90	HEX
		D	AGATGATTCAGATGGCCGGAGAGATTG		
IGF1	3	U	GAGTGCTGCTTCCGGAGCTGTGATCT	88	HEX
		D	AAGGAGGCTGGAGATGTATTGCGCAC		
	4	U	GTACATTTGAAGAACGCAAGTAGAGGGAGT	98	HEX
		D	GCAGGAAACAAGAACTACAGGATGTAGGAAGA		
GH1	4	U	CAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGA	116	FAM
		D	CCTCCTAAAGGACCTAGAGGAAGGCATCCAAA		
GHR	4	U	GCCACTGGACAGATGAGGTTCATCATGGTACAAAGAACC	112	FAM
		D	TAGGACCCATACAGCTGTTCTATACCAGAAG		
	10	U	GGTACTTTGGTGGCCACATAAGCCATTATTCACTAGTATGA	110	HEX
		D	CTAGTTGTGTCTGGCAGTTTATATTTAACTCTC		
SHOX	3	U	GGATTTATGAATGCAAAGAGAAGCGCGAGGACG	106	HEX
		D	TGAAGTCGGAGGACGAGGACGGGCAGACCAAGCTGAA		
	8	U	CCCTGAGTTTCTCTGGTGACGCCCTCATTCTCCTAA	108	HEX
		D	CGTTCAATAATCTCAATGTTGAGTTGCAGCAACAGA		
IGFBP1	1	U	CAAACTTATTTTGAACACTCAGCTCCTAGCGTGCG	102	HEX
		D	GCGCTGCCAATCATTAACCTCCTGGTGCAAG		
	4	U	GTGAGACATCCATGGATGGAGAGGCGGGACTCTGCT	104	HEX
		D	GGTGCGTCTACCCTTGGAATGGGAAGAGGATC		
IGFBP2	2	U	CACTCAGAAGGAGGCCTGGTGGAGAACCACGT	100	FAM
		D	GGACAGCACCATGAACATGTTGGGCG		
	4	U	CAAGATGTCTCTGAACGGGCAGCGTGGGGAGTGCT	108	FAM
		D	GGTGTGTGAACCCCAACACCGGGAAGCTGAT		
IGFBP3	2	U	GCTAAAGACAGCCAGCGCTACAAAGTTGACTACG	100	HEX
		D	AGTCTCAGAGCACAGATACCCAGAACTTCT		
	5	U	CTGGAGCTCACAGCCTTCTGTGGTGTCATT	96	HEX
		D	TCTGAAACAAGGGCGTGGATCCCTCAACCA		
IGFBP4	3	U	GACCTCTACATCATCCCCATCCC	84	HEX
		D	CAACTGCGACCGCAACGGCAACTTC		

	4	U	CAGAGTCAGAGGAGAAGAGACATGTACCTTGACCATCGTCC	114	HEX
		D	TTCCTCTCAAGCTAGCCAGAGGGTGGGAGCCTAAGGA		
IGFBP5	2	U	GATCTTCCGGCCCAAACACACCCGCATCTCCGAGCTGAAGGCTGAAG	120	HEX
		D	CAGTGAAGAAGGACCGCAGAAAGAAGCTGACCCAGTC		
	3	U	GTGCTGTGTACCTGCCCAATTGT	86	HEX
		D	GACCGCAAAGGATTCTACAAGAGAAAG		
IGFBP6	2	U	CCACAGGATGTGAACCGCAGAGA	92	FAM
		D	CCAACAGAGGAATCCAGGCACCTCTAC		
	3	U	CAAACACTCTACGTGCCCAATTGTG	90	FAM
		D	ACCATCGAGGCTTCTACCGGAAG		
ALS	2	U	GAACCTCTCTGGGAACTGTCTCCGG	88	FAM
		D	AACCTTCCGGAGCAGGTGTTC		
STAT5B	2	U	GATACAAGCTCAGCAGCTCCAAGGAGAAGCCCT	102	FAM
		D	TCATCAGATGCAAGCGTTATATGGCCA		
	14	U	GAGAATTTACCAGGACGGAATTACACTTTCTG	104	FAM
		D	GCAATGGTTTGACGGTGTGATGGAAGTGTT		
SOCS3	2	U	CTTCAGCATCTCTGTCGGAAGACCGTCAACG	98	FAM
		D	GCCACCTGGACTCCTATGAGAAAGT		
GRB10	3	U	GATGTGGACCTGGAAGCCCTGGTGAACGAT	96	FAM
		D	ATGAATGCATCCCTGGAGAGCCTG		
	10	U	CAGAGGACGAGCAAACCAGGACGTGCT	94	FAM
		D	GGATGACAGCGTTCAGACTCCTCAA		
NSD1	3	U	GAGATCTCATCTGGGCAAAATTCAAGAGACGCCCATGGTGGCCCT	116	HEX
		D	GCAGGATTTGTTCTGATCCGTTGATTAACACACAT		
	13	U	CACTGTTATGCAGAACAAGGGCTTCCGGTGCTCCCTCCACATCTGTA	118	HEX
		D	TAACCTGTCATGCTGCTAATCCAGCCAATGTTTCT		
	20	U	CTCGGTTCATGAATCATTGCTGCCAGCCCAACTGTGAAACACAGAAG	120	FAM
		D	TGGTCTGTGAATGGAGATACCCGTGTAGGCC		
IGF2	2	U	GAATCCCAATGGGGAAGTCGAT	86	FAM
(11p15)		D	GCTGGTGCTTCTCACCTTCTTG		
	4	U	CATCCTGCAGCCTCCTCCTGACCACGGACGTTTCCATCAGGTT	114	FAM
		D	CCATCCCGAAAATCTCTCGGTTCCACGTC		
SOCS2	1	U	GCCGCGGCCTCAACTAAAAGTGGCCATTGACCTTTCAA	110	FAM
		D	GCTTTCGAGCAGTGATGCAATAGAATAGAA		

* The Upstream hybridizing sequence (U) was extended with a labeled primer 5'-GGGTTCCCTAAGGGTTGGA-3'; the downstream hybridizing sequence was extended with an unlabeled primer 5'-GTGCCAGCAAGATCCAATCTAGA-3'.

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Genetic analysis of short children with apparent growth hormone insensitivity

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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.

chapter

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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 $0.72 \times$ 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/m²/day. In case of an insufficient response (defined as a change of <1 SDS), the dose was doubled (1.4 mg/m²/day), and if the response was still too low, a third series of GH injections (2.8 mg/m²/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<u>a</u>tgcctgacaaagtg-3' and reverse 5'-cactttgtcaggca<u>t</u>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/m²/day (equivalent to 25 µg/kg/day at a body surface area of 1 m²). 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	107	-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 m² body surface area 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 m² 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/m² 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	-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 m² 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/m²/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])

Patient	Age years	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	
		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.

chapter

6

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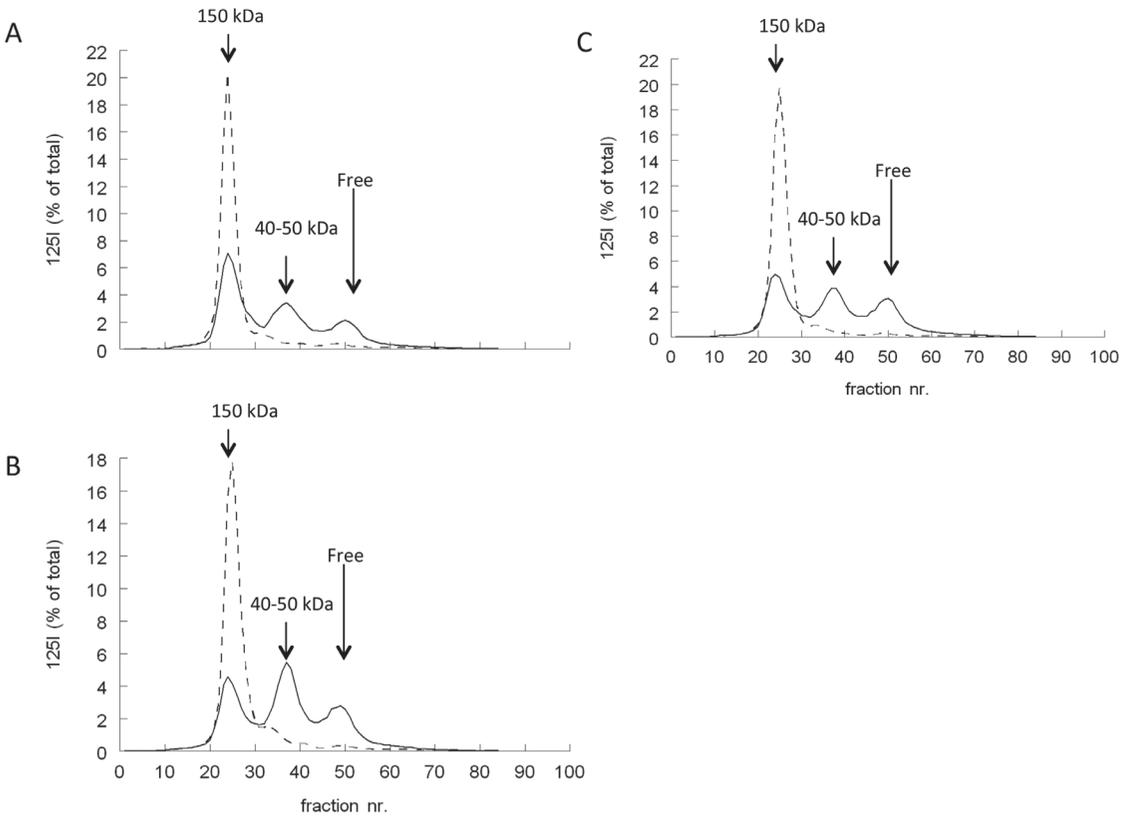
Figure 1 S200 gel filtration column chromatography. **a** Representative column profile for normal adult serum. **b** Serum RZ. **c** Serum IZ. Solid line: without addition; dotted line: addition of purified hIGFBP-3 (2.0 μg/ml serum).

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.

A.

GH (ng/ml)	0			100	
	F-STAT5b	F-V498M	pcDNA3.1	F-STAT5b	F-V498M
μg cell lysates	20	40	40	20	40

α-pY-STAT5

α-STAT5b

α-FLAG
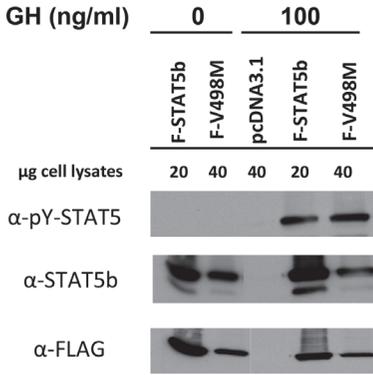
B.
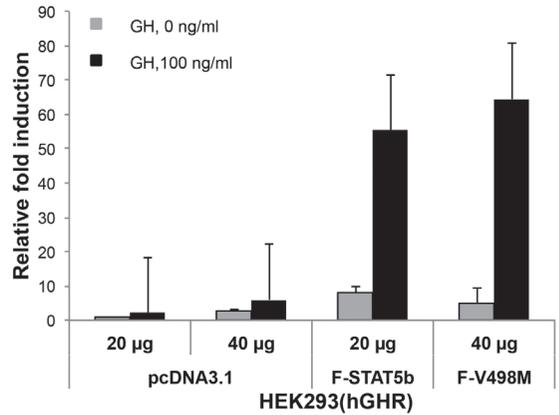
Figure 2 Reconstitution studies of the heterozygous *STAT5B* p.V498M mutation identified in CH. Reconstitution studies were performed in HEK293(hGHR). To compare immunologically equivalent amounts of FLAG-tagged STAT5b protein, loading of cell lysates from cells transfected with F-V498M was twice that 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.
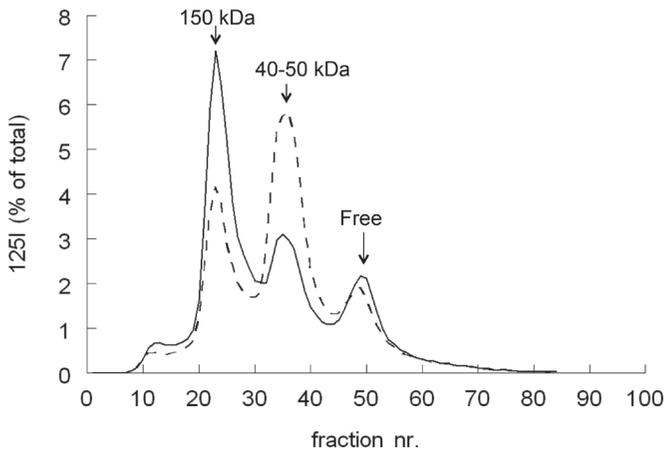
Figure 3 S200 gel filtration column chromatography. Column profile for serum of CH (dotted line) compared with normal adult serum (solid line).

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					arr5q24.2q24.3(SNP_A-4204149→SNP_A-4240707)×3 mat containing *C15orf27, ETFA, ISL2* and *SCAPER*
MV[a]		het POL c.558A>G, p.Gly86Gly			
NV[a]					arr6q12.1(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 *RABGAP1L*
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
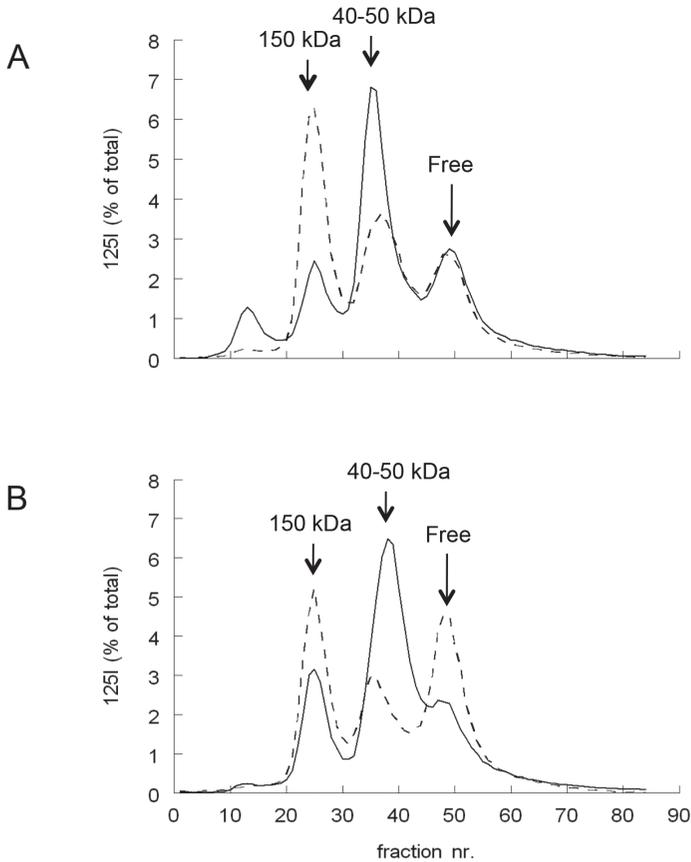
Figure 4 S200 gel filtration column chromatography. **a** Column profile of JB before GH treatment (solid line) and after 4 years of GH treatment (dotted line). **b** Column profile of mother (solid line) and father (dotted line).

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/m², 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.

chapter

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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.

Part C

Whole genome approach

Copy number variants in patients with short stature

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Abstract

Height is a highly heritable and classic polygenic trait. Recent Genome-Wide Association studies (GWAS) have revealed that at least 180 genetic variants influence adult height. However, these variants explain only about 10% of the phenotypic variation in height. Genetic analysis of short individuals can lead to the discovery of novel rare gene defects with a large effect on growth.

In an effort to identify novel genes associated with short stature, genome-wide analysis for copy number variants (CNVs), using Single Nucleotide Polymorphism arrays, in 162 patients (149 families) with short stature was performed. Segregation analysis was performed if possible, and genes in CNVs were compared with information from GWAS, gene expression in rodents' growth plates, and published information.

CNVs were detected in 40 families. In six families a known cause of short stature was found (*SHOX* deletion or duplication, *IGF1R* deletion), in two combined with a *de novo* potentially pathogenic CNV. Thirty-three families had one or more potentially pathogenic CNVs (n = 40). In 24 of these families segregation analysis could be performed, identifying 3 *de novo* CNVs and 9 CNVs segregating with short stature. Four were located near loci associated with height in GWAS (*ADAMTS17*, *TULP4*, *PRKG2/BMP3* and *PAPPA*).

Besides six CNVs known to be causative for short stature, 40 CNVs with possible pathogenicity were identified. Segregation studies and bioinformatics analysis suggested various potential candidate genes.

Introduction

Height is a highly heritable and classic polygenic trait. In order to discover genes involved in growth regulation, there are basically two approaches. The first approach is to carry out genome-wide association studies (GWAS) for common variants in large populations of individuals. This has led to the discovery of at least 180 loci associated with adult height. However, the contribution of each locus is small, each locus contains various genes, and cumulative loci only explain about 10% of the phenotypic variation [1]. Alternatively, when using all Single Nucleotide Polymorphisms (SNPs) identified in a GWAS approach as predictors simultaneously, up to 40% of the variance in height can be explained [2]. The second approach is to perform genetic studies in patients with extremely short or tall height, and search for causative variants [3]. With this approach one can either test for gene defects that were previously described or that appear plausible based on observations in knockout mice (candidate gene approach), or perform a genome-wide analysis for copy number variants (CNVs) or whole exome sequencing (WES) for mutations. The candidate gene approach has led to the detection of a substantial number of genes that are involved in monogenic defects associated with short or tall stature, such as *IGF1*, *STAT5B*, *IGFALS*, and *IGF1R* [4-10], but obviously does not result in finding novel genes involved in growth regulation.

In two previous papers from our group [11,12] we have described the results of a candidate gene approach in children with short stature, either associated with a low birth size (small for gestational age, SGA) [13] or with a normal birth size (idiopathic short stature, ISS) [14]. In the present paper we describe the results of a genome-wide analysis for CNVs using SNP arrays in short children, in an effort to identify novel gene variants associated with short stature.

Subjects and Methods
Patients

We studied 191 patients from 173 unrelated families with short stature of unknown origin, either born with a normal birth size or born small for gestational age (SGA). DNA was sent to our laboratory for analysis because of short stature between 2008 and 2011. Twenty-nine were excluded from the present analysis: eight because of a height standard deviation score (SDS) > –2.0, fifteen because of insufficient or low quality DNA or no parental consent, and six cases belonging to one family were separately described with a heterozygous *IGF1* mutation and an additional 435.7 Kb deletion (arr 3q26.1(162,681,814 –163,117,547)×1) [6]. This resulted in an analyzable group of 162 patients from 149 families. Height standard deviation score (SDS) was calculated for Dutch population references [15], except for one patient (I.6/II.2) for whom the reference for children of Turkish ethnicity was

used [16]. With consent of the Medical Ethical Committee of the Leiden University Medical Center, clinical data were collected and anonymized for all patients.

SNP array*s*

In 103 cases the Affymetrix GeneChip Human Mapping 262K *NspI* or 238K *StyI* arrays (Affymetrix, Santa Clara, CA, USA) was used, containing 262,262 and 238,304 25–mer oligonucleotides, respectively, with an average spacing of approximately 12 kb per array. An amount of 250 ng DNA was processed according to the manufacturer's protocol. Detection of SNP copy number was performed using copy number analyzer for GeneChip (CNAG) version 2.0 [17].

In 54 cases the Illumina HumanHap300 BeadChip (Illumina Inc., San Diego, CA, USA) was used, containing 317,000 TagSNPs, with an average spacing of approximately 9 kb, and in 5 cases the Illumina HumanCNV370 BeadChip (Illumina Inc., Eindhoven, The Netherlands), containing 317,000 TagSNPs and 52,000 non-polymorphic markers for specifically targeting nearly 14,000 known CNVs. This array has an average spacing of approximately 7.7 kb. A total of 750 ng DNA was processed according to the manufacturer's protocol. SNP copy number (log R ratio) and B-allele frequency were assessed using Beadstudio Data Analysis Software Version 3.2 (Illumina Inc., Eindhoven, The Netherlands).

Evaluation of CNVs

Deletions of at least five adjacent SNPs and a minimum region of 150 kb and duplications of at least seven adjacent SNPs and a minimum region of 200 kb were evaluated [18], except for 3 families in which a prominent, but smaller duplication than 200 kb (although consisting of ≥10 adjacent SNP probes) was observed. The CNVs were classified into four groups: I, known pathogenic CNVs (known microdeletion or microduplication syndromes); II, potentially pathogenic CNVs, not described in the Database of Genomic Variants (DGV; The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada, http://projects.tcag.ca/variation/); III, CNVs not described in the DGV, but not containing any protein-coding genes; and IV, known polymorphic CNVs described in the DGV or observed in our in-house reference set, whereby at least three individuals must have been reported with the same rearrangement. Type IV CNVs were not further evaluated. All type II CNVs were assessed with Ensembl (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK, http://www.ensembl.org: Ensembl release 63 – June 2011) and the DECIPHER database (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) for gene and microRNA (miRNA) content and similar cases, respectively. If DNA from the parents was available, segregation analysis was performed by SNP array.

The type I CNVs were confirmed with multiplex ligation-dependent probe amplification
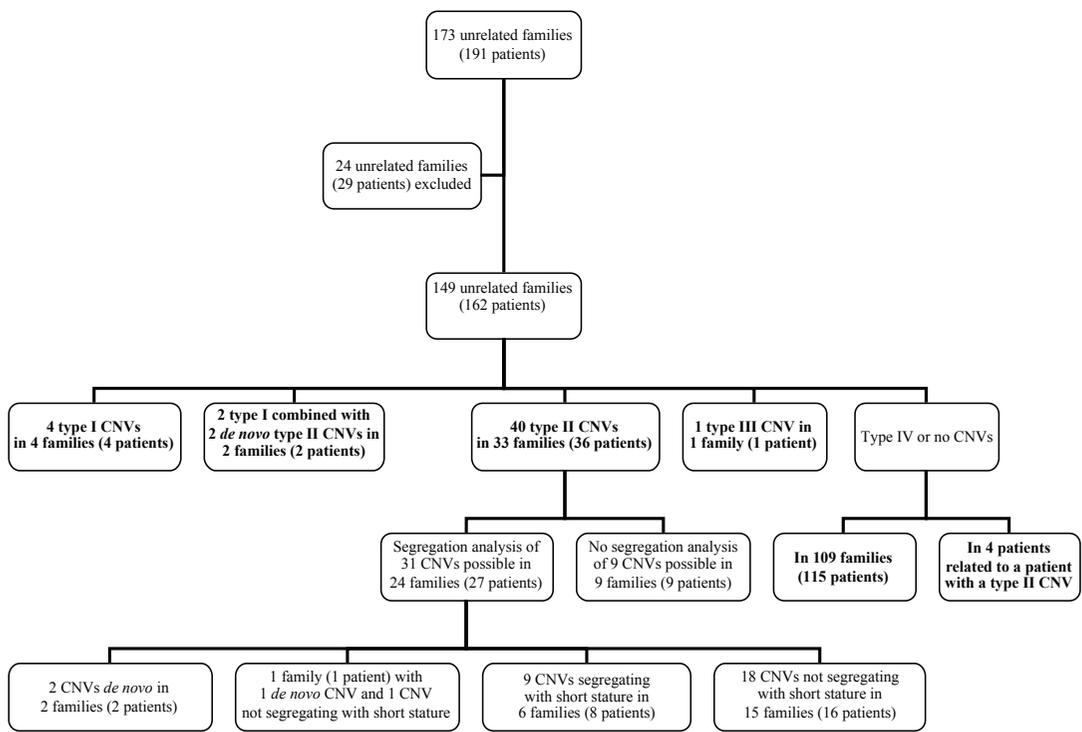
Figure 1 Organization Chart Organization chart illustrating the identified CNVs. The 149 unrelated families (162 patients) divided in the different subcategories are depicted in bold. A total of 49 CNVs were found in 40 families (43 patients).

(MLPA), using Salsa MLPA P018 probemix for *SHOX* and P217 for *IGF1R* analysis (MRC Holland, Amsterdam, The Netherlands). Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Fragment analysis was performed using GeneMarker (SoftGenetics, State College, USA). Thresholds for deletions and duplications were set at 0.75 and 1.25 respectively [19].

Bioinformatics approach

We checked for all CNVs whether they were located in one of the chromosomal regions associated with height in GWAS [1]. For genes in deleted or duplicated regions in cases with *de novo* CNVs, we used three additional approaches. First, the rodent homologues were checked for three criteria: 1) higher expression in 1 week old mouse growth plate than in 1 week old mouse lung, kidney, and heart; 2) spatial regulation: significant difference between zones in the 1 week old rat growth plate; and 3) temporal regulation: significant difference between 3 and 12 weeks of age in the rat growth plate using previously established mRNA expression profiles [20,21]. Second, associations were investigated for mouse growth plate-related phenotypes. Third, associations with human growth plate-related phenotypes were investigated. For details, see Lui *et al.* [21].

Results

CNVs

An organization chart illustrating the identified CNVs is shown in figure 1. In the 162 patients belonging to 149 unrelated families, a total of 49 CNVs were found in 40 families (43 patients).

In six families (4.0%, 6 patients) a type I CNV was observed and in two of them an additional *de novo* type II CNV. Table 1 shows the clinical and genetic findings of these 6 patients, including 2 microdeletions (I.1 and I.2) and 2 microduplications (I.3 and I.4) containing *SHOX*, and two terminal 15q deletions containing *IGF1R* (I.5/II.1/mi.3 and I.6/II.2). All these CNVs were confirmed with MLPA.

One or more type II CNVs (n = 40) were found in 33 unrelated families (22.1%, 36 patients). Five of these potentially pathogenic CNVs contained besides protein-coding genes also miRNAs (Table 2). In 24 families (27 patients) segregation analysis could be performed, which led to a total of 5 *de novo* CNVs (Table 3) and 9 CNVs segregating with a height below −1.5 SDS of a carrier family member (Table 4). For 19 CNVs the lack of segregation with short stature makes a causative role of the CNV unlikely (Supplementary Table 1). In 9 patients (9 CNVs) no information on segregation could be obtained (Supplementary Table 2). In two non-related patients (cases II.24 and II.25) a similar CNV (a deletion containing *DCAF12L2*, alias *WDR40C*) in the X-chromosome was identified, but both children inherited the deletion from a normal parent.

In one family (0.7%, 1 patient) a type III CNV was found encompassing a 192.3 Kb deletion of chromosome 13 (arr 13q31.1(86,733,645–86,925,974)×1). The girl (case III.1) was born SGA, had poor food intake and severe postnatal growth failure (length −8.2 SDS at 2.5 years). Screening for *IGF1* and the *IGF1R* for mutations or deletions was negative. The function of this region is unknown.

No potential pathogenic CNVs (only type IV or no CNVs) were found in 109 families (73.2 %, 119 patients).

Bioinformatics approach

Five CNVs encountered in our study are close to the loci associated with height in GWAS [1]. Four of these CNVs were *de novo* or segregating with short stature, including loci close to *ADAMTS17* (case II.5), *PRKG2/BMP3* (cases II.11 and II.13), *PAPPA* (cases II.11 and II.13) and *TULP4* (case II.7). However, none of the deletions included genes tightly linked ($r^2 < 0.5$) to a GWAS SNP implicated in human height variations. The fifth CNV is close to the *MKL2* locus (case II.37/mi.4) but did not segregate with short stature (Supplementary Table 1).

Table 1 Type I CNVs

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (Mb)	Protein-coding genes[a]	Additional CNV (type)	Known gene
I.1 F	−2.9	arr Xp22.33(1–1,522,908)×1 mat	1.32	9 protein-coding genes; from *PLCXD1* to *ASMTL*	–	*SHOX*
I.2 F	−4.0	arr Xp22.33(1–2,320,027)×1 dn	2.12	13 protein-coding genes; from *PLCXD1* to *DHRSX*	–	*SHOX*
I.3 M	−2.3	46,XY,t(8;13)(q13;q12).arr Xp22.33(1–727,565)×2 mat	0.52	*PLCXD1* *GTPBP6* *PPP2R3B* *SHOX*	–	*SHOX*
I.4 M	−2.8	46,X,psu idic(Y)(q11.22) dn.arr Yp11.32p11.31(1–2,640,827)×2 dn	2.49	16 protein-coding genes; from *PLCXD1* to *XG*	–	*SHOX*
I.5/II.1/mi.3 F	−3.1	arr 15q26.2q26.3(98,374,491–102,531,392)×1 dn	4.00	23 protein-coding genes; from *ARRDC4* to *OR4F15*	Gain 15q26.1q26.2 dn (Type II)	*IGF1R*
I.6/II.2 M	−5.9	arr 15q26.3(99,131,989–102,531,392)×1 dn	3.24	21 protein-coding genes; from *IGF1R* to *OR4F15*	Gain 9p24.3p24.2 dn (Type II)	*IGF1R*

dn = *de novo*; mat = maternally inherited; pat = paternally inherited.

a For CNVs containing ≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene is given.

Table 2 miRNAs

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (kb)	Protein-coding genes[a]	miRNA	Additional CNV (type)
II.19/mi.1 F	−4.6	arr 7q36.3(158,183,050−158,692,049)×3	509.0	PTPRN2 NCAPG2 ESYT2 WDR60	MIR595	–
II.32/mi.2 F	−2.8	arr 8p23.1(7,690,325−9,040,305)×3 pat, 8p23.1p22(2,242,033−13,046,661)×3 pat	8p23.1: 1,350.0 8p23.1p22: 804.6	Chr8p23.1: 10 protein-coding genes; from DEFB104A to PPP1R3B Chr8p23.1p22: FAM86B2 LONRF1 KIAA1456 DLC1	8p23.1: MIR548I3	–
I.5/II.1/mi.3 F	−3.1	arr 15q26.1q26.2(91,199,026−98,456,575)×3 dn	7 257.6	19 protein-coding genes; from BLM to SPATA8	MIR1469	Loss 15q26.2q26.3 (type I)
II.37/mi.4 F	−2.5	arr 16p13.12p13.11(14,760,735−16,633,360)×1 pat	1 872.6	17 protein-coding genes; from BFAR to NOMO3	MIR1972-1[b] MIR484[b]	–
II.22/mi.5 M	−2.1	arr 22q11.21(21,011,217−21,928,915)×1	917.7	16 protein-coding genes; from POM121L4P to UBE2L3	MIR649[b]	–

dn = *de novo*; mat = maternally inherited; pat = paternally inherited.

[a] For CNVs containing ≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene in the CNV are given.

[b] miRNA 484, 649, and 1972 have been predicted to bind to various isoforms of SHOX, accordingly contributing to the regulation of SHOX expression [45].

We reasoned that some of the identified CNVs might cause short stature because they contain genes that are expressed and function in the growth plate. We therefore used existing expression microarray data to identify genes that show greater expression in mouse growth plate than in soft tissues, temporal regulation in rat growth plate, or spatial regulation in rat growth plate. Within *de novo* CNVs, this approach implicated 5 genes (*Aldh1a3*, *Fam3c*, *Furin*, *Lrrk1*, and *Chsy1*), and within segregating CNVs, this implicated 7 genes (*Col14A1*, *Dscc1*, *Enpp2*, *Ezr*, *Prelid2*, *Taf2*, and *Trim32*) (Table 5). This information, in combination with other bioinformatic data, was used to formulate the arguments pro and contra an association of these genes with short stature (summarized in Tables 3 and 4). Potential candidate genes in *de novo* CNVs associated with short stature (Table 3) include *FURIN*, *DOCK8* and/or *KANK1*, *NLRP3*, *FAM3C*, *SLC13A1*, *ADAMTS17*, *ALDH1A3*, *LRRK1* and *CHSY1*. Potential candidate genes in CNVs segregating with short stature (Table 4) include *FHIT*, *PTPRG*, *TULP4*, *EZR*, *ENPP2*, *TAF2*, *COL14A1*, *DSCC1*, *LPPR1*, *ZNF675*, *C4orf22* (or *PRKG2/BMP3*), *PRELID2*, and *ASTN2* and *TRIM32* (or *PAPPA*).

For the CNVs for which insufficient information was available about segregation with short stature, the *in silico* analysis provided support for four potential candidate genes (*TBL1X*, *ROBO2*, *CHD8* and *TOX4*), as well as a candidate region (distal part of common 22q11 deletion syndrome) (Supplementary Table 2).

chapter
7

Table 3 *De novo* type II CNVs

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (Kb)	Protein-coding genes[a]	Arguments pro pathogenicity	Arguments against pathogenicity
I.5/II.1/ mi.3 F	−3.1	Type II: arr 15q26.2(91,199,026−98,456,575)×3 dn Type I: arr 15q26.2q26.3(98,374,491−102,531,392)×1 dn	7 257.6	19 protein-coding genes; from *BLM* to *SPATA8*, including MIR1469	*Furin* higher expressed in murine GP and upregulated from PZ to HZ.	*IGF1R* deletion can explain short stature [26].
I.6/II.2 M	−5.9	Type II: arr 9p24.3p24.2(1−2,612,433)×3 dn Type I: arr 15q26.3(99,131,989−102,531,392)×1 dn	2 612.4	9 protein-coding genes; from *FOXD4* to *SMARCA2*	2 short children with overlapping 9p duplication (*DOCK8* and *KANK1*, DECIPHER #256751 and #261831). Shorter than usual for IGF1R deletion [26].	*Dock8* and *Kank1* not overexpressed in murine GP.
II.3 M	−2.4	arr 1q44(246,715,197−247,652,602)×3 dn, 2q24.3(165,611,363−165,769,050)×3 pat	Chr1: 937.4 Chr2: 157.7	Chr1: 12 protein-coding genes; from *TFB2M* to *OR2B11* Chr2: *COBLL1* *SLC38A11*	Activating *NLRP3* mutations associated with short stature (NOMID). Constitutively activated *Nlrp3* in mice causes growth retardation [46].	*NLRP3* duplication described in three patients without short stature, with overlapping, smaller duplications inherited from a normal parent (DECIPHER #263423, #258032 and #253572), *Nlrp3* not overexpressed in murine GP.

				Protein-coding genes[a]		
II.4 M	−3.5	arr 7q31.31q31.32(119,770,125–123,600,606)×1 dn	3 830.5	21 protein-coding genes; from *KCND2* to *SPAM1*	*Fam3c* higher expressed in murine GP and downregulated from RZ to PZ. Homozygous mutations in *Slc13a1* in sheep and mice cause dwarfism [27,28].	Only 2 out of 9 patients with bigger overlapping deletions reported with short stature (DECIPHER).
II.5 M	−3.5	arr 15q26.3(101,003,122–102,374,592)×1 dn	1 371.5	13 protein-coding genes; from *CERS3* to *OR4F15*	*ADAMTS17* associated with height in human and dog (GWAS) [1,29]. Short child with overlapping 15q deletion (DECIPHER #251400). Mutations cause chondrodysplasia [30-33]. Associated with fibrillin-1 function [31,33]. *Aldh1a3* and *Lrrk1* higher expressed in murine GP; *Chsy1* highly expressed in HZ and downregulated with age.	Deletion is located 244 Kb downstream of the *ADAMTS17* locus.

dn = *de novo*; mat = maternally inherited; pat = paternally inherited.

GP = Growth Plate; RZ = Resting zone; PZ = Proliferative zone; HZ = Hypertrophic zone.

[a] For CNVs containing≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene in the CNV are given.

Table 4 Type II CNVs segregating with short stature

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (Kb)	Protein-coding genes[a]	Arguments pro pathogenicity	Arguments against pathogenicity
II.6 M	−2.0	arr 3p14.2(59,235,764–61,832,828)×3 pat	2 597.1	*FHIT* *PTRG*	*FHIT* acts as a repressor of beta-catenin transcriptional activity[34]. *PTPRG* possibly inhibits cell growth[35]. Height father −1.8 SDS.	Not overexpressed in murine GP.
II.7 M	−2.9	arr 6q25.3(159,026,380–159,929,652)×3 mat	903.3	8 protein-coding genes; from *TMEM181* to *FNDC1*	*TULP4* associated with height (GWAS)[1]. *Ezr* downregulated with age in murine GP.	Duplication is located 94 Kb downstream of the *TULP4* locus. Height mother −1.5 SDS.
II.8 M	−2.9	arr 8q24.12(120,463,609–121,849,380)×3 mat	1385.8	8 protein-coding genes; from *ENPP2* to *SNTB1*	*ENPP2* encodes for a lysophospholipase D, producing lysophosphatidic acid involved in cell proliferation[36]. *Enpp2* highly expressed in murine kidney and GP, and highly upregulated from PZ to HZ. *Tgf2* upregulated from PZ to HZ. *Col4a1* downregulated with age in murine GP and upregulated from RZ to PZ. *Dscc1* higher expressed in murine GP and downregulated from PZ to HZ.	Height mother −1.6 SDS.

II.9 F	–2.5	arr 9q31.1(103,493,752–104,059,876)×1 mat	566.1	LPPR1(PRG3)	*Prg1* knockout mice are small [37]. Maternal height –3.3 SDS. Height non-carrier maternal relative –0.1 SDS.	Height carrier maternal sister –1.7 SDS. Height non-carrier maternal relative –2.3 SDS. *LPPR1* not overexpressed in murine GP.
II.10 M	–3.7	arr 19p12(23,661,801–24,041,650)×1 pat	379.8	ZNF675 ZNF681 RPSAP58	*ZNF675* possibly involved in osteoclast differentiation [38]. Paternal height –2.6 SDS.	Not overexpressed in murine GP.
II.11[b] F	–3.0	arr 1p31.1(72,546,864–72,940,272)×1, 4q21.21(81,301,396–81,775,934)×1, 5q32(145,109,219–145,250,730)×3, 9q33.1(119,411,013–119,601,157)×1	Chr1: 393.4 Chr4: 494.4 Chr5: 141.5 Chr9: 190.1	Chr1: *NEGR1* Chr4: *C4orf22* Chr5: *PRELID2 GRXCR2* Chr9: *ASTN2 TRIM32*	*PRKG2/BMP3* (4q21.21) and *PAPPA* (9q33.1) associated with height (GWAS) [1]. *Prelid2* higher expressed in murine GP, downregulated with age and from PZ to HZ. *Trim32* downregulated from PZ to HZ.	4q21.21 Deletion is located 176 Kb upstream of the *PRKG2/BMP3* locus. 9q33.1 Deletion is located 246 Kb downstream of the *PAPPA* locus.
II.12[b] M	–2.0	arr 5q32(145,109,219–145,250,730)×3 mat	Chr5: 141.5	Chr5: *PRELID2 GRXCR2*		
II.13[b] M	–1.5	arr 4q21.21(81,301,396–81,775,934)×1 mat, 5q32(145,109,219–145,250,730)×3 mat, 9q33.1(119,411,013–119,601,157)×1 mat	Chr4: 494.4 Chr5: 141.5 Chr9: 190.1	Chr4: *C4orf22* Chr5: *PRELID2 GRXCR2* Chr9: *ASTN2 TRIM32*		

dn = *de novo*; mat = maternally inherited; pat = paternally inherited.

GP = Growth Plate; RZ = Resting zone; PZ = Proliferative zone; HZ = Hypertrophic zone.

[a] For CNVs containing ≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene in the CNV are given.

[b] Family; mother and 2 sons.

Table 5 Bioinformatic approach (mouse GP vs soft tissues expression, and spatial and temporal regulation of gene expression in the rat GP)

Gene	Growth Plate vs Soft Tissues (Mouse Array)						Growth Plate, 3 vs 12 wk; RZ vs PZ and PZ vs HZ at 1 wk (Rat Array)					
	GP vs Heart (FC)	p-value[a]	GP vs Kidney (FC)	p-value[b]	GP vs Lung (FC)	p-value[c]	3 vs 12 wk (FC)	p-value[d]	RZ vs PZ (FC)	p-value[e]	PZ vs HZ (FC)	p-value[f]
De novo CNVs												
Aldh1a3 [S]	15.9	<0.001	1.3	0.2	20.7	<0.001	1.3	0.2	1.0	0.7	**32.7**	**<0.001**
Fam3c [G,S]	**3.2**	**<0.001**	**3.0**	**<0.001**	**1.9**	**<0.001**	1.2	0.002	**-2.2**	**<0.001**	1.2	0.2
Furin [G,S]	**2.2**	**<0.001**	**1.9**	**<0.001**	**2.2**	**<0.001**	-1.4	0.03	-1.2	0.008	**1.6**	**<0.001**
Lrrk1 [S]	3.4	<0.001	2.2	<0.001	1.4	0.002	1.2	0.2	-1.4	0.02	**1.9**	**0.001**
Chsy1 [T,S]			no probe in mouse array				**-2.5**	**<0.001**	-1.2	0.3	**2.0**	**0.004**
Segregating CNVs												
Col4a1 [T,S]	-14.1	<0.001	-10.9	<0.001	-6.3	<0.001	**-2.5**	**<0.001**	**5.1**	**0.001**	-2.4	0.02
Dscc1 [G,S]	**2.4**	**<0.001**	**2.3**	**<0.001**	**2.0**	**<0.001**	1.2	0.2	-1.7	0.02	**-3.2**	**<0.001**
Enpp2 [S]	7.4	<0.001	-1.8	<0.001	2.3	<0.001	1.1	0.6	-1.2	0.4	**27.3**	**<0.001**
Ezr [T]	-6.8	<0.001	-15.4	<0.001	-9.1	<0.001	**-1.6**	**0.001**	-1.4	0.1	1.5	0.07
Prelid2 [G,T,S]	**2.4**	**<0.001**	**4.1**	**<0.001**	**6.0**	**<0.001**	**-2.8**	**<0.001**	1.1	0.3	**-1.9**	**<0.001**
Tdf2 [G,S]	**2.1**	**<0.001**	**1.6**	**<0.001**	**1.5**	**<0.001**	-1.2	0.1	1.0	1.0	**2.2**	**0.005**
Trim32 [S]	-2.2	0.007	-2.7	0.001	-1.9	0.02	1.2	0.2	-1.3	0.1	**-2.0**	**0.002**

GP = Growth Plate; FC = Fold change; RZ = Resting zone; PZ = Proliferative zone; HZ = Hypertrophic zone.

[a] P<0.0048 considered statistically significant (False Discovery Rate (FDR) <0.01).

[b] P<0.0048 considered statistically significant (FDR<0.01).

[c] P<0.0047 considered statistically significant (FDR<0.01).

[d] P<0.0042 considered statistically significant (FDR<0.05).

[e] P<0.0017 considered statistically significant (FDR<0.05).

[f] P<0.0086 considered statistically significant (FDR<0.05).

[G] Growth plate specific gene, defined as expression in GP vs Soft Tissue ≥ 1.5–fold and FDR<0.01 for all three soft tissues.

[T] Temporally regulated gene, defined as 3 vs 12 wk ≥ ±1.5–fold, FDR<0.05.

[S] Spatially regulated gene, defined as RZ vs PZ ≥ ±1.5–fold, FDR<0.05; and/or PZ vs HZ ≥ ±1.5–fold, FDR<0.05.

Genes fulfilling the criteria mentioned above are depicted in bold.

Discussion

Whole genome SNP array analysis in 162 patients with short stature from 149 unrelated families (Fig. 1) led to the detection of type I CNVs known to cause short stature (involving *SHOX* or *IGF1R*) in six families (in two of them combined with type II CNVs), and 40 potentially pathogenic CNVs (type II) in 33 families. Out of the total of 42 type II CNVs, five were *de novo* and nine others were associated with short stature in their families. In one severely short child a deletion without protein-coding genes was found, and in 5 CNVs 6 microRNAs were encountered.

A recent study on a genome-wide association analysis of copy-number variation and stature showed that children with short stature had a greater global burden of lower-frequency and rare deletions and a greater average CNV length than controls [22]. There were no significant associations with tall stature. These observations suggest that CNVs might contribute to genetic variation in stature in the general population. These authors also identified three preliminary candidate regions as having significant associations with stature; a duplication at 11q11 and deletions at 14q11.2 and 17q21.31. In our analysis these regions all display common CNVs, which have been often observed in our in-house database and in the DGV (type IV CNVs).

The two patients carrying a heterozygous deletion containing the *SHOX* gene had disproportionate short stature, but no Madelung deformity. Case I.1 (sitting height/height (SH/H) ratio +3.7 SDS) inherited the deletion from her mother, who also had disproportionate short stature (height –1.8 SDS, SH/H ratio +4.2 SDS). Case I.2 (SH/H ratio +3.8 SDS) carries besides a *de novo SHOX* haploinsufficiency also a heterozygous unclassified variant (UV) in the *IGFALS* gene (c.1555C>T, p.Arg519Trp) inherited from her father (height –1.1 SDS). IGFALS sequencing was performed because of a low circulating IGF-I and IGFBP-3 despite elevated GH secretion. While the referring physician had not suspected Leri-Weill syndrome, in retrospect the increased sitting height/height ratio would have been sufficient reason to directly test for *SHOX* defects. The two patients in whom a duplication of the *SHOX* gene including surrounding genes was observed (*de novo* and inherited via a normal statured parent, respectively), had a sitting height/height ratio of approximately +1.9 SDS. We and others have recently reported that a phenotype similar to Leri-Weill syndrome (including short stature) can be associated with *SHOX* duplication [11,23,24].

In two patients a heterozygous deletion on chromosome 15 containing the *IGF1R* gene was identified, a well-established cause of short stature [11,25,26]. In both patients an additional *de novo* CNV was present (Table 3). In case I.5/II.1/mi.3 this was a duplication in 15q26.1q26.2 (located upstream of the deleted area). Although this patient's growth failure is similar to that of other patients with IGF1R defects [26], duplication of *FURIN* may play an additional

role. In case I.6/II.2, considerably shorter than usual for *IGF1R* deletions [26], the terminal 15q deletion was combined with a terminal 9p24.3p24.2 duplication, suggesting the presence of an unbalanced reciprocal translocation. We suspect that one of the parents is a carrier of a balanced 9;15 translocation, but unfortunately parental chromosomes were not available for testing. The presence of two patients in the DECIPHER database with a similar 9q duplication and short stature suggests that there may be an association between the genes *DOCK8* and *KANK1*, and stature.

Bioinformatics analysis of the three other cases with *de novo* type II CNVs led to several candidate genes (Table 3). In case II.3 a duplication of *NLRP3* may be associated with short stature. The CNV in case II.4 (who has besides short stature also mental retardation, behavioral problems, strabismus, and various dysmorphic features) suggests that *FAM3C* and *SLC13A1* deletions may be associated with short stature, particularly because of the expression data of *Fam3c* in the murine growth plate and the dwarfism and skeletal deformities in Texel sheep and mice with loss of function of *Slc13a1* [27,28].

Case II.5, with a terminal *de novo* 15q deletion located 1.5 Mb downstream of *IGF1R* and 244 Kb downstream of the *ADAMTS17* locus on the reverse strand, had a normal birth size, but showed proportionate progressive growth failure (SH/H ratio +1.58 SDS) with a normal head circumference. Clinical characteristics included slight frontal bossing of the skull, a high pitched voice and slight abdominal adiposity and delayed bone age. GH secretion and circulating IGF-I were normal, but IGFBP-3 was low (–2 SDS). Several arguments are in favor of a role of *ADAMTS17* in growth regulation (for summary, see Table 3), including: 1) significant association with height in population GWAS [1]; 2) a short child with a similar terminal deletion in the DECIPHER database; 3) significant association with size in a GWAS in the domestic dog [29]; 4) human mutations in *ADAMTS17* causing the acromelic chondrodysplasia Weill-Marchesani-like syndrome (OMIM #277600 and #608328) [30-33]; and 5) association of members of the ADAMTSL/ADAMTS family with the modulation of fibrillin-1 function [31,33]. Unfortunately, expression of the rodent homologue of *ADAMTS17* could not be investigated, because the gene was not represented on the microarrays used. Besides *ADAMTS17*, this deletion contains three other genes, *ALDH1A3*, *LRRK1* and *CHSY1*, that might be implicated in short stature.

Nine CNVs in 6 families (5 families with one index patient each, and one family consisting of a mother and her 2 sons) segregated with a height of less than –1.5 SDS of a carrier family member (Table 4). The 3p duplication that case II.6 (height –2.0 SDS) inherited from his father (–1.8 SDS) contains *FHIT* and the first part of *PTPRG*. Both genes are considered tumor suppressors [34,35]. The 6q duplication that case II.7 inherited from his mother is located nearby (97 Kb downstream) a locus (*TULP4)* associated with height [1]. One of the duplicated genes (*ENPP2*) in case II.8 encodes for a lysophospholipase D, producing lysophosphatidic

acid (LPA) inducing cell proliferation [36]. The mouse homologs of *TAF2*, *COL14A1* and *DSCC1* are differentially expressed in the growth plate. In case II.9, the 9q deletion containing part of *LPPR1* (also known as *PRG3*) did not fully segregate with short stature in the family, but the observation that *Prg1* knockout mice are smaller compared to wild type littermates [37] suggests a role for this gene in height regulation. The 19p deletion that case II.10 inherited from his father includes *ZNF675*, associated with osteoclast differentiation [38]. Out of the four CNVs in cases II.11, 12 and 13 (the short members of one family), *C4orf22*, *ASTN2* and *TRIM32* are located close to loci (374 KB upstream *PRKG2/BMP3* and 289 Kb downstream *PAPPA*, respectively) associated with height [1], suggesting that the 4q and/or 9q deletion are associated with stature.

Four out of nine patients in whom no segregation analysis could be performed (Supplementary Table 2) carry a CNV suggestive for an association with short stature. One of the genes in the duplication of case II.14 is *TBL1X* (alias *TBL1*), encoding for transducin beta-like protein 1 (TBL1). TBL1 and its highly related family member *TBLR1* are required for Wnt-beta-catenin-mediated transcription [39]. Case II.17, described previously [12], carries a duplication of 3p12.3 containing part of *ROBO2*, as well as his younger brother (height −4.3), but his mother (height −3.6 SDS) does not carry the variant, while no DNA is available from the father. The encoded protein is a receptor for SLIT2 and probably SLIT1, which are thought to function in axon guidance and cell migration [40]. Case II.21 was born SGA, and at 1.2 years her length was −3.7 SDS and head circumference −3.1 SDS. Further clinical characteristics include clinodactily, a protruded tongue and delayed bone age. The mother does not carry this duplication, and DNA from her father is not available. A search in the DECIPHER database revealed 2 patients with (partially) overlapping duplications, one of whom was short (patient #258583) and one was not (#258497). Out of the 6 genes outside the overlapping region with patient #258497 *CHD8* and *TOX4* appear potential candidate genes [41,42]. Case II.22/mi.5 has a 22q deletion containing only the distal part of the common 22q11 deletion syndrome (Velocardiofacial/DiGeorge syndrome). His mother does not carry the duplication, and DNA from the father is not available. In 8 patients in the DECIPHER database with overlapping deletions short stature was observed. The common deleted region contains *PI4KA*, *SERPIND1*, *SNAP29*, *CRKL*, *AIFM3*, *LZTR1*, *THAP7*, and *P2RX6*.

Although non-coding DNA can play an important regulatory role [43,44], no supportive evidence could be obtained on a possible role of novel type III CNVs. Similarly, none of the 6 miRNAs (Table 2) identified in the type II CNVs could be directly linked to short stature, due to lack of segregation with short stature (data not shown), although miRNA 484, 649, and 1972 have been predicted to bind to various isoforms of SHOX, and contribute to the regulation of SHOX expression [45].

In conclusion, whole genome SNP array analysis in this exploratory study on 162 patients

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with short stature belonging to 149 unrelated families identified 6 CNVs in 6 families (4%) for which the association with short stature is virtually certain, and 40 CNVs in 33 families (22.1%) with possible pathogenicity. Several of the deleted or duplicated genes may be considered as potential candidate genes for growth disorders, including four genes associated with height in the genome-wide association studies (*ADAMTS17*, *PRKG2/BMP3*, *PAPPA*, *TULP4*). Future studies are needed to support the role of these and other genes in longitudinal growth regulation.

Acknowledgments

Reference List

1   Lango Allen H, Estrada K, Lettre G *et al*: Hundreds of variants clustered in genomic loci and biological pathways affect human height. *Nature* 2010; **467**: 832-838.

2   Yang J, Benyamin B, McEvoy BP *et al*: Common SNPs explain a large proportion of the heritability for human height. *Nat Genet* 2010; **42**: 565-569.

3   Cirulli ET, Goldstein DB: Uncovering the roles of rare variants in common disease through whole-genome sequencing. *Nat Rev Genet* 2010; **11**: 415-425.

4   Kant SG, Kriek M, Walenkamp MJ *et al*: Tall stature and duplication of the insulin-like growth factor I receptor gene. *Eur J Med Genet* 2007; **50**: 1-10.

5   van Duyvenvoorde HA, Kempers MJ, Twickler TB *et al*: Homozygous and heterozygous expression of a novel mutation of the acid-labile subunit. *Eur J Endocrinol* 2008; **159**: 113-120.

6   van Duyvenvoorde HA, van Setten PA, Walenkamp MJ *et al*: Short stature associated with a novel heterozygous mutation in the insulin-like growth factor 1 gene. *J Clin Endocrinol Metab* 2010; **95**: E363-E367.

7   Vidarsdottir S, Walenkamp MJ, Pereira AM *et al*: Clinical and biochemical characteristics of a male patient with a novel homozygous STAT5b mutation. *J Clin Endocrinol Metab* 2006; **91**: 3482-3485.

8   Walenkamp MJ, Karperien M, Pereira AM *et al*: Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. *J Clin Endocrinol Metab* 2005; **90**: 2855-2864.

9   Walenkamp MJ, van der Kamp HJ, Pereira AM *et al*: A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the insulin-like growth factor I receptor. *J Clin Endocrinol Metab* 2006; **91**: 3062-3070.

10   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.

11 Caliebe J, Broekman S, Boogaard M *et al*: IGF1, IGF1R and SHOX mutation analysis in short children born small for gestational age and short children with normal birth size (idiopathic short stature). *Horm Res Paediatr* 2012; **77**: 250-260.

12 Wit JM, van Duyvenvoorde HA, Scheltinga SA *et al*: Genetic analysis of short children with apparent growth hormone insensitivity. *Horm Res Paediatr* 2012; **77**: 320-333.

13 Clayton PE, Cianfarani S, Czernichow P, Johannsson G, Rapaport R, Rogol A: Management of the child born small for gestational age through to adulthood: a consensus statement of the International Societies of Pediatric Endocrinology and the Growth Hormone Research Society. *J Clin Endocrinol Metab* 2007; **92**: 804-810.

14 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.

15 Fredriks AM, van Buuren S, Burgmeijer RJ *et al*: Continuing positive secular growth change in The Netherlands 1955-1997. *Pediatr Res* 2000; **47**: 316-323.

16 Fredriks AM, van Buuren S, Jeurissen SE, Dekker FW, Verloove-Vanhorick SP, Wit JM: Height, weight, body mass index and pubertal development reference values for children of Turkish origin in the Netherlands. *Eur J Pediatr* 2003; **162**: 788-793.

17 Nannya Y, Sanada M, Nakazaki K *et al*: A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res* 2005; **65**: 6071-6079.

18 Hehir-Kwa JY, Egmont-Petersen M, Janssen IM, Smeets D, van Kessel AG, Veltman JA: Genome-wide copy number profiling on high-density bacterial artificial chromosomes, single-nucleotide polymorphisms, and oligonucleotide microarrays: a platform comparison based on statistical power analysis. *DNA Res* 2007; **14**: 1-11.

19 Lalic T, Vossen RH, Coffa J *et al*: Deletion and duplication screening in the DMD gene using MLPA. *Eur J Hum Genet* 2005; **13**: 1231-1234.

20 Lui JC, Andrade AC, Forcinito P *et al*: Spatial and temporal regulation of gene expression in the mammalian growth plate. *Bone* 2010; **46**: 1380-1390.

chapter

7

21 Lui JC, Nilsson O, Chan Y *et al*: Synthesizing genome-wide association studies and expression microarray reveals novel genes that act in the human growth plate to modulate height. *Hum Mol Genet* 2012; **21**: 5193-5201.

22 Dauber A, Yu Y, Turchin MC *et al*: 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.

23 Benito-Sanz S, Barroso E, Heine-Suner D *et al*: Clinical and molecular evaluation of SHOX/PAR1 duplications in Leri-Weill dyschondrosteosis (LWD) and idiopathic short stature (ISS). *J Clin Endocrinol Metab* 2011; **96**: E404-E412.

24 Iughetti L, Capone L, Elsedfy H *et al*: Unexpected phenotype in a boy with trisomy of the SHOX gene. *J Pediatr Endocrinol Metab* 2010; **23**: 159-169.

25 Ester WA, van Duyvenvoorde HA, de Wit CC *et al*: Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype. *J Clin Endocrinol Metab* 2009; **94**: 4717-4727.

26 Klammt J, Kiess W, Pfaffle R: IGF1R mutations as cause of SGA. *Best Pract Res Clin Endocrinol Metab* 2011; **25**: 191-206.

27 Zhao X, Onteru SK, Piripi S *et al*: In a shake of a lamb's tail: using genomics to unravel a cause of chondrodysplasia in Texel sheep. *Anim Genet* 2012; **43 Suppl 1**: 9-18.

28 Markovich D: Slc13a1 and Slc26a1 KO models reveal physiological roles of anion transporters. *Physiology (Bethesda)* 2012; **27**: 7-14.

29 Hoopes BC, Rimbault M, Liebers D, Ostrander EA, Sutter NB: The insulin-like growth factor 1 receptor (IGF1R) contributes to reduced size in dogs. *Mamm Genome* 2012.

30 Khan AO, Aldahmesh MA, Al-Ghadeer H, Mohamed JY, Alkuraya FS: Familial spherophakia with short stature caused by a novel homozygous ADAMTS17 mutation. *Ophthalmic Genet* 2012; **33**: 235-239.

31 Le Goff C, Cormier-Daire V: The ADAMTS(L) family and human genetic disorders. *Hum Mol Genet* 2011; **20**: R163-R167.

32 Morales J, Al-Sharif L, Khalil DS *et al*: Homozygous mutations in ADAMTS10 and ADAMTS17 cause lenticular myopia, ectopia lentis, glaucoma, spherophakia, and short stature. *Am J Hum Genet* 2009; **85**: 558-568.

33 Sengle G, Tsutsui K, Keene DR *et al*: Microenvironmental regulation by fibrillin-1. *PLoS Genet* 2012; **8**: e1002425.

34 Weiske J, Albring KF, Huber O: The tumor suppressor Fhit acts as a repressor of beta-catenin transcriptional activity. *Proc Natl Acad Sci U S A* 2007; **104**: 20344-20349.

35 Wang Z, Shen D, Parsons DW *et al*: Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 2004; **304**: 1164-1166.

36 van Meeteren LA, Moolenaar WH: Regulation and biological activities of the autotaxin-LPA axis. *Prog Lipid Res* 2007; **46**: 145-160.

37 Trimbuch T, Beed P, Vogt J *et al*: Synaptic PRG-1 modulates excitatory transmission via lipid phosphate-mediated signaling. *Cell* 2009; **138**: 1222-1235.

38 Shin JN, Kim I, Lee JS, Koh GY, Lee ZH, Kim HH: A novel zinc finger protein that inhibits osteoclastogenesis and the function of tumor necrosis factor receptor-associated factor 6. *J Biol Chem* 2002; **277**: 8346-8353.

39 Li J, Wang CY: TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 2008; **10**: 160-169.

40 Yue Y, Grossmann B, Galetzka D, Zechner U, Haaf T: Isolation and differential expression of two isoforms of the ROBO2/Robo2 axon guidance receptor gene in humans and mice. *Genomics* 2006; **88**: 772-778.

41 Thompson BA, Tremblay V, Lin G, Bochar DA: CHD8 is an ATP-dependent chromatin remodeling factor that regulates beta-catenin target genes. *Mol Cell Biol* 2008; **28**: 3894-3904.

42 Lee JH, You J, Dobrota E, Skalnik DG: Identification and characterization of a novel human PP1 phosphatase complex. *J Biol Chem* 2010; **285**: 24466-24476.

chapter

7

43 Shabalina SA, Spiridonov NA: The mammalian transcriptome and the function of non-coding DNA sequences. *Genome Biol* 2004; **5**: 105.

44 Stamoulis C: Estimation of correlations between copy-number variants in non-coding DNA. *Conf Proc IEEE Eng Med Biol Soc* 2011; **2011**: 5563-5566.

45 Durand C, Roeth R, Dweep H *et al*: Alternative splicing and nonsense-mediated RNA decay contribute to the regulation of SHOX expression. *PLoS ONE* 2011; **6**: e18115.

46 Bonar SL, Brydges SD, Mueller JL *et al*: Constitutively activated NLRP3 inflammasome causes inflammation and abnormal skeletal development in mice. *PLoS ONE* 2012; **7**: e35979.

Supplemental Tables

Supplementary Table 1 Type II CNVs with lack of segregation with short stature

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (kb)	Protein-coding genes[a]	miRNA
II.23 M	−2.1	arr Xq22.3(105,062,645→105,739,894)×2	677.2 Kb	*NRK* *SERPINA7* *MUM1L1*	-
II.24 F	−2.7	arr Xq25(125,001,006→125,318,345)×1 pat	317.3	*DCAF12L2 (WDR40C)*	-
II.25 M	−4.5	arr Xq25(125,263,911→125,536,426)×0 mat	272.5	*DCAF12L2*	-
II.26 M	−2.4	arr 1q25.1(173,834,684→174,140,227)×1 mat	305.5	*ZBTB37* *SERPINC1* *RC3H1* *RABGAP1L*	-
II.27 F	−8.5	arr 1q43q44(243,546,954→243,821,364)×1 mat	274.4	*SDCCAG8* *AKT3*	-
II.3 M	−2.4	arr 1q44(246,715,197→247,652,602)×3 dn, 2q24.3(165,611,363→165,769,050)×3 pat	Chr1: 937.4 Chr2: 157.7	Chr1: 12 protein-coding genes; from *TFB2M* to *OR2B11* Chr2: *COBLL1* *SLC38A11*	-
II.28 F	−4.7	arr 2p25.3(1,101,473→1,742,700)×3 pat	641.2	*SNTG2* *TPO* *PXDN*	-
II.29 F	−3.2	arr 2q31.1(169,703,120→170,064,498)×3 mat, 2q31.1(172,454,934→172,657,695)×3 mat	Gain: 361.4 Gain2: 202.8	Gain: 6 protein-coding genes; from *NOSTRIN* to *LRP2* Gain2: *DYNC1I2* *SLC25A12*	-
II.30 M	−1.4	arr 5q33.2(152,375,974→153,460,042)×3 mat, 7p14.1(40,298,879→40,528,146)×1 mat	Chr5: 1,084.1 Chr7: 229.3	Chr5: *GRIA1* *FAM114A2* *MFAP3* Chr7: *C7orf10*	-
II.31 F	−2.6	arr 7q34q35(142,491,575→143,864,670)×3 mat	1,373.1	29 protein-coding genes; from *EPHB6* to *OR2A4*	-

II.32/mi.2 F	−2.8	arr 8p23.1(7,690,325–9,040,305)×3 pat, 8p23.1p22(12,242,033–13,046,661)×3 pat	8p23.1: 1,350.0 8p23.1p22: 804.6	Chr8p23.1: 10 protein-coding genes; from *DEFB104A* to *PPP1R3B* Chr8p23.1p22: *FAM86B2* *LONRF1* *KIAA1456* *DLC1*	8p23.1: MIR548I3
II.33 F	−2.9	arr 9q31.3(111,555,994–111,711,514)×1 mat	155.5	*ACTL7B* *ACTL7A* *IKBKAP* *C9orf6* *CTNNAL1*	-
II.34 F	−3.0	arr 9q34.13q34.2(134,789,097–136,484,291)×3 mat	1,695.2	31 protein-coding genes; from *MED27* to *FAM163B*	-
II.35[b] F	−2.1	arr 15q24.2q24.3(76,314,543–76,727,022)×3 mat	412.5	*NRG4* *C15orf27* *ETFA* *ISL2* *SCAPER*	-
II.36[b] F	−3.4	arr 15q24.2q24.3(76,314,543–76,727,022)×3 mat	412.5	*NRG4* *C15orf27* *ETFA* *ISL2* *SCAPER*	-
II.37/ mi.4 F	−2.5	arr 16p13.12p13.11(14,760,735–16,633,360)×1 pat	1,872.6	17 protein-coding genes; from *BFAR* to *NOMO3*	MIR1972-1 MIR484
II.38 F	−2.5	arr 16q12.1(49,088,824–49,615,386)×3 mat	526.6	*CBLN1* *C16orf78* *ZNF423*	-

a For CNVs containing ≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene in the CNV are given. b Family; 2 sisters.

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Supplementary Table 2 Type II CNVs with no information about segregation

ID M/F	Height (SDS)	Karyotype (ISCN 2009)	Size (Kb)	Protein-coding genes[a]	miRNA	Arguments pro pathogenicity
II.14 F	−4.1	arr Xp22.2(9,594,546−10,046,186)×3	451.6	TBL1X GPR143 SHROOM2 WWC3	–	*TBL1X* required for WNT signalling
II.15 M	−4.7	arr 1q24.2(169,296,745−169,474,353)×3	177.6	NME7 BLZF1 C1orf114 SLC19A2	–	–
II.16 F	−2.5	arr 3p24.3(21,756,554−22,231,872)×3	475.3	ZNF385D	–	–
II.17 M	−5.2	arr 3p12.3(76,153,037−77,581,256)×3	1,428.2	ROBO2	–	Brother carrying same CNV short (−4.3 SDS); function in axon guidance and cell migration
II.18 M	−4.6	arr 7q21.11(80,071,944−80,768,261)×3	696.3	CD36 GNAT3 SEMA3C	–	–
II.19/mi.1 F	−4.6	arr 7q36.3(158,183,050−158,692,049)×3	509.0	PTPRN2 NCAPG2 ESYT2 WDR60	MIR595	–
II.20 F	−4.4	arr 9p23(9,503,942−10,018,120)×3	514.2	PTPRD	–	–
II.21 F	−3.7	arr 14q11.2(21,530,059−22,022,116)×3	492.1	13 protein-coding genes; from NDRG2 to SALL2	–	*CHD8* negative regulator of Wnt. *TOX4* involved in cell cycle progression
II.22/mi.5 M	−2.1	arr 22q11.21(21,011,217−21,928,915)×1	917.7	16 protein-coding genes; from POM121L4P to UBE2L3	MIR649	Distal part of common 22q11 deletion syndrome

[a] For CNVs containing ≤ 5 protein-coding genes, all protein-coding genes are depicted. For CNVs containing ≥ 6 protein-coding genes, the number, and the first and last protein-coding gene in the CNV are given.

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Part D

Combined whole genome approach and functional studies

8

A novel activating mutation in the kinase homology domain of natriuretic peptide receptor-2 causes extremely tall stature without skeletal deformities

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Abstract

C-type natriuretic peptide (CNP)/natriuretic peptide receptor 2 (NPR2) signalling is essential for long bone growth. In response to CNP, NPR2 synthesizes cyclic GMP, which stimulates proliferation, growth and differentiation of chondrocytes. Homozygous and compound heterozygous inactivating *NPR2* mutations cause acromesomelic dysplasia, Maroteaux type, a severe form of short-limbed dwarfism. Here we describe a novel heterozygous *NPR2* mutation (c.1963C>T, p.Arg655Cys) in an extremely tall (221 cm) male proband. CNP stimulated cyclic GMP production by the mutant NPR2 was markedly increased in skin fibroblasts of the proband as well as in transfected HEK 293 cells. Furthermore, the stimulatory effects of ATP on CNP-dependent guanylate cyclase activity were markedly enhanced, suggesting that this novel mutation enhances both the responsiveness of NPR2 to CNP and its allosteric modulation/stabilization by ATP. Coimmunoprecipitation showed that wildtype and mutant NPR2 can form stable heterodimers, suggesting a dominant positive effect. In accordance with augmented endogenous receptor activity, plasma concentration of NTproCNP (a marker of CNP production in tissues) was reduced in the proband. In summary, we report the first activating mutation within the kinase homology domain of NPR2, emphasizing the important role of this domain in the regulation of guanylate cyclase activity and bone growth by CNP.

Introduction

Natriuretic peptide receptor 2 (NPR2), also known as NPR-B or guanylate cyclase B, and encoded by the gene *NPR2*, is one of five human transmembrane receptors that catalyse the formation of cyclic GMP (cGMP) from GTP. These receptors consist of an extracellular ligand binding domain (ECD), a short membrane-spanning region and intracellularly a kinase homology domain (KHD), a helical hinge region and the C-terminal cGMP-synthesizing guanylate cyclase (GC) domain. *NPR2* is expressed by different types of cells (e.g. chondrocytes, cardiomyocytes, neurons, fibroblasts and vascular smooth muscle cells), in different tissues (e.g. in bone, brain, heart, vasculature, lung, ovary) [1]. Its ligand is C-type natriuretic peptide (CNP), a paracrine hormone of the natriuretic peptide family. In the absence of CNP, NPR2 exists as a homodimer or homotetramer, and ligand binding does not lead to further aggregation [1,2]. Multiple extra- and intracellular domains mediate this oligomerization. The intracellular oligomerization interface region has been mapped to the amphipathic sequence that bisects the KHD and GC domains [1]. Based on structural studies of the homologous protein NPR1 (also known as NPR-A or guanlyate cylcase A, the receptor for atrial natriuretic peptide (ANP), which exhibits 78% sequence identity to NPR2 [3]) it is postulated that CNP-induced rotation of the ECD is transmitted across the membrane and reorients the two intracellular domains into the active conformation, thereby enabling cGMP synthesis.

Studies in genetic mouse models revealed that CNP-NPR2-cGMP signalling is essential for stimulation of endochondral ossification required for long bone growth, for oocyte maturation and for sensory axon bifurcation in the spinal cord [4-6]. In humans, homozygous and compound heterozygous inactivating *NPR2* mutations cause acromesomelic dysplasia, Maroteaux type (AMDM) [7], and heterozygous inactivating mutations have been associated with short stature [8]. Enhanced production of CNP caused by chromosomal translocations results in tall stature, a Marfanoid phenotype and skeletal abnormalities [9,10]. Concordantly, an activating *NPR2* mutation located in the GC domain also causes skeletal overgrowth [11].

Here we describe a novel heterozygous activating *NPR2* mutation located within the KHD, in a proband with extremely tall stature but with only mild skeletal deformities. We show an increased cGMP response of the mutant NPR2 to CNP in the proband's skin fibroblasts and in transfected HEK 293 cells, and suppressed markers of tissue CNP production in the proband's serum, in accordance with increased NPR2 activity *in vivo*.

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Materials and methods

GH and prolactin secretion studies

The healthy volunteers of the present study participated in previous studies on pituitary hormone secretory dynamics; testing conditions were previously described [12, 13]. Blood samples (2.0 ml) were withdrawn at 10-min intervals for 24 h, and stored at -20°C for hormone measurements. Plasma GH concentrations were measured with a sensitive time-resolved fluoroimmunoassay (Wallac, Turku, Finland). The assay is specific for 22-kDa GH and was previously described in detail [14]. Plasma prolactin concentrations were measured with a sensitive time-resolved fluoroimmunoassay (Wallac Oy, Turku, Finland), details were previously described [13]. Hormone concentration time series were analyzed via a recently developed automated deconvolution method, empirically validated using hypothalamo-pituitary sampling and simulated pulsatile time series [15, 16].

DNA analysis

Genomic DNA was isolated from peripheral blood samples using the AUTOPURE LS Instrument (Gentra Systems). Cytogenetic microarray analysis was performed using the Affymetrix CytoScan HD Array according to the manufacturer's procedures. Copy number was assessed in the proband using ChAS software (Chromosome Analysis Suite) (Affymetrix, California, USA). Whole exome sequencing was performed on DNA fragmented into 200-400 bp fragments using Covaris Adaptive Focused Acoustics (AFA) shearing according to the manufacturer's instructions (Covaris, Inc., Woburn, MA). The exome was captured by Nimblegen SeqCap EZ V2 kit (Roche Nimblegen, Inc., Madison, WI) in combination with Illumina paired end library preparation and 2x 100 bp sequencing with at least 70x mean coverage. Downstream analyses included demultiplexing (CASAVA software, Illumina), sequence quality control, capture quality control, SNP calling and indel (insertions and deletions) calling using different software applications as described by Santen et al [17]. The *NPR2* mutation was confirmed by Sanger sequencing using standard procedures (primer sequences available on request).

Site-directed mutagenesis and intracellular cGMP responses of transfected HEK 293 cells

The expression plasmid encoding a FLAG-tagged NPR2 Arg655Cys mutant protein was generated by PCR-mediated mutagenesis using FLAG-tagged NPR2 in pFLAG-CMV1 [18]. Two oligonucleotides were synthesized; Arg655Cys-forward: 5'- cc aac tgt gtg gtg gat agt tgc ttt gtg ctc aaa ata aca g -3' and Arg655Cys-reverse: 5'- ct gtt att ttg agc aca aag caa cta tcc acc aca cag ttg g -3' (the underlined nucleotides represent the site of Arg655Cys mutation), to change residue arginine$_{655}$ to cysteine. Mutagenesis was performed using a Quick-Change site-directed mutagenesis kit (Agilent Technologies). The mutation and the absence

of unwanted mutations were verified by sequencing. HEK 293 cells were transiently transfected with the FLAG- or Myc-tagged wildtype or mutant NPR2 expression constructs using FuGene (Roche). Transfected cells were serum-starved for 4 h prior to CNP exposure (48 h after transfection). Cells were pretreated with 0.1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 15 min, and then exposed to various concentrations of CNP (Bachem, Heidelberg, Germany) for another 10 min. Intracellular cGMP contents were determined by radioimmunoassay [19, 20].

Guanylate cyclase assays

GC activity of crude membranes prepared from transfected HEK 293 cells was essentially assessed as described before [19]. Activity assays were carried out in 50 mM HEPES buffer, pH 7.4, containing 50 mM NaCl, 5% glycerol, 0.05% BSA, 1 mM IBMX, 2 mM GTP, 30 mM creatine phosphate, 1.5 U/ml creatine phosphokinase, and different concentrations of ATP (as indicated in the results section) [19]. To stimulate GC activity, membranes (20 µg protein) were incubated with 0.01 to 1000 nM CNP (ligand dependent activity), or with 1% (v/v) Triton X-100 (detergent stimulated, maximal activity) during 10 min [19]. cGMP formation was measured by radioimmunoassay [19, 20]. The basal and CNP-stimulated cGMP responses were calculated as percentage of the maximal, Triton-stimulated activity. NPR2 expression levels in the cytosol versus membrane fractions were assessed by Western blotting using an antibody against the FLAG epitope (Cell Signaling).

Isolation and culture of human skin fibroblasts and cGMP determinations

Skin biopsies were taken from the proband and a control donor, and a culture of dermal fibroblasts was established as previously described [21]. Experiments were performed with cells with a passage number ranging between 5 and 7 in mitogen-free, serum-reduced DMEM (0.5% fetal calf serum during 3 h prior to experimentation). Cells were pretreated with 0.1 mM IBMX for 15 min, and then exposed to CNP (0.1-1000 nM) for another 10 min. Intracellular cGMP contents were determined by RIA [19, 20].

Coimmunoprecipitation and Western blot analyses

For co-expression of FLAG-tagged wildtype NPR2 and Myc-tagged mutant NPR2, the FLAG (DYKDDDDK) and Myc (EQKLISEEDL) epitopes were positioned directly after the cleavage site of the NPR2 signal peptide by PCR-mediated mutagenesis. HEK 293 cells were prepared in 10 cm dishes and (co)transfected with 10 µg plasmid as described above. After 48 h cells were lysed at 4°C for 30 min in 250 µl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.5), with protease and phosphatase inhibitors (Roche). After centrifugation (2000 g, 10 min, 4°C), the supernatant was incubated with 25

µl pre-equilibrated anti-FLAG M2 affinity gel beads (Sigma) at 4°C for 2 h. The beads were washed three times, resuspended in 100 µl of electrophoresis sample buffer (200 mM Tris·HCl, pH 6.8, 6% (w/v) SDS, 15% glycerol, 10% ß-mercaptoethanol, 0.01% bromophenol blue) and boiled for 10 min. For Western blot analyses, protein samples were resolved by 10% SDS-PAGE. Electrophoresis and immunoblotting were performed as previously described [20]. Antibodies were anti-FLAG (Cell Signaling) or anti-Myc (Bioscience).

Modeling of the NPR2 mutant Arg655Cys

The full-length model of NPR2 was built from homology models of the individual domains on the basis of structure templates for the NPR2 ECD (protein data bank (PDB) entries 1DP4, 1T34, crystal structure of free and ligand-bound NPR1), the KHD (2EVA, 3P86, 2PHK) and the GC domain (3ET6). Since no structural templates are available for the linker regions, secondary structure prediction was applied using the amino acid sequence. A *de novo* modeling approach was applied to connect the individually modeled domains, ECD, KHD and GC. As the structure of the linker domains is purely speculative, the full-length model serves only representative purposes and was not used to predict or propose any activation mechanism. The most suited templates for modeling of the individual domains were identified by Basic Local Alignment Search Tool (BLAST) searches using the amino acid sequence of the individual domains (KHD and GC) and the PDB (http://www.rcsb. org). For modeling of the KHD, the best match of the BLAST search (PDB entry 3P86, crystal structure of the Crt1 kinase, a member of the raf-like kinase family, [22]) was used to obtain a 3D model. In addition, to minimize model bias, structures of kinases with lower E-values and amino acid sequence identity (i.e. PDB entries 2EVA: Kinase TAK1, a member of the MAPKKK family [23], and 2PHK: Phosphorylase kinase-γt [24]) were also used to build models for the KHD of NPR2. The model for the GC domain of NPR2 was obtained using the crystal structure of a soluble eukaryotic guanylyl cyclase, which was shown to exist as homodimer and is specific for cGMP (PDB entry 3ET6). The models were obtained either from automatic modeling using the SWISS-MODEL server [25] providing a sequence alignment and the structure template or by manually exchanging residues of the structure template using a multiple sequence alignment of target and template sequences using the software Quanta2008 (MSI Accelrys). Deletion and insertions between target and template molecule were modeled by manual model building. Sequence alignments were made using the software CLUSTALW.

CNP, ANP and BNP Assays

Plasma ANP, BNP, CNP and NTproCNP, following extraction over C18 SepPac cartridges (Waters Corp., Milford, MA), were measured by RIA as previously described [26, 27]. Recoveries

for CNP22 and NTproCNP over SepPac cartridges were 98% and 108% respectively. Crossreactivity of human BNP (at 100 pmol/L) in the CNP assay is approximately 4%. CNP concentrations were not corrected for BNP crossreactivity. Crossreactivity of CNP and human BNP in the NTproCNP assay was <0.05% for both.

Data Analysis
Statistical comparisons were done using Student's t test ($p < 0.05$). Data are given as mean ± S.E.

Results
Case - clinical description and investigations
Growth and skeletal phenotype. The proband was born at term with a weight of 4 kg (+ 1 standard deviation score (SDS)) and a length of 62 cm (+ 6 SDS). He continued to be tall in infancy, childhood and adolescence. Psychomotor development was normal but puberty started late, at the age of approximately 15 yr. At age 16 yr his height was 216 cm; on X-ray his epiphyses were 'wide open' but the exact bone age was not documented. He underwent epiphysiodesis of the distal femur and proximal tibia and fibula. His adult height is 221 cm (+ 5.2 SDS), sitting height is 107.8 cm (+3.7 SDS), sitting height/height ratio 0.49 (-1.3 SDS), arm span 232 cm, weight 155 kg, and head circumference 60 cm (+1.2 SDS) (Figure 1A). He has suffered several fractures, mainly due to sports accidents, and one metatarsal fracture after a long hike. Physical examination showed long fingers and mild thoracic kyphosis (Figures 1B and C). He did not have particularly long halluces (Figure 1D) or a Marfanoid habitus, which were described in the previously reported cases with increased CNP production or an activating *NPR2* mutation [9-11]. A skeletal survey demonstrated mild scoliosis and secondary degenerative changes of the skeleton, coxa valga with slightly flat femoral heads and mild right hip dysplasia (Figures 1E-I). Bone mineral density was normal (Z-score lumbar spine +1.9, hips +1.6 and +1.4, as determined by dexa scan (Hologic Discovery A)).

Cardiovascular system. Chronic venous insufficiency of the lower extremities was present since the age of 36 yr. Blood pressure was 130/80 mmHg, with a regular pulse. Echocardiography showed normal cardiac size (corrected for BMI) and contractile functions, with the exception of paroxysmal atrial fibrillation.

Endocrinology. Non-insulin dependent diabetes mellitus (NIDDM) was diagnosed at age 53 yr and is well controlled with metformin. Testicular volumes were normal, with a cystic abnormality of 4 mm in the left testis and a solitary microcalcification in the right testis on ultrasound.
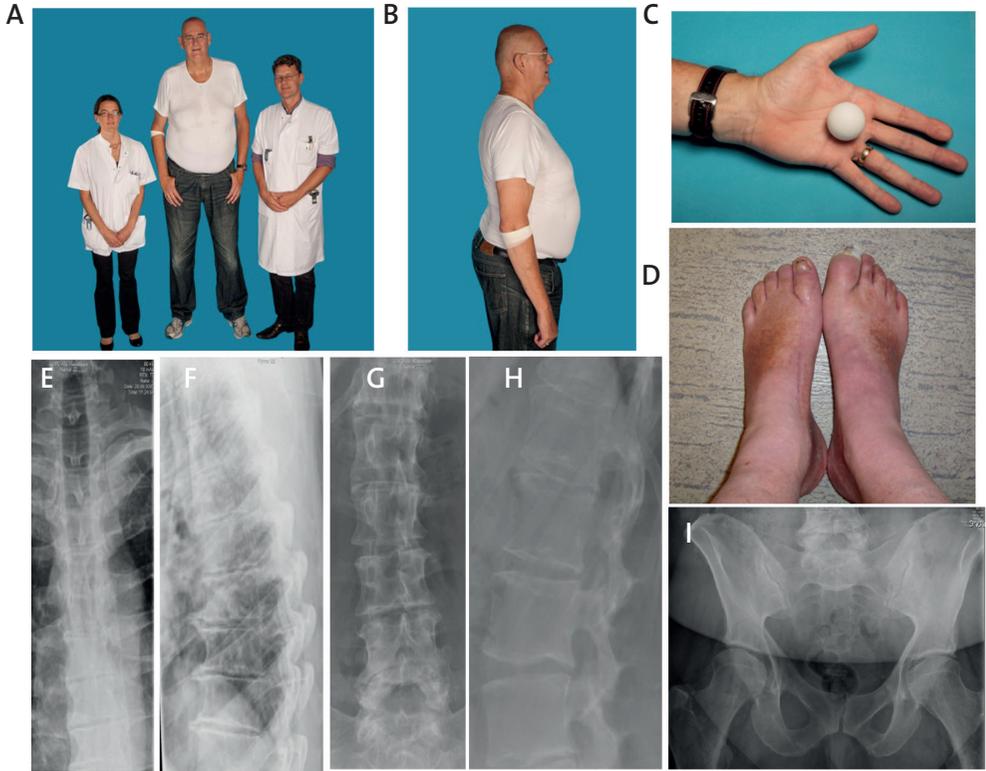
Figure 1 Clinical features of the proband

(A-D) Photographs of the proband, showing no Marfanoid habitus. **(A)** Proband with two of the authors (SEH, 173 cm, AMP, 192 cm). **(B)** Lateral photograph showing increased thoracic kyphosis and lumbar lordosis. **(C)** Proband's hand, holding a ping-pong ball for reference, showing long fingers. **(D)** Proband's feet, halluces are not particularly long. **(EF)** Posterioanterior and lateral radiographs of the thoracic spine. (GH) Posterioanterior and lateral radiographs of the lumbar spine, showing mild scoliosis. **(I)** Pelvic radiograph showing coxa valga, slightly flat femoral heads, mild right hip dysplasia and degenerative changes. The proband gave permission for publication of these photographs.

He has bilateral gynaecomastia and serum prolactin was elevated on two occasions. At age 55 yr the 24 h growth hormone (GH) and prolactin secretion rates were quantified showing low-normal GH secretion compared to control subjects (nine healthy males aged 47-61 yr, BMI 21-31 kg/m^2) (Supplemental figure 1A). Both basal and pulsatile prolactin secretion, however, were markedly increased with a total secretion of 679 µg/l distribution volume per 24 h compared to 183 µg/l distribution volume per 24 h (range 99-325) in the controls (Supplemental figure 1B and Table 1 of supplemental data). The only medication used at the time of sampling was lactulose (to treat constipation), which is not known to alter prolactin secretion. Although a recent prolactin level, at age 61 yr, was within the normal range (Table 1), this does not necessarily indicate that prolactin secretion has normalised since the serum levels were not constantly elevated during the 24 h profile either. Levels of other relevant hormones were in the normal range (Table 1).

Neurology. Axonal sensorimotor polyneuropathy was diagnosed at age 41 yr and was later attributed to NIDDM.

Identification of *NPR2* mutation and microdeletion on chromosome 15q

Because the tall stature was not due to GH overproduction and the proband did not show features of any known syndrome, a SNP array analysis was performed. This showed a heterozygous microdeletion in chromosome 15 (karyotype: array 15q25.2q25.3(85,084,470-85,724,984)x1 (Human Genome 19)), containing 8 genes (*ZSCAN2*, *WDR73*, *NMB*, *SEC11A*, *ZNF592*, *ALPK3*, *SLC28A1* and *PDE8A)*, encoding the following proteins: zinc finger and SCAN domain-containing protein 2, WD repeat-containing protein 73, neuromedin-B, signal peptidase complex catalytic subunit SEC11A, zinc finger protein 592, alpha-kinase 3, sodium/nucleoside cotransporter 1 and cAMP-specific phosphodiesterase 8A, respectively). Sequencing of this region on the remaining allele showed no mutations. Since this microdeletion cannot explain the tall stature, we subsequently performed whole exome sequencing and identified a heterozygous c.1963C>T mutation in the *NPR2* gene, predicted to exchange arginine at position 655 to cysteine within the KHD of NPR2. The mutation and microdeletion were not found in the proband's sister or son, who are not as tall as the proband (Figure 2). Unfortunately, we were unable to investigate other family members.

Site-directed mutagenesis shows that the substitution Arg655Cys enhances the guanylate cyclase activity of NPR2

We tested the function of the mutant NPR2$_{Arg655Cys}$ by transfecting HEK 293 cells and measuring cGMP production under baseline conditions and after stimulation with CNP.

Table 1 Blood biochemistry tests

Parameter	Age 55 yr	Age 61 yr	Unit	Normal range
Urea		10.7	mmol/l	2.5-7.5
Creatinin	58	70	µmol/l	64-104
SGOT	35	38	U/l	0-35
SGPT	32	52	U/l	0-45
Calcium	2.25	2.46	mmol/l	2.15-2.55
Phosphate	0.83	0.88	mmol/l	0.9-1.5
Alkaline phosphatase	79	87	U/l	0-115
P1NP	36		ng/ml	<59
Beta crosslaps		0.17	ng/ml	<0.704
HbA1c	6.3		%	4.3-6.3
HbA1c		45	mmol/mmol Hb	20-42
Cholesterol	5.19	4.86	mmol/l	3.9-7.3
Triglycerides	1.88	1.03	mmol/l	0.8-2.3
TSH	2.96	1.99	mU/l	0.3-4.8
FT4	14.3	15.5	pmol/l	10-24
IGF-I	15.3 (+0.5 SDS)	28.5 (+3.1 SDS)	nmol/l	7-83
IGFBP-3	1.6	3.8	mg/l	1.5-6
Prolactin	17.1	8.1	µg/l	4-15
Cortisol (8:00)	0.422		µmol/l	0.1-0.6
Androstenedione	4.1	1.7	nmol/l	2-10
DHEA-S	3.09	1.7	µmol/l	2-15
FSH	4.1	6.9	U/l	2-10
LH	3.2	4.6	U/l	2-9
Testosterone	9.1	13	nmol/l	8-31
Estradiol		71	pmol/l	70-200
Estrone		227	pmol/l	100-200
AMH		1.6	µg/l	5.1-9.1
Inhibin B		112	ng/l	150-400
ANP		32.8	pmol/l	4-27
BNP		7.8	pmol/l	3-12
CNP		0.9	pmol/l	0.5-1.2
NTproCNP		8.1	pmol/l	12-25
cGMP		5.8	nmol/l	2.5-7.7
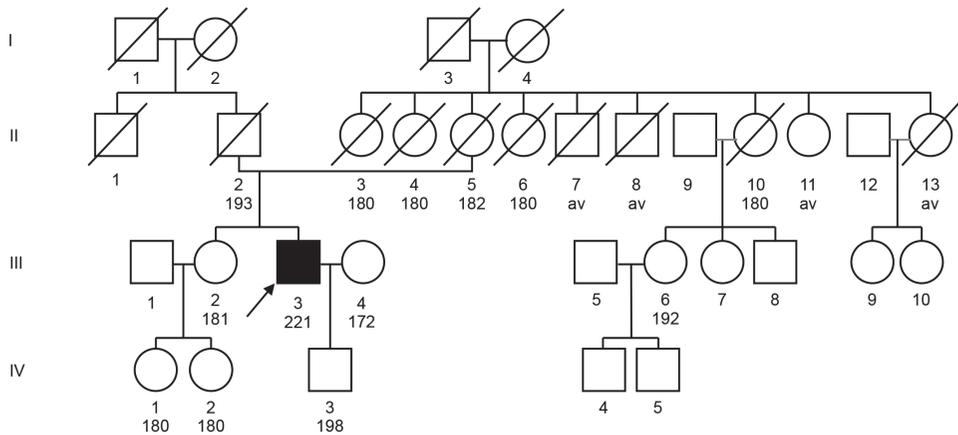
Figure 2 Family pedigree

The proband, III-3, is indicated with an arrow. The *NPR2* mutation and microdeletion on chromosome 15 were absent in his sister and son, other family members have not been investigated. Heights of family members (in cm), as reported by the proband, are indicated below the symbols. Heart valve pathology is indicated with hatching. Av = average height.

Transfection with cDNA encoding the FLAG-tagged mutant NPR2 produced equivalent amounts of membrane protein, compared with FLAG-tagged wildtype NPR2, suggesting that the mutation does not alter protein synthesis and membrane localization (see Western blots depicted in Figures 3A and B). However, basal cGMP contents of cells expressing mutant NPR2 were ~2.1 ± 0.3-fold higher in comparison with cells expressing wildtype NPR2 (n = 3). Intracellular cGMP levels were increased by CNP in a concentration-dependent manner, and these responses were much greater in cells expressing the mutant protein as compared to cells expressing wildtype NPR2 (Figure 3A). Intriguingly, co-expression of wildtype and mutant NPR2 resulted in cGMP responses to CNP which were almost as high as those observed with mutant NPR2 alone (Figure 3A).

Additionally, the effect of this amino acid substitution on NPR2 activity was evaluated in guanylate cyclase assays performed with crude membranes from transfected HEK 293 cells expressing either the wildtype or the mutated receptor, or both (Figure 3B). The membranes were incubated with CNP, and cGMP formation was measured by RIA [19]. Assays were performed in the presence of 2 mM ATP to mimic cytoplasmic ATP levels, which are in the range of 1-10 mM [28]. Again we confirmed, by immunoblotting, that mutant and wildtype FLAG-tagged NPR2, and their combination, were expressed in similar amounts (see inset

of Figure 3B). In addition, to account for small differences in the expression level of the two variants, we normalized the CNP-stimulated activity data to the respective maximal, Triton-stimulated NPR2 activity [19]. Wildtype NPR2 responded to CNP with a concentration-dependent increase in cGMP production (Figure 3B). In comparison, CNP-stimulated cGMP production by membranes containing mutant NPR2 or both mutant and wildtype NPR2 was markedly enhanced (Figure 3B).

Wildtype NPR2 can form heterodimers with mutant NPR2

Although the NPR2 contains a single cyclase catalytic site per polypeptide chain, a dimeric receptor assembly is essential for the activation of the catalytic domain [1]. The introduction of the Arg655Cys mutation in individual subunits could therefore lead to the formation of hyperfunctional dimers. The observation that co-expression of wildtype and mutant NPR2 resulted in cGMP responses to CNP close to those observed with mutant NPR2 alone suggested this "dominant positive" effect. Indeed, co-immunoprecipitation of FLAG-tagged wildtype NPR2 and Myc-tagged mutant NPR2 confirmed a tight interaction of both proteins (Figure 3C).

Arg655Cys substitution results in increased activity of native NPR2 in skin fibroblasts

To study the mutant NPR2 in primary cells we used cultured fibroblasts harvested and expanded from a skin biopsy from the proband's forearm. Intracellular cGMP levels were compared to levels in control skin fibroblasts obtained from a healthy 48 yr-old male donor with a height of 192 cm. Basal cGMP contents were similar in proband and control fibroblasts but the responses to CNP were markedly increased in the former (Figure 4A). In contrast, the cGMP responses to ANP, which are mediated by NPR1, were not different between proband and control fibroblasts (Figure 4B). This confirms increased CNP-stimulated activity of the mutant NPR2 not only when transfected into HEK 293 cells but also when naturally expressed in fibroblasts.
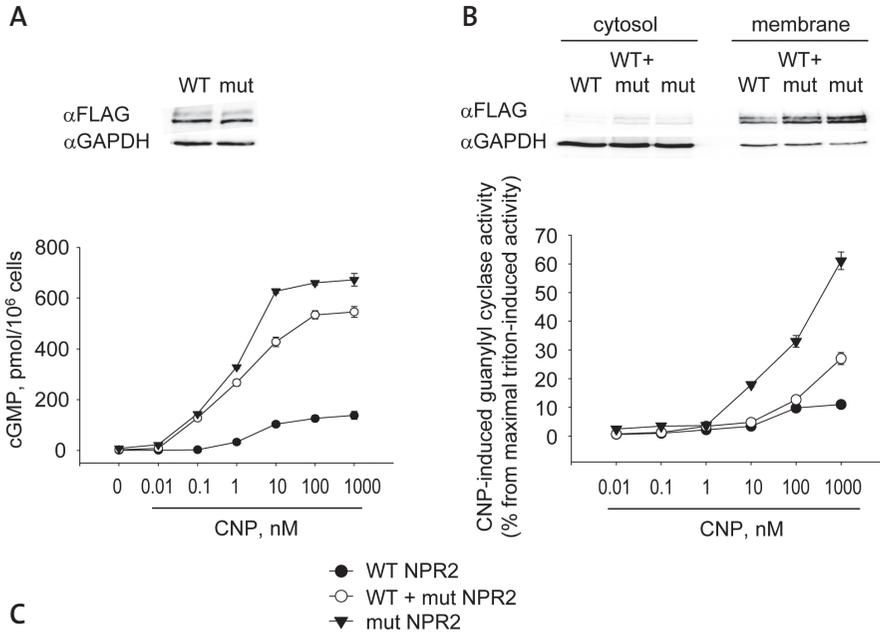
Figure 3 Increased cGMP responses to CNP of HEK 293 cells expressing mutant NPR2

(A) HEK 293 cells expressing FLAG-tagged wildtype (WT) or mutant (mut) NPR2, or both, were incubated with CNP (10 pM – 1 mM, 10 min). Whole cell cGMP contents were quantified by RIA (n=3 per condition). **(B)** Crude membranes prepared from HEK 293 cells expressing FLAG-tagged wildtype (WT) NPR2, mutant NPR2 (mut), or both, were incubated with vehicle, CNP or detergent (1% Triton X-100). cGMP production was measured by RIA in [fmol cGMP·(µg protein)−1·min−1]. All values were calculated as *X*-fold of the maximal Triton-induced activity (n=4 per condition). *Insets in A,B*: Western blots demonstrating similar expression levels of WT and mutated NPR2 in transfected HEK 293 cells (all 50 µg protein per lane). **(C)** Heterodimer formation of wildtype and mutant NPR2. Extracts from cells transfected with FLAG-NPR2 and pCMV5 (Mock), cells with FLAG-NPR2 and Myc-tagged mutant-NPR2, or cells with pCMV5 and Myc-tagged mutant NPR2 were immunoprecipitated (IP) with anti (α)-FLAG M2 affinity gel beads (Sigma). Aliquots of cell lysates (before IP: input) as well as the IP proteins were analyzed by Western blot and subsequent FLAG- and Myc-specific immunodetection. Results show that Myc-tagged mutant NRP2 is precipitated with FLAG-tagged wildtype NPR2, indicating interaction between these two isoforms. Similar data were obtained in two independent experiments.
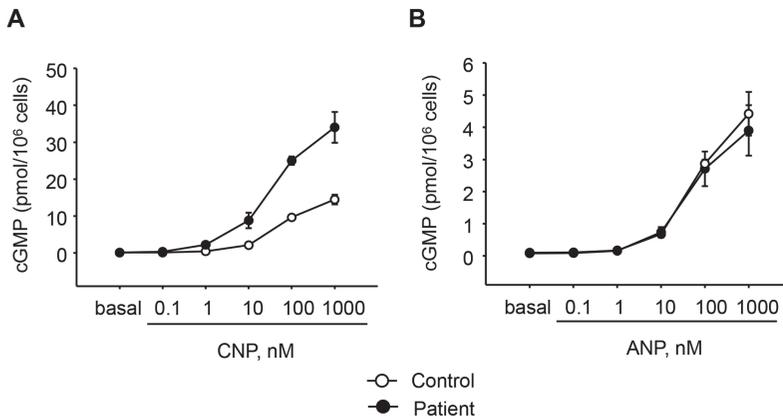
Figure 4 Increased effects of CNP on cGMP levels of proband´s skin fibroblasts
Proband and control fibroblasts were incubated with vehicle, CNP or ANP (both 0.1 nM – 1 mM) for 10 min, and intracellular cGMP contents were determined by RIA (n=8). (**A**) In comparison to the control fibroblasts, the CNP-induced cGMP-responses of the proband's fibroblasts were markedly increased. (**B**) The responses to ANP remained unaltered.

Homology modeling studies

As mentioned above, in contrast to previously published mutations directly affecting the GC domain, the novel mutation that we describe here is localized within the KHD of NPR2. Although the KHD binds ATP [29], kinase activity has never been demonstrated [30]. Modeling indeed revealed that although all elements required for ATP binding are present [31], the lack of kinase activity is likely linked to the loss of the conserved aspartate in the activation loop which is replaced by a serine (Ser644) in NPR. This abstracts the proton from the phospho-group acceptor required for the γ-phosphate transfer from ATP [32]. However, experiments by Duda *et al.* have shown that ATP binding to the KHD is coupled to a conformational rearrangement which possibly results in allosteric modulation of the GC domain [31].

To obtain insights into the molecular mechanism by which the Arg655Cys substitution influences NPR2 activation, we performed homology modeling to derive 3D models of the different protein domains (Figure 5). First a model of the mutation-carrying KHD was prepared (Figure 5A). Several different structure templates of protein kinases from the protein structure data bank RCSB were used to minimize structural bias of the KHD model towards a particular kinase template. In all models prepared, residue Arg655 is located at the solvent-accessible surface of the KHD (Figure 5B and C). The residue shares no direct contact with the ATP binding pocket or the dynamic linker connecting the upper and lower lobe of the kinase (Figure 5B and C). Thus, our modeling study suggests that the mutation does not directly influence ATP binding. Furthermore, an influence of the mutation Arg→Cys on protein folding seems unlikely due to the solvent-exposed position of Arg655.
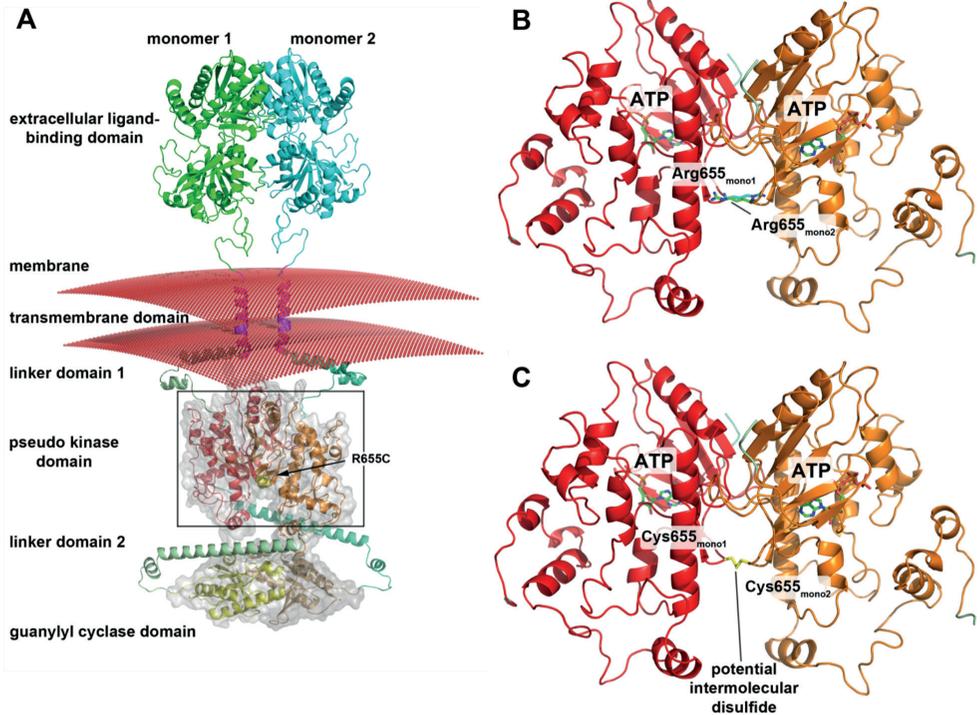
Figure 5 Homology modeling

(**A**) A theoretical model of NPR2 consisting of the ECD connected to the KHD and GC domains via a single transmembrane domain. The structurally characterized domains were modeled on the basis of structure templates obtained from the PDB databank, the linker regions were built on the basis of secondary structure predictions. Thus the full-length model only serves illustrative purposes. The area within the rectangle is shown in more detail in figures B and C. (**B**) A 3D model of the KHD of wildtype NPR2. The dimer assembly was obtained by docking the monomeric model structure of KHD onto the kinase structure of Crt1 (PDB entry 3P86). The side chain of arginine at position 655 (indicated as sticks) is located close to the dimer interface thereby possibly restraining the conformational rearrangement of the dimer assembly due to steric and electrostatic forces. (**C**) Replacement of the bulky, positively charged arginine side chain by a small and neutral cysteine could potentially relieve these restraints. Alternatively, the close proximity of two Cys655 residues in a homodimeric NPR2 mutant assembly might allow the formation of an intermolecular disulfide bridge leading to a semiactivated receptor.

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Arg655Cys substitution enhances the ATP-dependent activation of NPR2

To test whether the mutation of Arg655 to cysteine influences the modulatory effect of ATP on cyclase activity, we performed CNP concentration-response assays in the absence and presence of three different concentrations of ATP (10, 100 and 1000 nM). NPR2 activity was evaluated in guanylate cyclase assays performed with crude membranes prepared from transfected HEK 293 cells. Again we confirmed that mutant and wildtype FLAG-tagged NPR2 receptors were expressed in similar amounts (see Figure 6, upper part) and we normalized the CNP-stimulated activity to the maximal, Triton-stimulated NPR2 activity [19]. In the absence of ATP, the cGMP-responses of wildtype NPR2 to CNP were small: 1.1-fold (in response to 1 nM CNP), 2-fold (10 nM CNP) and 6-fold (100 nM CNP) increases of baseline activity. Basal and CNP-stimulated activities of mutant NPR2 were only slightly enhanced. More precisely, baseline activity of mutant NPR2 was 2-fold higher as compared to wildtype NPR2. In response to CNP baseline cGMP production raised 3-fold (1 nM CNP), 4.6-fold (10 nM CNP) and 6-fold (100 nM CNP) (n=4 per condition). In the absence of CNP, ATP (10-1000 nM) barely influenced cyclase activities of the wildtype and mutant receptors (Figure 6). However, increasing concentrations of ATP enhanced the ligand-dependent activation of wildtype and mutant NPR2. Notably, ATP increased the potency of CNP much more for the mutant as compared to the wildtype receptor (Figure 6).

Decreased level of NT-proCNP in proband's plasma

To assess whether increased NPR2 activity *in vivo* leads to reduced production of its ligand we measured the proband´s plasma levels of the CNP prohormone (NTproCNP) and the processed peptide (CNP) by respective radioimmunoassays. When renal function is normal, plasma NTproCNP level is a better reflection of CNP production in tissues than plasma CNP [33, 34]. While the concentration of NTproCNP was diminished (8.1 pmol/l, which is 3.3 SD below the mean for a subject of his age and sex; normal range in healthy donors of the same age: 12-25.0 pmol/l [34]), the plasma CNP concentration was normal (0.9 pmol/l; reference range 0.5-1.2 pmol/l). Plasma cGMP concentration in the same sample was within normal limits (5.8 nmol/l, normal range 2.5-7.7 nmol/l). Plasma ANP was mildly elevated (32.8 pmol/l, normal 4-27) and brain natriuretic peptide (BNP) level was 7.8 pmol/l (normal 3-12 pmol/l).
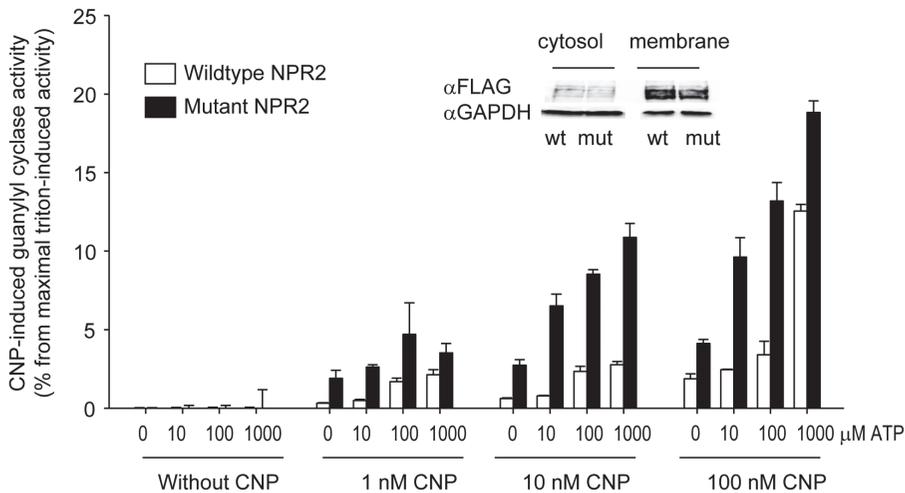
Figure 6 Enhanced modulatory effect of ATP on CNP-dependent guanylyl cyclase activity of mutant NPR2

GC activity was determined in crude membranes prepared from HEK 293 cells (expressing wildtype or mutant NPR2) in the absence or presence of ATP (10-1000 nM) and the indicated concentrations of CNP (n=4). *Inset:* Western blots demonstrating similar membrane location and expression of WT and mutated NPR2 in transfected HEK 293 cells (all 50 μg protein per lane).

Discussion

We identified a novel heterozygous *NPR2* mutation in a proband with extremely tall stature. *In vitro* assays with transfected HEK 293 cells showed that the NPR2 variant Arg655Cys can form stable heterodimers with the wildtype receptor and responds to CNP with markedly enhanced cGMP production. *Ex vivo* studies with the proband's fibroblasts confirmed that the endogenously expressed NPR2 variant mediates markedly enhanced CNP/cGMP signalling. Because this pathway is critically involved in bone development by stimulating growth plate chondrocyte differentiation and proliferation [4] and an activating *NPR2* mutation was previously found to result in tall stature [11], we conclude that the Arg655Cys mutation is responsible for the observed skeletal overgrowth in the proband.

Reduced production of CNP, as reflected by low NTproCNP level in the proband's plasma, is consistent with increased NPR2 activity *in vivo*. Low NTproCNP levels were also reported in two of the three Japanese patients with an activating mutation in the GC domain of

NPR2 [11] and are opposite to the marked increase in plasma CNP levels in subjects with loss of function mutations in *NPR2* [8, 34]. Together, these findings suggest the presence of a negative feedback mechanism whereby increased receptor activity leads to decreased CNP production and vice versa. NPR2-mediated cGMP production in response to CNP was markedly increased in the proband's skin fibroblasts, suggesting that the responsiveness of the mutant NPR2 expressed in the bone and many other tissues of the proband is also enhanced. Nevertheless, plasma cGMP concentrations were normal. However, in our experience even very high plasma levels of active CNP, achieved by intravenous infusion of synthetic peptide, have little if any effect on plasma cGMP levels in healthy controls [35]. Compared with cGMP increments evoked by equimolar concentrations of ANP and BNP, those induced by CNP were less than 10%. Other studies showed that plasma cGMP is mainly derived from vascular endothelial cells, which express high levels of NPR1 (the ANP receptor) but very low levels of NPR2 [36]. Hence, it is not surprising that plasma cGMP concentrations were normal in the proband.

The tall stature (height +5.2 SDS) is more severe in this case compared to the reported cases in the Japanese family with an activating mutation within the GC domain of NPR2 (height +2.7 to +3.8 SDS) [11], especially considering the fact that the proband would have been even taller without epiphysiodesis (his arm span of 232 cm suggests that without intervention adult height would have been approximately 230 cm). Of note, excessive length was already evident at birth. Other features, however, like a Marfanoid phenotype and skeletal deformities were much less pronounced than in the Japanese patients and in patients with CNP overproduction [9-11]. What causes this difference in phenotype is currently unclear; it might be related to the location of the mutation within the NPR2 (KHD versus GC domain) or perhaps to the different genetic background. Importantly, the findings in the proband imply that an activating *NPR2* mutation should be considered as a possible cause of isolated tall stature without dysmorphic features or skeletal deformities and could be screened for by measuring NTproCNP, provided renal function is normal.

How does the heterozygous Arg655Cys substitution within the conserved region of the KHD enhance NPR2 activity? As mentioned above, the function of this domain is not well understood. Although being inactive with respect to phosphorylating substrates [30], it binds ATP and modulates the enzymatic activity of the distal GC domain [37]. Furthermore, several studies showed that in the absence of ligand the KHD represses the activity of the GC domain [2, 30]. NPR activation possibly follows a ligand-induced rearrangement mechanism [38] and ATP binding to the KHD seems to allosterically modulate the ligand-stimulated catalytic activity of the GC domain [29, 37].

As illustrated in Figures 5B and C, homology modeling of the KHD suggests that Arg655 is solvent-exposed and does not affect ATP binding. For these modeling studies we used

different kinase structure templates, some of which form dimeric assemblies in the crystal. Sequence comparisons indicated that the KHD of NPR2 showed the highest homology (33% identity, 51% similarity on amino acid sequence level) to the serine/threonine kinase Ctr1 (constitutive triple response 1 kinase), which is a member of the raf-like kinases [22]. In the crystal structure Ctr1 has a dimeric assembly with a rather large interface between the two kinase subunits. An identical dimer architecture is also seen in crystal structures of the Raf-kinases C-Raf and B-Raf indicating that the dimer arrangement is of functional relevance. The monomeric model of the NPR2 KHD could be docked onto this dimer arrangement forming a similar large interface (see Figure 5). In our dimer model of the KHD of NPR2, Arg655 is located close to the dimer interface with the Cα atoms of Arg655 in either subunit distanced by about 6 Å only. Thus, the exchange of this arginine by a cysteine could bring the two thiol groups of a homodimeric mutant NPR2 in close proximity allowing formation of an intermolecular disulfide bond, thereby possibly leading to a semiactivated state of NPR2. Although disulfide bond formation inside the cytoplasm is rare, a recent study using a fluorescence resonance energy transfer (FRET)-based thiol-containing sensor showed that despite the reducing environment a significant amount of the sensor contained disulfide bonds [39]. Alternatively, the close proximity of Arg655 to the dimer interface could point to a mechanism relying on a relief from steric/electrostatic repulsion. Substitution of the bulky and positively charged arginine by a small and neutral cysteine might favor or ease the conformational rearrangement induced by extracellular CNP binding and facilitate the allosteric activation by intracellular ATP. This hypothesis is supported by our observation that the stimulatory effect of ATP on CNP-dependent guanylate cyclase activity of the mutant NPR2 was markedly enhanced.

Besides stimulating skeletal growth, CNP/NPR2 signalling is involved in the modulation of cardiovascular, neuronal and reproductive functions [40]. It is therefore important to mention that the proband has several medical problems outside the skeleton. In particular he has gynaecomastia and 24 h prolactin secretion was increased, which could not be attributed to medication. Increased prolactin secretion might be related to the activating *NPR2* mutation, as CNP injected into the third ventricle of castrated male rats and ovariectomised female rats increased plasma prolactin [41]. CNP does not seem to act directly on lactotrophs but may act at the hypothalamic level, where both CNP and NPR2 are expressed [42]. In fact, CNP and NTproCNP are present in human cerebrospinal fluid at levels that greatly exceed those in plasma [43].

In addition, the proband has a polyneuropathy which was attributed to diabetes mellitus but was diagnosed more than 10 years before diabetes mellitus was apparent. It is interesting to speculate on a role of the *NPR2* mutation because NPR2 was shown to be essential for sensory axon bifurcation in the spinal cord of mice [6] and CNP knockout mice

display a decreased response to pain [44]. In addition, the foot ulcers in the proband were regarded to be secondary to the diabetes and venous insufficiency, but again it is tempting to speculate on a direct effect of altered NPR2 function, since NPR2 has been suggested to play a role in wound healing [45].

Lastly, CNP, via NPR2, was reported to counteract experimental hypertensive cardiac remodeling and to inhibit valve interstitial cell differentiation [46, 47]. Tricuspid and mitral valve pathologies were reported in a 14 yr old girl with CNP overexpression [9]. However, the proband did not exhibit changes in cardiac size or function, with exception of atrial fibrillation.

It is unclear whether the heterozygous microdeletion on chromosome 15 found in the proband has any clinical consequences. Two of the proteins affected by this deletion have been studied in genetic mouse models. The *Pde8a* knockout mouse did not show an obvious change in body size or bone length (personal communication of Prof. Beavo), neither did the alpha-kinase 2 knockout mouse [48]. In humans, a homozygous mutation in *ZNF592* causes CAMOS (cerebellar ataxia with mental retardation, optic atrophy and skin abnormalities) and proportionate short stature [49]. Overall, these data suggest that haploinsufficiency of the genes affected by the heterozygous microdeletion on chromosome 15 does not contribute to the tall stature of the proband.

In conclusion, we report a novel activating *NPR2* mutation located in the KHD, which results in extremely tall stature without a Marfanoid phenotype or severe skeletal abnormalities. This activating mutation indicates that the KHD, whose function is still poorly understood, is crucially involved in regulating guanylate cyclase activity of NPR2. Whether the hyperprolactinemia, diabetes mellitus and polyneuropathy of the proband are also related to the *NPR2* mutation or to the microdeletion on chromosome 15, awaits further investigation.

Acknowledgements

Reference List

1. Potter LR (2011) Guanylyl cyclase structure, function and regulation. Cell Signal 23 (12):1921-1926

2. Tremblay J, Desjardins R, Hum D, Gutkowska J, and Hamet P (2002) Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. Mol Cell Biochem 230 (1-2):31-47

3. Misono KS, Philo JS, Arakawa T, Ogata CM, Qiu Y, Ogawa H, and Young HS (2011) Structure, signaling mechanism and regulation of the natriuretic peptide receptor guanylate cyclase. FEBS J 278 (11):1818-1829

4. Yasoda A, Ogawa Y, Suda M, Tamura N, Mori K, Sakuma Y, Chusho H, Shiota K, Tanaka K, and Nakao K (1998) Natriuretic peptide regulation of endochondral ossification. Evidence for possible roles of the C-type natriuretic peptide/guanylyl cyclase-B pathway. J Biol Chem 273 (19):11695-11700

5. Zhang M, Su YQ, Sugiura K, Xia G, and Eppig JJ (2010) Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science 330 (6002):366-369

6. Schmidt H, Stonkute A, Juttner R, Schaffer S, Buttgereit J, Feil R, Hofmann F, and Rathjen FG (2007) The receptor guanylyl cyclase Npr2 is essential for sensory axon bifurcation within the spinal cord. J Cell Biol 179 (2):331-340

7. Bartels CF, Bukulmez H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, Mundlos S, Chitayat D, Shih LY, Al-Gazali LI, Kant S, Cole T, Morton J, Cormier-Daire V, Faivre L, Lees M, Kirk J, Mortier GR, Leroy J, Zabel B, Kim CA, Crow Y, Braverman NE, van den Akker F, and Warman ML (2004) Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am J Hum Genet 75 (1):27-34

8. Olney RC, Bukulmez H, Bartels CF, Prickett TC, Espiner EA, Potter LR, and Warman ML (2006) Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. J Clin Endocrinol Metab 91 (4):1229-1232

9. Bocciardi R, Giorda R, Buttgereit J, Gimelli S, Divizia MT, Beri S, Garofalo S, Tavella S, Lerone

M, Zuffardi O, Bader M, Ravazzolo R, and Gimelli G (2007) Overexpression of the C-type natriuretic peptide (CNP) is associated with overgrowth and bone anomalies in an individual with balanced t(2;7) translocation. Hum Mutat 28 (7):724-731

10. Moncla A, Missirian C, Cacciagli P, Balzamo E, Legeai-Mallet L, Jouve JL, Chabrol B, Le MM, Plessis G, Villard L, and Philip N (2007) A cluster of translocation breakpoints in 2q37 is associated with overexpression of NPPC in patients with a similar overgrowth phenotype. Hum Mutat 28 (12):1183-1188

11. Miura K, Namba N, Fujiwara M, Ohata Y, Ishida H, Kitaoka T, Kubota T, Hirai H, Higuchi C, Tsumaki N, Yoshikawa H, Sakai N, Michigami T, and Ozono K (2012) An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. PLoS One 7 (8):e42180

12. Veldhuis JD, Erickson D, Wigham J, Weist S, Miles JM, and Bowers CY (2011) Gender, sex-steroid, and secretagogue-selective recovery from growth hormone-induced feedback in older women and men. J Clin Endocrinol Metab 96 (8):2540-2547

13. Roelfsema F, Pijl H, Keenan DM, and Veldhuis JD (2012) Prolactin secretion in healthy adults is determined by gender, age and body mass index. PLoS One 7 (2):e31305

14. Roelfsema F, Biermasz NR, Veldman RG, Veldhuis JD, Frolich M, Stokvis-Brantsma WH, and Wit JM (2001) Growth hormone (GH) secretion in patients with an inactivating defect of the GH-releasing hormone (GHRH) receptor is pulsatile: evidence for a role for non-GHRH inputs into the generation of GH pulses. J Clin Endocrinol Metab 86 (6):2459-2464

15. Liu PY, Keenan DM, Kok P, Padmanabhan V, O'Byrne KT, and Veldhuis JD (2009) Sensitivity and specificity of pulse detection using a new deconvolution method. Am J Physiol Endocrinol Metab 297 (2):E538-E544

16. Keenan DM, Roelfsema F, Biermasz N, and Veldhuis JD (2003) Physiological control of pituitary hormone secretory-burst mass, frequency, and waveform: a statistical formulation and analysis. Am J Physiol Regul Integr Comp Physiol 285 (3):R664-R673

17. Santen GW, Aten E, Sun Y, Almomani R, Gilissen C, Nielsen M, Kant SG, Snoeck IN, Peeters EA, Hilhorst-Hofstee Y, Wessels MW, den Hollander NS, Ruivenkamp CA, van Ommen GJ, Breuning MH, den Dunnen JT, van HA, and Kriek M (2012) Mutations in SWI/SNF chromatin

remodeling complex gene ARID1B cause Coffin-Siris syndrome. Nat Genet 44 (4):379-380

18. Chao YC, Cheng CJ, Hsieh HT, Lin CC, Chen CC, and Yang RB (2010) Guanylate cyclase-G, expressed in the Grueneberg ganglion olfactory subsystem, is activated by bicarbonate. Biochem J 432 (2):267-273

19. Schroter J, Zahedi RP, Hartmann M, Gassner B, Gazinski A, Waschke J, Sickmann A, and Kuhn M (2010) Homologous desensitization of guanylyl cyclase A, the receptor for atrial natriuretic peptide, is associated with a complex phosphorylation pattern. FEBS J 277 (11):2440-2453

20. Klaiber M, Dankworth B, Kruse M, Hartmann M, Nikolaev VO, Yang RB, Volker K, Gassner B, Oberwinkler H, Feil R, Freichel M, Groschner K, Skryabin BV, Frantz S, Birnbaumer L, Pongs O, and Kuhn M (2011) A cardiac pathway of cyclic GMP-independent signaling of guanylyl cyclase A, the receptor for atrial natriuretic peptide. Proc Natl Acad Sci U S A 108 (45):18500-18505

21. Kamp GA, Ouwens DM, Hoogerbrugge CM, Zwinderman AH, Maassen JA, and Wit JM (2002) Skin fibroblasts of children with idiopathic short stature show an increased mitogenic response to IGF-I and secrete more IGFBP-3. Clin Endocrinol (Oxf) 56 (4):439-447

22. Mayerhofer H, Panneerselvam S, and Mueller-Dieckmann J (2012) Protein kinase domain of CTR1 from Arabidopsis thaliana promotes ethylene receptor cross talk. J Mol Biol 415 (4):768-779

23. Brown K, Vial SC, Dedi N, Long JM, Dunster NJ, and Cheetham GM (2005) Structural basis for the interaction of TAK1 kinase with its activating protein TAB1. J Mol Biol 354 (5):1013-1020

24. Lowe ED, Noble ME, Skamnaki VT, Oikonomakos NG, Owen DJ, and Johnson LN (1997) The crystal structure of a phosphorylase kinase peptide substrate complex: kinase substrate recognition. EMBO J 16 (22):6646-6658

25. Arnold K, Bordoli L, Kopp J, and Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22 (2):195-201

26. Florkowski CM, Richards AM, Espiner EA, Yandle TG, and Frampton C (1994) Renal, endocrine, and hemodynamic interactions of atrial and brain natriuretic peptides in normal men. Am J Physiol 266 (4 Pt 2):R1244-R1250

27. Olney RC, Permuy JW, Prickett TC, Han JC, and Espiner EA (2012) Amino-terminal propeptide of C-type natriuretic peptide (NTproCNP) predicts height velocity in healthy children. Clin Endocrinol (Oxf) 77 (3):416-422

28. Beis I and Newsholme EA (1975) The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. Biochem J 152 (1):23-32

29. Duda T, Yadav P, and Sharma RK (2011) Allosteric modification, the primary ATP activation mechanism of atrial natriuretic factor receptor guanylate cyclase. Biochemistry 50 (7):1213-1225

30. Potter LR and Hunter T (1998) Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. J Biol Chem 273 (25):15533-15539

31. Duda T, Yadav P, Jankowska A, Venkataraman V, and Sharma RK (2000) Three dimensional atomic model and experimental validation for the ATP-Regulated Module (ARM) of the atrial natriuretic factor receptor guanylate cyclase. Mol Cell Biochem 214 (1-2):7-14

32. Schwartz PA and Murray BW (2011) Protein kinase biochemistry and drug discovery. Bioorg Chem 39 (5-6):192-210

33. Prickett TC, Lynn AM, Barrell GK, Darlow BA, Cameron VA, Espiner EA, Richards AM, and Yandle TG (2005) Amino-terminal proCNP: a putative marker of cartilage activity in postnatal growth. Pediatr Res 58 (2):334-340

34. Prickett T, Olney R, Cameron V, Ellis M, Richards A, and Espiner E (2012) Impact of Age, Phenotype and Cardio-Renal Function on Plasma C-Type and B-Type Natriuretic Peptide Forms in an Adult Population. Clin Endocrinol (Oxf)

35. Hunt PJ, Richards AM, Espiner EA, Nicholls MG, and Yandle TG (1994) Bioactivity and metabolism of C-type natriuretic peptide in normal man. J Clin Endocrinol Metab 78 (6):1428-1435

36. Stasch JP, Kazda S, and Neuser D (1989) Different effects of ANP and nitroprusside on cyclic GMP extrusion of isolated aorta. Eur J Pharmacol 174 (2-3):279-282

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37. Duda T, Yadav P, Jankowska A, Venkataraman V, and Sharma RK (2001) Three dimensional atomic model and experimental validation for the ATP-Regulated Module (ARM) of the atrial natriuretic factor receptor guanylate cyclase. Mol Cell Biochem 217 (1-2):165-172

38. Ogawa H, Qiu Y, Ogata CM, and Misono KS (2004) Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: rotation mechanism for transmembrane signal transduction. J Biol Chem 279 (27):28625-28631

39. Ostergaard H, Tachibana C, and Winther JR (2004) Monitoring disulfide bond formation in the eukaryotic cytosol. J Cell Biol 166 (3):337-345

40. Kuhn M (2009) Function and dysfunction of mammalian membrane guanylyl cyclase receptors: lessons from genetic mouse models and implications for human diseases. Handb Exp Pharmacol (191):47-69

41. Huang FL, Skala KD, and Samson WK (1992) C-type natriuretic Peptide stimulates prolactin secretion by a hypothalamic site of action. J Neuroendocrinol 4 (5):593-597

42. Jankowski M, Reis AM, Wang D, and Gutkowska J (2004) Postnatal ontogeny of natriuretic peptide systems in the rat hypothalamus. Brain Res Dev Brain Res 152 (1):39-46

43. Schouten BJ, Prickett TC, Hooper AA, Hooper GJ, Yandle TG, Richards AM, and Espiner EA (2011) Central and peripheral forms of C-type natriuretic peptide (CNP): evidence for differential regulation in plasma and cerebrospinal fluid. Peptides 32 (4):797-804

44. Kishimoto I, Tokudome T, Horio T, Soeki T, Chusho H, Nakao K, and Kangawa K (2008) C-type natriuretic peptide is a Schwann cell-derived factor for development and function of sensory neurones. J Neuroendocrinol 20 (11):1213-1223

45. Abbey-Hosch SE, Cody AN, and Potter LR (2004) Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B). Hypertension 43 (5):1103-1109

46. Soeki T, Kishimoto I, Okumura H, Tokudome T, Horio T, Mori K, and Kangawa K (2005) C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction. J Am Coll Cardiol 45 (4):608-616

47. Yip CY, Blaser MC, Mirzaei Z, Zhong X, and Simmons CA (2011) Inhibition of pathological differentiation of valvular interstitial cells by C-type natriuretic peptide. Arterioscler Thromb Vasc Biol 31 (8):1881-1889

48. Van S, I, Ding ZM, Shi ZZ, Read RW, Hansen G, and Vogel P (2012) Cardiomyopathy in alpha-Kinase 3 (ALPK3)-Deficient Mice. Vet Pathol 49 (1):131-141

49. Nicolas E, Poitelon Y, Chouery E, Salem N, Levy N, Megarbane A, and Delague V (2010) CAMOS, a nonprogressive, autosomal recessive, congenital cerebellar ataxia, is caused by a mutant zinc-finger protein, ZNF592. Eur J Hum Genet 18 (10):1107-1113

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Supplemental Figure + Table
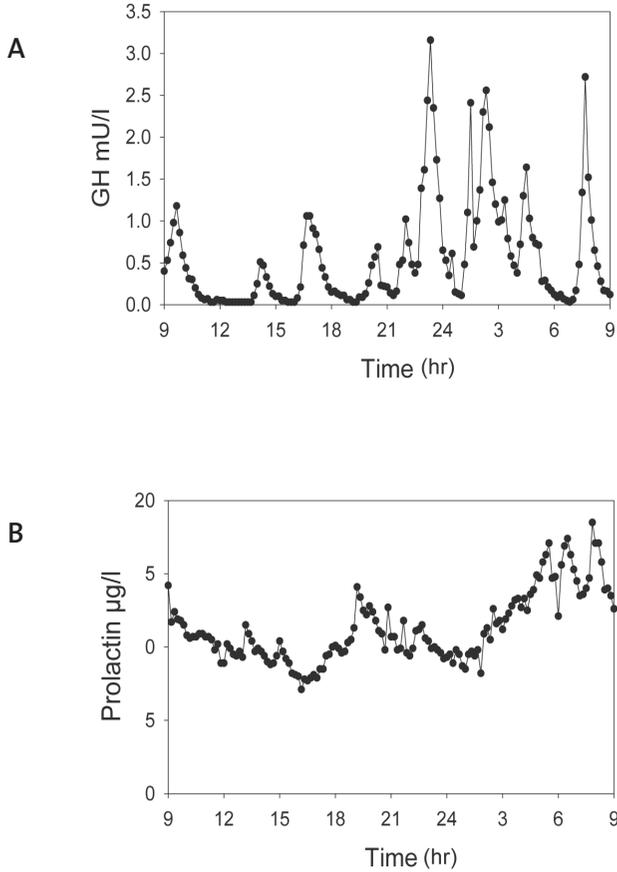
Supplemental figure 1 **Profiles (24 h) of growth hormone and prolactin secretion.**
(A) Pulsatile GH secretion of the proband. Compared to healthy controls of similar age GH secretion
is low, consistent with his increased BMI. For details of the secretion see supplemental table 1.
(B) Prolactin secretion was clearly elevated (see supplemental table 1).

Table 1 Deconvolution analysis of 24 h serum hormone profiles in controls and proband

	GH secretion in controls	Patient	PRL secretion in controls	Patient
Pulse frequency (no/24 h)	13 (10-21)	14	20 (16-24)	19
Half-life (min)	14.3 (8-25)	17.7	32 (20-42)	23.8
Pulse mode day (min)	14.2 (4.6-30)	13.9	12.1 (6.4-24)	3.79
Pulse mode night (min)	16.7 (6.9-30)	16.5	5.3 (3-14.6)	5.14
Basal secretion (units/liter)	4.2 (1.5 -42)	3.8	83 (44-201)	491
Pulsatile secretion (units/liter)	72 (27-277)	40.3	83 (30-125)	188
Total secretion (units/liter)	73 (32-319)	44.1	183 (99-325)	679
Pulse regularity (unitless)	1.40 (1.13-1.70)	1.28	1.99 (1.41-2.80)	2

Data are median and range. GH is expressed in mU/liter and prolactin as µg/liter.
Secretion rates are in unit/liter distribution volume per 24 h.

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9

General discussion

General discussion

The aim of this thesis was to investigate the genetic causes of growth disorders, following either the candidate gene or the whole genome approach and subsequently establish genotype-phenotype correlations, in order to acquire more insight in the regulation of longitudinal growth. In this chapter the results of our findings are discussed, as well as future perspectives.

A. Candidate gene approach

Since the introduction and optimization of the polymerase chain reaction (PCR) and sequencing techniques, a rollercoaster ride started in the genetics field, such as the start of the 13-year Human Genome Project in 1990 to determine the sequences of the 3 billion chemical base pairs that make up human DNA and to identify all the approximately 20,000-25,000 genes in human DNA. All this new information and the novel techniques, together with the already established knowledge, led to detection of genetic defects causing a certain phenotype or disease. Thus introducing the candidate gene approach. Meaning that by thoroughly investigating the clinical and biochemical phenotype of the patient and compare this to the medical literature, one or more candidate genes can be selected. Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis to identify mutations and copy number variations (CNVs; deletions and duplications) of these genes can be performed to investigate the presence of a genetic defect.

The candidate gene approach proved to be successful in the diagnosis of several patients with short stature. In *chapter 2 & 3, and 4* two families are described in whom the genetic defect (in *IGF1* and *IGFALS*, respectively) was identified following the candidate gene approach. The first family with a heterozygous mutation in the *IGF1* gene associated with severe short stature is described in *chapter 2*. The two children had severe short stature (height SD score -4.1 and -4.6), microcephaly and low circulating IGF-I levels. The flowchart created by our Leiden Growth Genetics Working Group (1) suggested in the oldest index case a homozygous *IGF1* missense mutation as a possible cause, and suggested mutation analysis of *IGF1*. However, in the youngest index case the flowchart did not suggest mutation analysis of *IGF1*, due to the normal birth weight and length. Just before these patients came to our attention, a patient exhibiting intrauterine and postnatal growth failure, microcephaly, mild intellectual impairment, but normal hearing caused by a homozygous *IGF1* mutation was described (2). This led us to sequence *IGF1*, but surprisingly we identified a heterozygous mutation. Until that point there was some evidence that heterozygosity for an *IGF1* defect was associated with a modest decrease of height, but it had never been associated with extreme short stature. Studies on the structural and

functional characteristics of the putative truncated IGF-I protein (synthetically derived mutant IGF-I protein), described in *chapter 3*, revealed that the severe short stature was not caused by a dominant negative effect of the truncated protein. Based on the clinical, structural and functional data we speculated that the growth failure was caused by a combination of partial IGF-I deficiency, placental IGF-I insufficiency, and other (not yet identified) genetic factors. We also speculated that such growth disorder can be treated successfully with growth hormone (GH), which indeed was confirmed in the following years (Noordam, personal communication). This study indicates that heterozygous mutations in *IGF1* can also result in short stature. This expanded the phenotype spectrum caused by an *IGF1* defect and amongst other findings led to adjustment of the flowchart (3).

The benefit of the identification of a genetic defect in a (large) family is that the effect of the mutation against the same genetic background can be investigated, as well as the possible effect of carrying a heterozygous mutation. An example of this is described in *chapter 4*. Investigation of the various family members carrying a homozygous or heterozygous mutation in the *IGFALS* gene compared to their family members that did not carry the mutation led to the hypothesis that there might be a gene-dosage effect, resulting in some negative effect on height and head circumference in heterozygous carriers. The international acid-labile subunit consortium investigated the impact of heterozygosity for *IGFALS* gene mutations on short stature in as many affected patients and families with mutations in *IGFALS* as possible, including the family we described, and concluded that heterozygosity indeed resulted in approximately 1.0 SDS height loss in comparison with family members that did not carry the mutation (4). Thus, this is in line with our hypothesis of a gene-dosage effect.

B. Combined candidate gene and whole genome approach

Although the candidate gene approach was successful in the two families described above, this was not always the case. The candidate gene approach can be very labor intensive, particularly if the phenotype is not typical for a certain gene defect. Furthermore, with this approach no novel genes involved in growth can be identified. We hypothesized that the phenotype caused by various genetic defects can be more heterogeneous than expected. If this is the case the candidate gene approach is not always the best approach to use. Therefore, we performed a diagnostic procedure in which the candidate gene approach was combined with a whole genome approach. The results are presented in *chapters 5 and 6*. In *chapter 5* we investigated 100 children born small for gestational age (SGA) with persistent short stature for intragenic (small) CNVs in 18 growth-related genes with MLPA. In *chapter 6* the experiment was extended by investigating short children with apparent GH insensitivity through combining the candidate gene approach, sequence analysis and

MLPA of 5 candidate genes, with whole genome single-nucleotide polymorphism (SNP) array analysis to identify (larger) CNVs with a size >150 kb, to identify novel genes involved in the regulation of longitudinal growth.

MLPA analysis of 18 growth-related genes in 100 SGA children with persistent short stature resulted in 2 patients with a *de novo* heterozygous 15q terminal deletion. The terminal deletion contained the complete *IGF1R* gene in patient A and exons 3-21 of the *IGF1R* in patient B. In patient A, serum IGF-I was low (-2.78 SDS), probably because of a coexisting GH deficiency. Functional studies in skin fibroblast cultures demonstrated similar levels of IGF1R autophosphorylation compared with controls, a tendency toward reduced total IGF1R protein expression, and reduced intracellular activation of protein kinase B/Akt upon a challenge with IGF-I. These results are in line with data observed in an earlier report on a patient with *IGF1R* haploinsufficiency (5) and suggest that this condition is characterized by a lower number of IGF-I receptors on the cell surface, resulting in decreased signal transduction. Haploinsufficiency would theoretically lead to a reduction of 50% of fully functional IGF-I receptors. However, in our fibroblast model this was not the case, although expression of the IGF1R tended to be lower. Still, downstream signaling was reduced. Probably the fibroblast model used to investigate the *IGF1R* haploinsufficiency is not the optimal model. It may be better to use other tissue, ideally growth plate chondrocytes, which are responsible for longitudinal growth. This tissue, however, is not available for study. We hypothesize that the consequence of haploinsufficiency may be cell type dependent, with possibly a relatively strong effect in growth plate chondrocytes, whereas the effect in the fibroblast model is less pronounced. In this study only MLPA was used to detect CNVs. Since sequence analysis of the 18 growth-related genes was not performed, mutations in these genes could not be excluded.

The combined search strategy in short children with apparent GH insensitivity (primary IGF-I deficiency), described in **chapter 6**, led to the detection of three patients with two novel heterozygous *STAT5B* mutations, in two of them combined with novel heterozygous *IGFALS* variants. The heterozygous mutations of *STAT5B* appeared to be involved in the observed GH insensitivity; the associations between the genetic variants in *IGFALS* and short stature remains uncertain. It is however conceivable that primary IGF-I deficiency resulting in short stature can also be associated with the cumulative effect of digenic or oligogenic defects, as height is determined by more than 180 genes (6) and possibly even up to 700 genes (Visser, personal communication). In this study we show that in severely short children with a low circulating IGF-I, for which the updated flowchart recommended genetic testing (3), the yield in terms of established diagnoses was 33%. This indicates that the clinical algorithm proposed in the flow chart may serve as a helpful tool for choosing the genetic tests to be performed in children with apparent GH insensitivity. 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.

C. Whole genome approach

In *chapter 7* we describe a large study to identify novel gene variants associated with the regulation of longitudinal growth, by performing whole genome SNP array analysis in 149 unrelated families (162 patients) with short stature of unknown origin (height more than 2 standard deviations (SD) below the corresponding mean height for the individual's age, sex and ethnicity). This method of patient selection is called an extreme-trait design; a carefully selected population at one end of the extremes of a phenotype. The idea is that variants that contribute to the trait will be enriched in frequency in such population, therefore even small sample sizes may suggest many candidate variants. For confirming potentially pathogenic variants, co-segregation data obtained via family members of the extreme individuals are invaluable. The potentially pathogenic variants that remain after co-segregation analysis can then be genotyped, for example by MLPA or sequence analysis, for confirmation in a much larger group of samples (7).

In this study we identified 6 copy number variations (CNVs) in 6 families for which the association with short stature is virtually certain (4.0%), and 40 CNVs in 33 families (22.1%) with possible pathogenicity, several of whom may be considered as potential candidate genes for growth disorders. Genome-wide association study (GWAS) and bioinformatics analysis identified various potential candidate genes in *de novo* and co-segregating CNVs. However, future studies will be needed to support the potential role of these genes in longitudinal growth regulation.

D. Combined whole genome approach and functional studies

Finally, in *chapter 8* we describe the use of whole exome sequencing (WES) and subsequent functional studies in the identification and characterization of a novel activating *NPR2* gene mutation which results in extremely tall stature of 221 cm in a healthy male. GH hormone overproduction had been excluded as the cause of tall stature and the patient did not show features of any known syndrome. SNP array analysis showed a deletion in chromosome 15q25.2q25.3, containing 8 protein-coding genes *(ZSCAN2, WDR73, NMB, SEC11A, ZNF592, ALPK3, SLC28A1 and PDE8A)*. Sequencing showed no mutations in these genes on the remaining allele. Since this deletion could not explain the tall stature, we subsequently performed WES which identified a heterozygous missense mutation in *NPR2* in a conserved region of the gene. The *NPR2* mutation and 15q25.2q25.3 deletion were not found in the patient's sister or son. Transfection studies of the mutant NPR2 protein in

HEK293 cells resulted in increased basal and stimulated NPR2 activity and co-expression of wild-type and mutant NPR2 resulted in increased activity, almost as high as those with mutant NPR2 alone, suggesting a dominant positive effect. Co-immunoprecipitation studies confirmed heterodimer forming of wild-type and mutant NPR2. Studies using skin fibroblasts of the patient showed increased NPR2 activity after stimulation with CNP, compared to control skin fibroblasts.

This study is a good example of the use of WES in the identification of a possibly pathogenic genetic defect. The challenge is how to prove the involvement of the genetic defect in the observed phenotype of the patient. First, an *in silico* analysis is performed to get more information about the protein in humans, but also in animals, and a possible linkage to the phenotype. Second, to really prove if the protein is involved in the manifestation of the phenotype, functional studies are necessary. Examples of functional studies are studies using the mutant protein, such as transfection studies with the mutant gene in an expression plasmid, or even synthesize the mutant protein and use this as a stimulant compared to the wild type protein. Expression studies can be carried out in different cell systems, such as HEK293 (Human Embryonic Kidney 293) which is a cell line originally derived from cultured human embryonic kidney cells. These cells are very easy to culture and transfect, therefore they have been widely used in research for many years, mainly for analysing protein expression after gene transfection. For investigating synthesized mutant protein expression compared to wild type protein expression, different cell systems can be used. For example, a stably transfected cell line containing characteristics that are relevant for the research question, or a patient derived cell line, for example skin fibroblasts which are easy to obtain and culture. In case of the stably transfected cell line a disadvantage is that the genetic environment does not mimic the patients' situation; the results only give an indication of the effect *in vitro* and it cannot be ruled out that the situation is different. In case of a patient derived cell line, not all cell types are easily obtainable and can be kept in culture. Skin fibroblasts can be easily obtained and cultured, but as described above and in **chapter 5**, this model may not always be representative for the results in other tissues due to tissue specific expression of genes. To investigate a genetic defect in the same genetic background of the patient, induced pluripotent stem cells (iPS cells) of the patient can be produced. iPS cells are a type of pluripotent stem cells artificially derived via reprogramming of a non-pluripotent cell (a somatic cell, for example blood, liver, lung, skin and stomach cells), by overexpression of specific genes (8-10). These patient iPS cells can then be differentiated to the desired cell type for investigating the genetic defect, thus the cells in the tissue important for the patient's phenotype, in our case growth plate chondrocytes. After obtaining the differentiated cells, further experiments on these cells

to prove the involvement of the genetic defect in the observed phenotype of the patient can be performed.

Conclusion and future perspectives

In this thesis we describe the results of genetic studies in patients with growth disorders, in order to detect the causal genetic defect. The candidate gene approach proved to be successful for identifying genetic defects in the GH-IGF-I axis, although we have to keep in mind that the phenotype caused by various genetic defects may be more heterogeneous than assumed on the first observations. Therefore we combined the candidate gene approach with the whole genome approach. This led to the hypothesis that short stature can be associated with the cumulative effect of digenic or oligogenic defects, as proposed in other genetic studies (11;12). In the large whole genome approach study, the SNP array technique was successfully used for the detection of novel CNVs. However, the clinical significance of detected potentially pathogenic CNVs is not immediately clear. Future studies will be needed to support or discard the role of these genes in longitudinal growth regulation. The combined whole genome approach and functional studies is a nice example of the identification of a potentially pathogenic gene mutation using WES and subsequent characterization of the mutation using functional studies, to prove the involvement of the genetic defect in the observed phenotype of the patient.

Since a few years next generation sequencing has been used to identify genetic defects. With this technique it is possible to detect mutations as well as deletions, duplications, inversions and translocations. Using this technique, similar problems will be encountered as using the whole genome SNP arrays, probably on an even larger scale. When novel variants are identified, the challenge is how to distinguish between non-causal and causal variants, and how to identify multiple variants contributing to a disease in a cumulative fashion, thus identifying causal digenic or oligogenic variants. We predict that studies using next generation sequencing will result in a higher and probably more convincing yield of genes associated with short stature.

chapter

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Reference List

1. Walenkamp MJ, Wit JM. Genetic disorders in the growth hormone - insulin-like growth factor-I axis. Horm Res 2006; 66(5):221-230.

2. Netchine I, Azzi S, Houang M et al. Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. J Clin Endocrinol Metab 2009; 94(10):3913-3921.

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

4. Fofanova-Gambetti OV, Hwa V, Wit JM et al. 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(9):4184-4191.

5. Walenkamp MJ, de Muinck Keizer-Schrama S, de Mos M et al. Successful long-term growth hormone therapy in a girl with haploinsufficiency of the insulin-like growth factor-I receptor due to a terminal 15q26.2->qter deletion detected by multiplex ligation probe amplification. J Clin Endocrinol Metab 2008; 93(6):2421-2425.

6. Lango Allen H, Estrada K, Lettre G et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 2010; 467(7317):832-838.

7. Cirulli ET, Goldstein DB. Uncovering the roles of rare variants in common disease through whole-genome sequencing. Nat Rev Genet 2010; 11(6):415-425.

8. Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318(5858):1917-1920.

9. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126(4):663-676.

10. Marchetto MC, Carromeu C, Acab A et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 2010; 143(4):527-539.

11. van Blitterswijk M, van Es MA, Hennekam EA et al. Evidence for an oligogenic basis of amyotrophic lateral sclerosis. Hum Mol Genet 2012; 21(17):3776-3784.

12. Sykiotis GP, Plummer L, Hughes VA et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci U S A 2010; 107(34):15140-15144.

chapter

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Summary

Samenvatting

Summary

Growth in humans, primarily longitudinal growth, is a complex process which starts at conception and proceeds through various developmental stages, mainly controlled by genetic factors and to a lesser degree by environmental, psychosocial and nutritional factors. The GH-IGF-I axis is an important regulator of longitudinal growth, what is evident from the observation that genetic defects in this axis have been shown to be responsible for abnormal growth. These mutations, however, are rare and do not explain the 'normal' variation in height among people. This thesis focuses on the detection of genetic defects in the GH-IGF-I axis that may explain growth disorders. Initially the so-called 'candidate gene approach' was used, examining various genes in the GH-IGF-I axis. After that, a whole genome approach was used to identify novel genes involved in aberrant growth using whole genome microarray studies (SNP arrays) and next-generation sequencing. Also, for some genes genotype-phenotype correlations were established. With this we have tried to acquire more insight in the regulation of longitudinal growth.

Chapter 1 presents a general introduction and the outline of this thesis.

In **part A** the candidate gene approach in the GH-IGF-I axis is described. In *Chapters 2 and 3* the first family with a heterozygous mutation in the *IGF1* gene associated with severe short stature and subsequent studies on the structural and functional characteristics of the mutant IGF-I protein are described. The two children presented with severe short stature (height SD score -4.1 and -4.6), microcephaly and low circulating IGF-I levels. Genetic analysis revealed a heterozygous duplication of four nucleotides in exon 4, resulting in a frame shift and a premature stopcodon. Until that point there was some evidence that heterozygosity for an *IGF1* defect was associated with a modest decrease of height, but it had never been associated with extreme short stature. We hypothesized that the putative truncated mutant IGF-I protein had a dominant negative effect on the wild type IGF-I protein, resulting in the extreme short stature observed in this family. The mutant IGF-I protein was synthetically derived to study the structural and functional characteristics of this putative truncated IGF-I protein. These studies revealed that the severe short stature was not caused by a dominant negative effect of the truncated protein, rejecting our hypothesis. Based on the clinical, structural and functional data we speculated that the growth failure was caused by a combination of partial IGF-I deficiency, placental IGF-I insufficiency, and other (not yet identified) genetic factors. In the following years, this growth disorder proved to be successfully treated with growth hormone. This study was the first to indicate that besides homozygous *IGF1* mutations, heterozygous mutations can also result in short stature.

Another candidate gene in the GH-IGF-I axis is *IGFALS*. Homozygous molecular defects of *IGFALS* can cause moderate short stature, pubertal delay and insulin insensitivity. Since the first report of a homozygous *IGFALS* mutation, a total of 16 unique homozygous or compound heterozygous mutations in 21 patients with ALS deficiency have been described. In **Chapter 4** the family members of a large Kurdish family carrying a homozygous, a heterozygous or no mutation in the *IGFALS* gene are described. The three index cases (brothers) presented with short stature (height SD score -4.2, -3.6 and -4.4), microcephaly, low circulating IGF-I and IGFBP-3, and undetectable ALS levels. Two were known with a low bone mineral density and one of them had suffered from two fractures. Genetic analysis revealed a homozygous duplication of one nucleotide in exon 2, resulting in a frame shift and a premature stopcodon. The IGF-I, IGFBP-3, and ALS 150 kDa ternary complex was absent in the sera of the three patients, and ALS proteins were not detected with Western blot. IGFPB-1 and IGFPB-2 plasma levels were low and there was a mild insulin resistance. Five heterozygous carriers of the *IGFALS* mutation tended to have a lower height and head circumference than five non-carriers, and had low plasma ALS and IGFBP-3 levels. Bone mineral (apparent) density was low in two out of three homozygous carriers, and also in four out of nine relatives. A benefit of this study was that the effect of the mutation could be investigated against the same genetic background, as well as the possible effect of carrying a heterozygous mutation. This led to the hypothesis that there might be a gene-dosage effect, resulting in a small negative effect on height and head circumference in heterozygous carriers. The international acid-labile subunit consortium investigated the impact of heterozygosity for *IGFALS* gene mutations on short stature in as many affected patients and families with mutations in *IGFALS* as possible, including the family we described, and concluded that heterozygosity indeed resulted in approximately 1.0 SDS height loss in comparison with wild type family members. An important lesson learned from these studies on ALS deficiency is that local IGF-I appears to be more important for growth than circulating IGF-I.

Part B describes the candidate gene approach in the GH-IGF-I axis in combination with a whole genome approach using single-nucleotide polymorphism (SNP) array analysis. The candidate gene approach was extended, because this strategy can be very labour intensive, no novel genes involved in growth can be identified, and we hypothesized that the phenotype caused by various genetic defects can be more heterogeneous than expected. In **Chapter 5** we investigated 100 children born small for gestational age (SGA) with persistent short stature for intragenic (small) CNVs in 18 growth-related genes with MLPA. This resulted in 2 patients with a *de novo* heterozygous 15q terminal deletion containing the complete (patient A) and partial (patient B) *IGF1R* gene. In patient A, serum IGF-I was

low (-2.78 SDS), probably because of a coexisting GH deficiency. Functional studies using skin fibroblast demonstrated similar levels of IGF-I receptor (IGF1R) autophosphorylation compared with controls, a tendency toward reduced total IGF1R protein expression, and reduced intracellular activation of protein kinase B/Akt upon a challenge with IGF-I. Functional studies using skin fibroblasts to test the theory that haploinsufficiency leads to a 50% reduction of fully functional IGF1Rs revealed that this was not the case, although IGF1R expression tended to be lower. However, other functional studies showed that downstream signaling was reduced. We hypothesize that the consequences of *IGF1R* haploinsufficiency may be cell type dependent, with possibly a relatively strong effect in growth plate chondrocytes, whereas the effect in the fibroblast model is less pronounced. In this study only MLPA was used to detect CNVs. Since sequence analysis of the 18 growth-related genes was not performed, mutations in these genes could not be excluded. In *Chapter 6* we investigated short children with apparent GH insensitivity (low IGF-I and normal GH secretion) using the candidate gene approach (sequence analysis and MLPA of 5 candidate genes in the GH-IGF-I axis) in combination with SNP array analysis to detect (larger) CNVs with a size >150 kb. Patients were divided into three groups based on height and IGF-I SDS, with a low height SDS and IGF-I level in group 1, moderate short stature associated with a low IGF-I in group 2 and short stature with serum IGF-I levels in the lower half of the normal range in group 3. This led to the detection of three patients in group 1 with two novel heterozygous *STAT5B* mutations, in two of them combined with novel heterozygous *IGFALS* variants. In groups 2 and 3 the association between genetic variants and short stature was uncertain. The heterozygous *STAT5B* mutations appeared to be involved in the observed GH insensitivity, but the association between the genetic variants in *IGFALS* and short stature remains uncertain. It is however conceivable that primary IGF-I deficiency resulting in short stature can also be associated with the cumulative effect of digenic or oligogenic defects. Besides the variants in the candidate genes, novel CNVs in 6 children were identified. It is possible that in some of these patients the CNVs are associated with the phenotype, but no clear candidate genes were present. This finding is in line with a recent report on an association between the presence of low-frequency genomic deletions in children with short stature. In this study we show that in severely short children with a low circulating IGF-I, genetic testing is advised. The yield in terms of established diagnoses was 33%. 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.

In **part C** the whole genome approach solely is described, using SNP array analysis in 149 unrelated families (162 patients) with short stature of unknown origin to identify novel

genes associated with short stature (**Chapter 7**). If possible, analysis of co-segregation of the CNV with the phenotype in families was performed, for confirming potentially pathogenic variants. In this study CNVs were detected in 40 families. In 6 families (4%) a known cause of short stature was found (*SHOX* deletion or duplication, *IGF1R* deletion), in two combined with a potentially pathogenic CNV. In 33 families (22.1%) one or more potentially pathogenic CNVs (n=40) were identified; in 9 families these CNVs occurred *de novo* or segregated with short stature. Several of the deleted or duplicated genes may be considered as potential candidate genes for growth disorders, including 4 genes previously associated with height in the genome-wide association studies (*ADAMTS17*, *PRKG2/BMP3*, *PAPPA*, *TULP4*). However, future studies will be needed to support the role of these and other genes in longitudinal growth regulation.

Part D describes the combined whole genome approach using whole exome sequencing (WES) and subsequent functional studies in the identification and characterization of a novel activating *NPR2* gene mutation which results in extremely tall stature of 221 cm in a healthy male (**Chapter 8**). GH overproduction, known syndromes, and a deletion in chromosome 15q25.2q25.3 containing 8 protein-coding genes and mutations in these genes on the remaining allele had been excluded as the cause of tall stature. WES was performed and a heterozygous missense mutation in the *NPR2* gene was detected. Resulting in an amino acid change at position 655 from Arginine to Cysteine, in the kinase homology domain of NPR2. The mutation was not present as a known variant in all available databases. The *NPR2* mutation and 15q25.2q25.3 deletion were not found in the patient's sister or son, who are not as tall as the proband. Transfection studies of the mutant NPR2 protein in HEK293 cells resulted in increased basal and CNP stimulated NPR2 activity and co-expression of wild type and mutant NPR2 resulted in increased activity, almost as high as those with mutant NPR2 alone, suggesting a dominant positive effect. Co-immunoprecipitation studies confirmed heterodimer forming of wild type and mutant NPR2. Studies using skin fibroblasts of the patient showed increased NPR2 activity after stimulation with CNP, confirming that the mutant NPR2 enhances CNP-NPR2-cGMP signalling. This pathway is critically involved in bone development by stimulating growth plate chondrocyte differentiation and proliferation. Based on these results and a previously found activating *NPR2* mutation resulting in tall stature, we conclude that the identified *NPR2* mutation in our patient is responsible for the extremely tall stature.

In **Chapter 9** the major findings of this thesis are summarized and critically reviewed and future perspectives are discussed. First it is discussed that the candidate gene approach proved to be successful in the diagnosis of several patients with short stature. However, the

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candidate gene approach also has its disadvantages. It is very labour intensive, particularly if the phenotype is not typical for a certain gene defect, no novel genes involved in growth can be identified, and if the phenotype caused by a genetic defect is more heterogeneous than expected, it will not be recognized. Second, a new strategy is discussed combining the candidate gene and whole genome approach. This did not lead to the identification of novel genes, but did identify mutations in the GH-IGF-I axis and led to the hypothesis that short stature can also be associated with the cumulative effect of digenic or oligogenic defects. Third, whole genome SNP array analysis proved to be a good method to identify potential candidate genes for growth disorders, although future studies will be needed to support the potential role of these genes. Finally, using a new activating *NPR2* mutation which results in extremely tall stature, it was illustrated that the use of WES combined with functional studies is successful in the identification and characterization of novel mutations in growth related genes.

Samenvatting

De groei van de mens, waarmee primair de lengtegroei wordt bedoeld, is een complex proces dat begint bij de conceptie en verloopt via verschillende ontwikkelingsstadia. Groei is voornamelijk gereguleerd door genetische factoren en in mindere mate door omgevings-, psychosociale en voedingsfactoren. De GH-IGF-I as is een belangrijke regulator van lengtegroei, wat onder andere blijkt uit de observatie dat genetische afwijkingen in deze as verantwoordelijk zijn voor abnormale groei. Deze mutaties zijn echter zeldzaam en kunnen de 'normale' variatie in lengte tussen mensen niet verklaren. Dit proefschrift richt zich op de detectie van genetische defecten die een groeistoornis kunnen verklaren. Aanvankelijk is hiervoor de zogenaamde 'kandidaatgen aanpak' gebruikt, waarbij diverse genen in de GH-IGF-I as zijn onderzocht. Daarna is met een genoom-brede aanpak gezocht naar nog onbekende genen die betrokken zijn bij afwijkende groei met behulp van genoom-brede microarray studies (SNP arrays) en 'next-generation sequencing'. Ook zijn voor enkele genen genotype-fenotype correlaties vastgesteld. Wij hebben geprobeerd om hiermee meer inzicht te verkrijgen in de regulatie van longitudinale groei.

In *Hoofdstuk 1* wordt een algemene introductie en de opbouw van dit proefschrift gepresenteerd.

In **deel A** wordt de kandidaatgen aanpak in de GH-IGF-I as beschreven. In de *Hoofdstukken 2 en 3* wordt de eerste familie beschreven met een heterozygote mutatie in het *IGF1* gen welke geassocieerd is met een zeer kleine lengte en de hierop volgende studies naar de structurele en functionele eigenschappen van het mutant IGF-I eiwit. De twee kinderen presenteerden met zeer kleine lengte (lengte SD score -4,1 en -4,6), microcefalie (een geringe hoofdomtrek) en lage circulerende IGF-I spiegels in het bloed. Genetische analyse toonde een heterozygote duplicatie van vier nucleotiden in exon 4, resulterend in een frame shift en een prematuur stopcodon. Tot dat moment waren er weliswaar aanwijzingen dat heterozygotie voor een *IGF1* defect een kleine afname van de lengte zou kunnen veroorzaken, maar het was nog nooit in verband gebracht met extreem kleine lengte. We veronderstelden dat het verkorte mutant IGF-I eiwit een dominant negatief effect op het wild type IGF-I eiwit zou kunnen hebben, wat de extreme kleine lengte in deze familie zou kunnen veroorzaken. Het mutant IGF-I eiwit werd gesynthetiseerd om de structurele en functionele kenmerken van dit verkorte IGF-I eiwit te bestuderen. Deze studies toonden aan dat de zeer kleine lengte niet werd veroorzaakt door een dominant negatief effect van het verkorte eiwit, en daarmee werd onze hypothese dus verworpen. Gebaseerd op de klinische, structurele en functionele gegevens speculeren we

dat de groeiafwijking is veroorzaakt door een combinatie van partiële IGF-I deficiëntie, placentaire IGF-I insufficiëntie en andere (nog niet geïdentificeerde) genetische factoren. In de hierop volgende jaren bleek dat de groeistoornis succesvol behandeld kon worden met groeihormoon. Deze studie was de eerste die liet zien dat naast homozygote *IGF1* mutaties, ook heterozygote *IGF1* mutaties kunnen resulteren in een kleine lengte.

Een ander kandidaatgen in de GH-IGF-I as is *IGFALS*. Homozygote moleculaire defecten in *IGFALS* kunnen een matig kleine lengte, vertraging van de puberteit en insuline ongevoeligheid veroorzaken. Sinds het verschijnen van de eerste rapportage over een homozygote *IGFALS* mutatie, zijn er in totaal 16 unieke homozygote of compound heterozygote mutaties in 21 patiënten met ALS deficiëntie beschreven. In *Hoofdstuk 4* worden de familieleden van een grote Koerdische familie beschreven, die een homozygote, een heterozygote of geen mutatie in het *IGFALS* gen dragen. De drie index patiënten (broers) presenteerden met kleine lengte (lengte SD score -4,2, -3,6 en -4,4), microcefalie, lage bloedspiegels van IGF-I en IGFBP-3, en onmeetbaar lage ALS spiegels in het bloed. Twee van de jongens waren bekend met een lage botmineraaldichtheid en één van hen had twee botbreuken gehad. Genetische analyse toonde een homozygote duplicatie van één nucleotide in exon 2, resulterend in een frame shift en een prematuur stopcodon. Het 150 kDa grote ternaire complex van IGF-I, IGFBP-3 en ALS was afwezig in de sera van de drie patiënten en er werden geen ALS eiwitten gedetecteerd met Western blot. IGFPB-1 en IGFBP-2 plasmaspiegels waren laag en er was een milde insulineresistentie. Vijf heterozygote dragers van de *IGFALS* mutatie neigden naar een kleinere lengte en een geringere hoofdomvang dan vijf niet-dragers, en hadden lage ALS en IGFBP-3 waarden in het bloed. De botdichtheid was laag in twee van de drie homozygote dragers, en ook in vier van de negen familieleden. Een voordeel van deze studie was dat het effect van de mutatie onderzocht kon worden tegen dezelfde genetische achtergrond, evenals het mogelijke effect van het dragen van een heterozygote mutatie. Dit leidde tot de hypothese dat er mogelijk een gen-dosis effect is, resulterend in een klein negatief effect op lengte en hoofdomvang in heterozygote dragers. Hierna heeft ook het internationale ALS consortium onderzoek gedaan naar de impact van heterozygotie voor *IGFALS* mutaties op kleine lengte in zoveel mogelijk aangedane patiënten en families, met inbegrip van de familie die wij hebben beschreven. In deze publicatie kon worden bevestigd dat heterozygotie inderdaad resulteerde in een lengteverlies van ongeveer 1,0 SDS in vergelijking met familieleden zonder een afwijking van het *IGFALS*. Deze en andere studies naar patiënten met ALS deficiëntie hebben laten zien dat het lokaal geproduceerde IGF-I belangrijker is voor groei dan circulerend IGF-I.

Deel B beschrijft de kandidaatgen aanpak in de GH-IGF-I as in combinatie met een genoom-brede aanpak. Hierbij hebben we gebruik gemaakt van de zogenaamde "single-nucleotide polymorphism (SNP) array analyse". De belangrijkste reden om de kandidaatgen aanpak uit te breiden was dat deze strategie zeer arbeidsintensief kan zijn, en dat hiermee geen nieuwe genen betrokken bij groei kunnen worden geïdentificeerd. Daarnaast realiseerden wij ons dat het fenotype veroorzaakt door verschillende genetische defecten veel heterogener kan zijn dan verwacht. In *Hoofdstuk 5* onderzochten we 100 kinderen, die klein werden geboren voor de zwangerschapsduur ('small for gestational age', SGA) waarbij de achterstand in lengtegroei vervolgens niet werd gecompenseerd door 'inhaalgroei'. Wij hebben hiervoor de zogenaamde MLPA techniek gebruikt (Multiplex Ligation-dependent Probe Amplification), waarmee intragene (kleine) 'copy number variants' (CNVs) in 18 groei-gerelateerde genen konden worden onderzocht. Dit resulteerde in 2 patiënten met een *de novo* heterozygote terminale 15q deletie, welke het volledige (bij patiënt A) en gedeeltelijke (bij patiënt B) *IGF1R* gen bevatte. Serum IGF-I was laag (-2,78 SDS) bij patiënt A, waarschijnlijk als gevolg van een eveneens aanwezige GH deficiëntie. Functionele studies met huidfibroblasten lieten een vergelijkbare IGF-I receptor (IGF1R) autofosforylering zien als in huidfibroblasten van controles, een tendens tot verminderde IGF1R eiwitexpressie en een verminderde intracellulaire activering van proteïne kinase B/ Akt na stimulering met IGF-I. Met functionele studies met behulp van huidfibroblasten hebben wij daarna de hypothese getoetst dat haploinsufficiëntie van *IGF1R* tot een 50% reductie van volledig functionele IGF-I receptoren leidt. Dit bleek niet het geval te zijn, hoewel de IGF1R expressie wel lager leek te zijn. Andere functionele studies lieten echter zien dat de intracellulaire signalering verminderd was. Onze hypothese is, dat de gevolgen van *IGF1R* haploinsufficiëntie afhankelijk kan zijn van het celtype, met mogelijk een relatief sterk effect in groeischijf chondrocyten, terwijl het effect in het fibroblast model minder uitgesproken is. In dit onderzoek werd alleen MLPA gebruikt om CNVs te detecteren. Aangezien er geen sequentieanalyse van de 18 groei-gerelateerde genen werd uitgevoerd kunnen mutaties in deze genen niet worden uitgesloten. In *Hoofdstuk 6* hebben we kinderen met een kleine lengte en aanwijzingen voor GH ongevoeligheid (een lage bloedspiegel van IGF-I bij een normale groeihormoon secretie) onderzocht met de kandidaatgen aanpak (sequentieanalyse en MLPA van 5 kandidaatgenen in de GH-IGF-I as) in combinatie met SNP (single nucleotide polymorphism) array analyse om (grotere) 'copy number variants' (CNVs) op te sporen, met een afmeting van > 150 kb. Patiënten werden verdeeld in drie groepen op basis van lengte en IGF-I SDS. Kinderen met een lage lengte SDS en IGF-I waarde werden ondergebracht in groep 1; kinderen met een matig kleine lengte die wel een laag IGF-I hadden werden ondergebracht in groep 2; en kinderen met kleine lengte met serum IGF-I waarden in de onderste helft van het normale gebied kwamen in groep 3.

Hierbij werden drie patiënten in groep 1 gevonden met twee nieuwe heterozygote *STAT5B* mutaties, in twee daarvan gecombineerd met nieuwe heterozygote *IGFALS* varianten. In de groepen 2 en 3 was de associatie tussen genetische varianten en kleine lengte onzeker. Er waren voldoende aanwijzingen dat de heterozygote *STAT5B* mutaties betrokken zijn bij de waargenomen GH ongevoeligheid, maar de associatie tussen de genetische varianten in *IGFALS* en kleine lengte blijft onzeker. Het is echter denkbaar dat IGF-I deficiëntie resulterend in kleine lengte ook geassocieerd kan worden met het cumulatieve effect van een defect in twee (digeen) of meer genen (oligogeen). Naast de varianten in de kandidaatgenen zijn er nieuwe CNVs in 6 kinderen geïdentificeerd. Het is mogelijk dat in sommige van deze patiënten de CNVs geassocieerd kunnen worden met het fenotype, maar er waren geen duidelijke kandidaatgenen aanwezig. Deze bevinding is in lijn met een recent rapport over een associatie tussen de aanwezigheid van laag frequente genomische deleties in kinderen met een kleine lengte. In deze studie hebben we aangetoond dat het raadzaam is om bij kinderen met een zeer kleine lengte en een laag circulerend IGF-I genetisch onderzoek te verrichten. De opbrengst in termen van vastgestelde diagnoses was 33%. Bij kinderen met minder kleine lengte en/of matig verlaagde serum IGF-I waarden, is de waarschijnlijkheid van het vinden van varianten in deze genen veel lager, wat suggereert dat andere, nog onbekende, genen een rol spelen.

In **deel C** wordt de genoom-brede aanpak beschreven, gebruik makend van SNP array analyse in 149 niet-verwante families (162 patiënten) met een kleine lengte door onbekende oorzaak. Hiermee kunnen nieuwe genen worden gevonden die zijn geassocieerd met kleine lengte (*Hoofdstuk 7*). Indien mogelijk werd co-segregatie analyse van de CNV met het fenotype in de familie uitgevoerd, voor het bevestigen van potentieel pathogene varianten. In deze studie werden CNVs gedetecteerd in 40 families. In 6 families (4%) werd een bekende oorzaak van kleine lengte gevonden (*SHOX* deletie of duplicatie, *IGF1R* deletie), in twee daarvan gecombineerd met een potentieel pathogene CNV. In 33 families (22,1%) werden één of meer potentieel pathogene CNVs (n = 40) geïdentificeerd, en in 9 families ontstonden deze CNVs *de novo* of segregeerden ze met kleine lengte. Verschillende van de gedeleteerde of gedupliceerde genen kunnen worden beschouwd als potentiële kandidaatgenen voor groeistoornissen, met inbegrip van 4 genen die eerder in verband werden gebracht met lengte in genoom-brede associatie studies (*ADAMTS17*, *PRKG2*/*BMP3*, *PAPPA*, *TULP4*). Er zijn echter aanvullende studies nodig om de rol van deze en andere genen in de regulatie van longitudinale groei te ondersteunen.

Deel D beschrijft de gecombineerde genoom-brede aanpak door middel van exoom-brede sequencing (whole exome sequencing, WES) en de hierop volgende functionele studies.

Deze aanpak was succesvol voor de identificatie en karakterisering van een nieuwe activerende *NPR2* mutatie, welke resulteert in een zeer grote lengte van 221 cm in een verder gezonde man (*Hoofdstuk 8*). Groeihormoon overproductie, bekende syndromen en een deletie in chromosoom 15q25.2q25.3 met 8 eiwit-coderende genen en mutaties in deze genen op het andere allel waren uitgesloten als de oorzaak van de grote lengte. De heterozygote missense mutatie in het *NPR2* gen resulteert in een aminozuurverandering op positie 655 van Arginine naar Cysteine, in het kinase homologie domein van NPR2 en is niet aanwezig als bekende variant in alle beschikbare databases. De *NPR2* mutatie en de 15q25.2q25.3 deletie werden niet gevonden in de zus en de zoon van de patiënt, die niet zo lang waren als de patiënt. Transfectie studies van het mutant NPR2 eiwit in HEK293 cellen resulteerde in verhoogde basale en CNP gestimuleerde NPR2 activiteit. Co-expressie van wild type en mutant NPR2 resulteerde in verhoogde activiteit, bijna even hoog als die met mutant NPR2 alleen. Co-immunoprecipitatie studies bevestigden heterodimeer vorming van wild type en mutant NPR2. Studies met huidfibroblasten van de patiënt toonden een toegenomen NPR2 activiteit na stimulatie met CNP, hetgeen bevestigt dat de NPR2 mutant de CNP-NPR2-cGMP signalering versterkt. Deze signaleringsroute is kritisch betrokken bij botontwikkeling door stimulering van chondrocyt differentiatie en proliferatie in de groeischijven van de lange pijpbeenderen. Op basis van deze resultaten en een eerder door Japanse onderzoekers gevonden activerende *NPR2* mutatie welke resulteerde in grote lengte, concluderen we dat de geïdentificeerde *NPR2* mutatie in onze patiënt verantwoordelijk is voor de extreem grote lengte.

In *Hoofdstuk 9* worden de belangrijkste bevindingen van dit proefschrift samengevat en kritisch beoordeeld en worden toekomstperspectieven besproken. In de eerste plaats is gebleken dat de kandidaatgen aanpak succesvol kan zijn bij de diagnose van patiënten met een kleine lengte. Deze aanpak heeft echter ook nadelen. Het is zeer arbeidsintensief, vooral als het fenotype niet typisch is voor een bepaald gendefect, er kunnen geen nieuwe genen betrokken bij groei mee worden geïdentificeerd en wanneer het fenotype veroorzaakt door een genetisch defect heterogener is dan verwacht, dan wordt het niet altijd herkend. Vervolgens wordt een nieuwe strategie bediscussieerd die de kandidaatgen en genoombrede aanpak combineert. Deze heeft niet geleid tot de identificatie van nieuwe genen, maar heeft wel mutaties geïdentificeerd in de GH-IGF-I as. Tevens werden aanwijzingen gevonden dat kleine lengte ook kan worden veroorzaakt door een cumulatief effect van digene of oligogene defecten. Daarna wordt bediscussieerd dat een genoom-brede SNP array analyse een goede methode is om potentiële kandidaatgenen voor groeistoornissen te identificeren, hoewel aanvullende studies nodig zullen zijn om de mogelijke rol van deze genen bij de afwijkende lengtegroei te ondersteunen. Tenslotte wordt aan de hand van een

nieuwe activerende *NPR2* mutatie welke resulteert in zeer grote lengte geïllustreerd dat het gebruik van WES gecombineerd met functionele studies succesvol is in de identificatie en karakterisering van nieuwe mutaties in groei gerelateerde genen.

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Curriculum Vitae

List of publications

Curriculum Vitae

Hermine van Duyvenvoorde werd geboren te Katwijk op 11 september 1981. Ze behaalde haar HAVO diploma in 1998 aan het Andreas College (locatie Pieter Groen) te Katwijk. Aansluitend begon zij met de studie Biologie en Medisch Laboratoriumonderzoek aan de Hogeschool Leiden. In de jaren 2000 – 2002 specialiseerde zij zich in Moleculaire Biologie. De afstudeerstage werd verricht bij de afdelingen Kindergeneeskunde (Willem-Alexander Kinderziekenhuis) en Moleculaire Celbiologie van het Leids Universitair Medisch Centrum (LUMC), onder leiding van Dr. Lonneke de Boer, Dr. Marcel Karperien en Prof. Dr. Jan-Maarten Wit. In juni 2002 behaalde ze de titel Ingenieur. In september van dat jaar won ze de Hogeschool Leiden Prijs 2002 voor haar afstudeerscriptie genaamd 'Onderzoek naar de etiologie van het Sotos syndroom'.

In augustus 2002 werd zij aangesteld als research analist bij het LUMC op het project 'genetica van groei', een samenwerkingsverband van de afdelingen Klinische Genetica, Endocrinologie & Stofwisselingsziekten en Kindergeneeskunde. In september 2006 startte zij haar promotietraject met als onderwerp het opsporen van genetische afwijkingen bij patiënten met groeistoornissen. Zij ontving diverse prijzen voor wetenschappelijk presentaties op nationale en internationale conferenties. In december 2011 trad zij in dienst als wetenschappelijk medewerker bij het Laboratorium voor Diagnostische Genoomanalyse (LDGA) van de afdeling Klinische Genetica in het LUMC en sinds 1 oktober 2012 is zij werkzaam als Laboratoriumspecialist Klinische Genetica in opleiding.

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List of publications

1. Wit JM, **van Duyvenvoorde HA**, Scheltinga SA, de Bruin S, Hafkenscheid L, Kant SG, Ruivenkamp CA, Gijsbers AC, van Doorn J, Feigerlova E, Noordam C, Walenkamp MJ, Claahsen-van de Grinten H, Stouthart P, Bonapart IE, Pereira AM, Gosen J, Delemarre-van de Waal HA, Hwa V, Breuning MH, Domené HM, Oostdijk W, Losekoot M. Genetic analysis of short children with apparent growth hormone insensitivity. Horm Res Paediatr. 2012;77(5):320-33.

2. Mul D, Wu S, de Paus RA, Oostdijk W, Lankester AC, **van Duyvenvoorde HA**, Ruivenkamp CA, Losekoot M, Tol MJ, De Luca F, van de Vosse E, Wit JM. A mosaic de novo duplication of 17q21-25 is associated with GH insensitivity, disturbed in vitro CD28-mediated signaling, and decreased STAT5B, PI3K, and NF-κB activation. Eur J Endocrinol. 2012 Apr;166(4):743-52.

3. Rensing KL, **van Duyvenvoorde HA**, Cramer MJ, Teske AJ, Prokop M, Stroes ES, Wit JM, Hermus AR, Twickler TB. Case report: low circulating IGF-I levels due to Acid-Labile Subunit deficiency in adulthood are not associated with early development of atherosclerosis and impaired heart function. Growth Horm IGF Res. 2011 Aug;21(4):233-7.

4. **van Duyvenvoorde HA**, van Doorn J, Koenig J, Gauguin L, Oostdijk W, Wade JD, Karperien M, Ruivenkamp CA, Losekoot M, van Setten PA, Walenkamp MJ, Noordam C, De Meyts P, Wit JM. The severe short stature in two siblings with a heterozygous IGF1 mutation is not caused by a dominant negative effect of the putative truncated protein. Growth Horm IGF Res. 2011 Feb;21(1):44-50.

5. **van Duyvenvoorde HA**, van Setten PA, Walenkamp MJ, van Doorn J, Koenig J, Gauguin L, Oostdijk W, Ruivenkamp CA, Losekoot M, Wade JD, De Meyts P, 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 Nov;95(11):E363-7.

6. Fofanova-Gambetti OV, Hwa V, Wit JM, Domene HM, Argente J, Bang P, Högler W, Kirsch S, Pihoker C, Chiu HK, Cohen L, Jacobsen C, Jasper HG, Haeusler G, Campos-Barros A, Gallego-Gómez 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 Sep;95(9):4184-91.

7. Ester WA, **van Duyvenvoorde HA**, de Wit CC, Broekman AJ, Ruivenkamp CA, Govaerts LC, Wit JM, Hokken-Koelega AC, Losekoot M. Two short children born small for gestational age with insulin-like growth factor 1 receptor haploinsufficiency illustrate the heterogeneity of its phenotype. J Clin Endocrinol Metab. 2009 Dec;94(12):4717-27.

8. Domené HM, Hwa V, Argente J, Wit JM, Camacho-Hübner C, Jasper HG, Pozo J, **van Duyvenvoorde HA**, Yakar S, Fofanova-Gambetti OV, Rosenfeld RG; International ALS Collaborative Group. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res. 2009;72(3):129-41.

9. **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 Aug;159(2):113-20.

10. Walenkamp MJ, de Muinck Keizer-Schrama SM, de Mos M, Kalf ME, **van Duyvenvoorde HA**, Boot AM, Kant SG, White SJ, Losekoot M, Den Dunnen JT, Karperien M, Wit JM. Successful long-term growth hormone therapy in a girl with haploinsufficiency of the insulin-like growth factor-I receptor due to a terminal 15q26.2->qter deletion detected by multiplex ligation probe amplification. J Clin Endocrinol Metab. 2008 Jun;93(6):2421-5.

11. van Bon BW, Koolen DA, Borgatti R, Magee A, Garcia-Minaur S, Rooms L, Reardon W, Zollino M, Bonaglia MC, De Gregori M, Novara F, Grasso R, Ciccone R, **van Duyvenvoorde HA**, Aalbers AM, Guerrini R, Fazzi E, Nillesen WM, McCullough S, Kant SG, Marcelis CL, Pfundt R, de Leeuw N, Smeets D, Sistermans EA, Wit JM, Hamel BC, Brunner HG, Kooy F, Zuffardi O, de Vries BB. Clinical and molecular characteristics of 1qter microdeletion syndrome: delineating a critical region for corpus callosum agenesis/hypogenesis. J Med Genet. 2008 Jun;45(6):346-54.

12. Walenkamp MJ, Vidarsdottir S, Pereira AM, Karperien M, van Doorn J, **van Duyvenvoorde HA**, Breuning MH, Roelfsema F, Kruithof MF, van Dissel J, Janssen R, Wit JM, Romijn JA. Growth hormone secretion and immunological function of a male patient with a homozygous STAT5b mutation. Eur J Endocrinol. 2007 Feb;156(2):155-65.

13. Vidarsdottir S, Walenkamp MJ, Pereira AM, Karperien M, van Doorn J, **van Duyvenvoorde HA**, White S, Breuning MH, Roelfsema F, Kruithof MF, van Dissel J, Janssen R, Wit JM, Romijn JA. Clinical and biochemical characteristics of a male patient with a novel homozygous STAT5b mutation. J Clin Endocrinol Metab. 2006 Sep;91(9):3482-5.

14. Walenkamp MJ, van der Kamp HJ, Pereira AM, Kant SG, **van Duyvenvoorde HA**, Kruithof MF, Breuning MH, Romijn JA, Karperien M, Wit JM. A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the insulin-like growth factor I receptor. J Clin Endocrinol Metab. 2006 Aug;91(8):3062-70.

15. 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 May;90(5):2855-64.

16. Denley A, Wang CC, McNeil KA, Walenkamp MJ, **van Duyvenvoorde H**, Wit JM, Wallace JC, Norton RS, Karperien M, Forbes BE. Structural and functional characteristics of the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development. Mol Endocrinol. 2005 Mar;19(3):711-21.

17. De Boer L, **Van Duyvenvoorde HA**, Willemstein-Van Hove EC, Hoogerbrugge CM, Van Doorn J, Maassen JA, Karperien M, Wit JM. Mutations in the NSD1 gene in patients with Sotos syndrome associate with endocrine and paracrine alterations in the IGF system. Eur J Endocrinol. 2004 Sep;151(3):333-41.