

**Exploring the Spectrum of Pituitary Hormone
Deficiencies: Genotype, molecular mechanisms and
phenotypic variability.**

Verkenning van het spectrum van hypofyse hormoon deficiënties:
genotype, moleculaire mechanismen en fenotypische variabiliteit

Thesis

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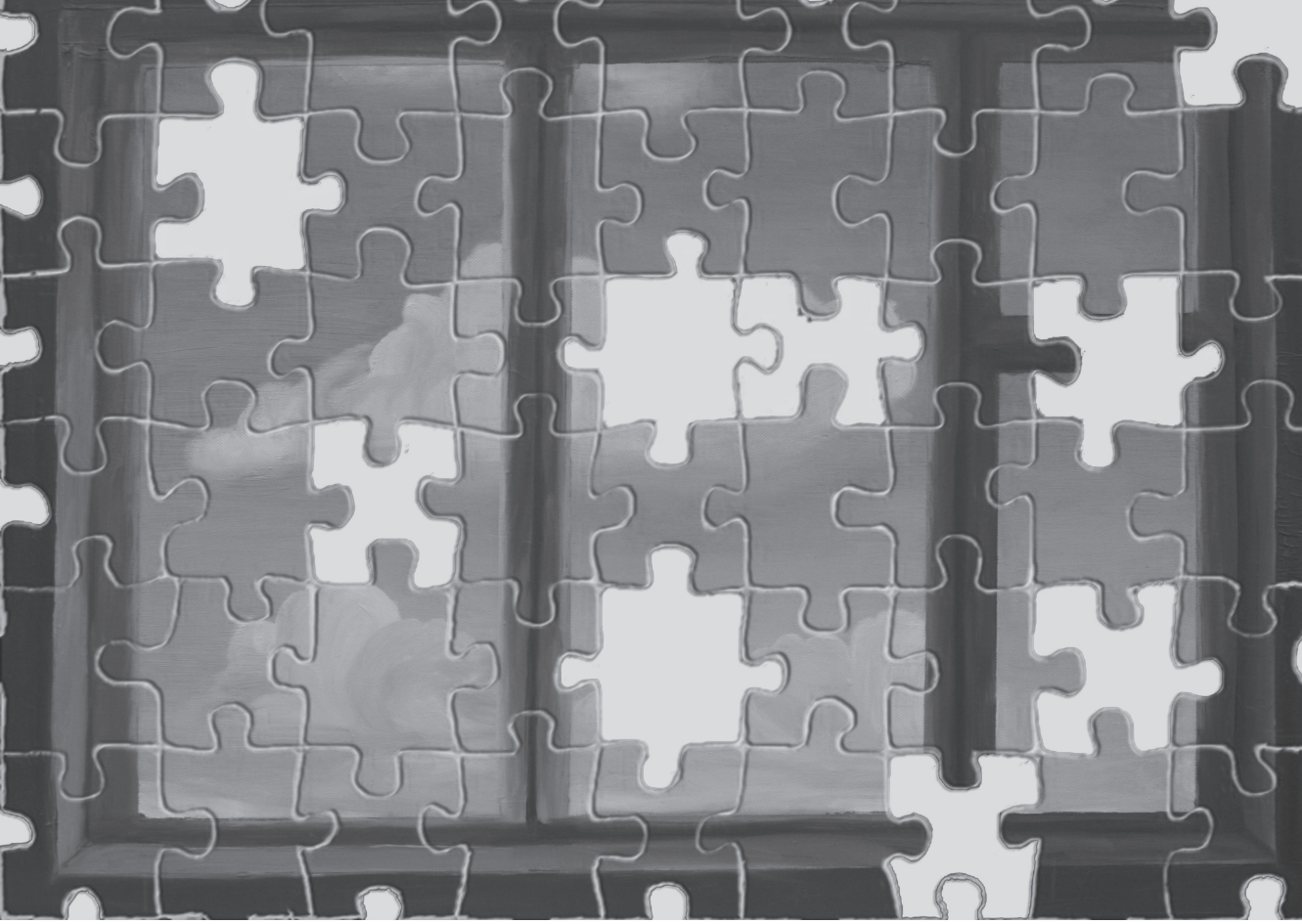
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Chapter 1

General Introduction

Preface: Hypothalamus and Pituitary

Important functions in our body, such as development, growth, reproduction, metabolism, temperature or response to stress are regulated by molecules called hormones. The hypothalamus and the pituitary gland are the main regulators of all hormone signaling pathways and endocrine glands of the body, like the thyroid, adrenals and gonads.

I. Anatomy

The hypothalamus is a neural structure located in the middle of the base of the brain (Fig. 1). Hypothalamic neural cells synthesize specific releasing and inhibiting hormones that control pituitary hormone secretion.

The pituitary gland is a small organ of about 600 mg, which lies within the sella turcica at the base of the brain (Fig. 1). The pituitary consists of two anatomically and functionally different lobes: the posterior and anterior pituitary.

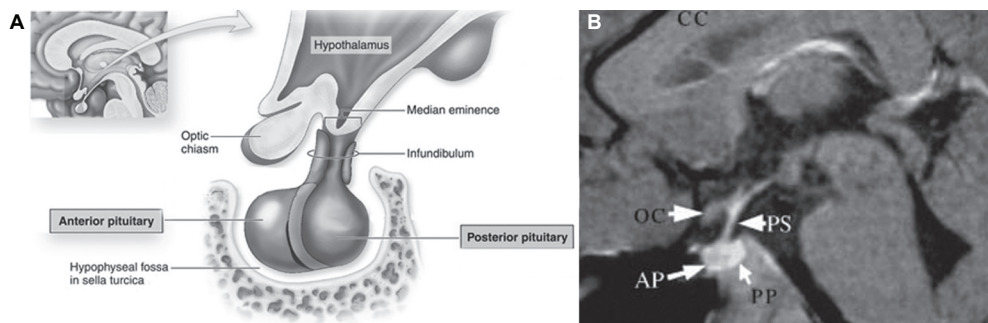


Figure 1. Hypothalamus and pituitary gland. (A) Schematic representation of the hypothalamus and pituitary and their localization in the brain. (B) Sagittal MRI scan showing corpus callosum (CC), optic chiasm (OC), anterior pituitary (AP), posterior pituitary (PP) and infundibulum lor pituitary stalk (PS). [Adapted with permission from Dattani *et al* [1]].

The posterior pituitary contains the terminal axons of neurons from the hypothalamus, and secretes vasopressin (antidiuretic hormone, ADH) and oxytocin. The anterior pituitary comprises five different cell types, each secreting their corresponding hormones: somatotrophs (secreting growth hormone, GH), thyrotrophs (secreting thyroid-stimulating hormone, TSH, also called thyrotropin), corticotrophs (secreting adrenocorticotropin, ACTH), lactotrophs (secreting prolactin, PRL) and gonadotrophs (secreting luteinizing hormone, LH and follicle-stimulating hormone, FSH) [2]. These pituitary hormones act at the corresponding target glands, being the liver, thyroid, adrenal glands and the gonads respectively. These glands, in turn, secrete the peripheral hormones IGF-1, thyroid hormone, cortisol, estradiol and testosterone, which act in peripheral target tissues and produce feedback control at hypothalamus and pituitary level (Fig. 2).

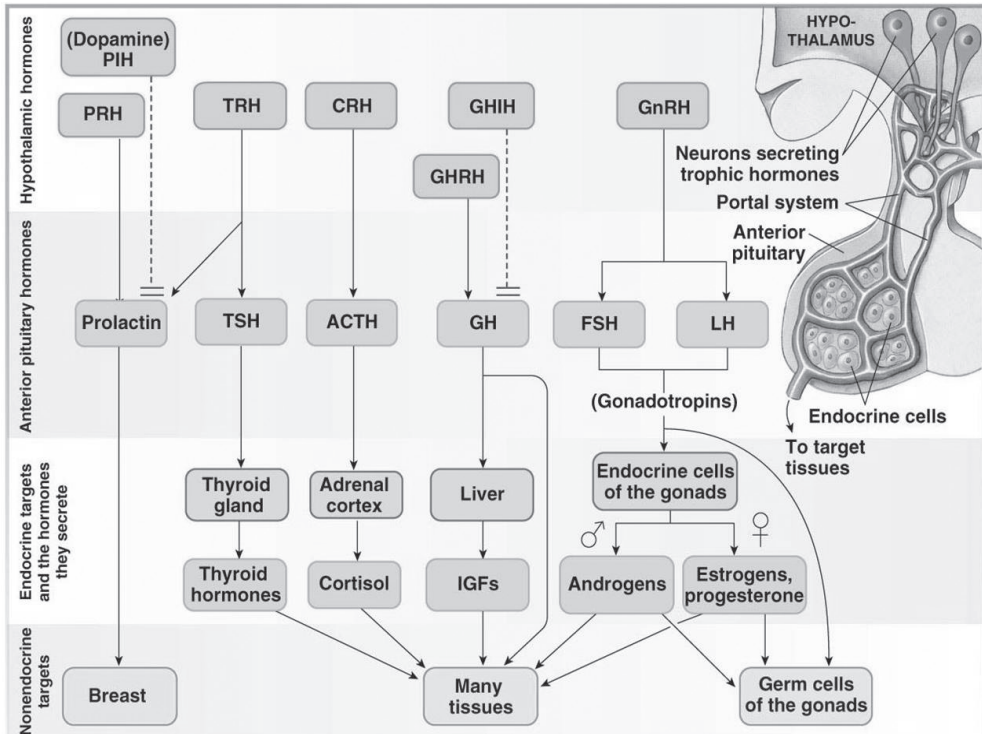


Figure 2. Overview of the endocrine axes studied in this thesis.

II. Pituitary development

The pituitary lobes develop from separate embryonic origins: the anterior pituitary derives from the oral ectoderm, whereas the posterior pituitary derives from the neural ectoderm (Fig. 3a) [3]. Normal pituitary development depends on spatial and sequential expression of multiple transcription factors such as *PROP1*, *HESX1*, *POU1F1*, *LHX3* or *LHX4* (review [3] (Fig. 3b). Defects in these transcription factors have been described in relation to various grades of combined pituitary hormone deficiency (CPHD), as well as macroscopic abnormalities in the development of the pituitary gland, hypothalamus, or surrounding structures [4-7].

Apart from *PROP1*, *HESX1*, *POU1F1*, *LHX3* and *LHX4*, the Hedgehog (Hh) signaling pathway is known to be indispensable for pituitary development. There are three human hedgehog genes: *Sonic Hedgehog* (*SHH*), *Indian Hedgehog* (*IHH*), and *Desert Hedgehog* (*DHH*). A simplified representation of the Hh signaling, including the actions of transmembrane receptors Patched1 (PTCH1), Smoothened (SMO) and transcription factors GLI is shown in Figure 4. In the absence of Hh ligand, PTCH1 represses the activity of SMO and inhibits the effect of GLI, repressing the expression of Hh target genes. The Hh pathway is activated when the ligand binds to PTCH1,

relieving the inhibiting effect of SMO on GLI, thereby permitting the transcription of Hh target genes. When HHIP (Hedgehog interacting protein) binds to SHH, the binding of SHH to PTCH1 is blocked, which leads to inhibition of the Hh pathway.

Shh expression is directly required for the differentiation of the pituitary gland during embryonic development [10]; *Shh* null mice lack a hypothalamus as well as a pituitary [11]. Genetic alterations of several Hedgehog genes have been described in patients with holoprosencephaly (HPE; MIM 236100), a disorder with congenital malformation of the brain and midface, characterized by pituitary agenesis [12-21].

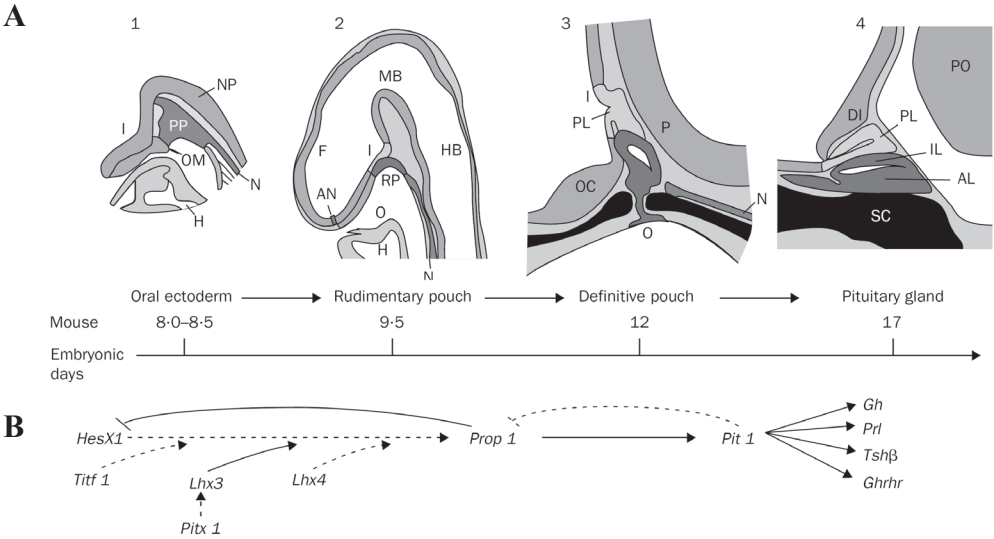


Figure 3. Pituitary development. (A) Drawings of rodent pituitary development: 1, growth of pre-infundibular portion of neural plate and establishment of presumptive Rathke's pouch area; 2, formation of a rudimentary pouch; 3, formation of definitive pouch; 4, adult pituitary gland. Abbreviations: AL, anterior lobe; AN, anterior neural pore; DI, diencephalon; F, forebrain; H, heart; HB, hindbrain; I, infundibulum; IL, intermediate lobe; MB, midbrain; N, notochord; NP, neural plate; O, oral cavity; OC, optic chiasma; OM, oral membrane; P, pontine flexure; PL, posterior lobe; PO, pons; PP, prechordal plate; RP, Rathke's pouch; SC, sphenoid cartilage. [Adapted with permission from Sheng *et al* [8]]. (B) Cascade of transcription factors that regulate anterior pituitary development. Arrows represent activation and truncated lines represent repression; solid lines indicate effects supported by analysis of pituitary mutants; dashed lines represent hypothetical interactions. [Adapted with permission from Dattani *et al* [9]].

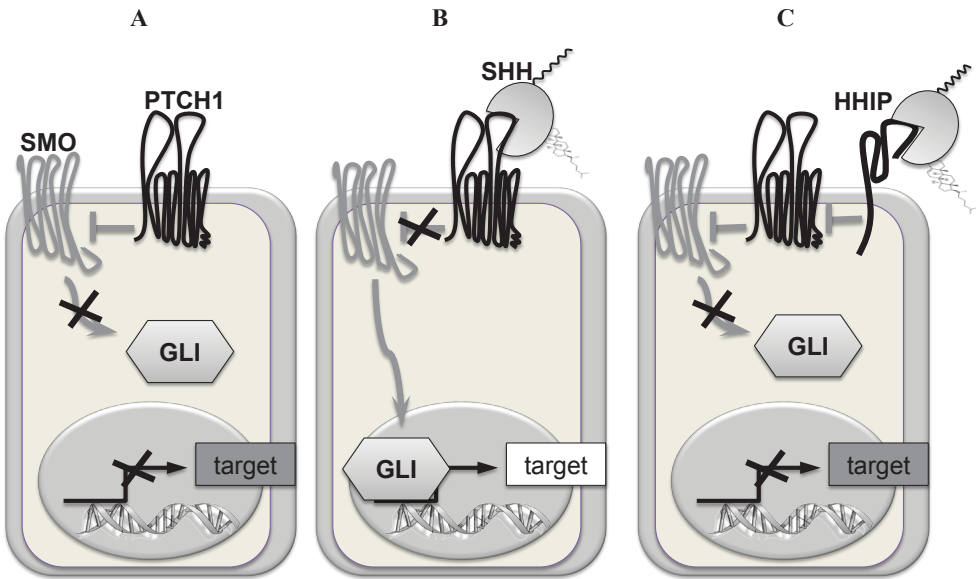


Figure 4. Schematic representation of the Hedgehog signaling pathway. **(A)** in the absence of Hh ligand, transmembrane receptor Patched1 (PTCH1) represses the activity of Smoothened (SMO) and inhibits the effect of transcription factors GLI, repressing the expression of Hh target genes. **(B)** The Hh pathway is activated when SHH binds to PTCH1, relieving SMO's inhibiting effect on GLI and thereby permitting the transcription of Hh target genes. **(C)** HHIP (Hedgehog interacting protein) binds to SHH, blocking its binding to PTCH1, leading to inhibition of the Hh pathway.

1. Disorders of the Hypothalamic – Pituitary (HYPOPIT) axis

Congenital pituitary hormone deficiency shows a wide variation in severity and can have various underlying causes. Deficiencies can be either isolated or combined.

1.1. Isolated Deficiencies

i. **Isolated Growth Hormone Deficiency (IGHD)** is the most common endocrinopathy, the incidence is ranging from 1 in 3500 to 1 in 10000 births [22-25]. IGHD is a congenital disorder characterized by growth failure due to a lack of GH. Its most important clinical characteristics are proportionate short stature accompanied by retarded growth and delayed bone maturation, but mostly normal length and weight at birth. Other frequent findings include truncal obesity, a high-pitched voice and delayed puberty, but normal fertility [26-28]. The patients usually respond well to exogenous GH [29, 30].

ii. **Isolated Central Congenital Hypothyroidism (ICCH)** is a rare disease, with an incidence of 1 per 16,404 neonates [31]. ICCH is due to lack of stimulation of the normal thyroid gland by TSH. The hypothyroidism can be secondary, due to a pituitary defect, or tertiary, due to a hypothalamic defect. The diagnosis is usually suggested by low thyroid hormone (TH) concentrations with normal TSH levels. Although TSH levels are within the normal range, they are abnormally low, considering the low TH levels.

The clinical features of ICCH vary depending on etiology, severity of the thyroid impairment and other associated problems. In general, hypothyroidism is not severe. However, in case of severe diagnostic delay, irreversible neurological damage is caused [32]. Diagnostic delay is more frequent in countries where neonatal screening is based only on TSH levels in blood; the majority of the patients are missed because TSH levels are often normal.

Patients with ICCH are treated with levo-thyroxine (L-T4) therapy, with the goal to maintain serum TH levels within the normal range.

iii. **Isolated Hypogonadotropic Hypogonadism** is defined as a deficiency of the pituitary secretion of FSH and LH, and a defect of gonadal functions manifested by lack of puberty, partial pubertal development or infertility. The prevalence is between 1 in 10,000 [33] and 1 in 86,000 men [34]. The male-to-female ratio ranges from 4:1 to 5:1.

iv. **Isolated Adrenocorticotrophic Hormone Deficiency (IAHD)** is a rare cause of secondary adrenocortical insufficiency. IAHD is diagnosed by the demonstration of low cortisol production with low plasma ACTH, absent adrenal responses to stimulation of pituitary or hypothalamus with intact or reduced adrenal response to exogenous ACTH [35]. Patients with adrenocorticotropin deficiency are usually relatively well during unstressed periods, until stressing events cause an acute adrenal crisis. The symptoms of an adrenal crisis range from weakness and electrolyte disturbances to life-threatening shock.

v. **Central Diabetes Insipidus** is a heterogeneous condition characterized by the excretion of abnormally large volumes of dilute urine, due to a deficiency of vasopressin. In many patients, especially children and young adults, it is caused by the destruction or degeneration of neurons that originate in the supra-optic and paraventricular nuclei of the hypothalamus [36]. Symptoms include excessive thirst and excretion of large amounts of diluted urine which is not influenced by reduced fluid intake. Although central diabetes insipidus is rare, its prevalence is higher among patients with midline defects like holoprosencephaly and septo-optic-dysplasia.

vi. **Isolated Prolactin Deficiency** is very rare, only a few cases have been reported [37-40]. Prolactin is essential for lactogenesis, and its deficiency results in puerperal alactogenesis, that is absolute lactation failure.

vii. **Oxytocin deficiency** generally causes few symptoms, since oxytocin is only necessary for reproduction, uterine contractions during labor and milk ejection during nursing.

- 1.1. **Combined Pituitary Hormone Deficiency (CPHD)** is any combination of two or more pituitary hormone deficiencies mentioned above. In infancy, severe hypoglycemia can be seen from birth, micropenis in boys as well as distinctive facial features like a prominent forehead and midfacial hypoplasia, resulting in a depressed nasal bridge, deep-set eyes, and a short nose with anteverted nostrils. Birth weight and birth length are in most cases only mildly reduced. Patients with CPHD rarely have a normal MRI scan result (0 – 15%) [41-45].
- 1.2. **Growth hormone insensitivity (GHI)** is defined as a highly heterogeneous spectrum of disorders with short stature and resistance to the actions of GH. The phenotype resembles that of IGHD, but with normal / elevated levels of circulating GH. The clinical characteristics range from the most severe form, known as Laron syndrome [46], to a less severe form in which the partial growth hormone sensitivity presents as ‘idiopathic short stature’ (ISS).

2. Dutch Growth Foundation

The research presented in this thesis was supported by the Dutch Growth Research Foundation (DGRF, “Stichting Kind en Groei”). The Dutch Growth Research Foundation was founded in 1969 to initiate and stimulate research into the causes of disorders of growth and development of children, to evaluate existing and new methods of treatment and to advise doctors and patients. In the recent years, the DGRF has been conducting an increasing number of large national studies in collaboration with pediatric endocrinologists.

The clinical data of the growth hormone deficient patients presented in this thesis were available thanks to the existence of the National Registry of Growth Hormone Treatment (Landelijke Registratie Groeihormoonbehandeling or LRG) in children, coordinated and maintained by the DGRF, in collaboration with pediatric endocrinologists.

2.1. Studied patients

For the research described in this thesis, DNA and clinical data of 105 patients with IGHD and 93 with CPHD were available. All patients with childhood onset idiopathic IGHD and CPHD from the Endocrinology Departments of six university- and two non-university hospitals, who were registered in the LRG between 1992 and 2003, were included in the studies.

IGHD was defined as a peak GH response < 20 mU/l to arginine or clonidine test, or < 30 mU/l combined with serum IGF-I < -2 SDS and normal serum levels of other

pituitary hormones.

All CPHD patients had deficiencies of GH and one or more additional hormonal axes. Deficiencies of hypothalamic-pituitary-thyroidal, -adrenal, and -gonadal axes were defined as abnormal TRH test or TSH levels that were low or inadequate for low FT4; abnormal CRF / ACTH / glucagon test or ACTH levels which were low or inadequate for low cortisol and LH, FSH, estrogen / testosterone or LHRH test low for age or lack of spontaneous puberty after age 14 y. Prolactin deficiency was defined as abnormal prolactin during random or TRH testing. Exclusion criteria were: patients with known cause of CPHD, such as a brain tumor, brain surgery, brain radiation or syndromes.

2.2. HYPOPIT

The research described in this thesis was the continuation of the HYPOthalamic and PITuitary gene (HYPOPIT) study. The HYPOPIT study was designed by the DGRF in order to fulfill a growing need among the Dutch pediatric endocrinologists to explain the phenotype of their patients with IGHD and CPHD. The HYPOPIT study showed that only a small minority of the Dutch IGHD and CPHD cases could be explained by mutations in *GH1* and *GHRHR* in IGHD patients [47] and *PROPL*, *HESX1*, *POU1F1*, *LHX3* and *LHX4* in CPHD patients [48]. Since the majority of the Dutch IGHD and CPHD patients still had the unsatisfying diagnosis of idiopathic IGHD or CPHD, further investigations in new candidate genes were considered necessary to explain their condition.

3. Genetic analysis

The candidate genes we investigated in this thesis have been selected on the basis of several Genome Wide Associations studies (GWAS) and recent literature. Four genes were reported by three major GWAS, published up to the start of the study in October 2008, in relation with human height: *HMGA2*, *CDK6*, *HHIP* and *ZBTB38* [49-51] (Fig. 5). Based on other literature data about their role in important cellular processes, mice models and human disorders, we considered *HMGA2*, *CDK6* and *HHIP* promising candidate genes. However, we could not find any indication for a possible relationship between *ZBTB38* and pituitary dysfunction.

I. *HMGA2* and *CDK6*

Two genes, *HMGA2* and *CDK6*, from GWAS on height variability in the normal population, provided new candidate genes for Isolated Growth Hormone Deficiency [49-53].

The High Mobility Group A2 protein (HMGA2, MIM 600698) is an architectural transcription factor involved in several biological processes, including regulation of

gene expression and embryogenesis. The gene encoding this transcription factor is localized on the chromosome 12. Two animal models show the role of HMGA2 in growth: homozygous knockout mice have a classical pygmy phenotype [54], and transgenic mice overexpressing a truncated HMGA2 protein have a giant and obese phenotype [55]. In patients with the 12q14 microdeletion syndrome in whom the deletion affects *HMGA2*, one of the common characteristics is proportionate short stature [56-60]. In 2005, Ligon *et al* described a case of pericentric inversion of chromosome 12 with intragenic rearrangement of *HMGA2* in a boy with extreme somatic overgrowth [61]. Based on these published data, we selected *HMGA2* as a candidate gene for IGHD.

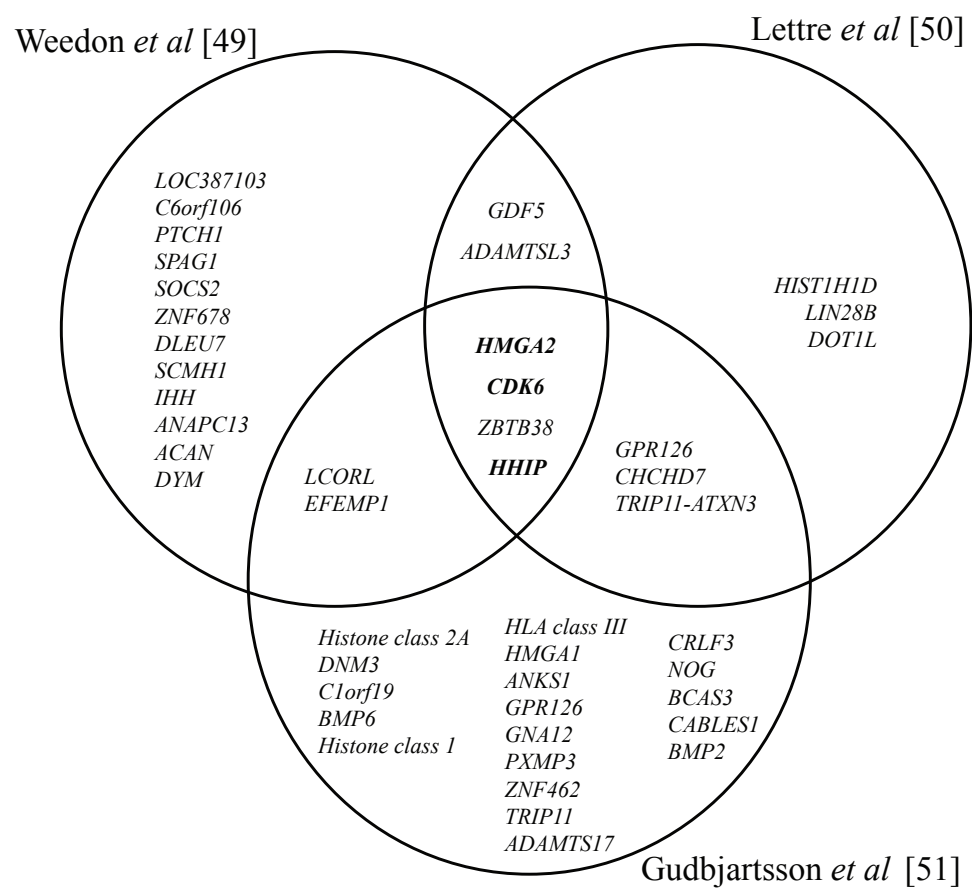


Figure 5. Overview of the loci associated with human height as revealed by GWAS published in 2008.

CDK6 (MIM 603368; localized on Chr. 7) is a member of the cyclin-dependent protein kinase (CDK) family. CDKs form complexes with cyclins in order to regulate cell-cycle progression. CDK6 and its counterpart CDK4 bind to their corresponding (D-type) cyclins and regulate the progression through the G1 phase in cells that con-

tinue proliferating [62]. *Cdk4*-null mice have phenotypes of dwarfism, infertility and resistance to human growth-hormone-releasing hormone (GHRH) [63]. Since it has been suggested that *CDK6* compensates for loss of *CDK4* and vice versa [64], mutations in *CDK6* might also affect growth.

II. *SHH* and *HHIP*

Based on previous studies showing involvement of the Hedgehog pathway in disorders associated with pituitary dysfunction (such as holoprosencephaly) we investigated two candidate genes from the Hedgehog pathway, *SHH* and *HHIP*.

The *SHH* gene (MIM 600725; localized on Chr. 7) encodes sonic hedgehog, a protein that is involved in the organization and morphology of the developing embryo. Shh is expressed in the Hensen node, the floor plate of the neural tube, the early gut endoderm, the posterior of the limb buds, and throughout the notochord [65, 66]. *Shh*-null mice show defects in the embryo axis patterning and dramatic distortion of head morphogenesis with malformation of midline structures, absence of Rathke's pouch and cyclopia [11]. Also in humans, cyclopia can be present as the most severe form of holoprosencephaly, a developmental disorder caused in some cases by mutations in *SHH* [16, 18-21]. The phenotypic heterogeneity of the *SHH* mutations is enormous; clinical variability has been described even between family members with the same *SHH* mutation [21, 67, 68].

Hedgehog Interacting Protein (HHIP; MIM 606178; localized on Chr. 4), is an inhibitor of Hh signaling pathway (Fig. 4C), and can bind all three Hedgehog ligands, namely *Sonic Hedgehog* (*SHH*), *Indian Hedgehog* (*IHH*), and *Desert Hedgehog* (*DHH*). Overexpression of Hhip in chondrocytes results in severe skeletal defects and short stature similar to those observed in *IHH* mutants [69]. In several GWAS, *HHIP* was related with adult height in the normal population [49-51,53] (Fig. 5).

III. *OTX2*

Recently, mutations and deletions in the *OTX2* gene have been detected in some patients with IGHD or CPHD, either with or without eye problems [70-75]. The orthodenticle *Drosophila* homolog 2 (*OTX2*; MIM 600037; localized on Chr. 14) is a homeobox family transcription factor, which is required for brain and eye formation. In the mouse, during early development, *Otx2* is expressed in the forebrain and mid-brain, and has a role in rostral brain and cranio-facial development [76-78]. Various genetic alterations in *OTX2* have been described, including interstitial deletions [79, 80], microdeletions [71, 81], frameshift and point mutations [82]. The majority of the alterations are found in patients with severe ocular malformations such as anophthalmia, microphthalmia, Leber congenital amaurosis or coloboma [81-84]. *OTX2* has also been implicated in the regulation of *HESX1* [85, 86], one of the transcription factors important for pituitary development (Fig. 3) which has become one of the 'classical CPHD genes'. Therefore we selected *OTX2* as a candidate gene for mutational

screening of our CPHD patients.

IV. *GHR*

Mutations in the growth hormone receptor (*GHR*) have been described as a genetic cause of growth hormone insensitivity syndrome (GHIS). The *GHR* gene (MIM 600946) is localized on chromosome 5 and has ten exons, of which nine encode the mature GHR protein. This mature GHR contains an extracellular domain, a single transmembrane domain and a cytoplasmic domain. The first *GHR* mutation, identified in 1989 [87], was a complex gene deletion and to date more than 70 mutations have been identified in more than 250 GHIS patients (reviewed in [46, 88, 89]). The most commonly described are nonsense, missense and splice site mutations. The majority of the identified genetic aberrations in *GHR* occur in the region encoding the extracellular domain of the receptor. The phenotype-genotype relationship of GHIS is widely variable. Patients with the same phenotype may show genetic heterogeneity, and the same mutation can be associated with wide variations in biological severity [90].

4. Aims of studies

The general aim of the research presented in this thesis was to identify genetic and/or molecular defect in several cohorts of patients with various growth disorders (CPHD, IGHD and GHIS).

Before the start of this study, in the majority of the patients with pituitary hormone deficiencies, the genetic cause was unknown. After ruling out mutations in the classical candidate genes for IGHD and CPHD, we suspected other genes might be involved in the pathogenesis of these disorders. Before the start of the genetic analysis, we performed an extensive literature investigation in order to select candidate genes. The first criterion for the selection of our candidate genes was that they should appear in several independent GWA studies associated with human height (*HMGA2*, *CDK6*, *HHIP* and *ZBTB38*, Fig. 5). The second criterion was that they should have possible clinical significance, based on other literature data including mouse models, human disorders and known importance in cellular processes. Therefore, in **Chapter 2** we investigated *HMGA2* and *CDK6* as candidates for IGHD. In **Chapter 3** we investigated the other genome-wide significant gene: *HHIP*, a gene that belongs to the Hedgehog family. Defects in genes from the Hedgehog pathway were previously described in disorders associated with pituitary dysfunction and/or CPHD. Therefore, we decided to investigate this gene in our cohort of Dutch HYPOPIT patients with CPHD. To complete this study we also investigated *SHH*, a gene that is reported in the literature as responsible for the majority of genetic cases of holoprosencephaly.

In **Chapter 4**, we investigated *OTX2*, another gene highlighted during our study of the literature as a new candidate for (some cases) of CPHD and IGHD.

In **Chapter 5**, following the pathways of clinical reasoning ('if the patients is not hormone deficient, he might be hormone resistant') and driven by the search for a satisfying diagnose in one of our patients, we describe our genetic and molecular studies in a patient with growth retardation due to Growth Hormone insensitivity.

5. Outline of the thesis.

Chapter 1 gives an introduction in the topics described in this thesis.

Chapter 2 describes the mutation screening and copy number analysis of *HMGA2* and *CDK6* in Dutch IGHD patients.

Chapter 3 presents the results of mutations screening of the Hedgehog genes *SHH* and *HHIP* in patients with CPHD.

Chapter 4 describes a new *OTX2* mutation in a patient with the combination of CPHD, pituitary malformation and optic nerve hypoplasia.

Chapter 5 presents a heterozygous *GHR* mutation, subject to nonsense mediated decay in a patient with Growth Hormone Insensitivity Syndrome.

Chapter 6 discusses our findings and presents the final conclusions of this thesis.

Chapter 7 summarizes our findings.

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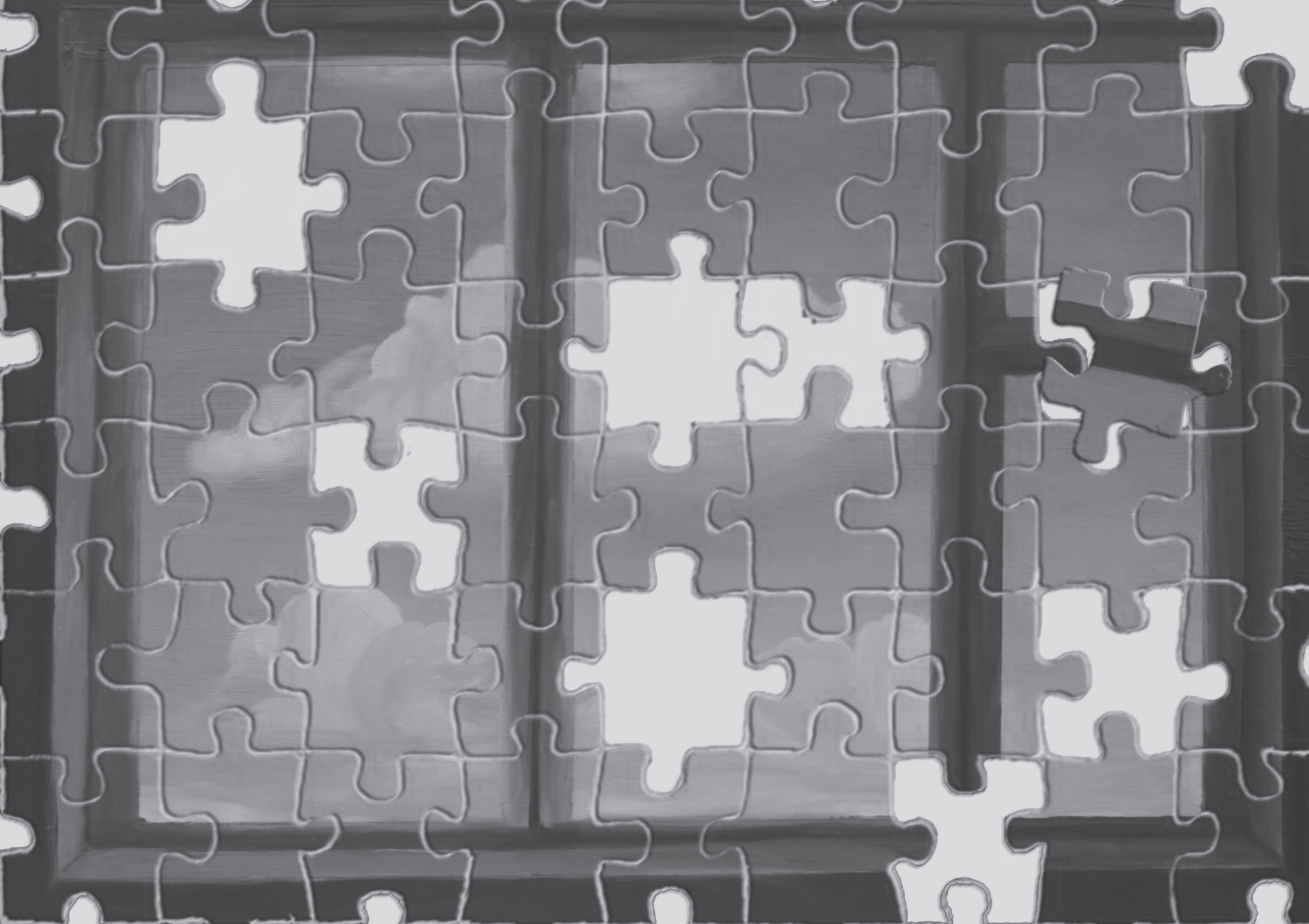
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Chapter 2

Isolated GH deficiency: mutation screening and copy number analysis of *HMG2* and *CDK6* genes.

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Abstract

In most patients, the genetic cause of isolated growth hormone deficiency (IGHD) is unknown. By identifying several genes associated with height variability within the normal population, three separate genome-wide association studies provided new candidate genes for human growth disorders. We selected two of them for genetic screening of our IGHD population.

We aimed to determine whether *HMGA2* and *CDK6* are involved in the pathogenicity of IGHD.

Methods: We directly sequenced coding regions and exon – intron boundaries of the genes *HMGA2* and *CDK6* in 105 Caucasian IGHD patients from the Dutch HY-POPIT study. In addition, we developed a new probe set of Multiplex Ligation-dependent Probe Amplification (MLPA) for both genes in order to detect copy number variations.

Results: In one patient, with the classical IGHD phenotype, we identified a new heterozygous 20 bp deletion in the intronic region of *HMGA2* (c.250-29_-9del), which was absent in databases and healthy controls. Together with recently published data concerning the 12q14 microdeletion syndrome, where patients with an *HMGA2* haploinsufficiency had proportionate short stature, this study provides further support of the important role of *HMGA2* in growth. In *CDK6*, we found only known polymorphisms.

Conclusions: This study provides the first report of a deletion in the *HMGA2* gene that might be related to IGHD. We suggest that this gene is investigated as a second screening in patients with a classical IGHD phenotype in whom mutations in classical candidate genes have been excluded.

Introduction

Isolated Growth Hormone Deficiency (IGHD) is a congenital disorder characterized by growth failure due to low levels of growth hormone (GH), not associated with other pituitary hormone deficiencies. Its most important clinical characteristics are proportionate short stature accompanied by retarded growth and delayed bone maturation, but mostly normal length and weight at birth. Other frequent findings include truncal obesity, a high-pitched voice and delayed puberty, but normal fertility (reviewed [1]). The patients usually respond well to exogenous GH [2].

The reported prevalence of IGHD ranges from 1 in 3480 to 1 in 10 000 live births [3]. Five to 30 % of cases have first-degree relatives with short stature [4], suggesting a genetic etiology. Even though mutations in *GHI* and *GHRHR* have been found as genetic cause of IGHD (reviewed [1]), the vast majority of patients do not carry mutations in these two genes. This suggests that other genes are involved and the identification of these genes is important to elucidate the pathogenesis of this complex condition.

In recent years, genome-wide associations (GWA) studies have revealed genes and biologic pathways that were not previously known to be involved in human growth. In 2007, the first GWA study by Weedon et al [5] identified *HMGA2* as a new gene associated with childhood and adult height in the general population. Then, in 2008, three separate studies together reported 44 SNPs that were associated with height variability within the normal population [6-8]. Only four of these loci were identified in all three studies, namely *HMGA2*, *CDK6*, *HHIP* and *ZBTB38* (Fig. 1) and they have been confirmed as truly associated in meta-analyses [9, 10]. Based on published data, we focused our research on the first two of these genes.

The High Mobility Group A2 protein HMGA2 (MIM 600698) is an architectural transcription factor which is involved in several biological processes, including regulation of gene expression and embryogenesis. HMGA2 has several isoforms; HMGA2a is the major isoform, with proven transcription factor function. The other five splice-variants (b-f) have been identified by Hauke *et al* and they lack exons 4 and 5. The expression of these isoforms has been detected in several normal and tumor tissues, and also in cultured cells [11, 12], however the function of these isoforms is currently unknown. HMGA2 is a transcription factor and one target gene has been described and characterized: insulin-like growth factor II mRNA-binding protein (*IGF2BP2*, MIM ID 608289; previously known as *IMP2*) [13, 14]. *IGF2BP2* is an mRNA-binding protein that is involved in the post-transcriptional regulation of IGF-II [15], a major fetal growth factor. Two animal models show the role of HMGA2 in growth: homozygous knockout mice have a classical pygmy phenotype [16], and transgenic mice overexpressing a truncated HMGA2 protein have a giant and obese phenotype [17]. In patients with the 12q14 microdeletion syndrome in whom the deletion affects *HMGA2*, one of the common characteristics is proportionate short stature [18-22]. In 2005, Ligon *et al* described a case of pericentric inversion

of chromosome 12 with intragenic rearrangement of *HMG42* in a boy with extreme somatic overgrowth [23].

The other gene we investigated, *CDK6* (MIM 603368), is a member of the cyclin-dependent protein kinase (CDK) family; CDKs form complexes with cyclins and regulate cell-cycle progression. CDK6 and its counterpart CDK4 bind to their corresponding (D-type) cyclins and regulate the progression through the G1 phase in cells that continue proliferating [24]. *Cdk4*-null mice combine dwarfism phenotypes with infertility and resistance to human growth-hormone-releasing hormone (GHRH) [25]. Since it has been suggested that *CDK6* compensates for loss of *CDK4* and vice versa [26], mutations in *CDK6* might also affect growth.

Based on the results of GWA studies and these other published data, we selected the candidate genes *HMG42* and *CDK6* for mutation screening and copy number variation analysis in 105 Dutch patients with IGHD.

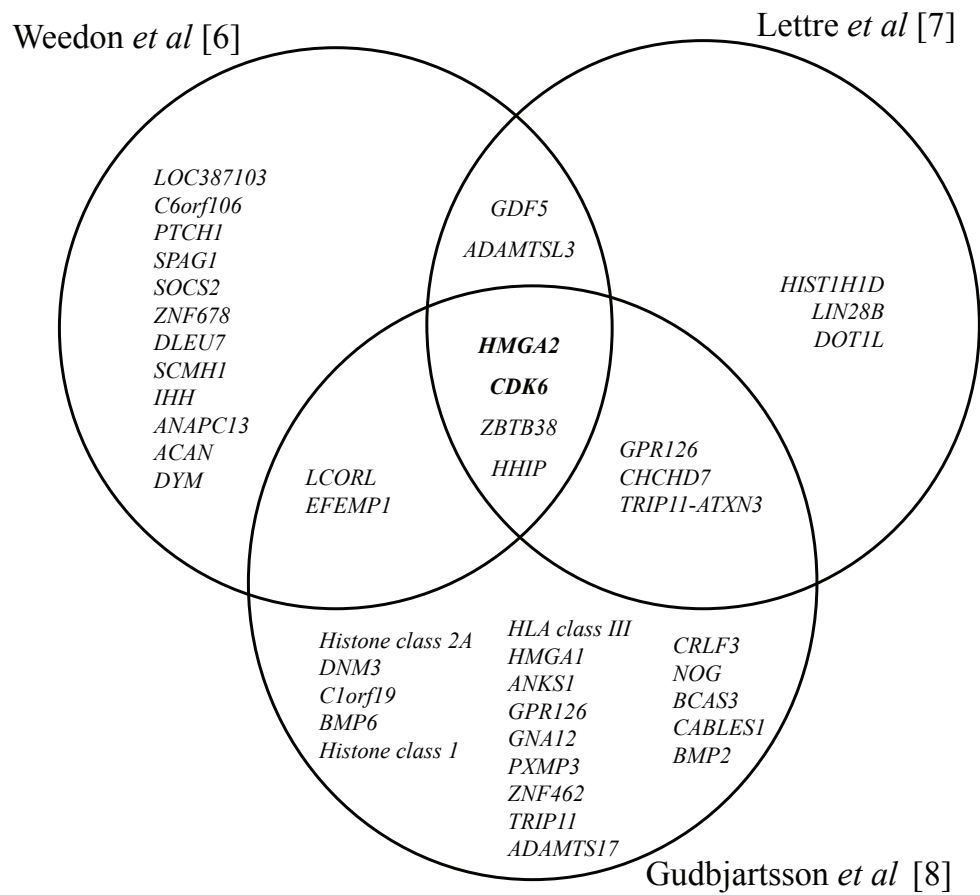


Figure 1. Overview of the loci associated with human height as revealed by GWA studies.

Subjects and Methods

Study subjects

DNA samples were collected from 105 patients with IGHD who had participated in the Dutch HYPOPIT study, which investigates the genetic causes of GH deficiency [27]. These patients had been recruited from the Endocrinology Departments at six university and two non-university hospitals, and had been registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003. IGHD was defined as a peak GH response < 20 mU/l to arginine or clonidine test, or < 30 mU/l combined with serum IGF-I < -2 SDS and normal serum levels of other pituitary hormones. Exclusion criteria were: GH deficiency of known cause, such as a brain tumor, brain surgery, brain radiation and known syndromes. Written informed consent was obtained from all participating patients and their parents or legal guardian(s).

Sequencing

DNA was isolated from whole blood collected in EDTA tubes using standard procedures.

In all patients, *HMGA2* (NM_003483.4) and *CDK6* (NM_001259.6) coding exons and exon–intron boundaries were PCR amplified using Qiagen reagents of 5 units/ μ l Taq DNA Polymerase, 10x PCR Buffer, 5x Q-Solution, 10 mM dNTPs, 25 mM $MgCl_2$ and primers (sequences available on request). Mixtures were incubated at 94 C for 3 min, followed by 35 cycles of 94 C for 30 sec and 60 C for 1 min and 72 C for 1 min, followed by a final incubation at 72 C for 10 min. The electrophoretic separation, sizing and quantification of PCR products were performed using LabChip GX (Caliper) microfluidics technology. The amplified products were purified using illustra GFX™ 96 PCR Purification Kit (GE Healthcare). Sequencing was performed using Big Dye Terminator reaction kit (Applied Biosystems). After purification with Dyex 96 kit (Qiagen) the products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems).

For each new variant, the degree of conservation of the affected residue was examined (Vertebral Multiz Alignment & Conservation from UCSC database) as an estimate of its potential pathogenicity.

TaqMan SNP Genotyping

The frequency of any newly identified variant was estimated in the normal population by screening 188 chromosomes of healthy Caucasian volunteers (with heights between -2 and +2 SDS) using Taqman® genotyping assay.

TaqMan® SNP Genotyping Assay was performed using an ABI PRISM 7900HT sequence-detection system following the manufacturer's instructions (Applied Biosystems). Two assays were used: C_58995818_10 targeting the SNP rs35654944, and Custom TaqMan SNP Genotyping Assay targeting the SNP rs73115423.

Multiplex ligation-dependent probe amplification (MLPA)

Copy number analysis was carried out using MLPA combined synthetic probes and the P200 kit with control sequences (MRC Holland, Amsterdam, Netherlands). We designed a home-made probe set in order to detect deletions or duplication in all coding exons in *HMGA2* (NM_003483.4) and *CDK6* (NM_001259.6), plus the 3'UTR region of *HMGA2* known to be regulated by miRNAs. The home-made probe set contained products ranging from 98 to 166 bp in size with a minimum product size difference of 4 bp, sufficient to be resolved by capillary electrophoresis (sequences available on request). The assay was performed according to the manufacturer's instructions and analyzed using Gene Marker software (SoftGenetics LLC).

Functional analysis of the HMGA2 mutant

After informed consent, a skin biopsy was taken from the patient with the *HMGA2* mutation and primary fibroblast cultures were established as previously described [28]. The fibroblasts were maintained in DMEM/F12 medium (Invitrogen) supplemented with 9% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin / streptomycin (Invitrogen).

Total RNA from primary fibroblast cells grown in a 75 cm² flask was extracted with High Pure RNA Isolation kit (Roche). cDNA from 1 µg of total RNA was synthesized using TaqMan RTreagent (Roche).

SYBR Green I (Eurogentec, Liège, Belgium) was used as the detector dye for quantitative PCR. IGF2BP2 expression analysis was performed as previously described [14]. Sense primers used for different *HMGA2* splice variants expression described by Cleynen *et al* [14] were combined with different antisense primers as described by Hauke *et al* [11, 12]. Melting curves of the PCR products were performed for quality control. Relative expression was calculated using the cyclophilin A housekeeping gene (Applied Biosystem). Fold changes was calculated using this comparative C_T method.

Statistical Analysis

All results are the mean of at least triplicate determinations from representative experiments. Values are expressed as mean ±S.E.M.

Results

We screened DNA of 69 patients with classical IGHD and 36 patients with partial IGHD, for mutations, deletions and duplications. The clinical characteristics of the patients are shown in Table 1.

Mutation Screening

We directly sequenced the complete coding region and intron-exon boundaries of

HMGA2 and *CDK6* in all 105 patients. In one patient, we identified a yet unknown heterozygous deletion in *HMGA2*. This was confirmed by the subcloning of the PCR product from the affected patient and the detection of two different fragments, the smaller fragment containing a 20 base pair deletion 9 bp before the start of exon 4 (c.250-29_-9del) (Fig. 2A). The deletion was detectable by electrophoresis of the PCR product from the patient's genomic DNA, showing a characteristic pattern of four bands (Fig. 2B). Taking advantage of this fact, we analyzed the PCR product of exon 4 in 94 healthy Dutch controls. The presence of only one wild-type band indicated that this deletion was absent in normal controls. The same analysis of genomic DNA from the phenotypically normal mother and two siblings of the patient also showed only the single wild-type band (Fig. 2B). Genetic material from the father with normal height of 185 cm was not available.

Table 1. Clinical data of 105 patients with IGHD. Data are expressed as median and interquartile range (IQR) unless indicated otherwise.

	IGHD (n=69)	pIGHD (n=36)
Gender (M/F)	47/22	25/11
Age (year)	20 (16 to 24)	21 (16 to 24)
BW SDS	-0.3 (-0.9 to 0.3)	-0.4 (-0.9 to 0.4)
BL SDS	-0.6 (-1.7 to 0.2)	-1.4 (-2.2 to -0.4)
Gestational age (w)	40 (38 to 40)	40 (38 to 40)
HSDS at start of GH treatment	-3.4 (-3.9 to -2.6)	-3.0 (-3.5 to -2.5)
GH peak during arginine test (mU/L)	6 (4 to 10) [normal > 20]	16 (9 to 19) [normal > 20]
GH peak during clonidine test (mU/L)	8 (4 to 12) [normal > 20]	16 (11 to 18) [normal > 20]
IGF-I SDS	-3.4 (-4.8 to -1.8)	-2.5 (-3.7 to -1.5)
IGFBP-3 SDS	-5.0 (-8.1 to -3.6)	-5.9 (-9.5 to -1.0)
MRI abnormalities	22 pts: normal 35 pts: abnormal (4 triad) ^a	13 pts: normal 15 pts: abnormal (1 triad) ^a
FDR with GHD	15%	3%
Micropenis	13%	6%
Neonatal jaundice ^b	20%	22%
Hypoglycaemia	12%	6%

pIGHD = partial IGHD; BW = birth weight; BL = birth length; SDS = standard deviation score; GH = Growth Hormone; FDR = first-degree relatives; HSDS, height SDS.

^aTriad = hypoplastic anterior pituitary, ectopic posterior pituitary and interrupted/invisible stalk.

^bProlonged neonatal jaundice: more than three weeks.

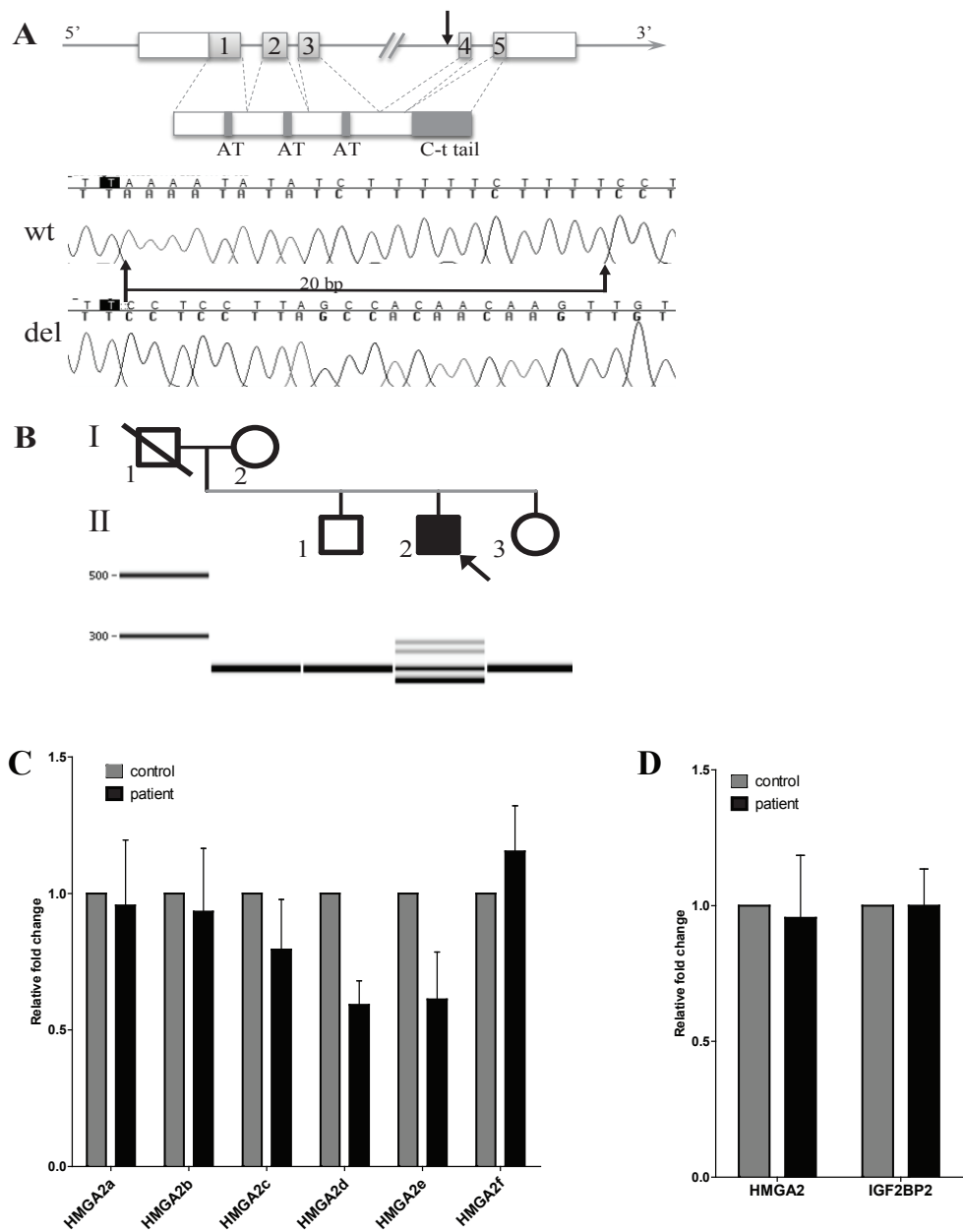


Figure 2. Deletion c.250-29_9del in *HMGA2*. (A) Human *HMGA2* gene and protein structure (adapted from Fusco and Fedele [36]; the vertical black arrow indicates the location of the deletion). Representative chromatograms of the wild-type sequence and the one carrying the deletion (arrows indicate the breakpoints). (B) Pedigree of the patient with the results of the PCR analysis of *HMGA2* exon 4 (the black arrow indicates index case). (C) Quantitative gene expression analysis of *HMGA2* splice-variants in RNA from patient and control fibroblasts (means \pm S.E.M.). (D) Quantitative gene expression analysis of *HMGA2* and *IGF2BP2* in RNA from patient and control fibroblasts (means \pm S.E.M.).

Additionally, we identified several known and unknown variants in *HMGA2*, all

outside the coding regions (Table 2). In five patients, we identified the known heterozygous intronic variant rs73115423. The *in silico* approach predicted a possible effect on the donor splice site of exon 3. In order to determine its nature as a mutation (frequency <1%) or a polymorphism (frequency >1%), we determined its prevalence using TaqMan SNP Genotyping Assay in 94 healthy Dutch controls. The fact that the variant was present in 2.7% of the healthy Dutch controls confirms that it is probably a non-pathogenic polymorphism rather than a functional mutation causing IGHD. We also found three minor genetic variations outside the coding regions of *HMGA2*. Their functional impact is probably small, based on their location.

In *CDK6*, we found several known polymorphisms (Table 2). However, we did not find any new variants.

Copy number analysis

Table 2. *HMGA2* and *CDK6* variants identified by sequencing in 105 patients

Gene	Exon	Position/rs number	Alleles	MAF		
				IGHD	controls	refSNP ^a
<i>HMGA2</i>	1	c.111+63C>G c.111+85G>C	C/G G/C	n.a. n.a.		n.a. n.a.
	2	-				
	3	rs73115423 rs3834468	T/A -/G	2.38% 8%	2.75%	1.7% n.a.
	4	c.250-29_-9del20		0.48%	0%	n.a.
	5	rs57800850	T/C	0.48%		n.a.
<i>CDK6</i>	2	-				
	3	rs35654944	C/T (p.Asp110Asn)	0.48%	1.10%	n.a.
	4	-				
	5	rs2301557	C/T	0.95%		2.7%
	6	-				
	7	rs3731373 rs42043	T/C G/A	5.7% 26.2%		5.0% 28.3%
	8	rs3731387	TGTAT/-	25.7%		n.a.

MAF, minor allele frequency; n.a., not available

^aMAF only shown if European population data were available at time of the study. The minor allele is indicated in bold.

In addition to mutation screening, we used MLPA for *HMGA2* and *CDK6* to screen DNA samples of all 105 (classical and partial) IGHD patients for deletions and duplications. In 101 patients, we did not detect any copy number variation; in 4 samples, the quality of DNA was not high enough for MLPA analysis, which is a very sensitive method. Because the probes were designed to cover the coding region, our MLPA kit did not cover the intronic 20 bp deletion previously detected by sequencing.

Clinical features of the patient with HMGA2 c.250-29_-9del deletion

The patient is the son of non-consanguineous Dutch parents, born after 40 weeks of gestation with normal birth weight (3400 g) and length (50 cm). At the age of 3 years, however, his height SDS had decreased to -4.8. He had various phenotypic characteristics typical for IGHD, such as frontal bossing and a high-pitched voice, as well as a doll's face and a micropenis. At the age of 3.5 years, his bone age was 1.3 years. He had an abnormal pituitary MRI with an undetectable anterior pituitary and an ectopic posterior pituitary, which was localized within the pituitary stalk (Fig. 3). The GH peak obtained during the arginine test was 3.0 mU/L (normal >20 mU/L). Serum IGF1 was 0.5 nmol/L (-6.0 SDS). All other pituitary hormones were normal. At the age of 3 years, GH treatment was started, to which the patient responded very well. His final adult height is 1.87 m (+0.6 SDS compared to the normal population).

Functional studies

We performed functional studies in order to assess whether the 20 bp deletion in *HMGA2* (c.250-29_-9del) is pathogenic or not. For this purpose we used the total RNA derived from patient's and control fibroblasts. In the first place, the presence of the 20 bp deletion was confirmed in the genomic DNA derived from the fibroblasts and the size and sequence of the major full-length *HMGA2* transcript (330 bp) was detected in both control and patient's samples (data not shown). Relative gene expression levels, determined from an average of triplicate real-time PCR experiments from fibroblasts cDNA did not show any changes in the expression of the major *HMGA2* transcript (*HMGA2a*) in the patient compared to the control. Expression levels of five additional splice-variants (*HMGA2b* – *f*) were measured; only the isoforms *HMGA2d* and *HMGA2e* showed a decreased expression in the patient compared to the control (Fig. 2C); the change in isoform d was statistically significant ($p = 0.02$). We also measured the expression of *IGF2BP2*, the only transcription target gene of *HMGA2* known to date and found similar levels for the control and the patient (Fig. 2D).

Discussion

We performed sequencing and copy number variation analysis in two new candidate genes, *HMGA2* and *CDK6*, in 105 Dutch IGHD patients. Our main finding was the identification of a new 20 base-pair intronic deletion (c.250-29_-9del) in *HMGA2*. This deletion was identified in one patient with classical IGHD and MRI abnormalities, from the cohort of 69 patients with classical IGHD, of whom 35 had MRI abnormalities. This deletion was absent in the mother and siblings of the patient, in the normal population screened in the present study as well as in the 1000 Genomes data-base, where 629 individuals (release November 2010) have been sequenced.

HMGA2 is an architectural transcription factor with three DNA-binding domains (AT-hooks) and a C-terminal acidic tail (Fig. 2A). Exons 1-3 encode the three AT-

hooks, exon 5 encodes the C-terminal tail. These two motifs are separated by a spacer encoded by exon 4. The function of the C-terminal tail is not yet completely understood, but it could be involved in protein-protein interactions or transcription activity enhancement [29, 30]. Hauke *et al* have described the complex alternative splicing pattern of *HMGA2*, in which the five additional splice-variants (b-f) differ from the major isoform (a) due to the replacement of exon 4 and 5 by sequences derived from the long intron 3 [11, 12]. The 20 bp deletion that we found in our patient is localized 9 bp before the start of exon 4, which could affect this complex alternative splicing pattern.

Studies on *Hmga2*-null mice with pygmy-phenotype provided the first target gene of *HMGA2*: insulin-like growth factor II mRNA-binding protein (*IGF2BP2*) [13, 14], a post-transcriptional regulator of IGF-II. In order to evaluate the possible del-

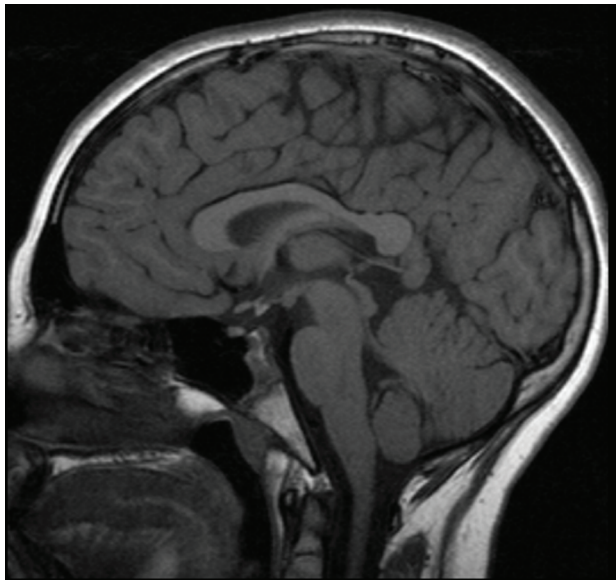


Figure 3. Pituitary structures on T1-weighted MRI scans of our patient with an undetectable anterior pituitary and an ectopic posterior pituitary.

eterious effect of the newly found 20 bp deletion, we performed real-time RT-PCR of fibroblast's RNA, and analyzed the expression pattern of the different *HMGA2* splice-variants as well as the *IGF2BP2* gene, comparing the results between the affected patient and a normal control. Our initial hypothesis was that the expression of the major full-length transcript (a) would be decreased, whereas expression of one or more of the other isoforms could be increased. However, in our experiments the expression levels of the full-length transcript (a) were similar for the patient and control; this could be explained by the fact that the normal allele is still present in the patient. Surprisingly, two of the other five isoforms (d and e) showed decreased expression (Fig. 2C). Currently, the function of these isoforms is unknown, but we cannot exclude the possibility that these low expression levels contribute to the patient's phenotype.

Although we expected to find decreased expression of *HMGA2(a)* and *IGF2BP2*, we did not find any changes during our experiments (Fig. 2D). One possible hypothesis for this is that the expression of these genes is only essential during embryogenesis [13], and could be less important in adult fibroblasts. It has been demonstrated that *HMGA* genes show high expression during human embryogenesis and fetal development and low expression levels in adult tissues [31, 32]. In mice, *Hmga2* is found to be preferentially expressed by stem cells, showing a progressive decline in expression with age [33]. Even though our patient was born with normal weight and height, the deficiency of an embryonic factor could cause an abnormal phenotype later in life. The deregulation and possibly decreased expression of *HMGA2* during the development of specific somatotroph cells in the pituitary might lead to low GH production after birth, contributing to the IGHD phenotype.

Previous reports demonstrate the role of *HMGA2* in human growth. Since 2007, several authors described a new microdeletion syndrome affecting the 12q14 genomic region [18-22]. A total of 13 patients with the 12q14microdeletion syndrome have been described and the common features include low birth weight, failure to thrive in infancy, short stature, learning problems in childhood and, in some cases, osteopoi-kilosis. The deletion covers several megabases and includes many genes, of which *HMGA2* is thought to be responsible for the growth problems among the patients carrying this deletion. Later reports confirmed this role: patients carrying the deletion including *HMGA2*, all had severe growth retardation. In the last report of Lynch *et al* four children with the deletion ending before *HMGA2* presented significantly better growth [22]. Interestingly, Buysse *et al* identified an intragenic *HMGA2* deletion in a boy with proportionate short stature without any other abnormalities, and found that the deletion co-segregated perfectly with reduced adult height in the extended family of the boy [19]. All these data provide evidence that a heterozygous deletion of *HMGA2* causes growth failure. On the other hand, the report by Ligon *et al* describes a boy with extreme somatic overgrowth due to pericentric inversion of chromosome 12 [23]. Taken together, these data and the 20 bp deletion described in the current study are consistent with an important role for *HMGA2* in growth and suggest that copy number variations in this gene could be a rare genetic cause of IGHD or other disorders characterized by growth failure. In this regard, it is important to note the relevance of duplication and deletion screening in addition to sequencing, in order to achieve maximum genetic coverage and understanding of the disease [34, 35].

Contrary to our expectation, we did not find any new pathological variants in *CDK6*. Although we did not examine the promotor and enhancer regions of either gene, we believe it is reasonable to assume that *CDK6* is not involved in the pathophysiology of IGHD, and may be excluded in future screening of IGHD patients.

In conclusion, this study provides the first report of a deletion in the *HMGA2* gene that might be a rare cause of IGHD. We suggest that this gene be investigated in patients with a classical IGHD phenotype, in whom mutations in the classical candidate genes *GHI*, *GHRHR*, have been previously excluded. At molecular level, further re-

search should be performed to better understand the possible involvement of this gene in the classical GH-IGF1 axis and to investigate how genetic alterations in *HMGA2* can affect human growth.

Acknowledgments

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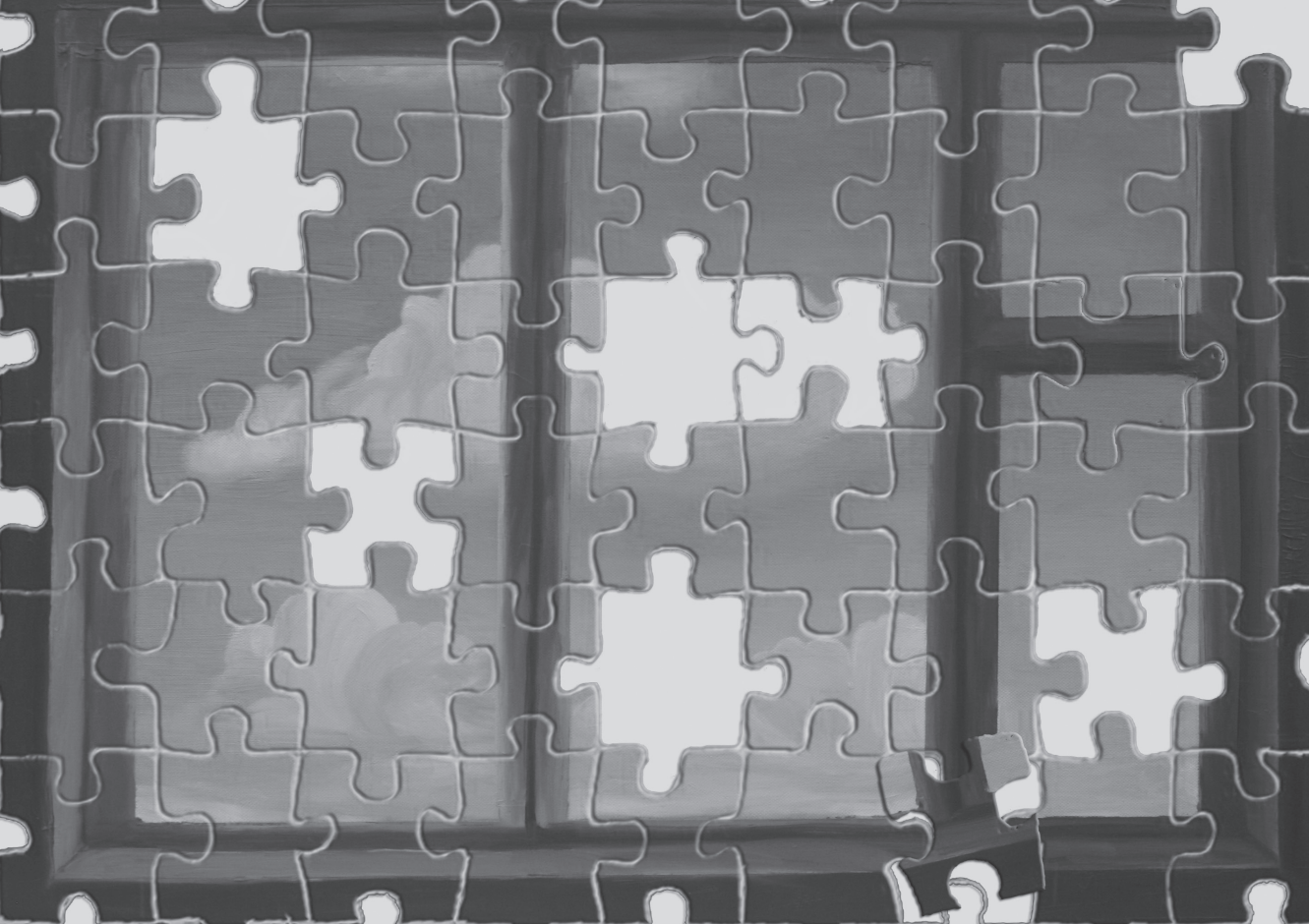
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Chapter 3

Mutations in two Hedgehog genes, *SHH* and *HHIP*, as the genetic cause of Combined Pituitary Hormone Deficiency.

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Abstract

Combined Pituitary Hormone Deficiency (CPHD) is characterized by deficiencies of two or more anterior pituitary hormones. Its genetic cause is unknown in the majority of cases. The Hedgehog (Hh) signaling pathway has been implicated in disorders associated with pituitary development. Mutations in Sonic Hedgehog (*SHH*) have been described in patients with holoprosencephaly (with or without pituitary involvement). Hedgehog Interacting Protein (*HHIP*) has been associated with variations in adult height in genome wide association studies. We investigated whether mutations in these two genes of the Hh pathway, *SHH* and *HHIP*, could result in 'idiopathic' CPHD.

Design / Patients. We directly sequenced the coding regions and exon – intron boundaries of *SHH* and *HHIP* in 93 CPHD patients of the Dutch HYPOPIT study in whom mutations in the classical CPHD genes *PROPI*, *POUIF1*, *HESX1*, *LHX3* and *LHX4* had been ruled out. We compared the expression of Hh genes in Hep3B transfected cells between wild-type proteins and mutants.

Results. We identified three mutations (p.Ala226Thr, c.1078C>T and c.*8G>T) in *SHH*. The function of the latter was severely affected in our *in vitro* assay. In *HHIP* we detected a new activating mutation c.-1G>C, which increases HHIP's inhibiting function on the Hh pathway.

Conclusions. Our results strongly suggest involvement of the Hedgehog pathway in CPHD. We suggest that screening of both *SHH* and *HHIP* be included in the future genetic analysis of patients with CPHD, after mutations in the classical CPHD genes have been ruled out.

Introduction

Combined Pituitary Hormone Deficiency (CPHD) denotes impaired production of growth hormone (GH) and one or more other anterior pituitary hormones. In infancy, severe growth deficiency can be seen from birth, as well as distinctive facial features like a prominent forehead and midfacial hypoplasia, resulting in a depressed nasal bridge, deep-set eyes, and a short nose with anteverted nostrils. An hypoplastic pituitary gland is often seen at MRI examination [1-4].

Normal function and development of the anterior pituitary is crucial for correct secretion of the pituitary hormones. Mutations in several genes involved in pituitary cell differentiation have been related with CPHD [5]. Although there is strong evidence of heritability [2, 6-9], mutant genes remain to be identified in the majority of cases.

The Hedgehog (Hh) signaling pathway plays a major role in embryonic development and has been implicated in disorders associated with pituitary development. A simplified scheme of the Hh signaling is shown in the Figure 1. Mutations within Sonic Hedgehog (SHH; MIM 600725) have been described in patients with holoprosencephaly (HPE; MIM 236100) [10-14], a congenital malformation of the brain and midface that occurs due to incomplete midline cleavage of the embryonic forebrain. Furthermore, mutations in other member of the Hh pathway such as the transcription factor *GLI2* [15-17], the transmembrane receptor *PTCH1* [18] and the ligand transporter *DISP1* [19] have been identified in patients with HPE and abnormal pituitary gland function.

In mammals, there are three ligands for the Hedgehog signaling pathway: Shh, Desert (Dhh) and Indian hedgehog (Ihh). Hedgehog Interacting Protein (HHIP; MIM 606178), can bind all three mammalian ligands, inhibiting their function. In recent Genome Wide Association studies (GWAs), variations in *HHIP* have been related with adult height in the normal population .

The Dutch HYPOPIT study is a nation-wide genetic study, which aims to obtain an overall picture of known and new genetic defects in CPHD patients. Since mutations in known CPHD related genes (*PROPI*, *HESX1*, *POU1F1*, *LHX3*, *LHX4* and *GHI* P89L and IVS3+1/+2) were identified in only a small minority of the participating patients [20], we searched for mutations in new candidate genes. Based on recent literature, we hypothesized that mutations in *HHIP* and *SHH* might explain the phenotype of some of our CPHD patients.

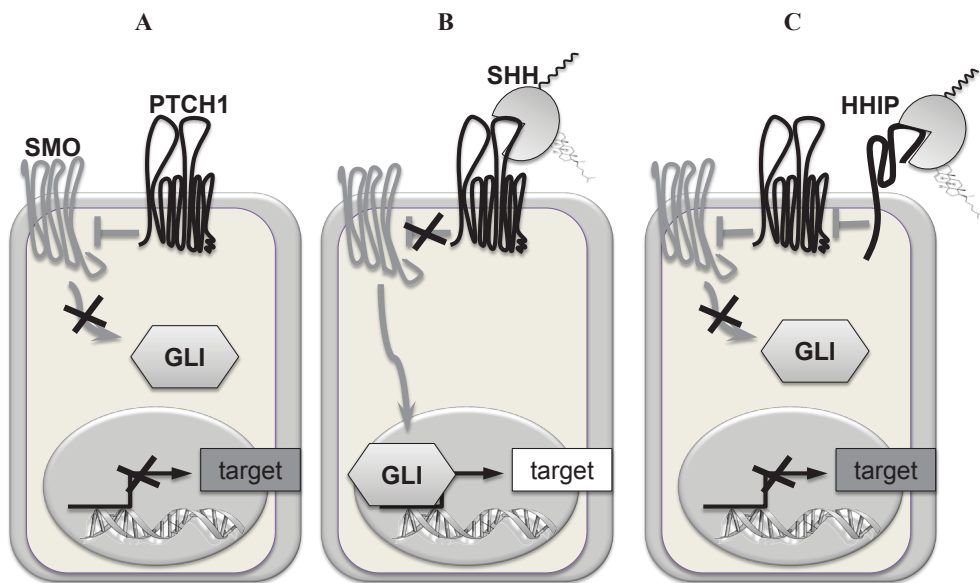


Figure 1. Schematic representation of the Hedgehog signaling pathway.

(A) in the absence of Hh ligand, transmembrane receptors Patched1 (PTCH1) represses the activity of Smoothened (SMO) and inhibits the effect of transcription factors GLI, repressing the expression of Hh target genes. (B) when the Hh pathway is active, SHH binds to PTCH1, this relieves the inhibition of SMO, leading to the activation of the GLI proteins and the transcription of Hh target genes. (C) HHIP (Hedgehog interacting protein) binds to SHH, which blocks the binding to PTCH1, leading to inhibition of the Hh pathway.

Subjects and Methods

Study subjects

DNA samples were collected of 93 patients with CPHD, who had participated in the Dutch HYPOPIT study and in whom mutations in the classical CPHD genes *PROPI*, *POUIF1*, *HESX1*, *LHX3* and *LHX4* had been ruled out [20]. These patients had been recruited from the Endocrinology departments of six University and two non-University Hospitals, and they had been registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003. All patients had deficiencies of GH and one or more additional hormonal axes. Deficiencies of hypothalamic-pituitary-thyroidal, -adrenal, and -gonadal axes were defined as abnormal TRH test or TSH levels that were low or inadequate for low FT4; abnormal CRF / ACTH / glucagon test or ACTH levels which were low or inadequate for low cortisol and LH, FSH, estrogen / testosterone or LHRH test low for age or lack of spontaneous puberty after age 14 y. Prolactin deficiency was defined as abnormal prolactin during random or TRH testing. Reference values of the individual hospitals were used. Exclusion criteria were: patients with known cause of CPHD, such as a brain tumor, brain surgery, brain radiation and known syndromes. Written informed consent was obtained from

all participating patients and their parents or legal guardian(s).

Sequencing

DNA was isolated from whole blood, collected in EDTA tubes according to standard procedures. Of all patients, *SHH* (NM_000193.2) and *HHIP* (NM_022475.2) coding exons and exon/intron boundaries were PCR amplified using Qiagen reagents of 5 units/ μ l Taq DNA Polymerase, 10x PCR Buffer, 5x Q-Solution, 10 mM dNTPs, 25 mM $MgCl_2$ and primers (supplementary table). Reactions were incubated for 3 min at 94 C, followed by 35 cycles of 30 sec at 94 C, 1 min at 60 C and 1 min at 72 C, followed by a final incubation for 10 min at 72 C. The electrophoretic separation, sizing and quantification of PCR products was performed using LabChip GX (Caliper) microfluidics technology. The amplified products were purified using illustra GFX™ 96 PCR Purification Kit (GE Healthcare). Sequencing was performed using Big Dye Terminator reaction kit (Applied Biosystems). After purification with Dyex 96 kit (Qiagen) the products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems).

Digestion with restriction enzyme

The presence of the newly identified variant *HHIP* c.-1G>C was examined in all available family members and 88 Dutch controls by restriction fragment length polymorphism, using the enzyme *CviAII* (New England BioLabs). For digestion, the following conditions were used: 15 μ l of *HHIP* Ex1 PCR product, 5 μ l of NEBuffer 4 and 1 μ l of the enzyme in a total volume of 30 μ l was incubated for 2 h at 25 C and heat-inactivated for 20 min at 65 C. The sizing of the PCR and digestion products was quantified using LabChip GX.

Generation of plasmids

The human full-length cDNA clone for *HHIP* (NM_022475.1) was obtained as transfection-ready DNA in the vector pCMV6-XL6 (OriGene Technologies). The human *SHH* (NP_000184.1) cDNA in the vector pUHD10 and the enhancer vector pUHD15 were a generous gift from Drs. Domenico Salvatore and Monica Dentice. Mutations were introduced according to the QuickChange Site-direct mutagenesis protocol (Stratagene), using the following primers:

HHIP -1G>C 5'CCTGCTGCTCTGGGCAGACcATGCTGAAGATGCTCTCC;
SHH Ala226Thr 5' CGGGGACCGCGTGCTGaCGGCGGACGACCAGGGC;
SHH c.C1078T 5'CCATTCTCATCAACCGGGTGtTGGCCTCGTGCTACGCGGTC;
SHH c.*8G>T 5'CAAGTCCAGCTGAAGCCGGGtGGCCGGGGGAGGGGCG.
DNA sequencing confirmed the presence of the introduced mutations.

Quantitative RT-PCR

Hep3B cells were cultured in six-well plates in DMEM/F12 with GlutaMax me-

dium supplemented with 10% fetal calf serum, 1% pen/strep (Invitrogen). After 24 h, cells were transiently transfected with a wild-type, mutant or corresponding empty vector. A typical reaction mixture contained pCMV6-HHIP (500 ng), pD10-SHH (25, 200 or 400 ng) and pD15 (10, 80 or 160 ng), 3 µl X-tremeGENE 9 (Roche), which were transfected according to the manufacturer's instructions.

Total RNA was extracted after 48 h from the transfected and the control mock cells with High Pure RNA Isolation kit (Roche). Reverse transcription of total RNA (3 µg) to single-stranded cDNA was performed using TaqMan RTreagent (Roche), in accordance with the manufacturer's instructions.

Quantitative PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems), in accordance with the manufacturer's instructions, employing the Applied Biosystems 7900HT Sequence detection system. The assays were performed with the following TaqMan Gene Expression Assays: *SHH* (Hs00179843_m1), *HHIP* (Hs00368450_m1) and *GLII* (Hs00171790_m1). Relative expression was calculated using the cyclophilin A housekeeping gene (Applied Biosystem). Fold changes were calculated using this comparative C_T method.

Table 1. Clinical data of 93 patients with CPHD.

Gender (M/F)	67 / 26
Age, years*	21.4 (8.7)
Birth weight, kg*	3.0 (0.8)
Birth length, cm*	49.5 (3.1)
Gestational age, weeks*	39.1 (2.6)
Age at start GH treatment, years*	4.9 (5.8)
Height SDS at start of GH, SDS*	-3.0 (1.3)
Hypothyroidism, N (%)	91 (98%)
Hypocortisolism, N (%)	71 (76%)
Hypogonadotrope hypogonadism, N (%)	43 (46%)
Hypoprolactinemia, N (%)	12 (13%)
Diabetes insipidus, N (%)	6 (6%)
MRI abnormalities, N	70 (19 triad) ^a
Micropenis, N (%)	25 (37%)
Prolonged neonatal jaundice, N (%)	51 (61%)
Hypoglycaemia, N (%)	39 (48%)

SDS, standard deviation score; GH, Growth Hormone; ^atriad, hypoplastic anterior pituitary, ectopic posterior pituitary and interrupted/invisible stalk, * numbers represent mean (SD)

Statistical Analysis

All results are the mean of at least triplicate determinations from representative experiments. Values are expressed as mean \pm SE. For statistical analysis, Student's t test was used (Excel, Microsoft office); $p < 0.05$ was considered significant.

Results

Mutation Screening

We directly sequenced the complete coding region and intron-exon boundaries of *SHH* and *HHIP* in DNA samples of 93 probands with CPHD. Clinical characteristics of the patients are shown in Table 1. We identified three mutations in exon 3 of *SHH*. In one patient, we identified a heterozygous missense mutation c. 676G>A, which causes the amino acid change p.Ala226Thr (Fig. 2A). This mutation was previously described in a patient with holoprosencephaly [21]. Our patient's mother and older sister were carriers of the same heterozygous mutation. In a second patient, we identified the new heterozygous synonymous variant c.1078C>T (p.=); patient's father and sister were carriers of the same heterozygous mutation (Fig. 2A). In a third patient, we identified a new heterozygous variant in the 3'UTR region c.*8G>T (Fig. 2A), localized at 8 bp after the stop codon in a very G-rich region; patient's mother carried the wild-type, the father was not available for genetic analysis.

In one patient, we identified a new heterozygous *HHIP* mutation c.-1G>C in the Kozak sequence which may be pathogenic (Fig. 2B and discussion's section). In order to determine its nature as a mutation (frequency <1%) or a polymorphism (frequency >1%), we examined its prevalence taking advantage of the fact that the mutation introduces a new restriction site for the *CviAII* enzyme. All 88 healthy Dutch controls as well as patient's mother displayed only the wild-type uncut band. The father was not available for genetic analysis.

Several other known and unknown variants were found outside the coding regions (Table 2).

Clinical Features

Patient 1, carrier of the *SHH* c.676G>A mutation, is a male, born from Dutch non-consanguineous parents after 42 weeks of gestation by vaginal breech delivery, followed by asphyxia. His birth length was 50 cm and birth weight 2850 g. At the age of 9 years and 4 months, his bone age was 8.0 years. He had a normal facial appearance. His MRI was clearly abnormal, with an ectopic posterior pituitary and an invisible stalk, with a small sella. Central hypothyroidism was diagnosed at the age of 8 years (FT4 8 pmol/L; reference 9 – 27 pmol/L), for which thyroid hormone treatment was started. He had undetectable GH levels during two arginine tests. After stopping GH treatment, he had a GH peak of 2 mU/L during retesting. Serum IGF-I

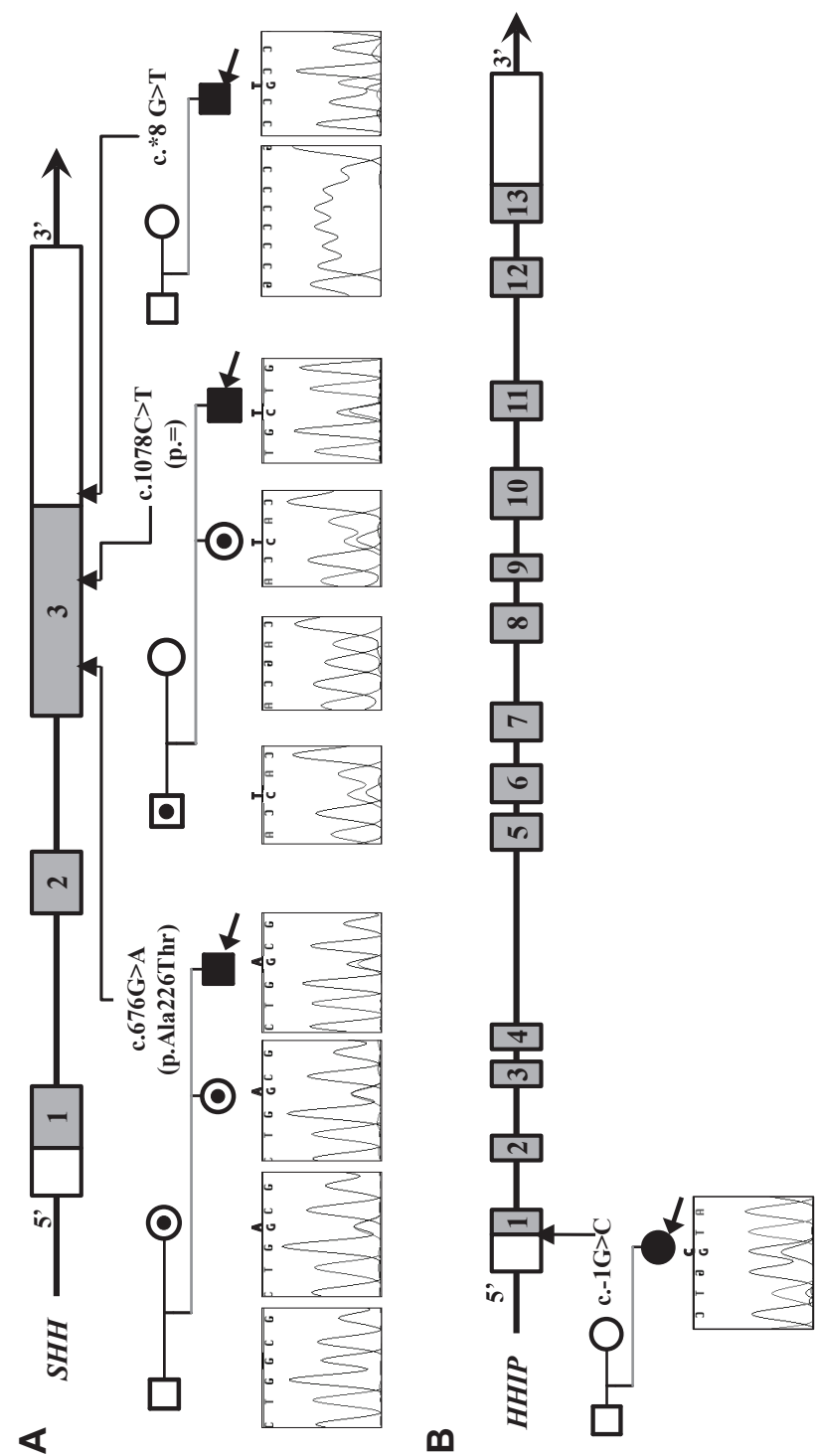


Figure 2. *SHH* and *HHIP* mutation analysis in patients with CPHD. Schematic diagram of the human *SHH* gene (**A**) and *HHIP* gene (**B**), and pedigrees with representative chromatograms of sequences. Vertical arrows show the position of each mutation. Blackened symbol, affected individuals; unblackened symbol, unaffected individuals; blackened dot in unblackened symbol, unaffected mutation carrier.

level before start of GH treatment was 2.0 nmol/L (-4.9 SDS) and 17.4 nmol/L (-2.2 SDS) after stop of GH treatment. At the age of 9 years, hydrocortisone treatment was started because he had low cortisol levels (at 8h 0.14 -0.19 μ mol/L, reference 0.2 – 0.8 μ mol/L) and undetectable ACTH levels during hypoglycemia. At age 14.3 years he was still prepubertal with low LH and FSH values, and LHRH treatment was started after an LHRH test without any increase in LH and FSH. Prolactin levels were normal. Father's height is 172 cm (-1.4 SDS), mother's height -153 cm (-2.5 SDS; carrier of the c.676G>A mutation). He has one sister with unremarkable phenotype (she is carrier of the c.676G>A mutation).

Patient 2, carrier of the *SHH* c.1078C>T mutation, is a male, born to Dutch non-consanguineous parents after 42 weeks of gestation by uncomplicated vaginal delivery with a birth length of 52.5 cm and birth weight of 3600 g. He was born with severe craniofacial encephalocele that was surgically removed during childhood. He had hypertelorism, septo optic dysplasia, and a broad nasal bridge. His MRI showed a partially empty sella and an absent posterior pituitary. At the age of 8 years he was diagnosed with central hypothyroidism (T4 65 nmol/L) and adrenal insufficiency was suspected (cortisol at 16 h repeatedly undetectable; ACTH test not available). Thyroxine and hydrocortisone treatment were started. The patient also developed diabetes insipidus, for which he was treated with desmopressin. Clonidin test at 7.4 years produced a low GH peak (4.5 mU/L) and an L-dopa propranolol test at 9.9 years, showed again a low peak (6 mU/L). At the age of 11 years he had a delay in bone age of 1.5 years. Although his height at the age of ten years was 143cm (-0.06 SDS), GH treatment was started based on the combination of the midline defect, CPHD and repeatedly low GH levels. At that age he had weight +2.77 SDS. He had a normal puberty and normal prolactin levels. Father's height is -1.71 SDS (170 cm; carrier of the c.1078C>T mutation), mother's height -0.7 SDS (165 cm). He has one sister with unremarkable phenotype (she is carrier of the c.1078C>T mutation).

Patient 3, carrier of *SHH* c.*8G>T mutation, is a male, born to Dutch non-consanguineous parents after 35 weeks of gestation by vaginal breech delivery with a birth weight of 2410 g. His MRI showed a small anterior pituitary and ectopic posterior pituitary. Thyroxine supplementation was started at the age of 6 years, because of the low FT4 levels (7 pmol/L, reference 9 – 27 pmol/L) and abnormal TSH response in TRH test. At the age of 6 years and 7 months, his bone age was 3.0 years. GH tests at the age of 6 years demonstrated low GH peaks (twice 3.5 mU/L). IGF-I was 6.0 nmol/L (-1.9 SDS) and IGFBP-3 was 0.8 mg/L (-6.6 SDS). GH treatment was started at the age of 6.7 years when his height was -3.5 SDS and his weight was -2.4 SDS. He has normal ACTH, cortisol and prolactin levels and had normal puberty. Father's height is 184.5 cm (0.3 SDS), mother's height is 176 cm (0.9 SDS). He has no siblings.

Patient 4, carrier of *HHIP* c.-1G>C mutation, is a female, born to Dutch non-consanguineous parents after 33 weeks of gestation by vaginal breech delivery followed

by severe asphyxia and resuscitation for 45 minutes. Her birth weight was 1490 g. She suffered from prolonged neonatal jaundice and her MRI showed a small anterior pituitary, invisible posterior pituitary and a very thin stalk. Thyroid hormone and hydrocortison treatment was started in the neonatal period, because of low T4 levels (54 nmol/L), very low cortisol (at 8h 0.002 μ mol/L) and undetectable ACTH. At the age of 4 months Arginine tests showed low GH peak (8 mU/L), as well as Clonidine test (6.5 mU/L). IGF-I before start of GH treatment was -1.5 SDS. At age of 13 years she was still totally prepubertal (Tanner stage M1 P1) and induction of puberty was started. Prolactin levels were normal. Father's height is 179 cm (-0.4 SDS), mother's height is 162 cm (-1.2 SDS). The patient has no siblings.

Table 2. *HHIP* and *SHH* variants identified by sequencing in 93 CPHD patients

Gene	Exon	Variant	Alleles	MAF	
				CPHD	refSNP ^a
<i>HHIP</i>	1	c.-1G>C	G/ C	0.5 %	n.a.
	2	rs3215015	T / -	41.9 %	n.a.
	3	rs6537301	G/ A	48.4 %	45.6 %
	5	c.983+34C>G	C/ G	1.1 %	n.a.
	11	rs2276933	C/ T	2.1 %	5.6 %
	12	rs11100867	C/ T	44.0 %	40.4 %
	13	rs11727676 (p.=)	T/ C	12.4 %	8.0 %
		rs62344037	C/ T	9.1 %	8.3 %
<i>SHH</i>	1	rs9333594	G/ A	3.8 %	8.4 %
		rs111495739	C/ G	0.5 %	0.2 %
	2	rs1233555	T/ C	38.7 %	40.0 %
		c.301-14C>G	C/ G	0.5 %	n.a.
	3	rs9333633 (p.=)	G/ A	0.5 %	0.6 %
		c. 676G>A (p. Ala226Thr)	G/ A	0.5 %	n.a.
		c.1078C>T (p.=)	C/ T	0.5 %	n.a.
		c.*8G>T	G/ T	0.5 %	n.a.

MAF, minor allele frequency; n.a., not available

^aMAF only shown if European population data were available at time of the study. The minor allele is indicated in bold.

Functional studies

We performed functional studies in order to assess whether the mutations identified in *SHH* and *HHIP* are pathogenic or not. For this purpose we used an *in vitro* system of transiently transfected Hep3B cells with different combination of wild-type, mutant and corresponding empty vectors. We determined the fold change of

SHH, HHIP and GLI1 (two of the known SHH target genes) mRNA expression in the cells (see also scheme in Fig. 1). Hep3b cells expressed endogenous levels of SHH and GLI1, but almost undetectable levels of HHIP. When we transfected Hep3B cells with different amounts of wild-type *SHH* (0, 25, 200 and 400 ng) we detected an increase in SHH expression, as expected (Fig. 3A). At the same time, there was an increase in Hh target GLI1 expression (Fig. 3C), but no differences in HHIP expression (Fig. 3B). When we compared these results with cells transfected with the *SHH* p.Ala226Thr mutant, we detected a significant difference in SHH expression at the highest concentration (Fig. 3A). The *SHH* c.*8G>T mutant, which affects the G-rich 3'UTR region, shows the most severely affected GLI1 expression at all concentration, although the significance is only seen at the highest concentration (Fig. 3C). The *SHH* c.1078C>T mutant showed only slightly decreased GLI1 expression at 25 and 200 ng SHH (Fig. 3C), without significant difference. HHIP expression for all three SHH mutants did not show any differences with wild-type, except for c.*8G>T that showed a high inter-experimental variability at 400 ng SHH (Fig. 3B).

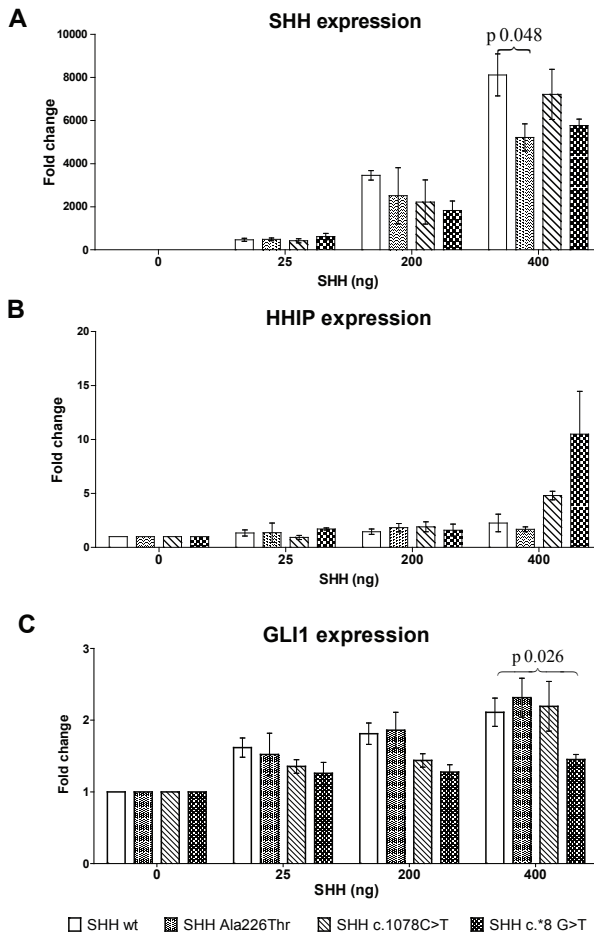


Figure 3. Functional studies of SHH mutations.

Hep3B cells were transiently transfected during 48 h with SHH wild-type or SHH mutants at different concentrations (25, 200 or 400 ng). RNA from the cells were extracted and analyzed by RT-PCR. Relative gene expression of SHH (A), HHIP (B) and GLI1 (C) was calculated using the cyclophilin A housekeeping gene. The results are the means of three replicates \pm S.E.M.). $p < 0.05$ was considered significant. wt, wild-type

Hep3B cells transfected with wild-type HHIP together with increasing amounts of SHH showed an increase of SHH expression, as expected. The same result was found in *HHIP* c.-1G>C mutant cells (Fig. 4A). Cells transfected with wild-type HHIP showed a decrease in HHIP expression (Fig. 4B), due to negative feedback effect of increasing amount of wild-type SHH. In all experiments, the *HHIP* c.-1G>C mutant had a significantly lower expression in comparison to the cells transfected with *HHIP* wild-type (Fig. 4B). Wild-type HHIP inhibit GLI1 expression when it is expressed together with SHH in comparison with cells transfected with SHH wild-type alone (Fig. 3C and 4C). As expected, the *HHIP* c.-1G>C mutant showed an even stronger inhibiting effect on GLI1 expression, with significant difference when transfected together with 25 ng of SHH wild-type (Fig. 4C).

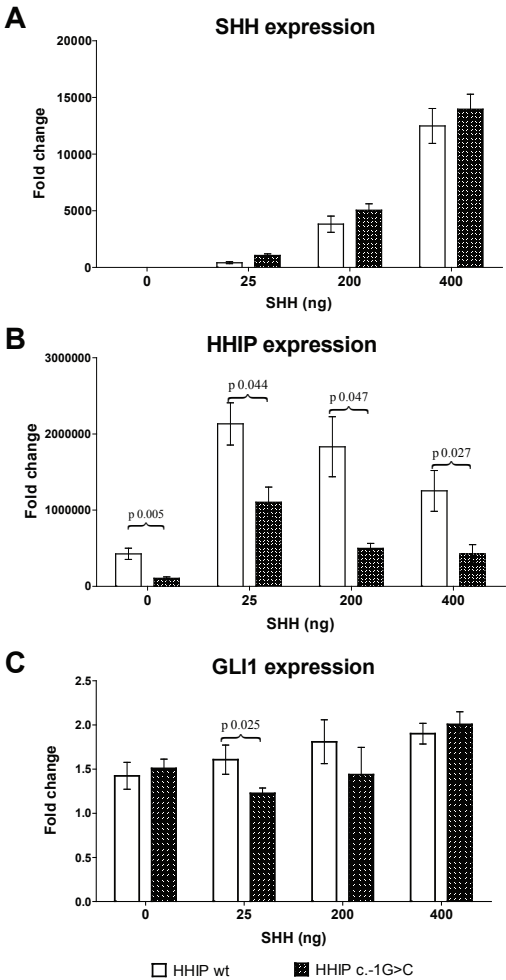


Figure 4. Functional studies of HHIP mutation.

Hep3B cells were transiently transfected during 48 h with different concentrations of SHH wild-type (25, 200 or 400 ng) and 500 ng of HHIP wild-type or HHIP mutant. RNA from the cells were extracted and analyzed by RT-PCR. Relative gene expression of SHH (A), HHIP (B) and GLI1 (C) was calculated using the cyclophilin A housekeeping gene. The results are the means of three replicates \pm S.E.M.). $p < 0.05$ was considered significant. wt, wild-type.

Discussion

We performed mutation screening by direct sequencing in two Hedgehog pathway genes, *SHH* and *HHIP*, in 93 Dutch CPHD patients. Our main findings in *SHH* include one missense mutation (p.Ala226Thr), one synonymous mutation in the coding region (c.1078C>T) and one mutation in the 3'UTR region (c.*8G>T). Additionally we found a new mutation affecting the Kozak sequence of the *HHIP*. All these mutations were absent in the 1000 Genomes data-base, sharing sequence data of 629 individuals (release November 2010).

SHH mutations have been described in relation to holoprosencephaly (HPE) [10-14], and the first mutation that we identified in *SHH* c.676G>A (p.Ala226Thr) was previously reported in relation to a familial case of HPE [21], in which the mutation was present in the female patient but also in her clinically unaffected father. In the present study, the unaffected mother and sister of the patient were also carriers of the same mutation. The *SHH* p. Ala226Thr mutation corresponds to highly conserved residues within the intein-like Hint motif, which gives SHH the intrinsic ability of self-cleavage. This mutation has previously been studied by Traiffort *et al*, whose functional studies did not demonstrate loss of function [22]. Our *in vitro* analysis showed that this mutation results in a significantly lower SHH expression when 400 ng of the plasmid containing the mutation was transfected in Hep3B cells (Fig. 3A), which suggests an mRNA stability problem. Posttranscriptional mRNA stability plays an important role in regulating gene expression. It influences the balance between mRNA synthesis and degradation, which determines the level of individual mRNAs in the cell. Reduced mRNA stability causes increased mRNA degradation and therefore decreased expression of the gene.

The second mutation in *SHH* was the synonymous change c.1078C>T, which is also located in a functionally conserved region. Our functional analysis showed a nonsignificant decrease in expression of the hedgehog transcription target *GLI1* (Fig. 3C).

The third mutation, *SHH* c.*8G>T, is localized 8 base-pare after the stop codon, in a very G-rich area. This means that the change of one G to T could modify the RNA structure. Our *in vitro* results show that the c.*8G>T mutation significantly decreases *GLI1* expression in Hep3B cells (Fig. 3C). Moreover, this mutation decreases SHH expression (Fig. 3A) and increases HHIP expression (Fig. 3B), although not significantly. These results indicate that the *SHH* c.*8G>T mutation leads to a loss of function.

SHH mutations, known to be associated with holoprosencephaly, show a broad genetic heterogeneity. Surprising clinical variability has been described between family members with the same *SHH* mutation [10, 23, 24], in many of this cases incomplete penetrance is described. The HPE phenotype itself shows also enormous variability, ranging from the most extreme form such as cyclopia or pronounced microcephaly to HPE microforms and normal facial appearance with normal MRI in

non-penetrant carriers [10, 11, 14]. Some patients with HPE have anterior pituitary hormone deficiencies such as hypothyroidism (11%), hypocorticism (7%), and GH deficiency (5%) [25, 26]. Our patients with *SHH* mutations show some overlap with a mild HPE phenotype: besides combined pituitary deficiency, they have pituitary abnormalities at MRI. Patient 2 carrying the c.1078C>T was born with a severe cranio-facial encephalocele for which he was operated during childhood. He also developed diabetes insipidus, which has a high incidence (up to 70%) in children with HPE.

Apart from *SHH*, we also studied another gene from the Hh pathway, *HHIP*, previously described to be associated with adult height variability [27]. We identified a mutation (c.-1G>C) in one patient with CPHD. This change is localized one nucleotide just before the ATG and alters the Kozak sequence. HHIP contributes to the negative regulatory feedback loop of the Hh signaling pathway. It binds the three mammalian Hh ligands, Dhh, Ihh and Shh, with similar affinity as the PTCH1 receptor. This receptor forms normally a complex with the ligands, activating the Hh pathway (Fig. 1B). The binding of these ligands by HHIP blocks the interaction with PTCH1, thereby inhibiting activation of the Hh pathway (Fig. 1C). Former research shows that overexpression of HHIP has similar effects as inactivation of the Hh ligands. For example, the overexpression of Hhip in chondrocytes results in severe skeletal defects similar to those observed in Indian hedgehog ligand mutants [28].

The mutation c.-1G>C, changes G to C. This base change makes the Kozak sequence more similar to the consensus sequence, which might increase the production of HHIP protein, inhibiting the Hh pathway. Our *in vitro* assays showed that, indeed, the HHIP c.-1G>C mutation inhibits the Hh pathway: the Hh target *GLI1* showed a significantly decreased expression in cells transfected with mutant HHIP (at 25 ng of SHH, Fig. 4C). This effect is overcome when the system is saturated with the SHH (400 ng of plasmid). Because HHIP inhibits Hh signaling, an increased expression of HHIP has a similar effect on the pathway as a loss-of-function mutation of the ligand. Our finding emphasizes the importance of fine-tuning of the Hh signaling pathway and shows that *HHIP* might be one of the modifiers leading to the enormous variability in the HPE phenotype. Further molecular research of the different Hh genes is warranted to better understand this complex family and its pathogenic implications.

In conclusion, we identified three new mutations in *SHH* and one in *HHIP* in a cohort of 93 patients with CPHD. Our results strongly suggest involvement of the Hedgehog pathway in CPHD. Based on our findings, we propose to perform genetic screening of both *SHH* and *HHIP* in patients with CPHD after mutations in the classical CPHD genes *PROPI*, *POUIF1*, *HESX1*, *LHX3* and *LHX4* have been ruled out.

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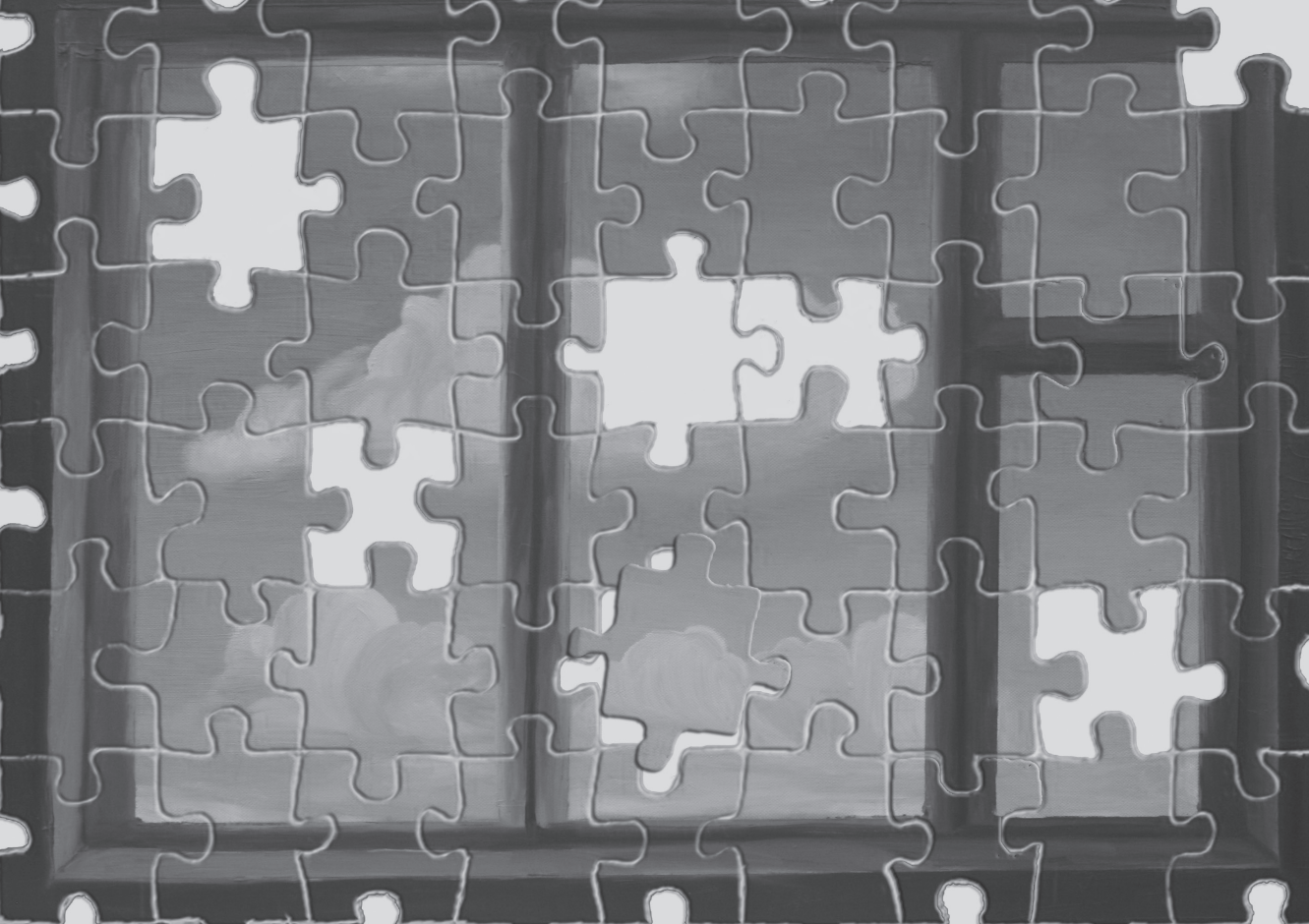
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Chapter 4

A novel *OTX2* mutation in a patient with Combined Pituitary Hormone Deficiency, pituitary malformation and an underdeveloped left optic nerve.

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Abstract

OTX2 is a homeobox family transcription factor, required for brain and eye formation. Various genetic alterations in *OTX2* have been described, mostly in patients with severe ocular malformations. In order to expand the knowledge of the *OTX2* mutation spectrum, we performed *OTX2* mutation screening in 92 patients with Combined Pituitary Hormone Deficiency (CPHD).

Design / Patients. We directly sequenced the coding regions and exon – intron boundaries of *OTX2* in 92 CPHD patients from the Dutch HYPOPIT study in whom mutations in the classical CPHD genes *PROPI*, *POU1F1*, *HESX1*, *LHX3* and *LHX4* had been ruled out.

Results. Among 92 CPHD patients, we identified a novel heterozygous missense mutation c.401C>G (p.Pro134Arg) in a patient with CPHD, pituitary malformation and an underdeveloped left optic nerve. Binding of both the wild-type and mutant OTX2 protein to bicoid binding sites were equivalent; however, the mutant OTX2 exhibited decreased transactivation. Furthermore, we identified a new silent mutation c.768C>T in a patient with CPHD and pituitary malformation.

Conclusions. We describe a novel missense heterozygous *OTX2* mutation which acts as a dominant negative inhibitor of target gene expression in a patient with CPHD, pituitary malformation and optic nerve hypoplasia. We provide an overview of all *OTX2* mutations described until date, which show that *OTX2* is a promising candidate gene for genetic screening of patients with CPHD or isolated growth hormone deficiency (IGHD). Since the majority of the *OTX2* mutations found in patients with CPHD, IGHD or short stature, have been found in exon 5, we recommend starting mutational screening in those patients in exon 5 of the gene.

Introduction

OTX2, the orthodenticle *Drosophila* homolog 2 (MIM 600037) is a homeobox family transcription factor, which is required for brain and eye formation. *OTX2* is located on chromosome 14q and has five exons of which three are coding. There are two known isoforms: a (NM_021728.2; NP_068374.) and b (NM_172337.1; NP_758840.1); the isoform b is the major product of the gene and has eight less amino acids than isoform a. In the mouse, during early development, *Otx2* is expressed in the forebrain and midbrain, and has a role in the development of brain, face and skull [1-3].

Various genetic alterations in *OTX2* have been described, including interstitial deletions [4, 5], microdeletions [6, 7], frameshift and point mutations [8]. The majority of the alterations are found in patients with severe ocular malformations such as anophthalmia, microphthalmia, Leber congenital amaurosis or coloboma [6, 8-10]. *OTX2* has been shown to be important for the regulation of *HESX1* [11, 12], one of the transcription factors involved in pituitary development. Since *HESX1* mutations have been described in patients with isolated growth hormone deficiency (IGHD) and combined pituitary hormone deficiency (CPHD), several recent studies also performed mutation and deletion screening of *OTX2* in patients with variable degree of pituitary dysfunction [7, 13-17]. In the majority of the patients, but not all [14], eye malformations were also present.

CPHD is any combination of two or more anterior pituitary hormone deficiencies. Mutations in several genes encoding the transcription factors involved in pituitary cell differentiation have been associated with CPHD (for review, see [18]). However, in the majority of CPHD patients, causative gene mutations remain unknown. Thus, in order to expand the mutation spectrum of CPHD, we performed mutation analysis of *OTX2* in a cohort of 92 CPHD patients from the Dutch HYPOPIT study. We describe a new *OTX2* mutation found in one of our patients with CPHD, who also had pituitary malformation and an underdeveloped left optic nerve. In addition to this new finding, we provide an overview of all phenotypic and genetic data reported to date.

Subjects and Methods

DNA samples were collected from 92 patients with CPHD, who participated in the Dutch HYPOPIT study [19]. These patients had been recruited from the Endocrinology Departments of six University and two non-University Hospitals, and they had been registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003. All patients had deficiencies of GH and of one or more additional hormonal axes. Deficiencies of hypothalamic-pituitary-thyroidal, -adrenal, and -gonadal axes were defined as abnormal TRH test or TSH levels that were low or inadequately low FT4; abnormal CRF / ACTH / glucagon test or ACTH levels which

were low or inadequately low cortisol and LH, FSH, estrogen / testosterone or LHRH test low for age or lack of spontaneous puberty after age 14 y. Prolactin deficiency was defined as abnormal prolactin during random sampling or TRH testing. Reference values of the individual hospitals were used. Exclusion criteria were: patients with known cause of CPHD, such as a brain tumor, brain surgery, brain radiation and known syndromes. Written informed consent was obtained from all participating patients and their parents or legal guardian.

DNA was isolated from whole blood collected in EDTA tubes according to standard procedures. *OTX2* (NM_021728.2) PCR amplification and sequencing was performed as previously described [20].

Generation of plasmids

The *OTX2* cDNA and multiple bicoid binding site luciferase reporter construct (pGL2) were cloned as previously described [14]. The *OTX2* Pro134Arg mutation was designed according to the QuickChange Site-direct mutagenesis protocol (Stratagene) after introducing a single base change using specific primers (available upon request). After DNA amplification and Maxiprep kit purification (Qiagen), the sequence, orientation, and presence of the mutation in the plasmid were confirmed by DNA sequencing.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed with *OTX2* recombinant proteins and ³²P-labeled DNA fragments as previously described [14]. Wild-type and Pro134Arg mutation proteins were synthesized using the TNT coupled transcription and translation reticulocyte lysate system with T7 polymerase, according to the manufacturer's protocol (Promega Corp., Madison, WI). To determine *OTX2*-DNA binding, a consensus *OTX2* DNA binding site was used. These oligonucleotides were γ ³²P-end labeled with T4 polynucleotide kinase. For each EMSA, *in vitro* translated proteins were mixed with radiolabeled probe for 30 min at room temperature, along with deoxyinosine-deoxycytosine, salmon sperm, and binding buffer [50 mM KCl, 20% glycerol, and 20 mM HEPES (pH 7.6–7.8)]. Each sample was then separated by gel electrophoresis on a 5% nondenaturing acrylamide gel and analyzed by autoradiography. The *OTX2* complexes were supershifted with a polyclonal *OTX2* antibody. The binding was competed by addition of the unlabeled oligonucleotide in excess. ³⁵S-Methionine was added to our reticulocyte lysate system to confirm the efficiency of protein synthesis. The radiolabeled proteins were resolved on a 10% denaturing acrylamide gel and analyzed by autoradiography.

Transient transfection and cell culture

Transient transfections were performed in 293T and GN cell lines. Cells were maintained in DMEM high glucose 1X (4.5 g/L D-glucose) (Life Technologies, Inc., Grand Island, NY), supplemented with L-glutamine, 1% antibiotic-antimycotic

(100X) (Life Technologies), 110 mg/L sodium pyruvate, and 10% fetal bovine serum (Life Technologies). Cells were grown at 37 C in 5% CO₂ and were transfected at 40–60% confluency. Total DNA was kept constant, and nonspecific effects of viral promoters were controlled using the empty pSG5 vector (EV). Luciferase activity in relative light units (RLUs) was measured at 48 h using the Lumat LB 9507 (Berthold Technologies, Oak Ridge, TN).

293T transient transfections were performed in six-well tissue culture plates using lipofectamine reagent (Invitrogen). For functional studies, the pSG5-mOTX2 cDNA was used. A total of 125 ng of a multiple bicoid binding site promoter luciferase vector (pGL2-control vector) was cotransfected with 125 ng WT OTX2, 62.5ng EV/62.5 ng WT OTX2, 31.25 ng EV/62.5 ng WT OTX2/31.25 ng MUT OTX2, 62.5 ng WT OTX2/62.5 ng MUT OTX2, or 125 ng MUT OTX2. This same experiment design was repeated using GN cells.

Transfections were performed in triplicate for each condition within a single experiment, and experiments were repeated at least three times using different plasmid preparations of each construct. The relative luciferase activity for each control (pGL2) was set to one, and results were expressed as fold-promoter activation and represented as the SEM of representative experiments.

Statistical methods

Transient transfection results are expressed as the standard error of mean (S.E.M.). Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). The data were normalized to empty vector expression and graphs depict fold change over empty vector. Group means were compared using single ANOVA and Tukey's multiple comparison test, with P values less than 0.05 considered statistically significant.

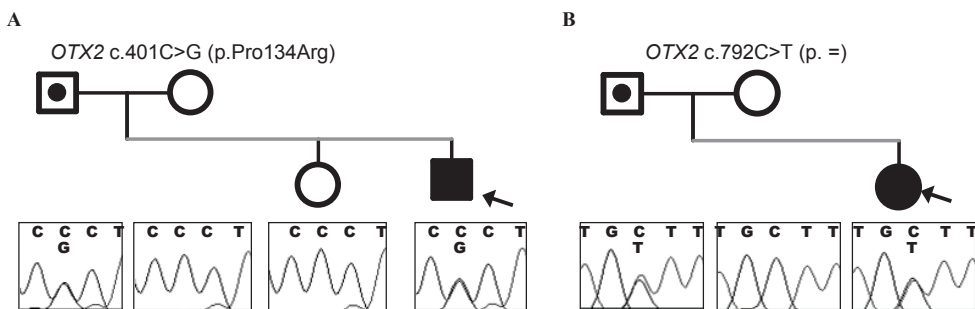


Figure 1. OTX2 mutation analysis in patients with CPHD. Pedigrees of the patients with missense and silent mutation (the black arrow indicates index case), and representative chromatograms of sequences.

Results

We directly sequenced the complete coding region and intron-exon boundaries of *OTX2* in DNA samples from 92 patients with CPHD, in whom mutations in the classical CPHD genes *PROPI*, *POUIF1*, *HESX1*, *LHX3* and *LHX4* had been ruled out [19]. In one patient, we identified a new missense mutation c.401C>G in exon 5, which replaces proline (CCC) with arginine (CGC) at amino acid position 134 (Fig. 1A). The mutation is heterozygous and inherited from the father. Additionally, we identified a synonymous change c.792C>T (p. =) in another CPHD patient (Fig. 1B). Finally, our sequencing results showed two known polymorphisms: rs2277499 in the exon 4 (minor allele frequency 36%) and rs171978 in the exon 5 (minor allele frequency 7%).

Table 1. Clinical data of five patients with hypopituitarism and eye phenotype.

Case	Gender	Hypopituitarism	Ocular lesion	MRI abnormalities	<i>OTX2</i>
1	Male	CPHD	Underdeveloped left optic nerve, blindness of one eye	Ectopic posterior pituitary, invisible stalk	MUT Pro134Arg
2	Male	CPHD	SOD suspected, low vision	Ectopic posterior pituitary, invisible stalk	WT
3	Male	CPHD	SOD, hypertelorism, strabismus, epicanthus	Partially empty sella, absent posterior pituitary	WT
4	Female	IGHD	Strabismus divergens alternans, very low vision, 'aspecific form of SOD'	Small anterior pituitary, ectopic posterior pituitary, very small sella	WT
5	Male	IGHD	Cavum septum pellucidum (described in SOD patients)	Very small anterior pituitary, ectopic posterior pituitary	WT

CPHD, combined pituitary hormone deficiency; IGHD, isolated growth hormone deficiency; SOD, septo-optic dysplasia; MUT, mutation; WT, wild-type

Clinical Features

Patient 1, carrier of the *OTX2* p.Pro134Arg mutation, is a male, born from Dutch Caucasian non-consanguineous parents after 40 weeks of gestation. His birth length was 50 cm and his birth weight 3600 grams. He had neonatal hypoglycemia. His MRI was clearly abnormal, with an ectopic posterior pituitary, an absent pituitary stalk and an underdeveloped left optic nerve. At the age of 8 years, Clonidine test showed a GH peak of 3.4 mU/L (normal peak above 20 mU/L). Before start of GH treatment, his IGF-I was 1.9 nmol/L (-5.3 SDS) and IGFBP-3 0.6 mg/L (-6.7 SDS). GH supplementation was started at the age of eight years when his height was 1.15

m (-3.2 SDS). At the start of GH treatment, his bone age delay was 3.5 years. Central hypothyroidism was detected at the age of nine years, based on a FT4 level of 8.0 pmol/L (normal range 12 – 26 pmol/L) and an abnormally low TSH for this low FT4 (2.7 mU/L); thyroid hormone supplementation was then started. Before the start of GH treatment, morning cortisol levels were normal (at 8h 0.22 μ mol/L), but at the age of 11 years the analysis was repeated and he had low cortisol levels: at 8h 0.04 μ mol/L (reference 0.2 – 0.8 μ mol/L) and at 14h 0.06 μ mol/L (reference 0.1 – 0.4 μ mol/L), ACTH was also low (12 ng/L, reference 20 – 80 ng/L); and therefore hydrocortisone treatment was started. At age of 13 years he was still totally prepubertal (Tanner stage G1 P1) with low LH (1.0 U/L) and FSH (1.8 U/L) values; at the age of

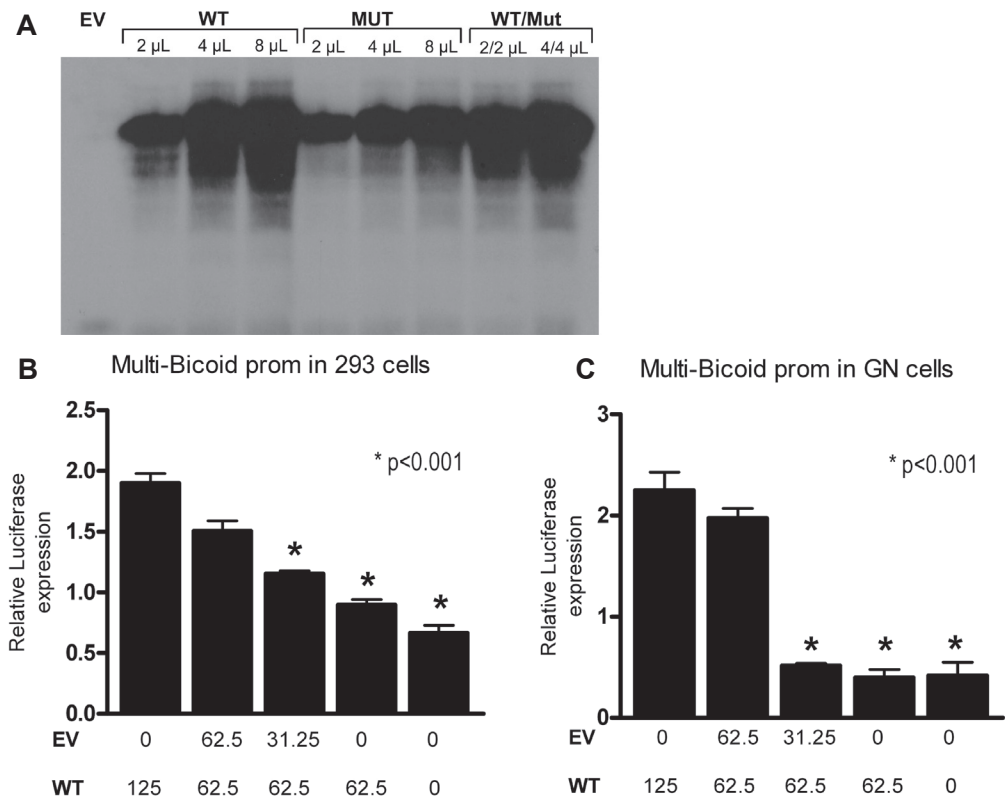


Figure 2. Functional studies. (A) EMSA was performed using the consensus OTX2 binding sequence incubated with *in vitro* transcribed and translated empty vector (EV), wild-type OTX2 (WT) or OTX2 Pro134Arg mutant (MUT) proteins. Transient transfection studies were performed in a heterologous cell line, 293T (B) or a GnRH expressing cell line, GN (C). A multiple bicoid binding site luciferase reporter construct (pGL2) was transfected along with expression vectors containing WT OTX2, MUT OTX2, or empty pSG5 (EV). The promoter activity seen with OTX2 expression or equal amounts of either EV/WT or WT/MUT OTX2 was compared with that seen with pGL2. In addition, in both cells lines, WT OTX2 concentration is held constant as that of the MUT OTX2 expression vector was increased. Percent expression of the multiple bicoid binding site reporter plasmid is calculated with respect to WT. Each independent experiment was performed in triplicate. The graphs show the mean \pm S.E.M. of the fold change from at least 5 representative experiments. For each experiment the coefficient of variation values were less than 10%, * p < 0.001.

13 years and 10 month he was still prepubertal, with a low testosterone for his age (< 0.1 nmol/L); induction of puberty was started. Patient has a complete pituitary hormone deficiency and continued GH treatment after reaching adulthood. He has severe behavioral problems. The father's adult height SDS is -1.0 (174 cm), and the mother's adult height SDS 0.8 (174 cm).

Patient 2, carrier of the *OTX2* c.792C>T (silent) mutation, is a female, born to Dutch Caucasian, non-consanguineous parents after 39 weeks of gestation. Her birth weight was 3120 g and birth length 52 cm. Her MRI showed an empty sella: absent anterior pituitary, ectopic posterior pituitary and absent stalk. Thyroid, growth hormone and hydrocortisone were supplemented at the age of 2 years and 7 months. Prolactin levels were normal. Due to the absence of spontaneous puberty, puberty was induced when the patient was fourteen years old. The father's adult height SDS is -0.8 (176.5 cm), and the mother's adult height SDS is -0.5 (166.5 cm). The patient has one healthy sister.

Functional studies

We performed structural and functional studies in order to determine the mechanism by which the *OTX2* Pro134Arg mutation affects target gene expression. EMSA showed that both mutant and wild-type proteins bind to the *OTX2* consensus site (Fig. 2A); the amount of protein synthesized was equivalent between wild-type and the mutant (data not shown).

The ability of wild-type and mutant Pro134Arg *OTX2* protein to activate gene expression was measured using luciferase reporter gene assay systems. A multiple bicoid binding site was fused to a luciferase reporter gene and co-transfected with expression vectors containing either wild-type or mutant DNA into a heterologous 293T human embryonal cell line (Fig. 2B) or GN cell line, a GnRH expressing neuronal cell line (Fig. 2C). In 293T cells, wild-type (WT) *OTX2* protein induced a 1.9 ± 0.08 fold increase (48%) in luciferase activity. Equivalent amounts of WT and empty vector similarly demonstrated a 1.5 fold increase (34%) in transactivation. While maintaining a constant WT DNA and introducing the mutant *OTX2* vector, there was a 40% decrease in luciferase gene expression compared to WT response. When equal amounts of WT and mutant *OTX2* DNA were cotransfected, we note a 53% decrease in expression. Further inhibition (65%) was seen with transfection of mutant alone. Cotransfection of WT and the multiple bicoid binding site reporter construct into GN cells induced a 2.25 ± 0.17 increase (56%) in transactivation. Equivalent amounts of WT and EV led to a 1.97 ± 0.09 fold increase. A decrease of activity was measured with the introduction of mutant vector during cotransfection, demonstrating a 77.4% decrease in expression compared to WT alone. Cotransfection of equal amounts of WT and mutant DNA demonstrated an 83% decrease in expression.

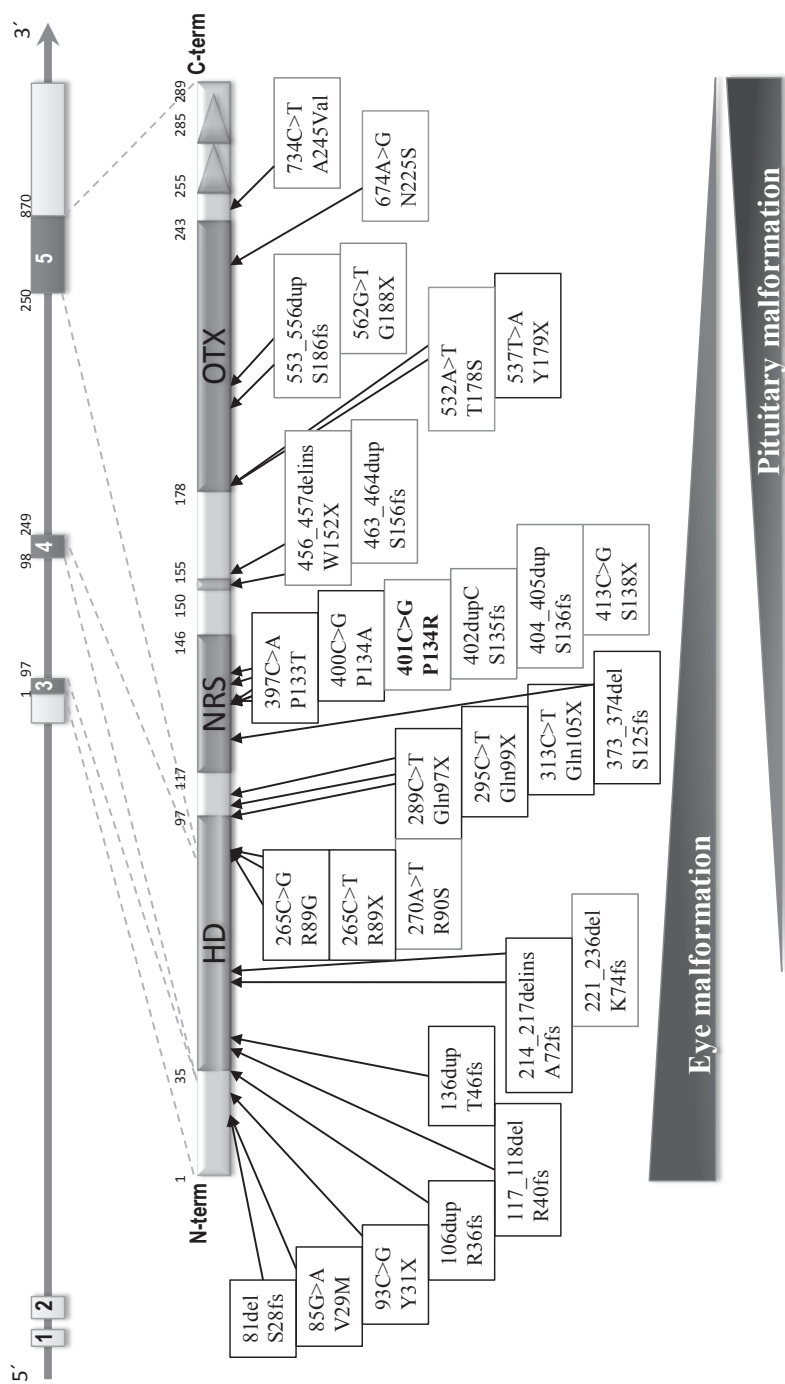


Figure 3. OTX2 structure and mutation mapping. Genomic and protein structure of human OTX2 major isoform b (NM_172337.1; NP_758840.1). Protein domains are defined as described in Chatelain *et al* [22] and the conserved domains. Domain abbreviations: HD (homeobox domain); NRS (nuclear retention signal); OTX (OTX family domain); orange box represents the SIWSPA conserved motif; the closed triangles represent two tandem repeated conserved transactivation motifs. OTX2 mutations in patients described with only eye malformation are indicated in black boxes, with additional pituitary malformations in green boxes, and exclusively with pituitary malformations in blue boxes. The novel mutation is presented in bold. Horizontal triangles represent the gradient of the eye and pituitary phenotypes described in the patients with OTX2 mutations.

Discussion

We performed *OTX2* mutation screening by direct sequencing in 92 Dutch CPHD patients. We identified a novel missense mutation, p.Pro134Arg, in a patient with pituitary malformation, complete pituitary hormone deficiency, an underdeveloped left optic nerve and severe behavioral problems. The same amino acid was mutated in a patient published by Ragge *et al* [8], but in that patient, the change was to alanine. The fact that the very similarly affected mother of Ragge's patient did not have the same mutation, made it highly unlikely that the p.Pro134Ala sequence variant was a major contributor to the phenotype. However, alanine is a neutral, non-polar and hydrophobic amino acid whereas arginine is basic, positively charged and hydrophilic. The difference in chemical characteristics between these amino acids means that the p.Pro134Arg mutation can have an entirely different functional impact than p.Pro134Ala.

Furthermore, we identified a new silent mutation c.768C>T, not described until date in any control nor patient population.

Our CPHD cohort included three CPHD patients with eye malformations. Apart from the CPHD cohort, the HYPOPIT study includes an IGHD cohort of which two patients had eye malformations. We directly sequenced all CPHD 92 patients, as well as the two IGHD patients with eye malformations and found that both were wild-type for *OTX2*. Table 1 shows the clinical characteristics of all five, CPHD and IGHD, patients. Only one patient from our study had an underdeveloped optic nerve; the other four patients had ocular malformation related with septo-optic dysplasia (SOD). It is remarkable that the *OTX2* mutation happened to be present in the only patient with an underdeveloped optic nerve and not among the four patients with other SOD-related ocular malformation.

The Pro134Arg mutation is located outside the binding domains of the *OTX2* protein, therefore, as would be expected, no alterations in binding affinity would be predicted. Indeed EMSA showed that binding to the consensus site by the mutant protein was similar to that of the wild-type protein (Fig. 2A). Functional expression studies clearly demonstrate that the *OTX2* Pro134Arg mutant protein was not able to activate target gene expression (Fig. 2B and C) and therefore delineate a etiology of pathogenesis leading to our patient's optic phenotype and hypopituitarism. Since the mutation was present in the heterologous state, studies to determine the mechanism of inhibition of gene activation were performed. A dominant negative effect was noted with increasing concentrations of mutant *OTX2* resulting in decreased gene expression. Interestingly, in the GnRH expressing cell line, mutant *OTX2* inhibition of reporter expression was noted at lower concentrations. Previous studies have shown that *OTX2* is required for GnRH expression [21]; therefore, our studies further supports the role for the mutant protein in the pathogenesis of the central hypogonadism seen in our patient.

To date, 29 different *OTX2* mutations have been reported in 32 unrelated


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MMSYLKQ-PPYAVNGLSLTTSGMDLLHPSVGYP-----ATPRKQRRE   41
MMSYLKQ-PPYAVNGLSLTTSGMDLLHPSVGYPGWASCPAATPRKQRRE   49
MMSYLKQ-PPYAVNGLSLTTSGMDLLHPSVGYPGWASCPAATPRKQRRE   49
MMSYLKQ-PPYAVNGLSLTTSGMDLLHPSVGYP-----ATPRKQRRE   41
MMSYLKQ-PPYTVNGLSLTTSGMDLLHPSVGYP-----ATPRKQRRE   41
MMSYLKQ-PPYGMGGLGLAGPAMDLLHPSVGYP-----ATPRKQRRE   41
MMAYMNPGRPHYSVNALASGPSVDLMHQAVPYP-----SAPRKQRRE   42
*****: * :*: *: *: *: *: *: *: *: *: *: *: *: *****

RTTFTTRAQLDLVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   91
RTTFTTRAQLDLVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   99
RTTFTTRAQLDLVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   97
RTTFTTRAQLDLVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   99
RTTFTTRAQLDLVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   91
RTTFTTRSQDLVLLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRA   91
RTTFTTRQLLEELEALFAKTQYPDVYAREEVALKINLPESRVQVWFKNRRA   92
*****: *: *****: *: *: *****: *****: *****: *****

KCR----QQQQQQGQGNKVPAKKKT--SP---AREVSSESGTSGGQF      131
KCR----QQQQQQGQGNKVPAKKKT--SP---AREVSSESGTSGGQF      139
KCR----QQQQQQGQGNKVPAKKKT--SP---AREVSSESGTSGGQF      139
KCR----QQQQQQGQGNKVPAKKKS--SP---AREVSSESGTSGGQF      131
KCR----QQQQQQGQGNKVPAKKKS--SP---AREASSESGSGGF       131
KCR----QQQQ--SGSGTSKRPAKKKS--SP---VRESSG-SGSSGQF     127
KCRQQRQQQQQQQPFGQAARPAKRKAGTSRPSTDVCDPLGISDSY      142
***          **    * ****: **    *          *          * ..

TPPSSTSVPPTIASS-----APVSIW      152
TPPSSTSVPPTIASS-----APVSIW      160
TPPDSTSVPTIASS-----AHGSIW      160
SPDSTSVPTIASS-----APVSIW      152
TPPSSTSVPAISSTTT-----APVSIW     152
TPPAVSSASSSSASSSANPAAAAAGLGPNVAASSLSTPAASS      177
SPPLPGFSGSPTTAV-----ATVSIW      163
**          : : :                               * ***

SPASISP-----LSDPLSTSS--SCMQR-----SYPMITYQT      181
SPASISP-----LSDPLSTSS--SCMQR-----SYPMITYQT      189
SPASISP-----LSDPLSTSS--SCMQR-----SYPMITYQT      189
SPASISP-----LSDPLSTSS--SCMQR-----SYPMITYQT      181
SPASISP-----LSDPLSTSS--SCMQR-----SYPMITYQT      181
SPASISPGAPASVSVEPLAAPNTSCMQRSVAGAATAAASYPMSYGO      227
SPASESP-----LPEAQRAGLVAGSPSLTS-----APYAMTYAP      197
**** *           : . :          *          * : * :

ASGYS---QGAYG-STSYFGGMDCGSYLTPMHHQLPGPGATLSPMGTNNAV   227
ASGYS---QGAYG-STSYFGGMDCGSYLTPMHHQLPGPGATLSPMGTNNAV   235
ASGYS---QGAYG-STSYFGGMDCGSYLTPMHHQLPGPGATLSPMGTNNAV   235
ASGYS---QGAYG-STSYFGGMDCGSYLTPMHHQLPGPGATLSPMGTNNAV   227
ASGYS---QGAYG-STSYFGGMDCGSYLTPMHHQLTPGSTLSPMSNNAV    227
GSGYG---QGYPTPSSSYFGGVDCSSYLAPMHSHHP--HQLSPMAPSSM    272
ASAFCSPPSAYGS-PSSYFSGLDP--YLSFMVQLGG--PALSPLSGSPSV    242
...      .*      .***.* *  **:* :          * : : :

TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD      261
TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD      269
TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD      269
TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD      261
TSHLNQS-----PASLST-----QGYGASSLGFNSTADCLDYKD      261
AGHHHHHHHAHHPLSQSGHHHHHHHHHHQHGYGSGGLAFNS-ADCIDYE    321
GPSLAQS-----PTLSLG-----QSYGAY-----SFVDSLDFDK      271
:          *          *          *          *          * :*. :*. :

-----QTASWKLNFN-ADCLDYKDQTSWWKFQVL 289
-----QTASWKLNFN-ADCLDYKDQTSWWKFQVL 297
-----QTASWKLNFN-ADCLDYKDQTSWWKFQVL 297
-----QTASWKLNFN-ADCLDYKDQTSWWKFQVL 289
-----QASSWKLNFN-ADCLDYKDQTSWWKFQVL 289
PGAAAASSAWKLNFNSPDCLDYKDQAS-WRFQVL 354
-----PTGTWFTYTMPDPLDYKDQSA-WKFQIL 299
.:*.*.*: * *****: *****: *****

```

Figure 4. Conservation annotation of known mutations in OTX2 and related proteins. A clustalW2 based multiple alignment of OTX2 human isoforms a and b, orthologs from *Macaca mulata*, *Mus musculus* and *Danio rerio*, and paralogs OTX1 and CRX proteins. Homeobox and SIWSPA domains are marked with upper line. Protein changes are highlighted in grey and bold (only, missense), italic (nonsense) and underline (frameshift deletion or insertions).

patients, including the new missense mutation found in our study (Table 2, Fig. 3 and 4). The first described genetic defects that affected the *OTX2* gene were interstitial deletions at 14q22, in association with anophthalmia and pituitary abnormalities [4, 5]. Using the candidate gene approach, Ragge *et al* screened a large cohort of 333 patients with anophthalmia, microphthalmia and/or coloboma identifying the first eight mutations [8]. In the following years, several other studies performed *OTX2* gene sequencing in patients with severe ocular malformations [6, 9].

HESX homeobox 1, or HESX1, is an important transcription factor involved in pituitary development. HESX1 has been shown to be a transcriptional target of *OTX2* [11, 12], thus our current screening of *OTX2* was a logical step following our previous search for *HESX1* mutations [19].

The pathogenicity of the *OTX2* mutations is not easy to interpret, although several studies performed a detailed analysis of the biological effects of these mutations [7, 13, 14, 22]. The inheritance pattern of the *OTX2* mutations is usually described as autosomal recessive with variable phenotype or incomplete penetrance [6-8, 17]. A relatively large number of alterations were found to be *de novo*, especially in all cases with whole gene deletions and translocations [4-8, 13, 16, 23]. The remarkable exception to this is the duplication 14q22.3 [24] that was inherited from the affected father and was present in several family members with less severe developmental delay. However, some cases add more complexity to this pattern, like two cases of gonosomal mosaicism inheritance described by Ragge *et al* [8] (Table 2: mutations 1, 25a and b). Moreover, Wyatt *et al* describe a family where normal parents without mutations had two affected children with the same mutation but different phenotypes [6] (Table 2: mutation 4); parental mosaicism was excluded in blood and buccal cells. In many cases, one or several relatives are carriers of the same mutation and have apparently normal or mild phenotype (Table 2: mutations 2, 5, 11, 12, 13, 15, 16, and 29). In this regard, it should be noted that all mutations and deletions found to date are strictly heterozygous, although several cases with additional mutations in other genes have been described [6, 25] (Table 2: mutations 2 and 25c). The above-mentioned cases suggest the existence of other genetic or environmental factors which influence the phenotype of patients with *OTX2* mutations. Similar phenomena are seen in mouse models; for example, the *Otx2*-null mice have a severe head phenotype, but heterozygous animals have a variable phenotype ranging from apparent normality to severe developmental eye and head abnormalities, dependent on genetic background [1-3].

It is known that prenatal exposure to teratogens can cause eye malformation, therefore environmental factors may also play a role in the phenotype and explain some of the differences between individuals with the same mutation. In at least two cases, the mothers of the patients reported exposure to probably damaging factors during pregnancy [8] (Table 2: mutations 1 and 13).

In order to draw global conclusions from the mutational analyses performed to date, we summarized the known 29 *OTX2* mutations together with reported pheno-

typic data in Table 2 and Figures 2 and 3. Mutations within *OTX2* are found all over the gene, affecting all three coding exons. However, the distribution seems to have several hotspots affecting functional domains like the homeobox, the nuclear retention signal or the highly conserved SIWSPA peptide sequence ('SIWSPA conserved motif', Fig. 4). There are several mutations affecting the same amino acid, in various unrelated patients with different phenotypes (Table 2: mutations 9 and 10; 17 and 18; 25, 27, 28). Most mutations are located in conserved residues (Fig. 4), which suggests functional relevance. However, their importance is not always functionally proven.

There seems to be a clear relationship between the localization of the reported mutations and clinical phenotype. Mutations in the N-terminus show very severe eye malformations without pituitary phenotype, while, mutations localized to the C-terminus are associated with pituitary malformation and CPHD. All patients reported to date with CPHD, IGHD or short stature have their mutations in exon 5, except for p.Lys74SerfsX30 (K74fs in Fig. 3). Although many patients have growth retardation or developmental problems during childhood, most of them are born with weights and lengths within the normal range. Patients with CPHD, IGHD or short stature are overrepresented in the nuclear retention signal and OTX family domain (Fig. 3). Additional research is needed to provide better understanding of the different functional regions of *OTX2*.

Another conclusion that can be drawn when reviewing the literature is that alterations in the *OTX2* affects both genders equally; furthermore, there does not seem to be any relationship between the localization of the mutations and their inheritance (paternal, maternal inheritance or *de novo*).

Although several investigators have already implicated mechanisms and potential additional factors by which *OTX2* mutations can cause the associated phenotype, further research is needed to more clearly define the role of *OTX2* in pituitary pathology. Our current studies provide further support for the important role of *OTX2* as a candidate gene in genetic screening in patients with CPHD or IGHD. In addition, we provide further evidence regarding the association between mutations in the C-terminus of the *OTX2* gene and clinical presentation of pituitary abnormalities; therefore emphasizing the need for a careful evaluation of the genetic sequence in this area of the *OTX2* gene.

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Table 2. OTX2 mutations reported until date.

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	Ref.
1 c.81delC	p.Ser28ProfsX23	Yes (totally inactive) [22]	Severe bilateral MO	Maternal gonosomal mosaicism (carrier)	Consanguineous parents; mother had affected fetus (bilateral MO) carrier of the same mutation; maternal polymorphic chromosome variant inv(2)(p11.2;q13)	[8]
2 c.85G>A	p.Val29Met	Not done	Donnai-Barrow syndrome (bilateral iris coloboma, cataract & retinal abnormalities)	Father carrier (no phenotype)	LRP2 homozygous deletion c.11469_11472delTTTG [26]	[6]
3 c.93C>G	p.Tyr31X	Not done	Left MO & right nystagmus	De novo		[6]
4 c.106dupC	p.Arg36ProfsX52	Not done	Siblings: (a) right MO (b) right AO & left coloboma	De novo	Both parents are wild-type (gonosomal parental mosaicism?)	[6]
5 c.117_118delCC	p.Arg40GlyfsX47	Yes (totally inactive) [22]	Bilateral AO, small optic nerves, thin chiasm; hypotelorism; DD	Mother mutation carrier (no phenotype)	Small stature	[8]
6 c.136dupA	p.Thr46AsnfsX42	Not done	(a) bilateral MO & severe optic nerve hypoplasia (b) father: unilateral MO, cataract, optic nerve aplasia, anterior segment dysgenesis	Affected father carrier	Caucasian; (a) sacral dimple & anteriorly placed anus	[9]
7 c.214_217delGCAC insCA	p.Ala72HisfsX15	Yes (no nuclear localization; no transactivation)	Bilateral MO; normal stature	De novo	Japanese; 1y old at the moment of the study	[7]

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	Ref.
8 c.221_236del	p.Lys74SerfsX30	Yes (no nuclear localization; no transactivation)	Right AO & left MO; DD; IGHD; pituitary hypoplasia; retractor testis	De novo	Japanese	[7]
9 c.265C>G	p.Arg89Gly	Yes (weak binding activity & reduced transactivation) [22]	Bilateral MO, optic-nerve aplasia, absent chiasm	De novo	Cognitive & language skills age-appropriate; mother had stillborn infant	[8]
10 c.265C>T	p.Arg89X	Not done	Bilateral AO	De novo		[10]
11 c.270A>T	p.Arg90Ser	Yes (inhibited DNA binding & transactivation)	Right unilateral AO; IGHD; pituitary hypoplasia; ectopic posterior lobe	Father carrier (short, no ocular phenotype)	Jewish family with different ocular phenotype (no genetic data)	[17]
12 c.289C>T	p.Gln97 X	Not done	Siblings: (a) extreme bilateral MO (b) right inferior iris coloboma & left retinal coloboma	Father carrier (reduced vision in one eye)	Dizygotic twins	[6]
13 c.295C>T	p.Gln99X	Yes (no transactivation activities) [22]	Bilateral MO, absent optic nerves & chiasm; asymmetry of the lateral ventricles; seizures	Father mutation carrier (no phenotype)	Intracerebral bleed in the newborn period	[8]
14 c.313C>T	p.Gln105X	Not done	Bilateral AO, bilateral dysplastic globes, optic nerve aplasia	De novo	Caucasian; latent Wolf-Parkinson-White syndrome; feeding difficulties	[9]
15 c.373_374delAG	p.Ser125TrpfsX11	Not done	Bilateral AO; mild DD	Mother carrier (no phenotype)		[6]

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	Ref.
16 c.397C>A	p.Pro133Thr	Yes (normal) [22]	Right MO & cataract, left sclerocornea	Mother & brother mutations carriers (no phenotype)		[8]
17 c.400C>G	p.Pro134Ala	Yes (normal) [22]	Left AO; early mild DD, attention-deficit/hyperactivity disorder	De novo	Mother with left AO is wild-type; half brother with learning difficulties	[8]
18 c.401C>G	p.Pro134Arg	Yes (dominant negative effect)	CPHD; underdeveloped left optic nerve; pituitary hypoplasia	Father carrier	Dutch; General GH deficient appearance; severe behavioral problems	This study
19 c.402dupC	p.Ser135LeufsX2	Yes (no transactivation activities)	Bilateral AO, short stature & partial IGHD; DD	De novo	MRI normal; Japanese	[13]
20 c.404_405dupCT	p.Ser136LeufsX43	Yes (not transactivation HESX1 & POU1F1)	Bilateral AO; CPHD; pituitary hypoplasia, ectopic posterior lobe & chori malformation; DD	De novo	Japanese	[16]
21 c.413C>G	p.Ser138X	Not done	GHD (low IGF1 & IGFBP3); alternating esotropia with mild torsional nystagmus	De novo	Failure to thrive & feeding	[15]
22 c.456_457delGAinsAT	p.Trp152X	Not done	Right AO & left MO, bilateral optic nerve hypoplasia, absent chiasm; CPHD; microcephaly; DD	De novo	Caucasian; consanguineous parents; feeding difficulties	[9]
23 c.463_464dupGC	p.Ser156LeufsX23	Yes (no transactivation activities) [22]	Right AO & left MO, iris & chorioretinal coloboma, total retinal detachment on the left; partial agenesis of corpus callosum, absent right & small left optic nerves, thin chiasm; growth delay; DD	De novo	Mother had hypopigmented macula & fundus; normal pituitary MRI and function	[8]

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	Ref.
24 c.532A>T	p.Thr178Ser	Yes (normal transactivation)	CPHD	parents re-fused molecular analysis	Japanese; without ocular anomalies	[7]
25 c.537T>A	p.Tyr179X	Yes (no transactivation activities) [22]	Siblings: (a) Bilateral MO & colobomata; severe DD & seizures (b) LCA, bilateral peripheral anterior synechiae	Maternal gonosomal mosaicism (with pigmentary retinopathy)	(a) Has bilateral fifth finger clinodactyly (b) has unilateral right-sided hearing loss Both with small stature(?)	[8]
26 c.553_556dupTATA	p.Ser186IlefsX2	Not done	(c) MO, aniridia, microcephaly & café au lait macules	Father with LCA	Also missense mut <i>PAX6</i> p.R38W & nonsense mut <i>NF-1</i> p.R192X	[25]
27 c.562G>T	p.Gly188X	Yes (50% reduced transactivation)	Bilateral MO, hypoplastic optic nerves, small optic chiasm; pituitary hypoplasia; microcephaly & DD (a) Bilateral MO; CPHD; pituitary hypoplasia; DD (b) Bilateral MO; DD; seizures	De novo	Hispanic; hypoplastic labia minora, hypotonia, failure to thrive	[9]
28 c.674A>G	p.Asn225Ser	Yes (dominant negative effect)	(a) CPHD; pituitary hypoplasia (b) CPHD; pituitary hypoplasia	Not available	Japanese	[7]
29 c.734C>T	p.Ala245Val	Yes (normal transactivation)	Bilateral optic nerve hypoplasia & short stature	Not reported	Japanese; normal stature, normal MRI	[7]
				Not reported	Without ocular anomalies	[14]
				Not reported	Without ocular anomalies	[14]
				Father carrier (normal phenotype)	Japanese	[7]

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	Ref.
Microdeletion chr 14, 46,XX,del (14Xq22q23)	Whole gene	n.a.	Bilateral AO; absent pituitary, optic nerves, chiasma and tracts; underdeveloped genitalia and micrognathia	De novo	Caucasian; fetus, forth abortion	
Microdeletion chr 14, 46,XY,del(14q22.1-q22.3)	Whole gene	n.a.	Bilateral AO, micrognathia, hypogonadism, hypothyroidism, growth retardation, DD, dentation delay	De novo		
Balanced translocation 46,XY,t(3;14)(q28;q23.2)	Whole gene	n.a.	AO, IGHD, pituitary hypoplasia, absence of optic nerves, chiasm & tracts; ear anomalies; undescended testes; DD	De novo	9.66 Mb deletion, including genes: <i>BMP4</i> , <i>OTX2</i> , <i>RTN1</i> , <i>SIX6</i> , <i>SIX1</i> & <i>SIX4</i> , Syndactyly & brachydactyly	[23]
Microdeletion Chr14:53758044-56834649	Whole gene	n.a.	Bilateral extreme MO	De novo	3.07 mb deletion	[6]
Microdeletion Chr14:56268037-57541514	Whole gene	n.a.	Bilateral AO; DD (maybe due to head trauma)	De novo	1.28 mb deletion	[6]
Microdeletion Chr14:56006531-58867091 (NC_000014.7)	Whole gene	n.a.	Right MO & left AO; DD, IGHD & pituitary hypoplasia	De novo	Japanese; 2.9 Mb deletion + 931bp addition, other 16 genes deleted	[7]
Duplication 14q22.3-q23.3 insertion in 13q21	Whole gene	n.a.	Branchiootorenal syndrome and Oculoauriculovertebral spectrum: growth delay, microcephaly, micrognathia, right-sided optic nerve hypoplasia, hearing loss, significant DD	Father	father & 2 other family members with same aberration and DD	[24]

Description of mutations is based on the NM_172337.1 reference sequence, follow HGVS recommendations (www.hgvs.org/mutnomen). Position +1 refers to the A position of the ATG initiation codon for that gene. Nomenclature may differ from the notation used in the original publication. The novel mutation is in bold type.

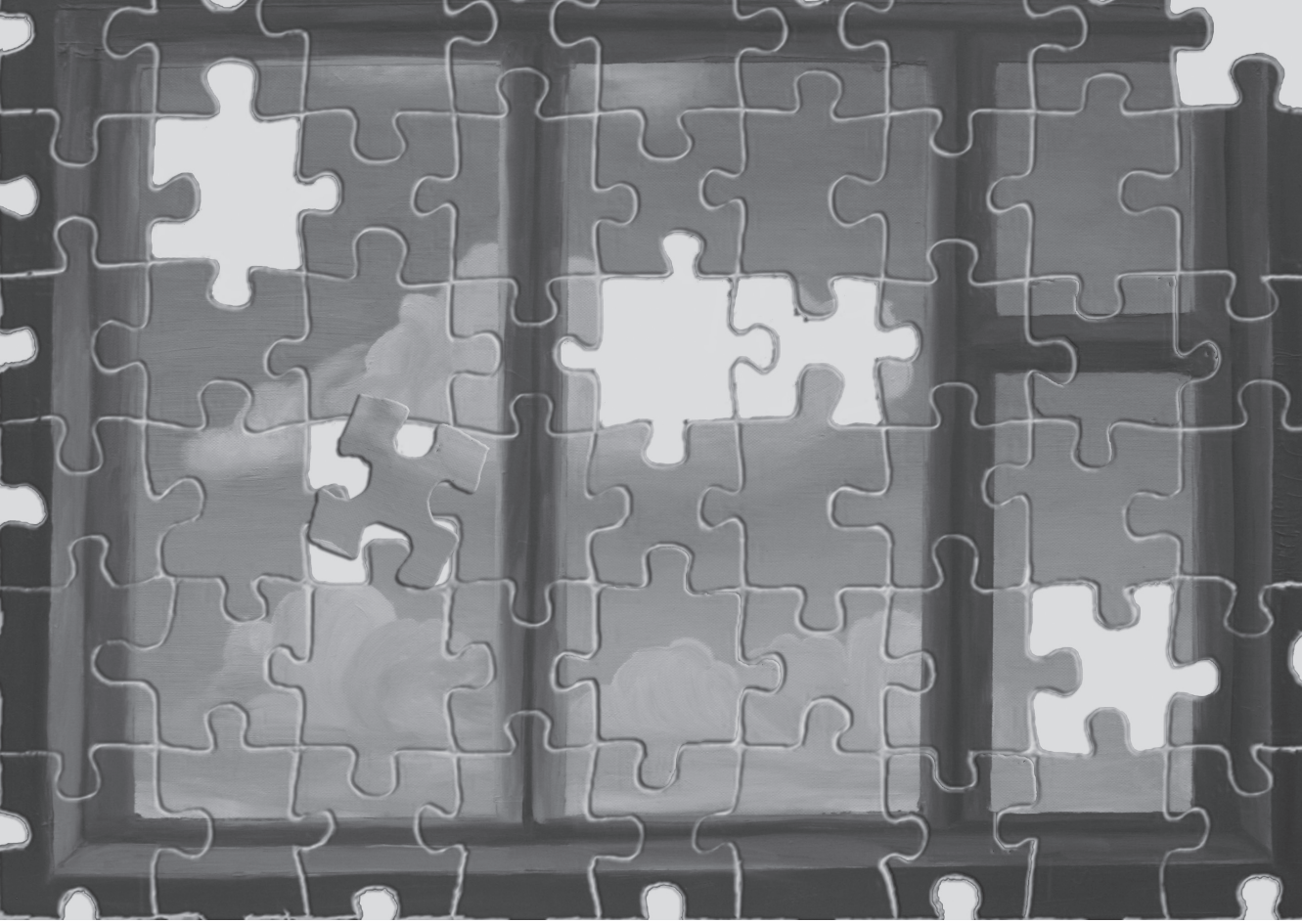
AO, anophthalmia; MO, microphthalmia; LCA, Leber congenital amaurosis; DD, developmental delay; IGHD, isolated growth hormone deficiency; CPHD, combined pituitary hormone deficiency; n.a., not applicable.

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Chapter 5

Growth Hormone Insensitivity Syndrome caused by a heterozygous *GHR* mutation: phe- notypic variability due to moderation by non- sense-mediated decay.

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Abstract

Growth hormone insensitivity syndrome (GHIS) is characterized by extreme short stature and resistance to the actions of growth hormone. The heterogeneity ranges from the most severe form, known as Laron syndrome, to less severe phenotypes like idiopathic short stature and partial growth hormone insensitivity. Here, we aimed to identify and characterize the molecular cause of severe short stature in a patient with resistance to GH treatment.

Patient: We describe a male patient born small for gestational age (38 weeks gestation, length 38.5 cm; -7.8 SDS, weight 1350 g; -4.84 SDS). At the age of seven years (109.7 cm; -2.89 SD) he received GH treatment (1 mg/m²/day) for one year without any increase in height SDS, IGF-I or IGFBP-3 levels. Double-dose GH treatment for another year did not result in any improvement of growth factor levels either. The patient does not have the typical Laron craniofacial and somatic features.

Results: Analysis of *GHR* showed a heterozygous nonsense mutation (c.703C>T; p.Arg217X). Extensive mutation screening as well as copy number variation analysis of other candidate genes in the GH-IGF1 axis excluded any additional genetic defects. Analysis of the patient's fibroblasts showed that GHR mRNA expressed from the mutant allele was degraded by a mechanism called nonsense-mediated mRNA decay (NMD).

Conclusions: GHIS in this patient is due to a heterozygous nonsense mutation in *GHR*. Our study is the first to demonstrate that nonsense-mediated decay is involved in the phenotypic variability of GHIS caused by *GHR* mutations.

Introduction

Growth hormone insensitivity syndrome (GHIS; OMIM #262500) is defined as a highly heterogeneous spectrum of disorders with short stature and resistance to the actions of growth hormone. The clinical characteristics range from the most severe form, known as Laron syndrome [1], to a less severe form in which the partial growth hormone sensitivity presents as “idiopathic short stature” (ISS).

The classical Laron syndrome (MIM 262500) is a rare autosomal recessive disorder resulting from a mutation in the growth hormone receptor (*GHR*). The phenotype is very similar to GH deficiency, but associated with normal to elevated serum concentrations of GH and abnormally low serum levels of IGF-I. Laron syndrome is characterized by severe postnatal growth failure and characteristic features such as midfacial hypoplasia. Other findings include poor musculature, delayed motor development, prominent forehead, laryngeal hypoplasia, hip dysplasia, osteopenia, thin skin, sparse and thin hair, and microphallus [2]. In contrast to the classical Laron syndrome, the term ISS describes a wide range of children with height more than two standard deviations below the mean for age and without specific endocrine defects. Some ISS patients have partial GHIS.

Several studies in children with ISS have identified heterozygous mutations in *GHR* [3-7]. Woods *et al.* hypothesized that some heterozygous mutations in *GHR* might cause partial GHIS and growth retardation, with evidence for a heterozygous effect in the parents of the affected children [8].

The *GHR* gene (MIM 600946) has ten exons, of which nine encode the mature GHR protein. Exon 1 and large part of exon 10 are untranslated regions, while most of exon 2 encodes the signal peptide. The mature GH receptor contains 638 amino acids and consists of an extracellular domain (encoded by exons 3 to 7), a single transmembrane domain (exon 8) and a cytoplasmic domain (exons 9 and 10).

The downstream signaling pathways mediated by the GHR include JAK2, IRS-1 and -2, SHC and members of the STAT family. They are activated upon binding of GH to the extracellular domain of the GHR. Recent studies show that GHR is predominantly expressed as a dimer on the cell surface [9]. Currently, the most accepted model is that one GH molecule asymmetrically binds to the receptor binding sites of preformed GHR dimers, which causes relative rotation resulting in activation of JAK2 [10].

The first *GHR* mutation, identified in 1989 [11], was a complex gene deletion and to date more than 70 unique mutations have been identified in more than 250 GHIS patients [1, 2, 12]. The most commonly described are nonsense, missense and splice site mutations. The majority of the identified genetic aberrancies, affecting the GHR, occur in the region encoding the extracellular domain of the receptor.

The phenotype-genotype relationship of GHIS is widely variable. Patients with the same phenotype may show genetic heterogeneity, and the same mutation can be

associated with wide variations in biological severity [8].

We now report the extensive investigation in a male with severe growth retardation and growth hormone insensitivity, without the typical Laron craniofacial appearance. We identified a heterozygous mutation c.703C>T causing a premature stop at codon 217, within the extracellular domain of the GHR. We demonstrate that the mutation p.Arg217X was subject to nonsense-mediated mRNA decay (NMD).

Material and Methods

Sequencing

Genomic DNA from whole blood and primary fibroblast cultures was isolated according to standard procedures. The genomic DNA from the following genes were investigated: *GHR* (NM_000163.2), *GHI* (NM_000515.3), *IGF-I* (NM_001111283.1 and NM_001111284.1), *IGF-IR* (NM_000875.3), *IGFBP-3* (NM_001013398.1), *STAT5b* (NM_012448.3; primers kindly provided by Dr. Vivian Hwa) and *GHR* pseudo-exon 6 (primers kindly provided by Prof. AJL Clark). All the primer sequences are available on request. Coding exons and exon/intron boundaries of all genes were PCR amplified using Qiagen reagents of 5 units/ μ l Taq DNA Polymerase, 10x PCR Buffer, 5x Q-Solution, 10 mM dNTPs, 25 mM MgCl₂ and primers. Mixtures were incubated at 94 C for 3 min, followed by 35 cycles of 94 C for 30 sec and 60 C for 1 min and 72 C for 1 min, followed by a final incubation at 72 C for 10 min. The electrophoretic separation, sizing and quantification of PCR products were performed using LabChip GX (Caliper) microfluidics technology. The amplified products were purified using illustra GFX™ 96 PCR Purification Kit (GE Healthcare). Sequencing was performed using Big Dye Terminator reaction kit (Applied Biosystems). After purification with Dyex 96 kit (Qiagen) the products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems).

Multiplex ligation-dependent probe amplification (MLPA)

The specific kit for Growth Hormone Insensitivity SALSA MLPA kit P262 (MRC Holland, Amsterdam, Netherlands) was used to detect copy number variations in *GHR*, *JAK2*, *IGF-I* and *STAT5B* genes. The assay was performed according to the manufacturer's instructions and analyzed using Gene Marker software (SoftGenetics LLC).

GHR transcripts analysis

Primary fibroblast cultures were established from a skin biopsy taken from patient and healthy controls, after informed consent. Establishment of normal human dermal fibroblasts has been described [13]. The fibroblasts were maintained in DMEM/F12 medium (Invitrogen) supplemented with 9% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin /streptomycin (Invitrogen).

Table 1. Height, IGF-I and IGFBP-3 data.

	Age (years)	Height (cm)	Height (SDS)	IGF-I (nmol/l)	IGF-I (SDS)	IGFBP-3 (mg/l)	IGFBP-3 (SDS)
Start GH treatment	7.8	109.70	-2.89	19.5	0.26	1.4	-1.53
1 year GH treatment	8.8	114.50	-2.95	21.2	0.11	2.2	-0.12
Stop GH treatment	9.8	120.30	-2.75	15.6	-0.99	2.2	-0.34
IGF-I generation test (before)	10.1			20.5	-0.43	1.3	-2.32
After 1 week 1 mg/m ² /day GH	10.1			22.5	-0.20	1.6	-1.52
6 w wash-out							
IGF-I generation test (before)	10.3			22.7	-0.22	2.5	0.08
After 1 month 1 mg/m ² /day GH	10.3			25.9	0.09	2.5	0.07
Start 2.5 mg/m ² /day GH treatment	14.9	137.60	-3.42	32.8	-0.42	3.5	0.69
Stop GH treatment	17.6	152.40	-3.96	44.9	0.28	3.8	0.91
Final height	18.6	153.50	-3.66				

Total RNA from primary fibroblast cells grown in a 75 cm² flask was extracted with High Pure RNA Isolation kit (Roche). Where indicated, 100 µg/ml cycloheximide (Sigma) was added 5 hours before harvesting. cDNA from 2 µg of total RNA was synthesized using TaqMan RTreagent (Roche). Complete cDNA PCR amplification was performed of the *GHR*. The region that includes the c.703C>T was sequenced with forward primer 5'-GTGCAACCAGATCCACCCATTGC-3' and reverse primer 5'-GTTACCTCCTCTAATTTTCC-3'. To test the presence or absence of exon 3 in *GHR* transcripts, PCR amplification was performed as described previously by Pantel *et al*, using primers located in exon 2 and 5 [14]. RT-PCR products were analyzed using LabChip GX (Caliper) microfluidics technology.

Results

Clinical data

The patient is a male, born small for gestational age with birth length 38.5 cm (-7.8 SDS) and birth weight 1350 g (-4.84 SDS) at 38 weeks of gestation. After birth he required artificial respiration for two weeks.

The patient first visited the outpatient clinic for short stature at the age of seven years, when his height was 109.7 cm (-2.89 SD, using the Spanish 1988 reference standards) (Fig. 1; Table 1). The patient's father from Cape Verdean origin was reported as short (between 155 and 160 cm); he was not available for detailed clinical or molecular investigation. The patient's mother was of Portuguese origin and her height was within the normal range (158.8 cm). The patient has no siblings. His target height was 168 cm, his growth velocity was 4 cm per year. The patient underwent a GH stimulation test with Arginine, during which his peak GH was 26 mU/L and IGF-I -1.4 SDS. At the age of seven years and nine months, the patient participated in a trial during which he blindly received GH treatment at either 1 or 2 mg/m²/day. After two years of GH treatment, his height was still -2.69 SD and there was no increase in IGF-I (Table 1). The patient did not have antibodies against GH and his thyroid hormones levels were normal. To exclude noncompliance or a bad injection technique as an explanation for the lack of response to GH, patient was injected subcutaneously by his family doctor for one week, which did not produce any increase in IGF-I levels. Patient underwent an IGF-I generation test during which he received a GH dose of 1 mg/m²/day for one week, followed by 2 mg/m²/day for one month, which failed to produce any increase in IGF-I or IGFBP-3 (Table 1). He received another episode of GH treatment (2.5 mg/m²/day) during two years together with GnRH and LHRH analogues in order to postpone his puberty. Patient's final height was 153.5 cm (-3.66 SD) at the age of 18 years and 7 months.

The patient had several developmental anomalies. He underwent operative correction of a ventricular septal defect at the age of nine months. At the age of seven

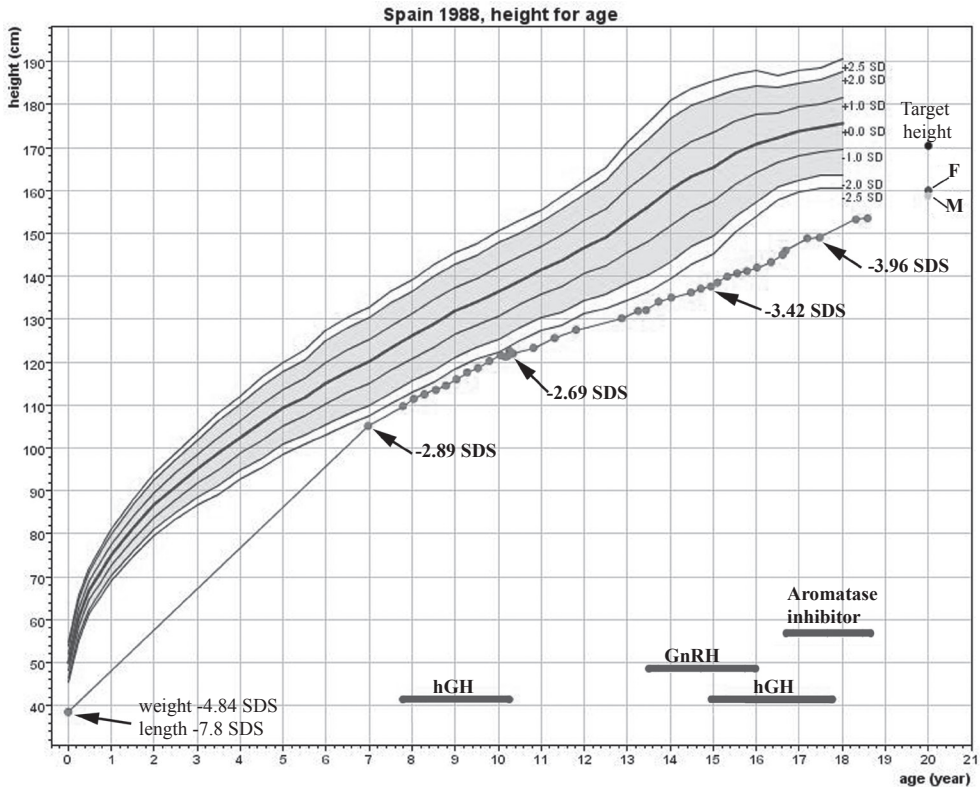


Figure 1. Growth chart. The Growth Analyser version 3.5 software (Dutch Growth Foundation, Rotterdam, The Netherlands) was used to calculate height SDS using the Spanish 1988 reference standards. SDS, standard deviation score; hGH, human recombinant growth hormone treatment; GnRH, gonadotropin releasing hormone analogue treatment; F, father; M, mother.

years he was examined by the pediatric cardiologist to exclude any cardiac cause of his growth retardation. His echocardiogram showed normal intracardial proportions, no abnormalities in pressure or volume. The valves moved normally and there was no remaining ventricular septal defect. Hence there was no cardiologic explanation for his growth retardation. The patient was also born with hypospadias, for which he underwent corrective surgery three times. After these corrections, the urologist concluded that reconstruction for hypospadias had been successful. Patient had cryptorchidism, for which he underwent orchidopexia. In the same year, he underwent corrective surgery for an umbilical hernia. Apart from these developmental anomalies, patient suffered frequent upper respiratory tract infections during infancy and he had some additional anomalies at physical examination. He had halluces valgus (both left and right) and minor facial dysmorphism like floppy ears, and his head circumference was small (-1.88 SD). He had relatively small hands and feet, but otherwise normal extremities and spine. He had normal body proportions and he had no signs of Silver-Russell or Aarskog Syndrome.

Mutation Screening

The direct sequencing of the complete coding region and intron-exon boundaries of *GHR* led to the identification of an heterozygous mutation c.703C>T causing a premature stop codon p.Arg217X within the extracellular domain of *GHR* (Fig. 2A). The patient's unaffected mother was wild-type for *GHR*. The p.Arg217X mutation has been previously reported in homozygosity in association with the classical Laron syndrome [15-17]. Hemizyosity was not excluded in these Laron patients, but it has been inferred that the parents were heterozygous with apparently normal phenotype. Therefore we continued our investigation in order to rule out other genetic defects that may contribute to the phenotype of our patient. We sequenced other candidate genes for GHIS, namely *IGF-I*, *IGF-IR*, *IGFBP-3* and *STAT5b*. We included the previously described pseudo-exon 6 in *GHR* in our analysis [18-20]. Additionally we sequenced the *GHI* under the hypothesis that *GHI* mutations might contribute to the GHI phenotype in a patient who already misses one wild-type allele of *GHR*. Our sequencing results revealed several previously known polymorphisms (Table 1) but no other changes.

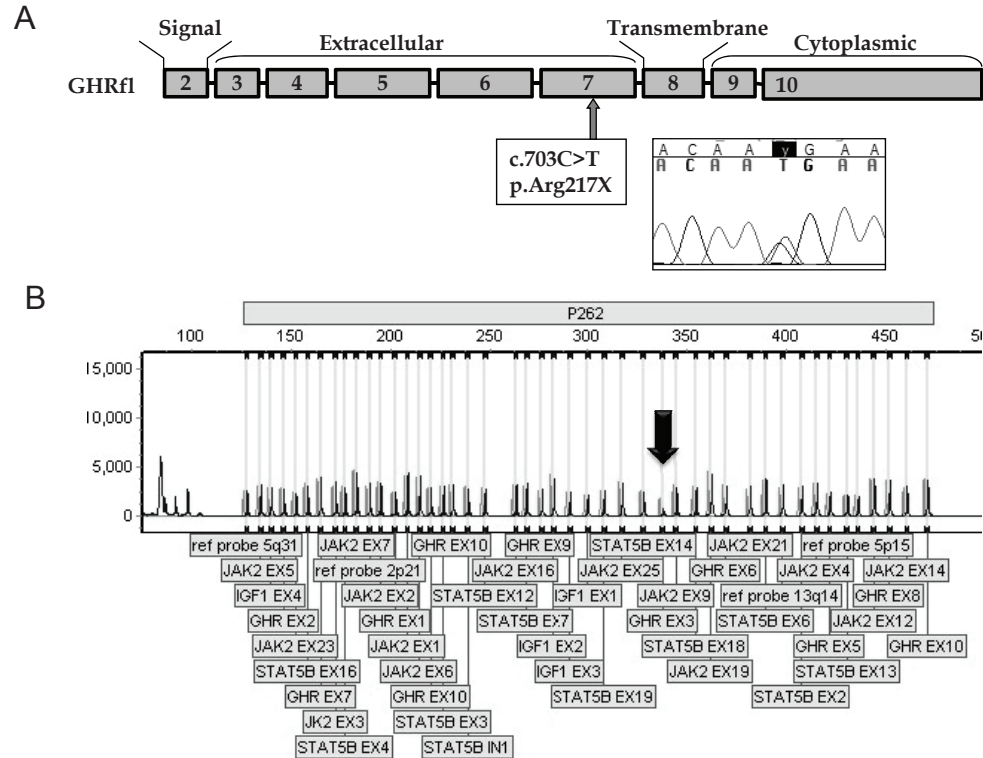


Figure 2. Genetic analysis. (A) Human *GHR* gene, the arrow indicates the location of the mutation. Representative chromatogram of the mutated sequence from whole blood genomic DNA. *GHR*fl, growth hormone receptor full-length. Amino acid numbering is based on mature *GHR* peptide numbering, which does not include the signal peptide. (B) Growth Hormone Insensitivity MLPA results. The arrow indicates *GHRd3* peak.

We also excluded copy number variations using a specific MLPA kit for growth hormone insensitivity that includes *GHR*, *IGF-I*, *JAK2* and *STAT5B*, and found no abnormalities (Fig. 2B). Our patient was heterozygous for the polymorphism *GHRfl*/*GHRd3*, defined as the retention or exclusion of exon 3.

In order to investigate the expression of the *GHR* transcripts, RNA extracted from the patient's fibroblasts was RT-PCR amplified. Because the patient is heterozygous for *GHRfl*/*GHRd3*, we amplified the region between exon 2 and 5 expecting two amplification products: a 361-bp and a 295-bp, corresponding to the *GHRfl* and *GHRd3* isoforms respectively. However, only the shorter fragment corresponding to *GHRd3* was strongly amplified with almost undetectable product from the *GHRfl* isoform (Fig. 3A blue line). The RT-PCR product was directly sequenced and compared to the genomic sequence from blood and fibroblasts. Whereas the patient's genomic sequence clearly shows equal abundance of the C and T 703 of the *GHR* cDNA,

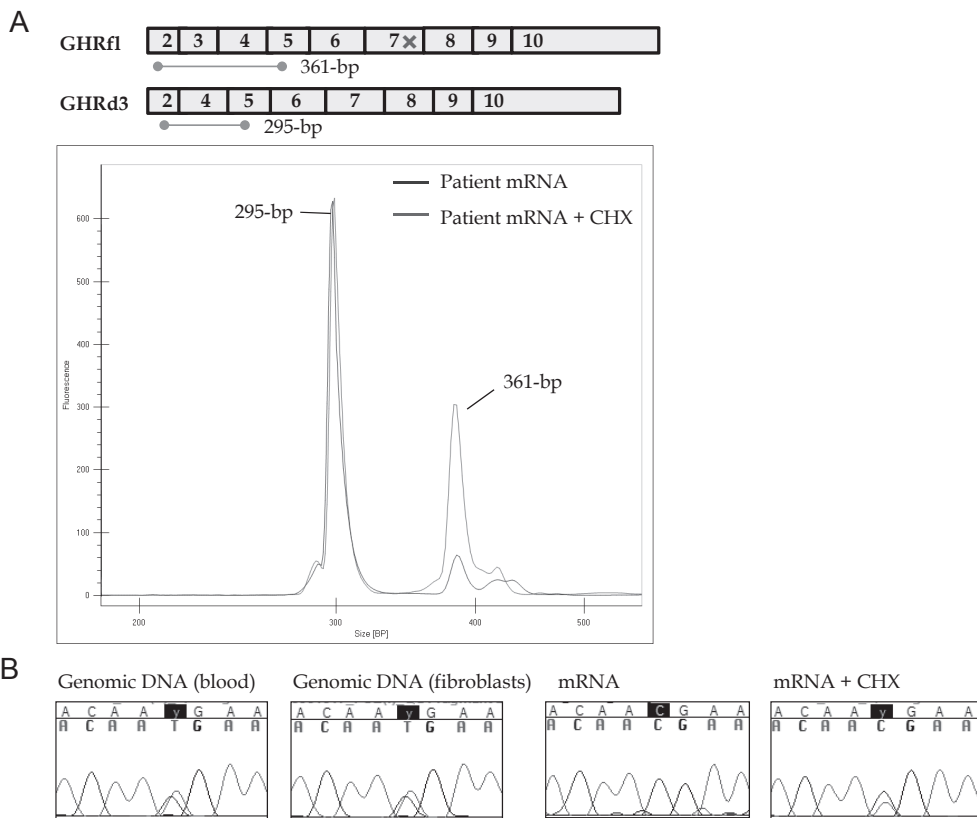


Figure 3. Nonsense-mediated decay. (A) Representation of the two different *GHR* transcripts identified in the patient. LabChip electropherogram of RT-PCR amplification of the *GHRfl* (361-bp) and *GHRd3* (295-bp) transcripts in the mRNA extracted from the patient's fibroblasts under standard conditions and after five hours of 100 µg/ml cycloheximide treatment. (B) Representative chromatograms of the genomic DNA from whole blood and fibroblasts with presence of heterozygous mutation, and mRNA with and without 100 µg/ml cycloheximide treatment. *GHRfl*, growth hormone receptor full-length; *GHRd3*, exon 3-deleted isoform; CHX, cycloheximide.

in the PCR product amplified from fibroblasts mRNA there was no evidence of the mutant sequence and only the wild-type allele was present (Fig. 3B). We treated the cells with cycloheximide for five hours, since this is known to inhibit a process called nonsense mediated decay (NMD). After treatment with this NMD inhibitor, amplification of *GHRfl* isoform was detected by PCR amplification (Fig. 3A red line) and the presence of the c.703C>T mutation was confirmed by sequencing (Fig. 3B). These results demonstrate that the mRNA carrying the premature termination codon (PTC) was subject to NMD.

Discussion

In this study, we report a patient with severe growth retardation, normal GH levels but low IGF-I/ IGFBP-3 levels and resistance to GH treatment, which resulted from a heterozygous *GHR* c.703C>T mutation causing a premature stop codon. Analysis of fibroblasts from the patient indicated that GHR mRNA expressed from the mutant allele was degraded by nonsense mediated decay (NMD). To our knowledge, this is the first time that NMD is described in *GHR* and in relation with GHIS.

NMD is an mRNA surveillance system that eliminates aberrant mRNAs containing PTC. In theory, all nonsense and frameshift mutations resulting in PTC more than 50–55 nucleotides upstream from the last exon-exon junction should be degraded through the NMD pathway (reviewed by [21]). There are many well-studied examples of human phenotypes resulting from nonsense or frameshift mutations that are modulated by NMD. NMD may cause variations in clinical severity or alter the pattern of inheritance. When mRNA containing a PTC mutation is degraded by NMD, the translation of protein with potentially dominant-negative effect or gain-of-function is prevented. However, exceptions of this mechanism have been reported [22, 23]. To date, fifteen different nonsense mutations have been reported in *GHR*, localized from exon 2 to 7, in patients with GHIS (reviewed in [2, 12]). NMD has been suggested in some of these mutations, but it was never directly demonstrated.

The p.Arg217X mutation in our patient is localized in the seventh of ten *GHR* exons, within the extracellular domain, and is predicted to cause a severely truncated protein. Since the p.Arg217X mutation has also been described in individuals with mild or normal phenotype, we considered it essential to verify the potential involvement of NMD experimentally, because this might explain the phenotypic variation associated with this mutation. In this regard, it is important to notice the use of RNA from primary fibroblast cultures for functional studies; intronless cDNAs normally used in *in vitro* systems are insensitive to NMD. In fibroblasts derived from the patient we could clearly show that, under normal conditions, only GHR wild-type allele was expressed; the allele carrying the PTC was absent unless NMD was inhibited by cycloheximide. A similar phenomenon has been described by Fang *et al* [24] in a family characterized by short stature, where the affected family members were carriers of a heterozygous frameshift duplication in *IGF-IR* which was subject to NMD and

caused IGF-IR haploinsufficiency.

The p.Arg217X mutation has been reported in association with the classical Laron syndrome [15-17]. The homozygous p.Arg217X mutation (with a potential lack of GHR protein) led to a very severe Laron phenotype (height from -5.4 to -7.04 SD) [8, 17]. The parents of these Laron patients had a nearly normal phenotype, with maternal heights ranging from -2.22 to -2.35 SD, but paternal height within the normal range (-0.63 to -1.31) [8]. In one patient, the parents were consanguineous and carried the mutation in heterozygosity [17]. In the other patients, hemizygosity was not excluded, but it has been inferred that the parents were heterozygous for the p.Arg217X mutation. Our patient has the same heterozygous mutation, a phenotypic severity “between Laron and ISS”, and he has a clear GH insensitivity.

Table 2. Genomic variations found in the GHIS index case.

Gene	Exon	rs number	Ancestral allele	Patients alleles	MAF (refSNP) ^a
<i>GH1</i>	Ex1	rs2005172	T	G/G	T=0.394
	Ex2	rs41295031	G	G/C	n.a.
<i>IGF-IR</i>	Ex1	rs34226328	C	C/T	n.a.
	Ex13	rs1464430	T	G/T	G=0.391
	Ex17	rs2293117	C	C/T	C=0.402
<i>IGFBP-3</i>	Ex1	rs2854746	C	G/C	C=0.441
<i>STAT5b</i>	Ex16	rs3054923	(TG)15/16/17/18 /19/-/GT (STR)	-/-	n.a.

MAF, minor allele frequency; n.a., not available; STR, short tandem repeat; n.a., not available.

^aMAF only shown if European population data were available at time of the study.

Previous reports about different *GHR* mutations have described a wide phenotypic variability among patients carrying the same mutation [18, 25], even among patient from the same family [26]. NMD could be one of the modulators to explain this variability in disease severity, because inter-individual variation in the efficiency of NMD might result in the different expression levels of the wild-type and mutant alleles, contributing to differences in phenotype as has been shown for other disorders [27-29].

In the past, two different publications have reported PTC-carrying *GHR* transcripts, which were detected in the material derived from the patient and parents: Sobrier *et al* mentioned that the allele with a nonsense mutation p.Trp80X in exon 5 was detected in the *GHR* transcripts obtained from lymphocytes [30]; and Pantel *et al* described a patient with compound heterozygous nonsense mutation p.Trp16X

in exons 3 and p.Cys38X in exon 4; each allele was inherited from a heterozygous parent and expressed in both lymphocytes and fibroblasts [14]. The fact that in those patients, the mutated allele remained present in fibroblasts and lymphocytes whereas in our patient it was eliminated by NMD, shows that some mutations escape from NMD whereas others do not. This suggests that differences in NMD efficiency might explain variations in GHIS phenotype if the product has function. The p.Arg217X mutation, localized in the last exon of the extracellular domain, might produce a protein with some function.

Alternatively, the variability in phenotype between our patient and other heterozygous individuals, as for example parents of Laron patients, could also be explained by the presence of compound heterozygous mutations for two or more genes involved in the GH-IGF-I axis. In order to exclude this possibility, we performed a comprehensive mutations and copy number variation analysis of several genes involved in the classical GH-IGF axis. We did not detect any potentially damaging changes; however we found several genomic variations (Table 2). The most remarkable are a homozygous G/G rs2005172 change in *GHI*, localized in the functional region of the promoter [31]; the heterozygous C/T polymorphism rs34226328 in *IGF-IR*, predicted to have a possible functional effect in the splicing regulation [32], and the heterozygous missense change G/C rs2854746 (p.Ala32Gly) in *IGFBP-3*, reported to be strongly associated with IGFBP-3 circulating serum levels in several studies [33-35]. Although these polymorphisms are present in a normal population, they might contribute to the GHIS phenotype when combined with other genetic and environmental modifiers that could influence GH signaling pathways.

In conclusion, we demonstrate that the mutation p.Arg217X in *GHR* was subject to nonsense-mediated mRNA decay. Our study is the first to provide support for the role of NMD in the phenotypic variability of GHIS caused by *GHR* mutations.

Acknowledgments

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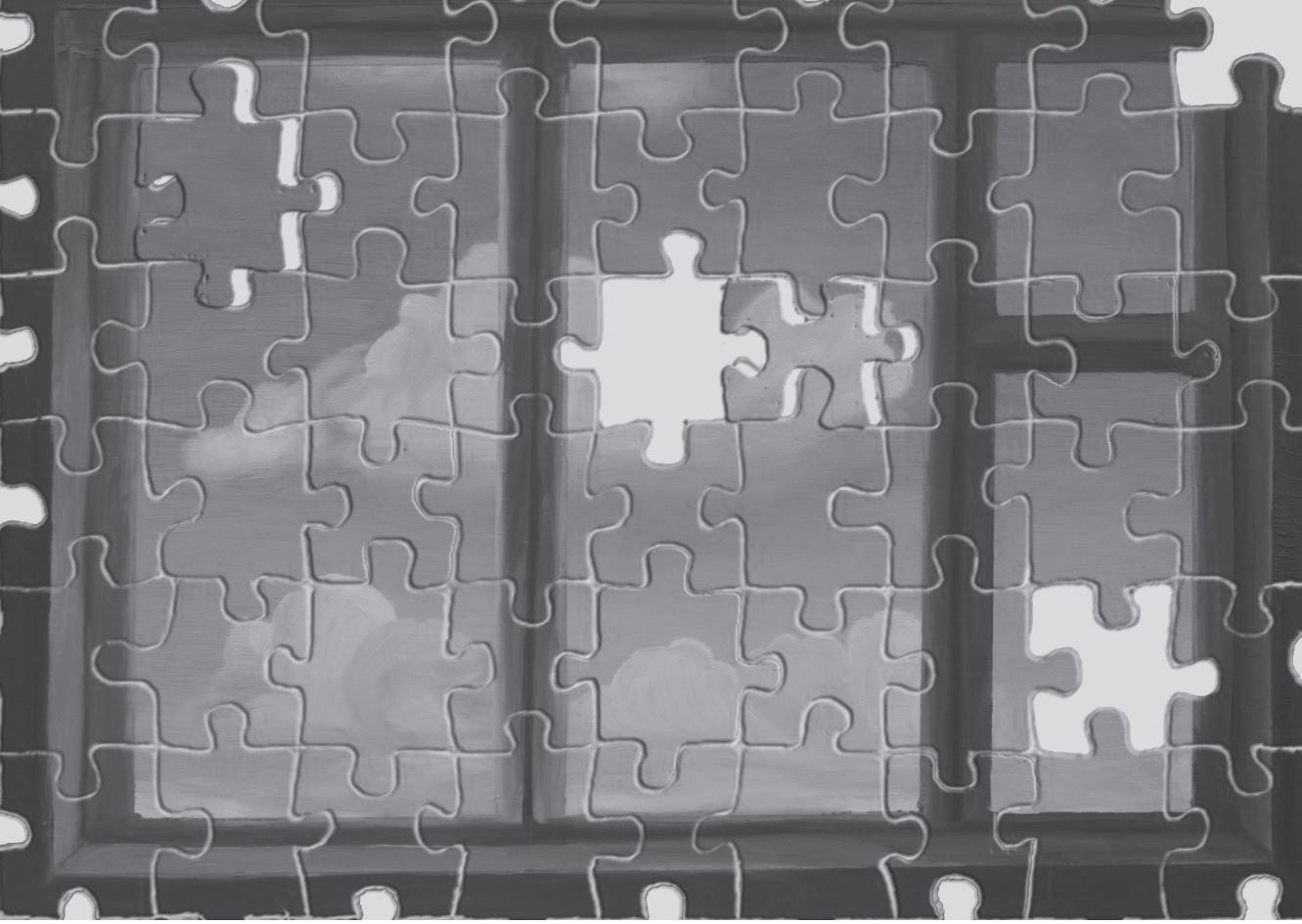
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Chapter 6

General discussion and conclusions

The main objective of the work described in this thesis was to identify genetic and/or molecular defects in several cohorts of patients with endocrine disorders, with especial attention to Growth Hormone Deficiency, isolated or in combination with other pituitary hormone deficiencies. This study, a continuation of the Dutch HYPOPIT study that started in 2003, was continued in 2008 and concluded in 2011. In this discussion, the main findings are summarized and discussed in the context of the most recent literature. Finally, conclusions, new hypotheses and potential future research are presented.

1. Isolated Growth Hormone Deficiency: mutation screening and copy number analysis of *HMGA2* and *CDK6* genes.

The aim of this study was to determine whether mutations or deletions in the *HMGA2* and *CDK6* genes are involved in the pathogenesis of isolated growth hormone deficiency (IGHD). Our main finding was the identification of a new 20 base-pair intronic deletion in the *HMGA2* gene (c.250-29_-9del), which was absent in databases and healthy controls. This deletion was identified in one patient with classical IGHD and MRI abnormalities, from the cohort of 69 patients with classical IGHD, of whom 35 had MRI abnormalities. Contrary to our expectation, we did not find any new pathological variants in *CDK6*.

Although we performed functional studies using fibroblasts from the patient with the *HMGA2* deletion, we could not fully prove the pathogenicity of this deletion. One possible explanation is that the expression of *HMGA2* and its transcription target, *IGF2BP2*, are only essential during embryogenesis [1], and could be less important in adulthood. It has been demonstrated that *HMGA* genes show high expression during human embryogenesis and fetal development and low expression levels in adult tissues [2, 3]. In mice, *Hmga2* is found to be preferentially expressed by stem cells, showing a progressive decline in expression with age [4]. We hypothesize that the deregulation and possibly decreased expression of *HMGA2* during the development of specific somatotroph cells in the pituitary might lead to low GH production after birth, contributing to the IGHD phenotype.

We believe that our results, together with recently published data concerning the 12q14 microdeletion syndrome where patients with an *HMGA2* haploinsufficiency had proportionate short stature [5-9], underline the relevance of *HMGA2* in human growth and therefore provide important information for pediatric endocrinologists.

In conclusion, our study is the first to report a deletion in the *HMGA2* gene that might be a rare cause of IGHD. We suggest that this gene be investigated in patients with the IGHD phenotype, in whom mutations and deletions in the classical candidate genes *GHI* and *GHRHR* have been excluded.

2. Combined Pituitary Hormone Deficiency: mutations screening in two Hedgehog genes, *SHH* and *HHIP*.

The aim of this study was to investigate whether mutations in *SHH* and *HHIP* could explain the phenotype of 'idiopathic' CPHD. Our main findings in *SHH* include 3 mutations in 93 patients with CPHD: one missense mutation (p.Ala226Thr), one synonymous mutation in the coding region (c.1078C>T) and one mutation in the 3'UTR region (c.*8G>T). In our *in vitro* assay, the function of all three mutants was affected, especially and consistently of the latter one. In *HHIP* we detected a new activating mutation c.-1G>C, which increases HHIP's inhibiting function in the Hedgehog pathway.

SHH mutations, known to be associated with holoprosencephaly (HPE), show a broad phenotypic heterogeneity. Clinical variability has been described even between family members with the same *SHH* mutation [10-12]. The HPE phenotype itself shows also enormous variability, ranging from the most extreme form such as cyclopia or pronounced microcephaly to HPE microforms and non-penetrant carriers with normal facial appearance and normal MRI [10, 13, 14]. Some patients with HPE have pituitary hormone deficiencies such as diabetes insipidus (up to 70%), TSH deficiency (11%), ACTH deficiency (7%), and GH deficiency (5%) [15, 16]. Our patients with *SHH* mutations show some overlap with a mild HPE phenotype: besides combined pituitary deficiency, they have pituitary abnormalities at MRI. One of our patients with *SHH* mutation was born with a severe craniofacial encephalocele. The same patient also developed diabetes insipidus. Moreover, the first mutation that we identified in *SHH* c.676G>A (p.Ala226Thr) was previously reported in relation to a familiar case of HPE [17].

HHIP is a less known member of the Hedgehog pathway. Variations in the HHIP gene have previously been described in association with adult height variability in the normal population [18-21]. We are the first to describe a *HHIP* mutation in relation to a human disorder (CPHD). In a mouse model, the overexpression of Hhip in chondrocytes results in severe skeletal defects similar to those observed in the mutants of another Hh ligand, Indian hedgehog [22]. Our finding emphasizes the importance of fine-tuning the Hh signaling pathway and shows that *HHIP* might be one of the modifiers contributing to the enormous variability in the HPE phenotype.

In conclusion, our study shows that mutations in the Hh pathway might be involved in CPHD. We suggest that screening of both *SHH* and *HHIP* be included in the future genetic analysis of patients with CPHD, after mutations in the classical CPHD genes have been ruled out.

3. A novel *OTX2* mutation in a patient with Combined Pituitary Hormone Deficiency, pituitary malformation and an underdeveloped left optic nerve.

In this study we aimed to expand the mutation spectrum of CPHD and to investigate how many of the 92 CPHD cases in the Dutch HYPOPIT study could be explained by mutations in *OTX2*. We identified one novel missense mutation (p.Pro134Arg) in a patient with complete pituitary hormone deficiency, pituitary malformation and an underdeveloped left optic nerve. Our luciferase reporter studies clearly demonstrated that the *OTX2* Pro134Arg mutant protein was not able to activate the multiple bicoid promotor. Furthermore, we identified a new silent mutation c.768C>T, so far undescribed in any control or patient population.

To date, 29 different *OTX2* mutations have been reported in 32 unrelated patients, including the new missense mutation found in our study. The majority of the alterations are found in patients with severe ocular malformations [23-26]. However, recently, *OTX2* mutations and/or deletions were also identified in IGHD or CPHD patients [27-32]. In the majority of the patients, but not all [29], eye malformations were also present. In our cohort, there are only three CPHD patients with eye malformations. In addition to the screening of our CPHD patients, we directly sequenced two patients with IGHD and eye malformation, both of whom were wild-type for *OTX2*. It is remarkable that the *OTX2* mutation happened to be present in the only patient with an underdeveloped optic nerve; the other four have ocular malformation related with septo-optic dysplasia.

From the summary of all *OTX2* mutations known to date, it is possible to draw some global conclusions. There seems to be a clear relation between the localization of the mutations on one hand, and phenotype on the other: mutations in the N-terminus show very severe eye malformations without pituitary phenotype. In contrast, mutations localized in the C-terminus are associated with pituitary malformation. All patients reported to date with CPHD, IGHD or short stature have their mutations in exon 5, except for p.Lys74SerfsX30 [28]. Moreover, the distribution of mutations seems to have several hotspots affecting functional domains, and most of them are located in conserved residues, which suggests functional relevance.

In conclusion, a novel missense *OTX2* mutation (p.Pro134Arg) was found in one patient with CPHD, pituitary malformation and optic nerve hypoplasia from the total cohort of 92 CPHD patients. We recommend *OTX2* as a candidate gene for genetic screening in patients with IGHD with ocular problems, and patients with CPHD (with or without ocular problems), starting the sequencing in exon 5 of the gene.

4. Growth Hormone Insensitivity Syndrome caused by a heterozygous GHR mutation: phenotypic variability due to moderation by non-sense-mediated decay.

Our aim in this study was to identify and characterize the molecular cause of severe short stature in a patient with resistance to GH treatment. We identified a heterozygous *GHR* c.703C>T (p.Arg217X) mutation causing a premature stop codon. This mutation, in homozygosity, has been reported previously in association with the classical Laron syndrome [33-35]. Since heterozygous parents carrying the same mutation have been reported as normal, we continued our investigation in order to search for additional genetic defects that may contribute to the phenotype of our patient. We performed an extensive mutation screening as well as copy number variation analysis of several candidate genes within the GH-IGF1 axis, namely *GHR*, *GHI*, *IGF-I*, *IGF-IR*, *IGFBP-3*, *STAT5b* and *JAK2*; we also included the pseudo-exon 6 in *GHR* in our analysis [36-38]. However, our genetic analysis revealed no other changes. Eventually, the analysis of fibroblasts from the patient showed that GHR mRNA expressed from the mutant allele was degraded by nonsense mediated decay (NMD). To our knowledge, this is the first time that NMD is described in *GHR* and in relation with GHIS.

NMD is a surveillance mechanism which eliminates aberrant mRNAs containing a premature termination codon. NMD may cause variations in clinical severity or alter the pattern of inheritance. To date, fifteen different nonsense mutations have been reported in *GHR*, localized from exon 2 to 7, in patients with GHIS (reviewed in [39, 40]). NMD has been suggested in some of these mutations, but it was never directly demonstrated. Previous reports about different *GHR* mutations have described a wide phenotypic variability among patients carrying the same mutation [36, 41], even among patients from the same family [42]. NMD could be one of the modulators to explain this variability in disease severity, because inter-individual variation in the efficiency of NMD might result in different expression levels of the wild-type and mutant alleles, contributing to differences in phenotype as has been shown for other disorders [43-45].

In conclusion, we demonstrate that the mutation p.Arg217X in *GHR* was subject to nonsense-mediated mRNA decay. Our study is the first to provide support for the role of NMD in the phenotypic variability of GHIS caused by *GHR* mutations.

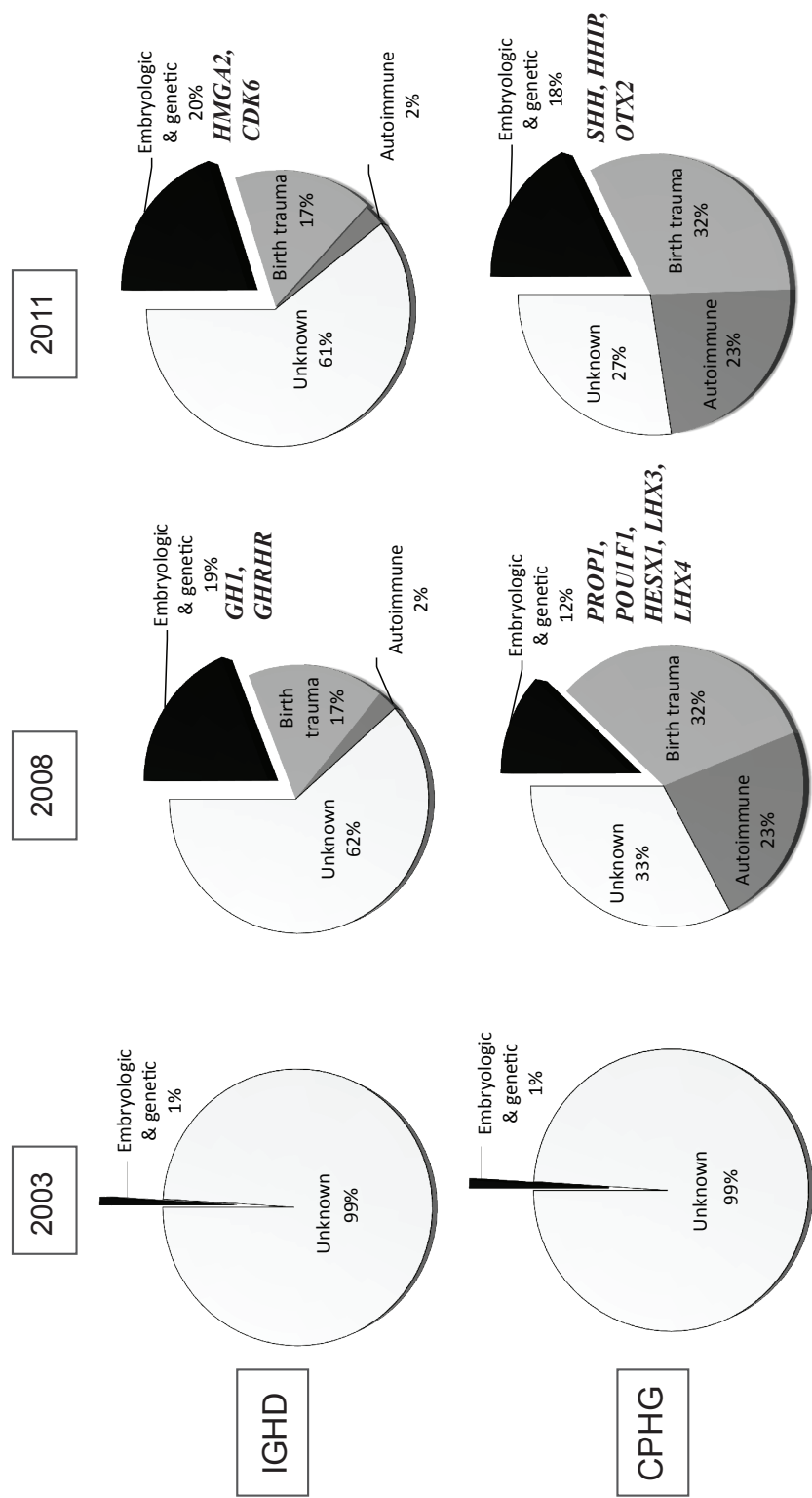


Figure 1. Progress in knowledge of underlying mechanisms of Dutch IGHD and CPHG cases, over the last eight years of our study.

5. Conclusions, practical implications and directions for future research.

In 2003, the HYPOPIT study started the search for genetic defects in Dutch patients with IGHD and CPHD, in order to reduce the number of patients carrying the unsatisfying diagnose of ‘idiopathic’ IGHD or CPHD.

At start of the HYPOPIT study, 99% of the participating patients had IGHD or CPHD of unknown origin (Fig. 1 [2003]). Apart from genetic research into the causes of IGHD and CPHD, the HYPOPIT study investigated possible other underlying disorders, like autoimmune mechanisms and breech or otherwise complicated deliveries. Clinical data of 19% of the IGHD patients and 12% of the CPHD patients pointed towards an embryologic or genetic cause. 2.5% of IGHD patients and 23% of CPHD tested for Anti Pituitary Antibodies (APA) were APA positive, suggesting an autoimmune process was involved in their condition. In 17% of the IGHD patients and 32% of the CPHD patients, clinical data suggested that birth trauma could have caused their deficiencies (Fig. 1 [2008]).

After the HYPOPIT study had shown that the classical genetic defects could only explain a small minority of the cases of IGHD and CPHD, we continued the search by expanding the genetic part of the study.

After very thorough genetic and molecular studies, we found novel, possibly pathogenic mutations in one patient with IGHD and 5 with CPHD. This means that 39% of IGHD cases and 73% of CPHD cases have now been explained, which is a major progress compared to 2003 (Fig. 1 [2011]).

Based on the findings presented in this thesis, we propose a flow chart for the candidate gene screening in patients with growth disorders (Fig. 2).

In the beginning of this thesis, we made a selection of candidate genes using the GWAS data on height variability in the normal population available at that moment (October 2008) [19-21]. In October 2010, the Genetic Investigation of ANthropo-centric Traits (GIANT) Consortium published a large GWA meta-analysis in which 180 genome-wide significant loci were associated with adult height [18]. The three genes we selected from the initial GWAS in 2008, which we analyzed in this thesis (*HMGA2*, *CDK6* and *HHIP*) were also present in the GIANT meta-analysis.

Apart from the three genes we already studied, the GIANT meta-analysis provides new candidate genes for future investigation of IGHD and CPHD, many of which are implicated in pathways known to be involved in growth or skeletal formation. For example, additional genes from the Hedgehog pathway (in this thesis shown to be involved in some cases of CPHD) have been highlighted in the GIANT height meta-analysis, like Indian Hedgehog *IHH* (another Hedgehog ligand) and *PTCH1*, a transmembrane receptor. Another example of a new genome-wide significant height-related gene (and therefore a new candidate gene for our research) is *HMGA1*. This gene belongs to the same high-mobility AT-hook group of proteins as *HMGA2*, studied in this thesis. Moreover, *IGF2BP2*, a *HMGA2* transcription target, is also genome-wide significant in the GWAS of 2010.

Apart from new candidate genes, new technologies such as next generation massive sequencing will be available for our future research.

The Dutch Growth Research Foundation will continue to encourage and support the search for pathogenic mutations, in order to reduce the number of patients carrying the unsatisfying diagnose of ‘idiopathic’ IGHD or CPHD.

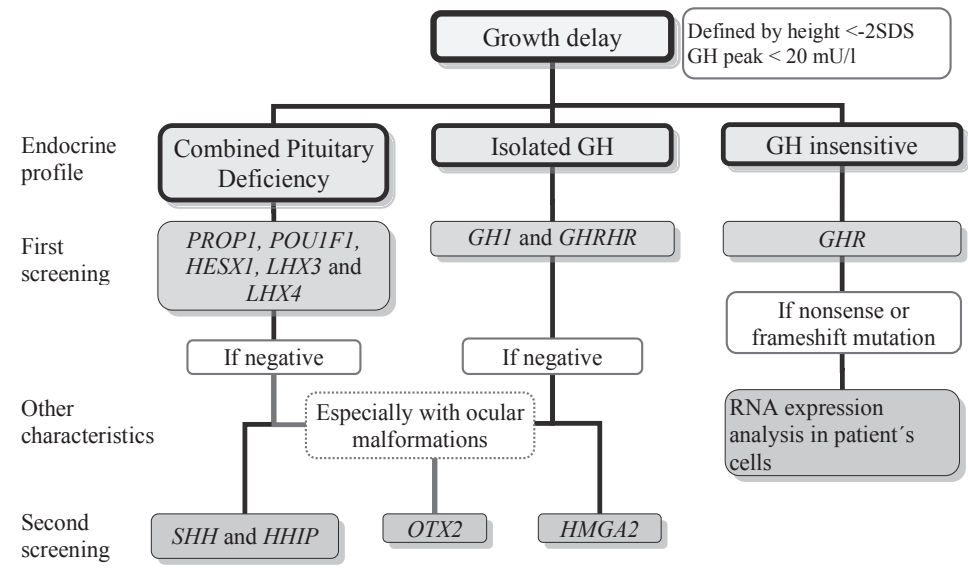


Figure 2: Flow diagram with our suggestions for genetic screening in patients with growth delay.

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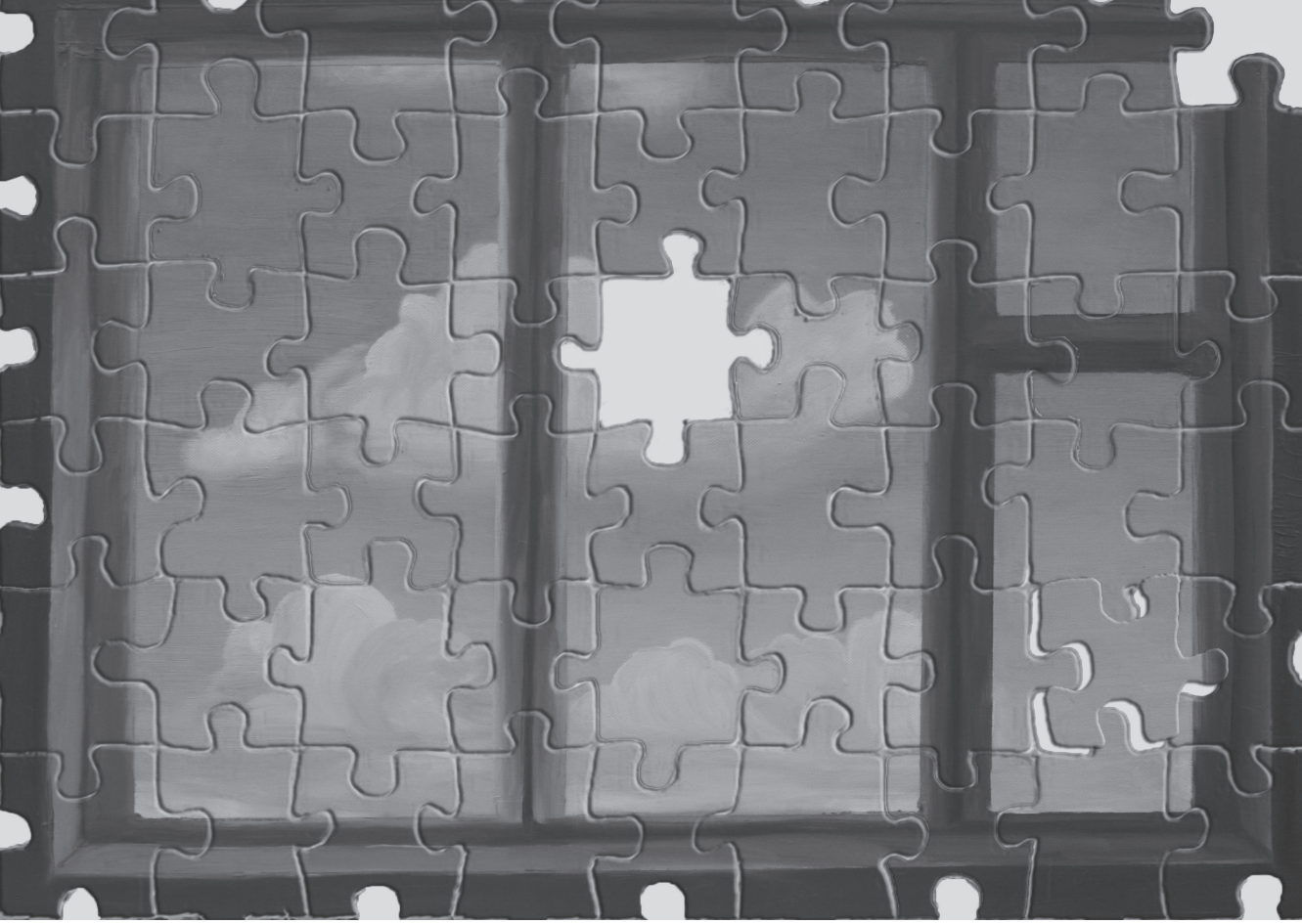
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Chapter 7

Summary

Samenvatting

Summary

Chapter 1 gives an introduction of the hypothalamus and pituitary axes and the disorders associated with disturbance of these axes. The chapter briefly describes the most important genes and pathways involved in pituitary development. It describes the candidate genes studied in this thesis and clarifies our choice for these candidate genes.

Chapter 2 focuses on Isolated Growth Hormone Deficiency (IGHD) and the genetic analysis performed in two new candidate genes: *HMG2* and *CDK6*. The aim of this study was to determine whether *HMG2* and *CDK6* are involved in the pathogenesis of IGHD. Our main finding was the identification of a 20 base-pair intronic deletion in *HMG2* (c.250-29_-9del), which was absent in databases and healthy controls. This deletion was identified in one patient with classical IGHD and MRI abnormalities, from the cohort of 69 patients with classical IGHD, of whom 35 had MRI abnormalities. We believe that our results, together with recently published data concerning the 12q14 microdeletion syndrome, where patients with an *HMG2* haploinsufficiency had proportionate short stature, provides important information for pediatric endocrinologists working in the field of growth. **In conclusion**, *HMG2* might be a rare cause of IGHD. We suggest that this gene be investigated in patients with a classical IGHD phenotype, in whom mutations in the classical candidate genes *GHI*, *GHRHR* have been previously excluded.

Chapter 3 describes the genetic study of new candidate genes from the Hedgehog pathway in our cohort of 93 patients with Combined Pituitary Hormone Deficiency (CPHD). We identified three mutations (p.Ala226Thr, c.1078C>T and c.*8G>T) in *SHH* and one activating mutation in *HHIP* (c.-1G>C). Our functional tests demonstrate abnormal function of the mutant proteins. **In conclusion**, mutations in the Hh pathway might be involved in CPHD. We suggest that screening of both *SHH* and *HHIP* be included in the future genetic analysis of patients with CPHD, after mutations in the classical CPHD genes have been ruled out.

Chapter 4 describes a novel *OTX2* mutation in a patient with CPHD, pituitary malformation and optic nerve hypoplasia. In this study we directly sequenced the coding regions and exon – intron boundaries of *OTX2* in 92 CPHD patients from the Dutch HYPOPIT study in whom mutations in the classical CPHD genes *PRO1*, *POU1F1*, *HESX1*, *LHX3* and *LHX4* had been ruled out. We identified the novel missense mutation c.401C>G (p.Pro134Arg) in a patient with the unusual combination of both CPHD, pituitary malformation and an underdeveloped left optic nerve. Furthermore, we identified a new silent mutation c.768C>T in a patient with CPHD and pituitary malformation. Our functional studies showed that the function of the p.Pro134Arg mutant protein was severely affected. Our overview of all mutations described until date showed that the majority of the patients with CPHD, isolate growth hormone

deficiency (IGHD) or short stature have their mutations in exon 5. **We conclude** that *OTX2* is a promising candidate gene for genetic screening of patients with CPHD or IGHD. We especially recommend mutational screening in those CPHD or IGHD patients with ocular problems, starting the sequencing in exon 5 of the gene.

Chapter 5 focuses on the Growth Hormone Insensitivity Syndrome caused by a heterozygous *GHR* mutation. This chapter describes a male patient born small for gestational age, who, at the age of seven years, received GH treatment for one year without any increase in height SDS, IGF-I or IGFBP-3 levels. Double-dose GH treatment for another year did not result in any improvement of growth factor levels either. Our aim was to identify and characterize the molecular cause of severe short stature in this patient with resistance to GH treatment. In order to achieve that, we performed an extensive mutation screening as well as copy number variation analysis of several candidate genes within the GH-IGF1 axis (*GHR*, *GH1*, *IGF-I*, *IGF-IR*, *IGFBP-3*, *STAT5b* and *JAK2*). We identified a heterozygous *GHR* c.703C>T (p.Arg217X) mutation causing a premature stop codon. Although this mutation has been reported previously in association with the classical Laron syndrome, which our patient did not have, the main interest of our investigation was the finding that the *GHR* nonsense mutation of our patient was subject to nonsense mediated decay (NMD). **In conclusion**, we demonstrate that the mutation p.Arg217X in *GHR* was subject to nonsense-mediated mRNA decay. Our study is the first to provide support for the role of NMD in the phenotypic variability of GHIS caused by *GHR* mutations.

Chapter 6 reviews our findings in the context of the most recent literature. The chapter ends with an overview of our findings, final conclusions and recommendations for future research.

Samenvatting

Hoofdstuk 1 geeft een overzicht van de hypothalamus-hypofyse assen en de problemen die ontstaan als deze assen niet goed werken. Het beschrijft de belangrijkste stappen in de ontwikkeling van de hypofyse. Daarnaast beschrijft het de kandidaatgenen die in dit proefschrift worden onderzocht en laat het zien waarom we juist deze kandidaatgenen hebben uitgekozen.

Hoofdstuk 2 beschrijft het genetisch onderzoek van twee nieuwe kandidaatgenen in onze groep van patiënten met geïsoleerde groeihormoondeficiëntie (IGHD): *HMGA2* en *CDK6*. Het doel van dit onderzoek was om betrokkenheid van *HMGA2* en *CDK6* in de pathogenese van IGHD aan te tonen of uit te sluiten. Onze belangrijkste nieuwe bevinding was een intronische deletie van 20 baseparen in *HMGA2* (c.250-29_-9del), die niet werd gevonden in gezonde controlepersonen of databases. Deze deletie werd gevonden in een patiënt met klassieke IGHD en MRI afwijkingen, uit het totale cohort van 69 IGHD patiënten (van wie er 35 MRI afwijkingen hadden). Wij zijn van mening dat onze bevindingen, samen met onlangs gepubliceerde gegevens over het 12q14 microdeletiesyndroom (waarin patiënten met *HMGA2* haploinsufficiëntie groeistoornissen hadden) belangrijke informatie vormen voor kinderendocrinologen die zich met groei bezig houden. **Concluderend** zouden *HMGA2* mutaties een zeldzame oorzaak van IGHD kunnen vormen. Wij adviseren om *HMGA2* te onderzoeken bij patiënten met klassieke IGHD, nadat mutaties in de traditionele IGHD genen *GHI* en *GHRHR* zijn uitgesloten.

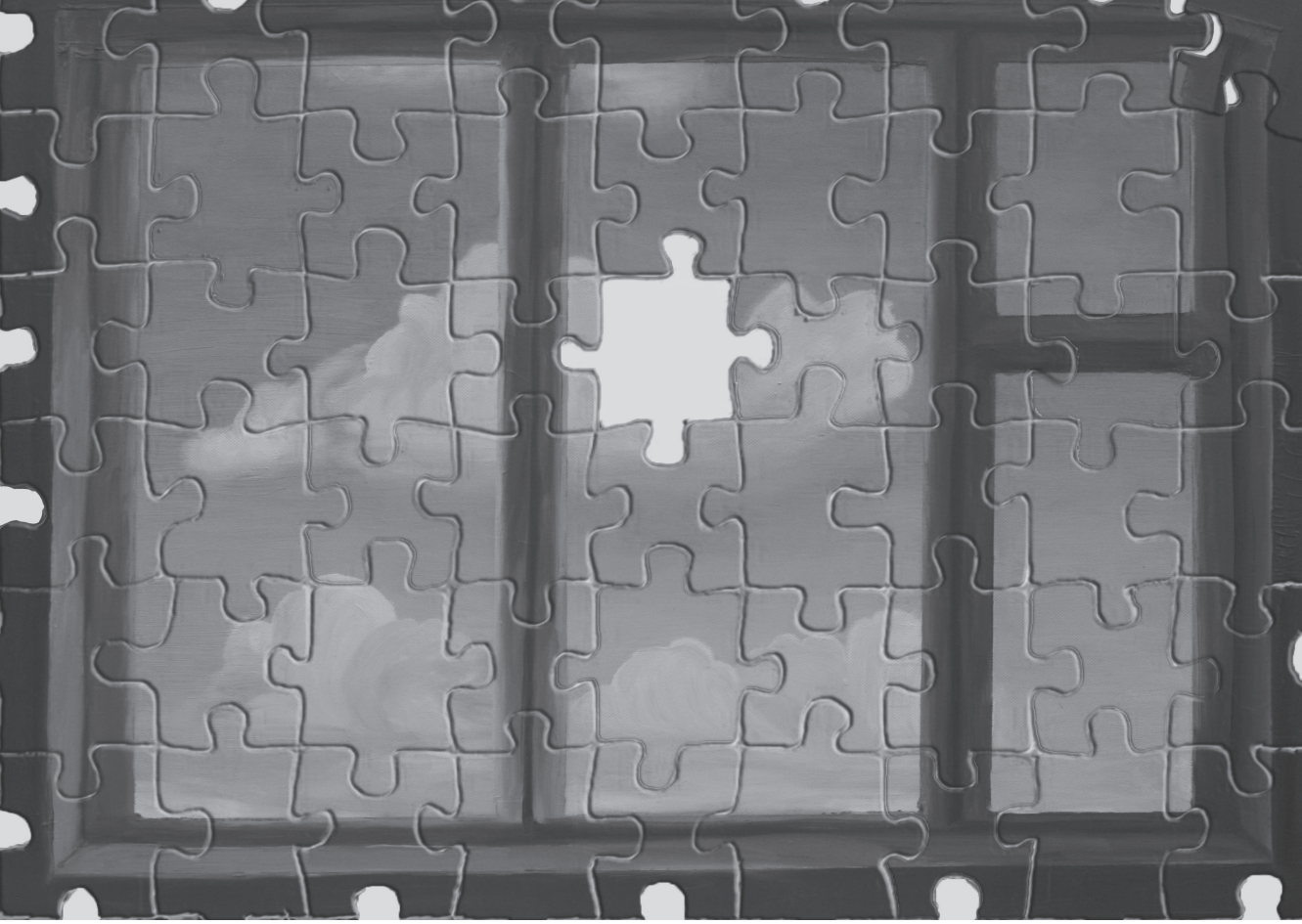
Hoofdstuk 3 beschrijft het genetisch onderzoek van nieuwe kandidaatgenen uit de Hedgehog (Hh) pathway bij onze 93 patiënten met gecombineerde uitval van hypofysehormonen (Combined Pituitary Hormone Deficiency of CPHD). We vonden drie mutaties (p.Ala226Thr, c.1078C>T en c.*8G>T) in *SHH* en een activerende mutatie in *HHIP* (c.-1G>C). Onze functionele proeven lieten zien dat de mutante eiwitten niet goed werken. **Concluderend**, kunnen mutaties in de Hh pathway betrokken zijn bij CPHD. Wij adviseren om *SHH* en *HHIP* te onderzoeken bij patiënten met CPHD, nadat mutaties in de traditionele CPHD genen zijn uitgesloten.

Hoofdstuk 4 beschrijft een nieuwe *OTX2* mutatie in een patiënt met CPHD, hypofyse afwijkingen en hypoplasie van de nervus opticus. We hebben de coderende regionen en intron-exon overgangen van *OTX2* bestudeerd in 92 CPHD patiënten uit de HYPOPIT studie, bij wie mutaties in de klassieke CPHD genen *PROPI*, *POUIF1*, *HESX1*, *LHX3* en *LHX4* reeds waren uitgesloten. We vonden de nieuwe c.401C>G (p.Pro134Arg) mutatie in een patiënt met de ongebruikelijke combinatie van zowel CPHD, hypofyse afwijkingen als hypoplasie van de nervus opticus. Daarnaast hebben we een nieuwe ‘stille’ mutatie gevonden (c.768C>T) in een patiënt met CPHD en hypofyse afwijkingen. Onze functionele proeven toonden aan dat de functie van het p.Pro134Arg mutante eiwit ernstig was aangetast. Ons overzicht van alle mutaties

die tot op heden beschreven zijn, laat zien dat de meerderheid van de patiënten met CPHD, IGHD of kleine gestalte hun mutaties in exon 5 hebben. Wij concluderen dat *OTX2* een veelbelovend kandidaatgen is voor het genetisch onderzoek van patiënten met CPHD of IGHD. Wij raden met name mutatie screening aan in CPHD en IGHD patiënten met oogafwijkingen, te beginnen met exon 5 van het gen.

Hoofdstuk 5 beschrijft een patiënt met groeihormoon ongevoeligheid, veroorzaakt door een heterozygote *GHR* mutatie. De patiënt, een jongetje dat bij de geboorte te klein was voor de zwangerschapsduur ('Small for Gestational Age' of SGA), werd op de leeftijd van zeven jaar behandeld met groeihormoon zonder dat dit enig effect had op lengte SDS, IGF-I of IGFBP-3 concentraties in serum. Een extra jaar GH behandeling, met dubbele dosis groeihormoon, gaf evenmin verbetering van deze groeiparameters. De patiënt had geen typisch Laron uiterlijk. Ons doel was om de moleculaire oorzaak van de zeer kleine gestalte te onderzoeken in deze patiënt met groeihormoon ongevoeligheid. Wij voerden een grondige mutatiescreening uit, evenals copy number variatie analyse van verschillende kandidaatgenen uit de GH-IGF1 as (*GHR*, *GHI*, *IGF-I*, *IGF-IR*, *IGFBP-3*, *STAT5b* en *JAK2*). We vonden een heterozygote *GHR* c.703C>T (p.Arg217X) mutatie, die een prematuur stopcodon veroorzaakt. Hoewel deze mutatie eerder is beschreven in associatie met het klassieke syndroom van Laron, was de belangrijkste bevinding van ons onderzoek het feit dat de *GHR* nonsense mutatie van onze patiënt onderhevig bleek te zijn aan nonsense mediated decay (NMD). **Concluderend**, laten we zien dat de p.Arg217X mutatie in *GHR* onderhevig is aan nonsense-mediated mRNA decay. Onze studie is de eerste die laat zien dat NMD een rol zou kunnen spelen in de fenotypische variatie van GHIS die veroorzaakt wordt door *GHR* mutaties.

Hoofdstuk 6 bespreekt onze bevindingen in de context van de meest recente literatuur. Het hoofdstuk eindigt met een overzicht van onze bevindingen, onze eindconclusies en onze aanbevelingen voor toekomstig onderzoek.



Chapter 8

List of publications

Acknowledgments

Curriculum vitae

PhD Portfolio

List of publications

Gorbenko Del Blanco D, de Graaff LC, Posthouwer D, Visser TJ, Hokken-Koelega AC. Isolated GH deficiency: mutation screening and copy number analysis of *HMGA2* and *CDK6* genes. *Eur J Endocrinol* 2011; 165: 537-544

Gorbenko Del Blanco D, de Graaff LC, Visser TJ, Hokken-Koelega AC. Mutations in two Hedgehog genes, *SHH* and *HHIP*, as genetic cause of Combined Pituitary Hormone Deficiency. *Submitted*

Gorbenko Del Blanco D, de Graaff LC, Visser TJ, Hokken-Koelega AC. Growth Hormone Insensitivity Syndrome caused by a heterozygous *GHR* mutation: phenotypic variability due to moderation by nonsense-mediated decay. *Submitted*

Gorbenko Del Blanco D, Romero C, de Graaff LC, Radovick S, Hokken-Koelega AC. A novel *OTX2* mutation in a patient with combined pituitary hormone deficiency, pituitary malformation and an underdeveloped left optic nerve. *Submitted*

Renes JS, van Doorn J, Breukhoven PE, **Gorbenko del Blanco D**, Lem AJ, Hokken-Koelega AC. *IGFALS* gene mutations in short children born Small for Gestational Age. *Submitted*

Benito-Sanz S, **D. G. del Blanco**, Aza-Carmona M, Magano LF, Lapunzina P, Argente J, Campos-Barros A, Heath KE. PAR1 deletions downstream of *SHOX* are the most frequent defect in a Spanish cohort of Léri-Weill dyschondrosteosis (LWD) probands. *Human Mutation* 2006; 27 (10): 1062.

Benito-Sanz S, **Gorbenko Del Blanco D**, Huber C, Aza-Carmona M, Thomas NS, Bunyan D, Maloney V, Argente J, Cormier-Daire V, Campos-Barros A, Heath KE. Characterization of *SHOX* deletions in Léri-Weill dyschondrosteosis (LWD) reveals genetic heterogeneity and no recombination hotspots. *Am J Hum Genet*: 2006; 79:409-414

Benito-Sanz S, Thomas NS, Huber C, **Gorbenko Del Blanco D**, Aza-Carmona M, Crolla JA, Maloney V, Argente J, Campos-Barros A, Cormier-Daire V, Heath KE. A novel class of pseudoautosomal region 1 (PAR1) deletions downstream of *SHOX* is associated with Léri-Weill dyschondrosteosis (LWD) *Am J Hum Genet* 2005; 77: 533-544.

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Dear Professor de Jong, thanks for your interest and advise in my work.

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Gracias a mi tío Miguel y Alisa, gracias por ser parte de la familia para lo bueno y para lo malo y sobre todo gracias por ayudarme con cruzar medio Europa y traer mis cosas de vuelta.

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Curriculum Vitae

Personalia

Name: Darya Gorbenko del Blanco
Date/place of birth: March 21, 1980, Nikolaev, Ukraine
Nationality: Spanish

Education

1995 – 1999 High school diploma in Natural Science and Health (I.E.S. Dámaso Alonso, Madrid, Spain)
1999 – 2004 Bachelor's degree in Biology, specialty Genetic and Molecular Biology (Universidad Autónoma, Madrid, Spain)

Work experience

2003 – 2004 Scholarship of scientific collaboration in the plant physiology laboratory of the Biology Department (Universidad Autónoma, Madrid, Spain), research project 'Molecular analysis of the response to a heavy metals in *Medicago spp.*'
2004 – 2008 PhD student position in the research laboratory of the Endocrinology department (Hospital Infantil Universitario Niño Jesús, Madrid, Spain), research project 'Genetic and functional analysis of *SHOX* and *SHOX2* in human growth'.
2008 – 2011 PhD student in the Department of Pediatric Endocrinology in Erasmus University (Rotterdam, Netherlands), research project 'Exploring the spectrum of pituitary hormone deficiencies: genotype, molecular mechanisms and phenotypic variability'. Collaboration with José C. Moreno (Thyroid molecular laboratory, INGEMM-Institute for Medical and Molecular Genetics, La Paz university hospital, Madrid, Spain) and Sally Radovick (Division of Pediatric Endocrinology, The Johns Hopkins University School of Medicine, Baltimore, US).



PhD Portfolio

Summary of PhD training and teaching

Name PhD student:	Darya Gorbenko del Blanco
Erasmus MC Department:	Internal Medicine
Research School:	MolMed
PhD period:	June 2008 – December 2011
Promotor:	Prof. dr. A.C.S. Hokken-Koelega
Co-promotor:	Dr. L.C.G. de Graaff

PhD training

	Year	Workload
General courses	2010	4 ECTS
- Biomedical English Writing and Communication		
Seminars and workshops		
- The workshop Applied Bioinformatics 'Finding your way in biological information'	2009	2 days
- Seminars at 'Get out of your lab days'	2009	2 days
- The Photoshop CS3 Workshop for PhD-students and other researchers	2010	2 days
- The workshop browsing genes and genomes with Ensembl VI	2010	2 days
Other		
- Award: third best poster at Internal Medicine Science Days 2011 for the work: TSH bioactivity in children with isolated central hypothyroidism and macroorchidism.	2011	

Presentations

- Molecular analysis of growth hormone receptor (<i>GHR</i>) gene in a patient with Idiopathic Short Stature and insensitivity to GH. <i>Spanish Society for Paediatric Endocrinology</i> .	2009	Poster
- Mutation screening of <i>HMGA2</i> and <i>CDK6</i> genes in Dutch patients with Isolated Growth Hormone Deficiency. <i>LWPES/ESPE 8th Joint Meeting</i> .	2009	Poster
- Mutation screening of <i>HMGA2</i> and <i>CDK6</i> genes in Dutch patients with Isolated Growth Hormone Deficiency. <i>Internal Medicine Science Days</i> .	2010	Poster
- TSH bioactivity in children with isolated central hypothyroidism associated with macroorchidism. <i>International Thyroid Congress</i> .	2010	Poster
- TSH bioactivity in children with isolated central hypothyroidism and macroorchidism. <i>European Society for Paediatric Endocrinology</i> .	2010	Oral
- TSH bioactivity in children with isolated central hypothyroidism and macroorchidism. <i>Internal Medicine Science Days</i> .	2011	Poster

(Inter)national conferences

- XXXI Congress of Spanish Society for Paediatric Endocrinology, Alicante, Spain	2009	3 days
- 13 th Molecular Medicine Day, Rotterdam, the Netherlands	2009	1 day
- Endo retreat, Rotterdam, the Netherlands	2009	1 day
- LWPES/ESPE 8 th Joint Meeting, New York city, USA	2009	4 days
- Internal Medicine Science Days, Antwerp, Belgium	2010	2 days
- 14 th Molecular Medicine Day, Rotterdam, the Netherlands	2010	1 day
- The XV th Annual Symposium of the Dutch Thyroid Club	2010	1 day
- 14 th International Thyroid Congress, Paris, France	2010	6 days
- 49 th Annual Meeting of the European Society for Paediatric Endocrinology, Prague, Czech Republic	2010	4 days
- Internal Medicine Science Days, Antwerp, Belgium	2011	2 days

Propositions (Stellingen)

' Exploring the Spectrum of Pituitary Hormone Deficiencies: Genotype, molecular mechanisms and phenotypic variability '

1. Isolated Growth Hormone Deficiency may be due to a mutation in *HMGA2* (this thesis).
2. Mutations in genes involved in the Hedgehog pathway may explain part of the phenotype of patients with Combined Pituitary Hormone Deficiency (this thesis).
3. *OTX2* is a good candidate gene for genetic screening in patients with Isolated Growth Hormone Deficiency or Combined Pituitary Hormone Deficiency, in whom mutations in the classical candidate genes have been ruled out, especially in patients with ocular problems (this thesis).
4. Heterozygous *OTX2* mutations may cause Combined Pituitary Hormone Deficiency by exerting a dominant negative effect (this thesis).
5. The same mutation in *GHR* may produce variable degrees in phenotypic severity due to nonsense mediated decay (this thesis).
6. Yet unidentified genetic variants may play a role in the etiology of Combined Pituitary Hormone Deficiency presently diagnosed as "idiopathic" (this thesis).
7. It is important to use primary cultures established from cells of patients for functional studies (Fang *et al*, J. Clin. Endocrinol. Metab. 2009).
8. Genetic defects may result in a range of clinical phenotypes depending on the degree of disturbance of key endocrine mechanisms (David *et al*, Endocr. Rev. 2011).
9. Next-generation exome sequencing has become a powerful new approach for identifying genes that underlie Mendelian disorders and perhaps for complex traits as well (Bamshad *et al*, Nat. Rev. Genet. 2011).
10. The factors involved in deciding human stature are numerous. They interact with each other to produce the single character we call height (William Bateson 1909).
11. We don't know a millionth of one percent about anything (Thomas A. Edison).

Rotterdam, December 13th, 2011

Darya Gorbenko del Blanco