Genetic Disorders in the Growth Hormone – IGF-I Axis

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ISBN: 978-90-9022266-0

Printed by: Pasmans Offsetdrukkerij BV, Den Haag

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For publication of this thesis financial support from Novo Nordisk Farma B.V., Pfizer B.V. Ferring geneesmiddelen B.V., Ipsen Farmaceutica B.V. and Eli Lilly Nederland B.V. is gratefully acknowledged.

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 8 november 2007
klokke 16.15 uur

door

Maria Josephina Elisabeth Walenkamp

geboren te Haarlem
in 1966

Promotiecommissie

Promotor	Prof. dr. J.M. Wit
Copromotores	Dr. A.M. Pereira Dr. M. Karperien
Referent	Dr. C. Camacho-Hübner (University of London)
Overige leden	Prof. dr. S.L.S. Drop (Erasmus Universiteit Rotterdam) Prof. dr. J.A.Romijn

If you can look into the seeds of time,
and say which grain will grow and which will not.....

William Shakespeare, *Macbeth*, Act 1 Scene 3

Aan mijn ouders,
Xavier, Fleur en Emilie

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General introduction

1

Growth is a complex process leading to an increase in size. On a cellular level growth is determined by an equilibrium between hyperplasia (increase in cell number), hypertrophy (increase in cell size), and apoptosis (programmed cell death). These cellular processes are regulated by multiple factors. External factors, including nutrition, psychosocial factors and physical environment interact with internal factors as genetic make-up, hormones and growth factors (1). Despite this complexity, most children grow in a remarkably predictable manner. Deviation from the normal growth pattern can be one of the first manifestations of a disruption of this growth process due to an underlying disorder. Accurate assessment of growth and knowledge of normal growth is therefore a prerequisite for optimal care of children (2).

Stages of growth

Four distinct stages of growth can be considered: fetal, infant, childhood and puberty.

With respect to fetal growth, the first trimester is characterized by forming of the organ systems, coordinated by the expression of various developmental genes. Major cellular hyperplasia takes place in the second trimester, in which peak growth velocity is reached (approximately 62 cm/year) (3). The third trimester is dominated by maturation of the organs and further body growth. The intrauterine environment, determined by maternal factors and placental function, has a large impact on fetal growth throughout gestation. The poor correlation between birth size (weight and length) and parental size reflects the dominant influence of this intrauterine environment over the genotype (3). Fetal factors associated with poor intrauterine growth consist of chromosomal abnormalities as trisomy 21, Turner syndrome and Cornelia de Lange syndrome. Endocrine factors that have been identified to play a role in intrauterine growth are IGF-I, IGF-II, and insulin.

In infancy (the first year of life) children grow rapidly (25 cm/year), but at a decelerating rate. Besides nutritional input the GH-IGF-I system, as well as genetic factors play a role in this stage. In the first two to three years the child establishes its own growth channel, which is highly correlated with target height (gender-corrected mid-parental height). By four years of age average growth velocity is 7 cm/year. At this stage GH, in addition to thyroid hormone, is the major hormonal determi-

nant of growth. Puberty is the last growth phase, characterized by a growth spurt followed by a rapid decrease of growth velocity due to fusion of the growth plate. Besides GH and IGF-I, estrogen is the main determinant of pubertal growth and epiphysial fusion in boys and girls (1).

As discussed above, various known and unknown factors play a role in the process of growth and development in different stages of life. This thesis will focus on the consequences of genetic defects in the GH-IGF-I axis on this complex process.

The GH-IGF-I axis – the historical perspective

Sixty years ago a method for measuring growth hormone activity in human plasma still had to be discovered. At present, the molecular mechanisms underlying GH and IGF-I action are topics of intense research. In the next paragraph the milestones in the history of the GH-IGF-I axis that lead to our current knowledge will be described (4). With this knowledge we were able to identify new genetic defects in patients with short stature, that were previously diagnosed as idiopathic short stature. Consequently, these patients have helped us to further unravel the role of the GH-IGF-I axis in growth and development.

Until 1956, GH activity could only be measured with the "tibia test": administration of GH increases the thickness of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats (5, 6). In 1957 Salmon and Daughaday measured the uptake of radioactive sulphate into costal cartilage in hypophysectomized rats and discovered that, if 10% normal rat plasma was added, there was a 200-300% increase in sulfate uptake. With the administration of increasing doses of GH, however, the sulphate uptake was only slightly increased (7). This laid the basis for their hypothesis that a GH dependent factor, which they termed sulfation factor (SF), was responsible for the stimulation of sulfate uptake. They found low levels of SF activity in patients with hypopituitarism, while patients with acromegaly had high levels of activity. Further proof came from administration of purified human GH to patients with hypopituitarism, which resulted in an increased serum SF level (8), while GH administered to a patient with Laron dwarfism failed to increase the low serum sulfation factor concentration (9). The findings that not only sulphate

uptake, but also protein and DNA synthesis was stimulated in a GH dependent way, and the observation that SF was active in muscle as well, led to the introduction of the more general term: somatomedin, which reflected the expanding scope of SF action (10). The original somatomedin hypothesis was formulated, proposing that GH stimulates somatomedin synthesis and release from the liver and that somatomedin reaches the main target organs via the circulation to act as an endocrine agent (Fig. 1, left panel) (10). In the meantime, another research field showed that non-suppressible insulin-like activity (NSILA) fractions demonstrated somatomedin activity, when added to hypophysectomized rats. On the other hand somatomedin had NSILA action. This raised the suspicion that somatomedin and NSILA were identical. The primary structure of two components of NSILA was published in 1978, which were termed Insulin-like Growth Factor-I and –II (IGF-I and IGF-II) (11, 12). In 1983, Klapper, and colleagues demonstrated that somatomedin-C was identical to IGF-I (13).

In the seventies, the IGF binding proteins (IGFBP's) were discovered (14, 15). After isolation of IGFBP-1 (16) Furlanetto *et al.* showed that the major IGF-BP complex in serum was composed of three elements: somatomedin, an acid stable and an acid-labile subunit (17). The latter two components were IGFBP-3 and ALS. The

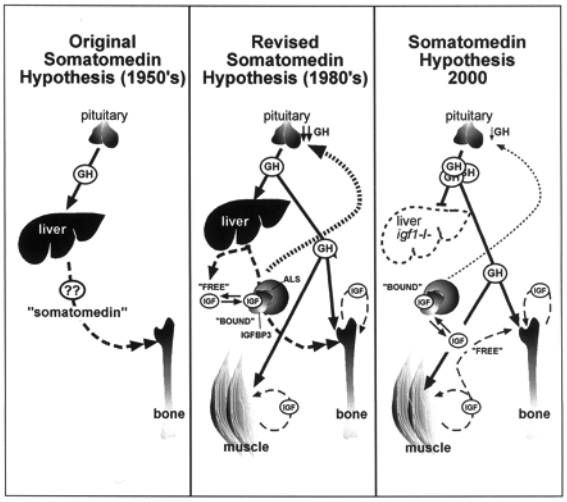

Figure 1. Evolving concepts of the somatomedin hypothesis (with permission from (21) copyright 2001, The Endocrine Society).

binding proteins appeared to act as carrier proteins, prolonging the half life of the IGF's by protecting them from proteolytic degradation, regulating the local action of IGF's and modulating IGF-I receptor activation. In addition, they seemed to regulate cell activity in various ways (18).

In the 1980's molecular biology allowed to determine that IGF-I was expressed in multiple tissues throughout embryonic and postnatal development and adult life, indicating that IGF-I also acts in a paracrine manner (19, 20). A revised version of the somatomedin hypothesis postulated that both endocrine and locally produced IGF-I are responsive to GH and therefore responsible for the effects of GH (Fig. 1, middle panel) (21). In addition, strong indications were found that GH also had a

Table 1. Characterization of genes in the GH-IGF-I axis and the first clinical report on a genetic defect.

Gene	Characterization	First clinical report on genetic defect
GH1	1979, Martial *et al*. Human growth hormone: complementary DNA cloning and expression in bacteria (31)	1981, Phillips *et al*. Molecular basis for familial isolated growth hormone deficiency. (32)
GHR	1987, Leung *et al*. Growth hormone receptor and serum binding protein: purification, cloning and expression (33)	1989, Godowski *et al*. Characterization of the Human Growth Hormone Receptor Gene and Demonstration of a Partial Gene Deletion in Two Patients with Laron-Type Dwarfism (34)
GHRH	1992, Mayo *et al*. Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone (35)	1996, Wajnrajch *et al*. Nonsense mutation in the human growth hormone-releasing hormone receptor causes growth failure analogous to the little (lit) mouse (36)
IGF-I	1983, Jansen *et al*. Sequence of cDNA encoding human insulin-like growth factor I precursor (37)	1996, Woods *et al*. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene (38)
IGF1R	1992, Abbott *et al*. Insulin-like growth factor I receptor gene structure (39)	2003, Abuzzahab *et al*. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation (40)
STAT5b	1996, Silva *et al*. Characterization and cloning of STAT5 from IM-9 cells and its activation by growth hormone (41)	2003, Kofoed *et al*. Growth hormone insensitivity associated with a STAT5b mutation (42)
ALS	1988, Baxter RC. Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex (44)	2004, Domene HM *et al*. Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene (45)
GHSR	1996, Howard *et al*. A receptor in pituitary and hypothalamus that functions in GH release (30)	2006, Pantel *et al*. Loss of constitutive activity of the GHSR in familial short stature (43)

direct effect on the epiphyseal growth plate (22). Experiments with IGF-I knockout mice, exhibiting a birth weight of only 60% of normal, indicated a direct, GH-independent effect of IGF-I on prenatal growth (23-25).

Tissue specific gene deletion experiments in mice resulted in the most recent, but undoubtedly not the final, revision of the somatomedin hypothesis, incorporating the role for IGF-I in glucose homeostasis and bone modeling (Fig. 1, right panel) (26). Mice with liver-specific IGF-I gene-deletion (LID) and consequently markedly reduced circulating IGF-I levels develop insulin resistance (27). In addition, these LID mice show a significant decrease in cortical bone volume (27).

Genes encoding the different components of the GH-IGF-I axis have now been identified and in the last few years mutations and deletions in these genes have been described in the human. Table 1 shows the original reports on the characterization of the genes involved in the GH-IGF-I axis and the first clinical description of the genetic defect.

The GH-IGF-I axis – present view

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. The release of GH is controlled by a wide range of other neurotransmitters and neuropeptides (28). The most potent GH secretagogue is ghrelin, a hormone predominantly produced by the stomach (29) whose plasma levels fluctuate with food intake. Ghrelin acts via the growth hormone secretagogue receptor (GHSR), which is highly expressed in the brain and in the pituitary (30).
The biological actions of GH are mediated by the transmembrane GH receptor (GHR). The GHR is a cytokine receptor, subject to various modifications during synthesis of which the generation of a soluble GH binding protein (GHBP), consisting of the extracellular domain of the GHR, is the most significant. The GHR uses the JAK-STAT signal transduction pathway (Fig. 2). Activation of the receptor ultimately results in transcription of target genes, including IGF-I, IGFBP-3, and ALS. Binding of IGF-I to the IGF-I receptor type I results in activation of this tyrosine kinase receptor leading to the physiological actions of IGF-I (Fig. 3).

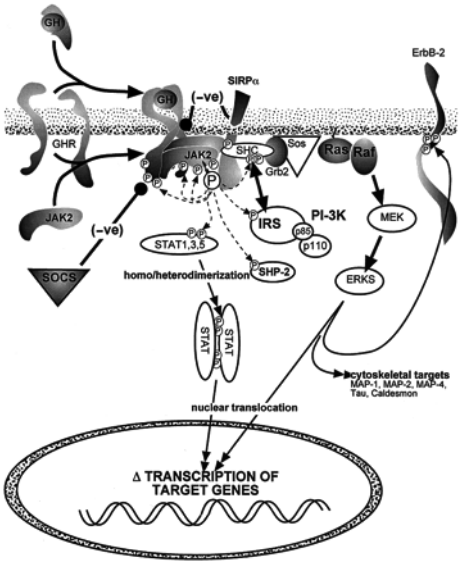

Figure 2. GH signal transduction pathway (with permission from (21) copyright 2001, The Endocrine Society).

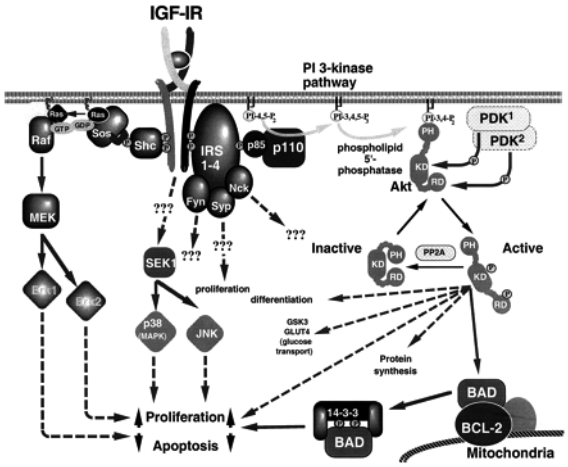

Figure 3. IGF-I signal transduction pathway (with permission from (21) copyright 2001, The Endocrine Society).

Outline of this thesis

Alert physicians, collaborating with geneticists and molecular biologists have presented many reports on patients with genetically determined causes of short stature. This thesis, describing the phenotypical and molecular characteristics of patients with genetic defects in various components of the GH-IGF-I axis is the result of such collaboration. The aim of this thesis was to study the genotype-phenotype relationship in these patients and to unravel the role of the GH-IGF-I axis in the complex process of growth and development throughout life.

Chapter 1 offers a general introduction and is followed by a review on genetic disorders in the GH-IGF-I axis, including a proposal for the diagnostic evaluation of patients with severe short stature in *chapter 2*.

Classical GH deficiency can be the result of mutations in the GHRH receptor gene, a defect in one of the genes involved in pituitary development or a mutation or deletion in the GH1 gene. *Chapter 3* describes two sibs with a GHRHR mutation and this report is focused on the positive effect of the combined treatment of GH and GnRH analogue on final height.

GH insensitivity is caused by a genetic defect of the GHR (Laron syndrome) or a post GHR signaling defect. The first male patient with GH insensitivity caused by a homozygous STAT5b mutation is described in *chapters 4 and 5*: the clinical and biochemical features in *chapter 4* and a detailed description of the growth hormone secretion pattern and immunological function in *chapter 5*.

The first patient with a homozygous missense mutation of the IGF-I gene, resulting in a bioinactive IGF-I protein is described in *chapter 6*, followed by the structural and functional analysis of the mutant IGF-I in *chapter 7*.

IGF-I resistance can be the result of genetic defect of the IGF-I receptor. In *chapter 8* a mother and daughter with a heterozygous missense mutation in the intracellular part of the IGF1R is described. A positive effect of GH treatment in a patient with a heterozygous terminal 15q deletion, including the IGF1R receptor, shows the clinical implications of this defect in *chapter 9*.

In *chapter 10* the significance of the findings is discussed, followed by a summary in *chapter 11*.

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Genetic disorders in the Growth Hormone – Insulin-like Growth Factor-I axis

2

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Hormone Research 2006;66:221-230

Abstract

In the last few years our knowledge of genetically determined causes of short stature has greatly increased by reports of challenging patients, who offered the opportunity to study genes that play a role in growth. Since the first paper that showed the etiology of Laron syndrome (1), many mutations in the growth hormone receptor have been identified. Recently, new mutations or deletions have been found in several components of the GH-IGF-I axis: a homozygous mutation of the GH1 gene, resulting in a bio-inactive GH; mutations in the STAT5b gene, which plays a major role in the GH signal transduction; a homozygous missense mutation in the IGF-I gene; heterozygous mutations in the IGF1R gene and a homozygous deletion of the ALS gene. In this mini review we describe the clinical and biochemical features of these genetic defects.

Genetic analysis has become essential in the diagnostic workup of a patient with short stature.

However, regarding the time consuming nature of molecular analysis, it is important to carefully select the patient for specific genetic evaluation. To help in this selection process we developed flowcharts, based on the recently described patients, that can be used as guidelines in the diagnostic process of patients with severe short stature of unknown origin.

Introduction

Body growth is regulated by many genes, of which only a few have been clarified. However, in the last years our knowledge of genetically determined causes of short stature has greatly increased and genetic analysis is becoming essential in the diagnosis of short stature.

A review article in this journal in 2003 described the most important genetically determined causes of short stature and the genes involved (2). Only two years later important papers were published presenting new diseases, caused by genetic defects in the GH-IGF-I axis. In this review we will give an overview of the clinical aspects and the biochemical parameters for these genetic defects in the GH-IGF-I axis and we present a flow chart for the diagnostic approach of these disorders.

We will focus on those children, whose height is more than 2.5 SDS below the mean of the population reference. The first discriminating step in the diagnostic process of short stature is the presence or absence of dysmorphic features or disproportionate stature. Hereditary causes of short stature in combination with dysmorphic or disproportionate features were reviewed by Kant *et al*. (2). In summary, in case of dysmorphic features a chromosomal abnormality (numeric, structural, mosaic or uniparental disomy (UPD)) is suspected and karyotyping is indicated. Dysmorphic features may be minor, as seen in patients with Silver-Russell syndrome, who have in 10% of the cases UPD of chromosome 7. One can consider to look for Noonan syndrome, Prader Willi syndrome or 22q11 deletion in patients with short stature and subtle dysmorphic features.
Disproportionate short stature is the result of skeletal dysplasia, a category of disorders affecting in most cases the epiphysis, metaphysis or diaphysis of the long bones, with specific radiological characteristics. The genetic basis of these disorders is emerging, as many skeletal dysplasia gene loci have been identified. More than half of all patients with skeletal dysplasias have a mutation at COL2A1 or FGFR3. Mutations in the SHOX gene are even more frequent, but do not always present with skeletal abnormalities. 2-3% of the children with idiopathic short stature have a SHOX deletion or mutation (3). It is particularly worthwhile to look for a SHOX deletion or mutation because treating these children with GH results in a similar catch-up growth as seen in girls with Turner syndrome treated with GH. Recently

deletions in the pseudoautosomal region downstream the SHOX gene were identified in patients with Leri Weill dyschondrosteosis (4). Phenotypically these patients were indistinguishable from patients with SHOX deletion.

The child with proportionate short stature should be screened for organic, systemic and endocrine disorders. In children born small for gestation age (SGA) and a small head circumference chromosome disorders, congenital infections or exposure to toxins should be considered. After excluding organic and systemic diseases, IGF-I and IGFBP-3 measurements serve to focus on disturbances in the GH-IGF-I axis. As further diagnostic procedures heavily depend on IGF-I and IGFBP-3 concentrations, we would like to stress the importance of a reliable IGF-I and IGFBP-3 assay.

GH-IGF-I axis

The GH-IGF-I axis plays a key role in regulating somatic growth. Genetic defects in one of the components (pituitary GH secretion, GH receptor (GHR), postreceptor signaling and IGF-I) of this axis usually result in proportionate growth retardation. In the last years several patients with new genetic defects in the GH-IGF-I axis have been identified. We will summarize the genetic, biochemical and clinical aspects of these new findings (Table 1).

Pituitary GH secretion
Classical GH deficiency can be the result of a mutation in the GH releasing hormone receptor (GHRH-R) gene (5), a genetic defect in one of the genes playing a role in the ontogenesis of the GH producing cells in the anterior pituitary (POU1F1, PROP1, HESX1, LHX3, LHX4 *etc.*) (6) or a mutation or deletion of the GH1 gene (6). Dysfunctional GH variants, caused by heterozygous missense mutations in the GH1 gene, have been described by Takahashi *et al*. (7). Recently, the first homozygous missense mutation in the GH1 gene (GH-C53S) has been described (8). This mutation leads to the absence of the disulfide bridge Cys-53 to Cys-165, resulting in reduced GHR binding and signaling. These genetic defects are comparatively rare causes of short stature.

Table 1. Clinical and biochemical features of genetic defects in the GH-IGF-I axis.

	Inactive GH promoter	GHR defect	Homozygous STAT5B defect	ALS defect	IGF-I deletion	IGF-I missense mutation	Heterozygous IGF1R mutation	Homozygous GH1 gene mutation
History								
Birth weight	LN	LN	LN	⇓	⇓	⇓	⇓	N
Birth length	LN	⇓ or LN	LN	⇓	⇓	⇓	⇓	LN
Birth head circ.	LN	LN	LN	?	⇓	⇓	⇓	?
Parental height	N	N	N	?	LN	LN	1 small parent or both N	N
Appetite as infant	N	N	⇓ or N	N	N	N	⇓	N
Milestones	N	N	N	N	⇓	⇓	N or ⇓	N
Psychomotor development	N	N	N	N	⇓	⇓	N or ⇓	N
Immuno-deficiency	-	-	+ or -	-	-	-	-	-
Physical exam								
Height	⇓	⇓	⇓	-2 SD	⇓	⇓	⇓	-3.6 SD
Weight for height	N	N	?	N	⇓	⇓	⇓	?
Head circ.	N or ⇓	N or ⇓	?	LN	⇓	⇓	⇓	?
Sitting height/height ratio	N	N	N	N	N	N	N	?
Other problems			Lymphoid interstitial pneumonia, pulmonary fibrosis, hemorrhagic varicella		deafness	deafness		
Biochemistry								
GH secretion	GH peak ⇓ or N, 12hr. profile ⇓	⇑	⇑	N	⇑	⇑	⇑ or N	⇑
IGF-I	⇓	⇓	⇓	⇓	⇓	⇑	N or ⇑	⇓
IGF-II	⇓	?	N	⇓	N	N	N	?
IGFBP-3	⇓	⇓	⇓	⇓⇓	N	N	N	⇓
Insulin	⇓	N	N	⇑	⇑	N	?	?
ALS	⇓	?	⇓	0	N	N	⇑	?
Prolactin	N	N	⇑	N	N	N	N	N
Radiology								
Skeletal age	⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓

N = normal, LN = lower normal range

Recent studies demonstrate high diversity in the proximal promoter region of the GH1 gene, resulting in some haplotypes that are associated with a reduced level of gene expression, while other haplotypes were associated with increased expression (9). One can speculate that a haplotype, associated with reduced expression of GH, results in a condition with low spontaneous GH secretion and thus low levels of IGF-I, while stimulated GH secretion may be normal.

Growth hormone receptor and GH signaling

The biological effects of GH can only be reached in the presence of a normal functioning GHR, and an intact postreceptor signaling pathway. Deletions and mutations in the extracellular domain of the GHR gene result in classical GH insensitivity (Laron syndrome). More recently, mutations in the transmembrane and intracellular domain of the receptor were identified, resulting in GH insensitivity syndrome with normal or high levels of GH binding protein (GHBP) (10-12).

The first report of a specific molecular defect in the GH signal transduction was published by Kofoed *et al*. in 2003 (13). The authors described a patient with a homozygous missense mutation in the highly conserved SH2 domain of the STAT5b gene, which is essential for the GH signaling cascade and IGF-I transcription. At the moment of writing this review, several patients with homozygous mutations in the STAT5b gene have been described: a frame shift mutation (14, 15), a nonsense mutation (16), another frameshift mutation (17), and a splice site mutation (18). All patients appear to show hyperprolactinaemia; some of them have a serious immunodeficiency, while others show no such clinical symptoms.

Recently, a heterozygous mutation of the IκB gene was described (19, 20). IκB is part of the NFκB signaling pathway, playing a major role in immune responses. Besides severe immune deficiency, this patient also had signs of partial GH insensitivity, suggesting that the NFκB pathway could play a role in the GH signal transduction.

IGF-I

One of the biological effects of GH is stimulating IGF-I production, which is mainly taking place in the liver, but also in all other cells of the body. IGF-I has endocrine, paracrine and autocrine functions. IGF-I secretion is under control of many other factors than GH (*e.g* nutrition). IGF-I, IGF-II and insulin are the most important

regulators of prenatal growth. Postnatally, IGF-I remains important, while the role of IGF-II is still unclear. In 1996 one patient with a homozygous deletion of exons 4 and 5 of the IGF-I gene was described. Phenotypically he showed intrauterine growth retardation, postnatal growth failure, microcephaly, mental retardation, sensorineural deafness and multiple dysmorphic features (21). In 2003, a patient with intrauterine growth retardation, short stature, delayed psychomotor development and sensorineural deafness was described, with a homozygous mutation, changing the normal amino acid sequence of the E domain of the IGF-I precursor, resulting in low circulating levels of IGF-I (22). In 2005, we described the first patient with a homozygous missense mutation of the IGF-I gene (23). The phenotype of this 55 year old patient was similar to that of the patient with an IGF-I deletion (21). Family members with a heterozygous IGF-I mutation were shorter and had lower head circumferences than family members without the mutation. Recently, a boy was presented with a partial IGF-I deficiency due to a homozygous missense mutation of the IGF-I gene, resulting in pre- and postnatal growth retardation, microcephaly, mild developmental delay and normal hearing tests (24).

In the circulation IGF-I is bound to binding proteins (IGF-binding proteins, IGFBP's). These proteins exhibit specific characteristics in relation to delivery of IGF-I to different tissues. IGFBP-3 production is strongly dependent on GH. IGFBP-3 forms with IGF-I and acid-labile subunit (ALS) a ternary complex in the circulation. A homozygous deletion of the ALS gene, resulting in a "circulating IGF-I deficiency", was described in 2004 (25). This patient was not very short, but had a very delayed puberty. Later, a boy with a similar phenotype was reported (26).
IGF-I receptors (IGF1R) are widely spread through the body. Children with a deletion of the distal long arm of chromosome 15, which includes the IGF1R gene, are short (27). It was assumed that specific mutations/deletions of the IGF1R gene could result in growth retardation.
The first report on mutations in the IGF1R gene was published in 2003 by Abuzzahab *et al*. (28): a compound heterozygous mutation of the IGF-IR gene, resulting in reduced IGF-I binding and decreased receptor phosphorylation and a nonsense mutation in exon 2, resulting in reduced expression of IGF1R. Recently, a heterozygous mutation in the cleavage site of the proreceptor of IGF1R was reported in a 6 year old Japanese girl and her mother (29). We described a mother and daughter with a heterozygous missense mutation in the intracellular part of

the IGF1R (30). A 13.6 year old girl was presented with a heterozygous missense mutation in the highly conserved N-terminal fibronectin type III domain of the IGF1R (31). Recently a new heterozygous missense mutation at the
α subunit of the IGF-I receptor was described in a 4 year old girl with short stature (-3.6 SDS) and her mother (32).

Proposal for a diagnostic flow chart for patients with severe short stature of unknown origin

Although we acknowledge that undoubtedly future studies will show additional cases of the genetic defects described above, as well as new genetic disorders, we think that developing a diagnostic algorithm might be helpful in the evaluation of severely short children. For this purpose, we developed some flowcharts, based on the recently described patients, in combination with theoretical considerations. The flowcharts can be used as guidelines in the diagnostic process of patients with idiopathic short stature. As our knowledge of genetic causes of short stature is increasing rapidly, these diagrams undoubtedly will be subject of adaptation in the coming years.

As main inclusion criterium for considering genetic evaluation we choose a height SDS of < -2.5, assuming that more pathology is found with a lower height SDS. We believe that deviation of growth is not a valuable parameter, as in some of the earlier described cases growth is far below, but parallel to, the –2.5 SDS line. Similarly, target height cannot be used as criterium, because in some cases parents are short due to the same genetic defect (as in the cases of heterozygous IGF1R mutations).
Fig. 1 shows the first diagnostic step in a child with a height < -2.5 SDS. Proportions should be measured and in case of disproportionate short stature a skeletal survey is performed and the child is referred to a clinical geneticist. Radiological abnormalities can point to a known skeletal dysplasia, requiring specific molecular analysis. If no or minimal radiological abnormalities are found, the SHOX and FGFR3 gene can be analysed, as in some cases mild disproportionate short stature is the only clinical feature (33, 34).

Karyotyping should be carried out, when dysmorphic features are found, but also in the absence of dysmorphic features karyotyping is usually carried out in all girls with unexplained short stature. Recently, it was argued that also in boys with short stature karyotyping should be considered, in order to diagnose a XY/X chromosomal pattern (35). In the dysmorphic child with a normal karyogram a genetic defect of the GH-IGF-I axis is still possible: *e.g.* the patient with a deletion of the IGF-I gene (21) and the patients with IGF1R gene mutation showed dysmorphic features (28). Obviously, if another cause for short stature is found by basic diagnostic screening, genetic analysis of the GH-IGF-I axis is not indicated.

The next criterium in a child with proportionate short stature is the presence or absence of being SGA, defined as a birth weight or length of <-2 SDS. In case of SGA, further investigations will be focused on genetic defects of IGF-I production or sensitivity. Indeed, children with classical GH deficiency or insensitivity are usually not born SGA. In case of SGA one should look for mutations or deletions in the IGF-I or IGF1R gene or a IGF-I signal transduction defect. In children with a normal birth weight and length additional testing should be focused on disturbances in GH secretion, GH sensitivity or GH signaling.

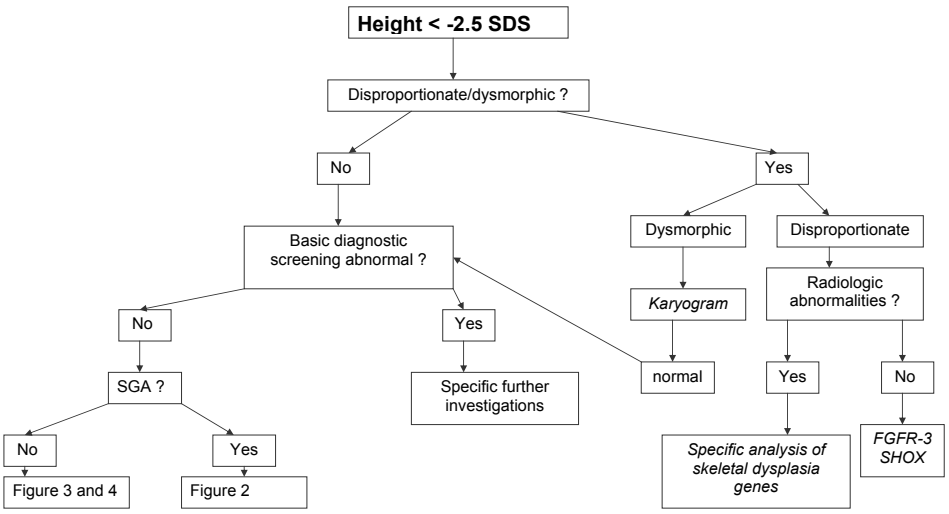

Figure 1. Flow chart for the diagnostic approach of a child with short stature (< -2.5 SDS).

In children with short stature, born SGA, measuring the head circumference is essential. IGF-I plays a key role in intrauterine growth and cerebral development and prenatally IGF-I secretion is GH independent. Therefore, in SGA children with a small head circumference primary IGF-I deficiency or insensitivity should be considered (Fig. 2). The IGF-I level will determine the differential diagnosis. Undetectable IGF-I levels will indicate a homozygous IGF-I deletion or nonsense mutation with absolutely no production of IGF-I. Theoretically one can expect very low IGF-I levels in cases of a homozygous missense mutation in the IGF-I gene resulting in an abnormal IGF-I protein that can only be partially detected by the assay. IGF-I levels between –2 and 0 SDS could be the result of heterozygous mutations or deletions of IGF-I. It is conceivable that in the future polymorphisms in the promoter region of the IGF-I gene will be found that may explain the short stature in some of these cases. In case of a heterozygous mutation of IGF1R plasma IGF-I is usually elevated, but it can be low if the child is malnourished by extremely poor appetite (30). In spite of these theoretical possibilities, at present, we do not advise further genetic analysis, at least with the current tools available, in patients with IGF-I levels in the lower normal range. The differential diagnosis in patients with normal or high IGF-I levels consists of a homozygous IGF-I missense mutation, resulting in an abnormal IGF-I molecule, or a heterozygous IGF1R mutation with decreased or absent binding of IGF-I to the mutated receptor. In all these conditions IGFBP-3 levels are within the normal range.

With a normal head circumference these genetic defects are less probable, but they cannot be completely ruled out. Heterozygous mutations or deletions of the IGF-I or IGF1R gene could present with short stature, SGA and a head circumference >-2 SDS. One may consider to perform in vitro experiments with fibroblasts of patients, that meet these criteria. Depending on the sensitivity of the fibroblasts to IGF-I, the IGF-I gene or the IGF1R gene can be sequenced.

We will now discuss the group of patients with short stature and a normal birth weight and length. The first diagnostic step in these patients is to determine the IGF-I and IGFBP-3 levels. If the IGF-I level is below the normal range (<-2 SDS) the interpretation of the GH peak in a stimulation test determines the next step (Fig. 3). An MRI of the pituitary-hypothalamus region should be performed to demonstrate or exclude anatomical defects.

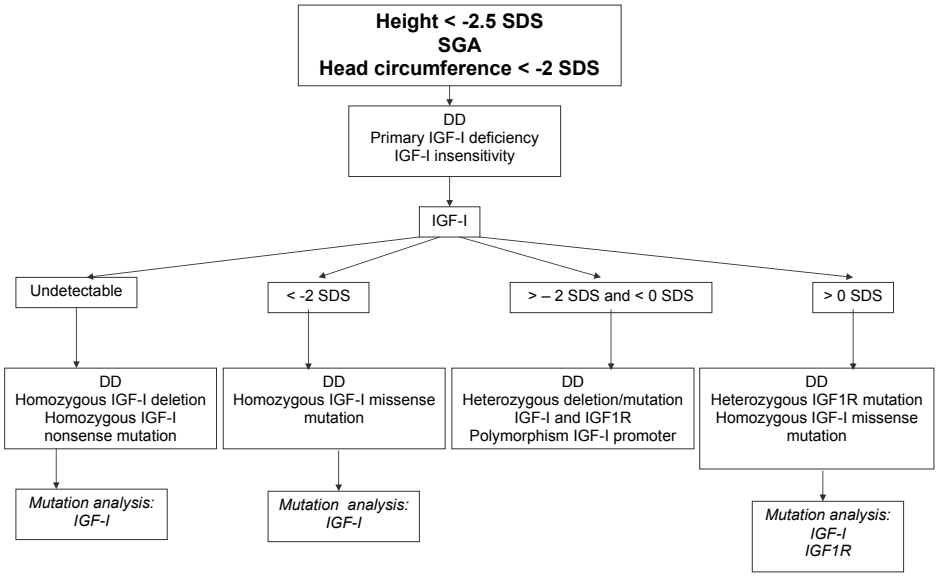

Figure 2. Flow chart for the evaluation of a child with proportionate short stature, born SGA and a head circumference < -2 SDS.

GH deficiency is usually diagnosed if the GH peak is below 20 mU/L (equivalent to 6.6, 7.7 or 10 µg/L, depending on the standard used) in two tests. Depending on the presence of other pituitary hormone deficiencies analysis of transcription factors as HESX-1, PROP-1, and Pit 1 is required. In special cases analysis of LHX3 or LHX4 can be considered. In case of isolated GH deficiency we advise to analyse the GH and GHRH-R gene, but one can argue that these tests could be restricted to those children in whom a positive family history for short stature or extremely short stature is found.

A GH peak within the lower normal range (20-30 mU/L) can be the result of a disturbance in the GH secretion and one can consider to analyze the GH promoter gene. In several countries, including the Netherlands, the combination of very low IGF-I and borderline GH peaks after stimulation, is sufficient indication for GH therapy. A low 12- or 24 hr profile, which has been termed "neurosecretory dysfunction" by several investigators, could be used as criterium for analysis of the GH promoter gene.

A normal GH peak (30-40 mU/L) in combination with low IGF-I levels in patients with clinical features of GH deficiency and retarded skeletal maturation can be present. In these cases a low 12- or 24 hr GH profile could reflect a relatively inactive GH promoter haplotype. One should note, however, that in some cases with a GH signaling disorder very low IGF-I levels in the presence of normal GH peaks have been found.

The differential diagnosis of a low IGF-I in combination with a high stimulated GH secretion (GH peak > 60 mU/L) consists of: bio-inactive GH, a GHR defect, a GH signal transduction defect (STAT5b mutation) or an ALS deficiency (Fig. 4). The response of IGF-I to increasing doses of GH (the IGF-I generation test, described in Table 2) will roughly distinguish the conditions characterized by an abnormal GH molecule from GH insensitivity states. We are aware that many different protocols for the IGF-I generation test have been described, and that the diagnostic value of all of them is still uncertain. Theoretically, in patients with a bio-inactive GH, IGF-I will reach normal levels with the lowest GH dose, while in patients with a GH

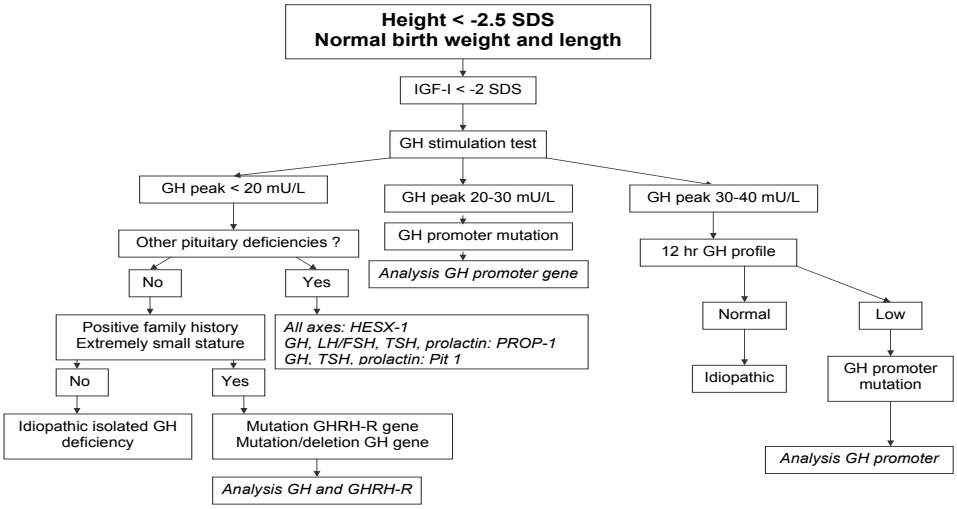

Figure 3. Flow chart for the evaluation of a child with proportionate short stature, normal birth weight and length, low IGF-I levels (< -2 SDS) and a GH peak in a stimulation test < 40 mU/L.

receptor or postreceptor problem IGF-I will not increase or only on the highest GH dose.

Theoretically, an inactive GH promoter or partial GH insensitivity can result in low IGF-I levels with a normal GH peak in the stimulation test (40-60 mU/l). At his moment, however, we do not propose genetic analysis in these patients, as we think the time and money investment will not be balanced by the results.

Conclusion

In patients with short stature a systematic diagnostic approach may reveal the cause of the growth disorder. The medical history, including birth weight, length and head circumference, and physical examination, including body proportions, are necessary for the first differential diagnosis. Biochemical evaluation will point to a more specific diagnosis, which can be confirmed with molecular techniques.

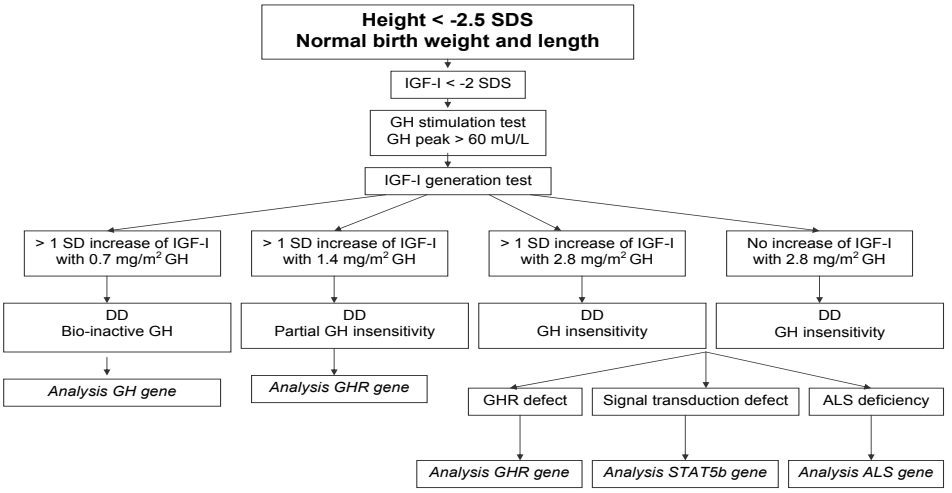

Figure 4. Flow chart for the analysis of a child with proportionate short stature, normal birth weight and length, low IGF-I levels (< -2 SDS) and a GH peak in a stimulation test > 60 mU/L.

Table 2. IGF-I generation test.

	Growth hormone dose	Biochemical evaluation
Week 1	0.7 mg/m2/day	IGF-I and IGFBP-3 at day 0 and day 8
Wash out period (at least 4 weeks)		
Week 2	1.4 mg/m2/day	IGF-I and IGFBP-3 at day 0 and day 8
Wash out period (at least 4 weeks)		
Week 3	2.8 mg/m2/day	IGF-I and IGFBP-3 at day 0 and day 8

The response criterium is defined as an increase of IGF-I of at least 1 SD on day 8.

In this review we discussed new genetic defects in the GH-IGF-I axis and proposed a practical flow chart for the diagnostic work-up.
The proposed diagnostic pathways will lead to maximum results when pediatric endocrinologists, adult endocrinologists, clinical geneticists and molecular biologists cooperate. An unusual presentation of a patient with a growth disorder should alert the clinician to look for new abnormalities in the GH-IGF-I axis or other genes involved in growth.

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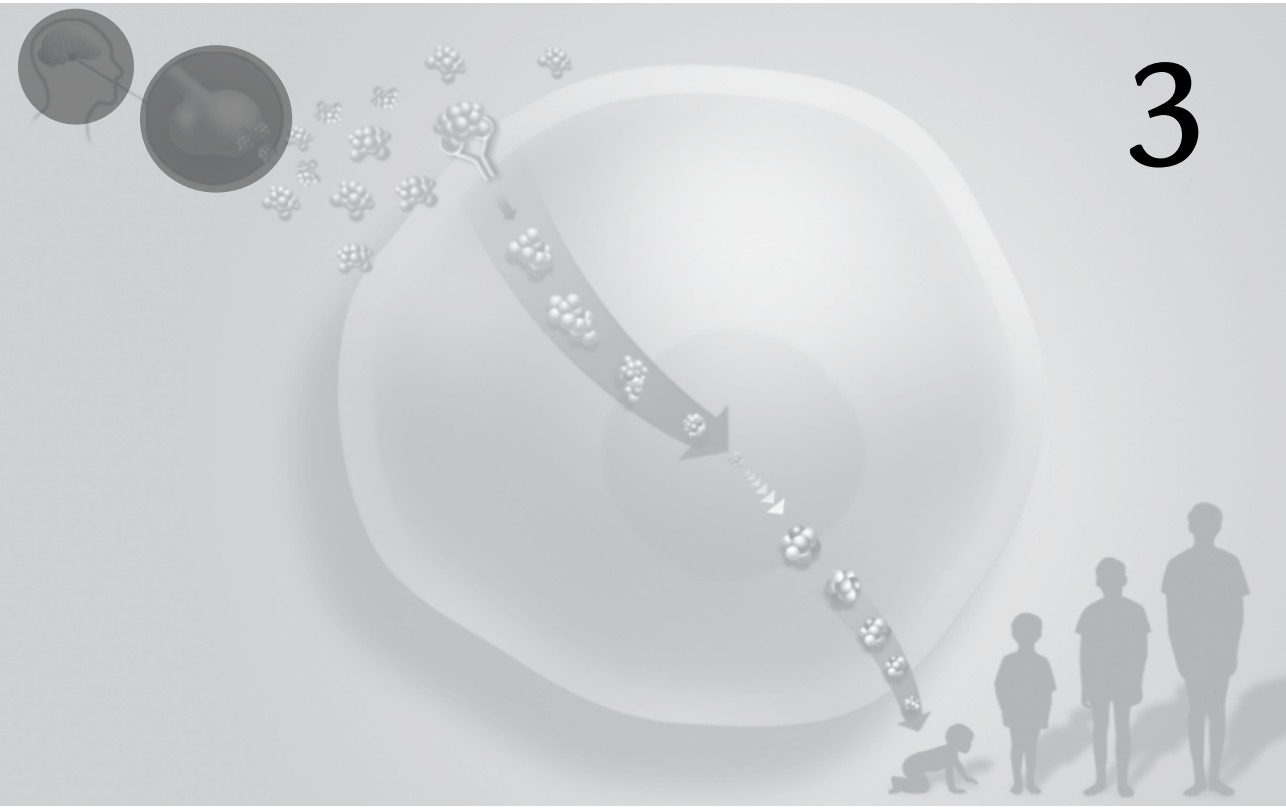
Large height gain by growth hormone therapy in combination with GnRH analog in two pubertal sibs with a GH-releasing hormone receptor mutation



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Submitted

Abstract

Context: Patients with GHRH receptor (GHRH-R) mutations present with familial isolated GH deficiency, which untreated leads to a severely compromised adult height. Few data are available about the efficacy of treatment with GH in combination with a gonadotropin-releasing hormone (GnRH) analog (GnRHa) in adolescence.

Objective: To describe the evolution of growth and skeletal age of a brother and sister of Moroccan descent with a homozygous GHRH-R mutation who presented at an advanced age (16 and 14.9 years, respectively) and pubertal stage (Tanner stage G4 and B3, respectively) with a height of -5.1 SDS and -7.3 SDS on treatment with a combination of GH and GnRHa for 2.5 and 3 years followed by GH alone.

Methods: GH was given in a dosage of 0.7 mg/m^2/day (25 μg/kg/day) sc and triptorelin in a dosage of 3.75 mg/4 weeks im. Height and pubertal stage were measured three-monthly, bone age yearly.

Results: Combined GH and GnRHa treatment resulted in a height gain of 24 cm and 28.2 cm respectively, compared to the initial predicted adult height by the method of Bayley-Pinneau. Adult height was within the population range and well within the target range.

Conclusions: Our patients demonstrate that, in case of isolated GH deficiency caused by a GHRH-R mutation, combined treatment of GH and GnRHa can be very effective in increasing final height, even at an advanced bone age and pubertal stage

Introduction

Growth Hormone Releasing Hormone (GHRH) and somatostatin are hypothalamic peptides that regulate the pulsatile GH secretion. GHRH stimulates GH synthesis and release, while somatostatin inhibits GH release (1). Binding of GHRH to the GHRH receptor (GHRH-R) activates the signaling cascade, resulting in cellular proliferation, GH synthesis, and secretion (2).

Several mutations in the GHRH-R gene have been identified, all showing an autosomal recessive inheritance pattern. Clinically, patients with homozygous GHRH-R mutations present with familial isolated GH deficiency (GHD), including proportionate short stature, variable anterior pituitary hypoplasia, low IGF-I and IGFBP-3 levels, subnormal stimulated GH levels and good response to exogenous GH (2-5).

In 2001, we described the GH secretion in two patients with a novel homozygous single base pair transition (G->C) at the splice donor site of intron VII of the GHRH-R gene (6). At the time of diagnosis, they were 14.9 and 16 years old and had reached an advanced stage of puberty. Both patients were treated with GH in combination with a gonadotropin-releasing hormone (GnRH) analog (GnRHa). We demonstrate that this combination can be highly effective in increasing final height, even at this advanced age and maturational stage.

Methods

Auxological measurements

Height, body mass index (BMI) and target height were expresssed as standard deviation score (SDS) based on Dutch references for children of Moroccon descent (7). Sitting height/height ratio and head circumference were expressed as standard SDS based on Dutch references (8, 9). Target height was calculated from the parents heights corrected for sex and secular trend: [height of father and mother +/- 13]/2 + 4.5) (8).

Biochemical measurements
Biochemical assays have been described previously (6). An arginine test was performed with 0.5 gr/kg arginine in 30 min.

Radiographic measurements
Bone age and predicted adult height was assessed by scoring the radius, ulna and short bones (RUS), using the Tanner and Whitehouse-2 (TW-2) method (10). In addition, bone age was assessed with the Greulich and Pyle method (11), and predicted adult height according to the tables of Bayley and Pinneau (12).

Patients

The patients were the third and fourth son and daughter of a Moroccan family with 6 children. The parents were not aware of consanguinity. The other four children were healthy. Father's height was 170 cm and mother's height was 156.4 cm, resulting in a target height of 174.2 cm (0 SDS) for the brother and 161.2 cm (0 SDS) for the sister
The boy was referred to our hospital because of short stature at the age of 16 yr. Three months before, he and his sister had immigrated from Morocco. No previous growth data were available. His medical history was uneventful and he had no other complaints. Auxological, biochemical, and radiological features are summarized in Table 1. Total 24-h GH production was greatly diminished, as has been described earlier in detail (6). The maximal rise of TSH after 200 µg TRH was 5.7 mU/liter after 20 min, which was considered a suboptimal response. A normal increase of prolactin was observed (from 4.5 to 15 µg/liter). Although computed tomography at the age of 16 yr showed a partial empty sella, a magnetic resonance imaging scan at the age of 25 yr showed no abnormalities of the pituitary and hypothalamus.

GH treatment was started at the age of 16.2 yr (Genotropin, Pharmacia, Stockholm, presently Pfizer, US) at a dosage of 0.7 mg/m^2/day (25 µg/kg/day). After six weeks of GH treatment, a low level of total thyroxine was found (56 nmol/liter (normal value 70-160 nmol/liter)) with a normal T3 and TSH level. Substitution with levothyroxine was initiated. Retesting 6 years later revealed a normal TSH response to

Table 1. Auxological, biochemical and radiological characteristics of the patients.
Tanner stages: PH: pubic hair, G: genital, B:breast.

Variable	Brother	Sister
At start of GH therapy		
Age (yr)	16	14.9
Height [cm (SDS)]	135.2 (-5.1)	116.9 (-7.3)
Weight (kg)	26.5	23
BMI [kg/m^2 (SDS)]	14.5 (-2.9)	16.8 (-1.8)
Head circumference [cm (SDS)]	49.7 (-3.8)	50.3 (-2.7)
Sitting height/height [ratio (SDS)]	0.52 (0.2)	0.51 (-0.4)
Tanner stage	PH4, G4	PH1, B3
IGF-I [ng/ml (SDS)]	14.5 (-7.0)	26.8 (-6.4)
GH maximum in exercise test (mU/liter)	2.8	0.5
GH maximum in arginine test (mU/liter)	5.0	1.3
Bone age - Greulich and Pyle (yr)	14	11.6
Predicted adult height – Bayley-Pinneau (cm)	144.2	127.4
Bone age – TW-2 (yr)	14.6	12.1
Predicted adult height – TW-2 (cm)	148.6	120.3
At final height		
Age (yr)	21.3	20.5
Height [cm (SDS)]	168.2 (-1)	155.6 (-1)
Target height [cm (SDS)]	174.2 (0)	161.2 (0)

TRH (6). Administration of 100 μg GnRH, showed a maximal increase of LH to 10.1 U/liter, which was considered as a pubertal response. Plasma testosterone was 32.4 nmol/liter (923 ng/dl). To prevent further progression of puberty and bone maturation, GnRH agonist (GnRHa) treatment was initiated one month after the start of GH (Decapeptyl CR, 3.75 mg every 4 wk im). GnRHa treatment resulted in prepubertal testosterone levels within one month and decreased the testicular volumes from 15 ml to 6 ml after 9 months of treatment. GnRHa was discontinued at the age of 18.7 yr (total duration of treatment 2.4 yr). Subsequently, the GH dose was increased to 1 mg/m^2/day (34 μg/kg/day). Adult height was reached at

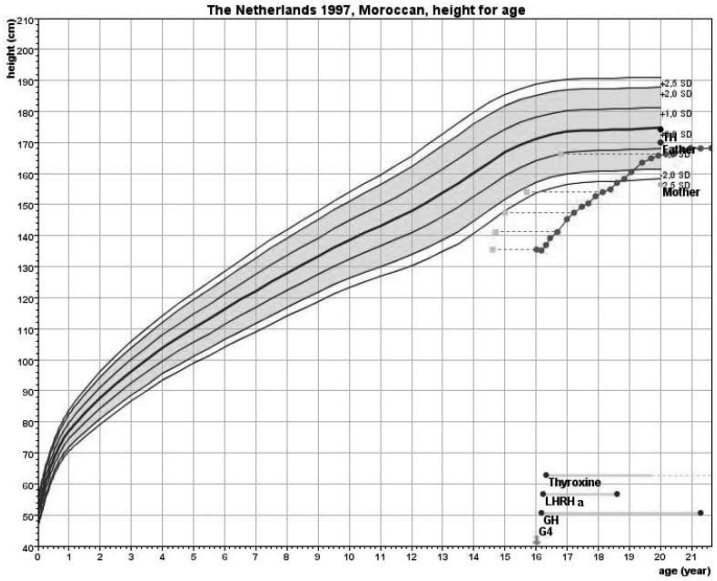

Figure 1A. Growth chart for Dutch children of Moroccon descent of the boy with a GHRH-R mutation. The squares represent the bone age (TW-2 RUS).

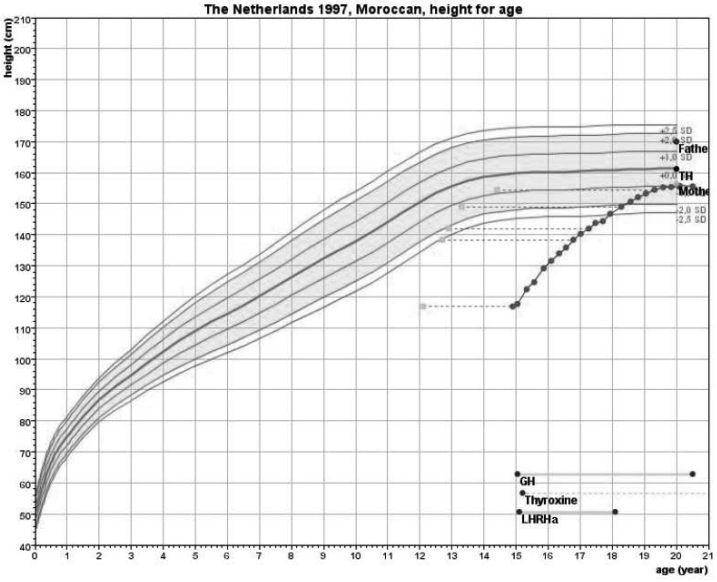

Figure 1B. Growth chart for Dutch children of Moroccon descent of the girl with a GHRH-R mutation. The squares represent the bone age (TW-2 RUS).

the age of 21.3 yr and was 168.2 cm (– 1.0 SDS), which was well within his target range (Fig. 1A). After reaching adult height he has received GH replacement at a dosage of 0.4 mg/day sc.

The girl was referred to our hospital at the same time as her brother, at the age of 14.9 years. Her medical history was also uneventful. Her auxological, biochemical, and radiological characteristics are summarized in Table 1. At physical examination she had a doll like face and increased abdominal fat. No dysmorphic features were noticed. Menarche had occurred, but it was uncertain at what age. She had regular menstrual cycles. Total 24-h GH production was greatly diminished, as was earlier described in detail (6). The maximal rise of TSH after 200 μg TRH was from 0.8 to 6.7 mU/liter after 20 min, which was considered as a subnormal response. Prolactin increased from 3.6 to 21 μg/liter (normal). Computed tomography at the age of 15 yr showed an empty sella, which was confirmed on the magnetic resonance imaging scan at 23 yr of age.

At the age of 15 yr GH treatment was started (Genotropin, 0.8 mg/m^2/day = 28 μg/kg/day sc) and one month after the start of GH a GnRHa was added (Decapeptyl CR 3.75 mg, every 4 wk im). Prior to treatment a GnRH test showed a pubertal response with a LH peak of 17.3 mU/liter 30 min after 100 μg GnRH iv. Plasma estradiol was 64 nmol/liter. On GnRHa treatment plasma estradiol was < 40 pmol/liter and menstrual cycles stopped. Total thyroxin levels decreased after 6 weeks of GH treatment (total thyroxine 61 nmol/liter), whereas T3 and TSH levels were normal, for which levothyroxine replacement therapy was initiated. Retesting at adult age showed a normal TSH response to TRH and levothyroxine was discontinued (6). The GnRHa was stopped after 3 yr of treatment at the age of 17.1 yr. At that moment bone age was 12.9 yr according to TW-2 and 12 yr according to Greulich and Pyle. Subsequently, the GH dose was increased to 1.3 mg/m^2/day (30 μg/kg/day). Puberty progressed normally after discontinuation of the GnRHa. At 20.5 yr she had Tanner stage B5 and regular menstrual cycles. Her final height was 155.6 cm (-1 SDS), which was well within the target range (Fig. 1B). After reaching final height, GH injections were discontinued for 4 yr, but after retesting GH treatment was reinstituted in a dosage of 0.4 mg/day. During uneventful pregnancies, at the age of 26 and 27 yr, GH was discontinued after the first trimester. She delivered two healthy babies, a girl and a boy, who are growing normally.

Results

Result of GH and GnRHa treatment

Treatment with the combination of GH and GnRHa during 2.5 yr in the male sib resulted in a height gain of 24 and 19.6 cm according to the pretreatment predicted adult height determined by Bayley/Pinneau and TW-2, respectively. In the female sib a height gain of 28.2 and 35.3 cm was established after 3 yr of combination treatment compared with the pretreatment predicted adult height determined by Bayley/Pinneau and TW-2, respectively.

Discussion

Mutations in the GHRH-R gene account for approximately 10% of the patients with familial isolated GHD. In both our patients a homozygous G-> C transition at the splice donor site of intron VII was found, altering the first basepair of the intervening intronic sequence after exon 7. This mutation interrupts the signal transduction of GHRH, causes GHD and consequently severe postnatal growth retardation. Our patients were diagnosed at the ages of 16 and 14.9 years, respectively, and they both showed signs of advanced puberty. We hypothesized that GH treatment alone at this advanced stage of puberty would accelerate bone maturation progressively and thereby limit the effect on final height, while administration of GnRHa might delay the process of epiphyseal fusion by inhibiting the secretion of gonadotropins and gonadal sex steroids. Our cases illustrate that severely GH deficient adolescents can greatly benefit from the combination GH and GnRHa treatment, even at an advanced age, bone age and pubertal stage.

The large height gain on GH and GnRHa treatment as we experienced in our patients is far beyond the average additional effect of GnRHa in group analyses (approximately 7 cm) (13-17). Also in individual patients with GH deficiency we are not aware of reports on such extremely good result. We speculate that, in our cases, who have a severe GH deficiency caused by a well-defined genetic disorder, the intact GH –IGF-I axis downstream the level of the GHRH-R resulted in maximal GH sensitivity. This is supported by the significant rise of IGF-I seen in the IGF-I generation test in patients with a GHRH-R mutation (18) and the good growth

response to GH therapy in those patients (2-5, 18). The good sensitivity allows optimal catch-up growth, while the absence of estrogen restricts epiphyseal maturation. The only report on GH and GnRHa therapy in a boy with a GHRH-R mutation also showed a positive effect on linear growth, but this was limited to a few centimeters (19).

The TSH response to TRH was suboptimal in our patients, suggesting mild secondary hypothyroidism. A blunted TSH response was also reported in other patients with a GHRH-R mutation (18). One could hypothesize that hypoplasia of the pituitary is one of the contributing factors, however the pituitary size was normal in the brother. Another possible explanation for the blunted TSH response is that GHRH is necessary for maximal TSH production, although the normal TRH test at adult age argues against this. The low T_4 levels after the start of GH therapy were probably the result of the positive effect of GH on 5'deiodinase activity, the enzyme converting T_4 into T_3 (20). In general, treatment is not necessary as T_4 levels recover spontaneously, but in our cases levothyroxine treatment was started to create optimal conditions for growth.

In conclusion, this report shows that in patients with isolated GH deficiency due to a GHRH-R mutation, combined treatment of GH and GnRHa can have a great effect on adult height, even if started at an advanced bone age and pubertal stage.

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Clinical and biochemical characteristics of a male patient with a novel homozygous STAT5b mutation

4

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The Journal of Clinical Endocrinology and Metabolism 2006; 91(9):3482-3485

Abstract

Context: GH insensitivity can be caused by defects in the GH receptor (GHR) or in the postreceptor signaling pathway. Recently, two female patients with severe growth retardation and pulmonary and immunological problems were described with a defect in STAT5b, a critical intermediary of downstream GHR signaling.

Objective: The objective was to determine the functional characteristics of a novel STAT5b mutation and describe the phenotype.

Patients: We describe an adult male patient with short stature (-5.9 SDS), delayed puberty, and no history of pulmonary or immunological problems. GH-binding protein level as well as GH secretion characteristics were normal. Plasma prolactin level was elevated. Extremely low levels of IGF-I (-6.9 SDS), IGF-binding protein-3 (-12 SDS), and acid-labile subunit (-7.5 SDS) were found.

Results: We found a homozygous frameshift mutation in the STAT5b gene (nucleotide 1102-3insC, Q368fsX376), resulting in an inactive truncated protein, lacking most of the DNA binding domain and the SH2-domain.

Conclusions: This report confirms the essential role of STAT5b in GH signaling in the human. We show for the first time that immunological or pulmonary problems or elevated GH secretion are not obligatory signs of STAT5b deficiency, whereas hyperprolactinemia appears to be part of the syndrome. Therefore, in patients with severe short stature, signs of GH insensitivity, and a normal GHR, analysis of the STAT5b gene is recommended.

Introduction

Primary GH insensitivity is characterized by severe postnatal growth failure, elevated levels of GH and decreased levels of IGF-I. The majority of patients with GH insensitivity have mutations in the extracellular domain of the GH receptor (GHR), resulting in reduced GH binding. The first report of a mutation concerning the GH signaling pathway was published by Kofoed *et al.* (1) describing a female patient with a homozygous missense mutation in the STAT5b gene. Recently, Hwa *et al.* (2) described another female patient with a homozygous frameshift mutation of the STAT5b gene. In both patients short stature is associated with pulmonary problems and severe immunodeficiency.

STAT5b is a component of the Janus kinase-STAT signal transduction pathway. Of the seven STAT proteins, the GHR preferentially uses STAT5b for signal transduction. In the absence of STAT5b, the ability of GH to induce the expression of IGF-I mRNA is almost completely abrogated both in mice and man (1-3).

We now describe the clinical and biochemical characteristics of the first male with a homozygous frameshift mutation in the STAT5b gene, in whom short stature was not accompanied by immunodeficiency.

Patients and Methods

Clinical and molecular studies were performed after obtaining written informed consent.

Biochemical assays

Plasma GH was measured with time-resolved immunofluorometric assay (Wallac/PE, Turku, Finland), using the WHO 80/505 as a standard (1 mg=2.6 IU). Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1, IGFBP-2, IGFBP-3, and IGFBP-6 were determined by specific RIAs (4).

Acid-labile subunit (ALS) was measured by an ELISA (Diagnostics Systems Laboratories, Inc., Webster, TX) (5). With the exception of IGFBP-1, smoothed references were available for all parameters, based on the LMS method (6), allowing

conversion of the data of the patient to SD score (SDS) values. Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of 6 healthy adult controls. Prolactin was measured by electrochemical luminescence immunoassay (Roche Diagnostics, Almere, Netherlands). GH-binding protein was measured with the ligand immunofluorometric method (7).

Analysis of STAT5b

A skin biopsy was taken, and a culture of dermal fibroblasts was established. Genomic DNA was isolated from whole blood. STAT5b mRNA isolated from fibroblasts and STAT5b coding exons were amplified by PCR and subjected to direct sequencing. Primer combinations are available on request.

Western blotting

Western blotting to detect phospho-Erk1, Erk2 and PKB/Akt was performed using fibroblast cultures from the patient and an age- and sex-matched normal subject as described in detail previously (8). Antibodies directed to inact STAT5b were obtained by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Results

Phenotype

The index patient was born in the Dutch Antilles. His parents were not aware of any consanguinity. Paternal and maternal heights were 164.3 cm (-2.8 SDS) and 165.6 cm (-0.8 SDS), target height was 176 cm (-1.1 SDS) (9). He was born after a full-term uncomplicated pregnancy. At birth, his weight was 3270 g (-0.7 SDS) and his length was 50 cm (-0.4 SDS) (10). Shortly after birth, congenital ichthyosis was diagnosed.

At the age of 16 yr, he emigrated to The Netherlands. Shortly after arrival, he was admitted to the hospital because of hemorrhagic chickenpox. He made a full recovery after treatment with acyclovir. He was referred to a paediatric endocrinologist, for evaluation of growth retardation. His body proportions were normal, although his pubertal development was delayed (Tanner stage G1P1), testicular volume being only 1 ml. Biochemical evaluation revealed a low IGF-I level (3.8 nmol/liter, -5.6 SDS) and

a slightly elevated prolactin level 1.4 U/liter (normal value < 0.3 U/liter). His maximal GH response to 50 μg GHRH iv was 25 μg/liter, which is considered to be a normal response. A computed tomography scan of the pituitary and hypothalamus revealed no abnormalities. His bone age was 9 yr. A 24-h plasma GH profile was normal. Nevertheless, a presumptive diagnosis of GH secretory dysfunction was made and a trial with recombinant human GH treatment (Genotropin) was started in a dose of 1.5 IU (0.5 mg)/day for 25 months, followed by a dose of 3.0 IU (1.0 mg)/day for an additional 3 months. However, this treatment did not significantly improve growth rate, except for a slight growth acceleration probably due to pubertal development. IGF-I levels increased only slightly during treatment with recombinant human GH (5.8 nmol/liter, -4.7 SDS). At the age of 19.8 yr, bromocriptin was started (2.5 mg/day) and serum prolactin decreased (<0.05 U/liter). Eight months later, he returned to the Dutch Antilles, and he was lost to follow-up for 10 yr.

At the age of 30 yr, he was referred to our clinic for evaluation of his short stature (Table 1). He did not have any complaints and reported normal libido, erections, and ejaculations. He used to shave every four days. There was no history of infectious diseases during the previous 16 yr. There was striking central obesity and pseudogynecomastia. He did not have galactorrhea. Secondary sexual characteristics were typical for a male, facial hair was present, and body hair distribution showed a male pattern, although scarce. The ichthyosis was not active. There were no dysmorphic features. Testicular volume was 12 ml. The stretched penile length was 8 cm (P10-P90, 10.2-16.4 cm) (11). Cardiovascular, respiratory, and abdominal examinations were normal.

Endocrine features at 30 yr of age

Circulating levels of IGF-I, IGF-II, IGFBP-3, and ALS were markedly reduced (Table 1). The concentration of prolactin was markedly elevated (*i.e.* five times the upper limit of normal). Basal GH level was normal (0.33 mU/liter). The maximal GH response after administration of insulin was 37.0 mU/liter (14.2 ng/ml), and after GHRH-arginine, 29.4 mU/liter (11.3 ng/ml), which are considered to be normal responses (12).

Mutational analysis

Sequence analysis of STAT5b mRNA derived from dermal fibroblasts revealed the

Table 1. Clinical and biochemical features of the patient at the age of 30 yr.

Parameter	Value
Age (yr)	30
Height [cm (SDS)]	141.8 (-5.9)
Weight (kg)	56
BMI (kg/m^2)	28.2
Head circumference [cm (SDS)]	54 (-1.4)
Glucose (<110 mg/dl)	74
Insulin (0-20 mU/liter)	11
Prolactin (0-22 μg/liter)	110
TSH (0.3-4.8 mU/liter)	3.8
Free T_4 (0.8-1.8 ng/dl)	0.84
LH (2-8 U/liter)	6.5
FSH (2-10 U/liter)	5.6
Testosterone (>230 ng/dl)	373
GH (mU/liter)	0.33
GHBP[a] (pmol/liter)	1524
IGF-I [ng/ml (SDS)]	8 (-8.2)
IGF-II [ng/ml (SDS)]	83 (-6.2)
IGFBP-1[b] (ng/liter)	31
IGFBP-2 [ng/ml (SDS)]	142 (-0.6)
IGFBP-3 [mg/liter (SDS)]	0.18 (-12.4)
IGFBP-6 [ng/ml (SDS)]	230 (1.3)
ALS [mg/liter (SDS)]	0.7 (-7.0)

Conversion factors: glucose x 0.0551 (millimoles per liter), free T_4 x 2.87 (picomoles per liter), and testosterone x 0.035 (nanomoles per liter)

[a] Normal range, 330-2437 pmol/liter

[b] Normal range for non-fasting subjects, 24-58 ng IGFBP-1/ml. After overnight fasting, there was an average 5-fold rise in normal individuals

insertion of an extra C between nucleotide positions 1102 and 1103 (Fig. 1A). The parents, brother, and sister were carriers of the mutation (Fig. 1A). This resulted in a frame shift and premature truncation of the protein (Q368fsX376). The presence of the frame shift mutation was confirmed in genomic DNA. No other mutations were detected in the open reading frame of the STAT5b gene. The truncated protein lacked a large part of the DNA binding domain and completely lacked the

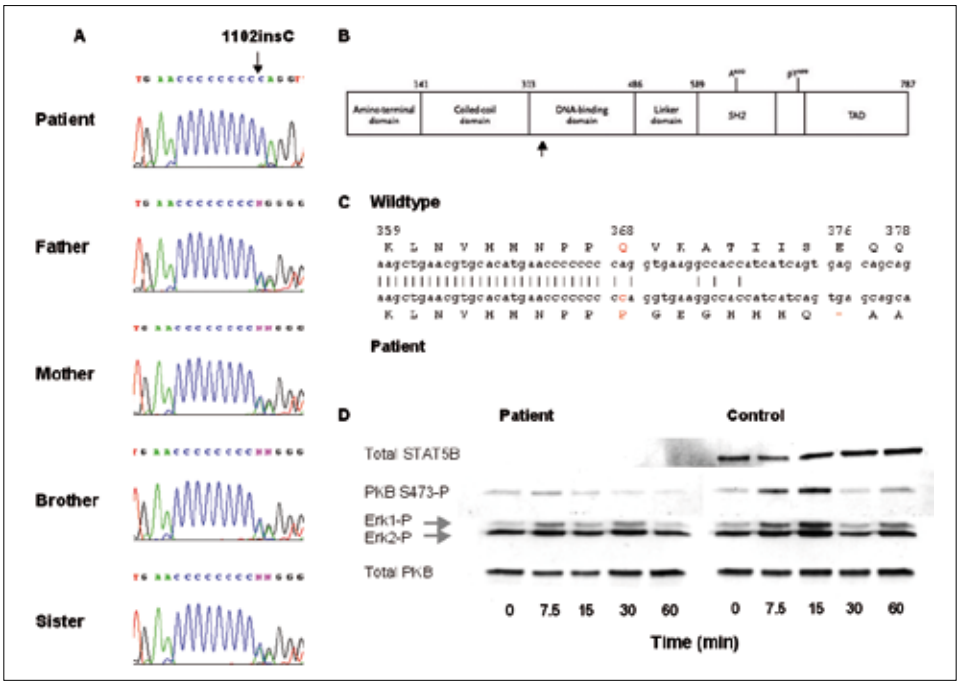

Figure 1: Identification of a homozygous frame shift mutation in STAT5b.

A. Electropherogram of the sequence analysis of the patient and his family members. The position of the extra C is indicated by the *arrow*.

B. Insertion results in a premature stopcodon in the N-terminal half of the protein. The truncated protein lacks the majority of the functional domains of STAT5b. The position of the mutation is depicted schematically by the *arrow*.

C. Alignment of part of the cDNA sequence corresponding to amino acids 359 to 378 of wild type (*upper part*) and patient's STAT5b (*lower part*) showing the consequence of the frame shift mutation for the open reading frame.

D. Western blot analysis using protein extracts isolated from fibroblasts from the patient and an age- and sex-matched control after a challenge with 500 ng/ml human GH. No STAT5b protein could be detected in the patient's cells. In addition, the activation of PKB/Akt and Erk1/2 was impaired. Total PKB was used to check loading efficiencies.

C-terminal SH2 domain required for dimerization with other STAT proteins and translocation to the nucleus, making this protein biologically inactive (Fig. 1, B and C). In line with this and in contrast to a normal control, total STAT5b could not be detected in protein extracts of the patient's fibroblast using Western blot even after stimulation with 500 ng/ml GH (Fig. 1D). Although no abnormalities were present in the GHR, absence of STAT5b also resulted in impaired activation of the

PKB/Akt pathway by decreased phosphorylation on serine 473 and Erk1 and -2 phosphorylation (Fig. 1D).
Because a stretch of eight consecutive Cs may be susceptible to replication errors, we carried out sequence analysis of this exon (PCR amplified from genomic DNA) of 71 individuals diagnosed with short stature. In four cases a heterozygous C>A transversion at the seventh C (nucleotide position 1101) was detected. This has no predicted effect on the coding sequence (Pro>Pro). No changes were found in any of the remaining 67 cases.

Discussion

This is the first report of a male patient, homozygous for a frame shift mutation in the STAT5b gene, which induces a premature stopcodon at position 376 (Q368fsX376). The resulting truncated protein lacks a functional DNA binding domain and misses the SH2 domain. The SH2 domain is critical for recruitment of STAT5b to the activated GHR complex and binding of STAT to the phosphorylated tyrosine residues. It also contains a conserved tyrosine residue at position 699, which is critical for STAT dimerization and its subsequent translocation to the nucleus, where it can act as a DNA binding transcription factor (13, 14). Based on this analysis, the mutation is presumed to result in inactivation of the protein. Indeed, we could not detect any STAT5b in fibroblasts of the patient, at least not with the antibody available to us.
The expression of IGF-I, IGF-II, IGFBP-3, and ALS is tightly controlled by GH. STAT5b appears to be the dominant transcription factor, mediating these effects of GH at the transcriptional level. In both the IGF-I and ALS genes, functional STAT5b response elements have been identified (15, 16). Fully in line with this, the serum levels of IGF-I, IGFBP-3, and ALS are extremely low in all patients with a STAT5b mutation (1, 2).

Our patient is the first known male with a STAT5b mutation. He shows the same degree of postnatal growth retardation as the two female patients (-7 and -7.8 SDS) (1, 2). Female STAT5b knockout mice are largely unaffected, whereas male knockouts, which are normally 30% larger than female mice, resemble females in size (17). Apparently, in humans, homozygous STAT5b mutations result in an equal pattern of growth retardation in males and females.

In line with the observations in STAT5b knockout mice (18), GHR gene-disrupted mice (19) and patients with Laron's syndrome (20), our patient showed markedly increased serum prolactin levels. In STAT5b-deficient mice the high serum prolactin concentrations could be suppressed by bromocriptine (dopamine D2 agonist), suggesting absence of endogenous dopaminergic inhibition. Hyperprolactinemia might also be the result of elevated hypothalamic GHRH secretion, under the diminished feedback restraint from IGF-I and GH. Indeed, the elevated prolactin levels in Laron's syndrome decrease upon IGF-I administration, in line with the inhibitory action of IGF-I on hypothalamic GHRH neurons (20).

The first reported patient with a STAT5b mutation had respiratory difficulties due to lymphoid interstitial pneumonia and a *Pneunocystis Carinii* infection (1). The second patient with a STAT5b mutation had primary idiopathic pulmonary fibrosis (2). In contrast, our patient has had neither pulmonary complaints nor signs of immunodeficiency. Our patient demonstrates that STAT5b deficiency is not obligatory resulting in a clinically immunodeficient state.

In conclusion, this report supports the essential role of STAT5b in GH signaling in the human and confirms that STAT5b deficiency leads to severe postnatal growth failure. In contrast with mice, the growth retardation in the human does not show a sexually dimorphic pattern. Hyperprolactinemia is apparently part of the syndrome, possibly by a deficient negative feedback loop in the hypothalamus. Although in earlier cases STAT5b deficiency was suggested to be associated with immunodeficiency, our patient shows that immunodeficiency is not an obligatory feature in patients with a STAT5b mutation. In cases with postnatal growth retardation, a low IGF-I, IGFBP-3, and ALS, high or normal GH secretion, and an absence of mutations or deletions in the GHR, genetic analysis of STAT5b is warranted. Our case illustrates that the heterogeneity of clinical manifestations of this syndrome is larger than suggested by the first two cases.

Acknowledgements

We thank Dr. Bidlingmaier for measuring GH-binding protein.

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Growth hormone secretion and immunological function of a male patient with a homozygous STAT5b mutation

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European Journal of Endocrinology 2007;156:155-165

Abstract

Objective: STAT5b is a component of the GH signaling pathway. Recently, we described a 31-year old male patient (height – 5.9 SDS) with a novel homozygous inactivating mutation in the STAT5b gene. The purpose of this study is to describe the phenotype in detail, including GH secretion and immunological function. In addition, we report four family members of this patient, all heterozygous carriers of the mutation.

Design and methods: Twenty-four hour GH and prolactin secretion characteristics were assessed by blood sampling at 10-min intervals. An IGF-I generation test was performed. Monocyte function was tested by stimulation of whole blood with lipopolysacharide (LPS) in the presence or absence of Interferon-γ (IFN-γ). In addition, T cell function was determined by measuring proliferative responses of peripheral blood mononuclear cells (PBMC) after stimulation by various polyclonal activators and Interleukin-2 (IL-2). Clinical and biochemical characteristics were determined in the carriers of the mutation.

Results: GH secretory parameters were comparable to that of healthy male controls (mean fat percentage 25), but likely increased in relation to the patient's 40% body fat. The regularity of GH secretion was diminished. Prolactin secretion was increased by sixfold. The IGF-I generation test showed a small increase of IGF-I and IGFBP-3 on lower GH doses and an increase of IGF-I to –2.4 SDS on the highest dose of GH. *In vitro*, IL-12p40, IL 10 and TNF-α production rates by PBMC increased to values within the normal range upon stimulation of LPS. Heterozygous carriers of the mutation did not show abnormalities, although the height of the males was below the normal range.

Conclusions: This report shows that GH and prolactin secretion was increased is this patient homozygous for a new STAT5b mutation. Although STAT5b plays a role in signaling within immune cells, clinical immunodeficiency is not an obligatory phenomenon of STAT5b deficiency *per se*. Heterozygous carriers of a STAT5b mutation show no signs of GH insensitiviy.

Introduction

The growth hormone receptor (GHR) uses the JAK-STAT proteins as signal transduction pathway. Out of the seven STAT proteins, STAT5b is preferred. Upon phosphorylation, STAT5b dissociates from the GHR, dimerizes and translocates to the cell nucleus, where it transcriptionally regulates the expression of a variety of target genes, including the gene coding for insulin-like growth factor-I (IGF-I) (1). In addition to the role of STAT5b in GHR signal transduction, clinical observations and studies in knock out mice have suggested that STAT5b is involved in the immune system (2, 3).

To date, two immunodeficient females homozygous for a missense mutation and a frameshift mutation respectively, in the STAT5b gene, have been described (4, 5). Recently, we reported the first male with a homozygous frameshift mutation, due to insertion of a C-residue at nucleotide position 1102-1103, that caused a premature truncation of the STAT5b protein (6). The patient presented with severe short stature (Fig. 1), low IGF-I levels and elevated prolactin levels. However, in contrast to the two previously reported females, he showed no overall signs of immunodeficiency.

In this report of our male patient, we present the GH and prolactin secretion patterns. We have evaluated his immunological status in more detail and, in addition, we investigated the clinical and biochemical features of four family members who appeared to carry the mutation.

Methods

All studies were performed after obtaining written informed consent from all subjects.

Clinical measurements and auxology

We measured height with a Harpenden stadiometer, and head circumference with a tape measure. Height and head circumference were expressed as standard deviation score (SDS) for the Dutch population (7).

Biochemical assays

Plasma GH was measured with time-resolved immunofluorometric assay (Wallac/PE, Turku, Finland), using the WHO 80/505 as a standard (1 mg = 2.6 IU). The detection limit was 0.03 mU/l. Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1, IGFBP-2, IGFBP-3, and IGFBP-6 were determined by specific RIAs (8). Acid-labile subunit (ALS) was measured by an ELISA (Diagnostics Systems Laboratories, Inc., Webster, TX, USA) (9). With the exception of free IGF-I and IGFBP-1, smoothed references were available for all parameters, based on the LMS method (10), allowing conversion of the data of the patient to SDS values. Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of six healthy adult controls. Prolactin was measured by a sensitive time-resolved immunofluorometric assay (Wallac, Turku, Finland). The standards were calibrated against the WHO third International Standard for prolactin 84/500. The detection limit of the assay was 0.04 µg/l.

Twenty-four hours plasma hormone profiles

Blood sampling protocol

Patients and controls were admitted to the hospital on the day of study. An indwelling i.v. cannula was inserted into a vein of the forearm 60 min before sampling began and blood samples were withdrawn at 10-min intervals starting at 0900 h and for the next 24 h. A slow infusion of 0.9% NaCl and heparin (1 U/ml) was used to keep the line open. The subjects were free to ambulate, but not to sleep during daytime. Meals were served at 0800, 1230, and 1730 h and lights were turned off between 2200 and 2400 h. Plasma samples were collected on ice in heparinized tubes. The samples were centrifuged at 4°C for 15 minutes; the plasma was then separated, frozen and stored at -20°C until the assays were performed.

Deconvolution analysis

Multiparameter deconvolution analysis was used to determine kinetic and secretory parameters of 24-h spontaneous GH secretion, calculated from GH plasma concentrations. Initial parameters were created with Pulse 2, an automated pulse-detection program. Subsequent waveform-dependent analyses were performed as described previously (11). This technique estimates the rate of basal release, the number and mass of secretory bursts, and the subject-specific half-life. Daily pulsatile GH secretion is the product of secretory burst-frequency and mean mass

of GH released per event. Total GH secretion is the sum of basal and pulsatile secretion.

Approximate entropy

Approximate entropy (ApEn) is a scale- and model-independent statistic, applicable to a wide variety of physiological and clinical time-series data. ApEn quantitates the orderliness or regularity of serial GH concentrations over 24 h. Normalized ApEn parameters of m = 1 (test range) and r = 20% (threshold) of the intraseries standard deviation were used, as described previously (12). Hence, this member of the ApEn family is designated ApEn (1, 20%). The ApEn metric evaluates the consistency of recurrent subordinate (nonpulsatile) patterns in a time series and thus yields information distinct from and complementary to cosinor and deconvolution (pulse) analyses (13). Higher absolute ApEn values denote greater relative randomness of hormone patterns. Data are presented as absolute ApEn values and normalized ApEn ratios, defined by the mean ratio of absolute ApEn to that of 1000 randomly shuffled versions of the same series (14).

Cluster Analysis

For the detection of discrete prolactin peaks, cluster analysis was used. This computerized pulse algorithm is largely model-free, and identifies statistically significant pulses in relation to dose-dependent measurement error in the hormone time series (15). A concentration peak is defined as a significant increase in the test peak cluster vs. the test nadir cluster. We used a 2 x 1 cluster configuration (2 samples in the test nadir and 1 in the test peak) and t-statistics of 2.0 for significant up- and down-strokes in prolactin levels to constrain the false positive rate of peak identification to less than 5% of signal-free noise. The locations and widths of all significant concentration peaks were identified, the total number of peaks was counted, and the mean inter-peak interval was calculated in minutes. In addition, the following pulse parameters were determined: peak height (highest value attained within the peak), incremental peak amplitude (the difference between peak height and prepeak nadir), and area under the peak. Interpulse valleys were identified as regions embracing nadirs with no intervening upstrokes. The total area under the curve was also calculated, as well as the summed pulse areas.

Immunologic investigations

Whole blood was collected in endotoxin-free tubes (Endotube ET; Chromogenix, Milan, Italy), diluted in the ratio 1:5 and stimulated overnight with 0.1–100 ng/ml lipopolysacharide (LPS)(*Escherichia coli* 0111 LPS) in the presence or absence of 100 IU IFN-γ/ml. In several experiments either growth hormone or prolactin was added to a final concentration of 50–500 ng/ml, and 10 ng, respectively. After 18 h, supernatants were collected and tested by ELISA for the presence of IL-12p40 (R&D Systems), IL-10 and TNF-γ (Sanquin Research, Amsterdam)(16). PBMCs were incubated overnight with increasing amounts of IFN-gamma (0–100 IU/ml) in hydron-coated wells to prevent adherence of the monocytes. Cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD64 and PE-conjugated anti-CD14 to analyze CD64 expression on CD14-positive cells using a FACSCalibur (16).

PBMCs were phenotyped by four color immunostaining using FITC-, PE-, PerCPCy5.5-, and activated protein C (APC)-conjugated antibodies, and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Baltimore, MD, USA). Data were analyzed using CELLQuest software (Becton Dickinson).

Results

Phenotype

The index patient was born in the Dutch Antilles. Paternal and maternal heights were 164.3 cm (-2.8 SDS) and 165.6 cm (-0.8 SDS), target height was 176 cm (-1.1 SDS) (7). He was born after a full-term uncomplicated pregnancy with a birth weight of 3270 g (-0.7 SDS) and birth length of 50 cm (-0.4 SDS)(17). During childhood severe postnatal growth retardation was noticed (Fig. 1), although evaluation did not take place until the age of 16 years, when he emigrated to The Netherlands.

Physical examination at 16 years revealed normal body proportions, although his pubertal development was delayed (Tanner stage G1P1), testicular volume being only 1 ml. His bone age was 9 years. He was treated with recombinant human GH (rhGH, Genotropin, Pharmacia) in a dose of 1.5 IU (0.5 mg)/day for 25 months, followed by a dose of 3.0 IU (1.0 mg)/day during an additional 3 months. However, this treatment did not significantly improve growth rate, except for a slight growth acceleration probably due to pubertal development (Fig. 1).

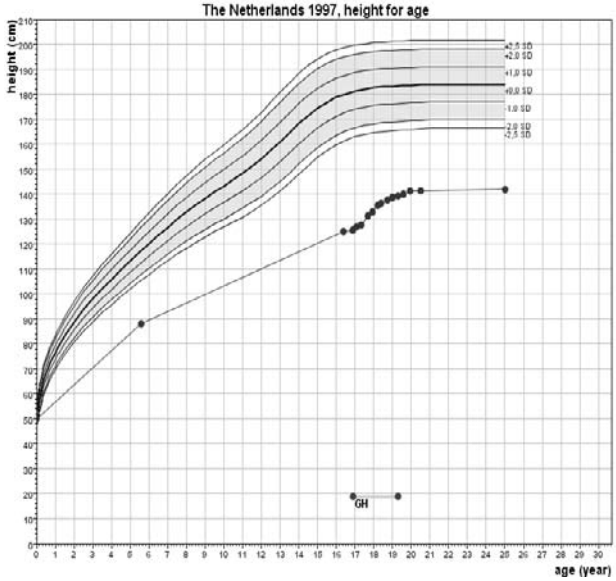

Figure 1. Height for age chart of index patient, based on Dutch references (7). In the absence of local growth standards, we have assumed that inhabitants of the Dutch Antilles of African descent have a similar height as the Dutch population.

At the age of 30 years, he was referred to our clinic for evaluation of his short stature. Auxological parameters are summarized in Table 3. Basal endocrinological parameters were within the normal range: free T4 10.8 pmol/l (10-24 pmol/l), TSH 3.8 mU/l (0.3-4.8 mU/l), LH 6.5 U/l (2-8 U/l), FSH 5.6 mU/l (2-10 U/l) and testosterone 13.1 nmol/l (8-50 nmol/l).

Twenty-four hour profiles of plasma GH and prolactin

The plasma GH concentration profile of the patient and that of a representative control subject are illustrated in Fig. 2. The results of the deconvolution analyis are shown in Table 1. Basal secretory rate and GH burst frequency were increased in the patient, but the other deconvolution parameters were comparable with that of the controls. It should be noted that although the body mass index (BMI) of the patient was within the range of that of the controls, his fat percentage was much higher. ApEn and ApEn ratio were increased in the patient, denoting decreased GH secretory regularity (Table 1).

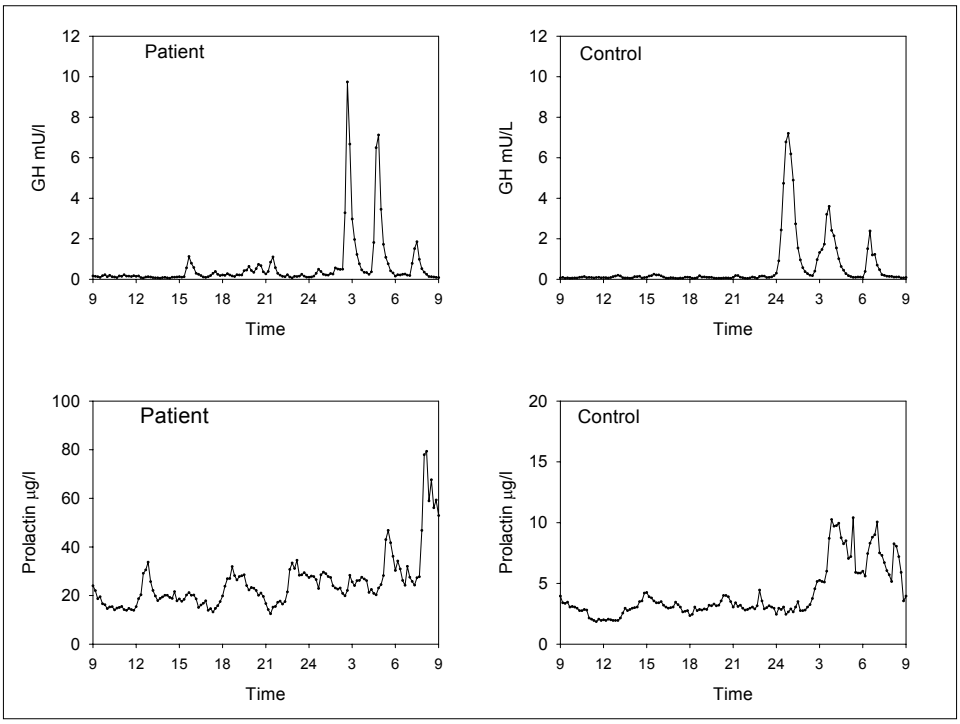

Figure 2. Plasma GH and prolactin profiles obtained by blood sampling at 10 min intervals in the patient and a representative healthy control subject.

The plasma prolactin profiles of the patient and a healthy control are displayed in Fig. 2. Cluster analysis revealed a five- to six fold increase in mean pulse height, mean pulse amplitude, mean pulse mass and pulsatile production. The prolactin secretory regularity was not decreased in the patient.

IGF-I generation test

At the age of 30 years an IGF-I generation test was performed with 12.5, 25.0 and 50.0 µg GH/kg per day for 7 consecutive days for each dose, interrupted by washout periods of 2 weeks and 9 months respectively. The results of the IGF-I generation test are shown in Fig. 3. On the low and intermediate dose, a slight increase of IGF-I and IGFBP-3 was noted. However on the highest dose IGF-I level increased from -6.6 SDS to -2.4 SDS, IGFBP-3 from -11.6 SDS to -6.4 SDS and ALS from –7.2 SDS to –5.3 SDS.

Table 1. Deconvolution analysis and approximate entropy of 24-h GH profiles in a male patient with STAT 5b mutation and eight male healthy controls.

	Patient	**Controls (n=8)**
Basal secretory rate (mU/l per min x 10^3)	3.25	1.6 (0.7-2.4)
Endogenous GH half-life (min)	13.0	16 (13.6-18)
No. of secretory bursts/ 24 h	16	10 (7-13)
Mean mass GH secreted/burst (mU/l)	2.3	4.4 (2.2-6.5)
Pulsatile GH secretion (mU/l per day)	37	36 (25-47)
Total GH secretion (mU/l per day)	41	38 (27-49)
Age (years)	30	33 (30-36)
ApEn (1, 20%)	0.383	0.241 (0.189-0.335)
ApEn ratio	0.46	0.32 (0.27-0.36)
BMI (kg/m^2)	28	26.0 (23.4-29.3)
Fat percentage	40	25 (21-28)

Data of the controls are shown as mean and (95% confidence interval). To convert mU/l into µg/l divide by 2.6. The fat percentage in the patient was measured by dual-energy X-ray absorptiometry (DEXA), and in the controls by BIA.

Table 2. Cluster analysis and approximate entropy of 24-h prolactin profiles in a male patient with STAT 5b mutation and eight male healthy controls.

	Patient	**Male adult controls (n=8)**
Pulse frequency (no./24 h)	17	15.8 (12-19.5)
Mean pulse height (µg/l)	20.5	4.4 (3.5-5.3)
Mean pulse amplitude (µg/l)	7.2	1.4 (1.0-1.7)
Mean pulse mass GH (µg/l)	340	67 (46-87)
Mean nadir concentration (µg/l)	19.1	2.9 (2.2-3.7)
Pulsatile production (µg/l per 24 h)	5750	1000 (700-1300)
AUC (µg/l/24 h)	36250	26.0 (23.4-29.3)
ApEn (1, 20%)	0.746	0.700 (0.590-0.810)
ApEn ratio	0.47	0.44 (0.38-0.50)
Age (years)	30	33 (30-36)

Data of the controls are shown as mean and (95% confidence interval).

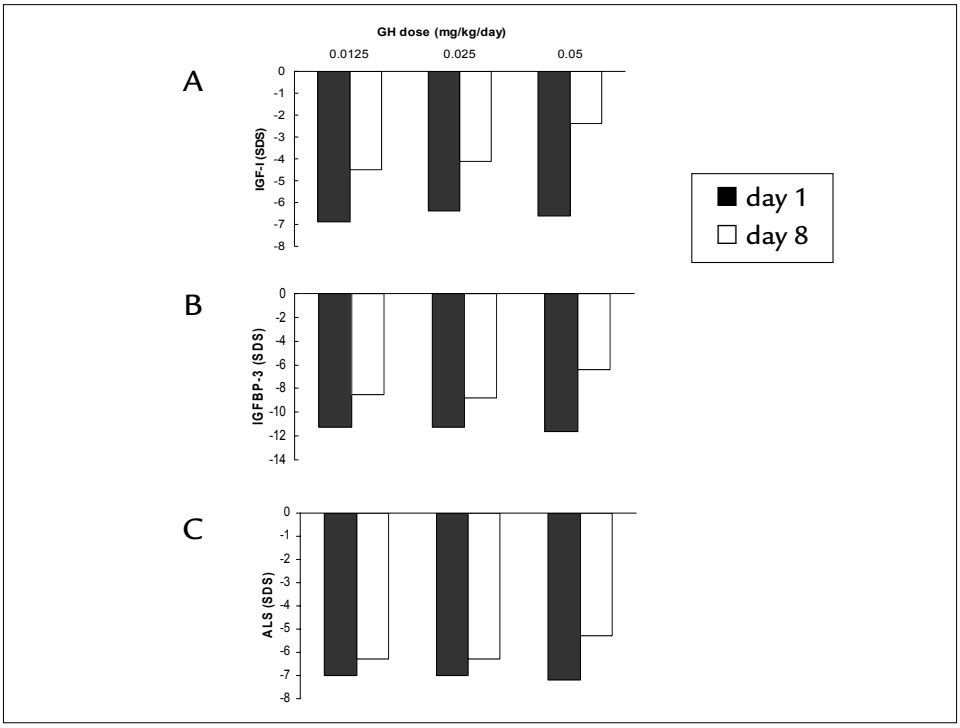

Figure 3. Results of the IGF-I generation test, performed in the index patient. (A) IGF-I (SDS), (B) IGFBP-3 (SDS), and (C) ALS (SDS).

Immunological evaluation

At least one of the first reported female patients with a STAT5b mutation was severely immunocompromised. The first suffered lymphoid interstitial pneumonia and had a *Pneumocystis Jiroveci* (previously, *Pneumocystis Carinii*) lung infection, and presented with severe hemorrhagic varicella and recurrent episodes of herpes zoster (4). The second female patient had primary idiopathic pulmonary fibrosis, a condition in which an infectious etiology cannot be ruled out (5).

Our male patient has had neither pulmonary complaints nor signs of immunodeficiency. Remarkably, he had a hemorrhagic varicella infection at the age of 16 years, but this could well be explained by lack of a history of chickenpox in the past and the fact that he developed the varicella secondary to his brother, who suffered a less severe case. Thus, we do not consider the severe case of chickenpox in this patient as sufficient evidence for immunodeficiency. Otherwise, the medical history of our

patient was unremarkable for childhood infections, and response to vaccinations. Since the two previously reported patients with a homozygous STAT5b mutation appeared to be immunocompromized, we investigated various immunological functions of PBMCs.

IL-12p40, TNF-γ and IL-10 production in response to LPS

Monocyte function of the patient was determined by culturing whole blood in the presence of LPS. There was a LPS-dose dependent increase in IL-12p40 production that was similar to that in the control (Fig. 4A, grey and black bars). Similarly, IL-10 production increased in response to LPS, to a level somewhat higher than observed in the control, but the response was well within normal values (Fig. 4B). These findings indicated that IL-12p40 and IL-10 production in response to LPS was not impaired in the patient. Similar data were obtained for TNF-α production (Fig. 4C). To investigate whether TNF-α and IL-12p40 production could be up-regulated and IL-10 production down-regulated by IFN-γ, the combination of LPS and a high dose of IFN-γ (100 IU/ml) was used to stimulate whole blood. In the patient, IL-12p40 and TNF-α production was increased, and IL-10 decreased, both in an LPS-dose-dependent fashion, and to a level similar to that observed in controls (Fig. 4A-C, hatched bars). Of note, incubation with neither GH nor prolactin influenced the IL-12p40, TNF-γ and IL-10 production after stimulation with LPS (data not shown).

IFN-γ responsiveness in PBMCs

To study the response to IFN-γ in more detail, monocytes were cultured in the presence of 0, 1, 10 and 100 U IFN-γ/ml and the increase in the expression of CD64 (FcγR1) was measured by FACS analysis. The CD64 promoter contains a STAT1 binding site and up-regulation of CD64, in response to IFN-γ stimulation, is a well-accepted marker of IFN-γ responsiveness of these cells (18). IFN-γ stimulation of the monocytes of the patient resulted in a dose-dependent increase in CD64 expression, and at the highest (100 IU IFN-γ/ml) concentration was about equal to the maximal increase in CD64 expression for the healthy control: the cell-surface expression increased upon increasing concentrations of IFN-γ 1.2-, 2.9- and 4.5-fold respectively, for monocytes of the patient and 1.3-, 2.9- and 5.3-fold respectively, for control monocytes, as compared to cell-surface expression without IFNγ. Neither GH nor prolactin influenced basal CD64 receptor expression (data not shown).

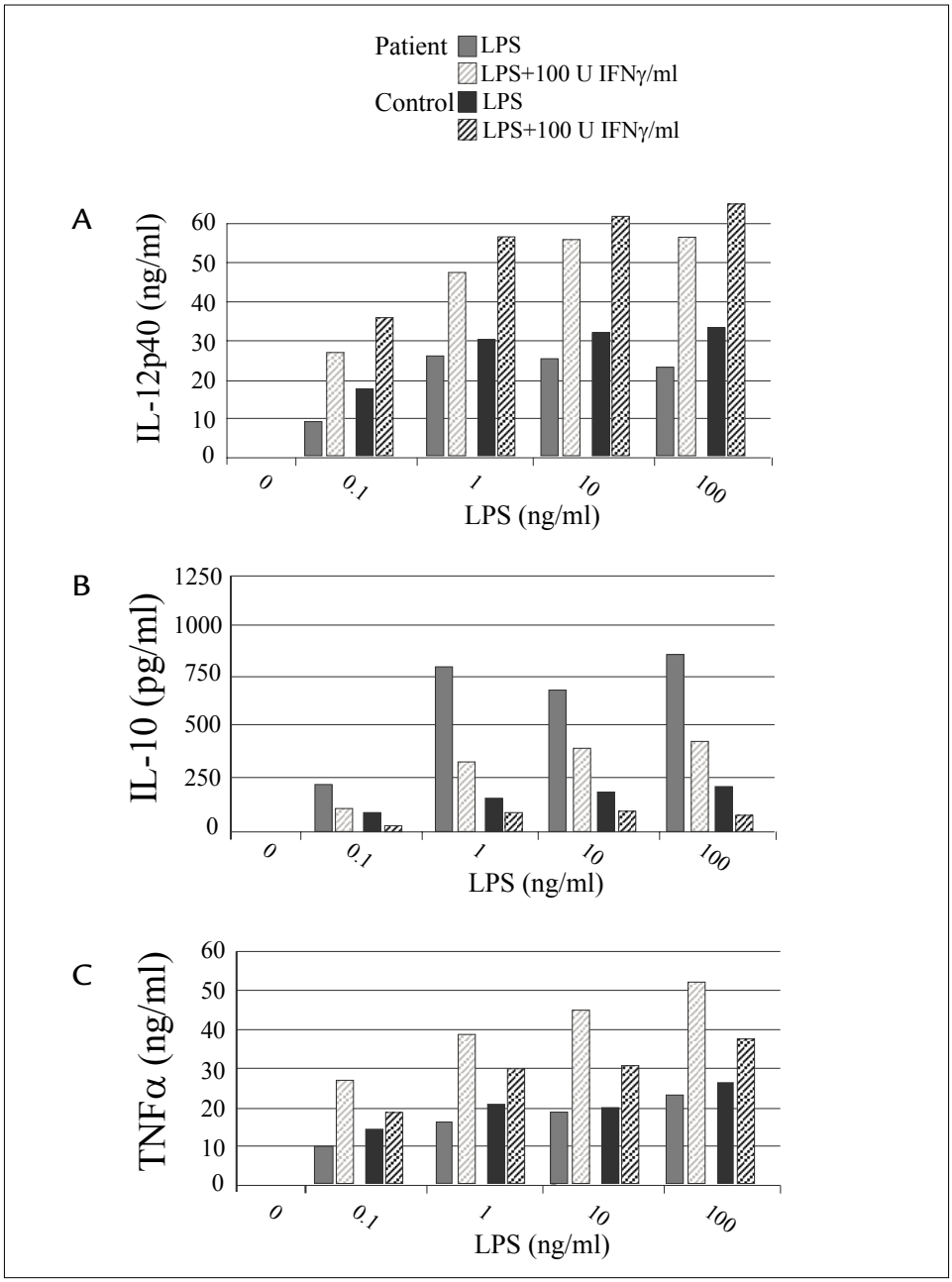

Figure 4. Production of (A) IL-12p40, (B) IL-10, and (C) TNF-α in whole blood of the patient and a healthy control. Whole blood was stimulated with lipopolysacharide (LPS) in the presence or absence of 100 U IFN-γ/ml. Supernatants were collected after 18 h and IL-12p40, IL-10 and TNF-α was measured by ELISA. Experiments were performed in duplicate.

Cell surface expression of IFN-γ-receptors and lymphocyte proliferation

The expression of IFN-γR1 and TNF-γR1 and TNF-γR2-receptors on peripheral blood mononuclear cells did not differ from that in cells of healthy controls, as evident by the relative cell-surface expression of these receptors of 1.14, 1.69, and 0.86 respectively, in the patient as compared with the control.

To determine T cell function, phytohemagglutinin (PHA) blasts were obtained after 2-week culture of PBMCs and proliferative responses were measured after stimulation with various polyclonal activators and Il-2. Stimulation by anti-CD2/28 mAbs or with phorbol-12-myristate-13-acetate (PMA) resulted in somewhat lower responses than in the controls, but this could be overcome by additional co-stimulation with IL-2 (Fig. 5). It should be noted that also between healthy controls, such reactivity can differ markedly. We hypothesize that the overall damping of

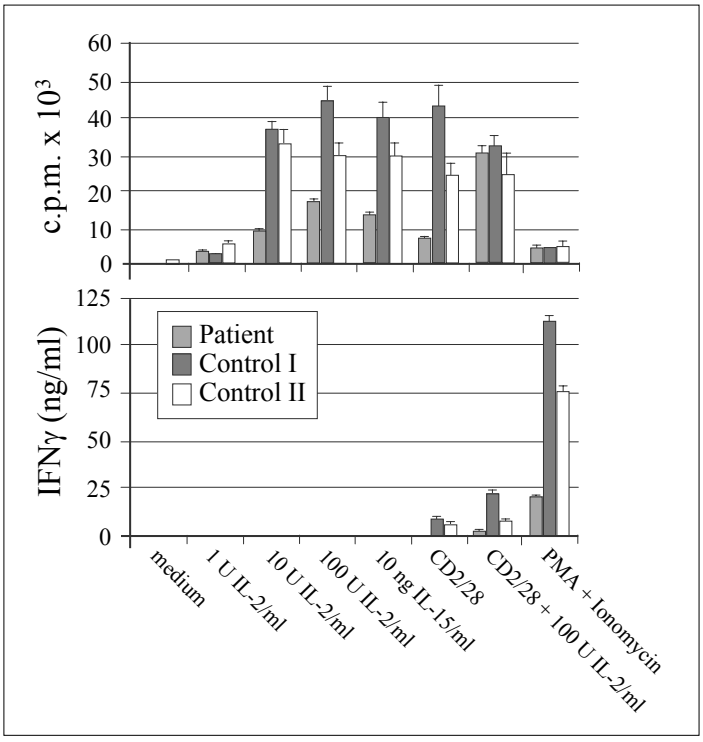

Figure 5. T-cell proliferative responses (top) and IFN-γ production (bottom). PHA blasts obtained after 14-day culture of PBMC were stimulated via TCR ligation by anti-CD2/28 mAbs or with PMA. The somewhat decreased response in the patient was well within normal range and could be overcome by additional co-stimulation with IL-2.

T cell reactivity may reflect the signaling defect due to the STAT5b mutation, but apparently the impaired intracellular signaling can be largely compensated by other mechanisms in the presence of a potent, combined stimulus like anti-CD2/28 with co-stimulation with IL-2. Although both the proliferative response and the IFN-γ production lagged behind the two controls used in this particular experiment, the patient's values are within normal range, in particular to the combined stimulation by CD2/28 and IL-2, or PMA plus Ionomycin.

Carriers of the STAT5b mutation

We investigated four family members of the index patient: his parents, brother and sister. They were all heterozygous carriers of the frameshift mutation in the STAT5b gene. All had a history of normal birth weight. Remarkably, his father and brother were short (height < -2 SDS; Table 3), while his mother and sister were not. They were all in good physical condition, except the father, who had type 2

Table 3. Clinical and biochemical features of the index patient and family members.

Parameter	Patient	Father	Mother	Brother	Sister
Age (years)	31	65	58	33	27
Height (cm) (SDS)	141.8 (-5.9)	164.3 (-2.8)	165.6 (-0.8)	167.4 (-2.3)	165.7 (-0.8)
Weight (kg)	56	87	71	78.5	56
BMI (kg/m^2)	28.2	32.2	25.9	28.0	20.4
Head circ (cm) (SDS)	54 (-1.4)	58 (0.1)	58 (1.6)	58.5 (0.4)	57 (1.0)
Hip/waist ratio[a]	0.94	1.05	0.86	0.94	0.68
GH (mU/l)	0.33	0.2	0.1	0.1	0.6
IGF-I (ng/ml) (SDS)	8 (-8.2)	102 (-0.3)	42 (-3.1)	147 (-0.3)	130 (-1.1)
IGF-II (ng/ml) (SDS)	83 (-6.2)	403 (-0.1)	396 (-0.3)	511 (0.9)	452 (0.2)
IGFBP-1[b] (ng/l)	31	68	18	4	26
IGFBP-2 (ng/ml) (SDS)	142 (-0.6)	247 (0.6)	193 (0.2)	102 [(1.3)	239 (1.0)
IGFBP-3 (mg/l) (SDS)	0.18 (-12.4)	1.58 (-0.8)	1.86 (-0.1)	1.72 (-1.1)	1.95 (-1.0)
IGFBP-6 (ng/ml) (SDS)	230 (1.3)	267 (1.2)	206 (0.8)	178 (0.3)	206 (1.5)
ALS (mg/l) (SDS)	0.7 (7.0)	9.5 (-1.9)	13.4 (-1.0)	16.3 (-0.2)	12.9 (-2.0)

[a] Normal value in men < 0.90, in women < 0.85 (WHO criteria).

[b] Normal range for non-fasting subjects: 24-58 ng IGFBP-1/ml. After overnight fasting there is an average fivefold rise in normal individuals.

diabetes mellitus and prostate carcinoma. None of the family members reported any infectious problems and their plasma prolactin levels and thyroid function were normal. Basal GH levels were within the normal range. Plasma IGF-I levels were also normal with exception of the mother of the index patient, who had a low IGF-I value (Table 3).

Discussion

The data described in this study indicate that total GH secretion is not obligatorily increased in patients with a mutation in the STAT5b gene when compared with healthy BMI-matched controls. In contrast, prolactin secretion was clearly enhanced. We also assessed immunological function in detail in the present patient, because the previous two patients with STAT5b mutations showed signs of immunodeficiencies. With the exception of chickenpox contracted in adolescence (see below), the patient did not suffer any infections, and the data obtained in our patient indicate that a homozygous deleterious STAT5b mutation is not necessarily accompanied by severe immunodeficiency. Finally, heterozygous carriers of an inactive STAT5b mutation are phenotypically normal.

All the three reported patients with STAT5b mutations (4-6) show the same growth pattern, resembling that of patients with classical GH insufficiency (GHI) (19). The degree of postnatal growth retardation is comparable to that of patients with an IGF-I deletion or mutation, who reached a final height of -6.9 and –8.5 SDS respectively (8, 20). The main difference is the intrauterine growth retardation that occurs in the patients with primary IGF-I deficiency, but not in those with STAT5b mutations, reflecting the GH-independent IGF-I gene expression *in utero*.

The pubertal growth spurt that occurred in our patient (15 cm) during GH therapy is about half the magnitude that would be expected in a boy (Fig. 1). We hypothesize, that this growth acceleration is mainly attributable to the rise in sex steroids during puberty, as GH treatment did not normalize IGF-I. This observation supports the hypothesis, that the full growth promoting action of testosterone can only be exerted in the presence of normal GH secretion (21).

The regulation of GH secretion is complex. Pulsatile secretion is important since most of the secretion occurs in bursts. The magnitude of GH bursts correlates with somatic growth and hepatic actions of the hormone. Decreased pulsatility may contribute to decreased IGF-I concentration as observed in aged subjects (22, 23). Pulses are generated by interactions among GHRH, ghrelin and somatostatin under negative feedback control by IGF-I and GH (24). A GH pulse autoregulates hypothalamic-pituitary outflow in a biphasic mode, imposing initial inhibition which is followed by disinhibition of GH secretion (25). In animal models, the increase in GH leads to prompt somatostatin release, which inhibits GHRH and sensitizes the somatotrope to the next GHRH stimulus. In this perspective, ghrelin further amplifies GH release (26).

The diminished negative feedback signaling by IGF-I and GH in GHI leads to amplified GH secretion in many, but not all patients with GHIS (27, 28). IGF-I administration in this syndrome diminishes GH secretion, indicating that feedback can be restored (28). The amplified GH secretion is likely mediated by increased hypothalamic GHRH output, while somatostatin release is not affected in view of the unchanged GH pulsatility (29-32).

The magnitude of GH secretion is determined by age, gender, sex hormones and adiposity (24). Especially, adiposity decreases spontaneous and stimulated GH secretion (33, 34). Detailed studies have established that particularly visceral fat mass is correlated with GH secretion (35, 36). It is therefore of interest that patients with Laron's syndrome have a greater total and regional fat mass than BMI- and gender-matched controls, showing that the normal relation between these two measures as found in the general population is lost (37-39). We hypothesize that the seemingly normal GH secretion in our patient was severely suppressed by his (visceral) adiposity. The relatively unchanged basal GH secretion and pulsatility argue against GH suppression by somatostatin. Detailed GH secretion studies in normal and adipose subjects point to decreased pituitary responsiveness to GHRH as a possible mechanistic explanation (36). Finally, the only moderately increased GH ApEn would fit with partly attenuated GHRH signaling.

It would seem that in our patient altered responsiveness only affected the somatotrophe but not the lactotrophe in view of the stimulated prolactin secretion. Increased prolactin secretion has also been found in other states of endogenous GHRH excess, such as ectopic GHRH-secreting tumors (unpublished data) and hGHRH-transgenic mice (40).

The IGF-I generation test in our patient showed a limited response of plasma IGF-I, IGFBP-3 and ALS levels on stimulation with the two lower doses of GH (Fig. 3). However, after administration of 0.05 mg GH/kg/day the concentration of IGF-I in plasma approached reference range values. Apparently, a high dose of GH can induce IGF-I transcription and secretion considerably, despite the absence of a functional STAT5b protein. Under this condition, possibly, other STAT proteins and/or alternative signal transduction pathways (e.g Microtubule-associated protein kinase (MAPK)/Erk or Protein Kinase B (PKB)/Akt) may play a compensatory role in the regulation of GH-dependent IGF-I expression. Indeed, we found some evidence for this *in vitro* (6). A high dose of GH could activate Erk1/2 and PKB/Akt although at a much lower level when compared with controls. Therefore, at high concentrations other mechanisms may partly compensate the loss of STAT5b, for example by recruitment of other STAT family members to the GHR (41). Since this effect is only seen at high GH concentrations, the affinity of other STAT proteins for the activated GHR appears much lower than of STAT5b.

The first reported female patient with a STAT5b mutation had respiratory difficulties due to lymphoid interstitial pneumonia and a *Pneunocystis Jiroveci* infection. At 8 years of age, she presented with severe hemorrhagic varicella and subsequently she had several episodes of herpes zoster (4). The second female patient with a STAT5b mutation also had pulmonary problems, characterized as primary idiopathic pulmonary fibrosis (5). In contrast, our male patient has had neither pulmonary complaints nor signs of immunodeficiency. Initially, we thought that the hemorrhagic varicella infection at the age of 16 years (6) could be a sign of immunodeficiency. However, the patient did not have a history of chickenpox in the past (a reliable surrogate of a lack of seroimmunity (42, 43)) and he developed the varicella secondary to his brother, who suffered a less severe case. Varicella secondary attack rate among susceptible household contacts is high and at higher age, e.g. above 10-14 years, the infection typically runs a more severe course than in infants (44, 45). For instance, adults with chickenpox, males in particular, have an about 25-fold higher risk of complications like pneumonitis or encephalitis, when compared with children with the disease (46). Thus, we do not consider the severe case of chickenpox in this patient as sufficient evidence for immunodeficiency, secondary to the STAT5b mutation. Moreover, with the exception of the chickenpox, the medical history of our patient was unremarkable for childhood

infections, and response to vaccinations. This was in line with additonal immunological investigations.
The *in vitro* proliferative responses of T-cells to IL-2 and CD2/28 and the production of IFN-γ upon IL-2 stimulation were within the normal range, although the latter cytokine response lagged somewhat behind those in controls. Although the present findings certainly cannot exclude some sort of subtle immunodeficiency, our patient demonstrates that STAT5b deficiency is not obligatory resulting in a severe immunocompromised state, which was suggested by at least one of the other two cases with STAT5b mutations, and findings in STAT5b -/- mice. This suggests a role for other factors in the expression of immunodeficiency in humans with STAT5b deficiency. Since our case is the first male patient described with STAT5b deficiency, it might be possible that the presentation of the immunophenotype of STAT5b deficiency is sex-dependent.

The heterozygous family members of the index patient did not exhibit signs of GH insensitivity. The short stature of the father and brother can not merely be explained by heterozygosity for the STAT5b mutation, as their IGF-I levels were normal. The mother had a low IGF-I level, but her normal IGFBP-3 and ALS levels argue against an effect of partial STAT5b deficiency.

In conclusion, STAT5b is essential for GH signaling and postnatal growth. GH secretion in our patient was likely attenuated by the visceral adiposity, but prolactin secretion was clearly amplified. Although STAT5b plays a role in signaling within the immune system, the clinical consequences of disrupting this signaling pathway appear limited, probably due to compensatory pathways, and immunodeficiency is not present in all patients with a STAT5b mutation. The heterozygous carriers show no signs of GH insensitivity. Other factors may contribute to the short stature of the male carriers.

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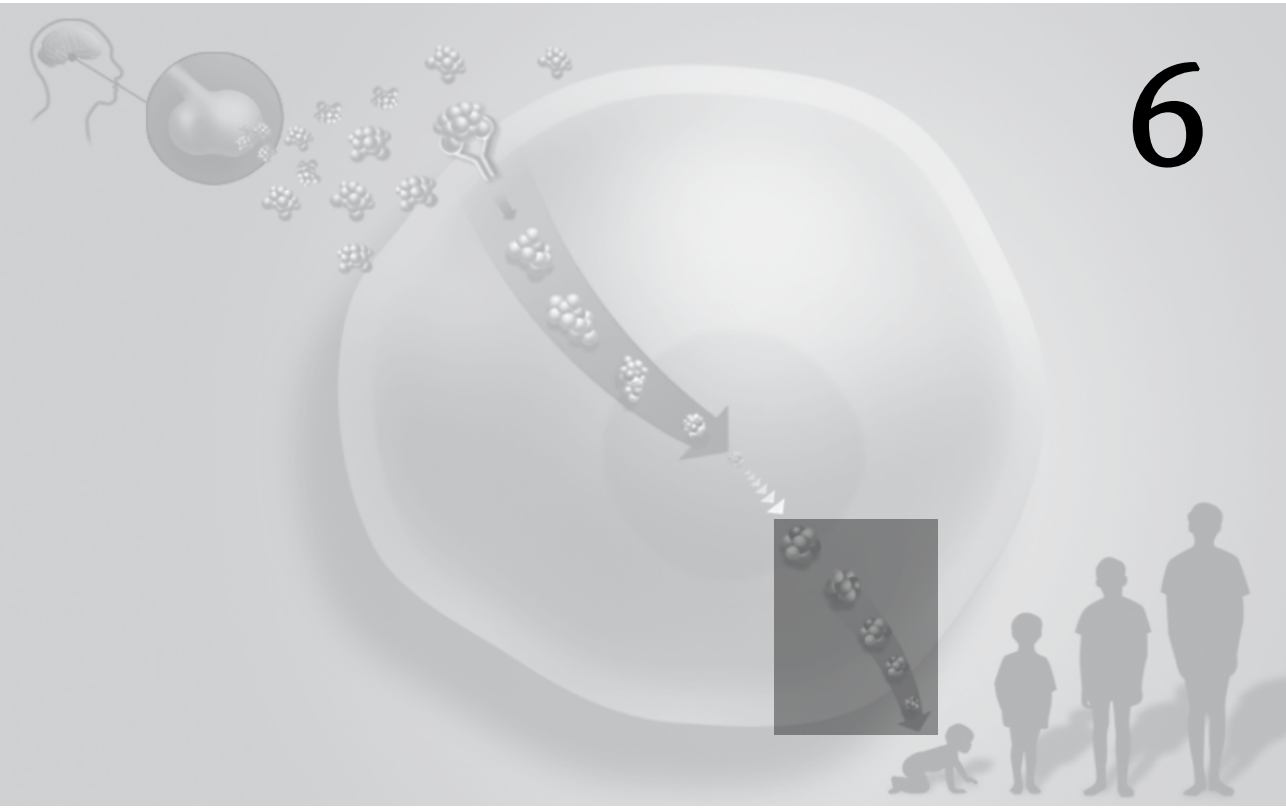
Homozygous and heterozygous expression of a novel IGF-I mutation



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The Journal of Clinical Endocrinology and Metabolism 2005;90(5):2855-2864

Abstract

IGF-I is a key factor in intrauterine development and postnatal growth and metabolism. The secretion of IGF-I *in utero* is not dependent on GH, whereas in childhood and adult life IGF-I secretion seems to be mainly controlled by GH, as revealed from studies on patients with GHRH-R and GH-receptor mutations.
In a 55-yr-old male, the first child of consanguineous parents, presenting with severe intrauterine and postnatal growth retardation, microcephaly, and sensorineural deafness we found a homozygous G to A nucleotide substitution in the IGF-I gene changing valine 44 into methione (V44M). The inactivating nature of the mutation was proven by functional analysis demonstrating a 90-fold reduced affinity of recombinantly produced V44M for the IGF-I receptor. Additional investigations revealed osteoporosis, a partial gonadal dysfunction, and a relatively well preserved cardiac function. Nine of the 24 relatives studied carried the mutation. They had a significantly lower birth weight, final height and head circumference than noncarriers.

In conclusion, the phenotype of our patient consists of severe intrauterine growth retardation, deafness, and mental retardation, reflecting the GH-independent secretion of IGF-I *in utero*. The postnatal growth pattern, similar to growth of untreated GH deficient or GH-insensitive children, is in agreement with the hypothesis that IGF-I secretion in childhood is mainly GH-dependent. Remarkably, IGF-I deficiency is relatively well tolerated during the subsequent four decades of adulthood. IGF-I haploinsufficiency results in subtle inhibition of intrauterine and postnatal growth.

Introduction

In mice, the GH-IGF-I system plays a key role in intrauterine development and postnatal growth and metabolism (1-3). Knockout models of the GH receptor (GHR) and IGF-I have indicated that *in utero* IGF-I, but not GH, is required for normal fetal growth (1, 3, 4). Postnatally, growth is mediated predominantly via GH-dependent IGF-I secretion. In mice the effects on bone accretion before puberty are mediated predominantly via mechanisms independent of GH whereas the pubertal and postpubertal changes are predominantly mediated via GH-dependent mechanisms (5).

So far in humans only one adolescent male has been reported with a total IGF-I deficiency, resulting from a homozygous deletion of exons 4 and 5 of the IGF-I gene (6). This patient exhibited severe intrauterine growth retardation (birth weight –3.9 SDS), severe postnatal growth failure (height in childhood –6.9 SDS), sensorineural deafness, and mental retardation. Recently two patients with mutations of the IGF-I receptor (IGF1R) were described (one compound heterozygote and one heterozygote) (7). Both had decreased intrauterine growth (birth weight -3.5 SDS), comparable with the birth weight of the IGF-I deficient boy, but postnatal growth failure was less severe (a final height of -4.8 SDS and a height in childhood of -2.6 SDS, respectively). The patient who was compound heterozygous for missense mutations had signs of a nonverbal learning disorder and psychiatric anomalies. The patient with the heterozygous nonsense mutation had dysmorphic features, microcephaly, and mild retardation of motor development and speech. The heterozygous parents had heights of -1.6, -2.8 and -2.6 SDS.

The severely compromised intrauterine growth of the patients with IGF-I deficiency and (partial) IGF-I insensitivity is in contrast with the slightly diminished intrauterine growth of patients with either congenital complete GH deficiency (GHD) (8) or GH resistance (9-11) suggesting that, as observed in mice, *in utero* the production of IGF-I is largely independent of GH. Postnatally, IGF-I insufficiency leads to a persistent and severe growth retardation, of approximately equal magnitude as observed in complete GHD or GH resistance, implying that postnatal growth is the result of GH dependent IGF-I production

We describe a 55-yr-old patient with severe pre-and postnatal growth retardation, deafness, and mental retardation. He and his younger brother were described in 1969 as a familial syndrome of prenatal dwarfism, associated with elevated GH levels and end-organ unresponsiveness (12). We show that this clinical picture is caused by a homozygous missense mutation in the IGF-I gene, resulting in an IGF-I polypeptide with strongly decreased binding affinity to the IGF-I receptor *in vitro* and a lack of bioactive IGF-I *in vivo*. This unique patient enables us to study the effects of nearly complete primary IGF-I deficiency in adulthood. Investigations in 24 family members, of whom nine proved heterozygous for the mutation, have also enabled us to characterize for the first time the phenotype of IGF-I haploinsufficiency in detail.

Methods

The guardian of the index case, and all relatives who underwent investigations, provided written informed consent.

Clinical measurements and auxology

Height and sitting height were determined with a Harpenden stadiometer, and head circumference was assessed with a tape measure. Height was expressed as standard deviation score (SDS) based on Dutch references (13), after correction for shrinking (age) and secular trend (birth cohort) (14). Sitting height, sitting height/height ratio and head circumference were also expressed as SDS for the Dutch population (13, 15). Blood pressure was measured with a Dynamap and testicular volume with the Prader orchidometer.

Radiological and sonographic measurements

Bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (QDR 4500; Hologic Inc., Bedford, MA) at the lumbar spine and femoral neck. Bone mineral apparent density (BMAD) of the lumbar spine was calculated using the formula BMAD=BMD x [4 / (π x width)]. The validity of this model has been tested using volumetric data obtained from magnetic resonance imaging of lumbar vertebrae (16). Cardiac ultrasound was performed (GE System 7, Vingmed, Milwaukee, WI) and routine images (for assessment of left ventricular

systolic function) and color Doppler data (to detect valvular abnormalities) were obtained. In addition, tissue Doppler imaging was performed for detailed evaluation of diastolic function (17).

Biochemical measurements

Plasma GH was measured with time-resolved immunofluorometric assay (Wallac/PE, Turku, Finland). Spontaneous GH secretion was assessed after an overnight fast by sampling every 20 min from 0900 to 1300 h. A fasting combined GHRH (1 µg/kg iv at 0 min) and arginine test (0.5 g/kg iv over 30 min, from 0 to +30 min) was performed.

Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6 were determined by specific RIAs (18-22). Acid-labile subunit (ALS) was measured by a commercially available ELISA (Diagnostics Systems Laboratories, Inc., Webster, TX) (23). With the exception of IGFBP-1 and -5, for all parameters smoothed references were available, based on the LMS method (24), allowing conversion of patient data to SDS values. Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of six healthy adult controls. We compared IGFBP-5 with a reference population (5). The proportion of intact IGFBP-3 was determined by a ligand immunofunctional assay (25). The different molecular-size classes of endogenous IGF-IGFBP complexes in plasma were determined by neutral gel filtration (26). IGF-I bioactivity was assessed by an IGF-I kinase receptor activation assay based on cells transfected with the human IGF1R gene (KIRA) (27).

Molecular studies

A skin biopsy was taken, and a culture of dermal fibroblasts was established. These cells were used for a thymidine incorporation assay in response to IGF-I (28). Total RNA was isolated and reversed transcribed into cDNA. Full length IGF-I cDNA was isolated by PCR using the following primer combination (forward 5'-GCTTCATTATTCCTGCTAAC-3'; reverse 5'-AACTCGTGAGAGCAAAGGATC-3'), cloned, and subjected to direct sequencing, using routine procedures. The V44M mutation was introduced into *Escherichia coli* codon optimised human IGF-I cDNA by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Expression and purification of recombinant V44M IGF-I was performed as previously described (29). Folding patterns of V44M IGF-I and native IGF-I were very similar. Purified

V44M IGF-I was shown to be more than 95% pure by N-terminal sequencing and of the correct mass by mass spectroscopy. IGF1R binding affinity was determined using an europium-labeled IGF-I binding assay, essentially as described previously for epidermal growth factor binding to the epidermal growth factor receptor (30) but using IGF1R from lysed P6 cells. 3T3 cells overexpressing IGF1R (gift from Professor Renato Baserga, Thomas Jefferson University, Philadelphia, PA) captured with antibody 24-31 (a gift from Professor Ken Siddle, University of Cambridge, UK). Europium labeled IGF-I (DELFIA Eu-labeling kit, Perkin Elmer, Norton, OH) was added with competing IGF-I or V44M IGF-I and the amount of labeled IGF-I bound was measured by time-resolved fluorescence.

IGF-I binding to bovine placenta membrane fractions was performed using acid Sep-Pak C18 extracts of the sera. Sep-Pak C18 extraction is a well-established technique that effectively removes interfering IGF-binding proteins from both normal and chronic renal failure patient sera or plasma (the latter contain elevated levels of various IGF-binding proteins) as we have verified previously by [^{125}I] IGF-I or [^{125}I] IGF-II probed Western ligand blots of chromatographed materials (31-33). In addition, experiments were performed in which ^{125}I radiolabeled (30,000 cpm) wild-type IGF-I was incubated (overnight at 4 °C) with an aliquot (200 µl) of pooled normal serum. In a similar way, other 200 µl-aliquots of pooled normal serum were incubated with the same amount of ^{125}I radiolabeled wild-type IGF-I in the presence of either 200 ng recombinant wild-type IGF-I or 200 ng recombinant V44M IGF-I. The distribution of [^{125}I]IGF-I among the various molecular-size classes (*i.e.* 150 kD, 40-50 kD, and free IGF-I) in each aliquot of serum was determined after separation by S200 gel filtration and counting of ^{125}I activity in each fraction.

Statistical analysis

Data were analyzed with SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL). Independent-samples *t* tests were used to compare the data of the carriers with the non-carriers. $P < 0.05$ was considered significant.

Results

Subjects

The index case, RV, was born in 1947 as the first of five children of a consanguineous marriage (Fig. 1). His grandfathers were brothers. At birth after 8 months' gestation, weight was 1420 g (-3.9 SDS) and length 39 cm (-4.3 SDS) (34). Postnatally there was a persistent progressive growth failure (Fig. 2) with normal proportions, retarded skeletal maturation, microcephaly, deaf-mutism, and severe mental retardation. At 11.4 yr, a 6-month trial with a testosterone depot preparation (75 mg im once a month) was given, which caused a slight acceleration of growth velocity, but also a clear advance in skeletal maturation. At 15 yr of age he had to be institutionalized because of severe mental retardation (IQ < 40) and motor unrest. Pubic hair and testicular growth occurred at the age of nearly 20 yr. Studies performed at the age of 21 yr (1968) revealed elevated basal [13 ng/ml (20 mU/liter)] and stimulated [127 ng/ml (191 mU/liter)] plasma GH levels in response to insulin-induced hypoglycemia (12). Adrenal and thyroid function was normal. He had a history of a right radial fracture following a minor trauma.

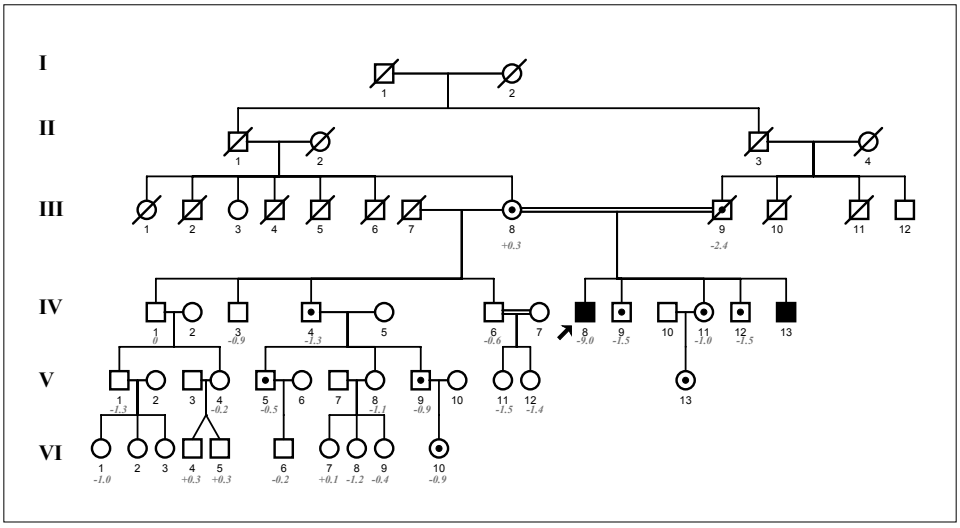

Figure 1. Pedigree of the family. The *arrow* points at the index case. Height SDS of the familymembers is added in *italic*. Heterozygotes are marked with a *central dot*.

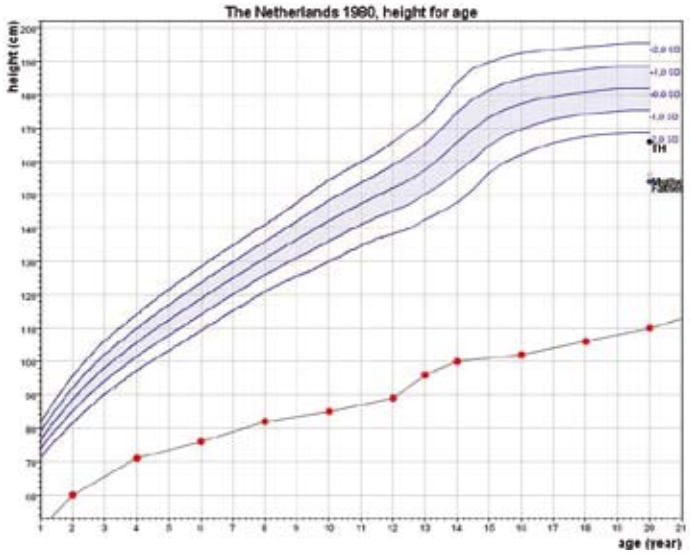

Figure 2. Growth chart of RV. Target height (TH) corrected for secular trend is calculated as: [height father + height mother + 13 cm] /2 + 4.5 cm.

Figure 3. RV at 55 yr of age, together with one of his brothers (168.2 cm).

At 55 yr of age he came again to our attention because of a request for genetic counseling by one of his healthy younger brothers (Fig. 3). The auxological findings are summarized in Table 1. Abdominal fat mass was increased. There were several dysmorphic features, including deep-set eyes, flat occiput, a columella extending beyond the alae nasi and a striking micrognathia. His extremities showed broad end phalanges and convex nails. There was hypermobility of the interphalangeal joints but not of the other joints. The mobility of both elbows was restricted. Blood pressure was 172/93 mm Hg. Neurological examination was normal. Audiological analysis demonstrated severe bilateral hearing loss confirmed by absent brainstem evoked potentials. Testicular volume was 7 (left) and 1 ml (right). The stretched penile length was 8 cm (P10-90: 10.2-16.4 cm) (35). Bilateral inguinal scars were seen. In 1975 an operation for a bilateral inguinal hernia with ileus had been performed. Cardiovascular, respiratory, and abdominal examinations were normal. His IQ was less than 40. Ophthalmological examination revealed cataracts of both eyes and shallow anterior chambers. Vision was 0.63.

His youngest brother, AV, was born at term with a birth weight of 1900 g (-4.5 SDS) (34). His clinical phenotype strongly resembled that of RV**.** He too showed deaf-mutism and severe retardation (12). At the age of 12 yr (1968) endocrine investigations were performed. At baseline GH was 3 ng/ml and after insulin-induced hypoglycemia, plasma GH exhibited an exaggerated response [maximum 206 ng/ml (309 mU/liter)] (36). An oral glucose tolerance test showed an elevated baseline plasma insulin (15 mU/liter) (37), an insulin peak of 46 mU/liter with a maximum glucose of 125 mg/dl (6.9 mmol/l) [normal according to the American Diabetes Association (38)], and a normal suppression of plasma GH (<1 ng/ml). Prolonged fasting resulted in a physiological rise of GH (up to 56 ng/ml) and free fatty acids. Human pituitary GH was administered im for 4 d in a dose of 5 mg (≈ 4 IU) once daily, which did not result in a reduction of urinary excretion of nitrogen, potassium, and phosphate or an increase of hydroxyl-proline excretion (12). He died at 32 yr of age of aspiration pneumonia and severe gastritis with reflux oesophagitis after orthopedic surgery (triple athrodesis of left ankle).

Radiological features

BMD at the right femoral neck was 0.57 gr/cm^2 (-3.7 SDS) and 0.51 gr/cm^2 at the left femoral neck (-4.3 SDS). Lumbar spine BMD (L2-L4) was 0.52 gr/cm^2

Table 1. Auxological and biochemical characteristics of RV at 55 yr of age

Variable	Value	SDS
Height (cm)	117.8	- 8.5[a]
BMI (kg/m^2)	17.9	- 2.0[b]
Height/sitting height ratio	0.51	- 0.3[b]
Head circumference (cm)	44.2	- 8.0[b]
GH maximum (ng/ml) after GHRH/arginine stimulation	91.9	
GH maximum (ng/ml) in 3-h GH profile	1.8	
Total IGF-I (ng/ml)	606	+ 7.3
IGF-II (ng/ml)	454	+ 0.5
ALS (µg/ml)	28.9	+ 3.4
IGFBP-1 (ng/ml)	12	Low[c]
IGFBP-2 (ng/ml)	106.3	- 1.0
IGFBP-3 (µg/ml)	1.98	+ 0.1
IGFBP-4 (ng/ml)	145.6	+ 0.6
IGFBP-5 (ng/ml)	347.7	- 0.4
IGFBP-6 (ng/ml)	121.8	- 1.5
		Normal value
Testosterone (ng/dl)	271	>230
Inhibin B (ng/liter)	64	>150
LH (U/liter)	3.5	2-8
FSH (U/liter)	19.2	2-10

To convert GH to milliunits per liter, multiply by 2.6; 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 nanograms per milliliter, multiply by 0.033; to convert IGFBP-2 to nanamoles per liter, multiply by 0.032; to convert IGFBP-3 to nanomoles per liter, multiply by 33.3; to convert IGFBP-4 to nanamoles per liter, multiply by 0.038; to convert IGFBP-5 to nanomoles per liter, multiply by 0.035; to convert IGFBP-6 to nanomoles per liter, multiply by 0.034; to convert testosterone to nanomoles per liter, multiply by 0.035

[a] See Ref. 60

[b] See Ref. 13

[c] Normal range for nonfasting subjects: 24 - 58 ng IGFBP-1 per liter. After overnight fasting there is an average 5-fold rise in normal individuals.

(-5.2 SDS*)*. BMAD was 0.2 gr/cm^3 (-3.5 SDS) (16). Cardiac ultrasound showed a left ventricular ejection fraction of 60%, without regional wall motion abnormalities. Left ventricular mass was normal. Diastolic function was impaired (abnormal relaxation), with an E (early flow) to A (late flow) ratio of 0.53, and the E' (early diastolic myocardial velocity assessed by tissue Doppler imaging) being 4.5 cm/sec (normal value >12 cm/sec).

Biochemical features

The results of the biochemical analysis are shown in Table 1. Maximum GH concentration after stimulation was in the upper normal range (36). Serum IGF-I was markedly elevated. After an overnight fast, the IGFBP-1 level remained low. Circulating levels of IGFBP-2, -3, -4, -5, and -6 were within the normal range whereas ALS was increased. The proportion of intact IGFBP-3 did not differ from that encountered for age- and gender-matched normal controls. As with normal plasma, in the patient's plasma, most of IGF-I (> 90%) (Fig. 4A) and IGF-II (>70%) (Fig. 4B) was associated with the 150 kD complex. The distribution of IGFBP-3 and IGFBP-5 over the various molecular weight classes in serum appeared normal as did IGFBP profiles analyzed by Western ligand blotting (data not shown).

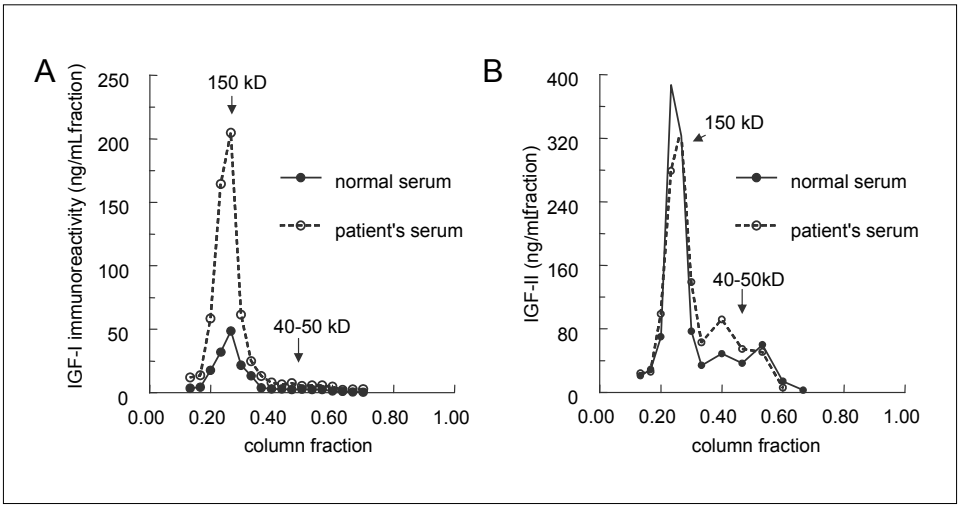

Figure 4. IGF-I (A) and IGF-II (B) in serum of RV are normally distributed over the various molecular weight classes. The majority of IGF-I and -II is present in the 150-kDa complex with IGFBP-3 and

Mutational analysis

Considering the increased level of IGF-I in our patient, we reasoned that the clinical symptoms could be caused by either an inactivating mutation in the IGF1R or in IGF-I itself. The wild-type (wt) IGF-I induced stimulation of ^{3}H-thymidine incorporation by cultured fibroblasts of the patient was comparable with the average response observed in cells obtained from 10 healthy control subjects. Considering the variation observed in IGF-I responses using dermal fibroblast cultures (28), the presence of an inactive IGF1R appeared unlikely (Fig. 5A). Subsequently, IGF-I cDNA was isolated by RT-PCR from fibroblasts. Sequence analysis identified a homozygous G>A nucleotide substitution at position 274, changing valine at position 44 of the mature IGF-I protein to methionine (Fig. 5B). The same nucleotide substitution was also present in the genomic DNA but not in a control panel of 87 individuals. V44 is located in the first α helix of the A domain of wt IGF-I and is exposed at the surface of the polypeptide (39). A normal male karyogram was found (46 XY).

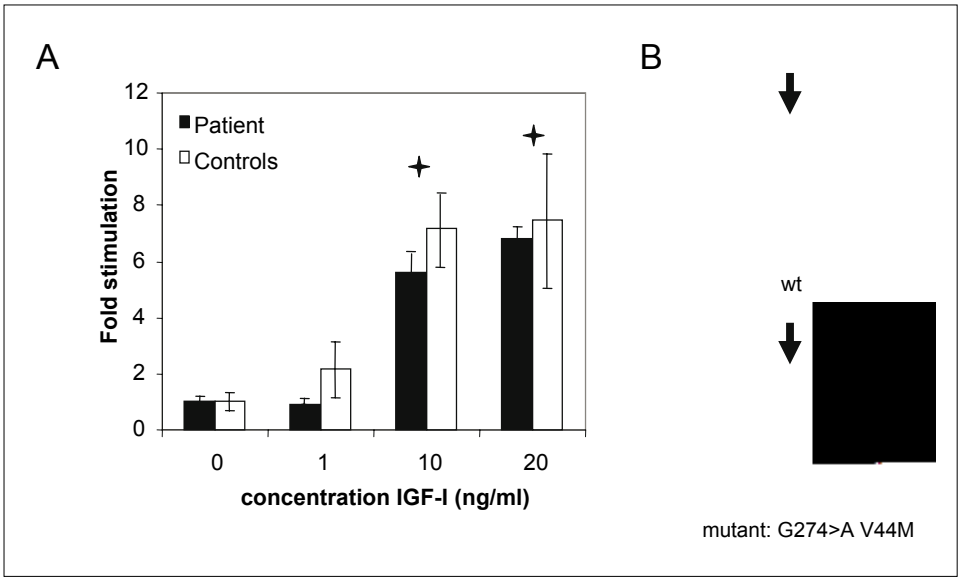

Figure 5.

A. Fibroblasts of RV respond normally to wt IGF-I in a thymidine incorporation assay. An *asterisk* indicates a statistical difference from unstimulated cells ($P < 0.01$).

B. Sequence analysis of wt IGF-I (*upper panel*) and IGF-I of RV (*lower panel*). *Arrow* indicates the homozygous G>A nucleotide alteration.

Functional analysis

We subsequently used the mutant cDNA for the synthesis of mutant IGF-I (V44M). V44M had an approximately 90-fold lower affinity for the IGF1R compared with wt IGF-I measured in both europium (Fig. 6A) and ^{125}I-labeled IGF-I competition receptor binding assays (Fig. 6B). Competition binding studies with ^{125}I-labeled IGF-I and serum of our patient or an age-and sex-matched control revealed that serum of the patient displaced ^{125}I-wt IGF-I with an ED_{50} of 11.3 μl serum equivalents (Fig. 6B). This volume of serum contained 5.3 ng IGF-II (serum value of 454 ng/ml, Table 1) that also competed for binding to the receptor. This is close to the deduced ED_{50} value of purified human IGF-II (4.3 ng) in this assay. Thus, these calculations demonstrate that the displacement of ^{125}I-wt IGF-I in the bovine placenta membrane assay can be explained by the presence of IGF-II and that V44M in serum did not significantly contribute to this process, in agreement with the data obtained for recombinant V44M providing further support for the inactivating nature of the mutation. In addition, a competition experiment was performed in which ^{125}I-wt IGF-I was incubated with normal serum in the absence or presence of unlabeled wt and V44M IGF-I. As shown in Fig. 6C wt IGF-I and V44M IGF-I competed with equal affinity for 150 kD and 40-50 kD complex forming with radiolabeled ligand. This suggests that the V44M mutation has only minor effect on binding to IGFBPs in marked contrast to binding to the IGF1R.

Carriers of the IGF-I mutation

We investigated a total of 24 relatives of the index patient. Nine of them carried the heterozygous V44M IGF-I mutation. Their birth weight and head circumference were lower in comparison with noncarriers (Table 2). Their height SDS was lower as shown in Fig. 7. Testicular volume was normal.

Except for one diabetic individual and the 87-yr-old mother, fasting glucose levels were normal. Fasting insulin levels were significantly higher in carriers (Table 2). Serum LH, FSH, testosterone, and estradiol were normal (data not shown). Basal GH levels (a single fasting sample) were within the normal range. Carriers had higher total IGF-I levels (Fig. 7) and lower IGF-I bioactivity than noncarriers (Table 2).

We measured BMD at the femoral neck and lumbar spine in all carriers. They had values within the normal range for their age and sex. In 21 relatives (three young children were excluded), we performed audiometry, revealing hearing abnormalities in seven individuals. However, no statistically significant association with carriership could be detected.

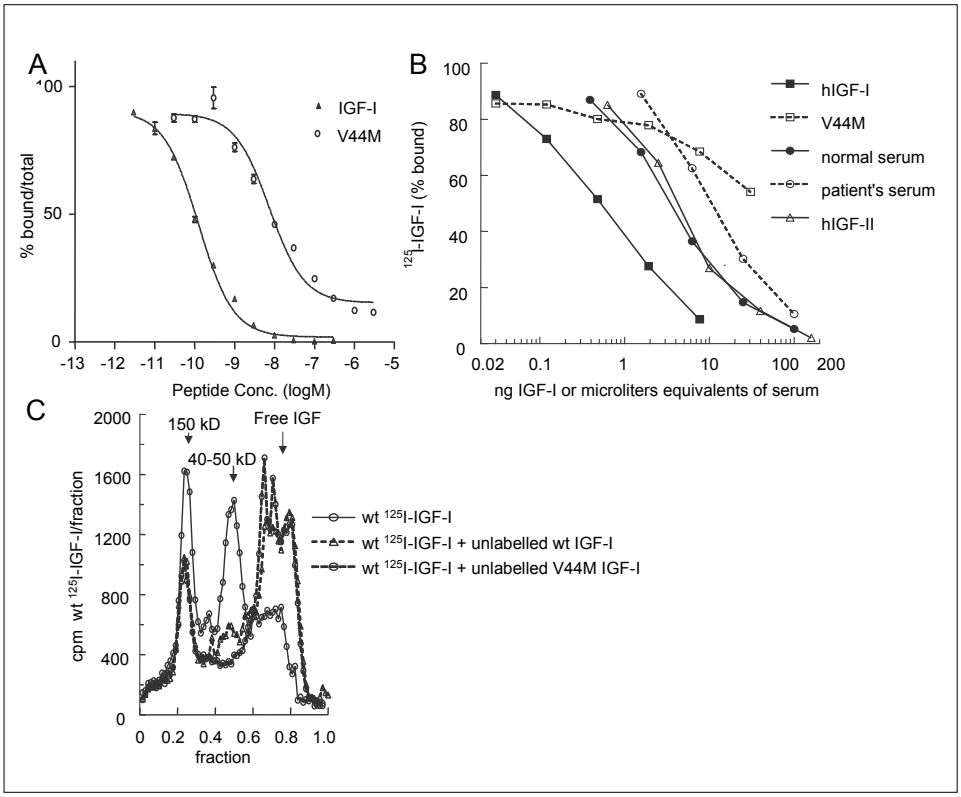

Figure 6.

A Receptor binding assay showing 90-fold reduced binding affinity of V44M IGF-I to the IGF-I receptor.

B. Competition binding assay using ^{125}I-IGF-I as tracer and IGF1R-expressing membranes of bovine placenta. Competition is performed with wt hIGF-I, wt hIGF-II, V44M, serum of the patient, or an age- and sex-matched control. The ED_{50} of a Sep-Pak C18 extract of RV is 11.3 µl, which corresponds to 5.3 ng IGF-II, being close to the deduced ED_{50} of IGF-II (4.3 ng). The values of IGF-I and IGF-II of the age- and sex-matched control were 185 ng/ml and 386 ng/ml.

C. ^{125}I radiolabeled wt IGF-I was incubated with an aliquot of pooled normal serum, in either the absence or presence of 200 ng recombinant wt IGF-I or 200 ng recombinant V44M IGF-I. wt IGF-I and mutant IGF-I efficiently competed for 150 kD and 40-50 kD complex forming with iodinated IGF-I.

Table 2. Auxological and biochemical characteristics of the carriers of the heterozygous IGF-I mutation (n = 9) *vs*. noncarriers (n = 15).

Variable	Carrier	Noncarrier	P value
Age (yr)	44.2 ± 7.8	27 ± 5.7	0.08
Male/female	5/4	8/7	
Height (SDS)[a]	-1.0 ± 0.2	-0.4 ± 0.2	0.04
Head circumference (SDS)	-1.0 ± 0.3	0.5 ± 0.3	< 0.01
BMI (SDS) [b]	0.5 ± 0.3	0.7 ± 0.3	0.62
Birth weight (g)[c]	3048 ± 101	3358 ± 85	0.04
Fasting insulin (mU/liter)[d]	16.5 ± 1.9	12.1 ± 0.7	0.03
Total IGF-I (SDS)	0.6 ± 0.3	-0.3 ± 0.2	0.04
Bioactive IGF-I (ng/ml)	0.9 ± 0.09	1.6 ± 0.16	< 0.01

Data are expressed as mean values ± SEM.
To convert insulin to picomoles per liter, multiply by 7.175; to convert bioactive IGF-I to nanomoles per liter, multiply by 0.131.
[a] For the calculation of mean height SDS, height of the father of the index cases was included (n=10).
[b] For the analysis of BMI, one noncarrier (obese and type 2 diabetes) was excluded.
[c] Birth weight data were available from eight carriers and 15 noncarriers.
[d] For the analysis of fasting insulin, one noncarrier (obese and type 2 diabetes) was excluded.

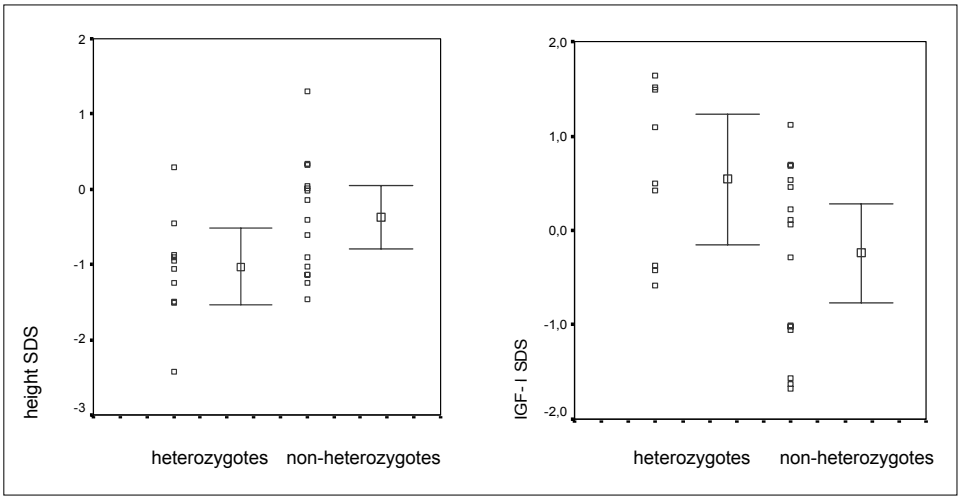

Figure 7: Height SDS and IGF-I SDS of heterozygous and nonheterozygous family members (*dots*) and 95% confidential interval (*bars*).

Discussion

There are two principal, novel findings in our study. It is the first report on a homozygous missense mutation in the human IGF-I gene resulting in an IGF-I protein that is hardly capable of interacting with the IGF1R but with relatively unaffected binding capacity for IGFBPs. This leads to severe effects on growth and development *in utero* and during childhood. It provides an opportunity to evaluate the effects of primary IGF-I insufficiency in adulthood, in comparison with the secondary IGF-I deficiency present in severe GH resistance or deficiency. Second, we have shown for the first time that IGF-I haploinsufficiency results in a subtle, but statistically significant, inhibition of intrauterine and postnatal statural and cranial growth and increased fasting insulin concentrations.

From observations on analogous peptides in the human as well as in other species, one can predict that a change in V44 should lead to a severe loss of function. V44 is not conserved only in IGF-I of all species except one (40) available in the National Center for Biotechnical Information database, but also in the two structurally related proteins IGF-II and insulin. In insulin, amino acid V^{A3}, structurally homologous to V44 in IGF-I, is involved in binding to the insulin receptor (41). Interestingly, a germline mutation of V^{A3} to leucine (insulin Wakayama) resulting in a variant that poorly binds to the insulin receptor has been associated with diabetes mellitus (42).

Proof for the inactivating nature of V44M was provided by demonstrating a 90-fold lower binding affinity for the IGF-I receptor in receptor binding assays using recombinantly produced protein. In addition, total receptor binding activity of the patient's serum was equivalent to a level that would be achieved by the same concentration of IGF-II found in his serum. This observation indicated that V44M did not contribute significantly to receptor binding despite the increased circulating levels of V44M in serum. Thus, the phenotype of our patient is caused by a complete lack of bioactive IGF-I. Furthermore IGF-II, although in the upper normal range, cannot compensate for IGF-I deficiency *in utero*, in childhood and neither in adulthood, in agreement with earlier reports (1-3, 6, 7).

Remarkably, gel filtration experiments revealed that the relative distribution of V44M over the various molecular weight classes in serum is not different from wt IGF-I in normal serum. This suggests normal binding of V44M to IGFBPs and is indicative of a relatively small impact on overall structure of V44M. It also implies that V44M can compete normally with IGF-II in the formation of complexes with the binding proteins and ALS. This was supported by our observation that V44M efficiently competed with wt IGF-I for 150- and 40-50 kD complex formation in serum, and was recently confirmed by BIAcore analysis showing normal binding of V44M to human (h)IGFBP-2, hIGFBP-3 or hIGFBP-6 biosensor surfaces. In addition, nuclear magnetic resonance analysis demonstrated only subtle changes in overall structure of V44M (43).

Considering the respective roles of IGF-I and GH *in utero* and in childhood, the clinical presentation of these two sibs with a dysfunctional IGF-I generally confirm the observations made in the single previously reported case of the IGF-I-deficient adolescent (6) and the two patients with partial IGF-I insensitivity (7). *In utero*, biologically active IGF-I is necessary for normal intrauterine growth, brain development, development of the inner ear, and mandibular growth, similarly to the phenotype of IGF-I knockout mice (44, 45). The functional effects of a partial IGF-I insensitivity on the brain are much milder, but the head circumference at birth of the heterozygote was severely decreased (-4.6 SDS). No effects were seen on inner ear development. This suggests that the heterozygous and compound heterozygous mutations in the IGF1R do not completely preclude IGF-I signaling. The variation in dysmorphic features in the different cases of IGF-I deficiency and IGF-I insensitivity may be partially due to a tissue-specific expression of IGF1R alleles (7). The absence of intrauterine growth retardation, mental retardation, hearing problems, and micrognathia in GH-deficient and GH-insensitive infants implies that in the fetal stage IGF-I secretion is GH-independent.

In childhood and adolescence, a complete lack of IGF-I function seems to lead to a qualitatively similar postnatal growth pattern as seen in a complete lack of GH effect: severe proportionate growth retardation accompanied by retarded skeletal maturation and delayed pubertal development. In our case and the case with IGF-I deletion growth was extremely retarded, and led in our male patients to a final height of 118 cm (about 8.5 SD below the mean). In the two patients with partial

IGF-I insensitivity short stature was less extreme (final height -4.8 SDS and a height in childhood of -2.6 SDS, respectively). Patients with GH insensitivity due to a GHR mutation reach an average height varying from –5.3 to -12 SDS (46-48). Mean height of patients with GHRH-R mutations is –7.2 SDS. So the effect of primary or secondary IGF-I insufficiency on postnatal growth is similar.

Whereas IGF-I deficiency leads to specific detrimental consequences for growth and development of various organs *in utero* and during childhood, IGF-I deficiency appears to be tolerated relatively well during the subsequent four decades. We found a low BMD and decreased testicular function in our patient, but both findings might be explained by other factors than IGF-I deficiency. The severe osteoporosis in our 55-yr-old patient could be explained by hypomuscularity and a low level of physical activity. Because our patient had a normal serum testosterone level, it is unlikely that his osteoporosis is due to hypogonadism. Our findings are in contrast with the absence of osteoporosis in the adolescent with the IGF-I deletion, and in the 12-yr-old girl with IGF1R mutation. They are also in contrast with the normal BMAD values observed in patients with Laron syndrome (GH insensitivity) and in patients with untreated GHD due to GHRH receptor mutations (49-51). Similar observations in future cases with primary IGF-I deficiency are needed before it can be assumed that maintenance of normal bone density is dependent on GH-independent IGF-I secretion.

At 55 yr of age, secondary sex characteristics and plasma testosterone level were normal, but FSH was elevated and inhibin was low in our patient. This suggests that mainly Sertoli cell function and spermatogenesis are impaired, probably resulting in infertility. The significance of the partial gonadal failure that we observed in our patient is most probably the result of testicular damage due to the bilateral inguinal hernia operation. This speculation is strengthened by the observation that the adolescent with IGF-I deletion has normal basal and GnRH-stimulated LH and FSH values. Furthermore, in patients with a secondary IGF-I deficiency due to a GHR or GHRH receptor mutation, fertility is normal.

IGF-I deficiency appears noncritical for adult cardiac function because only diastolic function was mildly impaired, a nonspecific finding observed frequently in individuals of 50 yr and older. The hypertension we found is in line with the

findings in IGF-I deficient mice. IGF-I has growth-promoting effects on smooth vascular muscle and has potent vasodilator effects, suggesting a possible role for IGF-I in the regulation of vascular tone (52). In our patient, with probably life-long exposure to increased GH levels, the absence of cardiac abnormalities is striking, compared to the findings in patients with untreated acromegaly who are exposed to GH and IGF-I excess for years before diagnosis. In these patients the development of both ventricular hypertrophy and valvular abnormalities are strongly dependent on the duration of exposure to increased GH production (53, 54). We speculate that the cardiomyopathic changes found in patients with acromegaly are caused by increased levels of IGF-I due to GH-excess.

One of the clinical characteristics of our patient is a restricted mobility of both elbows. This feature was also described in patients with GHR deficiency and GH deficiency caused by Prophet of Pit-1 (PROP 1) gene mutation (55, 56). This suggests that the effect of GH on the development of a normal joint mobility is mediated through IGF-I.

With regard to the IGFBPs, the normal IGFBP-3 level in RV and the patient with the IGF-I deletion supports the notion that the production of IGFBP-3 is controlled independently of IGF-I in humans (57). ALS is increased, which suggests that ALS is primarily GH dependent, and in this case may predominantly circulate in an unbound form. Also in the case of IGF-I deficiency, an elevated plasma ALS was reported, which decreased after IGF-I therapy (57).

In contrast to patients with GHD and GH insensitivity, in whom IGFBP-1 is usually elevated, IGF-I deficiency is associated with a low fasting IGFBP-1. IGFBP-1 has an inhibitory effect on the growth-promoting and anabolic effects of IGF-I. It is primarily regulated by insulin, but also IGF-I, IGF-II, and GH have specific inhibitory effects on IGFBP-1 expression (58). We speculate that the persistently low IGFBP-1 levels are caused by the direct suppressive effect of high GH levels (59).

Finally, it is of interest that heterozygous carriers of the mutation have a lower birth weight, height, and head circumference than noncarriers. Although height SDS was significantly lower than in the noncarriers, it was still within the normal range. The clinical findings are in line with the elevated IGF-I, and lower IGF-I bio-

activity. We hypothesize that a gene-dose effect may explain this mild effect on growth. The carriers had higher fasting insulin levels than the noncarriers supporting the role of IGF-I bioactivity in insulin sensitivity (57). Fertility in heterozygous carriers appears normal, considering the offspring of the obligate heterozygous parents of the index cases and the fact that four other adult heterozygotes had children. This observation is supported by normal testosterone, estradiol, LH, and FSH levels in carriers.

In conclusion, this homozygous missense mutation in the human IGF-I gene results in an abnormal IGF-I molecule with low receptor binding. In contrast to the severe effects on pre- and postnatal growth and development, IGF-I insufficiency is well tolerated in adulthood. IGF-I haploinsufficiency results in decreased IGF-I bioactivity, a subtle, but statistically significant, inhibition of intrauterine and postnatal statural and cranial growth, and elevated fasting insulin levels.

Acknowledgements

We are grateful to Dr. Bart Ballieux (Leiden) for biochemical advice; Ms Kerrie McNeil (Adelaide) for assistance in recombinant V44M production; and Mr. Cok Hoogerbrugge (Utrecht) for laboratory assistance.

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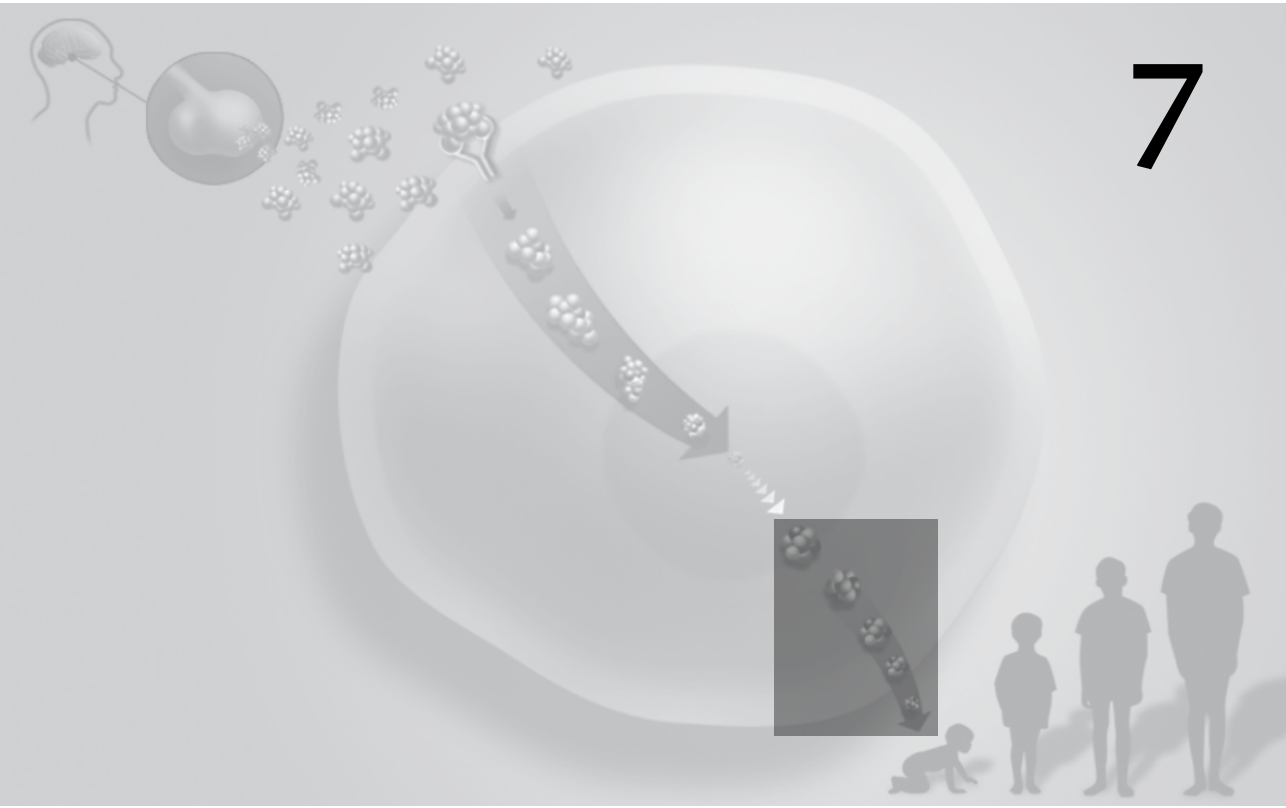
Structural and functional characteristics of the Val[44]Met IGF-I missense mutation: correlation with effects on growth and development



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Molecular Endocrinology 2005;19(3):711-721

Abstract

We have previously described the phenotype resulting from a missense mutation in the IGF-I gene, which leads to expression of IGF-I with a methionine instead of a valine at position 44 (Val^{44}Met IGF-I). This mutation caused severe growth and mental retardation as well as deafness evident at birth and growth retardation in childhood, but is relatively well tolerated in adulthood. We have conducted a biochemical and structural analysis of Val^{44}Met IGF-I to provide a molecular basis for the phenotype observed. Val^{44}Met IGF-I exhibits a 90-fold decrease in type 1 IGF receptor (IGF1R) binding compared with wild-type human IGFI and only poorly stimulates autophosphorylation of the IGF1R. The ability of Val^{44}Met IGF-I to signal via the extracellular signal-regulated kinase 1/2 and Akt/protein kinase B pathways and to stimulate DNA synthesis is correspondingly poorer. Binding or activation of both insulin receptor isoforms is not detectable even at micromolar concentrations. However, Val^{44}Met IGF-I binds IGF-binding protein 2 (IGFBP-2), IGFBP-3, and IGFBP-6 with equal affinity to IGF-I, suggesting the maintenance of overall structure, particularly in the IGFBP binding domain. Structural analysis by nuclear magnetic resonance confirms retention of near native structure with only local side-chain disruptions despite the significant loss of function. To our knowledge, our results provide the first structural study of a naturally occurring mutant human IGF-I associated with growth and developmental abnormalities and identifies Val^{44} as an essential residue involved in the IGF–IGF1R interaction.

Introduction

The IGF system plays an important role in normal growth and development. Activation of the type-1 IGF receptor (IGF1R) by IGF-I or IGF-II results in potentiation of growth, survival and differentiation. The action of IGFs is modulated by IGF binding proteins (IGFBPs), which regulate the availability to bind to the IGF1R.

The importance of IGF-I in normal growth has been demonstrated experimentally in mice with IGF-I knockout (1). These mice exhibit a deficiency in intrauterine growth, and those that survive continue to show restricted growth. At birth they are 60% of normal weight, but fall to 30% normal weight in adulthood (1, 2). The significance of IGF-I in normal growth is also demonstrated by disease states in which a disruption in circulating IGF-I levels occurs. Overexpression of IGF-I resulting from overproduction of GH leads to acromegaly, whereas low IGF-I levels resulting from an inactive GH receptor lead to Laron dwarfism (3, 4).

We have recently described the phenotype resulting from a homozygous missense mutation in the human IGF-I gene (5). The mutation (G274A) leads to the expression of IGF-I with a methionine instead of a valine at residue 44 (Val^{44}Met IGF-I). This was the first description of the effect of IGF-I deficiency in adulthood; the individual carrying the homozygous mutation is now 55 yr old. We observed several similarities between this individual and an earlier report of an IGF-I gene deletion described in a young male (6). Both patients suffered severe pre- and postnatal growth retardation, deafness and mental retardation. In adulthood, however, the lack of functional IGF-I is well tolerated, with effects mainly on bone mass and gonadal function (5).

In this study we describe biochemical and structural analysis of Val^{44}Met IGF-I and provide an explanation for the growth and developmental abnormalities observed. Native IGF-I is a single polypeptide chain of 70 amino acid residues, that contains three α-helical regions surrounding a hydrophobic core (7, 8). Residues 3-29 of IGF-I, which are homologous to the B chain of insulin, include helix 1 (Ala^{8}-Cys^{18}), and residues 42-60, which are homologous to the insulin A chain, include helices 2 (Ile^{43}-Cys^{48}) and 3 (Leu^{54}-Tyr^{60}). Residues 30-41 make up the C region loop, which is missing in insulin, and residues 61-70 make up the C-terminal D region

tail. We show that substitution of Met for Val at residue 44 of IGF-I results in a 90-fold reduced affinity for the IGF1R and a correspondingly lower activation of downstream signaling pathways. Remarkably, Val^{44}Met IGF-I binds with equal affinity to IGFBP-2, -3 and -6, suggesting maintenance of overall structure. This was confirmed by nuclear magnetic resonance analyses revealing only local side chain disruptions compared with IGF-I. Our study identifies Val^{44} as an essential residue involved in IGF-IGF1R interaction.

Results

Receptor binding [IGF1R and Insulin Receptor (IR)] and activation

Purified IGF-I and Val^{44}Met IGF-I were analyzed for their relative abilities to bind and activate the IGF1R and both isoforms of the IR (IR-A and IR-B). Competition binding curves for binding to the IGF1R are shown in Fig. 1A and 50% inhibitory concentration (IC_{50}) values are summarized in Table 1. As reported previously (5), the affinity of Val^{44}Met IGF-I is approximately 90-fold lower than that of IGF-I for the IGF1R. IGF1R activation on P6 cells was assessed using IGF-I, IGF-II, insulin and Val^{44}Met IGF-I (Fig. 1B). Although IGF-I activates the IGF1R with an IC_{50} of 3.9 ± 0.43 nM, IGF-II at the same concentration is only able to induce IGF1R phosphorylation equal to 35% that of IGF-I. In addition, activation of the IGF1R by insulin can only be detected at concentrations greater than 50 nM. Here we show that Val^{44}Met IGF-I is only slightly more potent than insulin in IGF1R activation as a result of decreased receptor binding affinity.

Competition binding curves for binding to the two isoforms of the IR (IR-A and IR-B) are shown in Fig. 2, and IC_{50} values are summarized in Table 2. No competition by Val^{44}Met IGF-I for europium-labeled insulin (Eu-insulin) binding is detected using either IR-A or IR-B, even at micromolar concentrations. IGF-I is a relatively poor binder to both IR isoforms and binds with a 3-fold higher affinity to the IR-A (IC_{50} = 120 nM) than to IR-B (IC_{50} = 366 nM). In contrast, insulin binds with high affinity to both IR isoforms, with a slightly higher affinity to the IR-B isoform in our assay (IR-A, IC_{50} = 1.4 nM ; IR-B, IC_{50} = 2.8 nM) (9). IGF-II also competes with high affinity (IC_{50} = 18nM) for Eu-insulin binding to the IR-A and has a 3.7-fold lower affinity for the IR-B. In addition, activation of the IR by concentrations of up

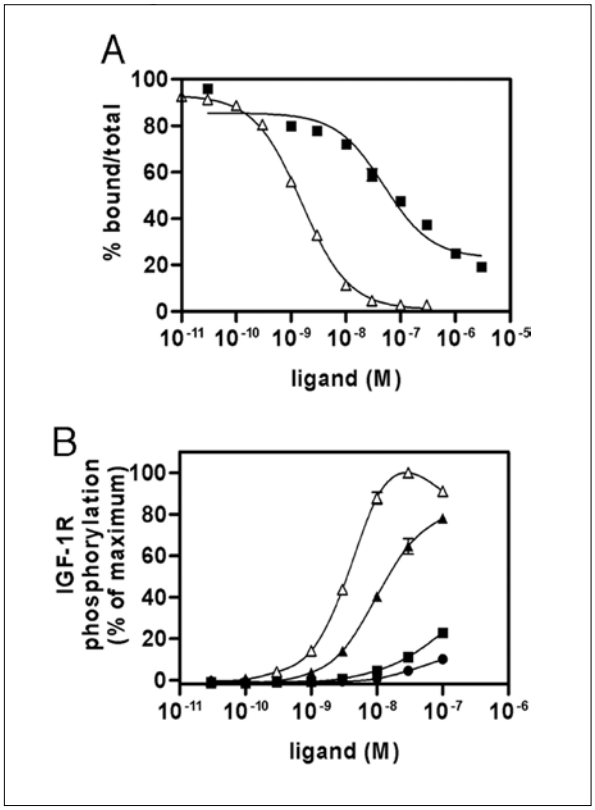

Figure 1. Binding and Activation of Human IGF1R by Val[44]Met IGF-I

A. Immunocaptured IGF1R was incubated with Eu-IGF-I in the presence or absence of increasing concentrations of IGF-I or Val[44]Met IGF-I as described in *Materials and Methods*. The graph shown is a representative of two independent experiments. Results are expressed as a percentage of Eu-IGF-I bound in the absence of competing ligand, and the data points are the mean ± SEM of triplicate samples. Errors are shown when they are greater than the size of the symbols. The ligands are as follows: A, IGF-I (△); Val[44]Met IGF-I (■);

B. IGF1R phosphorylation by IGF-I, IGF-II, insulin, and Val[44]Met IGF-I. P6 cells overexpressing the human IGF1R were serum-starved for 4 h, followed by stimulation with various concentrations of ligand for 10 min. Cells were lysed with ice-cold lysis buffer containing phosphatase inhibitors and activated receptors were immunocaptured with the anti-IGF1R antibody 24-31 as described in *Materials and Methods*. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY20 to detect phosphorylated tyrosines. The graph shown is a representative of three experiments and data points are the mean ± SEM of triplicate points. Errors are shown when they are greater than the size of the symbols. The ligands are as follows: B, IGF-I (△), IGF-II (▲), insulin (●), and Val[44]Met IGF-I (■).

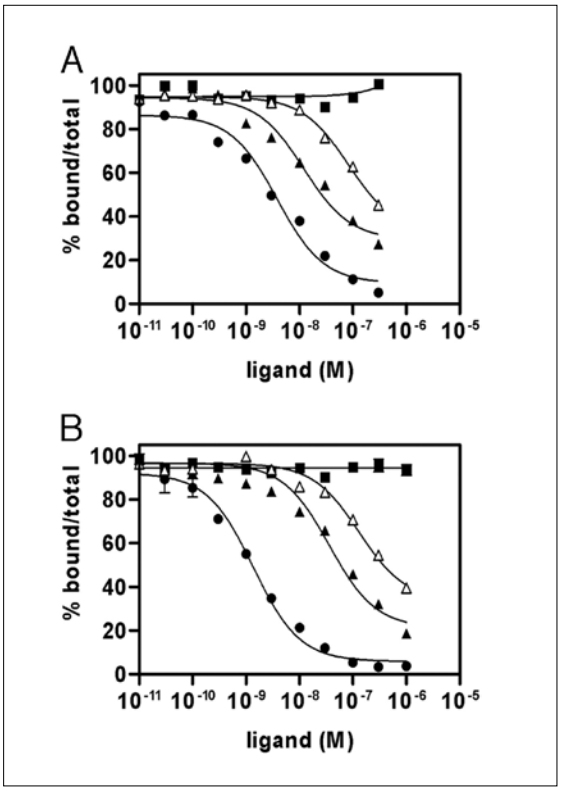

Figure 2. Competition Binding Curves of Eu-Insulin Binding to Immunopurified Human IR-A or IR-B. Immunocaptured IR-As or IR-Bs were incubated with Eu-insulin in the presence or absence of increasing concentrations of insulin, IGF-I, IGF-II, or Val^{44}Met IGF-I as described in *Materials and Methods*. The graphs shown are a representative of three experiments.

A. competition for binding to IR-A

B. competition for binding to the IR-B. Results are expressed as a percentage of Eu-insulin bound in the absence of competing ligand and the data points are the mean ± SEM of triplicate samples. Errors are shown when they are greater than the size of the symbols. The ligands are as follows in A and B, insulin (●); IGF-II (▲); IGF-I (△); Val^{44}Met IGF-I (■).

to 1 μM Val^{44}Met IGF-I is not detectable (data not shown), whereas the extent of IR phosphorylation by the other ligands correlates with receptor binding affinities (9).

In summary, IGF1R binding affinity of Val^{44}Met IGF-I is 90-fold lower than that of IGF-I and activation is correspondingly lower. IR binding and, therefore, activation are disrupted very significantly by substitution of valine for methionine at residue 44.

Table 1. Inhibition of Eu-IGF-I binding to the IGF1R by IGF-I and Val^{44}Met IGF-I

Ligand	IC_{50} (nM)	IC_{50} relative to IGF-I
IGF-I	1.7 ± 0.09	1
Val^{44}Met IGF-I	142 ± 43	83.8

The IC_{50} relative to that of IGF-I is also shown. Values are the mean ± SEM from two independent experiments.

Table 2. Inhibition of Eu—Insulin for binding to the IR-A and IR-B by insulin, IGF-I, IGF-II, and Val^{44}Met IGF-I

Ligand	IR-A		IR-B	
	IC_{50}(nM)	IC_{50} relative to IGF-I	IC_{50} (nM)	IC_{50} relative to IGF-I
IGF-I	120.4 ±34.1	1	366 ±15	1
Val^{44}Met IGF-I	>1000	ND	>1000	ND
Insulin	2.8 ±0.3	0.02	1.4±0.1	0.004
IGF-II	18.2 ±2.4	0.15	68 ±11	0.19

The IC_{50} relative to that of IGF-II binding to the IR-A is also shown. Values are the mean ± SEM from three independent experiments. ND, not determined.

Receptor signaling and biological activity in fibroblasts

To examine the effect of the Val^{44}Met-mutation on the ability to activate signal transduction in cells with a more physiological number of IGF1R, activation of the extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt/protein kinase B (PKB) pathways was analyzed in cultures of dermal fibroblasts. After a 10-min stimulation by IGF-I or Val^{44}Met IGF-I, a dose-dependent activation of Erk1/2 was detected (Fig. 3). Approximately 100-fold more Val^{44}Met- IGF-I was required to induce detectable Erk1/2 phosphorylation. The maximal stimulation reached with Val^{44}Met IGF-I was about half of the activation level reached by IGF-I. In contrast, approximately 200-fold more Val^{44}Met IGF-I was required to activate Akt/PkB on Ser^{473} and Thr^{308} compared with IGF-I. Again, the maximal stimulation of Akt/PKB reached with Val^{44}Met IGF-I varied between 70% and 40% of the levels induced by IGF-I (Fig. 3). In summary, the reduced activation of downstream signaling by Val^{44}Met IGF-I corresponds with its reduced affinity for the IGF1R compared with

IGF-I. However, there appears to be a greater effect on signaling via the Akt/PKB pathway than on Erk1/2 signaling, indicating a differential ability to stimulate signaling outcome after binding by Val[44]Met IGF-I.

Subsequently, the ability of Val[44]Met IGF-I to stimulate DNA synthesis was measured in primary cultures of skin fibroblasts isolated from the patient and a normal age- and sex-matched subject. IGF-I was able to stimulate DNA synthesis in both the patient and the normal fibroblasts to a similar extent. In contrast, Val[44]Met IGF-I was unable to stimulate DNA synthesis in either cell type in the

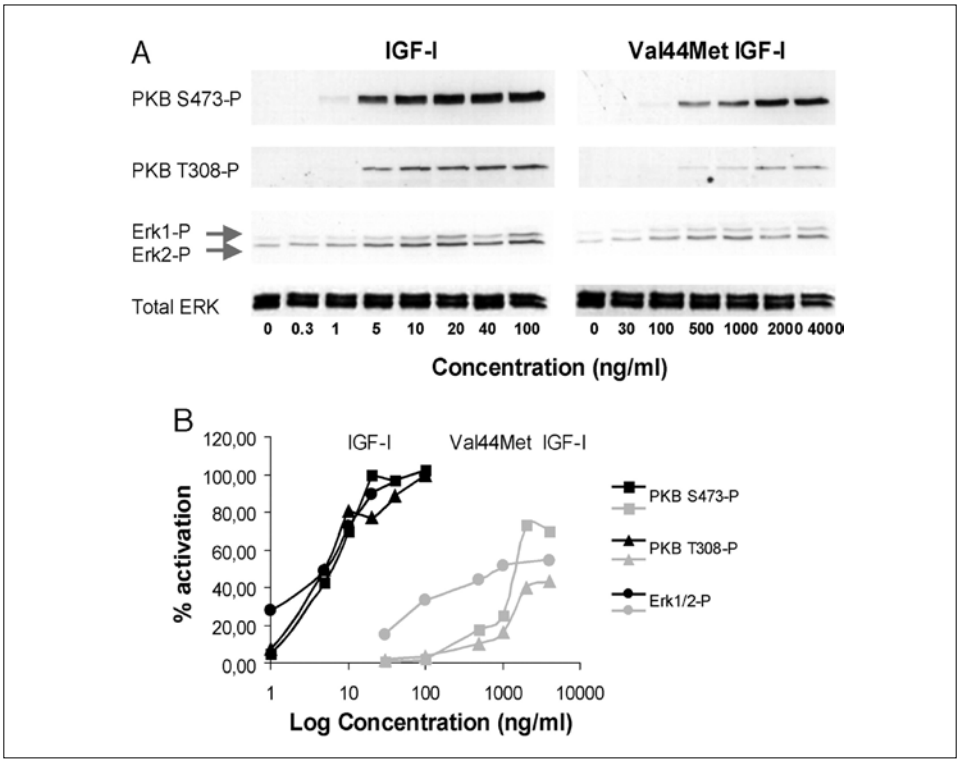

Figure 3. Activation of PKB/Akt and Erk1/2 in Skin Fibroblasts by Val[44]Met IGF-I.

A. Western blot analysis of fibroblasts stimulated for 10 min with a dose-response range of IGF-I and Val[44]Met IGF-I. The blots were probed with phospho-specific antibodies for activation of PKB at Ser[473] and Thr[308] and Erk1/2. Total Erk was used to check for equal loading. Pictures are a representative example of a duplicate experiment.

B. Quantification by densitometric scanning of the blots shown in A. Values were expressed as a percentage of the maximal activation level reached with IGF-I, which was set to 100%, and were corrected for loading efficiency using total Erk.

physiological dose-response range of IGF-I (1-100 ng/ml; Fig. 4). When 100-fold higher concentrations of Val^{44}Met IGF-I were used (>1000 ng/ml), small inductions of [^{3}H]thymidine incorporation were observed, which leveled off at about 45% of the levels reached by IGF-I. These experiments indicate that the patient's IGF-1Rs were functioning normally, but the Val^{44}Met IGF-I was unable to elicit a biological response in the normal dose-response range of IGF-I action.

IGFBP binding

We previously reported that neutral gel filtration of the patient's serum showed that endogenous Val^{44}Met IGF-I predominantly associates with the 150-kDa complex (comprised of Val^{44}Met IGF-I, IGFBP-3, and the acid labile subunit) as seen with wild type IGF-I in control serum (5). Additional IGFBP binding was assessed using BIAcore analysis with IGFBP-2, IGFBP-3 and IGFBP-6 biosensor surfaces (Fig. 5). There was no difference in binding affinities between IGF-I and Val^{44}Met IGF-I for any of the surfaces. IGFBP-2 and IGFBP-3 exhibit similar affinities for IGF-I and Val^{44}Met IGF-I (0.7 nM), whereas IGFBP-6 bound IGF-I and Val^{44}Met IGF-I with a much lower affinity (6.6 nM; see Fig. 5). Because IGFBP binding was not perturbed we can conclude that Val^{44}Met IGF-I is correctly folded.

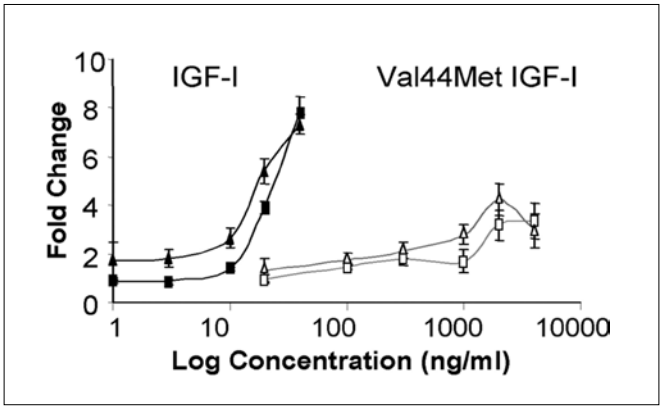

Figure 4. Stimulation of DNA synthesis in Normal and Patient Skin Fibroblasts by Val^{44}Met IGF-I. Increasing concentrations of Val^{44}Met IGF-I (*gray lines*) and IGF-I (*black lines*) were used to stimulate DNA synthesis in fibroblasts from the patient (△ and ▲) and a normal age- and sex-matched individual (□ and ■). Amount of incorporation of [^{3}H]thymidine is expressed as the fold change over that in unstimulated fibroblasts and represents the mean of a triplicate experiment ± SEM.

Structural analysis of Val^{44}Met IGF-I by NMR

To determine whether there were any significant structural differences between Val^{44}Met IGF-I and wild-type IGF-I, NMR spectra of mutant IGF-I were compared with those of the native protein (supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). ^{1}H and ^{15}N NMR resonance assignments for Val^{44}Met IGF-I were made from a three-dimensional nuclear overhauser effect spectroscopy heteronuclear single quantum coherence (NOESY-HSQC) spectrum. The assignment process was assisted by comparison of two-dimensional ^{15}N-^{1}H HSQC spectra of Val^{44}Met IGF-I with those of IGF-I in the presence of excess IGF-F1-1 peptide (8), long-[Arg^{3}]IGF-I (10) and long-[Leu^{60}]IGF-I (11), although some significant discrepancies exist among the

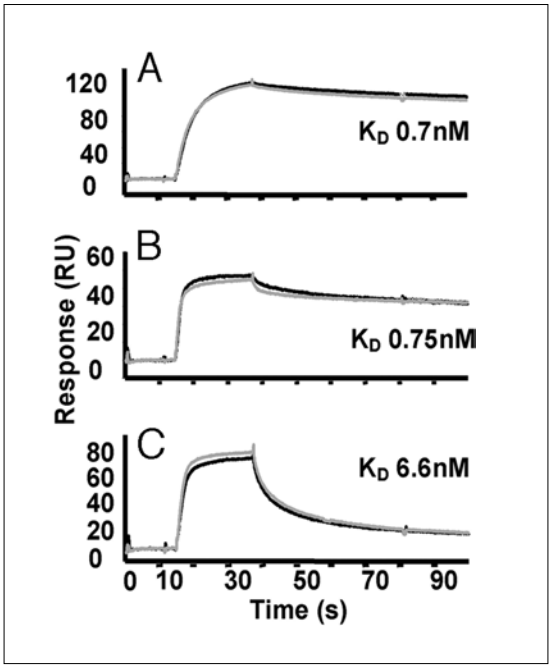

Figure 5. Surface Plasmon Resonance Analysis of Val^{44}Met IGF-I Binding to IGFBP-2, -3, and -6.
Sensorgrams represent binding to IGFBP-2 (A), IGFBP-3 (B), and IGFBP-6 (C) surfaces at 50 nM IGF-I (*black line*) or Val^{44}Met IGF-I (*gray line*). Kinetic studies with a range of analyte concentrations were determined at a flow rate of 40 µl/min to minimize mass transfer effects, allowing 300 sec for association and 900 sec for dissociation. Dissociation constants (K_d) were derived using BIAevaluation 3.2 software an a 1:1 Langmuir binding model.

assignments for these three proteins, as summarized in supplemental Table 1, published on The Endocrine Society's Journals Online web wite at http://mend.endojournals.org. One group of residues, including Cys^{6}, Gly^{7}, Leu^{10}, Val^{11}, Phe^{16}, Arg^{50}, and Leu^{54}, is affected by F1 peptide binding to IGF-I (8), so their chemical shifts differed from those of Val^{44}Met IGF-I, long-[Arg^{3}] IGF-I and long-[Leu^{60}] IGF-I. A second group, including Ala^{8}, Phe^{25}, Ile^{43}, Ser^{51}, Arg^{55}, and Tyr^{60}, differed in the Val^{44}Met IGF-I mutant as a direct consequence of the mutation. Resonances from Gly^{7}, Leu^{10}, Glu^{58}, and Cys^{61} were not found in spectra of Val^{44}Met IGF-I even at lower temperatures (15° and 20°C).

Chemical shift differences between Val^{44}Met IGF-I and IGF-I were small, except for Cys^{6}, Ala^{8}, Phe^{23}, Ile^{43}, Asp^{45}, Ser^{51}, Arg^{56}, Leu^{57} and Tyr^{60} (Fig. 6). The largest changes were for Ile^{43} and Asp^{45}, which flank the site of substitution, and Arg^{56}, which is located in the middle of second helix of the A region, about 9.1-11.2 Å away from Val^{44} (NH-NH distance) in the long-[Arg^{3}]IGF-I structure (12) and 11.5-12.6 Å away in the IGF-I plus F1 peptide structure (8). Two of the residues strongly affected, Phe^{23} and Tyr^{60}, are implicated in IGF-I binding to IGF1R (13, 14) (Fig. 7). Thus, although the structure of Val^{44}Met IGF-I is similar to that of native, chemical shift comparisons suggest that the mutation has caused local structural changes around the mutant site and in surrounding regions, some of which are involved in binding to the IGF-I receptor

This conclusion is supported by a detailed analysis of nuclear overhauser effects (NOEs) from the backbone amide resonances. If the distance between two protons is less than 6 Å in the structure, an NOE between those two protons should be observable in NMR spectra. Most of the observed NOEs to Met^{44} (Table S2) are consistent with the native structure, although the NOE between Met^{44} H$^{\gamma}$ and Thr^{41} NH is new, suggesting that the side-chain of Met^{44} has a different orientation from that of Val^{44} in native IGF-I. The relative intensities of the backbone NOEs to Met^{44} in Val^{44}Met IGF-I indicate that the native helix encompassing residues 43-48 is maintained, with HN-HN NOEs from Met^{44} to Ile^{43} and Asp^{45} being observed, as expected for an α-helix (15). One difficulty in making a detailed comparison with native IGF-I is that neither of the two high-quality solution structures for IGF-I corresponds precisely to IGF-I. Long-[Arg^{3}]IGF-I has a substitution at position 3 and an N-terminal extension (although this is not shown in Fig. 7), and the other has a peptide from phage display bound to it (again, not shown in Fig. 7). It is clear

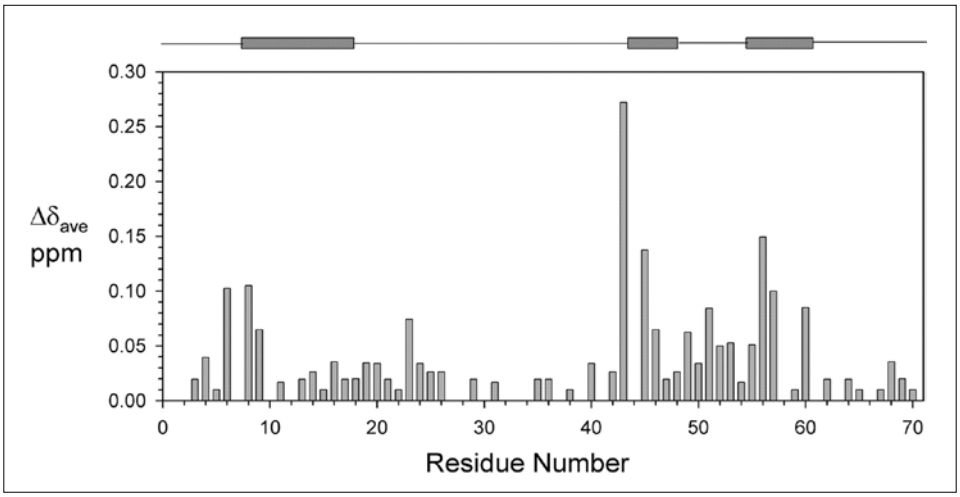

Figure 6. Weighted Average Chemical Shift Differences between Val[44]Met IGF-I and Native IGF-I
The average chemical shift differences derived from our spectra were calculated for ^{15}N and ^{1}H resonances using $\Delta\delta_{av} = (\Delta\delta_{NH}^2 + 0.17\Delta\delta_N^2)^{1/2}(66)$. Residues Gly[1], Gly[7], Leu[10], Glu[58], and Cys[61] were not assigned. Residues 2, 28, 39, 63 and 66 are proline and Asp[12], Lys[27], Gly[30], Gly[32], Ser[33], Ser[34], Arg[37], and Thr[41] had zero $\Delta\delta_{av}$ values. The locations of the three helices of native IGF-I are indicated above the plot.

from Fig. 7 that these structures are not identical. From inspection of the pattern of chemical shift perturbations and NOEs observed for Val[44]Met, it appears that the structure of long-[Arg[3]]IGF-I may be more representative of Val[44]Met.
Deviations from random coil chemical shifts for backbone ^{15}N, NH, and H^{α} resonances are a valuable indicator of ordered secondary structure in proteins (16). These plots for Val[44]Met IGF-I (Fig. S2) are consistent with the secondary structure of native IGF-I. Plots of ^{15}N backbone relaxation parameters R_1, R_2, and NOE for Val[44]Met IGF-I as a function of residue number (Fig. S3) are similar to those for long-[Arg[3]]IGF-I (12), with the regions of ordered secondary structure showing positive NOEs and faster spin-spin relaxation (larger R_2 values) and the N- and C-termini and the C region having smaller NOEs (in some cases negative) and smaller R_2 values. The mean R_1, R_2 and NOE values for Val[44]Met IGF-I are 1.40 ± 0.12 s^{-1}, 10.05 ± 0.46 s^{-1}, and 0.42 ± 0.02, respectively, compared with values of approximately 1.39 s^{-1}, 7.69 s^{-1} and 0.55 ± 0.12, respectively, in long-[Arg[3]]IGF-I. Detailed comparisons are difficult because of differences in protein concentration and state

of aggregation, but one significant difference between the two data sets occurs at residues 52 and 53, which have low NOEs and smaller R_2 values in Val[44]Met IGFI than long-[Arg[3]]IGF-I. This suggests that the loop connecting helices 2 and 3 in the native structure (arrowed in Fig. 7) may be somewhat more flexible in Val[44]Met.

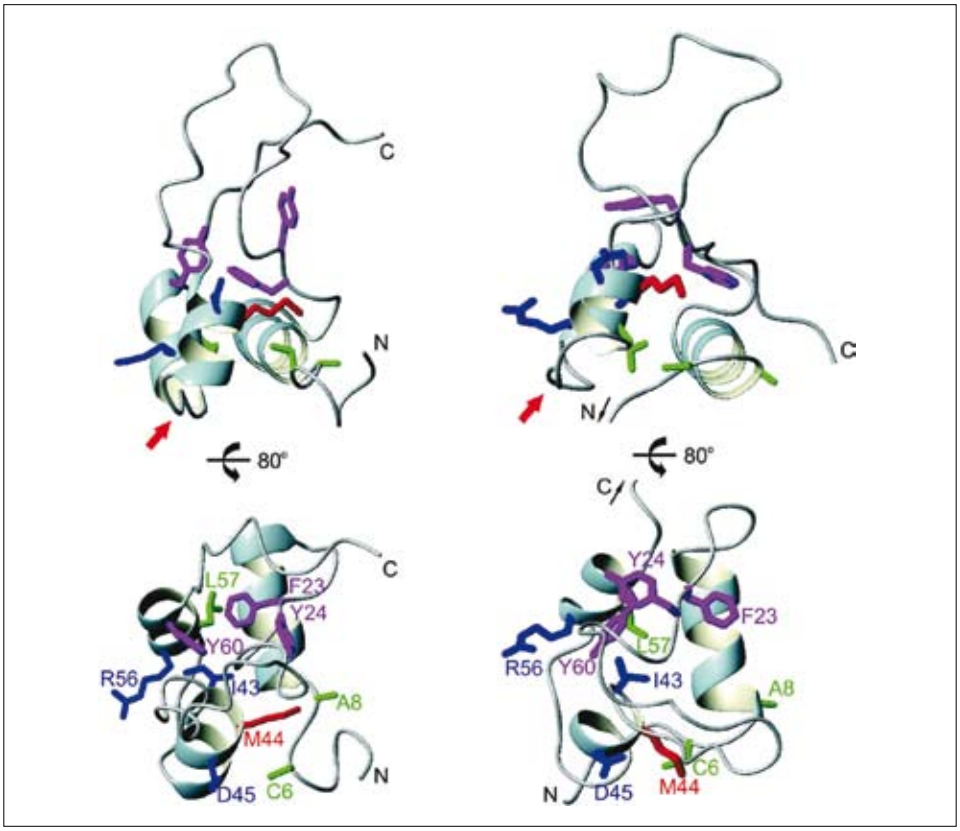

Figure 7. Backbone Ribbon View of IGF-I with Side-Chains of Key Residues Indicated
Long-[Arg[3]] IGF-I (12) (PDB accession no. 3LRI) is shown on the *left*, and IGF-I + F1 peptide (8) (PDB accession no. 1PMX) is shown on the *right* (with the peptide not shown for clarity); in each case the closest to average structure over the family is shown. Side chains are colored as follows: Met[44] in *red*; Ile[43], Asp[45], and Arg[56], which have the largest chemical shift changes between mutant and native IGF-I, in *blue*; Cys[6], Ala[8], and Leu[57], which have smaller chemical shift changes between mutant and native IGF-I, in *green*, and Phe[23], Tyr[24], and Tyr[60], which are implicated in IGF-I binding to the type 1 IGF receptor (13, 22, 27, 67) in *magenta*. The *upper* and *lower* views of each structure are related by an 80° rotation around the horizontal axis. The loop connecting helices 2 and 3 in the native structure is *arrowed*; note that the last five residues in the *lower* view of IGF-I + F1 peptide, and the first two residues in the *upper* view, are not shown in order to avoid overlap. The N-terminal extension in long-[Arg[3]]IGF-I is not shown, so the chain begins at the equivalent of Gly[1] of IGF-I.

Discussion

We have conducted a comprehensive biochemical and structural analysis of Val^{44}Met IGF-I in order to explain the phenotype described of a patient carrying a point mutation in the IGF-I gene. A similar phenotype was previously described resulting from a deletion in the IGF-I gene (6). The phenotype is not due to a defective IGF1R as IGF-I can stimulate the same biological response in fibroblasts derived from the patient or from a normal individual. However, we demonstrate that the Val^{44}Met mutation results in a significant reduction (~90-fold) in IGF1R receptor binding affinity and undetectable binding to either IR isoform. As a consequence, phosphorylation of the IGF1R and downstream-acting signaling proteins, *i.e.* Erk1/2 and Akt/PKB, is diminished. Remarkably, Val-Met substitution at position 44 seems to affect the Akt/PKB pathway to a greater extent than one would expect on the basis of receptor binding affinities. It is possible that these differences are a direct consequence of the changed kinetics of receptor-ligand interaction, which may have a greater impact on activation of the PKB/Akt-pathway than on the Erk1/2 pathway. This interesting finding is the subject of ongoing investigations.

Despite a large effect on receptor binding Val^{44}Met IGF-I is still able to bind IGFBP-2, IGFBP-3, and IGFBP-6 with equal affinity to IGF-I. This suggests that the common IGFBP-binding site is not disrupted. In support of this conclusion we see that Val^{44}Met IGF-I shows a normal association with the 150-kDa complex in serum (5). Dubaquie and Lowman (17) reported a small disruption in IGFBP-1 and IGFBP-3 binding by Val^{44}Ala IGF-I (2.3- and 1.4-fold lower binding than IGF-I, respectively), but did not report IGF1R binding. A recent crystal structure of IGF-I in complex with the N domain of IGFBP-5 shows that Val^{44} is not included in the N domain binding site (18). Headey *et al*. (19) reported that binding of IGFBP-6 C domain to IGF-II affects the two residues adjacent to Val^{43}, namely Ile^{42} and Glu^{44}. Although Val^{43} could not be assessed because of peak overlap, it seems that this region of the IGF-II surface is involved in interaction with the C domain of IGFBPs. Therefore, the lack of effect of the Val^{44}Met substitution in IGF-I on IGFBP binding may be attributable to the fact that the hydrophobic nature of the surface is preserved. The C domain of the IGFBPs is apparently less sensitive to the nature of the side-chain at position 44 than is the IGF-I receptor.

Interestingly, the results of the NMR analysis of the Val^{44}Met IGF-I structure suggest relatively little disruption of the overall structure. The marked effect on IGF1R binding could be explained by either local structural disruption around the mutation site and in surrounding areas or by a direct interaction of Val^{44} with the receptor. Analysis of chemical shift comparisons shows differences in local structure at residues Cys^{6}, Ala^{8}, Phe^{23}, Ile^{43}, Asp^{45}, Ser^{51}, Arg^{56}, Leu^{57}, and Tyr^{60}. Of these residues, Tyr^{60} has previously been implicated as important for IGF1R and IR binding (13, 20). Tyr^{60}Leu IGF-I has a 20-fold reduction in IGF1R binding affinity and Tyr^{60}Phe IGF-I has 2.6-fold reduced IR binding affinity. In addition, Maly and Luthi (21) showed that Tyr^{60} was protected from iodination in the presence of the IGF1R. Interestingly, iodination experiments with Val^{44}Met IGF-I revealed an approximately 10-fold reduced incorporation of ^{125}I compared with wild type (data not shown). These iodinations predominantly occur on tyrosine residues including Tyr^{60}. Reduced incorporation of ^{125}I is compatible with the local differences in structure at Tyr^{60}, which could make this residue less accessible for iodination. Phe^{23} has also been identified as important for IGF1R binding as mutation to Gly results in a 48-fold reduction in receptor binding affinity compared with IGF-I (14). Whether this mutation is causing a structural perturbation has not been investigated. The neighboring residue, Tyr^{24}, has been identified in several studies as being important for IGF1R binding (22). We have also previously demonstrated decreased IGF1R binding (~ 6-fold) by mutation of Ala^{8} to Leu (23). Only relatively small effects of mutating Ser^{51} and Arg^{5}6 have been reported (24, 25).

Val^{44} is conserved in all but one (catfish brain)(26) of the IGF-I sequences reported to date and is also found in the corresponding position in the two structurally related proteins, IGF-II and insulin. Interestingly, mutation of Val^{43} of IGF-II (which corresponds to Val^{44} in IGF-I) to Leu results in a 220-fold lower IGF1R binding affinity while maintaining IGFBP binding affinities similar to IGF-II (27). This observation confirms the importance of this residue in maintaining IGF1R binding.

A point mutation in the insulin gene (guanine to thymine at position 1298) resulting in the Val^{A3} to Leu mutation in the A chain has been termed insulin Wakayama (28, 29). Val^{A3} corresponds to Val^{44} of IGF-I. The expression of Val^{A3}Leu insulin leads to hyperinsulinemia and in some cases diabetes (29) resulting from severely defective IR binding. It has been suggested that Val^{A3} and Ile^{A2} make direct contact with the IR

after a structural change in insulin (30). Removal of contact between the beginning of the A chain and the C terminus of the B chain (involving residue B24) exposes residues Ile^{A2} and Val^{A3} and thereby allows their interaction with the IR (30-34). Substitution of Ile^{A2} with *allo*-Ile^{A2} leads to a 50-fold reduction in IR binding affinity while maintaining overall structure (32). Direct evidence for interaction with the receptor has recently been provided by a cross-linking study using a *p*-azido-Phe derivative of Val^{A3} and suggests an interaction with the insert domain (35).

Several substitutions have been made at Val^{A3} including Val^{A3}Leu insulin, which has only 0.14% of IR binding affinity compared with insulin (29, 36). Nakagawa and Tager (37) reported a similar helical content in Val^{A3}Leu insulin and native insulin after circular dichroism spectral analysis. Interestingly, NMR analysis of Val^{A3}Leu insulin revealed no significant change in structure (Weiss, M., unpublished observations) despite the significant effect on IR binding (29, 36). Structural analyses of Val^{A3}Ile (37) and Val^{A3}Thr (38) by far ultraviolet light circular dichroism show little disruption to the overall structure, whereas mutation to Gly leads to a complete disruption of the first A chain α-helix, as shown by NMR analysis (39). Furthermore, substitutions at residue Ile^{A2} highlight the importance of the beginning of the A domain helix in IR binding. Substitution of Ile^{A2} with Val reduces the helical content and destabilizes the first A domain helix (40). As with Val^{A3}Leu insulin (37), our data show that Val^{44}Met IGF-I maintains all helical structures. This is perhaps not surprising, because Met is a residue of reasonable helical propensity (41, 42) and is commonly found in the same position in proteins as Val (43). However, we did find that the loop connecting helices 2 and 3 in the native structure (Fig. 7) is somewhat more flexible in Val^{44}Met IGF. Despite this minor structural perturbation, both Val^{44}Met IGF-I and Val^{A3}Leu insulin have severely disrupted receptor binding properties. It seems likely that Val^{44} in IGF-I plays a similar role in IR binding to Val^{A3} in insulin.

In conclusion, we describe a biochemical and structural analysis of the first naturally occurring mutant of IGF-I. The mutant, Val^{44}Met IGF-I, exhibits large reductions in IGF1R and IR binding affinities and correspondingly lower potential to activate signaling events downstream of the IGF1R, while preserving native affinity to several binding proteins. Biological activities of Val^{44}Met IGF-I are only observed when supraphysiological concentrations (at least 100-fold higher) are used. In

the normal physiological dose-response range Val^{44}Met substitution is completely inactivating. These data led us conclude that the homozygous patient with the Val^{44}Met substitution is effective null for IGF-I. This fully explains the phenotype of our patient, and is in line with the observed similarities in developmental defects observed in our patient and in one previously described adolescent man with a homozygous IGF-I gene deletion, as well as in IGF-I knockout mice (1, 6). The lack of binding to the IR by Val^{44}Met IGF-I probably plays a minor role in the overall phenotype of our patient, because the affinity of IGF-I for either the IR-A or IR-B isoform is relatively low compared with that of insulin. Our structural analyses reveal only minor perturbations in the local structure of residues known to be involved in IGF1R binding and the overall structure is remarkably well preserved. Finally, our analysis identifies Val^{44} as a critical residue involved in receptor-ligand interactions, and further mutational analysis of this residue could provide valuable insight into the mechanism of IGF1R binding by IGF-I.

Materials and Methods

Materials

Oligonucleotides were purchased from Geneworks Pty Ltd. (Adelaide, Australia). Restriction enzymes were from New England Biolabs (Hitchin, UK) or Geneworks Pty Ltd. (Adelaide, Australia). ^{15}N-Labeled NH_4Cl was purchased from Sigma-Aldrich Corp. (Castle Hill, Australia). Human IGF-I for Eu labeling and human IGFBP-2 were purchased from GroPep Pty. Ltd. (Adelaide, Australia). Human IGFBP-3 and IGFBP-6 were from R&D systems (Minneapolis, MN). Human insulin was purchased from Novo Nordisk (Bagsværd, Denmark). Greiner Lumitrac 600 96-well plates were obtained from Omega Scientific (Tarzana, CA). DELFIA Eu labeling kit, DELFIA enhancement solution, and Eu-conjugated antiphosphotyrosine antibody PY20 were purchased from Perkin Elmer (Turku, Finland). Eu-IGF-I and Eu-insulin were produced as described by Denley *et al*. (9) according to the manufacturer's instructions.

Antibodies 83-7 and 24-31 were gifts from Prof. K. Siddle (Cambridge, UK). P6 cells (BALB/c3T3 cells overexpressing human IGF1R) (44) and R$^-$ cells (mouse 3T3-like cells with a targeted ablation of the IGF1R gene) (45) were gifts from Prof. R.

Baserga (Philadelphia, PA). Cells overexpressing the exon 11^-/IR-A and exon 11^+/IR-B isoforms of the IR (R^-IR-A and R^-IR-B cells, respectively) were created as previously described (9). Total Erk1/2, Phospho-p44/42 MAPK, Phospho-Akt (Ser^{473}) and Phospho-Akt (Thr^{308}) antibodies were obtained from Cell Signaling Technology (Beverly, CA).

Construction of expression plasmids encoding human IGF-I and Val^{44}Met IGF-I

Human IGF-I expression vector was developed by King *et al*. (46). The Quikchange site-directed mutagenesis kit was used to incorporate a G to A mutation in the IGF-I coding sequence at position 130 using the following oligonucleotides: Val^{44}Met forward 5' CCG CAG ACC GGA ATC ATG GAT GAA TGC TGC 3', Val^{44}Met reverse 5' GCA GCA TCC ATC CAT GAT TCC GGT CTG CGG 3'. The Val^{44}Met IGF-I-coding sequence was then subcloned using *Hpa*I and *Hind*III restriction enzymes into the pGH (1-11) expression vector (46).

Recombinant IGF-I and Val^{44}Met IGF-I production

IGF-I and Val44Met IGF-I were expressed and purified essentially as described by Shooter et al. (23). ^{15}N labeled Val^{44}Met IGF-I was expressed in minimal medium supplemented with ^{15}N-labeled NH_4Cl essentially as described previously (47). The purified proteins were analyzed by mass spectroscopy and N-terminal sequencing and were shown to have the correct masses (93% incorporation of ^{15}N) and to be greater than 95% pure. Quantitation of proteins was performed by comparing analytical C4 HPLC profiles with profiles of standard long-[Arg^3]IGF-I preparations (48).

Binding analysis of Val^{44}Met IGF-I to the IGF1R and IR isoforms

Receptor binding affinities were measured using an assay similar to that described for analyzing epidermal growth factor binding to the epidermal growth factor receptor (49) and outlined by Denley *et al*. (9). Briefly, R^-IR-A, R^-IR-B or P6 cells were lysed with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM EGTA, 1mM phenylmethylsulfonylfluoride, pH 7.5) for 1 h at 4°C. Lysates were centrifuged for 10 min at 3500 rpm at 4°C, then 100 µl were added per well to a white Greiner Lumitrac 600 plate previously coated with anti-IR antibody 83-7 (50) or anti-IGF1R antibody 24-31 (51). Approximately 100,000 fluorescent counts of Eu-insulin or Eu-IGF-I

were added to each well along with various amounts of unlabeled competitor and incubated for 16 h at 4°C. Wells were washed with 20 mM Tris, 150 mM NaCl, and 0.05% (vol/vol) Tween 20 (TBST) and DELFIA enhancement solution (100 µl/well) was added. Time-resolved fluorescence was measured using 340-nm excitation and 612-nm emission filters with a BMG Lab Technologies Polarstar Fluorometer (Mornington, Australia).

IR and IGF1R phosphorylation assays

Receptor phosphorylation was detected essentially as described by Denley *et al*. (9). R$^-$IR-A, R$^-$IR-B cells or P6 cells (2.5 x 10^4 cells/well; Falcon 96-well, flat-bottom plate) were washed for 4 h in serum-free medium before treating with IGF-I, IGF-II, insulin or Val^{44}Met-IGF-I in 100µl DMEM with 1% BSA for 10 min at 37°C, 5% CO_2. Lysis buffer containing 2 mM Na_3VO_4 and 1 mg/ml NaF was added to cells, and receptors from lysates were captured on 96-well plates pre-coated with antibody 83-7 or 24-31 and blocked with 1x TBST/0.5% BSA. After overnight incubation at 4°C, the plates were washed with 1 x TBST. Phosphorylated receptor was detected with Eu-antiphosphotyrosine antibody PY20 (10 ng/well; room temperature, 2 h). DELFIA enhancement solution (100 µl/well) was added and time-resolved fluorescence was detected as described above.

Cell culture, [^{3}H]thymidine incorporation assay, and Western blot

[^{3}H]Thymidine incorporation assays and Western blotting were performed using fibroblast cultures, which were established from skin biopsies of the patient and an age- and sex-matched normal subject, as described in detail previously (52, 53).

BIAcore analysis of IGFBP binding

Coupling of IGFBPs to CM5 BIAsensor chips via amine group linkage was achieved using standard coupling procedures (54-56). Briefly, IGFBPs were coupled to activated surfaces (2 µg IGFBP/210 µl in 10 mM sodium acetate, pH 4.5) at 5 µl/min. Unreacted groups were inactivated with 35 µl 1 M ethanolamine-HCl, pH 8.5. A sensor surface with 600 response units (RU) coupled IGFBP-2 would routinely result in a response of approximately 100 RU with 100 nM IGF-I. In addition, a surface with 470 RU IGFBP-6 would result in a response of 70 RU, and a surface with 400 RU IGFBP-3 would result in a response of 45 RU with 100 nM IGF-I. Kinetic studies with 6.25, 12.5, 25, 50, and 100 nM IGF-I or Val^{44}Met IGF-I were

determined at a flow rate of 40 µl/min to minimize mass transfer effects and by allowing 300 sec for association and 900 sec for dissociation. IGFBP biosensor surfaces were regenerated with 10 mM HCl. Analysis of kinetic data was performed with BIAevaluation 3.2 software (Uppsala, Sweden). For each binding curve, the response obtained using control surfaces (no protein coupled) was subtracted. IGF-I binding fitted a 1:1 Langmuir binding model using global fitting. This model describes a simple reversible interaction of two molecules in a 1:1 complex. Goodness of fit measured as a χ^2 value was not greater than 5 for all experiments. All binding experiments were repeated at least in duplicate, and biosensor chips coupled at different times yielded surfaces with identical binding affinities. The binding affinities of IGF-I to IGFBP-2 (K_d = 0.7 nM), IGFBP-3 (K_d = 0.75 nM), and IGFBP-6 (K_d = 6.6 nM) were comparable to the binding affinities reported by Hobba *et al*. (57) and Wong *et al*. (58) for bovine IGFBP-2 (K_d = 0.3 nM) and human IGFBP-2 (K_d = 0.45 nM) respectively, Heding *et al*. (59) for IGFBP-3, (K_d = 0.23 nM), and were 5-fold higher than those of Marinaro *et al*. (60) for IGFBP-6 (K_a = 0.028 nM^{-1}) using BIAcore technology.

NMR structural analysis

Lyophilized, uniformly ^{15}N-enriched Val^{44}Met IGF-I was dissolved in 95% H_2O / 5% ^2H_2O containing 10 mM sodium acetate-$^2H_4$ and 0.02 % sodium azide. The protein concentration was approximately 0.9 mM, and the pH was adjusted to 5.1 without correcting for the deuterium isotope effect. The after-spectra were recorded at 37°C on a DRX-600 spectrometer (Bruker, Karlsruhe, Germany) using a triple-resonance probe equipped with triple-axis gradients: two-dimensional ^1H-^{15}N-HSQC, three-dimensional ^1H-^{15}N-NOESY-HSQC with a 150 msec mixing time, and ^{15}N T_1, T_2, and NOE measurements. A series of ^1H-^{15}N-HSQC spectra was recorded at temperatures of 15, 20, and 37°C on a Bruker Avance500 spectrometer equipped with a cryoprobe. Two-dimensional ^1H-^{15}N-HSQC and three-dimensional ^1H-^{15}N-NOESY-HSQC spectra were also run at 600 MHz and 37°C on native IGF-I (0.5 mM, pH 4.9, in 95% H_2O/5% ^2H_2O containing 10 mM sodium acetate). Water was suppressed using the Watergate pulse sequence (61). All spectra were processed in XWINNMR, version 3.5 (Bruker) and analyzed with XEASY, version 1.4 (62). ^1H chemical shifts were referenced to sodium 4,4-dimethyl-4-silapentane-1-sulphonate at 0 ppm via the H_2O signal, and ^{15}N chemical shifts were referenced indirectly using the $^{15}N/^1H$ γratios (63). ^{15}N relaxation rates R_1 and R_2

were determined by fitting these measured peak intensities, respectively, to three- and two-parameter single-exponential decay curves using the program CURVEFIT (64). Steady-state ^{1}H-^{15}N NOE values were calculated from peak intensity ratios obtained from spectra acquired in the presence and absence of proton saturation. The SD of NOE values was determined from the background noise level of the spectra as described by Farrow *et al*. (65).

Acknowledgments

We thank Dr. Shenggen Yao (WEHI, Melbourne, Australia) for help with the NMR experiments, Dr. D.M. Ouwens and Prof. Dr. J.A. Maassen (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands) for helpful discussions on Western blot analysis, and Dr. Jaap van Doorn (Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Utrecht, The Netherlands) for critical reading the manuscript.

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Supplemental data

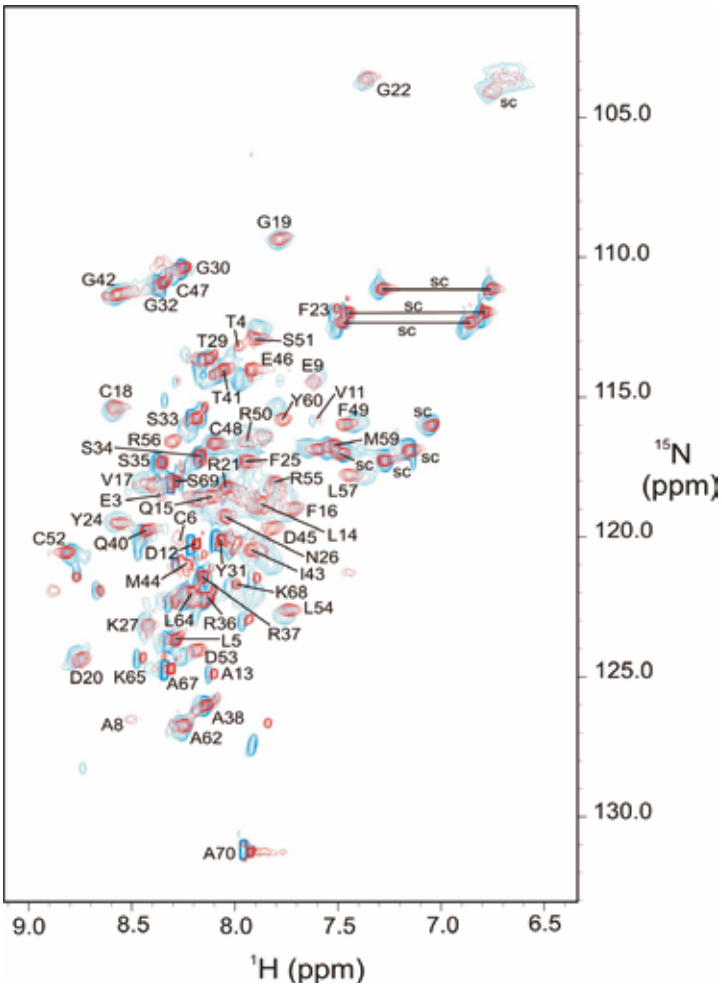


Figure S1. Contour plots of 600 MHz 2D ^{1}H-^{15}N-HSQC spectra with V44M IGF-I in red and native IGF-I in blue. The V44M IGF-I spectrum was acquired using a 0.9 mM sample at 37 °C and pH 5.1 in 95% H2O/5% $^{2}H_2O$ containing 10mM acetate- $^{2}H_4$ and 0.02% sodium azide, while the IGF-I spectrum was acquired on a 0.5 mM sample at 37°C and pH 4.9 in 95% H2O/5% $^{2}H_2O$ containing 10mM sodium acetate. Resonances are labelled with the corresponding sequence positions and side-chain amide resonances (Asn and Gln) are connected with a line. Other side chain resonances are labelled with a "sc" sign. Unlabelled peaks are not assigned.

Table S1. Residues showing significant differences in ^{1}H and ^{15}N chemical shifts ($\Delta\delta_{av}$>0.3) between V44M IGF-I and IGF-I plus F1 peptide (8), where $\Delta\delta_{av}=(\Delta\delta_{NH}^{2} + 0.17\Delta\delta_{N}^{2})^{1/2}$ (66). Chemical shifts for these residues in long-[Arg3] IGF-I (10) and long-[Leu60] IGF-I (11) are also included. The residues implicated in F1 peptide binding actions (8) are shown in black and others shaded in gray.

residue	V44M IGF-I		IGF-I plus F1		Long-[Arg3]IGF-I		Long-[Leu60] IGF-I	
	NH	N	NH	N	NH	N	NH	N
Cys 6	8.25	120.2	8.47	118.0	8.38	119.7	8.33	118.3
Gly7*	-------	-------	8.86	109.7	7.74	110.0	7.63	108.3#
Ala8*	8.49	126.5	8.77	128.5	8.98	130.3	8.83	128.2
Glu9	7.61	114.5	7.80	115.5	8.00	118.1	7.94	115.1#
Leu10*	-------	-------	6.74	119.9	6.88	121.7	6.87	120.3
Val11	7.59	115.7	7.18	118.1	7.29	119.3	7.38	117.9
Phe16	7.71	119.0	8.29	121.2	7.84	120.6	7.38	117.9#
Phe25	7.93	117.3	8.53	116.9	8.48	119.6	7.86	120.3#
Ile43	7.91	120.4	7.58	121.5	7.89	122.4	7.74	121.4
Phe49	7.45	115.9	7.70	116.1	7.72	117.3	7.89	113.7#
Arg50	7.93	116.5	7.42	117.1	7.53	119.9	7.95	119.4
Ser51	7.90	112.9	7.75	110.7	7.83	113.3	7.81	112.9
Leu54	7.73	122.6	8.67	127.0	7.59	123.6	7.57	122.5#
Arg55	7.80	118.0	8.22	117.5	7.86	119.9	7.92	119.4
Glu58*	-------	-------	7.40	112.5	8.09	114.8	8.15	113.7
Tyr60	7.76	115.8	7.81	117.5	7.98	117.0	7.76	117.9
Cys61*	-------	-------	7.09		7.26	116.9	7.22	115.3

* Gly7, Leu10, Glu58 and Cys61 are not found in any spectra of 15°C, 20°C and 37°C in V44M IGF-I.
+ Ala8 has a very weak peak in 2D HSQC.
#Chemical shifts show significant differences among the three published IGF-I data sets.

Table S2. NOE intensities observed in Val44Met IGF-I compared with inter-proton distances for the mutated residue 44 in A) long-[Arg3] IGF-I (12) and B) IGF-I plus F1 peptide (8). Relative intensities (RI), calculated using the integrated intensity divided by the average noise in the 3D NOESY-HSQC spectrum, are designated S (strong, RI>3.5), M (medium, 3.5>RI>2.0) and W (weak, RI<2.0). HN-HN and HN-HA NOEs and corresponding distances are shaded in grey. Note that the relative strengths of the sequential NOEs to Met44 HN correspond more closely with the distances in the long-[Arg3] IGF-I structure. Several backbone-to-backbone NOEs expected from the long-[Arg3] IGF-I structure are not seen in spectra of Val44Met IGF-I as follows: HN of Asn26, Gly42, Glu46, Cys47, Cys48 to Met44 HN; HN of Leu10, Val11, Glu46, Cys47, Cys48 to Met44 H^{α}. NOEs expected from the IGF-I+F1 structure but not seen in spectra of Val44Met IGF-I are: HN of Gly42, Glu46, Cys47, Cys48 to Met44 HN; HN of Glu46, Cys47, Cys48, Phe49 to Met44 H^{α}. However, a number of these expected NOEs are also not observed in our spectra of native IGF-I, probably as a result of the effects of conformational averaging and aggregation.

A.		NOEs observed in 3D ^{1}H-^{15}N-NOESY-HSQC spectrum of V44M IGF-I				Inter-proton distances in the native IGF-I structure (Å) for pairwise interactions giving rise to NOEs in the spectrum of Val44Met			
		Met44				**Val44**			
		HN	HA	HB	HG21	HN	HA	HB	HG21
Ile43	HN	W		W		2.55-2.84		5.55-5.96	
	HA	M				3.34-3.45			
	HG23	S				3.49-4.93			
Asp45	HN	S	S	S		1.90-2.18	3.59-3.63	2.79-3.18	
Thr41	HN				M				6.62-9.09

B.		NOEs observed in 3D ^{1}H-^{15}N-NOESY-HSQC spectrum of V44M IGF-I				Inter-proton distances in the native IGF-I structure (Å) for pairwise interactions giving rise to NOEs in the spectrum of Val44Met			
		Met44				**Val44**			
		HN	HA	HB	HG21	HN	HA	HB	HG21
Ile43	HN	W		W		2.06-2.71		4.23-4.94	
	HA	M				3.49-3.57			
	HG23	S				2.48-3.96			
Asp45	HN	S	S	S		2.76-2.99	3.57-3.59	3.53-3.73	
Thr41	HN				M				7.13-9.92

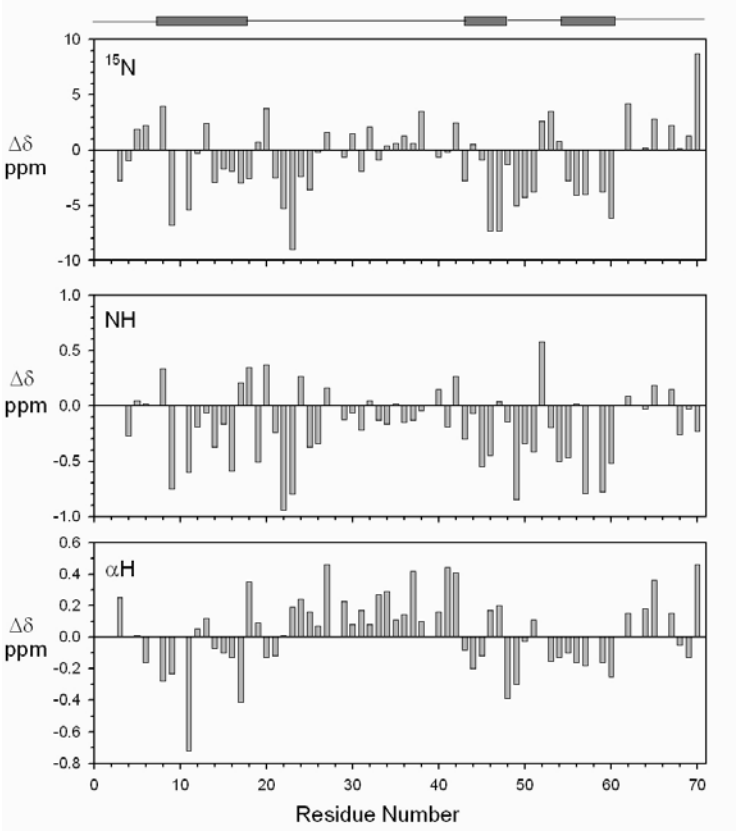


Figure S2. Deviations from random coil chemical shifts for ^{15}N, NH and H^{α} resonances in Val44Met IGF-I. Random coil values were taking from published data (16). Residues Gly1, Gly7, Leu10, Glu58 and Cys61 were not assigned. Residues 2, 28, 39, 63 and 66 are proline and the other gaps are calculated to be zero. The locations of the three helices of native IGF-I are indicated above the plot.

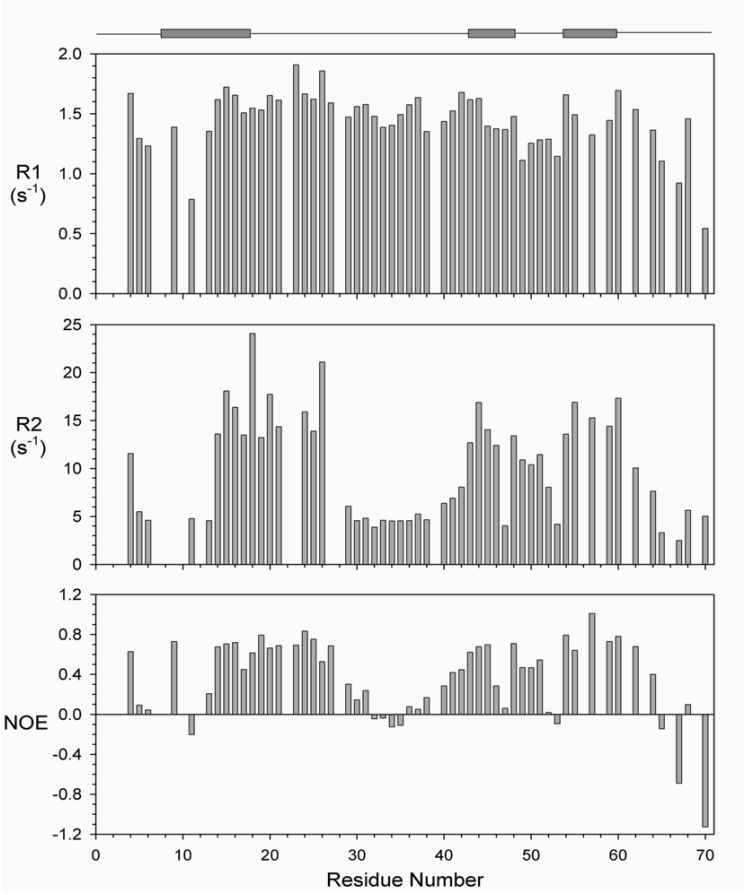
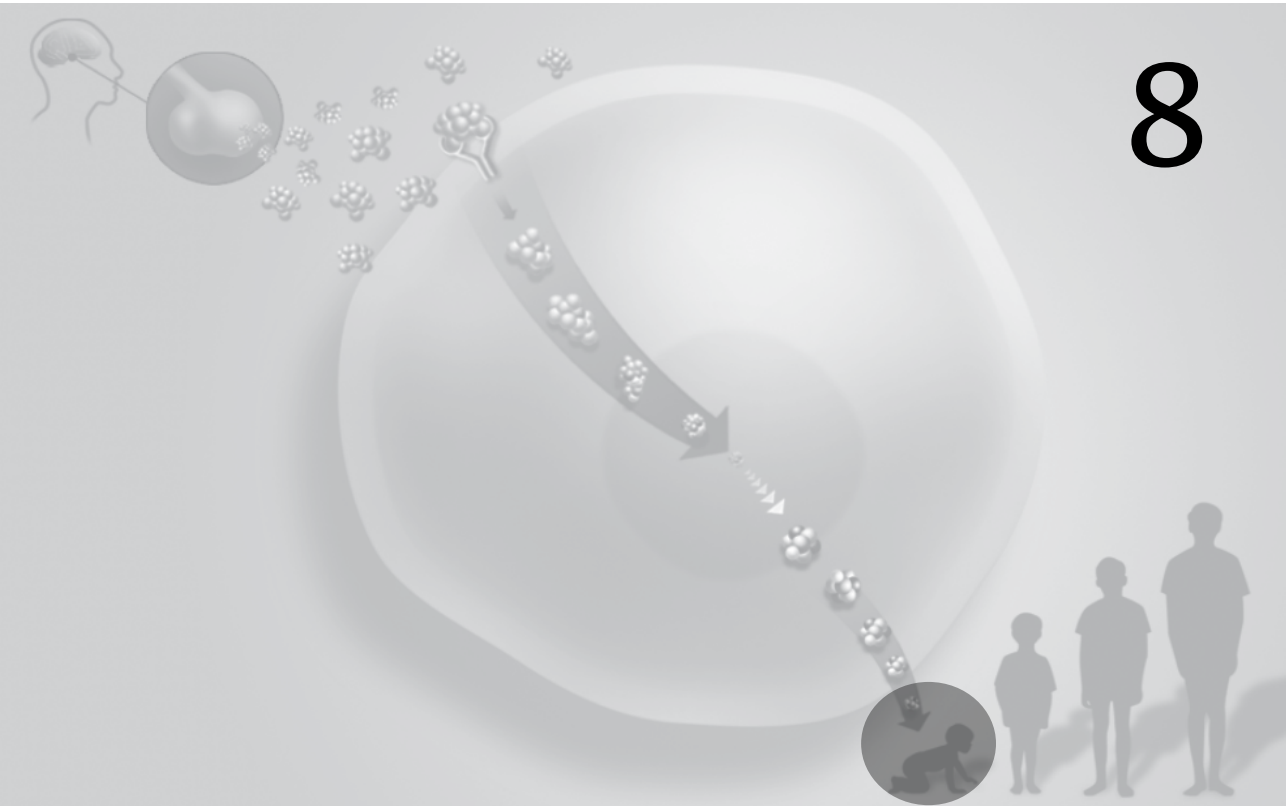


Figure S3. Summary of 15N backbone relaxation parameters R1, R2 and steady-state 1H-15N NOE for V44M IGF-I. Residues Gly1, Gly7, Leu10, Glu58 and Cys61 were not assigned. Residues Glu3, Ala8, Asp12, Gly22, Arg56 and Ser69 are not shown due to poor fitting and overlap. Residues Glu9, Phe23 and Lys27 are not shown in the R2 plot because of their very short T2 values. Residues 2, 28, 39, 63 and 66 are proline. The locations of the three helices of native IGF-I are indicated above the plot.

A variable degree of intrauterine and postnatal growth retardation in a family with a missense mutation in the IGF-I receptor



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The Journal of Clinical Endocrinology and Metabolism 2006;91(8):3062-3070

Abstract

Context: The type 1 IGF-I receptor (IGF1R) mediates the biological functions of IGF-I. Binding of IGF-I to the IGF1R results in autophosphorylation of the intracellular β-subunit and activation of intracellular signaling.

Objective: The objective of this study was to evaluate the functional characteristics of a novel IGF1R mutation and describe the phenotypic features of two patients with this mutation.

Design: The study was performed in a university hospital.

Patients: We describe a 35-yr-old female with mild intrauterine growth failure, progressive postnatal growth retardation, severe failure to thrive, and microcephaly. Her daughter was born with severe intrauterine growth retardation and also showed postnatal failure to thrive and microcephaly.

Results: We found a heterozygous G3148→A nucleotide substitution in the IGF1R-gene, changing a negatively charged glutamic acid at position 1050 into a positively charged lysine residue (E1050K). E1050 is a conserved residue in the intracellular kinase domain. Dermal fibroblasts of the mother showed normal binding of iodinated IGF-I, but autophosphorylation and activation of downstream signaling cascades upon challenging with IGF-I was markedly reduced. Consequently, the maximal [^{3}H]thymidine incorporation upon a challenge with a dose range of IGF-I was reduced compared with a panel of control cells (3.65 ± 1.79 *vs.* 6.75 ± 4.7-fold stimulation ($P < 0.01$)). These data suggest that the mutation result in the inactivation of one copy of the IGF1R gene.

Conclusions: These two patients support the key role for IGF-I in intrauterine and postnatal growth. The different phenotypes of these and earlier described patients may be associated with variability in IGF-I signaling. The degree of intrauterine growth retardation may be partially determined by the presence or absence of maternal IGF-I resistance.

Introduction

IGF-I plays a key role in intrauterine development and postnatal growth and metabolism. IGF-I deficiency due to a homozygous deletion or mutation of the IGF-I gene results in severe intrauterine and postnatal growth failure, mental retardation and deafness (1, 2). The biological functions of IGF-I are primarily mediated through the type 1 IGF receptor (IGF1R). The IGF1R gene is located on the distal long arm of chromosome 15 (15q26.3) and has a similar organization compared with the insulin receptor (IR) gene, with sequence homology varying from 41-84% depending on the domain (3). Both are heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoproteins, synthesized as a single chain preproreceptor and consisting of an α-subunit that is mainly involved in ligand binding and a β-subunit containing the tyrosine kinase domain. Ligand binding to the tyrosine kinase receptor results in receptor autophosphorylation on intracellular tyrosine residues and activation of the receptor's intrinsic tyrosine kinase, initiating distinct intracellular signaling pathways (4).

Although the IR gene and the IGF1R gene are homologous, genetic disturbances lead to a different phenotypical spectrum. Mutations of the IR gene in humans present with a heterogeneous phenotype, ranging from mild insulin resistance to leprechaunism (5). The clinical features of patients with IGF1R mutations are less well defined. So far, no cases have been found with a homozygous IGF1R mutation, and observations in mice suggest that such defect is lethal: IGF1R knock out mice die within min after birth due to respiratory failure (6). Heterozygous IGF1R mutations presenting with intrauterine and postnatal growth retardation have been observed in three families (7, 8). Heterozygous mutant mice are phenotypically normal (6), but targeted partial invalidation of the IGF1R-gene in mice causes postnatal growth deficiency (9).
Abuzzahab *et al*.(7) described a girl and a boy with mutations in the IGF1R. The girl was compound heterozygous for two missense mutations in exon 2, resulting in reduced ligand binding and decreased receptor phosphorylation on IGF-I stimulation. The boy had a nonsense mutation in exon 2, resulting in reduced expression of IGF1R. Both children had severe intra-uterine growth retardation and postnatal growth failure. Recently, a heterozygous mutation in the cleavage site of the proreceptor of IGF1R was reported in a 6 year old Japanese girl and her mother, present-

ing with mild intrauterine growth retardation and postnatal short stature (8). We now describe a mother and her daughter with the first missense mutation in the intracellular kinase domain of the IGF1R.

Methods

Patient A provided written informed consent for herself and her daughter (Patient B) for all investigations. The Medical Ethics Committee of the Leiden University Medical Center approved the protocol.

Clinical measurements and auxology

Height and sitting height were determined with a Harpenden stadiometer, and head circumference was assessed with a tape measure. Height and head circumference were expressed as standard deviation score (SDS) based on Dutch references (10). Sitting height and sitting height/height ratio were also expressed as SDS for the Dutch population (11).

Radiological and sonographic measurements

BMD (g/cm^2) of the lumbar spine and total body was measured by dual-energy x-ray absorptiometry (DXA) (Lunar, DPXL/PED, Lunar Radiation Corporation, Madison, WI). Ancillary DXA-derived data were used to calculate lumbar spine volumetric BMD [bone mineral apparent density (BMAD)] with the model BMAD = BMD x [4/(x width)], as validated before (12). BMD and BMAD results were compared with age- and sex-matched reference values and expressed as SDS.
Cardiac ultrasound in patient A was performed (GE System 7 – Vingmed, Milwaukee, WI, USA) and routine images (for assessment of left ventricular systolic function) and color Doppler data (to detect valvular abnormalities) were obtained.

Biochemical measurements

Plasma GH in both patients was measured with time resolved IFMA (Wallac/PE, Turku, Finland), using the WHO 80/505 as a standard (1 mg = 2.6 IU). Spontaneous GH secretion was assessed in case A after an overnight fast by sampling every 20 min from 0900-1200 (3-hr GH profile). An arginine stimulation test (0.5 g/kg iv over 30 min, from 0-30 min) and a combined GH-Releasing-Hormone (GHRH) (1

μg/kg iv at 0 min) and arginine test was performed in case A. An arginine stimulation test (0.5 g/kg iv over 30 min) was performed in case B.

Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1 and IGFBP-3, were determined by specific RIAs (13, 14) With the exception of IGFBP-1 smoothed references based on the LMS method were available for all parameters allowing conversion of patients data to SDS values (15). Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of six healthy adult controls.

A 75-g oral glucose tolerance test performed after a 10-h overnight fast was used to classify the patient as having normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes on the basis of the American Diabetes Association 1997 criteria (16). Glucose was assayed by an automated glucose oxidase method. Plasma insulin and C-peptide levels were measured by specific RIAs. The insulin secretion ratio or insulinogenic index was calculated as (30- to 0-min plasma insulin)/30-min glucose, which correlates well with direct measures of stimulated insulin secretion (17, 18). The homeostatic model assessment for insulin resistance index (HOMA-IR = fasting glucose (mmol/l) × Fasting insulin (mUI/ml)/22.5) was used for estimating insulin action. High HOMA-IR index denotes low insulin sensitivity and thus insulin resistance (19).

Molecular and functional studies

A skin biopsy from patient A was taken, and a culture of dermal fibroblasts was established. Total RNA was isolated and reverse transcribed into cDNA. The coding regions of the IGF1R were amplified by PCR using overlapping primer combinations (Table 1) and subjected to direct sequencing as described previously (2). Genomic DNA was isolated from whole blood of the patients and of 87 unrelated individuals with proportionate growth retardation, of whom 19 also had intrauterine growth retardation. All coding exons of the IGF1R were PCR amplified and subjected to direct sequencing. Primer combinations are indicated in Table 2. Fibroblast cultures of the patient and of eight healthy donors were used for a thymidine incorporation assay in response to IGF-I as described previously (20). Each of these cultures was used in at least two independent thymidine incorporation assays performed in quadruplicate. The interexperimental variance for each dose of IGF-I varied between 10 and 25%. For Western blotting, cells were stimulated for 10 min with a dose

Table 1. Overlapping primersets used for PCR amplification and sequencing of the IGF1R cDNA.

Primer	Sequence (5'-3')	Nucleotide[a]
hIGF1R 1 F	5'- TTTGAGAAAGGGAATTTCATCC - 3'	12 - 33
hIGF1R 2 R	5'- AGACACCGGCATAGTAGTAGTGG - 3'	823 - 845
hIGF1R 3 F	5'- TTGCCGCCACTACTACTATGC - 3'	795 - 815
hIGF1R 3 R	5'- GTTATGATGATGCGATTCTTCG - 3'	1568 - 1589
hIGF1R 4 F	5'- GACATAAACACCAGGAACAACG - 3'	1471 - 1492
hIGF1R 4 R	5'- CTTCTCGGCTTCAGTTTTGG - 3'	2172 - 2191
hIGF1R 5 F	5'- ATTGAGGAGGTCACAGAGAACC - 3'	2083 - 2104
hIGF1R 5 R	5'- GGAAGACGTACAGCATAATCACC - 3'	2920 - 2942
hIGF1R 6 F	5'- CACATCTCTCTCTGGGAATGG - 3'	2766 - 2786
hIGF1R 6 R	5'- CCGAGTAAGTGGTGAAGACTCC - 3'	3625 - 3646
hIGF1R 7 F	5'- TGGAGTCTTCACCACTTACTCG - 3'	3603 - 3624
hIGF1R 7 R	5'- TAAAGGCCCATGTCAGTTAAGG - 3'	4390 - 4411

F, Forward; R, Reverse

[a] The numbers correspond to nucleotide position in the IGF1R mRNA with accession number NM_000875

range of IGF-I, 1 x 10^{-6} M insulin or 10 ng/ml epidermal growth factor (EGF). Blots were probed with an anti-phospho-PKB/Akt, total PKB/Akt, anti-phospho-ERK-1 and -2, anti-phospho-IGF1R (Biosource International, Camarillo, CA), and a total IGF1R β (Cell Signaling technology, Beverly, MA) antibody as described previously (21). Binding studies were performed using iodinated IGF-I in the presence of an IGF-I analogue that is bound by IGFBPs but not by the IGF1R (Ala ^{31}Leu^{60}-IGF-I, GroPep) (22). In short, fibroblasts of the patient and controls were incubated at 4°C with 30,000 cpm [^{125}I]IGF-I, 250 ng/mL Ala^{31}Leu^{60}-IGF-I and graded amounts of unlabeled native IGF-I in 250 μL of HEPES binding buffer (100 mM HEPES (pH 7.8), 0.5% fatty-acid-free BSA, 120 mM sodium chloride, 1.2 mM magnesium sulfate, 5 mM potassium chloride, 15 mM sodium acetate, and 10 mM dextrose) as previously described (7, 22). After 18 h, cells were washed and solubilized in 1 M NaOH. Radioactivity was determined using a γ-counter.

Table 2. Primer sets for the amplification and sequencing of the coding exons of the IGF1R.

exon	name	primer sequence	exon	name	primer sequence
1	ex01-F	aaggggaatttcatcccaaa	11	ex11-F	aagtcatagaaaagacaaaagagg
	ex01-R	aggaaaagttcccgcagtg		ex11-R	ccactaggttgtgaggaagg
2	ex02a-F	tccttctaactgagacgtttacc	12	ex12-F	gaacccaaatccaactttgt
	ex02a-R	gcatttttctcaatcctgatg		ex12-R	catatgctgtcaatggatgg
2	ex02b-F	ttctacaactacgccctggt	13	ex13-F	ctgcattcatgggaaattg
	ex02b-R	cagaagagaagggaggtcaa		ex13-R	accctgctttcagttttacc
3	ex03-F	tcatctccgtctctcctctc	14	ex14-F	tgtgaagaaatgaaatgagca
	ex03-R	gggcgggtagtgaccaca		ex14-R	accactcagccacagaaagt
4	ex04-F	cagactcaattatgtgtgttttg	15	ex15-F	atgtatggaggtggggtttt
	ex04-R	aagcccatattttagtggtga		ex15-R	cttcctttcccaaattagca
5	ex05-F	agcacacagtgacacaatcc	16	ex16-F	cgttctgtctaagggcttgt
	ex05-R	gcacgctgcttattttgtaa		ex16-R	caaaggcaagacaccaaac
6	ex06-F	gcaggtgcgctaacatcg	17	ex17-F	caacgaagcttctgtgatga
	ex06-R	gctgttatcatgatggttgg		ex17-R	tttccttgcagggagattat
7	ex07-F	aagcaagacaggtgcttttc	18	ex18-F	aagaaattggcatggaaaaa
	ex07-R	cgtagccctgtcaacagaat		ex18-R	taatgccaacaaagtcctca
8	ex08-F	tgagggttttgatgtcagag	19	ex19-F	gctccagcgtgtgactct
	ex08-R	caggcatagctcactgctaa		ex19-R	agactgagctggtggaaagt
9	ex09-F	ctgttggcttgccagagtat	20	ex20-F	ttgttcagtccatccctttc
	ex09-R	acaggaatgaacggtcaca		ex20-R	cccaagaaaacaggtatttga
10	ex10-F	gctttcattcccactcttgt	21	ex21-F	agggctgtgttcagtgct
	ex10-R	agtgggttttgccaactg		ex21-R	aggcttgtgaatggattgtt

To facilitate sequence analysis, oligonucleotides included an M13 (tgt-aaa-acg-acg-gcc-agt) or M13 rev (cag-gaa-aca-gct-atg-acc) sequence primer.
F, Forward and R, Reverse

Results

Case reports

Patient A was born after 40 wk gestation as the second daughter of a nonconsanguineous marriage. This pregnancy was complicated by hyperemesis gravidarum. Her birth weight was 2.6 kg (-2.1 SDS), and birth length was 49 cm (-0.34 SDS) (23). The height of her father was 171.5 cm (-1.0 SDS) and of her mother 158.0 cm (-1.3 SDS). Her target height was 162.8 cm (-1.2 SDS) (10). Postnatally severe failure to thrive and poor appetite were noticed. She needed nasogastric tube feeding during the first year of life. Psychomotor development was normal. At 3.3 yr of age her height was 88 cm (-2.9 SDS) and her weight was 9.9 kg (weight for height, – 3.3 SDS). Additional investigations excluded hypothyroidism and renal failure. A normal female karyotype (46 XX) was found. In 1975, an arginine stimulation test was performed with a maximum GH response of 62 mU/liter. In 1981, an exercise test showed a peak GH concentration of 109 mU/liter. Bone age was 10 yr at the chronological age of 12.1 yr.

Recently, she came again to our attention through her daughter, who had similar problems (patient B). At present, patient A is 35 yr of age. At physical examination her height was 144.6 cm (-4.0 SDS), weight 47 kg (-1.7 SDS), sitting height/height ratio 0.52 (-0.1 SDS), body mass index 22.5 kg/m^2 and head circumference 50.2 cm (-3.0 SDS) (10, 11). Blood pressure was 135/85 mm Hg. There were no dysmorphic features (Fig. 1). Cardiovascular, respiratory and abdominal examinations were all normal. Menarche had occurred at 18 yr. She has worked as administrative assistant after completing high school. Neuropsychological tests showed an above average intelligence. Biochemical characteristics are summarized in Table 3. Plasma IGF-I was in the upper normal range and IGFBP-1 was low. Ingestion of 75 g glucose suppressed GH secretion (GH nadir, 0.2 mU/liter). Glucose tolerance was slightly impaired: fasting glucose, 4.8 mmol/liter; 2-h glucose, 8.5 mmol/liter; fasting insulin, 14 mU/liter. Stimulated insulin secretion, as reflected by the insulinogenic index, was normal: 12.3 (mU/liter x mmol/$liter^{-1}$). HOMA-IR index indicated mild insulin resistance: index: 3.1 (mU/liter x mmol/$liter^{-1}$)

BMD at the lumbar spine (L2-L4) was 1.07 gr/cm^2 (-0.5 SDS), BMAD (L3) was 0.43 gr/cm^3 (+1.3 SDS). Abdominal ultrasound revealed no abnormalities of liver,

Table 3. Biochemical characteristics of case A at 35 yr of age and case B at 15 months of age.

	Case A		Case B	
Variable	**Value**	**SDS**	**Value**	**SDS**
GH max. (mU/liter) after arginine stimulation	62[a]		222	
GH max. (mU/liter) in 3-h GH profile	3.4			
GH max. (mU/liter) after GHRH/arginine	131			
Total IGF-I (ng/ml)	239	1.6	145[b]	2.9
IGF-II (ng/ml)	656	-0.5	430	0.9
IGFBP-1 (ng/ml)	< 5 [c]		21[c]	
IGFBP-3 (μg/ml)	2.63	0.1	1.9	1.0

[a] performed at the age of 5.7 yr

[b] Before placing the gastrostoma, IGF-I was 68 ng/ml (-0.1 SDS)

[c] Normal range for nonfasting subjects is 24-58 ng IGFBP-1/ml. After overnight fasting, there is an average 5-fold rise in normal individuals.

spleen, kidneys, pancreas, uterus, and ovaries. Cardiac ultrasound was normal.

Patient B, the daughter of patient A, was born after 39 + 1/7 wk gestation. Her father was from Hindoestani descent. This pregnancy was complicated by hyperemesis gravidarum and oligohydramnion. Birth weight was 2100 g (-3.3 SDS), length 42 cm (-4.2 SDS) (23), and placental weight was 290 gr. (-2.4 SDS) (24). Head circumference was 33.3 cm at 2 months of age (-5.6 SDS). At physical examination, she had a triangular face, brachycephaly, mild hypotelorism, a small mouth with thin lips, and prominent ears (Fig. 1). Because of extremely poor appetite a percutaneous gastrostoma was positioned at the age of 10 months. Failure to thrive persisted (Fig. 2). Biochemical characteristics at 16 months of age are summarized in Table 3. Most noteworthy is the elevated plasma IGF-I after realimentation and the low IGFBP-1. An arginine stimulation test was performed at 14 months of age. Bone age was 1.5 yr at the chronological age of 1.05 yr and 1.75 yr at the age of 1.6 yr . Psychomotor development was determined with the Bayley test at 9 months and showed a motor delay of 3 months. After feeding through the gastrostoma and with physical therapy, motor skills progressed rapidly. At the age of 15 months a normal mental and slightly delayed motor development was found.

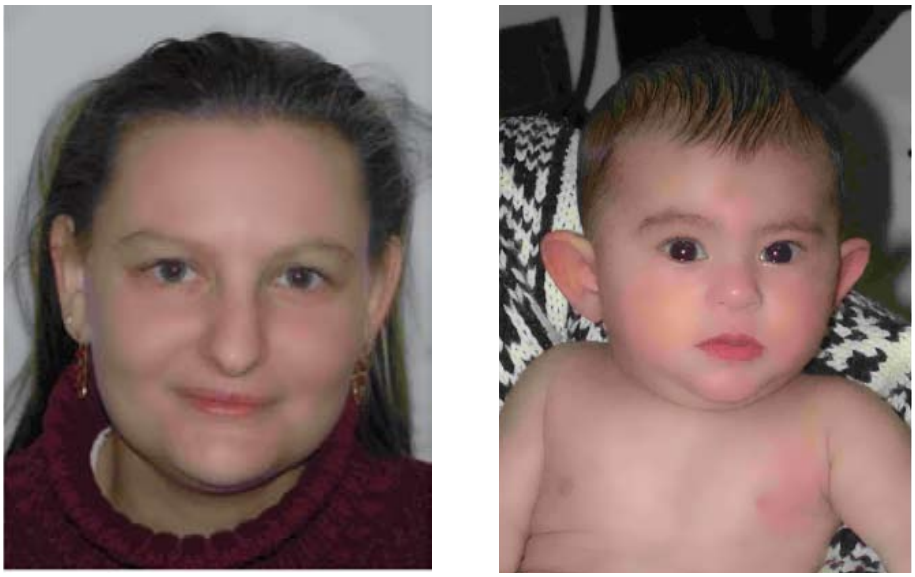


Figure 1. Patient A at the age of 35 yr and her daughter at the age of 4.5 months (patient B).

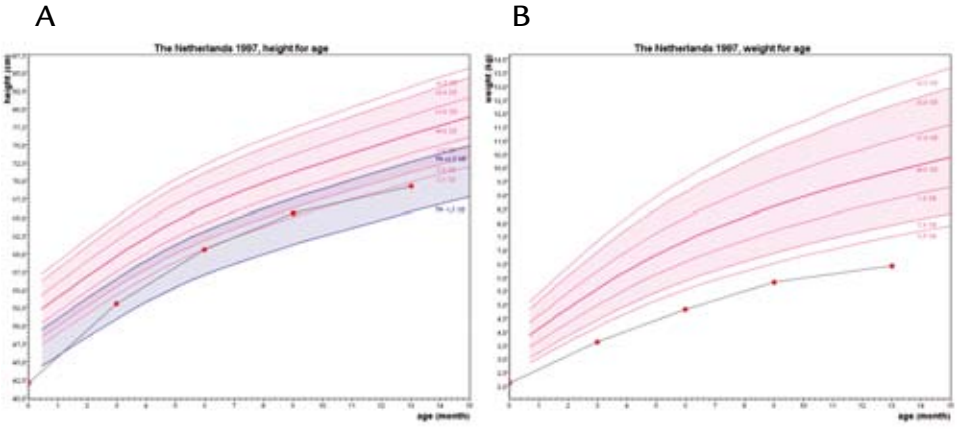


Figure 2. A, Length for age of patient B, with the pink area showing the normal distribution (-2 to +2 SDS) and the blue area showing the target range; B, weight for age.

Mutational analysis

Considering the increased IGF-I levels in both patients, we hypothesized that the clinical symptoms could be caused by a deletion or an inactivating mutation in the IGF1R-gene. IGF1R cDNA was isolated by RT-PCR from fibroblasts of patient A. Sequence analysis showed, besides a known polymorphism, a heterozygous G >A nucleotide substitution at position 3148, changing glutamic acid to lysine at position 1050 of the mature IGFIR protein (E1050K) (Fig. 3). No other mutations were found after sequence analysis of all coding exons of the IGF1R-gene. In genomic DNA of patient B, the same mutation was found. Patient B did not carry the polymorphism. In addition, we sequenced all coding exons of a panel of 87 growth-retarded patients of whom 19 also had intrauterine growth retardation. In none of these patients was the G3148A substitution or another mutation in the IGF1R present. This excluded that the G3148A substitution was a common polymorfism and provided further support for its pathogenicity.

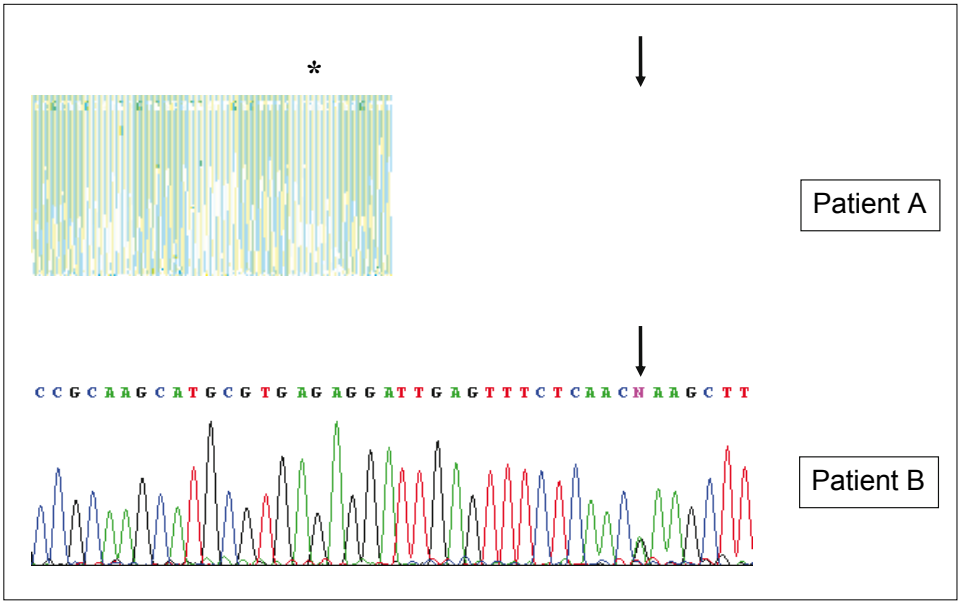
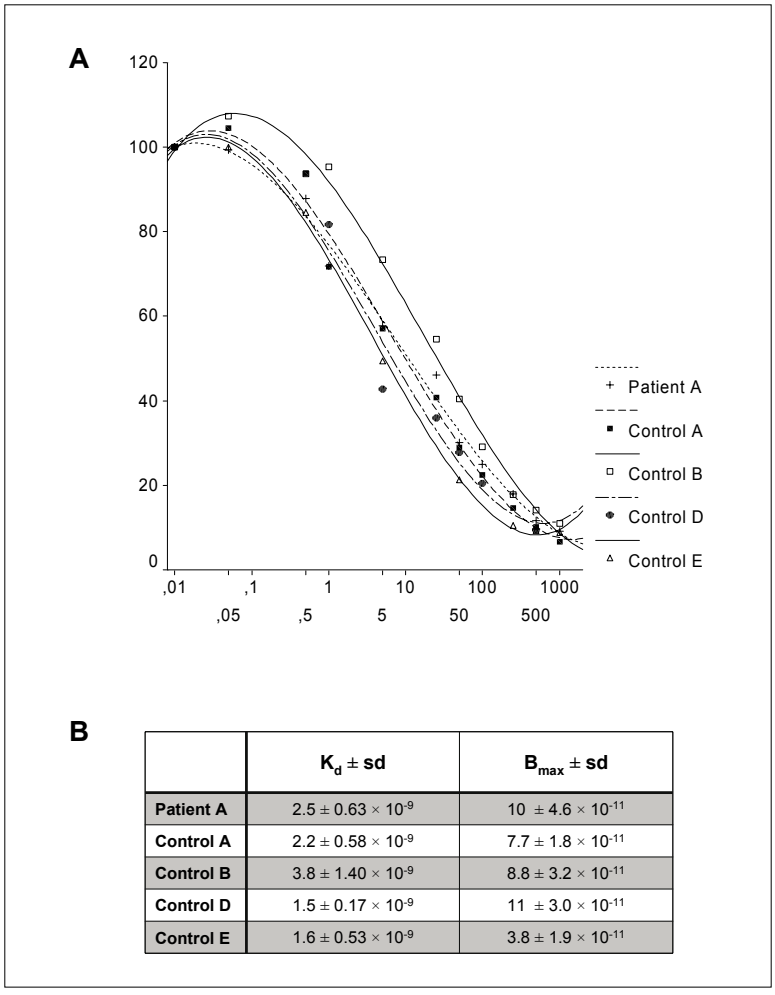


Figure 3. Sequence analysis of IGF1R cDNA of patient A (*top*) and genomic DNA of patient B (*bottom*). The *asterisk* indicates the polymorphism. The *arrow* indicates the heterozygous G→A nucleotide alteration. The wild-type and mutant alleles are both expressed in fibroblasts of patient A.



B

	K_d ± sd	B_{max} ± sd
Patient A	2.5 ± 0.63 × 10^{-9}	10 ± 4.6 × 10^{-11}
Control A	2.2 ± 0.58 × 10^{-9}	7.7 ± 1.8 × 10^{-11}
Control B	3.8 ± 1.40 × 10^{-9}	8.8 ± 3.2 × 10^{-11}
Control D	1.5 ± 0.17 × 10^{-9}	11 ± 3.0 × 10^{-11}
Control E	1.6 ± 0.53 × 10^{-9}	3.8 ± 1.9 × 10^{-11}

Figure 4. IGF-I binding studies.

A. Equal amounts of cells of patient A and four controls were seeded in 24-well plates. At confluency, cells were incubated with [^{125}I]IGF-I in the presence of 250 ng Ala^{31}Leu^{60} IGF-I and increasing amounts of unlabeled IGF-I. After 18 h, cells were washed and binding of [^{125}I]IGF-I was determined. Data represent the mean of four quadruplicate experiments and are expressed as percentage of total binding in the absence of competition with unlabeled IGF-I, which was set to 100% after correction for nonspecific binding. The displacement curve of the patient's cells was indistinguishable from controls.

B. Scatchard analysis was performed, with GraphPad for Windows, for the calculation of the binding affinity (K_d) and binding capacity (B_{max}) of the patient's cells and controls. Both values were in the normal range. Values represent the mean of four quadruplicate experiments ± SD.

Functional analysis

E1050 is a highly conserved amino acid residue located in the intracellular tyrosine kinase domain. The charge change induced by the amino acid substitution is presumed to result in inactivation of the IGF1R. Based on PCR and sequence analysis, both mutant and wild-type alleles were equally well expressed in fibroblasts of patient A (Fig. 3). In addition, we did not observe a difference in the expression of the IGF1R mRNA in the patient's *vs.* control cells (data not shown). Binding studies showed that the mutation did not affect binding affinity or total binding of iodinated IGF-I to patient's cells in comparison with a panel of control cell lines (Fig. 4). Western blot demonstrated a comparable level of total IGF1R protein expression in the patient compared with controls; however, autophosphorylation of the IGF1R and activation of PKB/Akt upon a challenge with IGF-I for 10 min were markedly reduced (Fig. 5, A and B). Stimulation with a dose range of IGF-I also showed that autophosphorylation of the IGF1R and activation of the downstream targets PKB/Akt and to a lesser extent Erk1/2 were reduced in cells of the patient compared with an age- and sex-matched healthy control (Fig. 5C). This reduction in activation of intracellular signal transducers was specific for IGF-I, because it was not seen when EGF or insulin were used as stimuli (Fig. 5D), excluding global unresponsiveness of the cells of the patient. Finally we determined the incorporation of [^{3}H]thymidine after a challenge with a dose range of IGF-I and compared the response in the patient's cells with the average response in a panel of fibroblast cultures of eight non-growth-retarded individuals. As shown in Fig. 5E, the maximal response was almost 50% reduced compared with controls (3.65 ± 1.79 *vs*. 6.75 ± 4.7-fold stimulation ($P< 0.01$). These results are in line with the inactivation of one copy of the IGF1R caused by the E1050K mutation.

Table 4. Clinical features of the four families with heterozygous IGF1R mutations.

nr	Subject	Mutation	Origin of mutation	Birth weight	Birth length	Head circ.	Last reported height	Dysmorphic features	Development
1A	Index case	R108Q K115N	father and mother	-3.5			-4.8	no	Verbal IQ 134 Perf. IQ 89 Psychiatric anomaly
1B	Mother	K115N		-2.0			-1.6		
1C	Father	R108Q		-2.0			-2.8		
2A	Index case	R59stop	mother	-3.5	-5.8	- 4.6 at birth	-2.6	yes	Mild motor and speech retardaton
2B	Brother	R59stop	mother	-2.7	-2.1				
2C	Mother	R59stop	father or de novo	-2.4	-1.6		-2.6		
3A	Index case	R709Q	mother	-1.5	-1.0		-2.1		IQ 60
3B	Mother	R709Q		-1.6			-2.9		Normal
4A	Mother	E1050K	father or de novo	-2.1	-0.3	- 3.0 at 35 yr	-4.0	no	Verbal IQ 110 Perf. IQ 112
4B	Daughter	E1050K	mother	-3.3	-4.2	- 5.6 at 2 months	-2.3	yes	normal

Families 1 and 2 are described by Abuzzahab *et al*. (7). Family 3 is described by Kawashima *et al*. (8). Family 4 is described in this article.
Data are expressed as SDS.

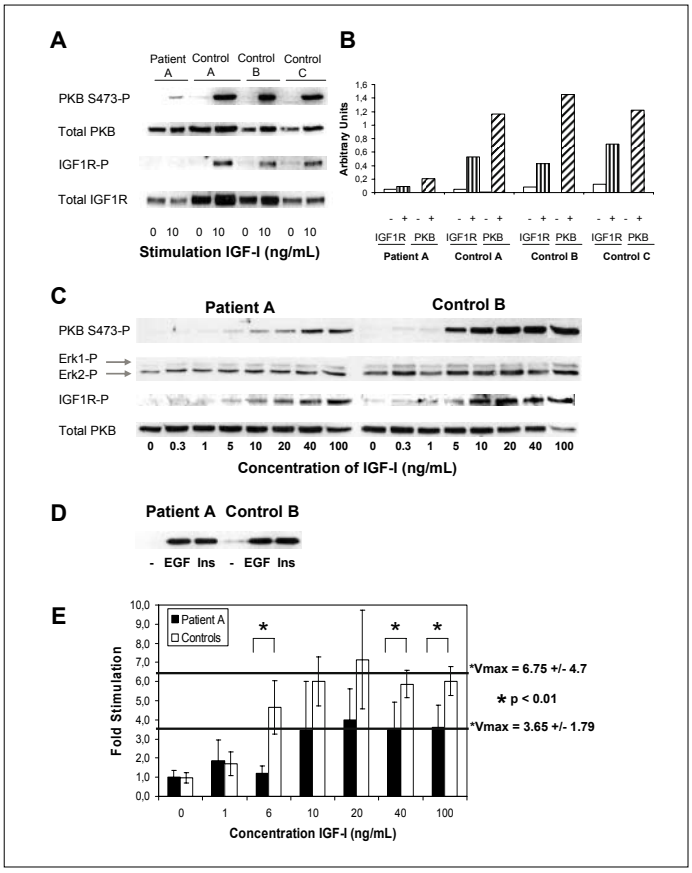


Figure 5. The E1050K missense mutation results in partial IGF-I resistance.

A. Dermal fibroblasts of patient A and controls were stimulated with 10 ng/ml IGF-I for 10 min. Protein lysates were collected and 25 μg of protein was used for Western blotting using phosphospecific IGF1R and PKB/Akt (Ser473) antibodies and total IGF1R and PKB/Akt antibodies. A representative picture of a triplicate experiment is shown.

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.

C. Activation of PKB/Akt by phosphorylation on Ser473 and of phosphor-Erk-1 and –2 as well as autophosphorylation of the IGF1R in fibroblasts of patient A and an age- and sex-matched control was determined by Western blot after a challenge with a dose range of IGF-I. Protein lysates were collected after 10-min stimulation. Total PKB/Akt was used to check for equal loading.

D. To exclude global unresponsiveness, fibroblasts of patient A and the age- and sex-matched control were challenged with 10 ng/ml EGF and 1 x 10^{-6} M Insulin (Ins). Protein lysates were collected and equal amounts were loaded on a gel and subjected to Western blot to determine phosphorylated PKB/Akt. Both stimuli were equally potent in activating PKB/Akt in cells of the patient and the control.

E. Fibroblasts of patient A and fibroblasts of eight healthy donors were stimulated with a dose range of IGF-I and the incorporation of [^{3}H]-thymidine was determined. The maximal response expressed as fold stimulation (Vmax) was calculated by averaging the responses observed at concentrations of IGF-I of 10, 20, 40, and 100 ng/ml at which the plateau phase was reached. Data are expressed as fold stimulation ± SEM. *, $P < 0.05$).

Discussion

In this study, we describe two cases, a mother and daughter, with the first heterozygous missense mutation in the intracellular tyrosine kinase domain of the IGF1R. This IGF1R mutation results in partial resistance to IGF-I, which presents with intrauterine and postnatal growth retardation and failure to thrive. These cases, together with the three previously described families with IGF1R mutations (7, 8), and two cases with homozygous IGF-I defects (1, 2) have provided a unique opportunity to study the role of the IGF-I/IGF1R system in human physiology.

Our patients have a mutation in exon 16, coding for the intracellular tyrosine kinase domain of the receptor. The tyrosine kinase (catalytic) domain is part of the cytoplasmatic portion of the β-chain of the IGF1R. Binding of IGF-I to the extracellular α-chain induces a conformational change in the structure of the receptor leading to autophosphorylation of three tyrosines in the activation loop of the catalytic domain of the β chain. Phosphorylation of the tyrosine residues results in a dramatic conformational change (25, 26). Glutamic acid at position 1050 (E1050) is extremely well conserved in all tyrosine kinase domains available in the database of the National Center for Biotechnology Information (NCBI) and is believed to play an important role in stabilizing the active conformational state.

The equivalent of E1050 in the IR is E1047. Upon binding insulin, the intracellular kinase domain is activated and conformational changes are induced. The change in three-dimensional structure places E1047 in close proximity of K1030 (K1033 in the IGF1R). This lysin residue is also highly conserved in tyrosine kinase domains and is located in the ATP-binding site. Hydrogen bonding between the negatively charged E1047 and the positively charged K1030 is believed to stabilize the structure of the activated receptor upon binding of insulin (25). Similarly, hydrogen bonding between E1050 and K1033 is believed to stabilize the active conformation of the IGF1R upon IGF-I binding. Due to the charge change induced by the E1050K substitution, it seems likely that the mutant receptor cannot preserve its active conformation. Consequently, the substitution should result in inactivation. This was confirmed by functional studies showing a dramatic reduction in autophosphorylation of IGF1R and in activation of downstream signaling pathways in cells of patient A compared with a panel of controls upon a challenge with IGF-I. The mutation did not have an

effect on IGF-I binding or on IGF1R mRNA and protein expression. Compared with the PKB/Akt pathway, the reduction in activation of the Erk1/2 pathway was less pronounced. This can be explained by assuming that less kinase activity is required to saturate activation of the Erk1/2 pathway compared with the PKB/Akt pathway. Finally, the maximal response determined in a [^{3}H]thymidine incorporation assay was almost 50% reduced compared with a panel of fibroblasts derived from healthy controls. Taken together, these data are fully in line with the inactivation of one copy of the IGF1R as a result of the E1050K substitution and suggests that the IGF-I resistance in our patients is caused by IGF1R haploinsufficiency. They furthermore confirm the important function of E1050 in stabilizing the active conformation of the tyrosine kinase domain as predicted by structural models (25, 26).
Although we believe that the best explanation of the phenotype observed in our two patients is the heterozygous IGF1R mutation leading to partial IGF-I resistance, we acknowledge that the results cannot be viewed as definitive proof. Even if one accepts that the mutated IGF1R must be dysfunctional based on its structure and that in *in vitro* studies of cultured fibroblasts stimulation of the the PKB/Akt pathway is reduced, one still has to assume that one functional allele is not sufficient for a full biological activity, at least with respect to growth of the skull, trunk, and extremities. Clinical observations on more patients, combined with detailed *in vitro* studies, are needed before it can be concluded that IGF1R haploinsufficiency indeed causes a clinical syndrome of pre- and postnatal growth failure.

The most striking clinical characteristics of patients with primary IGF-I resistance are intrauterine and postnatal growth retardation. The growth data of the reported patients with a mutation of the IGF1R gene are summarized in Table 4. Most patients have intrauterine growth retardation, but the degree of growth failure is variable. The patients with an affected mother (patients 2A, 2B, and 4B, and possibly 1A) seem to be more severely growth retarded than the patients with an apparently unaffected mother (patients 2C and 4A). One can hypothesize that maternal IGF-I resistance during pregnancy may affect placental size and, as a consequence, fetal growth. This hypothesis is supported by a strongly positive correlation between the rate of IGF-I increase during pregnancy and placental weight (27, 28) and by the finding that placentas from IUGR pregnancies are characterized by decreased expression of IGF1R and signal transduction proteins (29).

Postnatally, we saw a stimulated GH response to arginine in patient B, reflecting the state of IGF-I resistance. The direct IGF-I independent effects of GH could account for the better postnatal than prenatal growth in patient B. Another explanation could be that later in life the body compensates for the lack of IGF-I effect by producing more IGF-I receptor per cell. In the adult patient, GH secretion was normal, while it had been high in childhood and adolescence. This suggests that the GH-IGF-I feedback loop may change with age.

Concerning puberty, our patient A had a late onset of puberty. In the earlier reported patients, timing of puberty was not described in detail, except for the compound heterozygous patient in the report of Abuzzahab *et al*. (7) who had a normal pubertal development. The patients with IGF-I deletion and mutation had delayed puberty (1, 2). In Laron syndrome puberty is delayed, more so in boys than in girls (30). In patients with ALS deficiency puberty is extremely delayed (31). Taking all data together, it appears that IGF-I plays a role in pubertal onset.

So far, all patients with heterozygous IGF1R mutations with a reported head circumference have been microcephalic (Table 4). This is in line with the severe microcephaly found in both patients with primary IGF-I deficiency (-8 SDS in the case of IGF-I mutation and – 5.3 SDS in the case of IGF-I deletion) (1, 2). Carriers of the IGF-I mutation had a lower head circumference than noncarriers (-1.0 SDS *vs*. – 0.4 SDS) (2). This confirms the important role of IGF-I in intrauterine brain development as is known from IGF-I knockout mice (32). In contrast, the findings regarding intellectual and emotional development seem to vary substantially (Table 4).

Both our patients needed tube feeding due to poor appetite and failure to thrive, so it is tempting to believe that the IGF1R mutation is associated with this phenomenon. Observations in rats lend some support to this speculation: in rats, IGF-I receptors are localized in the hypothalamic arcuate nucleus (33), whereas the arcuate nucleus integrates signals regulating appetite (34). Insulin is known to penetrate the blood-brain barrier, where it acts as an anorexigenic signal, decreasing intake and body weight (34). Although data on the role of IGF-I on appetite are limited, we hypothesize that IGF-I has a similar effect. In our case B we observed that plasma IGF-I levels can be within the normal range in a state of poor nutrition. After restoring the nutritional state by a gastrostoma, IGF-I increased from -0.1 SDS to 2.9 SDS.

In our adult patient, an increased HOMA-IR was found, reflecting a moderate degree of insulin resistance, which could not be explained by an increased fat mass, since body mass index as well as stimulated insulin secretion were normal. Primary IGF-I deficiency is associated with insulin resistance (35) and treatment with recombinant human IGF-I improved insulin sensitivity in a patient with homozygous IGF-I mutation (36). Administration of recombinant human IGF-I to patients with severe insulin resistance and to patients with type 2 diabetes improves insulin sensitivity (37). These observations support the hypothesis that IGF-I is necessary for normal insulin sensitivity.

Comparing the phenotypes of the affected individuals in the four families with an IGF1R mutation, the degree of mental performance, dysmorphic features, and failure to thrive varies substantially (Table 4). This may reflect a spectrum of remaining IGF-I signaling activity. This is supported by our finding in patient A, that [^{3}H]thymidine can be incorporated in fibroblasts that are stimulated with high doses of IGF-I, although the maximal response is significantly reduced compared with controls. With regard to therapy, this *in vitro* response to high-dose IGF-I is an important argument for the potential benefits of GH or IGF-I treatment. Our patient B has just started GH treatment (1.4 mg/m2/day).

In conclusion, the novel heterozygous mutation in the intracellular tyrosine kinase domain of the IGF1R leads to intrauterine and postnatal growth retardation to a similar extent as the previously described mutations in the extracellular ligand binding part of the receptor. The degree of remaining IGF-I signaling may explain the different phenotypes seen in patients with IGF1R mutations. Maternal IGF-I resistance may affect placental size and explain part of the variance in birth weight of carriers of an IGF1R mutation. Heterozygous IGF1R mutations can be expected in patients born small for gestational age with a small head circumference in the presence of relatively high levels of IGF-I. Genetic analysis in such patients may provide important information on genotype-phenotype relations and the role of the IGF-I system in intrauterine and postnatal growth and development in human.

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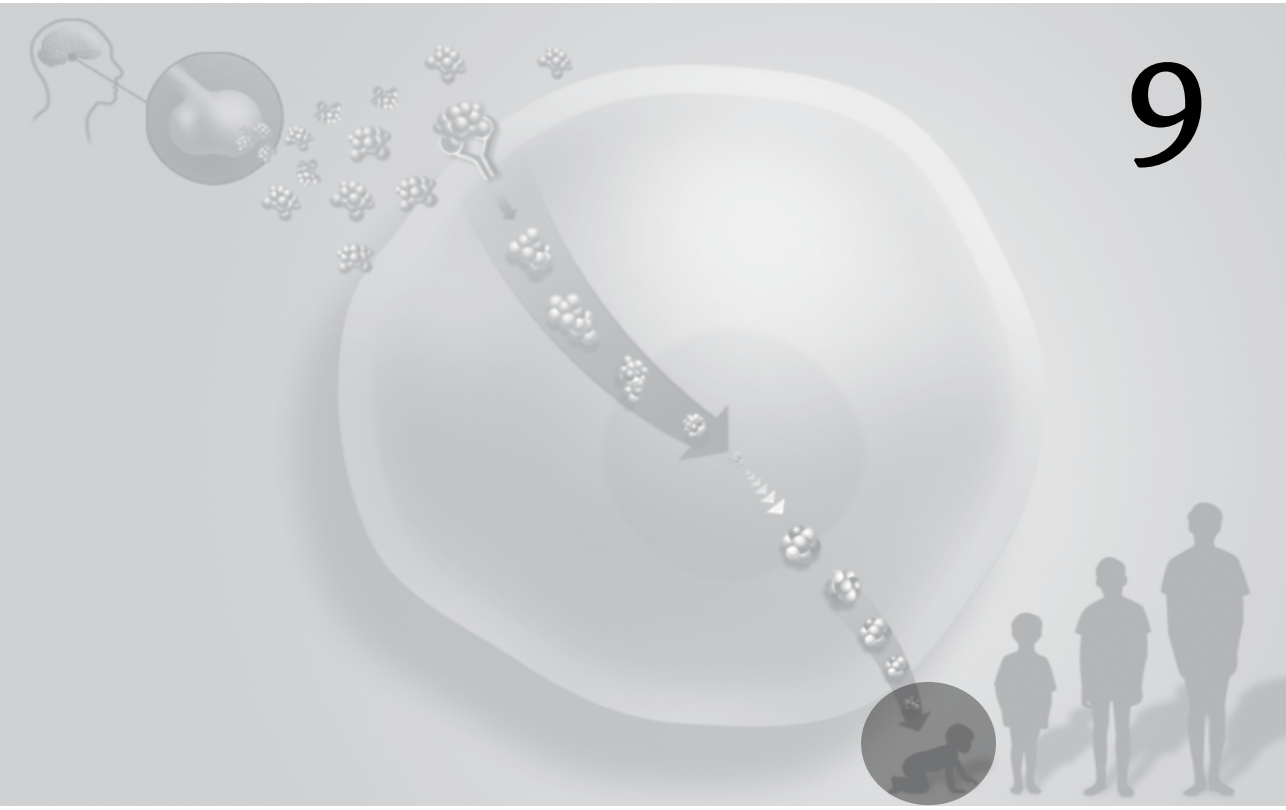
Succesful long-term growth hormone therapy in a girl with haploinsufficiency of the IGF-I receptor due to a terminal 15q26.2 → qter deletion



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Submitted

Abstract

Context: Microscopically visible heterozygous terminal 15q deletions encompassing the IGF1R gene are rare and invariably associated with intrauterine growth retardation and short stature. The incidence of submicroscopic deletions is unknown, as well as the effect of growth hormone therapy in this condition.

Objective: To describe the use of a novel genetic technique [multiplex ligation probe amplification (MLPA)] to detect haploinsufficiency of the IGF1R gene in a patient suspected of an IGF1R gene defect and evaluate the effect of long-term GH therapy.

Patient: We describe a 15 yr old adolescent, born small for gestational age, who showed persistent postnatal growth retardation, mild developmental delay and elevated IGF-I levels. She had been treated with GH since the age of 5 yr.

Methods: Mutation analysis of the IGF1R gene was performed by DNA sequencing followed by MLPA and array Comparative Genomic Hybridization (aCGH) to examine gene copy number changes. Dermal fibroblast cultures were used for functional analysis.

Results: Sequence analysis revealed no abnormalities in the IGF1R gene. With MLPA, a deletion of one copy of the IGF1R gene was detected, which was defined by aCGH to a loss of 15q26.2→qter. In concordance with IGF1R haploinsufficiency, IGF1R mRNA expression was decreased and activation of IGF1R and PKB/Akt after a challenge with IGF-I was decreased in the patient's fibroblasts, though to a lesser extent. IGF-I binding assays showed normal binding affinity and maximal binding of the IGF1R. GH treatment resulted in a good growth response and final height within the normal range.

Conclusions: The phenotype of a heterozygous terminal 15q deletion resembles that of a heterozygous inactivating IGF1R mutation with respect to intrauterine growth retardation, microcephaly, short stature, and elevated IGF-I levels. Long term growth hormone therapy is well tolerated and causes growth acceleration in childhood resulting in a normal adult height. MLPA and aCGH are useful tools to detect submicroscopic deletions of the IGF1R gene in patients born small for gestational age with persistent growth failure.

Introduction

Insulin-like growth factor I (IGF-I) is required for normal intrauterine and postnatal growth. The biological functions of IGF-I are mediated through the type 1 IGF receptor (IGF1R). The IGF1R gene is located on the distal long arm of chromosome 15 (15q26.3). Heterozygous inactivating mutations of the IGF1R gene result in a phenotype of intrauterine and postnatal growth failure and microcephaly, with a variable degree of psychomotor retardation (1-4). Mild dysmorphic features have been described in some cases (1, 3). An isolated homozygous or heterozygous deletion of the IGF1R gene has not yet been described.

Heterozygous terminal deletions of the distal long arm of chromosome 15, including the IGF1R, have been reported in only a few cases: 6 patients with a15q26.1 → 15qter deletion (5-9) and 2 patients with a 15q26.2→15qter deletion (10-12). Intrauterine growth retardation (IUGR) is present in almost all cases, with a birth weight varying between -1.8 SDS and –5.6 SDS and a birth length between -1.3 SDS and -5.5 SDS. In all cases, except one, the karyotype was abnormal, showing the terminal deletion of 15q.

We report a female patient with pre- and postnatal growth failure and elevated plasma IGF-I levels. The karyotype was normal. This clinical picture resembles the presentation of patients with a heterozygous mutation in the IGF1R gene (1-4). However, sequencing of the IGF1R gene did not reveal any abnormalities. Since the clinical picture may also be caused by haploinsufficiency of the IGF1R gene, we performed multiplex ligation-dependent probe amplification (MLPA) and found loss of one copy of the IGF1R gene. The borders of the deletion were mapped using array-comparative genomic hybridization (aCGH). 15q26.2→qter was deleted, including IGF1R gene. We show that long term growth hormone therapy in a patient with IGF1R haploinsufficiency improves growth considerably and leads to a normal adult height without notable side-effects.

Methods

The patient and her parents provided written informed consent

Clinical measurements and auxology
Height and sitting height were determined with a Harpenden stadiometer, and head circumference was assessed with a tape measure. Height and head circumference were expressed as standard deviation score (SDS) based on Dutch references (13). Sitting height and sitting height/height ratio were also expressed as SDS for the Dutch population (14).

Bone Mineral Density (BMD)
BMD (g/cm^2) of the lumbar spine and total body was measured by dual-energy x-ray absorptiometry (DXA) (Lunar, DPXL/PED, Lunar Radiation Corporation, Madison, WI). Ancillary DXA-derived data were used to calculate lumbar spine volumetric BMD [bone mineral apparent density (BMAD)] with the model BMAD = BMD x [4/(x width)], as validated before (15). BMD and BMAD results were compared with age- and sex-matched reference values and expressed as SDS (16, 17).

Biochemical measurements
Plasma GH was measured with Plasma GH was measured with Immulite 2000 a solid-phase, two-site chemiluminescent immunometric assay (DPC, Los Angeles, CA) using the WHO NIBSC 1st international standard 80/505 (1mg=2.6 IU). An arginine stimulation test was performed with 0.5 g/kg arginine iv over 30 min and blood samples at 0 and 30 min. A clonidine stimulation test was performed with 0.15 mg/m^2 clonidine orally, collecting blood samples every 30 minutes until 150 min.

Plasma IGF-I, IGF-II, IGF-binding-protein (IGFBP)-1 and IGFBP-3 were determined by specific RIAs (18, 19). With the exception of IGFBP-1 smoothed references based on the LMS method were available for all parameters allowing conversion of patients data to SDS values (20). Plasma IGFBP-1 concentration after an overnight fast was compared with a reference group of 6 healthy adult controls. An IGF-I generation test was performed with 1 mg/m^2 body surface GH (Humatrope, Lilly) during 4 days, followed by 2 mg/m^2 during 3 days.

Genetic analysis

Fluorescence In Situ Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was performed on cultured dermal fibroblasts according to standard procedures. The probes used were the 15q subtelomeric PAC clone 154P1 (GS-154P1, (21) and the BAC clone 342L10 (RP11-342L10, "Cancer_1E9", located in 15q26.3, BACPAC Resource Center (http://bacpac.chori.org/order.php).

Sequence analysis

Total RNA was isolated from cultured fibroblasts and reverse transcribed into cDNA. The coding regions of the IGF1R were amplified by PCR using overlapping primer combinations and subjected to direct sequencing as described previously (22). Genomic DNA was isolated from whole blood according to the salting out procedure described by Miller et al (23). All coding exons of the IGF1R were PCR amplified and subjected to direct sequencing as described previously (3).

Multiplex ligation-dependent probe amplification (MLPA)

MLPA probes for the IGF1R were designed according to the criteria described by White et al (24) and were directed to exons 2, 8 and 18 of the IGF1R gene, which are conserved exons in different species (Table 1). The oligonucleotide probes were ordered from Illumina Inc. (San Diego, CA) and used without purification.

Table 1. Sequence of the MLPA probes.

Gene	exon	Upstream (U) / downstream (D)	Sequence (5'-3')	Primer length (bp)	Fragment length (bp)	label
IGF1R	2	U	GATGTGTGAGAAGACCACCATCAACA	44	92	HEX
		D	ATGAGTACAACTACCGCTGCTGGACCACAA	48		
	8	U	CTACATGGGCTGAAGCCCTGGACTCAG	45	94	HEX
		D	TACGCCGTTTACGTCAAGGCTGTGACCCTCA	49		
	18	U	CAGTCCTAGCACCTCCAAGCCTGAGCA	45	90	HEX
		D	AGATGATTCAGATGGCCGGAGAGATTG	45		
Labeled primer			GGGTTCCCTAAGGGTTGGA			
Unlabeled primer			GTGCCAGCAAGATCCAATCTAGA			

All reagents for the MLPA were obtained from MRC Holland (Amsterdam, the Netherlands). Reactions were performed according to White *et al*. (24) and the manufacturer's instructions. In short, 50 ng of genomic DNA in a final volume of 1 µl was heated at 98°C for 5 min and subsequently cooled at 25°C. 0.375 µl probe mix (4 fmol/µl), 0.25 µl H_2O and 0.375 µl SALSA MLPA buffer were added to each sample, heat denatured at 95°C for 2 minutes and followed by hybridization at 60°C for 2.5-3 hours. Samples were kept at 54°C and 8 µl Ligase-65 mix (0.75 µl buffer A, 0.75 µl buffer B, 0.25 µl Ligase and 6.25 µl H_2O) was added to each sample. After 10-15 min, the reaction was stopped by heat inactivation at 98°C for 5 min and cooled at 4°C. The MLPA product (10 µl) was added to 20 µl PCR mix at 60°C and 33 cycles of 20 sec. 95°C, 30 sec at 62°C and 60 sec. at 72°C were carried out. From each PCR reaction 2 µl of product was mixed with 15 µl deionized formamide and 0.5 µl ROX-500 Genescan. Product separation was performed using capillary electrophoresis on an ABI 310 (Applied Biosystems). The data analysis was performed as described by White *et al.* (24).

Array comparative genomic hybridization (aCGH)

To determine the boundaries of the deletion we performed array-based comparative genomic hybridization (aCGH) using the 44B Human Genome CGH Microarrays (Agilent, Santa Clara, CA). These arrays contain $43x10^3$ 60-mer oligonucleotide probes (mostly exonic) that span the human genome with an average spacing of 35 Kb. aCGH analysis was performed according to the manufacturer's protocols. After hybridization and washing, slides were dried and scanned using a microarray scanner (Agilent, Santa Clara, CA). Images were analyzed with Agilent's CGH Analytics software.

Functional analysis

Real Time PCR was performed with the Biorad iQ5 multicolor real-time PCR detection system using Hs_IGF1R_SG Quantitect Primer Assay primers (Qiagen, Valencia, CA). Fibroblast cultures of the patient and of two healthy donors were used for Western blotting. Cells were stimulated for 10 min with or without 10 ng/ml IGF-I. Blots were probed with an anti-phospho-PKB/Akt, total PKB/Akt (Cell Signaling Technology, Beverly, MA), anti-phospho-IGF1R (Biosource International, Camarillo, CA) and total IGF1R (Cell Signaling Technology, Beverly, MA) antibodies as described previously (22). Binding studies were performed using iodinated

IGF-I in the presence of an excess of an IGF-I analog that is bound by IGFBPs but not by the IGF1R ($Ala^{31}Leu^{60}$-IGF-I, GroPep, Adelaide, Australia) (23). In short, fibroblasts of the patient and controls were incubated at 4°C with 30,000 cpm [^{125}I]IGF-I, 250 ng/ml $Ala^{31}Leu^{60}$-IGF-I, and graded amounts of unlabeled native IGF-I in 250 µl HEPES binding buffer (100 mM HEPES (pH 7.8), 0.5% fatty-acid-free BSA, 120 mM sodium chloride, 1.2 mM magnesium sulfate, 5 mM potassium chloride, 15 mM sodium acetate, and 10 mM dextrose) as previously described (23). After 18 h, cells were washed and solubilized in 1 M NaOH. Radioactivity was determined using a γ-counter.

Results

Case report

A 4.5 year old girl presented to the growth clinic of the Sophia Children's Hospital with severe growth retardation. She was born after 39 wk gestation as the third child of healthy unrelated parents. The pregnancy was uneventful. Her birth weight was 2.1 kg (-3.0 SDS), birth length was 47 cm (-1.3 SDS), and head circumference 33 cm (-2 SDS)(25). Father's height was 182 cm (-0.3 SDS) and the height of her mother was 165.5 cm (-0.8 SDS). Her target height corrected for secular trend (4.5 cm per generation) was 171.8 (0.2 SDS) (13). Her sister's height was 0 SDS, her brother's height was +1.3 SDS. Motor development was normal: she started sitting at the age of 10 months and walking at 17 months. Speech development was slow.

At the age of 4.5 yr her height was 93.5 cm (-3.5 SDS) (Fig. 1), weight 14.8 kg (weight for height +0.7 SDS), BMI 17 kg/m^2 (1.0 SDS), sitting height 51.6 cm (sitting height/ height ratio 0.55 = 0.1 SDS). At physical examination she had a puppet face and a high-pitched voice. Her upper legs were strikingly muscular and there was increased abdominal fat distributed in a lobular pattern. She wore strong glasses because of extreme myopia (-10 and -7.5). There were no dysmorphic features and no abnormalities at further physical examination.

IgG and IgA anti-gliadin antibodies were negative, virtually excluding celiac disease. Thyroid function was normal. The karyogram showed a normal female pattern

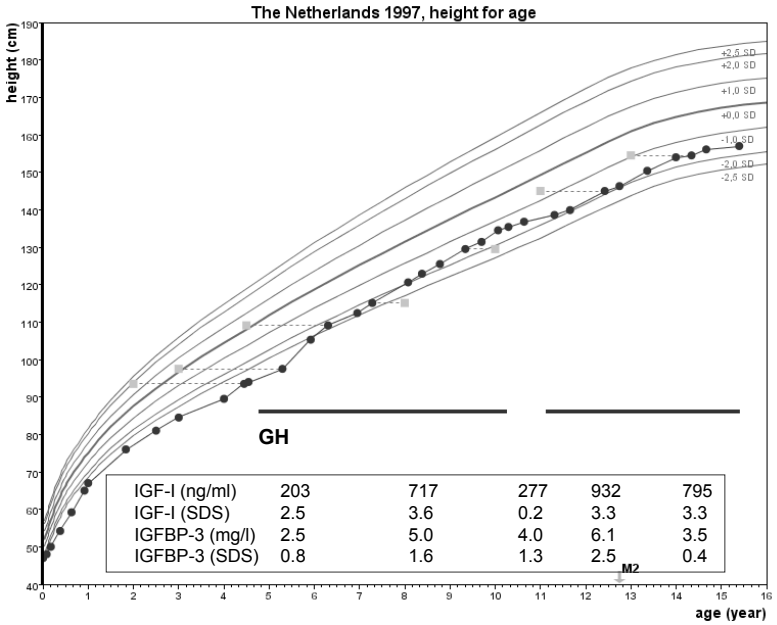

Figure 1. Growth curve
The black dots represent height measurements, the gray squares the bone age, M2 indicates Tanner breast stage 2. The black horizontal lines indicate the periods of GH treatment.

(46,XX). IGF-I was 203 ng/ml (+2.5 SDS), IGFBP-3 2.49 mg/l (+0.8 SDS), IGFBP-1 46.6 ng/ml (normal). GH stimulation tests were performed with clonidine and arginine on separate days, resulting in peak GH concentrations of 8.4 and 25 IU/liter, respectively. At the start of the IGF-I generation test IGF-I was 224 ng/ml, after 4 days of GH injections (1 mg/m^2 body surface) IGF-I was 375 ng/ml, after another 3 days of GH (2 mg/m^2) IGF-I was 480 ng/ml (normal range 115-329 ng/ml).
Bone age was delayed by 2.5 years. BMD was 0.56 g/cm^2 (-1.80 SDS), BMAD was 0.25 g/cm^3 (-1.09 SDS) and total body BMD was 0.77 g/cm^2 (-1.39 SD). Lean body mass SDS was –2.45 and percentage body fat SDS was 0.75.

At the age of 5.3 years GH therapy was started in a dosage of 1 mg/m^2.day sc (Humatrope, Lilly) (equivalent to 0.26 mg/kg body weight/week). A rapid catch-up growth occurred, followed by a stabilisation at -2 SDS (Fig. 1). During GH

therapy IGF-I was elevated (Fig. 1). BMD and lean body mass SDS increased. After two years of GH-treatment lumbar spine BMD SDS was –1.55, BMAD SDS –0.62, total body BMD SDS –0.95, and lean body mass SDS –0.96. GH therapy was interrupted at the age of 10.8 years for 6 months, resulting in a decrease in height velocity and IGF-I level (Fig. 1). Puberty started at the age of 12.8 years and final height was reached at 15 years (157 cm, -1.6 SDS), 1.8 SD lower than target height SDS. Head circumference was 53 cm (-1.1 SDS). She had a regular menstrual cycle. She has recently completed high school.

Genetic and functional analysis

Sequence analysis revealed no mutation in the coding exons of the IGF1R gene. MLPA showed a heterozygous deletion of the three exons (2, 8 and 18) of the IGF1R gene that were included in the MLPA assay (Fig. 2). This deletion was not present in the DNA of the parents. FISH confirmed the IGF1R deletion, together with a deletion of the 15q telomere. aCGH revealed that the deletion comprises 5.2 Mb of the terminal part of the long arm of chromosome 15 (15q26.2 → 15qter), starting from the SPATA8 gene, spanning 34 genes.

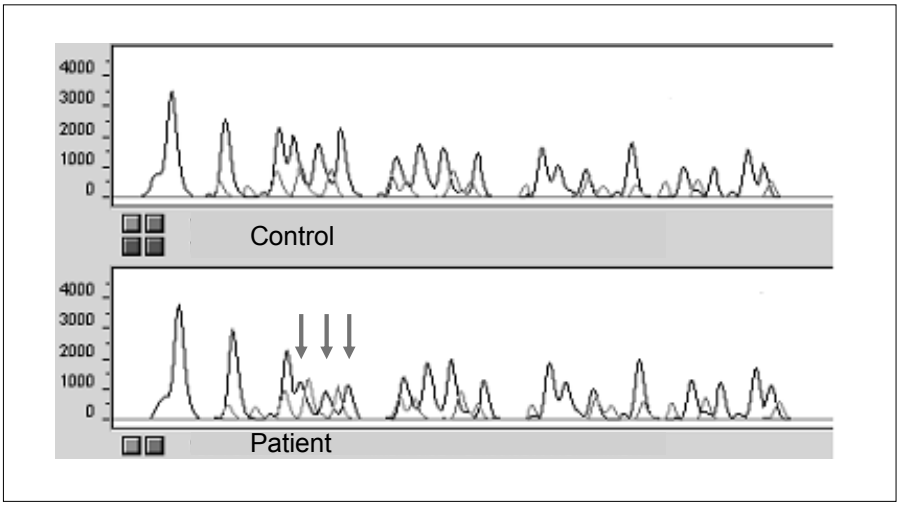

Figure 2. Genescan of the MLPA analysis of the IGF1Rgene.
The arrows in the *lower* panel show the lower peaks of the 3 IGF1R probes in the patient compared with the control in the *upper* panel, indicating a deletion of IGF1R.

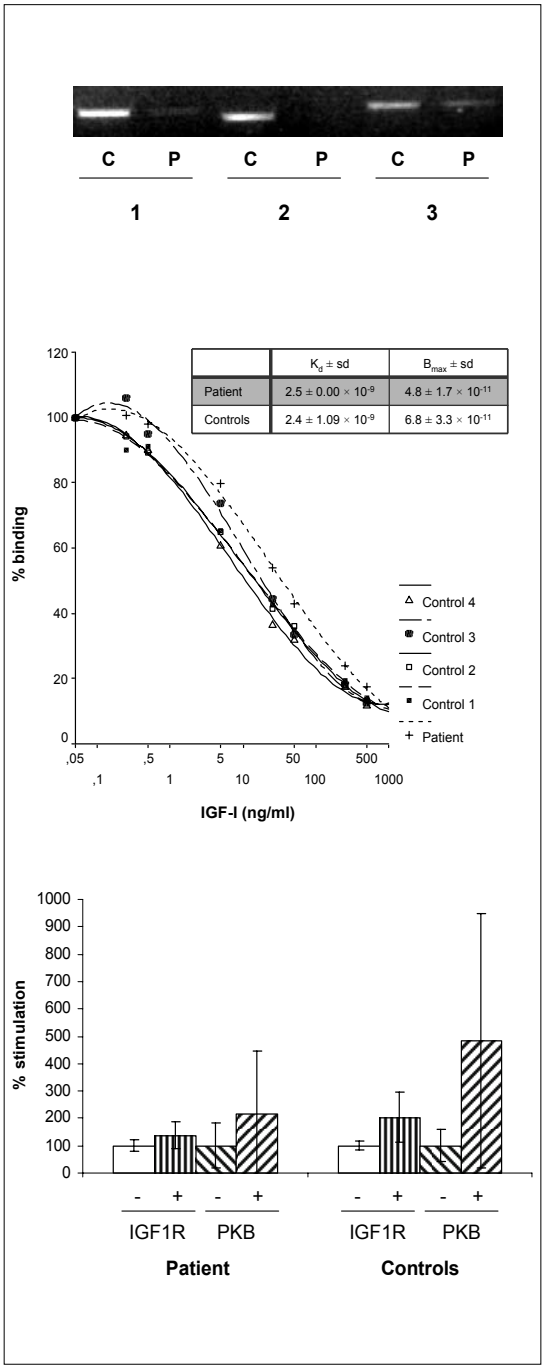

Figure 3. Functional analysis

A. Expression of three IGF1R mRNA fragments (P = patient, C = control). Cultured dermal fibroblasts showed decreased expression of IGF1R mRNA in the patient *vs* a random control.

B. Equal amounts of cells of the patient and four controls were seeded in 24-wells plates. At confluency, cells were incubated with [^{125}I]IGF-I in the presence of 250 ng/ml Ala^{31}Leu^{60}-IGF-I and increasing amounts of unlabeled native IGF-I. After 18 h, cells were washed and binding of [^{125}I]IGF-I was determined. Data represent the mean of two quadruplicate experiments and are expressed as percentage of total binding in the presence of competition with the lowest concentration IGF-I (0.05 ng/ml), which was set to 100% after correction for non-specific binding. The displacement curve of the patient's cells was indistinguishable from controls.

Scatchard analysis was performed for the calculation of the binding affinity (K_d) and binding capacity (B_{max}) of the patient's cells and controls. The K_d and B_{max} of our patient showed no significant differences compared with various controls. Values represent the mean of two quadruplicate experiments ± SD.

C. Dermal fibroblasts of the patient and controls were stimulated with 10 ng/ml IGF-I for 10 min. Protein lysates were collected and 25 µg of protein was used for Western blotting using phosphospecific IGF1R and PKB/Akt (Ser473) antibodies and total IGF1R and PKB/Akt antibodies (picture not shown). Densitometric quantification of the Western blots was performed. Data are expressed as a ratio of phosphor specific IGF1R or PKB/Akt and total IGF1R or PKB/Akt, respectively. The ratio of the unstimulated lysates was set at 100%. The activation of the IGF1R and PKB/Akt tended to be lower in the patient, but this did not reach significance.

RT-PCR analysis using various fragments of the IGF1R gene showed decreased expression of IGF1R mRNA in fibroblasts of the patient versus a normal control (Fig. 3A). This was confirmed by quantitative PCR (qPCR) showing an approximately 5 times reduction in IGF1R mRNA expression compared to a panel of normal controls (data not shown). Binding studies showed normal binding affinity and a trend towards decreased total binding (not significant) of iodinated IGF-I to patient's cells in comparison with a panel of control cell lines (Fig. 3B). Western blot demonstrated a comparable level of total IGF1R protein expression in the patient compared with controls (data not shown); however, autophosphorylation of the IGF1R and activation of PKB/Akt upon a challenge with 10 ng/ml IGF-I for 10 min were reduced (Fig. 3C), although this did not reach significance.

Discussion

Partial IGF-I resistance due to heterozygous deletions or inactivating mutations of the IGF1R gene is a rare cause of short stature. We present a girl with a heterozygous terminal deletion of the long arm of chromosome 15, including the IGF1R gene.

The IGF1R is a tyrosine kinase receptor, consisting of a heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein. Binding of IGF-I to the receptor results in autophosphorylation of three intracellular tyrosine residues and activation of the receptor's intrinsic tyrosine kinase. Subsequently, distinct intracellular signaling pathways are activated, which induce amongst others protein synthesis and glucose transport and regulate cell proliferation, differentiation and apoptosis (26). A deletion of one copy of the IGF1R may result in haploinsufficiency due to lower expression of IGF1R mRNA and protein. Indeed, in dermal fibroblasts of the patient IGF1R mRNA expression was decreased compared with a panel of controls. However, this resulted not in reduced protein expression, diminished IGF-I binding or decreased activation of downstream signaling upon a challenge with IGF-I. Maximal binding and phosphorylation of IGF1R and PKB/AKT tended to be lower but this did not reach significance. Also others have reported inconsistent results regarding IGF1R haploinsufficiency due to a 15q deletion in dermal fibroblasts and it was suggested that dermal fibroblasts were less suited to study the functional consequences of IGF1R haploinsufficiency (6, 10). This contrasts findings in a family with a missense

mutation in the intracellular kinase domain of the IGF1R, which resulted in strongly decreased activation of downstream signaling in dermal fibroblasts (3). It may well be that the consequences of haploinsufficiency are cell type dependend, with little effect in dermal fibroblasts but strong effects in growth plate chondrocytes that are responsible for longitudinal growth.

A pure terminal deletion of 15q, without the presence of a ring chromosome, has only been described in a few cases (5-11). In all cases, except one, the deletion was detected by regular karyotyping. Pinson et al. diagnosed a patient with a terminal 15q deletion unexpectedly: looking for a maternal 15q11-q13 deletion to exclude Angelman syndrome, they accidently observed that the specific telomeric control probe on one chromosome 15 was missing. In our case the deletion was not diagnosed with regular karyotyping, but detected through the MLPA technique. We have developed a MLPA probe-mixture directed against 3 different exons of the IGF1R and several other genes for the evaluation of children with unexplained short stature. In our patient MLPA proved successful in detecting the IGF1R deletion, which was further characterized with aCGH. Since the MLPA assay can be easily extended to include as many as at least 40 different probe sets, this technique is ideally suited for the evaluation of copy number changes of multiple genomic regions simultaneously. We, therefore, expect that these techniques will significantly contribute to the elucidation of genetic causes of unexplained short stature in the future.

Some of the phenotypical features can be attributed to the loss of one copy of the IGF1R gene, while other features are probably the result of the absence of other genes in the deleted region.
In all cases of terminal 15q deletion, except in one child with a diabetic mother (9), IUGR was present. IUGR is also a common feature in patients with inactivating IGF1R mutations (1-4), indicating that IGF1R haploinsufficiency is responsible for the poor prenatal growth. Also, the postnatal growth pattern in our patient and in other cases with this condition is comparable with the growth in patients with a heterozygous inactivating mutation in the IGF1R gene, suggesting that the growth retardation in patients with terminal 15q deletion is the result of partial IGF-I resistance.

Another common feature in patients with partial IGF-I resistance is microcephaly with or without delayed psychomotor development. The normal development in our patient, our earlier observations of a normal intellectual development in an adult woman with a heterozygous missense mutation (3), in combination with the normal intelligence reported for three other cases with IGF1R missense mutations (1, 2, 4) suggest that the degree of developmental delay is predominantly determined by the deletion of other genes on 15q26.2→qter.

The dysmorphic features described in the patients with terminal 15q deletion, including our own patient, mainly include craniofacial characteristics and anomalies of hand and feet. These features are uncommon in patients with IGF1R mutations and probably result from the loss of other genes in the gene-rich 15q subtelomeric region. Our patient had extreme myopia, which has not been reported in other patients with IGF-I resistance. Although the IGF1R is expressed in the lens (27), it is unlikely that myopia is the result of IGF-I resistance, as one would expect to find this more frequently in other patients with IGF-I resistance. We have now shown that the absence of major dysmorphic features does not exclude a terminal 15q deletion.

We have shown that the growth retardation caused by IGF1R haploinsufficiency can successfully be treated with GH: height at start of therapy was -3.5 SDS and final height was -1.6 SDS, within the population range, but 1.8 SD below target height. This observation is partially consistent with the effects of GH therapy in two patients with IGF1R haploinsuffiency (6, 10). One of them (6) was treated from 3.5 years onwards during three periods with different dose regimes. During the first two periods catch-up growth was observed, but this was not sustained and at the age of 12 years height was -5.5 SDS, similar to height SDS at start. Okubo et al. (10) treated a patient with 1.7 U/day from the age of 22 months resulting in improved growth (-4.9 SDS to -3.2 SDS at the age of 9 years). The result of two years of GH treatment (30 µg/kg/day) administered to a patient with an inactivating mutation of the IGF1R gene was reported by Raile et al (28). Height SDS increased from -2.5 to -1.5. Thus, we conclude that patients with partial IGF-I resistance due to heterozygous IGF1R deletions or mutations may benefit from GH therapy in terms of longitudinal growth, possibly due to the direct effect of increased concentrations of GH in combination with strongly elevated plasma IGF-I levels that may partially overcome the decreased IGF-I sensitivity.

In conclusion, the characteristics of a heterozygous terminal 15q deletion are dominated by partial IGF-I resistance, due to IGF1R haploinsufficiency, while the dysmorphic features and the developmental delay are probably the result of haploinsufficiency of other genes in this chromosomal region. GH treatment considerably improves growth during childhood and leads to a normal adult height, though still below the genetic target. A normal karyogram in patients with features suggestive for IGF-I resistance does not exclude small deletions. MLPA followed by aCGH is a powerful diagnostic strategy to detect submicroscopic deletions in children with short stature.

Acknowledgements

The authors thank Kerstin Hansson for technical assistance.

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General discussion

Genetic disorders in the GH-IGF-I axis in mouse and man

10

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European Journal of Endocrinology 2007;157(suppl 1):S15-26

Abstract

Animal knockout experiments have offered the opportunity to study genes that play a role in growth and development. In the last few years, reports of patients with genetic defects in the GH-IGF-I axis have greatly increased our knowledge of genetically determined causes of short stature. In the various chapters of this thesis, patients are described with a genetic defect in the GHRH receptor, the GH signaling pathway, IGF-I or the IGF-I receptor, all of which are components of the GH-IGF-I axis. In this final chapter the animal data and human reports of genetic disorders in the GH-IGF-I axis will be reviewed, in order to describe the role of the GH-IGF-I axis in intrauterine and postnatal growth. In addition, the effects of the GH-IGF-I axis on development and function of different organ systems as brain, inner ear, eye, skeleton, glucose homeostasis, gonadal function and the immune system will be discussed. The number of patients with genetic defects in the GH-IGF-I axis is small and a systematic diagnostic approach and selective genetic analysis in a patient with short stature are essential to identify more patients. Finally, the implications of a genetic defect in the GH-IGF-I axis for the patient and the therapeutic options will be discussed.

Introduction

Reports of patients with genetic defects in the growth hormone – Insulin-like Growth Factor-I (GH-IGF-I) axis, in addition to animal knockout experiments, have increased our knowledge on the role of the GH-IGF-I axis in growth and development throughout life considerably. By means of these reports we will describe the factors playing a role in intrauterine as well as postnatal growth and the effect of the GH-IGF-I axis on the development and function of different organs. Finally, the tools for the diagnostic approach for the physician and the clinical implications for the patients will be discussed.

Intrauterine growth

The first evidence that intrauterine growth is determined by GH-independent IGF-I secretion, comes from animal experiments. Mice with severe GH deficiency due to spontaneous mutations in the genes encoding GHRHR (little mouse), Pit 1 (Snell dwarf) and Prop-1 (Ames dwarf) have a normal birthweight (1). The GH insensitive Laron mouse is born with a normal body size and weight (2). In contrast, IGF-I and IGF1R knockout mice have birthweights of 60% and 45% of normal, respectively. From these observations it was concluded that IGF-I is a growth determinant factor for intrauterine growth, independently of GH (3-5). The effects of IGF-I are mediated through the IGF-I receceptor (IGF1R), which is in line with the observation that the IGF1R -/- mice and the IGF1R-/- IGF-I-/- mice are equally growth retarded at birth (45 % of normal) (3). IGF1R-/- knockout mice die within minutes after birth due to respiratory failure (3). IGF1R+/- mice are phenotypically normal (3). Disruptions in the IGF-I signaling pathway as described in mice deficient in Akt1, insulin receptor substrate-1 (IRS-1) or IRS-2 result in reduced intrauterine growth (6-9).

In the human, GH deficiency and insensivity result in a normal birth size (10-12). In contrast, an IGF-I gene deletion or mutation results in severe intrauterine growth retardation as is demonstrated in the patient described by Woods *et al*. (birth weight and length –3.9 SDS and –5.4 SDS, respectively), Walenkamp *et al*. (birth weight and length –3.9 SDS and –4.3 SDS, respectively) and Bonapace *et al*. (birth weight and length –4 SDS and –6.5 SDS, respectively) (13-15). The finding

that genetically determined low IGF-I levels, due to polymorphisms in the IGF-I promoter region, result in a reduced birth weight and length support the role of IGF-I in fetal growth (16, 17).
There are also indications of an IGF-I dose effect on intrauterine growth. IGF-I heterozygous mouse are 10-20% smaller at birth (4) and in the human we found that carriers of an IGF-I missense mutation have a 10% lower birth weight than non-carriers (14).
In contrast to the observations in mice, heterozygous mutations of the IGF1R result in intrauterine growth retardation in the human (18-22). The degree of IUGR varies between -1.5 SDS and -3.5 SDS for birth weight and between -0.3 SDS and -5.8 SDS for birth length. The different mutations result in a variable degree of remaining IGF-I signaling, which may explain the wide range of birth size. Another hypothesis is that maternal IGF-I resistance during pregnancy in the mothers with a heterozygous IGF1R mutation contribute to more severe growth retardation. This is supported by the finding that children with an affected mother are more growth retarded at birth than children with an apparently non-affected mother (22).
IGF1R haploinsufficiency is associated with a variable degree of intrauterine growth retardation (-1.8 SDS to –5.6 SDS) as is demonstrated in patients with a terminal 15q deletion, including the IGF1R gene (23-30). The report of a patient with three copies of the IGF1R gene supports the concept that a gene dose effect plays a role in intrauterine growth. This patient is born at 42 weeks gestational age with a birth weight of 5140 gr, a birth length of 60 cm and a head circumference 38.5 cm (28).
So far, genetic defects of the IGF-I signaling pathway are not described in human. However, in a recent study of Laviola *et al*. the IRS-2 and Akt pathway were down-regulated in human placentas from pregnancies complicated by IUGR, indicating an important role for IGF-I signaling in the appropiate development of the fetoplacental unit (31).

ALS (acid-labile subunit) forms a ternary complex with IGF-I or IGF-II and with IGFBP-3 or IGFBP-5 in the circulation to increase the half-life of the IGFs. ALS deficiency appears to have no effect on fetal growth in ALS knockout mice (32). Data on intrauterine growth of ALS deficient patients are limited, as the first described patient is adopted and gestational age is not available. At the age of one week

weight was 2500 gr and length was 47 cm, which is considered normal (33). Also from the second patient no data regarding gestation, birth weight and length are available (34). As expression of ALS occurs late in fetal life (35), severe effects on intrauterine growth are unlikely.

Although this review focuses on genetic defects of the GH-IGF-I axis, the importance of the role of IGF-II in intrauterine growth cannot be left unmentioned. IGF-II acts as an important modulator of placental cell proliferation and maturation (36). A positive correlation has been demonstrated between cord blood IGF-II and placental weight (37). However, controversy exists with regard to the relation between fetal serum IGF-II levels and fetal growth. Some studies report reduced cord blood IGF-II levels in IUGR babies (38-40), others report similar levels of IGF-II in IUGR and normal fetuses (41). Human mutations in the gene encoding IGF-II, located on 11p15, have not been identified sofar. However, alterations in imprinting status of the IGF-II gene are associated with severe IUGR as part of the Silver Rusell syndrome (a condition characterized by severe IUGR, postnatal growth failure, dysmorphic facial features and body assymetry). The IGF-II gene is paternally expressed. A maternal duplication of 11p15 or demethylation of the telomeric imprinting centre ICR1 on 11p15, resulting in under-expression of IGF-II is found in 50 % of patients with Silver Russell syndrome (42-44).

Postnatal growth

The first two weeks of postnatal life growth of the Snell dwarf, the Ames dwarf, the little and Laron mouse is indistinguishable from the wild type littermates (1). However, at postnatal day 40, their size is about 50% of normal, which confirms the increasing role of GH in postnatal life. The size of the IGF-I knockout mice decreases progressively from 60% of normal at birth to 30% of normal at 8 weeks. Double knockout mice (GHR-/- and IGF-I-/-) have a postnatal growth pattern of 17% of normal (45). These findings demonstrate that GH dependent IGF-I action is the main determinant of postnatal growth, but that GH and IGF-I have also independent effects (45). Targeted gene deletion of liver-specific IGF-I and ALS (LID/ALSO mouse), resulting in a 85-90% reduction of circulating IGF-I, shows a 20% lower body weight than control mice. This implicates that, while endocrine

IGF-I is important in postnatal growth, tissue IGF-I also plays a role since the total IGF-I-/- mouse is more growth retarded (46).
The classical heterozygous IGF1R-/+ mice are phenotypically normal, with normal expression of IGF1RmRNA, suggesting that the intact wild-type allele is up-regulated and implying that a single functional IGF1R allele is sufficient to assure normal growth (3). However, later experiments inducing reduced availability of the IGF1R (41% less than normal) showed a growth deficit of 13% in males and of 6% in females, implying that a partial reduction in IGF-I signaling reduces the growth potential, at least in the male mouse (47).

In the human, severe isolated GH deficiency leads to a final height ranging from -3.9 to -6.1 SDS (mean -4.7 SDS) (48). GH deficiency due to GHRHR mutations results in a mean final height of –7.2 SDS (49). Patients with GH insensitivity due to a GHR mutation reach a final height varying from -3.2 SDS to -12 SDS (12, 50, 51) and due to a STAT5b mutation from -5.9 SDS to -7.8 SDS (52-54). Complete IGF-I deficiency due to a deletion of the IGF-I gene results in a final height of -6.9 SDS (13). The final height of the patient with an inactivating mutation of the IGF-I gene is -8.5 SDS (14). The height of a patient with a mutation resulting in extremely low IGF-I levels at the age of 19 months is -6.2 SDS (15). These results show that GH deficiency results in a similar growth pattern as primary IGF-I deficiency, which emphasizes the predominant role of GH dependent IGF-I production in postnatal growth. Treating GH deficient patients with GH results in a better growth response than treating GH insensitive patients with recombinant human (rh)IGF-I, suggesting that a direct GH effect is necessary for optimal postnatal growth (55).

GH insensitivity caused by a STAT5b mutation results in severe postnatal growth retardation in males and females. In contrast, the homozygous ablation of STAT5b in mice results in loss of the sexual dimorphism in body growth: the postnatal growth curves of female wild type, female STAT5b-/- and male STAT5b-/- mice show no significant difference (56). STAT5b gene disruption in mice leads to a loss of the sexually dimorphic pituitary pulsatile GH secretion pattern and liver gene expression. It is uncertain how this difference with regard to the interaction between sex steroids and the GH-STAT5b-IGF-I axis in mouse *vs* human is can be explained. One explanation may be that in the mouse the growth promoting effect of the typical male pattern of GH secretion (high GH peaks, low throughs) is trans-

mitted by STAT5B, while the irregular GH profile of female rats, with lower peaks and higher troughs, is primarily transmitted by another signal transduction route. Knocking out STAT5B in the mouse can then be expected to abolish the sexual dimorphism in growth in the rat. In the human, the GH profile is not different between sexes, and is similar to the pattern of GH secretion seen in male rats. A STAT5B defect would then be expected to have a similar effect in both sexes 56, 57)
Patients with IGF-I insensitivity due to IGF1R mutations show more variety in postnatal growth (-0.9 SDS to -4.8 SDS) than patients with IGF-I deficiency. The phenotypical variability in IGF-I insensitivity is most probably caused by differences in remaining IGF-I signaling or by a compensatory mechanism that up-regulates the expression of the normal IGF1R allele. In addition, a gene dose effect seems to play a role in postnatal growth as is demonstrated by the severe postnatal growth failure (-3.5 SDS to –6.3 SDS) of patients with IGF1R haploinsufficiency due to a terminal 15q deletion, including the IGF1R gene (23, 25, 26, 28-30). The finding that trisomy of terminal 15q resulting in duplication of the IGF1R gene is associated with tall stature supports this hypothesis (58).

Two patients with ALS deficiency due to a mutation in the ALS gene have been described (33, 34) and we have recently reported two affected sibs (59). They present with a variable degree of growth retardation: height at 14.6 years of age –2.05 SDS and final height -0.8 SDS in the patient described by Domene *et al*, -2.1 SDS at 15.5 years of age in the patient described by Hwa *et al*, final height was -4.2 SDS in one of the sibs and height was -4.3 SDS at the age of 16.5 years in the other sib. Extremely low IGF-I and IGFBP-3 levels is a common finding in all these patients. A high flux of free IGF-I into the tissues and rapid proteolysis of IGF-I can explain this feature. Another possibility is that paracrine and autocrine IGF-I effects, stimulated by increased GH production, compensate for the deficiency of circulatory IGF-I (34).

Regulation of GH secretion

The regulation of GH secretion is complex. Shortly, GHRH stimulates GH synthesis and determines the amplitude of the pulses, while the intermittent withdrawal of

somatostatin regulates the timing of the GH pulses. The low amplitude spontaneous GH release in patients with a GHRHR mutation supports this hypothesis (60). The autofeedback mechanism of GH and IGF-I suppresses GHRH and stimulates somatostatin expression.
Age, gender, sex hormones and adiposity influence the magnitude of GH secretion (61). Ghrelin, a hormone predominantly produced in the stomach, stimulates GH release and appetite. Ghrelin -/- mouse have a normal phenotype, suggesting that ghrelin itself is not required for growth (62). Human mutations have not yet been described. Ghrelin acts via the GH secretagogue receptor (GHSR). Recently, two families with a GHSR missense mutation resulting in impairment of the constitutive activity of the receptor were identified. The heights of the homozygous probands were –3.7 SDS and –3.2 SDS, supporting the hypothesis that ghrelin signaling via the GHSR plays a role in the GH-IGF-I axis (63).
In patients with GH insensitivity low IGF-I levels result in loss of negative feedback and as a consequence increased GH secretion. IGF-I therapy restores GH secretion to normal, implying that the sensitivity of the feedback mechanism is intact in these patients (64). In addition to the increased GH secretion, prolactin levels are slightly elevated in patients with GHI due to a GHR mutation (65). This may be the result of increased GHRH secretion as a consequence of the diminished negative feedback of IGF-I and GH, which also stimulates the prolactin secretion from somatolactotroph cells in the pituitary. Indeed, the elevated prolactin levels are suppressed upon IGF-I administration, in line with the inhibitory action of IGF-I on hypothalamic GHRH neurons (65). In patients with a STAT5b mutation prolactin levels are even more elevated (52, 54, 66). This can be explained by the obligatory role of STAT5b in mediating the negative feedback action of prolactin on tuberoinfundibular dopamine neurons; in the absence of STAT5b the signal transduction in the hypothalamic dopamine neurons is impaired (67).

Patients with IGF-I insensitivity are characterized by elevated IGF-I levels as a result of increased GH production due to pituitary and hypothalamic IGF1R deficiency. This is supported by the finding that IGF1R-/- mice exhibit signs of somatotropic stimulation in the pituitary (61). However, in patients with poor caloric intake IGF-I can be in the normal range, which should be considered as relatively high for a malnourished infant or child. Restoring the nutritional status increases the IGF-I level above the normal range (22).

Patients with IGF1R haploinsufficiency have normal or elevated IGF-I levels (23, 25, 26, 30). Stimulated GH secretion was normal in all tested patients (24, 26, 28-30). Apparently, IGF1R haploinsufficiency has no major impact on GH and IGF-I secretion.

IGF binding proteins

IGF-I is secreted in the circulation and associated with soluble high-affinity binding proteins, the IGFBPs. IGFBP-3 and IGFBP-5 form a complex with IGF-I and ALS. This ternary complex slows the clearance of IGF-I. Changes in IGFBP expression have an important role in modulating the growth-promoting actions of the IGFs.

In mice, reports of IGFBP knockout models are limited and demonstrate only few phenotypic manifestations (68). Overexpression of IGFBP-1 in transgenic mice result in a modest and transient impairment of fetal growth. Also maternal IGFBP-1 excess is associated with reduced fetal growth indicating placental insufficiency (69). In human there is a striking inverse correlation between maternal and fetal circulating levels of IGFBP-1 and fetal size (39, 44, 70, 71). It is postulated that IGFBP-1 inhibits the growth promoting effect of IGF-I by binding fetal IGF's in IUGR. Indeed, IGFBP-1 levels and mRNA expression are markedly elevated in the umbilical cord blood of babies with profound and prolonged hypoxia, which is considered as a leading cause of IUGR (72). In a recent study the key role for IGFBP-1 in mediating the effects of hypoxia on fetal growth was confirmed in zebrafish, suggesting this is a conserved physiological mechanism to restrict IGF stimulated growth under hypoxic conditions (73).

IGFBP-1 is synthesized in the liver, where its expression is under control of insulin which suppresses its production (74). It has also been suggested that GH directly regulates IGFBP-1 secretion (75). In the patient with the IGF-I deletion IGFBP-1 was low. This patient was severely insulin resistant and a combination of high insulin levels, the absence of IGF-I and increased GH levels probably contributes to the low IGFBP-1 levels. Treatment of this patient with rhIGF-I resulted in beneficial effects on insulin resistance, decreased GH secretion and higher IGFBP-1 levels (76). The patient with the inactivating mutation of the IGF-I gene was not severely

insulin resistant and had also low IGFBP-1 levels, suggesting an important role for a direct suppressive effect of GH on IGFBP-1 (14). The patient with ALS deficiency also had low IGFBP-1 levels, which can also be attributed to stimulated GH levels (33).
IGFBP-2 levels were low in the patient with the IGF-I gene deletion. rhIGF-I treatment increased the IGFBP-2 levels (76). IGFBP-2 in the patient with the IGF-I mutation was -1 SDS (14). The patient with ALS deficiency had low IGFBP-2 levels (33). These data suggest that GH plays a role in regulating IGFBP-2 production.

In the absence of ALS, IGFBP-3 is cleared very fast, resulting in extremely low serum concentrations (33, 77). The transcription of IGFBP-3 is induced by activation of the GH signal transduction pathway, including STAT5b. Therefore, IGFBP-3 is a valuable biochemical parameter in differentiating a GH receptor or postreceptor defect from an IGF-I or IGF1R defect. In the latter IGFBP-3 levels are normal (13, 14, 22), while in patients with a GHR defect or a STAT5b mutation IGFBP-3 is low (51, 52, 54, 77).

The GH-IGF-I axis brain development

IGF-I plays a key role in the development of the central nervous system, stimulating neurogenesis and synaptogenesis, facilitating oligodendrocyte development, promoting neuron and oligodendrocyte survival and stimulating myelination (78). In addition, IGF-I appears to be a potent agent for rescuing neurons from apoptosis. As systemic IGF-I is not readily transported through the blood-brain-barrier local production of IGF-I is considered to be responsible for these effects (79).

Psychomotor development is normal in patients with GH deficiency or insensitivity (80). Patients with primary IGF-I deficiency due to a deletion or mutation of the IGF-I gene are severely mentally retarded and microcephalic with a head circumference at birth of -4.9 SDS (13) and -5.7 SDS (15) and in adulthood of -5.3 SDS (13) and -8 SDS (14), emphasizing the essential role of GH-independent IGF-I production for prenatal brain development. The head circumference of carriers of the inactivating IGF-I mutation is within the normal range. However, carriers have a lower head circumference than non-carriers (-1 SDS vs 0.5 SDS). Microcephaly is

a common feature in patients with IGF-I insensitivity due to a heterozygous IGF1R mutation, however less severe than in complete IGF-I deficiency (-3 SDS to -5.6 SDS)(22). Psychomotor development in these patients varies from retarded with an IQ of 60 to completely normal. Head circumference in patients with IGF1R haploinsufficiency due to a terminal 15q deletion is not well documented. In two patients head circumference was measured: -5.3 SDS (25) and -3 SDS (26). In most cases psychomotor development was delayed, but the possible contribution of other genes in the deleted region makes conclusions on a causal relation difficult.

Hearing

Recently, the role of IGF-I in auditory function was evaluated. Auditory brainstem responses were analized in IGF-I-/- mice, showing an all-frequency involved bilateral sensorineural hearing loss. The delayed response to acoustic stimuli along the auditory pathway indicates the contribution of the central nervous system to the hearing loss in IGF-I deficiency (81). At a cellular level a significant decrease in number and size of auditory neurons, increased apoptosis of cochlear neurons, a significant reduced volume of the cochlea and cochlear ganglion result in abnormal differentiation and maturation of the cochlear ganglion cells and abnormal innervation of the sensory cells in the organ of Corti (82).
Audiograms of the three patients with complete IGF-I deficiency due to a homozygous deletion or mutation of the IGF-I gene demonstrate severe bilateral sensorineural deafness (13-15). This is confirmed by absent brain stem evoked potentials in one of the patients (14). Seven of the 21 family members of the patient with the inactivating IGF-I mutation, including 9 carriers of the inactivating IGF-I mutation, revealhearing abnormalities. However, no significant association with the carriership could be detected (14). In patients with GH deficiency or insensitivity or heterozygous IGF1R receptor mutations hearing problems have not been reported.
In conclusion, IGF-I is a key factor in development and postnatal differentiation and maturation of the inner ear.

Vision

In a recent study, the ocular dimensions of patients with Laron syndrome were compared with reference values. Patients with Laron syndrome have significantly shorter axial length of the eye and shallower anterior chambers. Treatment with IGF-I increases the axial length of the eye (83). The patient with an inactivating IGF-I mutation, has a shallow anterior chamber, suggesting that IGF-I may play a role in ocular growth (14).
Retinal vascularization is significantly reduced in patients with defects of the GHR, IGF-I and IGF1R indicating that IGF-I plays an important role in this phenomenon (84). A strong association has been found between reduced IGF-I levels in preterm children and development of retinopathy of prematurity. Deficient IGF-I levels after premature birth result in initial poor retinal vascular development and a large area of avascular retina (85).

Skeletal features

Limited elbow extensibility is seen in 85% of patients with Laron syndrome over 5 years of age, with increasing severity with age (50). In addition, 6 of 8 patients with GH deficiency due to a PROP1 mutation show symmetrical limitation of elbow extensibility, correlated with age (86). In the patient with the inactivating mutation of the IGF-I gene we also found this feature but it was not described in the other patients with IGF-I defects at 19 months and 15.5 years, suggesting that IGF-I plays a role in elbow extensibility later in life. The mechanism is unknown.

Micrognathia is a striking feature in two patients with IGF-I defects. Diewert *et al*. found that major growth movements and developmental changes in craniofacial tissues take place between 7 and 12 weeks of gestation (87). Significant alterations in growth during this period may produce significant irreversible effects on postnatal craniofacial morphology. One can hypothesize that prenatal IGF-I deficiency may disturb this process and result in micrognathia.

Bone Mineral Density

Studies on skeletal structure in IGF-I -/- mice show a 17% decrease in cortical bone but an increase in trabecular bone (23% in male and 88% in female) (88). This phenomenon is also observed in IGF-I deficient double knockout mice (liver-specific IGF-I and ALS deletion) (89). Yakar *et al.* postulated that the ternary complex (IGF-I, IGFBP-3 and ALS) influences bone acquisition in a compartment-specific manner (i.e.cortical vs trabecular bone). The finding that IGF-I deficient mice exhibit greater impairment in bone accretion than GH deficient mice, implies a GH-independent effect of IGF-I on bone formation during postnatal growth (90). Dual-energy X-ray absorptiometry (DEXA) is a method to assess BMD (in g per cm^2). However, the method does not correct for antero-posterior depth and is therefore greatly influenced by bone size. Bone mineral apparent density (BMAD) calculates volumetric density to minimize the effect of bone size on BMD values (91). This method greatly contributes to the interpretation of BMD in patients with short stature.

Patients with GH deficiency due to GHRHR mutations or GH insensitivity due to GHR mutations have a normal BMAD (92-94). BMAD in the patient with an IGF-I gene deletion is mildly reduced. Treatment with rhIGF-I resulted in a 7% increase of BMAD, compared to a 17% increase of BMD, suggesting that the increase in BMD is partly attributable to an increase in bone size (95). In contrast, severe osteoporosis is demonstrated in the patient with an inactivating IGF-I mutation (14). Two patients with a heterozygous mutation of the IGF1R gene have a normal BMD (18, 22). In one patient with ALS deficiency severe osteoporosis is found at the age of 16 years (BMD at lumbar spine -4.6 SDS), with a partial recovery at 19 years of age (BMD at lumbar spine –2.1 SDS) (96). In the patient we recently described a similar pattern was observed with an increasing BMD from -5.2 to -4.1 and -2.5 SDS at the age of 16, 17 and 19 years, respectively. This suggests that sex steroids can reduce the osteoporotic effects of ALS deficiency during puberty.

Glucose homeostasis

It has been well documented that GH exerts direct effects on insulin secretion as well as indirect effects through increased lipolysis, resulting in elevated free fatty

acid (FFA) levels and impaired insulin sensitivity, the "lipotoxic effect" (97). Acute administration of GH has an insulin-like effect, mediated by the JAK-2 signaling pathway. Activation of insulin receptor substrate (IRS)-1 and IRS-2 leads to recruitment of PI3 kinase and, analogous to the post-receptor events for insulin, results in increased glucose uptake (98). GH therapy is often associated with impaired insulin sensitivity. Prevention of lipolysis by coadministration of GH with FFA regulators can partially prevent the deterioration of insulin sensitivity, indicating that insulin resistance is a consequence of enhanced GH-induced lipolysis (99). In addition, the GH plus FFA regulator combination treatment significantly enhances linear body growth in SGA and control rats. The precise mechanism of this observation remains to be elucidated (100). Chronic excess of GH, as in acromegaly, is associated with insulin resistance and impairment of insulin receptor signal transduction. Treatment of acromegalic patients with somatostatin analogues improves insulin resistance (101). Post receptor cross talk between the insulin receptor and GH receptor signaling pathway is believed to play a key role in this process (102).

IGF-I, which has 48% amino acid sequence identity with proinsulin, enhances insulin sensitivity. Epidemiological studies have demonstrated that lower baseline IGF-I levels are associated with a higher risk of insulin resistance (103). Genetically determined low IGF-I levels are associated with an increased risk of insulin resistance (104). Treatment with rhIGF-I improves insulin sensitivity in normal individuals (105), in patients with insulin resistance (106) and in those with diabetes type I (107) or type II (108). The patient with IGF-I deficiency due to an IGF-I deletion had severe insulin resistance, which improved with rhIGF-I treatment (76). Patients with a heterozygous inactivating mutation of the IGF-I gene had higher fasting insulin levels than non-carriers (14). Two patients with a heterozygous IGF1R mutation had a moderate degree of insulin resistance (22, 109). The first described patient with ALS deficiency was insulin resistant (33). These results demonstrate a role for IGF-I in glucose homeostasis. Whether IGF-I directly affects insulin sensitivity or by regulating endogenous GH levels is a topic of intense research.

Body composition

GH is known to have a lipolytic effect, which is illustrated by the finding that GH treatment in GH reduces adiposity and improves lipid profiles (110). Patients with GH resistance have a markedly decreased ratio of lean mass to fat mass, indicating the lack of the direct antilipolytic effect of GH (50). In the patient with the IGF-I gene deletion, in whom the direct GH effects are intact, a low body fat content was found (19.9%), which is in line with a direct antilipolytic effect of GH (95). Total body fat content increased in this patient after 1 year of treatment with rhIGF-I, which can be explained by the fall in GH levels induced by rhIGF-I. IGF-I has no direct effects on lipolysis or lipogenesis.

Puberty and gonadal function

Puberty is delayed in all conditions associated with IGF-I deficiency: GH deficiency due to a GHRHR mutation (49), GH insensitivity caused by a GHR mutation (50, 111), or a STAT5b mutation (54), IGF-I gene deletion (76) and an inactivating IGF-I mutation (14). One female with a heterozygous IGF1R mutation had menarche at the age of 18 years (22), but one of the patients described by Abbuzahab *et al*. had a normal onset of puberty (18). The first patient with a mutation of the ALS gene had a delayed onset of puberty (112), but a later report showed a normal onset of puberty (34). These findings suggest that IGF-I plays a role in pubertal onset.

The pubertal growth spurt is decreased in patients with GH deficiency or resistance with normal testosterone levels. This was initially interpreted by speculating that testosterone needs the presence of normal GH secretion to exert its full growth promoting effect (113). Later, evidence was provided that the growth stimulating effect of testosterone in puberty may be primarily caused by conversion to estrogens and that estrogens are responsible for the pubertal growth spurt and epiphysial closure (114).

IGF-I-/- mice are infertile. Males have drastically reduced testosterone levels, caused by a significant developmental delay of Leydig cells. Females fail to ovulate and possess an infantile uterus with hypoplastic endometrium (115). It is difficult

to hypothesize on the role of GH and IGF-I in reproductive function in the human, as most reported patients with GH-IGF-I defects are too young. Patients with GHRHR mutations (116) and GHR mutation are reported to be fertile. Gonadal function of a 30 year old patient with STAT5b mutation was normal (54). The 55 year old patient with an inactivating IGF-I gene mutation had a small testicular volume, low inhibin B levels and elevated FSH levels, indicating compromised Sertoli cell function and impaired spermatogenesis (14). The role of IGF-I deficiency in the partial gonadal failure is unclear, as this patient underwent a bilateral inguinal hernia operation, with possible damage to the testicles. Patients with a heterozygous deletion or mutation of the IGF-I (13, 14) or IGF1R gene (22) are fertile. More patients and accurate follow-up at adult age will contribute to unravel the role of IGF-I in human reproduction.

Immune system

It has been well established that GH and IGF-I affect the development and function of the immune system (117). Most defects of the GH-IGF-I axis are not associated with immune disorders, except the STAT5b mutation. STAT proteins are involved in the signaling pathway of cytokine receptors (118) and STAT5b-/- mouse exhibit a severe immunologic phenotype (119). So far, 5 females and 1 male with a STAT5b mutation have been reported (52-54, 66, 77). Five patients have signs of immune deficiency. Clinically this results in lymphoid interstitial pneumonia due to Pneumocystis carinii (52), recurrent pulmonary infections (53, 77), and recurrent infections of skin and respiratory tract, severe chronic lung disease and herpetic keratitis (66). One patient suffers from juvenile idiopathic arthritis (53). The 30 year old male patient has no history or signs of immune deficiency (120). Thus, in the human an intact STAT5b is not obligatory for a normal immune phenotype.
We have described a patient with partial GH insensitivity and severe immune deficiency due to a mutation of I-κBγ, disturbing the NF-κB signaling pathway (121). In vitro studies have shown that GH binding to the GHR can promote the NF-κB signaling pathway (122). Another patient with severe combined immunodeficiency (SCID) and GH insensitivity has been described (123). This patient has a mutation of the IL2Rγchain gene, suggesting a common underlying pathogenic mechanism for the endocrinological and immunological problems.

More reports on patients with combined growth and immune disorders are needed to increase our knowledge on the interaction between the immune system and the GH-IGF-I axis.

Longevity

Several lines of evidence suggest an inverse relationship between body size and lifespan in mice (124). For example, Laron mice live up to 55% longer than normal mice (125). Genetic studies in various experimental models suggest that the ageing process is regulated by genes that encode proteins from the GH-IGF-I axis. Exciting studies in IGF1R +/- mice with 50% reduction in receptor level, show that females live 33% longer and males 16% longer. The longer lifespan could not be attributed to other factors and the authors conclude that IGF-I may be a central regulator of mammalian lifespan (126). At a cellular level, fibroblasts with a reduced number of receptors are better able to survive to oxidative stress. By causing damage to DNA, protein, lipids and cellular components oxidative stress is the major determinant of the ageing process.. At a molecular level the intracellular signaling pathway is downregulated. Repressing intracellular signals of insulin and IGF-I is an evolutionarily conserved mechanism for extending lifespan.
Caloric restriction also has a positive effect on lifespan. Mild food restriction in normal and the Ames dwarf mice demonstrates changes in the expression of genes related to insulin and IGF-I signaling pathways (127) . These changes may cause the animals to become more sensitive to insulin. Since insulin sensitive mutant animals live longer, it is suggested that these genes may play a role in aging and life span determination. Studies in the human are limited.

Diagnostic approach

The number of patients with defects in the GH-IGF-I axis is still small, but we believe that they represent a tip of the iceberg. Future studies aimed at detecting known or unknown molecular defects in patients with short stature will undoubtedly make the tip of the iceberg grow.
The diagnostic process to reveal an abnormality in the GH-IGF-I axis begins with an

alert physician, who is not satisfied with the diagnosis of idiopathic short stature. The medical history should include birth weight, length and head circumference, as low values of these parameters for gestational age are features of a genetic defect in IGF-I, the IGF1R or a not yet described IGF-I signaling disorder. It cannot be stressed enough that measurement of length at birth is very important to detect underlying pathology and the fear that stretching the legs could be harmful for the hip joint is unjustified (128). Careful evaluation of milestones in development is necessary to have an impression of the psychomotor development. Family history should include height of other family members and if possible birth data. The presence of hearing abnormalities in a family should alert the physician to consider an IGF-I defect.

After excluding organic and systemic disorders like celiac disease and Turner syndrome the IGF-I and IGFBP-3 level will determine the follow up. We recently presented guidelines for the diagnostic process of patients with severe short stature of unknown origin (129).
The procedure for detailed functional and genetic analysis of a patient with short stature and a possible defect in the GH-IGF-I axis depends on the local setting. In the Leiden University Medical Center the Leiden Growth Genetics Working group, consisting of pediatric and adult endocrinologists, clinical and molecular geneticists, molecular biologists and interested physicians meet to discuss the locally, nationally and internationally referred patients. The referring physician is asked to complete a form (Appendix A) to register all the information that is necessary to make a presumptive diagnosis, including phenotypic features of the patient and his or her family and biochemical measurements (IGF-I, IGFBP-3, GH stimulation test). One of the members of the group assesses the information and can advise on additional testing, for example an IGF-I generation test, measurement of other binding proteins, GH-binding protein or ALS. After presentation of the patient and discussion the decision for the next diagnostic step is made, which can be sequencing a specific gene, Multiplex Ligation-dependent Probe Amplification (MLPA) analysis, Single Nucleotide Polymorphism (SNP) array or detailed functional experiments.

Implications for the patient

Idiopathic short stature is an unsatisfactory diagnosis for the physician as well as for the patient. To find the cause of short stature is important for the patient for several reasons. First, with a definite diagnose the often long-lasting diagnostic process will come to an end. Second, it will be possible to give information on the specific defect and accompanying problems. For example, life style advices in case of higher risk of insulin resistance in patients with a heterozygous IGF1R mutation or prevention of osteoporosis in patients with ALS deficiency . Finally, therapeutic options can be discussed. It has been reported that GH therapy in patients with a heterozygous IGF1R mutation or deletion improves growth and head circumference(18, 30, 109, 130). In the late 1980's trials with rhIGF-I started in patients with GHR defects. Approximately 60 children have been treated with rhIGF-I injections for 2 years or longer. Height SDS increases, although not as much as GH treatment in GH deficient patients (131). The explanation for the modest growth response to rhIGF-I is probably the absence of direct GH effects at the level of the growth plate, including enhancement of local IGF-I production. Recently an rhIGF-I-rhIGFBP-3 complex, with a longer half life, has been developed as therapeutic agent. Trials in patients with GH insensitivity are currently in progress (132).

Future perspectives

Future research should be focused on identifying patients with established defects in the GH-IGF-I axis and to evaluate carefully the clinical and biochemical features. Genetic defects in the GH-IGF-I axis are rare and international collaboration will increase the knowledge on the role of the GH-IGF-I axis in growth and development. Finally, it will be a challenge to find new defects in the GH-IGF-I axis in order to unravel the molecular mechanisms that are responsible for the effects of GH and IGF-I on pre-and postnatal growth and development.

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Appendix A

Application form for genetic analysis of a patient with a growth disorder

LUMC

Patient details

Name

Birth date

Gender

Referring physician

Name

Address

City Zip Code

Country

E-mail

Date

Medical history

Neonatal period

Gestational age weeks days

Birth weight gr = SDS

Birth length cm = SDS

First length after birth: date cm = SDS

Head circumference at birth cm = SDS

First year

Feeding problems No ☐ Yes:

Psychomotor development

Deafness No ☐ Yes: indicate type and severity

Other diseases

Date Diagnosis

Date Diagnosis

Date Diagnosis

Symptoms of immune deficiency No ☐ Yes:

Medication No ☐ Yes:

Family history

Are the parents related ? Yes ☐ No ☐

Father Genetic background

Height cm = SDS

Sitting height cm Head circ. cm

Puberty

Mother Genetic background

Height cm = SDS

Sitting height cm Head circ. cm

Puberty Menarche yr

Siblings

Sib 1, gender Height

Sib 2, gender Height

Sib 3, gender Height

Sib 4, gender Height

Grandparents

Father's father yr of birth Height cm

Father's mother yr of birth Height cm

Mother's father yr of birth Height cm

Mother's mother yr of birth Height cm

Physical examination

Dysmorphic features No ☐ Yes:

Syndrome ? No ☐ Yes:

Date

Height cm = SDS

Weight kg = SDS

BMI kg/m2 = SDS

Sitting height cm

Sitting height/height = SDS

Head circ. cm = SDS

Radiological examination

Date

Bone age yr

Method

Signs of skeletal dysplasia ? No ☐

Yes:

Biochemical results

Date

IGF-I nmol/L = SDS

IGF-II nmol/L = SDS

IGFBP-3 mg/L = SDS

ALS nmol/L = SDS

Date

Free T4 pmol/L

TSH mU/L

Prolactin microgr/L ref range

Celiac disease excluded ? No ☐ Yes ☐

GH stimulation tests

Date Type of the test GH peak mU/L Priming ?

Date Type of the test GH peak mU/L Priming ?

IGF-I generation test

Date GH dose (mg/m2) for days

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

Date GH dose (mg/m2) for days

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

Date GH dose (mg/m2) for days

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

IGF-I at day : nmol/L = SDS IGFBP-3 mg/L = SDS

Genetic analysis

Karyogram

DNA analysis

Which genes have already been analyzed ?

Gene Method Result

Gene Method Result

Gene Method Result

Conclusion and presumptive diagnosis

Additional remarks

Instructions for sending the form and the material

Save the completed form and send as attachment to: m.walenkamp@lumc.nl
or
Print the completed form Print Form and send to:
M.J. Walenkamp
Dept. of Pediatrics J6-S
Leiden University Medical Center
PO Box 9600
2300 RC Leiden
the Netherlands

Include the growth charts and pedigree !

Send 2 tubes EDTA blood (7-10 ml), couriered at room temperature or a DNA sample,
complete the separate requisistion form for molecular diagnostic analysis
and send this to:
Center for human and clinical genetics, dept. of molecular genetics (LDGA)
Leiden University Medical Center, Building 2 zone S-06-P
PO Box 9600
2300 RC Leiden
the Netherlands

Summary

11

Growth is a complex process, regulated by multiple external and internal factors. Deviation from the normal growth pattern can be one of the first manifestations of an underlying disorder, disrupting the normal growth process. The GH-IGF-I axis plays a key role in regulating the growth process. This thesis focuses on growth disorders as a result of genetic defects in the GH-IGF-I axis.
The aim of this thesis is to study the genotype-phenotype relationship in patients with a documented genetic defect in a component of the GH-IGF-I axis and to unravel the role of the GH-IGF-I axis in the complex process of growth and development throughout life.

Chapter 1 describes the milestones in the history of the GH-IGF-I axis that lead the basis for our current knowledge. In addition, the present view on the GH-IGF-I axis is summarized.

Chapter 2 gives an overview of the clinical aspects and biochemical parameters of the various genetic defects in the GH-IGF-I axis. Classical GH deficiency, resulting in decreased or absent pituitary GH secretion, can be the result of a mutation in the GH releasing hormone receptor (GHRH-R) gene, a defect in the ontogenesis of the GH producing cells or a mutation or deletion of the GH1 gene. Since the first paper that showed the etiology of Laron syndrome, many mutations in the GH receptor have been identified. Several patients have been described with a STAT5b mutation, which disrupts the GH signal transduction. More recently, genetic defects in the IGF-I gene or the IGF-I receptor gene have been described. All these defects result in proportionate short stature. Therefore, in the workup of a patient with proportionate short stature genetic analysis is essential. However, careful selection of the patients is a prerequisite for optimal results. Therefore, we developed flowcharts, based on the described patients in combination with theoretical considerations. These flowcharts can be used as guidelines in the diagnostic process of patients with idiopathic short stature.

Chapter 3 describes a brother and sister with a mutation of the GHRH-R gene. They presented with a height of -5.8 SDS and -7.6 SDS at an advanced age of 16 and 14.9 years, respectively, and an advanced pubertal stage. Genetic analysis revealed a homozygous single base pair transition at the splice donor site of intron 7. Both patients were treated with GH. In order to combine the growth promoting effect of

GH with postponing puberty and delaying skeletal maturation, GH treatment was combined with the administration of a gonadotropin-releasing hormone analog (GnRHa). This combination treatment was highly effective in increasing final height. Sitting height/height ratio decreased in both patients during treatment. Although this ratio was within the normal population range, one could conclude that combined treatment with GH and GnRHa may result in relatively longer legs. Bone mineral density was in the lower normal range in both patients at adult age. However, both patients developed severe vitamin D deficiency as adults, which confounds the effects of GnRHa treatment on the acquisition of peak bone mass.

GH insensitivity can be caused by defects in the GH receptor (Laron's syndrome) or a defect in the postreceptor signaling pathway. Recently, reports on two female patients with severe postnatal growth retardation, pulmonary problems and immunodeficiency were published. These patients had a homozygous defect in the STAT5b gene. In **chapter 4 and 5** of this thesis the first male patient is described with a homozygous frameshift mutation in the STAT5b gene, resulting in an inactive truncated protein, lacking most of the DNA binding and SH2-domain. The phenotype consisted of severe short stature (final height -5.9 SDS), delayed puberty and high body fat percentage (40%), but no history or signs of pulmonary problems or immunodeficiency. Biochemically extremely low levels of IGF-I (-6.9 SDS), IGFBP-3 (-12 SDS), and ALS (-7.5 SDS) were present. In addition, prolactin levels were elevated. 24-h GH and prolactin secretion characteristics were assessed. The GH secretory parameters were comparable with healthy male controls. However, one could hypothesize that GH secretion is severely suppressed by his visceral adiposity. Prolactin secretion was increased by six-fold. High doses of GH in the IGF-I generation test showed a response of IGF-I to levels approaching reference range values. Evaluation of the monocyte and T-cell function revealed no abnormalities. The heterozygous family members of the patient showed no signs of GH insensitivity.
In conclusion, STAT5b deficiency causes a disruption of the GH signaling pathway, resulting in severe short stature. Although STAT5b plays a role in signaling processes in immune cells, apparently, immunodeficiency is not an obligatory symptom of STAT5b deficiency, whereas hyperprolactinemia appears to be part of the syndrome.

Chapter 6 reports on the first patient with an inactivating homozygous missense mutation of the IGF-I gene. The phenotype consisted of severe intrauterine growth retardation (birth weight -3.9 SDS and birth length -4.3 SDS), reflecting the GH-independent IGF-I secretion *in utero*. Severe mental retardation, microcephaly (head circumference -8 SDS) and sensorineural deafness were observed and considered as a consequence of IGF-I defciency on intrauterine brain development. The postnatal growth pattern was comparable with untreated GH deficient or GH-insensitive patients (final height -8.5 SDS), which is in line with the hypothesis that IGF-I secretion in childhood is mainly GH-dependent. Apparently IGF-I deficiency is well tolerated after adolescence, considering the relative healthy condition of the 55-yr-old patient. Biochemically, the patient had high IGF-I levels (+7.3 SDS) and a stimulated GH secretion in the upper normal range.
In addition, 24 relatives of the patient were studied: nine heterozygous carriers were identified, which had a significantly lower birth weight, final height and head circumference than the noncarriers.

The structural and functional characterization of the IGF-I mutation is described in detail in **chapter 7**. The mutation lead to the expression of IGF-I with an aminoacid substitution of methionine instead of a valine at residue 44 of the protein(val^{44}met IGF-I). Val^{44}met IGF-I exhibited a 90-fold decrease in IGF1R binding compared with wild type IGF-I. An indirect argument for the important role of this part of the molecule is provided by observations that a point mutation in the insulin gene (val^{A3}leu insulin, also termed insulin Wakayama, corresponding with Val^{44} of IGF-I.) result in hyperinsulinemia due to severely defective insulin receptor binding. Activation of downstream signaling by val^{44}met IGF-I was reduced, corresponding with the reduced affinity for the IGF1R. Also, val^{44}met IGF-I was unable to stimulate DNA synthesis. Binding or activation of both insulin receptor isoforms was not detectable. However, val^{44}met IGF-I bound IGFBP-2, IGFBP-3 and IGFBP-6 with equal affinity to IGF-I, suggesting the maintenance of the overall structure. Nuclear magnetic resonance studies confirmed retention of near-native structure with only local side-chain disruptions.

In **chapter 8** we describe a mother and daughter with a heterozygous missense mutation in the intracellular tyrosine kinase domain of the IGF1R. The phenotype of the mother consisted of mild intrauterine growth retardation (birth weight -

2.1 SDS, birth length -0.3 SDS) and progressive postnatal growth failure (final height -4 SDS), microcephaly (head circumference -3 SDS) and failure to thrive. The daughter suffered from severe intrauterine growth retardation (birth weight -3.3 SDS, birth length -4.2 SDS), microcephaly (head circumference -5.2 SDS) and postnatal growth failure. In both patients IGF-I levels were elevated (+1.6 SDS and +2.9 SDS, respectively). Functional characterization revealed normal binding of IGF-I to the IGF1 receptor, but marked reduction of autophosphorylation and activation of the downstream signaling cascade, suggesting inactivation of one copy of the IGF1R gene. A summary of the clinical features of the other patients described in the literature leads to the hypothesis that the degree of intrauterine growth retardation may be determined by the presence or absence of maternal IGF-I resistance.

In **chapter 9** the phenotype of a heterozygous terminal 15q deletion is described, consisting of intrauterine growth retardation, postnatal growth retardation, microcephaly, and elevated IGF-I levels. These phenotypic features are similar to the features found in patients with an inactivating IGF1R mutation and can therefore be ascribed to the loss of one copy of the IGF1R gene. This was diagnosed with a novel genetic technique: multiplex ligation-dependent probe amplification (MLPA). Subsequently, array comparative genomic hybridization was used to define the deleted area, which was 15q26.2->qter. The patient was effectively treated with GH, reaching a final height of -1.8 SDS, which is within the population range, although not in her genetic target range.

Chapter 10 reviews the reports of patients with a genetic defect in the GH-IGF-I axis and summarizes the data of animal knockout experiments. The role of the GH-IGF-I axis in intrauterine and postnatal growth is described. In addition, the effects of the GH-IGF-I axis on the development and function of different organ systems as brain, eye, skeleton, glucose homeostasis, gonadal function and immune system are discussed. In this chapter a systematic diagnostic approach and selective genetic analysis in a patient with short stature is advocated in order to identify more patients with a genetic defect in the GH-IGF-I axis and thereby increasing the knowledge on genes that play a role in the complex process of growth and development.

Samenvatting

12

Groei is een complex proces, dat wordt gereguleerd door verschillende externe en interne factoren. Een afwijking van het normale groeipatroon kan een aanwijzing zijn voor een onderliggende afwijking, die het normale groeiproces verstoort. De groeihormoon (GH)-Insuline-achtige groeifactor I (IGF-I) as speelt een belangrijke rol in de regulatie van groei. Dit proefschrift is gericht op groeistoornissen als gevolg van een genetisch defect in de GH-IGF-I as.
Het doel van dit proefschrift is genotype-fenotype relaties te beschrijven in patiënten met een genetisch defect in één van de componenten van de GH-IGF-I as en om de rol van de GH-IGF-I as in het complexe proces van groei en ontwikkeling gedurende het leven te bestuderen.

In **Hoofdstuk 1** worden de mijlpalen in de geschiedenis van de GH-IGF-I as beschreven, die hebben geleid tot onze huidige kennis. Tevens wordt de meest recente kijk op de GH-IGF-I as samengevat.

Hoofdstuk 2 geeft een overzicht van de klinische aspecten en de biochemische parameters van de verschillende genetische defecten in de GH-IGF-I as. Klassieke GH deficiëntie, met als gevolg verminderde of afwezige hypofysaire GH secretie, kan het gevolg zijn van een mutatie in het GH releasing hormoon receptor (GHRH-R) gen, een defect in de ontogenese van de GH producerende cellen of een mutatie of deletie van het GH1 gen. Sinds het eerste artikel dat de etiologie van het syndroom van Laron aantoonde zijn er vele mutaties in de GH receptor beschreven. Er zijn meerdere patiënten beschreven met een mutatie in het STAT5b gen, waardoor de GH signaaltransductie wordt verstoord. Meer recent zijn er genetische defecten in het IGF-I gen of het IGF-I receptor gen beschreven. Al deze defecten resulteren in geproportioneerde kleine lengte. In de evaluatie van een patiënt met geproportioneerde kleine lengte is genetische analyse dan ook een essentieel onderdeel. Echter, zorgvuldige selectie van de patiënt is van belang voor een optimaal resultaat. Tevens worden flow-diagrammen gepresenteerd, gebaseerd op de beschreven patiënten in combinatie met theoretische overwegingen. Deze flow-diagrammen kunnen als richtlijn worden gebruikt in het diagnostisch proces van patiënten met idiopathisch kleine gestalte.

Hoofdstuk 3 beschrijft een broer en zus met een mutatie van het GHRH-R gen. Zij presenteerden zich met een lengte van -5,8 SDS en -7,6 SDS op de leeftijd van

respectievelijk 16 en 14,9 jaar in een vergevorderd puberteitstadium. Genetische analyse toonde een homozygote transitie van een enkel basepaar aan, ter hoogte van intron 7. Beide patiënten werden behandeld met groeihormoon. Om het groeibevorderend effect van groeihormoon te combineren met het uitstellen van de puberteit en daarmee het vertragen van de botrijping, werd een gonadotropine releasing hormoon analoog (GnRHa) toegevoegd aan de GH behandeling. Deze combinatie leidde tot een positief effect op de eindlengte.

GH ongevoeligheid kan het gevolg zijn van een defect in de GH receptor, zoals in Laron syndroom of een defect in de postreceptor signaaltransductie. Recent werden twee artikelen gepubliceerd waarin vrouwelijke patiënten werden beschreven met ernstige postnatale groeivertraging, pulmonale problemen en immuundeficiëntie. Deze patiënten hadden een homozygoot defect in het STAT5b gen.
In **hoofdstuk 4 en 5** van dit proefschrift wordt de eerste mannelijke patiënt beschreven met een homozygote frameshift mutatie in het STAT5b gen, leidend tot een inactief, getrunceerd eiwit, waarbij het grootste deel van het DNA bindend domein en het gehele SH2 domein ontbreekt. Het fenotype van de patiënt bestond uit extreem kleine lengte (eindlengte -5.9 SDS), late puberteit en een hoog percentage lichaamsvet (40%), maar er waren geen aanwijzingen voor pulmonale problemen of immuundeficiëntie. Biochemisch had hij extreem lage IGF-I waarden (-6,9 SDS), IGFBP-3 (-12 SDS) en ALS (-7. SDS). Tevens had hij verhoogde prolactine waarden. Bij deze patient werden ook 24-uurs groeihormoon en prolactine profielen verricht. De GH secretie was vergelijkbaar met gezonde mannelijke controles. In het licht van het verhoogde vetpercentage in de patiënt zou deze normale secretie als verhoogd kunnen worden beschouwd, aangezien bekend is dat de GH secretie wordt onderdrukt door visceraal vet. De prolactine secretie was zesvoudig verhoogd. Hoge dosis GH in de IGF-I generatietest liet een respons van IGF-I zien tot bijna normale referentiewaarden. Evaluatie van de monocyt en T-cel functie liet geen afwijkingen zien. De heterozygote familieleden van de patiënt lieten geen aanwijzingen zien voor GH ongevoeligheid. Concluderend veroorzaakt STAT5b deficiëntie een verstoring in de GH signaaltransductie, leidend tot kleine lengte. Hoewel STAT5b een rol speelt in de signaaltransductie van immuuncellen, is immuundeficiëntie geen obligaat symptoom van STAT5b deficiëntie. Hyperprolactinemie, daarentegen, lijkt een essentieel onderdeel van een STAT5b defect.

In **hoofdstuk 6** wordt de eerste patiënt beschreven met een inactiverende homozygote missense mutatie van het IGF-I gen. Het fenotype bestond uit ernstige intrauteriene groeivertraging (geboortegewicht -3,9 SDS en geboortelengte -4,3 SDS). Deze bevinding reflecteert de GH-onafhankelijke IGF-I secretie *in utero*. Verder bestond het fenotype uit ernstige mentale retardatie, microcefalie (hoofdomtrek -8 SDS) en perceptiedoofheid. Deze kenmerken zijn het gevolg van IGF-I deficiëntie op de intrauteriene hersenontwikkeling. De postnatale groei was vergelijkbaar met die van onbehandelde GH-deficiënte of GH-resistente patiënten (eindlengte was -8,5 SDS). Dit is overeenkomstig de hypothese dat IGF-I secretie na de geboorte voornamelijk GH-afhankelijk is. Blijkbaar wordt IGF-I deficiëntie goed verdragen in de rest van het leven, gezien de relatief gezonde conditie van deze 55 jarige patiënt. Biochemisch had de patiënt zeer hoge IGF-I waarden (+7,3 SDS) en een gestimuleerde GH secretie in de hoog normale range.
Tevens werden 24 familieleden van de patiënt onderzocht, waarvan negen familieleden heterozygoot waren voor de mutatie. Zij hadden een significant lager geboortegewicht, eindlengte en hoofdomtrek dan de familieleden die geen drager waren.

De structurele en functionele eigenschappen van de IGF-I mutatie worden gedetailleerd beschreven in **hoofdstuk 7**. De mutatie leidt tot de expressie van IGF-I met een methionine in plaats van een valine op positie 44 (val^{44}met IGF-I). val^{44}met IGF-I heeft een 90-voudig verminderde capaciteit om aan de IGF1R te binden, vergeleken met wild type IGF-I. Een indirect argument voor de belangrijke rol van dit deel van het molecuul is dat een punt mutatie in het insuline gen (val^{A3}leu insulin, ook wel bekend als insuline Wakayama, dat overeenkomt met Val^{44} van IGF-I.) resulteert in hyperinsulinisme als gevolg van een verminderde binding aan de insuline receptor. Activatie van de signaaltransductie-route door val^{44}met IGF-I was verminderd, overeenkomend met de verminderde affiniteit voor de IGF1R. Tevens was val^{44}met IGF-I niet in staat om DNA synthese te stimuleren. Ook was er geen binding en activatie van de insuline receptor detecteerbaar. Echter, val^{44}met IGF-I was wel in staat IGFBP-2, IGFBP-3 en IGFBP-6 te binden, met dezelfde affiniteit als IGF-I. Dit suggereert dat de globale structuur behouden is gebleven. Dit werd bevestigd met nucleaire magnetische resonantie studies, waarbij de oorspronkelijke structuur onveranderd was en alleen locale veranderingen in de zijketen werden gezien.

In **hoofdstuk 8** beschrijven wij een moeder en haar dochter met een heterozygote missense mutatie in het intracellulaire tyrosine kinase domein van de IGF1R. Het fenotype van de moeder bestaat uit milde intrauteriene groeiretardatie (geboortegewicht -2,1 SDS, geboortelengte -0,3 SDS), progressieve postnatale groeivertraging (eindlengte -4 SDS), microcefalie (hoofdomtrek -3 SDS) en slechte gewichtstoename ("failure to thrive"). Haar dochter werd geboren met een geboortegewicht van -3,3 SDS en een geboortelengte van -4,2 SDS, dus een ernstige intrauteriene groeivertraging. In beide patiënten was het IGF-I verhoogd (respectievelijk +1,6 SDS en +2,9 SDS). Functionele karakterisatie liet een normale binding van IGF-I zien aan de IGF-I receptor, maar een duidelijk verminderde autofosforylatie en activatie van de signaaltransductie cascade. Dit suggereert inactivatie van één kopie van het IGF1R gen. De overige patiënten die in de literatuur beschreven zijn, worden samengevat in dit hoofdstuk, waarbij geconcludeerd wordt dat de mate van intrauteriene groeivertraging ten dele afhankelijk zou kunnen zijn van de aan- dan wel afwezigheid van maternale IGF-I resistentie.

In **hoofdstuk 9** wordt het fenotype beschreven van een patiënte met een heterozygote deletie van het terminale deel van chromosoom 15q. Dit fenotype bestaat uit intrauteriene groeiretardatie, postnatale groeivertraging, microcefalie en verhoogde IGF-I waarden. Dit fenotype is gelijk aan het fenotype dat beschreven is bij de inactiverende IGF1R mutatie en kan dus worden toegeschreven aan de afwezigheid van één kopie van het IGF1R gen. Dit werd gediagnosticeerd met een nieuwe genetische techniek: multiplex ligatie-afhankelijke probe amplificatie (MLPA). Vervolgens werd door middel van array comparatieve genomische hybridisatie de grootte van het missende deel vastgesteld: 15q26.2->qter. De patiënt werd behandeld met groeihormoon met goed resultaat. Zij bereikte een eindlengte van -1.8 SDS. Dit is binnen de populatierange, maar niet binnen haar genetisch bepaalde lengte.

In **hoofdstuk 10** worden de publicaties over patiënten met een genetische afwijking in de GH-IGF-I as beschreven en de data van dierexperimenteel onderzoek samengevat. De rol van de GH-IGF-I as in intrauteriene en postnatale groei wordt beschreven. Tevens worden de effecten van de GH-IGF-I as op ontwikkeling en functie van verschillende orgaansystemen als hersenen, ogen, skelet, glucose-metabolisme, gonadale functie en immuunsysteem bediscussieerd. In dit hoofdstuk

wordt een systematische diagnostische benadering en selectieve genetische analyse van patiënten met een groeistoornis voorgesteld om zo meer patiënten met een genetisch defect in de GH-IGF-I as te kunnen identificeren. Hierdoor zal de kennis over de genen die een rol spelen bij het complexe proces van groei en ontwikkeling toenemen.

Curriculum vitae

The author of this thesis was born on august 31st 1966 in Haarlem, the Netherlands. She attended secondary school at "College Blaucapel" in Utrecht and passed her gymnasium β exam in 1984. From 1984 to 1993 she studied Medicine at the State University Utrecht. In 1993 she obtained her medical degree. During her study she spend 6 months at the University of Minnesota, USA, performing a research project on selective deficient immune response in children with recurrent upper airway infections (head: Prof. dr. S. Giebink). After one year as pediatric resident (AGNIO) in the "Antonius Ziekenhuis" in Nieuwegein she started her Pediatric training at the department of Pediatrics in the Leiden University Medical Center (Head: prof.dr. J.M. Wit) and "Juliana Children's Hospital" in the Hague (Head: prof.dr. A.J. van der Heijden). From 2000-2004 she was a fellow pediatric endocrinology at the Leiden University Medical Center (Head: prof. dr. J.M. Wit). During this period she started her research project on genetic disorders in the GH-IGF-I axis which resulted in this thesis and the establishment of the Leiden Growth Genetics Working group. At this moment she is working as a pediatric endocrinologist at the department of pediatrics of the Leiden University Medical Center and she is involved in the implementation of the new training program for pediatric residents.

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