Genetic causes of growth disorders

Duyvenvoorde, H.A. van

1

General introduction

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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.***

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