GENETIC ANALYSIS OF *GH1*, *GLI2* AND *SHOX* GENES IN PATIENTS WITH GROWTH IMPAIRMENT

By

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A thesis submitted to

The Department of Health Sciences of the

University of Eastern Piedmont

In partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

in

Molecular medicine

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UNIVERSITÀ DEL PIEMONTE ORIENTALE

Year 2015

XXVII CYCLE

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Prof. Mara Giordano for giving me the opportunity to carry out this research. I appreciate all her contributions of time, advice, patience and funding to make my PhD possible.

I would also like to thank my fellow PhD student Ileana Fusco for her constant support, help and friendship during the last three years.

I wish to express my sincere gratitude to all the members of my group, both present and past, for providing a wonderful environment to work in. I gratefully acknowledge the contributions of Michela Godi, Simona Mellone, Ranjith Muniswamy and Gillian Walker to this work.

I would like to extend my special thanks to Alessandra Bagarotti and Lucia Corrado for their valuable encouragement, help and friendship. I know that I could always go to them for advices and opinions and they were most supportive whenever I needed. I will never forget the fun activities we have done together.

Laboratory of Genetica has been a place of friendships and fun. I would like to thank Nadia Barizzone, Antonella Fanelli, Clarissa Locci, Elena Corsetti, Giulia Predebon, Miriam Zuccalà, Sabrina Botta and all the other present and past members of the laboratory for their friendship and support.

Lastly, I would like to thank my family and friends for all their love and encouragement. I am grateful to my parents and my sister for their unconditional support in all my pursuits, to whom I dedicate this thesis.

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LIST OF ABBREVIATIONS

SDS	Standard Deviations Score
rhGH	recombinant human Growth Hormone
GHD	Growth Hormone Deficiency
CPHD	Combined Pituitary Hormone Deficiencies
GH1	Growth Hormone gene
GHRHR	Growth Hormone Releasing Hormone Receptor
IGHD	Isolated Growth Hormone Deficiency
ESE	Exonic Splicing Enhancer
ISE	Intronic Splicing Enhancer
IVS	Intervening Sequence
ISS	Idiopathic Short Stature
SHOX	Short stature Homeobox containing gene
CNE	Conserved non-coding elements
MLPA	Multiplex Ligation-dependent Probe Amplification assay
PCR	Polymerase Chain reaction
SHH	Sonic Hedgehog Signalling
HPE	Holoprocensephaly
aCGH	array Comparitive genomic Hybridisation
PAR1	Pseudo autosomal region 1
TS	Turner Syndrome
PRL	Prolactin
TSH	Thyroid stimulating Hormone
CGDP	Constitutional delay of growth and puberty
UTR	Untranslated region

I. INTRODUCTION

I.1 Growth disorders

Longitudinal growth is a complex process in which genetic, nutritional, hormonal and environmental factors among others are involved. Growth abnormalities resulting in short stature is one of the most common conditions affecting the childhood growth. The cause of short stature can be either a variant of normal growth or a pathological condition. Variants of normal growth includes familial short stature, constitutional delay of growth and puberty and small gestational age with catch-up growth. Pathological causes of growth defects can be systemic diseases and their treatment, pituitary hormone deficiencies (isolated or combined) or a series of genetic syndromes. Short stature can also be found in individuals where no cause can be identified (idiopathic short stature).

It is assumed that the human height follows a normal distribution (Gaussian distribution) where the height is distributed along a bell shaped curve. The mean height of the population is located at the centre of the curve with shorter and the taller stature distributed towards both the sides. The standard height deviation (SDS) is obtained by subtracting the mean value of the reference population from the observed value and dividing by the standard deviation (SD) value of the reference population. For example, Height SDS = (Child's height – normal population mean for children of comparable age and sex) / SD of the height of children of comparable age and sex. Height SDS of >-2 SD is used to identify children with short stature. However, it is more likely that stature does not fit a perfect Gaussian distribution and a variety of conditions such as Growth hormone deficiency (GHD), Hypopituitarism, chronic diseases and malnutrition contribute to the extreme cases within the Gaussian distribution.

Successful treatment with rhGH (recombinant human growth hormone) has been developed to achieve the target height in selected children with small gestational age without catch up growth, growth hormone deficiency, idiopathic short stature due to *SHOX* mutations and other

genetic syndromes like Turner syndrome. The accurate and early diagnosis of the abnormal growth patterns are highly important as it allows appropriate treatment and improved clinical outcomes.

I.2 Growth Hormone Deficiency (GHD)

Growth hormone Deficiency (GHD) refers to conditions associated with childhood growth failure due to the lack of growth hormone action. It can be isolated (IGHD) or in combination with other pituitary hormone deficiencies. The incidence of GHD is estimated to be 1/4000 – 1/10000 births (1). While majority of cases are sporadic, 3-30% has an affected relative, suggesting a genetic etiology. In most of the sporadic cases, no cause of GHD can be identified, even though mutations have been identified up to 4% of patients with sporadic growth hormone deficiency. It is assumed that significantly higher proportion of sporadic cases may have genetic causes. Several mutations, including *de novo*, mainly in the GH encoding gene (*GH1*) or in some cases, the receptor of growth hormone releasing hormone (*GHRHR*) have been detected in both sporadic and familial cases of IGHD. The deletion of the entire gene, missense and frameshift mutations produce severe growth hormone deficiency whereas the splicing mutations produce milder forms (2-4). In addition to *GH1* mutations, other mutations that cause growth hormone deficiency has been reported in the growth hormone receptor (*GHR*) gene and in a series of transcription factor genes involved in the pituitary development such as Pit-1, Prop-1, Hesx-1,Sox-2, Sox-3, Lhx-3, Lhx-4 (5).

I.2.1 *GH1* gene

Human growth hormone gene *(GH1)* is located on chromosome 17q23 within a cluster of five highly homologous (92-98%) genes consists of *GH1, CSHP* (chorionic somatomammotropin pseudogene), *CSH1* (chorionic somatomammotropin gene), *GH2* and *CSH2* (Figure 1) (6,7) . Despite the high degree of sequence homology, they express in a tissue-specific manner. While

GH1 gene express exclusively in the somatotrophic cells of the anterior pituitary gland, *GH2*, *CSH1* and *CSH2* all are expressed in placental level (8,9). All the five genes in this cluster are located in the same transcriptional orientation and consists of 5 exons separated by 4 introns. *GH1* encode a 217 aminoacid prehormone which is cleaved to yield a mature hormone with 191aminoacods and a molecular weight of 22KDa.

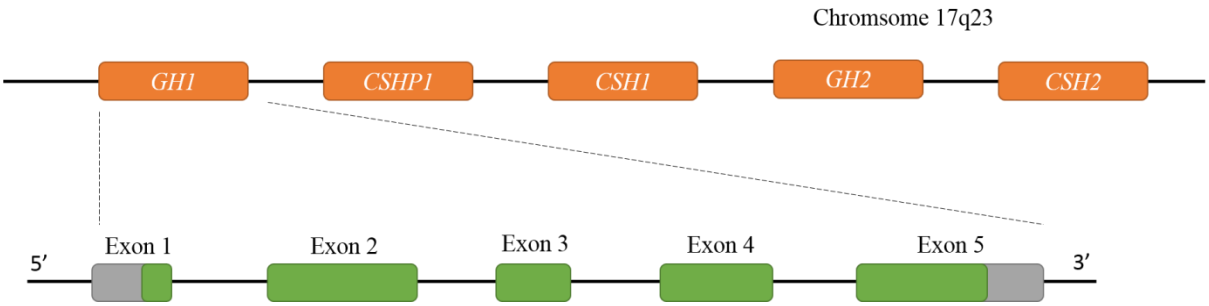


Figure 1: Schematic representation of the GH gene cluster and the *GH1* gene.

I.2.2 *GH1* mutations and Classification of IGHD

On the basis of clinical characteristics, severity and mode of inheritance familial IGHD has been classified into four types; Type IA and IB, Type II and Type III (Table 1). IGHD Type 1A is the most severe form of IGHD with patients showing early and profound growth failure due to the lack or extremely low levels of serum GH. It has an autosomal recessive mode of inheritance and is caused by deletions removing the entire *GH1* or a nonsense mutation leading to a premature stop codon (10-12). IGHD Type IB is a milder form with low but detectable serum GH and positive response and immunological tolerance to treatment with exogenous GH. This condition is inherited as autosomal recessive trait due to the splice site mutations of the GH gene or mutations within gene encoding the GHRH receptor (5). IGHD Type II, a very common form of IGHD which are inherited in an autosomal dominant pattern. They are mainly caused by the GH1 mutations affecting the mRNA splicing and subsequent loss of exon 3. A 17.5 kDa isoform is produced as result of the skipping of Exon 3, which exerts a dominant

negative effect on the wild type isoform. The most common cases include mutations within the first 6 bp of intervening sequences 3(IVS3)(13). Several mutations causing skipping of exon3 in patients with IGHDII have also been reported within the Intron Splice Enhancer (ISE) and Exon Splice Enhancer (ESE) sequences (Table 2) (14-16). Missense mutations that affect growth hormone secretion or action or both were also identified to be causing IGHD. Type III is X-linked, recessively inherited disorder. Previous studies have shown that some individuals have an associated X-linked agammaglobulinemia. It is also suggested that the disorder may be caused by mutations and/or deletions of a portion of the X-chromosome containing two loci, one necessary for normal immunoglobulin production and the other for GH expression (17,18).

Table 1: Genetic forms of IGHD and the mutations associated with them.

IGHD Type	**Inheritance**	**Phenotype**	**GH Level**	**Associated gene**	**Mutations**
IA	Autosomal recessive	Severe Short stature; Anti-GH antibodies develop during treatment	Absent	*GH1*	Large deletions Frame shift Nonsense
IB	Autosomal recessive	Less severe short stature than type IA	Low	*GH1*, GHRHR	Frameshift, Nonsense, missense and splice site
II	Autosomal dominant	Variable Height(Severe short stature to normal height	Low	*GH1*	Splice site mutations, splicing enhancer mutations or missense
III	X-linked Recessive	GH deficiency with agammaglobulinemia	Low	SOX3/other genes	Deletions, Expansions/other mutations

I.2.3 Alternative splicing in *GH1*

Alternative splicing is the event responsible for the production of multiple mature mRNAs with different sequence compositions from a single gene. In most of the cases, Alternative splicing is a very common and crucial mechanism, responsible for the complexity and diversity of the proteome. It is also well understood that alternative splicing isoforms are differently expressed among tissues (19). Majority of the human genes are subject to alternative splicing and genes that code for ten to hundreds of transcripts are common in the genome. Alternative splicing

studies on chromosome 22 indicated that about 60% of genes are represented by two or more transcripts (20-22).

Alternative splicing is recognized by several modes of splicing patterns. Some common mechanisms involve; use of alternative 5' or 3' splice sites (cryptic splice sites), exon skipping or inclusion, selection between mutually exclusive exons and intron retention. Other important modes for alternative splicing also includes the use of alternative promoters and alternative poly (A) signals. The mechanism of accurate recognition of the exons are significant to maintain the splicing fidelity. For example, a "weaker" splice site is harder to recognize by the splicing machinery and leads to alternative splicing. Alternative splicing has both positive and negative impact on the gene expression. While alternative splicing maintain the greater diversity in proteome, its misregulation underlies several genetic diseases.

When correctly spliced *GH1* produces the biologically active 22 KDa protein comprises of all the exons (Figure 2). It accounts for the majority of the circulating GH (10). Despite the correct processing, even under normal conditions, small percentages of at least other four kinds of alternatively spliced isoforms are produced. The presence of an in frame cryptic splice site within exon 3 (Figure 2) gives rise to a transcript lacking the first 45 base pairs of exon 3 and encodes a shorter active isoform of 20KDa representing 5-10% of GH transcripts(23). A 17.5 KDa isoform is produced by the complete skipping of exon 3, which acts as dominant negative isoform (Figure 2). This isoform lacks the entire loop connecting helix 1 and helix 2 in the tertiary structure of GH. This biologically inactive isoform accounts for 0.1 – 5% of GH transcripts and prevents the secretion of the wild type GH (24-26). Trace amounts of transcripts skipping exon 3-4 (11.3 KDa) and exons 2-4 (7.4 KDa) have also been identified (Figure 2). These severely truncated isoforms are biologically inactive (27).

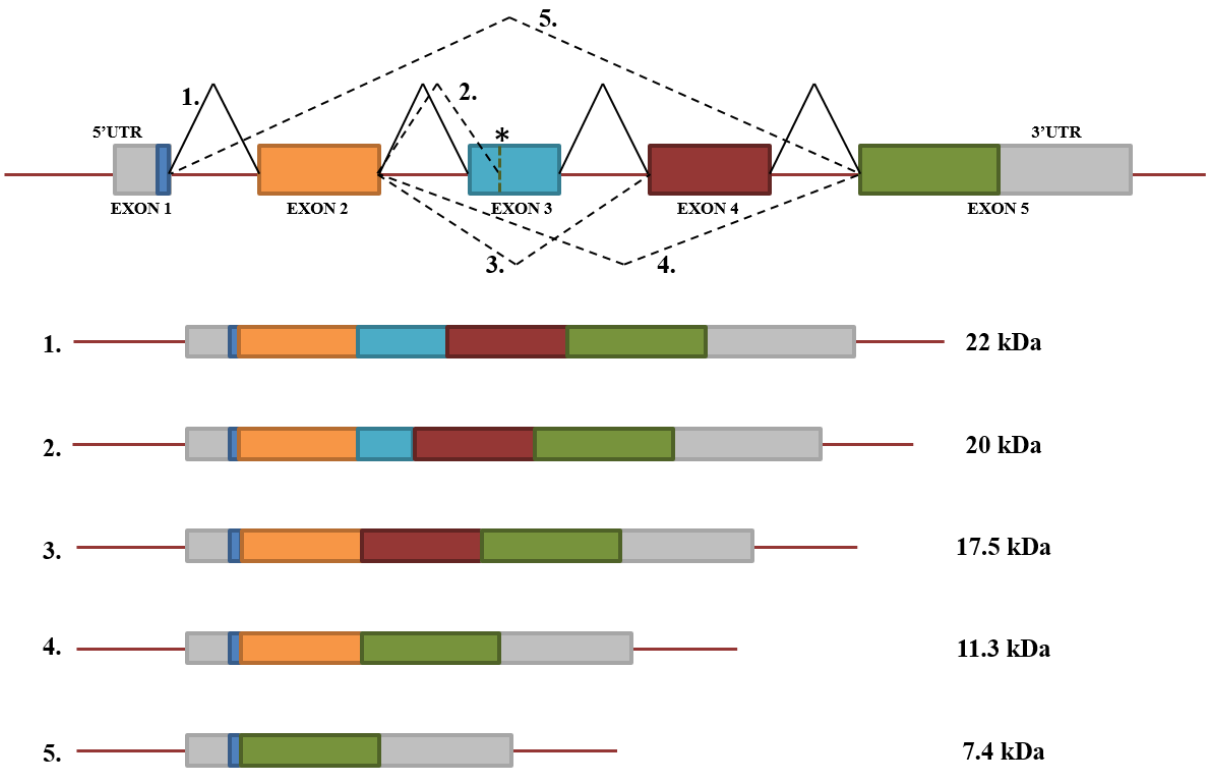


Figure2: Alternative splicing in *GH1*

The classical splicing elements (5' and 3' splice sites) are not sufficient enough for the correct identification of the splice sites and maintain splicing fidelity. Additional cis acting regulatory elements are necessary to guide the spliceosome to the splice sites. These elements are capable of stimulating (enhancers) or repressing (silencers) splicing and have an important role in the regulation of alterative splicing. Based on their location and function they are mainly characterised into exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). Among these splicing regulatory elements, ESEs are the most prevalent, widely present and intensively studied. ESEs are short 6-10 nucleotide elements reside within exons, recognised by SR proteins to promote spliceosome assembly (20,28). SR proteins are a large family of structurally related and highly conserved splicing factors which can stimulate the exon definition either by directly recognising the splicing machinery or by antagonising the action of nearby silencer elements (20,28,29). Most of the identified enhancers are purine rich motifs (GC rich), although it is

proved that high purine rich composition by itself is not sufficient to promote splicing. Several ESEs are identified with no purine rich elements, mostly AC rich elements (30-32). Although most of the ESEs are studied in the context of alternative splicing(28,32), it is likely that they are also important for constitutive splicing (33).

The canonical splice sites surrounding *GH1* exon 3 are relatively weak and require the multiple cis acting splicing elements to maintain the exon 3 definition. Two ESEs residing within exon 3 and an ISE within intron 3 have been well characterised. ESE1 comprises the first 7 base pairs of exon 3 and is essential to maintain the proper recognition of upstream 3' splice site and silencing the cryptic splice site (14,34). ESE2 is found 12 nucleotides upstream of the cryptic splice site and comprises of 15 nucleotides(14). ISE is a nine nucleotide sequence within intron 3(IVS3+26-34).

Table 2: Mutations affecting *GH1* splicing.

***GH1* mutation**	**Type**	**Comment**	**Location**	**Reference**	**IGHD type**
Mutations within Exon Splice Enhancers					
Glu32X; E3+1G>T	Heterozygous	ESE1; loss of exon 3 (amino acids 32–71)	Exon 3, c.172G>T*	Takahashi *et al.*(35)	II
Glu32Lys; E3+1G>A	Heterozygous	ESE1	Exon 3, c.172G>A*	Shariat *et al.* (24)	II
Glu32Ala; E3+2A>C	Heterozygous	ESE1; 17.5 kDa (68%), 20 kDa (22%)	Exon 3, c.173A>C*	Petkovic *et al.* (16)	II
Glu33Gly; E3+5A>G	Heterozygous	ESE1; 17.5 kDa (62%), 20 kDa (27%)	Exon 3, c.176A>G*	Moseley *et al.* (34)	II
Lys41Arg	Heterozygous	ESE2; 20% exon skipping	Exon 3, c.200A>G*	Millar *et al.*(36)	II
Splice site Mutations					
IVS2 –1G>A	Heterozygous	3' acceptor splice site	Intron 2	Millar *et al.* (36)	II
IVS2 –2A>T	Heterozygous	3' acceptor splice site	Intron 2	Fofanova *et al.* (37)	II
IVS3+1G>A	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Cogan *et al.* (38)	II
IVS3+1G>C	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Binder&Ranke (39)	II
IVS3+2T>C	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Fofanova *et al.* (40)	II
IVS3+5G>A	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Hayashi *et al.* (41)	II
IVS3+5G>C	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Hayashi *et al.* (42)	II
IVS3+6T>C	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Cogan *et al.* (43)	II
IVS3+6T>G	Heterozygous	Skipping of exon 3, del32–71 GH	Intron 3	Katsumata *et al.* (44)	II
IVS4+1G>C	Homozygous	Loss of amino acids 103-126 in exon 4; frame shift in exon 5	Intron 4	Cogan *et al.* (12)	
IVS4+1G>T	Homozygous	Loss of amino acids 103-126 in exon 4; frame shift in exon 5	Intron 4	Phillips & Cogan (2)	IB
IVS4+5G>C	Homozygous	Loss of amino acids 103-126 in exon 4; frame shift in exon 5	Intron 4	Leiberman *et al.* (45)	IB
IVS4 –1G>A	Heterozygous	No amino acid change; assumed to affect splicing	Intron 4;c.456G>A	Fofanova *et al.* (46)	II
Mutations affecting ISE elements or BPS					
IVS3+28–45del	Heterozygous	18 bp deletion; skipping of exon 3	Intron 3, ISEm2	Cogan *et al.* (47); McCarthy & Phillips (15)	II
IVS3+56–77del	Heterozygous	removes BPS in intron 3; skipping of exon 3	Intron 3	Vivenza *et al.* (48)	II
IVS3+28G>A	Heterozygous	Abnormal splicing	Intron 3, ISEm1	Cogan *et al.* (47); McCarthy & Phillips (15)	II

I.2.4 Mutations in *GH1* Splicing elements cause IGHD II

Several mutations in *GH1* have been reported in IGHDII patients producing increased levels of exon 3 skipped transcripts represents the 17.5 kDa isoform (Table 2). Majority of these mutations are present within the splice sites (3' and 5') bordering exon 3 or in cis regulating splicing elements (splicing enhancers). The splice sites flanking exon 3 are particularly weaker than the cryptic splice site present within exon 3. Any disruption in the natural splice sites thereby increases the aberrant splicing and skipping of exon 3(2,36-38,42,49,50). Besides, the mutations identified in ESE1 (E3+1G→T, E3+2A→C, E3+5A→G; Figure 3) and ESE2 (E3+29A→G; Figure 3) affects the splicing enhancer functions and exon 3 definition (14,34,35,47,51). These enhancers activate the 3' splice sites of intron 2 and inactivate the cryptic splice site and promote the exon 3 inclusion. Two families with mutations in Intron splicing Enhancer (IVS3+28G→A, IVS3+del28-45; Figure 3) were also identified to produce abnormal levels of transcripts encoding the 17.5kDa isoform (15,38). In addition to these, missense mutations at amino acid positions 89(P89V), 110(V110F), 183(R183H) and a 22- bp deletion including the branch point site within intron 3 (IVS3 del+56-77; Figure 3) have also been reported leading to Exon 3 skipping (5,39,48,52). However, splice site mutations which produce 17.5KDa isoforms are understood to cause severe impact on the patients than ESE mutations.

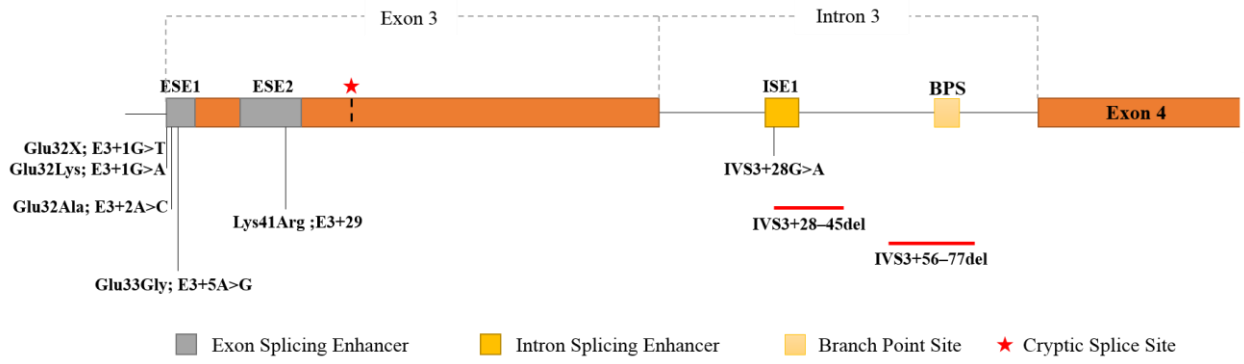


Figure3: Location of Exonic and Intronic splicing enhancer mutations identified in *GH1*.

Cell culture and transgenic mice experiments have proved that the 17.55 KDa isoform exhibit a dominant negative effect on the secretion of the wild type 22 KDa isoform (25,26,41). This dominant negative isoform is retained in the endoplasmic reticulum and impairs the secretory pathway and trafficking of GH and other hormones (53). Under normal conditions, the 17.5KDa isoforms are targeted by ER and undergoes controlled degradation by proteasomal pathway. When the amount of 17.5KDa exceeds the limit of the proteasome degradation mechanism, it accumulates in the cytoplasm leading to reduced cell proliferation *in vitro* (16). Moreover, transgenic mice overexpressing the 17.5 KDa isoform exhibited a loss of majority of somatotropes and subsequent defects in the maturation of GH secretory vesicles and anterior pituitary hypoplasia (14,25).

I.3 Combined pituitary hormone deficiency (CPHD)

Pituitary development is dependent upon a complex genetic cascade of transcription factors and signaling molecules acting as activators or repressors which dictate organ commitment, cell differentiation and cell proliferation that produces five distinct pituitary cell types (54,55). These cells in the in the pituitary gland are specialized to produce and secrete specific hormones, including growth hormone (GH), prolactin (PRL), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and adrenocorticotropic hormone (ACTH). Combined Pituitary Hormone Deficiency (CPHD) is diagnosed when the production of GH and one or more of the pituitary hormone is insufficient or absent. The incidence of CPHD is estimated to be approximately 1:8000 births and is usually sporadic but, familial forms have also been described (56). Clinically, CPHD is mainly characterised by short stature, hypothyroidism, impaired sexual development and hypocorticolism. More distinctive facial features with prominent forehead, marked midfacial hypoplasia with depressed nasal bridge, deep-set eyes, short nose with anteverted nostrils and hypoplastic pituitary gland by MRI examination were also have been reported along with growth deficiency (57). Some cases were also presented with mental retardation along with other typical phenotypical features (58).

The aetiology of CPHD is considered to be multifactorial which includes environmental and genetic factors. However, in the majority of cases the aetiology of CPHD cases remains unexplained. So far, the genetic aetiology is explained by the perturbation of expression or function of several developmental genes which are involved in different stages of pituitary development.

I.3.1 Pituitary Transcriptional factors associated with CPHD

The identification and characterization of the pituitary developmental factors *in vitro and from animal models* has enabled us to clarify a genetic basis for combined pituitary hormone deficiency (CPHD) in humans (59,60). To date detected genes include *PROP1, PIT1* (also named *POU1F1*), *HESX1, LHX3, LHX4, OTX2, GLI2, SOX2,* and *SOX3* (Table 3). Numerous studies demonstrated that mutations of these transcription factor genes cause a wide range of pituitary phenotypes, from severe life-threatening CPHD to isolated GH deficiency (59-61).

Numerous studies demonstrated that mutations of these transcription factor genes cause a wide range of pituitary phenotypes, from severe life-threatening CPHD to isolated GH deficiency (59-61). Transcriptional factors that are involved in the formation of pituitary primordial structures are not pituitary-specific but also play a role in the development of other organs and structures. Mutations within these "early factors" may present extra-pituitary manifestations: syndromic hypopituitarism with craniofacial defects such as septo-optic dysplasia (SOD) or holoprosencephaly (HPE) in the case of *HESX1* or *GLI2* mutations respectively. In some instance *HESX1* and *GLI2* mutations have been found associated to milder phenotypes with pituitary hormone deficiency without a syndromic phenotype (62,63). Other extra-pituitary manifestations such as Chiari malformation, corpus callosum hypoplasia, hearing impairment and skeletal abnormalities are associated with *LHX3* and *LHX4* mutations (59).

Conversely, *PROP1* and *PIT1* are homeodomain transcription factors specifically involved in pituitary development. Accordingly, mutations of these later-acting factors, are responsible of a pituitary specific phenotype (59) characterized by multiple hormone deficiencies without relevant extrapituitary findings. The hormonal phenotype in patients with *PROP1* mutations is characterized by deficiency of GH, TSH, PRL and gonadotropins with an extremely variable phenotype both within and between families with respect to the severity of hormone deficiency,

age of onset, adrenal function and height at diagnosis. In magnetic resonance imaging (MRI) posterior pituitary and infundibulus are normal, while anterior pituitary is often hypoplastic; a hyperplastic expansion of the intermediate lobe that usually involves during the second decade of life has been described (64-66).

PIT1 mutations present a phenotype characterized by profound deficiency of GH and PRL, variable degree of TSH deficit, severe proportional short stature, a typical facies, feeding difficulties in infancy and variable degree of mental delay. Neuroimaging usually shows a normal or hypoplastic anterior gland and a normal pituitary stalk and posterior pituitary.

In many CPHD patients genetic screening failed to detect mutations within any of these genes whereas in some CPHD cohorts the majority of the tested patients carried mutations, mostly affecting *PROP1*.

Table 3: Mutation frequencies within PROP1, PIT1, HESX1, LHX3, LHX4, and *GLI2* in different CPHD cohorts

Gene	Geographical origin	Sporadic cases		Familial cases		Total mutation rate (sporadic and familial)	Reference
		Analysed patients, n	Patients with mutation, n (%)	Analysed families, n	Families with mutation, n (%)		
PROP1	*Italy*	*118*	*2 (1.7)*	*8*	*1 (12.5)*	*2.4%*	*Unpublished*
	Lithuania	43	25 (58.1)	11	10 (90.9)	64,8%	Navardauskaite *et al.* (67)
	Turkey	51	0	1	1 (100)	1.9%	Kandemir *et al.* (68)
	*Japan	77	0	0	-	0	Takagi *et al.* (69)
	*Japan	71	0	0	-	0	Dateki *et al.* (70)
	Germany (mostly)	75	0	1	0	0	de Graaff *et al.* (71)
	Various (UK mostly)	119	1 (0.8)	0	-	0.8%	Mehta *et al.* (72)
	*USA	19	0	0	-	0	Diaczok *et al.* (73)
	Brazil	24	0	5	5 (100)	17.2%	Vieira *et al.* (56)
	Spain	36	0	0	-	0	Coya *et al.* (74)
	Various	92	12 (13.2)	17	8 (47)	18.3%	Reynaud *et al.* (75)
	Portugal	29	2 (6.9)	7	7 (100)	25%	Lemos *et al.*(76)
	Hungary	35	15 (42.8)	0	-	42.8%	Halasz *et al.*(77)
	UK	20	0	6	0	0	Rainbow *et al.*(78)
	*Czechia	66	12 (18.2)	4	3 (75)	21.4%	Lebl *et al.* (79)
	φ Various (UK minority)	189	2 (1)	25	6 (24)	3.7%	Turton *et al.*(65)
	Australia	31	0	1	0	0	McLennan *et al.*(80)
	Korea	12	0	0	-	0	Kim *et al.*(81)
	Russia	7	3 (42.9)	4	2 (50)	45.5%	Fofanova *et al.* (82)
	Various	21	2 (9.5)	10	5 (50)	22.6%	Cogan *et al.*(83)
	**Various	0	-	36	18 (50)	50%	Deladoey *et al.*(84)
	Total	**1135**	**76 (6.7)**	**136**	**66 (48.5)**	**11.2%**	
PIT1	*Italy*	*24*	*1 (4.2)*	*0*	-	*4.2%*	*Unpublished*
	*Japan	77	1 (1.3)	0	-	1.3%	Takagi *et al.* (69)
	*Japan	71	0	0	-	0	Dateki *et al.* (70)
	Germany (mostly)	75	0	1	1	1.3%	de Graaff *et al.* (71)
	Various (UK mostly)	55	1 (1.8)	0	-	1.8%	Mehta *et al.* (72)
	*USA	19	0	0	-	0	Diaczok *et al.* (73)
	Brazil	5	0	0	-	0	Vieira *et al.* (56)
	Spain	36	0	0	-	0	Coya *et al.* (74)
	Various	13	0	4	1 (25)	5.9%	Reynaud *et al.* (75)
	*Czech	66	1 (1.5)	4	0	1.4%	Lebl *et al.* (79)
	φ Various (UK mostly)	105	4 (3.8)	17	3 (17.7)	5.7%	Turton *et al.*(65)
	UK	20	0	6	2 (33.3)	7.7%	Rainbow *et al.*(78)
	Australia	31	2 (6.5)	1	0	6.3%	McLennan *et al.*(80)
	Korea	12	0	0	-	0	Kim *et al.*(81)
	Russia	8	0	4	1 (25)	8.3%	Fofanova *et al.* (82)
	Total	**617**	**10 (1.6)**	**37**	**8 (21.6)**	**2.8%**	

HESX1	*Italy*	*126*	*1 (0.8)*	*7*	*0*	*0.8%*	*Unpublished*
	*Japan	77	0	0	-	0	Takagi *et al.* (69)
	*Japan	71	0	0	-	0	Dateki *et al.* (70)
	Germany (mostly)	75	0	1	0	0	de Graaff *et al.* (71)
	Various (UK mostly)	119	0	0	-	0	Mehta *et al.* (72)
	*USA	19	0	0	-	0	Diaczok *et al.* (73)
	Brazil	24	0	5	0	0	Vieira *et al.* (56)
	Spain	36	2 (5.6)	0	-	5.6%	Coya *et al.* (74)
	Various	16	0	0	-	0	Reynaud *et al.* (75)
	*Czechia	66	0	4	0	0	Lebl *et al.* (79)
	UK	20	0	6	0	0	Rainbow *et al.*(78)
	Korea	12	0	0	-	0	Kim *et al.*(81)
	Total	**661**	**3 (0.5)**	**23**	**0**	**0.4%**	
LHX3	*Italy*	*95*	*0*	*7*	*0*	*0*	*Unpublished*
	*Japan	77	0	0	-	0	Takagi *et al.* (69)
	*Japan	71	0	0	-	0	Dateki *et al.* (70)
	Germany (mostly)	75	0	1	0	0	de Graaff *et al.* (71)
	UK	119	0	0	-	0	Mehta *et al.* (72)
	*USA	19	0	0	-	0	Diaczok *et al.* (73)
	Brazil	13	0	5	0	0	Vieira *et al.* (56)
	* Various	300	2 (0.7)	2	2 (?)	1.3%	Pfaeffle *et al.* (85)
	Various	17	0	3	0	0	Reynaud *et al.* (75)
	Korea	12	0	0	-	0	Kim *et al.*(81)
	Total	**798**	**2 (0.3)**	**18**	**2 (11.1)**	**0.5%**	
LHX4	*Italy*	*94*	*0*	*7*	*0*	*0*	*Unpublished*
	*Japan	77	2 (2.6)	0	-	2.6%	Takagi *et al.* (69)
	*Japan	71	1 (1.4)	0	-	1.4%	Dateki *et al.* (70)
	Germany (mostly)	75	0	1	0	0	de Graaff *et al.* (71)
	UK	119	0	0	-	0	Mehta *et al.* (72)
	Various	126	0	7	2 (28.6)	1.5%	Castinetti *et al.*(86)
	* Various	244	2 (0.8)	1	1 (?)	1.2%	Pfaeffle *et al.* (85)
	*USA	19	0	0	-	0	Diaczok *et al.* (73)
	Brazil	11	0	0	-	0	Vieira *et al.* (56)
	Total	**916**	**5 (0.5)**	**16**	**3 (18.8)**	**0.9%**	
GLI2	*Brazil	136	4 (2.9)	0	-	2.9%	Franca *et al.* (87)
	*Various	165	1 (0.6)	0	-	0.6%	Flemming *et al.* (63)
	*Japan	77	0	0	-	0	Takagi *et al.* (69)
	Total	**430**	**7 (1.6)**	**0**	**-**	**1.6%**	

*** Subjects considered as sporadic if otherwise not specified**

**** All subjects considered as familial**

ϕ IGHD patients were also considered because it was not possible to obtain the number of CPHD sporadic and familial patient

I.3.2 Human GLI2

GLI2 is a member of GLI family of transcription factors along with GLI1 and GLI3 known as Glis super family. These transcription factors are recognized by a conserved DNA binding domain X3-Cys-X4-Cys-X12-His-X4-His-X3, where X is any amino acid. GLI2 is specifically recognized and bind to GAACCACCCA motif in the target genes. GLI2 is a 1586 –aminoacid protein (197 kDa) which is encoded by 13 exons on chromosome 2q14. The functional domains of GLI2 are not yet fully understood. In addition to the central zinc finger DNA binding domain consisting of 5 fingers, GLI2 proteins also contains an amino terminal (N-terminal) repressor domain and carboxyl terminal (C-terminal) transactivation domain. GLI2 protein has been identified to be present in at least four different splice variants, known as α (133kDa), β (131kDa), γ (88 kDa) and δ (86kDa). A human variant lacking the N-terminal repressor domain (GLI2ΔN) showed a 30-fold higher activity compared with the full length protein *in vitro* (Figure 4).

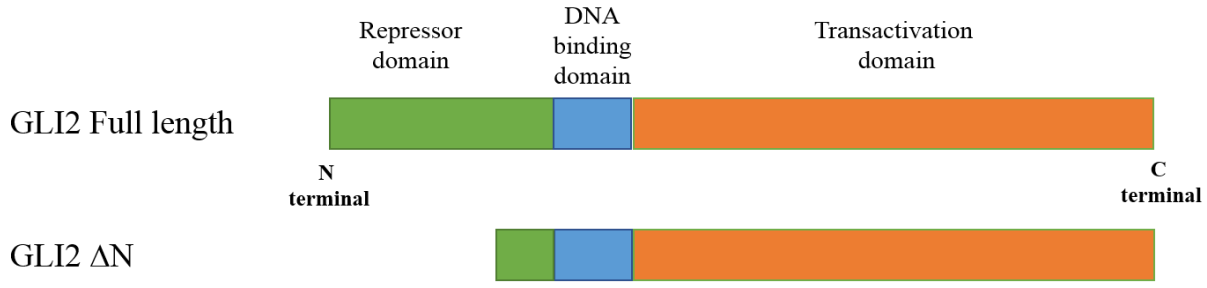


Figure 4: Schematic representation of different domains identified in the human GLI2. GLI2ΔN represents the isoform lacking the N-terminal repressor domain (below) compared with the Full length isoform (above).

I.3.3 Role of GLI2 in the development of pituitary

GLI2 is an important component in the developmental programs. As an effector molecule of the sonic hedgehog (Shh) signalling pathway, GLI2 has a fundamental role in the pituitary development. Sonic hedgehog is a morphogen expressed in the early steps of pituitary otogenesis by exerting effects on both proliferation and cell-type determination. *Sonic hedgehog* is expressed in the ventral diencephalon and throughout the oral ectoderm except Rathke's pouch (88,89). However, the *patched* receptor (PTCH1) as well as the GLI family of transcription factors (GLI1, GLI2 and GLI3) are expressed in the Rathke's pouch, indicating the developing gland is competent to receive and respond to SHH signalling (90).

There are three different Hedgehog proteins in humans, each with different cellular responses and distribution in the body: Sonic hedgehog (SHH), Indian Hedgehog (IHH) and desert hedgehog (DHH). The Hh pathway is considered as the canonical pathway through which GLI2 activity is regulated (Figure 5). The Hedgehog ligands binds to and activate the transmembrane receptor called *patched* (PTCH). When the Hh ligand is absent, PTCH exerts a consistent inhibitory effect on transmembrane G-protein coupled receptor *smoothened* (SMO). When the Hh ligand is present and binds to PTCH, inhibition over SMO is released (91,92). The GLI transcription factors are bound with SuFu which keeps GLI2 tethered in the cytoplasm (93). Activated SMO triggers the dissociation of SuFu/GLI complex and allowing the nuclear translocation and activation of GLI. This translocation promotes the subsequent DNA binding and transcription of a series of Hh pathway target genes (Figure 5).

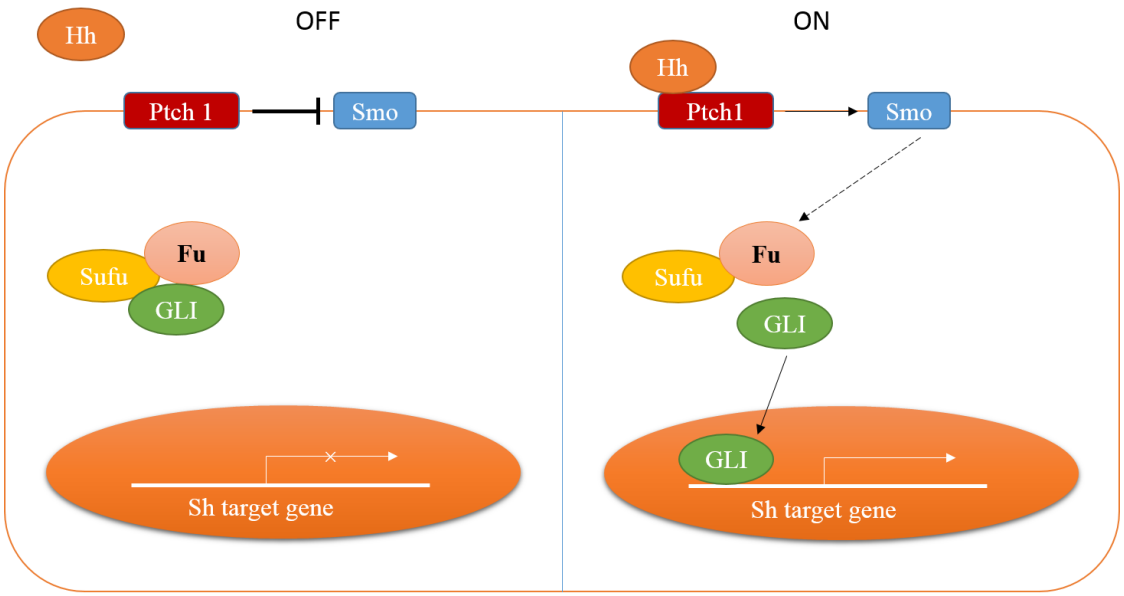


Figure 5: Schematic representation of hedgehog signalling pathway

Multiple studies using knockout mice has been performed to study the importance of GLI2 in development. Mice with homozygous loss of functioning GLI2 resulted in lethal phenotype later in development while the heterozygous mice developed normally. The phenotypic evaluation of abnormalities in the knock out mice showed severe skeletal abnormalities including absence of vertebral body and intervertebral disc, truncated mandibles with absent incisors, shortened limbs and sternum, missing tympanic ring bones of the inner ear and severe cleft palate malformations (90). *GLI2* deficient mice also showed defects in the pituitary development including partial loss of anterior and complete loss of posterior pituitary (94,95). These defects were attributed by the loss of expression of GLI2 target genes *BMP4* and *FGF8* (95).

I.3.4 *GLI2* Mutations

A spectrum of disorders and diseases has been described involving the loss of expression and deregulation of GLI2. The loss of function *GLI2* mutations are generally responsible for congenital malformations while gain of function in adult cells to tumerogenesis. The status of *GLI2* as an oncogene is well established.

Apart from Carcinogenesis, Heterozygous *GLI2* loss of function mutations were initially reported in patients with holoprocensephaly (HPE), a condition of incomplete or failed forebrain separation, or HPE-like phenotypes with pituitary anomalies and postaxial polydactyly (96,97). Several *GLI2* variations were since reported in patients with HPE characterized by a wide spectrum of phenotypes including craniofacial abnormalities, brancial arch anomalies, polydactyly and variable degree of pituitary malformations (98-101). Recently, Franca et al (87,102) reported novel heterozygous frame shift or nonsense *GLI2* mutations and high frequency of non-synonymous *GLI2* variants in patients with congenital hypopituitarism without HPE and most of these patients presented with CPHD and an ectopic posterior pituitary lobe. These patients were also presented with a wide spectrum of phenotype including polydactyly, hypoglycemia, seizures, midline facial defects and hypoplastic anterior pituitary. More recently, Bear et al (103) reported that individuals with truncating *GLI2* mutations were more likely to have typical pituitary anomalies, polydactyly and subtle facial features rather than HPE. These individuals are more likely to have higher penetrance than individuals with missense mutation.

I.4 Idiopathic Short stature

Short stature is a condition where the height of an individual is less than 2 standard deviation (SD) from the mean height, where the normal height is considered within more or less 2 SD, for a given age, sex and population. In most of the cases the aetiology of short stature is unknown and this is referred as Idiopathic Short Stature (ISS) (104,105). Thus, ISS can be defined as a condition in which the height of an individual is more than 2 SD below the corresponding average height for a given age, sex and population with no recognizable endocrine or systemic diseases and no known genetic causes. It has been estimated that around 80% of the short children presented to a paediatric clinic are classified as ISS (105). ISS can be subdivided into Familial Short Stature or FSS (when the height of an individual is below the

mean height compared to the reference population but remain within the target height of the family and Non Familial Short Stature) and Non Familial Short Stature or NFSS (when the height of a child is short compared to both reference population and the target height of the family). In FSS children, with the absence of pathological causes of parental shortness, full set of diagnostic screening may not be necessary and they attain their genetic potential. However, if one of the parent is short (<-2 SDS) attention should be given to the possibility of a dominant genetic effect. Similarly, in NFSS children with a positive family history of constitutional delay of growth and puberty (CGDP), and the experienced clinician may follow an expectant course (105).

Thus, ISS is a diagnosis based on careful exclusion of recognizable conditions such as chromosomal abnormalities, dysmorphic syndromes, low birth size (small for gestational age, SGA), systemic, endocrine or nutritional disorders and skeletal dysplasias (104,105). But, how far these criteria should be considered before a condition could be labelled as ISS is still remain to be completely agreed. It is widely considered that analysis to exclude turner syndrome should be done in all females presented with short stature. *SHOX* is another gene now widely agreed to be screened in the ISS patients. *SHOX* haploinsufficiency, which is caused by heterozygous mutations or deletion of *SHOX* or abnormalities of the enhancer region, is reported in 2-15% of children previously diagnosed as ISS (106-110). *SHOX* screening is also relevant, since GH therapy is reported as an effective treatment of short stature associated with *SHOX* defects(111). *SHOX* defect is now an approved indication for GH treatment in US and Europe.

I.4.1 *SHOX* gene

SHOX (short stature homeobox containing gene) was first discovered by deletion mapping of the short arm of chromosome as a gene responsible for growth failures in idiopathic short stature and Turner Syndrome (TS) patients (108). The gene is located in the pseudoautosomal region 1 (PAR1), a region of 2.6Mb, on the very tip of the short arms of both X and Y chromosomes, Xp22 and Yp11.3 (Figure 6). All characterized genes residing in PAR1 region escapes X inactivation. Because of this, *SHOX* is expressed on both X and Y chromosomes as two functional copies. PAR1 is a hotspot for recombination events between the X and Y chromosomes during the meiosis pairing of spermatogenesis (112).

The *SHOX* gene contains two characteristic domains: a homeodomain encoded by the homeobox and an OAR domain (otp, aristaless and rax) at the carboxyl terminal. The homeobox enables specific DNA binding and act as transcriptional activators. This domain is also relevant for nuclear translocation and dimerization of *SHOX*. The OAR domain is also relevant in keeping transactivation capacity of *SHOX*.

The SHOX mRNA is encoded by 7 exons (one non-coding and six coding) encompassing about 40 kb of genomic DNA. The homeobox is coded by exons 3 and 4. Two alternative forms of exon 6 (6A and 6B), leads to different spliced transcripts named SHOXa and SHOXb (Figure 6). Both the transcripts are identical at the 5' end but differ at the 3' end in exon 6 and are translated into two different proteins of 292 (SHOXa) and 225 (SHOXb) aminoacids.

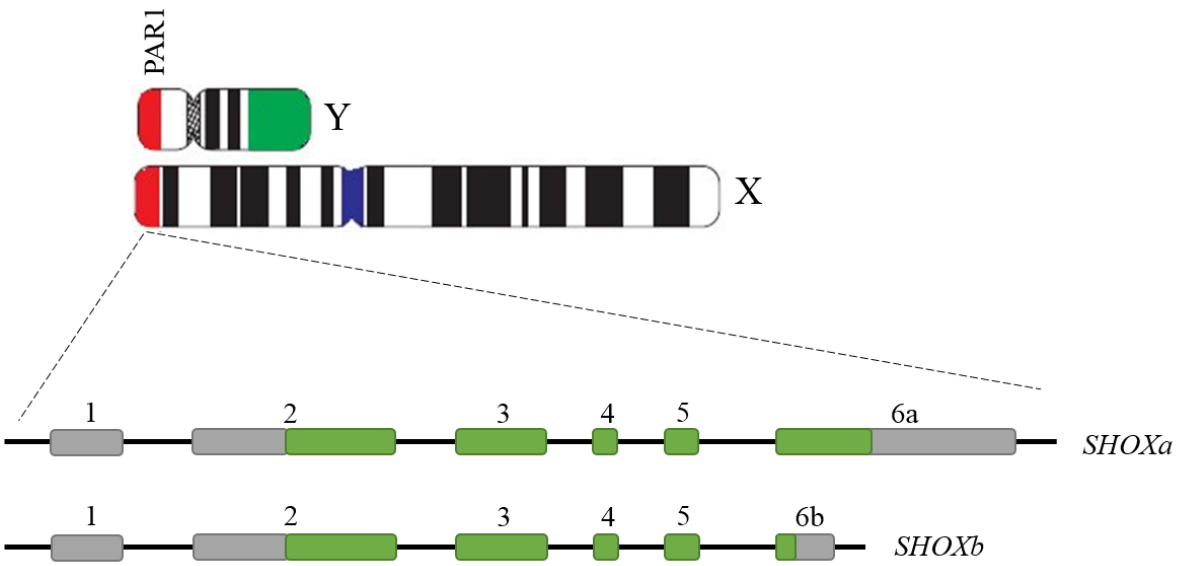


Figure 6: Chromosomal Localisation and genomic structure of *SHOX* gene

I.4.2 SHOX Protein and Expression

SHOX is a nuclear protein and acts as a transcriptional activator. SHOX is expressed during fetal life in the development of bone tissue in the distal humerus, radius, ulna, wrist, limbs and pharyngeal arches. The SHOX expression is restricted to limbs, first and second pharyngeal arches and it can be detected in osteoblasts of human embryos from the second month post consumption. This expression pattern resembles the locations of the anatomic structures which are affected when the SHOX is deficient such as the forearm, lower legs, maxilla, mandible and the external ear tract (113). The role of SHOX in bone development is also indicated by the finding that SHOX protein is detected in the hypertrophic chondrocytes of the growth plate. The overexpression SHOX causes blockage of the cell cycle with proliferation arrest and apoptosis which indicate the role of SHOX in the processes regulating chondrocytes differentiation (114,115). Even though the highest expression of SHOX is identified in skeletal muscle and bone marrow fibroblasts, SHOXa and SHOXb are independently expressed in a different array of tissues. SHOXa is also expressed in placenta, pancreas and heart while low

level of SHOXb expression is also identified in fetal kidney. Natriuretic peptide (NPPB) gene which encode brain natriuretic peptide (BNP) is identified as the first transcriptional target of SHOX. The significance of this finding is still not fully understood (116).

I.4.3 *SHOX* mutations

The correct *SHOX* gene function is dosage dependant. The loss-of-function mutation of one *SHOX* allele results in haploinsufficiency and the disorder of SHOX deficiency causing growth failure. The haploinsufficiency of *SHOX* causes a wide range of short stature phenotypes. The mutations in *SHOX* were frequently reported as responsible for short stature in patients with Leri-Weill syndrome. The prevalence of *SHOX* mutations in individuals with Leri-Weill syndrome is estimated around 50- 90% (117-120). The deletion at the end of the short arm of the X chromosome including *SHOX* gene (loss of one allele) is almost always associated with Turner syndrome (121). The loss of both the *SHOX* alleles causes the complete lack of SHOX and results in extreme phenotype of osteodysplasia called Langer syndrome (122). A relatively smaller percentage of *SHOX* mutations were also identified as the cause of growth retardation in 2-15% individuals with ISS (Table 4).

The high frequency of repeats within the PAR1 region make this genomic region highly prone to the recombination and this explains the high frequency of deletions identified in affected individuals. The deletions which encompass *SHOX* gene itself or the regulatory enhancer regions account for 80% of all the *SHOX* mutations. The other gene defects found were point mutations which were reported all over the gene, but predominantly in exon 3 and 4 encoding homeodomain. The missense mutations in the homeodomain fundamentally impairs the *SHOX* key functions such as DNA binding, dimerization and nuclear translocation (123). Partial and complete *SHOX* duplications of varying sizes were also reported in the short stature patients (124,125).

Table 4: Summery of *SHOX* defects in ISS patients

Study	Patients	Mutation (%)
Rao *et al*. (108)	91	1.1
Binder *et al*. (109)	68	1.5
Musebeck *et al*.(126)	35	-
Rappold *et al*.(106)	900	2.4
Ezquieta *et al*.(127)	73	-
Stuppia *et al*.(128)	56	12.4
Binder *et al*.(129)	140	2
Schneider *et al*.(130)	>1500	2
Huber *et al*.(110)	84	4.2
Rappold *et al*.(107)	1534	2.2
Jorge *et al*.(119)	63	3.2
Chen *et al*.(117)	735	4.2
Benito-Sanz *et al*.(124)	613	1
Benito-Sanz *et al*. (131)	576	1.9
Hirschfeldova *et al*.(132)	51	13.7
Sandoval *et al*.(133)	62	8.1

A phenotypic scoring system assisting the identification of the most suitable subjects for the *SHOX* genetic testing has been developed by Rappold et al. (107) by integrating clinical and anthropometric data of the patients. This score combines three anthropometric variables; arm span/height ratio, sitting height/height ratio and BMI with five clinical variables; cubitus valgus, short forearm, bowing of forearm, muscular hypertrophy and dislocation of the ulna at the elbow. Each of these variable represents at least two points in the scoring system. This system recommends *SHOX* testing in the presence of a score greater than 4 or 7 (increased threshold) out of a total possible score of 24.

I.4.4 *SHOX* Enhancers

Enhancer elements are cis acing regulatory elements located upstream or downstream of an associated gene. When bound by specific transcription factors these elements enhance the transcription of the gene. Enhancers can reside at considerable distance away from the coding parts of the gene and can be the site of mutations in genetic diseases.

Along with comparative genomics and functional analysis, mutational screening in affected individuals demonstrated the presence of multiple evolutionary conserved sequences (ECS) both upstream and downstream of *SHOX* that have variable effects on transcription (Figure 7). Three conserved non coding elements (CNE4, CNE5 and CNE9) within the downstream enhancer region have been shown transcriptional activity in vitro and or in vivo (117,123,134). The presence of another 3' enhancer (CNE7) has been recently identified by Benito-Sanz et al.(131) and the in vitro functional analysis using luciferase reporter assay showed its enhancer activity. All these enhancers reside within 250 kb downstream from the start codon. Three Enhancers (CNE-5, CNE-3 and CNE-2; Figure 7) upstream of *SHOX* within 300kb has also been identified as functionally active (134).

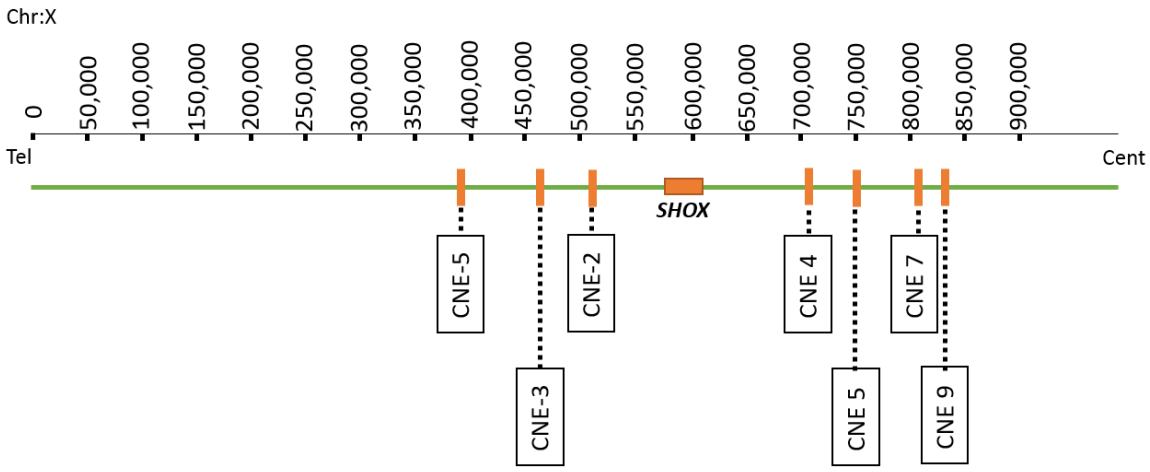


Figure 7: Schematic representation of the genomic location of the *SHOX* enhancers.

Almost all the *SHOX* deletions/duplications identified were either included whole/partial *SHOX* gene or 3' (downstream) enhancers. The deletions identified in the downstream region had highly variable in extension and location. Only one case has been reported involving the 5' (upstream) enhancer region in which two of the three upstream enhancer regions were deleted in a female patient and her father with ISS (135). A deletion within enhancer region could affect transcription simply by the removal of an important regulatory region or indirectly by altering the DNA folding thereby the known regulatory enhancers becomes inaccessible. Partial tandem duplications may affect the gene expression by changing the distance between the coding sequence of the gene and the regulatory elements (131).

Recently, the presence of an additional putative long range regulatory region further downstream of currently known enhancers has been suggested by the deletions identified in individuals with mild phenotypic effect (125,136). This may be more likely due to the presence of one or more additional downstream transcription regulators. A highly conserved transcription factor was also identified within this region and is the most likely candidate for the critical part of these deletions (125).

I.4.5 Phenotypic Variability associated with *SHOX* mutations

All classes of SHOX mutation are associated with broad phenotypic variability even within families. In the case of the enhancer deletions, they generates a phenotype indistinguishable from that of patients with mutations in the SHOX coding region and the size of the deletion is not considered to be related to the severity of the clinical phenotype (137). Kant et al. (138)reported a case in which an enhancer deletion was associated with normal stature although below target height. In many previous studies on SHOX deficiency, not all index parents had short stature and is not always the leading clinical sign to request a SHOX analysis by clinicians, who consider other clinical characteristics of the patients and the family.

II. AIMS

1. **Functional analysis of *GH1* mutations identified in Italian IGHD patients.**

   The exon 3 *GH1* contains two known exon splicing enhancers (ESEs) which are necessary for the correct splicing. The aim of this study was to perform functional analysis on the novel point mutations identified in exon3 outside of known ESEs to determine their involvement in splicing.

2. **Screening of *GLI2* gene in CPHD patients**

   The involvement of *GLI2* gene in the aetiology of CPHD has been recently identified. The aim of this study was to determine the frequency of *GLI2* mutations in a cohort of Italian CPHD patients that resulted negative for mutations in other causative genes encoding pituitary transcription factors (*PIT1, PROP1, HEXS1, LHX3 and LHX4*).

3. **Screening of *SHOX* gene in Idiopathic Short Stature patients**

   SHOX mutations are the most frequent monogenic defects identified in idiopathic short stature patients. The aim of this study was to screen for the *SHOX* deletions, duplications and point mutations in large cohort of ISS patients and to establish a phenotype-genotype correlation in patients carrying mutations, in order to identify new criteria for patient selection.

III. SUBJECTS

III.1 IGHD Cohort

A total of 103 sporadic patients with IGHD and 205 normal stature individuals, all belonging to the Italian population, were included in the genetic analysis. The subjects were referred to the clinical centers because they had a height less than or equal to -2 SDS (Standard Deviation Score) or a height less than or equal to -1.8 SDS in combination with a height velocity over 1 year less than -1.5 SDS using the criteria of Tanner-Whitehouse(139). Patients with a known postnatal cause of acquired hypopituitarism were excluded. Skeletal maturation was estimated as bone age (radius, ulna and short bone) with the TW2 (Tanner –Whitehouse 2nd edition) method by a pediatric endocrinologist (140). They were all evaluated for GH serum levels after two provocative tests (with arginine or clonidine or insulin or glucagon or with GHRH+arginine (141). Traditionally, a diagnosis of GH deficiency (GHD) is supported by GH peaks less than 10 ng/ml both after the two different stimuli (142), or less than 20 ng/ml after the double provocative test with GHRH + arginine. The GHD patients futfill these criteria and had a mean (±SD) secretion peak of 4.4 ± 2.5 ng/ml after the classical stimuli (N=78) or 9.4 ± 5.8 ng/ml after the test with GHRH+arginine (N=25). None of the GHD patients was deficient for other pituitary hormones and none had a documented family history of the disease or consanguineous parents. All the patients have been also screened for mutations in *GHRHR* (data not shown). Patients carrying mutations in this gene were not included in the IGHD cohort described here. Normal stature controls included University and Hospital staff, as well as medical students who were not tested for GH secretion levels. A written informed consent was obtained from the patient's parents, as they were all aged less than 18 and from the normal stature controls.

III.2 CPHD Cohort

The CPHD patients were recruited based on the following criteria:1) they presented with a clinical and hormonal evidence of childhood-onset GH deficiency combined with at least one other pituitary defect in the absence of an identified cause of hypopituitarism (e.g. cerebral tumors, cranial trauma, documented asphyxia, or other injuries at delivery), 2) they had a negative family history for pituitary dysfunction or apparent or declared consanguinity and were thus considered as sporadic cases, and 3) mutations in the coding sequences of genes associated with multiple pituitary hormone dysfunctions (PIT1, PROP1, HEXS1, LHX3, and LHX4) were excluded (data not shown). Mean height SDS for chronological age was calculated using the criteria of Tanner-Whitehouse(140). The mean height of the patients at diagnosis was −2.26 SDS ± 2.3 sd. Morphological evaluation of the hypothalamus-pituitary area and/or of the central nervous system was performed in 136 patients by magnetic resonance imaging, using precontrast coronal spin echo T1-weighted images followed by postgadolinium T1-weighted imaging. Among the 136 CPHD index cases, 8 (5.8%) were the probands of pedigrees with more than one affected individual (familial cases). Four patients were born from consanguineous parents but they were considered as sporadic cases since they were the only affected subject in their families. The mean height of these patients at diagnosis was -2.81 ± 1.83 SDS and the mean delay in bone age relative to chronological age was 2.57± 2.36 years. GHD was present in all the patients, TSH deficiency in 78.6% (107/136) and ACTH deficiency in 61% (83/136). Thirty-nine subjects were prepubertal at the time of diagnosis. Among the remaining 97 subjects that could be evaluated in terms of pubertal age, 81 (83.5%) presented with FSH/LH deficiencies. Eight male patients presented neonatal micropenis and/or cryptorchidism. Five patients (3.5%) had diabetes insipidus. We obtained MRI data from 101 patients (74% of the total). Among these, abnormalities (ectopy of the neurohypophysis, pituitary hypoplasia and empty sella) were found in 81 (80%) subjects; in particular anterior

pituitary hypoplasia or aplasia was the most frequent abnormality and was present in 61 patients (60.4%), while pituitary stalk interruption and/or neuropituitary ectopia were observed in 35 patients (34.6%), 16 of them presenting both abnormalities. Eleven patients (10.8%) presented also extra-pituitary abnormalities such as SOD, other midline defects or cerebellar abnormalities.

Patients or parents of the patients under 18 years of age gave their written informed consent to participate to this study, which was approved by the local ethical committee of each contributing auxological center.

III.3 ISS Cohort

Two hundred and ninety ISS patients with growth failure of varying severity were screened for *SHOX* abnormalities. The mean height SDS of the cohort was -1.8±0.9. The patients were generally selected with a height <-2SDS or with growth velocity of <-2SDS. Some patients with >-2SDS were also considered based on other clinical characteristics. The detailed description of the ISS patients selected for this study are shown in table 5. Patients with recognizable conditions such as chromosomal abnormalities, dysmorphic syndromes, low birth size (small for gestational age), systemic, endocrine or nutritional disorders and skeletal dysplasias were excluded from this analysis.

Table 5: Clinical characteristics of the ISS patients screened for *SHOX* defects.

	Mean ± SD	
	Females (n=149)	Male (n=140)
Age at Diagnosis	8.1±3.73	9.2±4.08
Height SDS	-1.9±0.79	-1.8±0.97
Growth Velocity	-1.2±1.75	-0.9±2.1
Target SDS	-0.8±1.07	-1±0.95
BMI	16.5±1.75	16.8±3.35

IV. MATERIALS AND METHODS

IV.1 Hormonal investigations

Hormonal assays were performed using several commercial kits and results of biochemical investigations at diagnosis were recorded including basal free T4, TSH, cortisol, and ACTH levels and basal and peak levels of GH, TSH, LH, FSH, and cortisol in response to pituitary stimulation tests. GH deficiency was diagnosed in the presence of low-normal IGF-I levels according to sex and age cutoffs and impaired response to two consecutive classical provocative tests (with arginine or clonidine or insulin; GH peaks <10 ng/ml) or one double stimulus with GHRH + arginine (GH peaks <20 ng/ml)(141). A diagnosis of TSH deficiency was made if serum free T4concentration was under the normal cutoff level(<10 pmol/liter) with normal or low TSH levels. ACTH deficiency, in presence of low or normal ACTH levels, was suspected when fasting morning serum cortisol was less than 193 nmol/liter and was confirmed by an impaired response to the 1-μg tetracosactide or insulin tolerance test (<497 nmol/liter). Gonadotroph axis was investigated only in patients of postpubertal age. FSH-LH deficiency was diagnosed on the basis of delayed or absent pubertal development and no increase in serum FSH and LH in response to the GHRH test.

IV.2 Genomic DNA extraction

Genomic DNA was extracted from whole blood samples using either the salting out method based on Miller et al. (143) or QIAGEN blood mini kit according to the manufactures instructions.

IV.3 Polymerase Chain Reaction (PCR)

Standard PCR reactions were performed using *Taq Polymerase* (Promega) for the amplification of the genomic DNA for the sequencing analysis of *GH1, GLI2* and *SHOX*. For the screening of *GH1*, Genomic DNA was initially amplified by PCR using a proof reading Taq polymerase (Finnzymes). The primers (Appendix 1) were designed specifically for the *GH1* gene and do

not amplify other genes in the cluster The resulting 2.7 Kb product, including the whole *GH1*, was used as template for a series of nested PCRs using internal primers for the proximal promoter, five exons, four introns and the untranslated regions of the *GH1*.

A standard reaction was carried out using relevant primers (sequence showed in appendix 1), dNTPs, *Taq Polymerase* and 5x Buffer (Promega). The final volume of PCR reactions were 25µl unless otherwise specified. The reactions were set up as follows:

Table 6: PCR conditions for *Taq Polymerase*

Reagents	Concentration		Reaction Mix
	Initial Concentration	Final Concentration	
Go Taq® Flexi Buffer	5X	1X	5 µl
MgCl2	25 mM	1,5 mM	1,5 µl
dNTPs	2,5 mM	0,2 mM	2 µl
Primers F+R	10 pmol/µl	0,4 pmol/µl	1 µl
Go Taq® (Promega)	5 U/µl	0,02 U/µl	0.1 µl
DNA	25 ng/µl	50 ng	2 µl
H_2O	-	-	13,4 µl
Volume Totale	25 µl	25 µl	25 µl

Cycling Parameters used were: denaturation at 94°C for 2 min, 30cycles consisting of 30s denaturation at 94°C, 30s annealing at 55-58°c (optimized passed on the primers) and 30s extension at 72°c, followed by a final extension at 72°C for 10 min.

The Reactions using proof reading Taq polymerase were set up as follows:

Table 7: PCR conditions for *DyNAzyme* EXT DNA Polymerase

Reagents	Concentration		Reaction Mix
	Initial Concentration	Final Concentration	
Mg Free 10X Buffer	10X	1X	2,5 µl
MgCl2	50 mM	1,5 mM	0,75 µl
dNTPs	2,5 mM	0,25 mM	2,5 µl
Primers F+R	10 pmol/µl	0,4 pmol/µl	1 µl
DyNAzyme EXT DNA Polymerase	1 U/µl	0,03 U/µl	0.75 µl
DNA	25 ng/µl	100 ng	4 µl
H_2O	-	-	13,5 µl
Volume Totale	25 µl	25 µl	25 µl

Cycling conditions used were: denaturation at 94°C for 2 min, 30cycles consisting of 30s denaturation at94°C, 30s annealing at 58°c and 3min extension at 72°c, followed by a final extension at 72°C for 10 min.

After the reaction all the PCR products were visualized on a 2% agarose gel and purified using ExoSAP-IT enzymatic PCR clean up system (Affymetrix).

IV.4 Sequencing

The purified products were directly sequenced in the forward or reverse direction with Big Dye Terminator kit (Applied Biosystems) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The sequencing Reactions were set up as follows:

- 2 µL of purified PCR product
- 1 µL primer (3.2 µM)
- 1 µL Big Dye terminator mix
- 6 µL H_2O

Cycling conditions used were: 25cycles consisting of 15s denaturation at96°C, 05s annealing at 50°c and 4 minutes extension at 60°c.

IV.5 T/A cloning

T/A cloning (Thermo Scientific InsTAclone PCR cloning kit) has been performed to understand whether the double mutations were in the compound heterozygous state. The PCR products containing the variations in the heterozygous state were cloned into the plasmid vector pTZ57R/T (Appendix 3) using the InsTAclone PCR cloning kit (Fermentas) and the two alleles were separately sequenced.

IV.6 Site-directed mutagenesis

The 2.7-kb fragment containing *GH1* was inserted into the pcDNA 3.1(+) expression vector (Appendix 3). The constructs bearing the single variants, namely 246C, 255A, 261T, and 272T, and the variants combined as in the patients, namely 261T/272T (patient 1) and 255A/261T (patient 2), were generated from the GH(wt)-pcDNA 3.1 plasmid by the QuikChange Site-Directed Mutagenesis kit from Stratagene using mismatch complementary primers (Appendix 1) containing the desired mutations. Reaction mixtures were denatured at 94^0C for 5 minutes, cycled 25 times at 94^0C for 15 sec, 58^0C for 1 min, 68^0C for 8 minutes.

The reaction was set up as follows:

Buffer (10X)	5µl
Primer 1 (100ng/µl)	1.25 µl (100ng/µl)
Primer 2 (100ng/µl)	1.25 µl (100ng/µl)

dNTPs	1 µl
dsDNA template	1-5 µl (50 ng)
Dd H_2O	To a final volume of 50 µl
Pfu Turbo®	2.5 U/ µl

After the reaction, the products were digested with DpnI. DH5a competent cells were transformed with the different constructs and grown on Luria Broth/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using Maxiprep kit (QIAGEN). The desired mutation was confirmed by sequencing.

IV.7 Cell culture, transfection, and isolation of RNA

The GH4C1 rat pituitary cell line was used for the transfection experiments. The stock culture was grown in Ham's F10 medium (Gibco-Life Technologies) supplemented with 15% horse serum, 2.5% FBS, 100-U/mL penicillin, and 100-µg/mL streptomycin in a 5% CO2. A day before transfection, 4 $X10^5$ cells were seeded into each well of a 6-well tissue culture plate in 2.5-mL medium. The wells were previously treated with 1:10 diluted poly-L-lysine solution (Sigma-Aldrich) to allow the cells to completely attach to the plate. At 50%–70% confluency, cells were transfected with 2.5-µg DNA of the wt-GH or the mutated constructs using the Trans IT-LT1 transfection reagent (Mirus Bio LLC). A GFP control was used to test transfection efficiency. Forty-eight hours after transfection, totalRNAwas isolated and purified from the cells using the QIAGEN RNA mini kit (QIAGEN).

IV.8 cDNA synthesis

cDNA was synthesized from 1.5 µg of RNA by the High CapacitycDNAReverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. The different transcripts produced by alternative splicing were analyzed using primers specific for *GH1* cDNA (Figure 2A). The RT-PCR was performed with primers GH2 (5'-

CGTCTGCACCAGCTGGCCTTT-3') and GH7 (5'-AAGCCACAGCTGCCCTCCACAGA-3'), which amplify part of exon 2, exon 3, exon 4, and part of exon 5, allowing detection of both exon 3- and exon 4-skipped products.

IV.9 Western immunoblot analysis

CHO cells were transiently transfected with wt-GH and mutated constructs, as described above. After 48 hours, whole-cell lysates were collected using the standard RIPA lysis buffer containing 0.1% SDS. A total of 20 µg of whole-cell lysates were separated on 15% SDS-PAGE gel and blotted on Immun-Blot PVDF membrane (Bio-Rad). Membranes were probed with a polyclonal rabbit antihuman GH antibody (Abnova) and detected with a secondary HRP-conjugated goat antirabbit IgG (Millipore). A polyclonal antiactin antibody (Sigma-Aldrich) was used to normalize the protein loading. Protein bands were visualized using enhanced chemiluminescence reagent (Thermo Scientific) with image capture performed using a CCD camera linked to ChemiDoc apparatus (Bio-Rad). CHO cells were used instead ofGH4C1cells for protein analysis, because the GH4C1 showed many unspecific bands after Western blotting, likely due to cross-reactions with endogenous proteins.

IV.10 MLPA Analysis

The MLPA analysis was performed using the MRC-Holland MLPA kit (SALSA P-018 G1, MRC-Holland, Netherlands) according to the manufacture's instruction. The probe mix contains 48 MLPA probes among them 26 are located in the *SHOX*+Xp22 area, including *SHOX* and its regulatory regions (Figure 8). 13 probes are located elsewhere on the X-chromosome and other 9 probes detecting autosomal chromosomes are also used. Seven of the probes are specific for each exon of the *SHOX* promoter region. Furthermore, several probes are located on the previously identified *SHOX* regulatory regions, upstream and downstream of *SHOX*. The amplified fragments were submitted to a capillary electrophoresis in ABI PRISM 3100 Genetic Analyzer and were analyzed by GeneMapper software (Applied

Biosystems, Foster City, CA). The data analysis was performed by MRC Holland Coffalyser v9.4 software. This software calculates the odds ratios for each probe. An odd ratio of less the 0.7 was considered as deletion and an odd ratio of more than 1.3 was considered duplication. All patients were compared with the DNA from three controls.

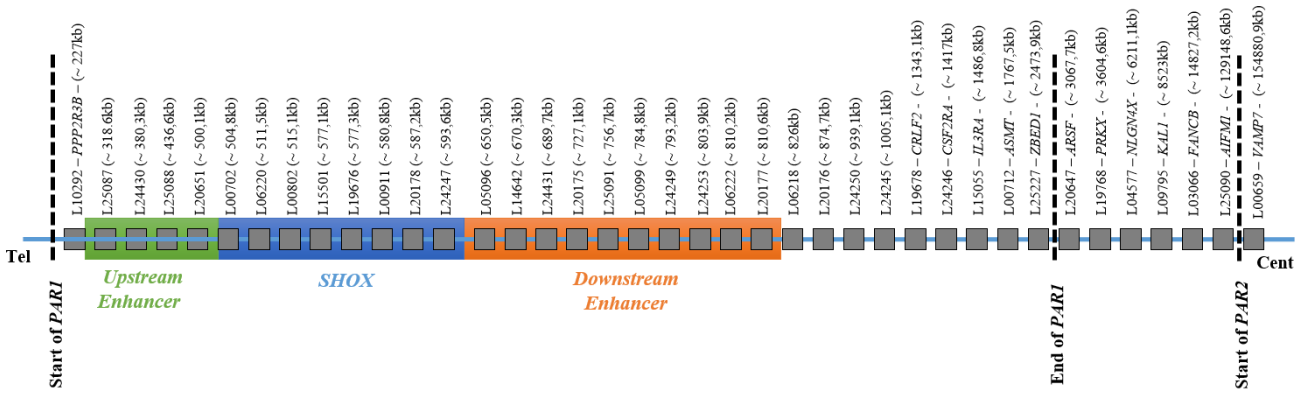


Figure 8: Schematic representation of PAR1 with the location of the MLPA probes

The MLPA reaction was set up as follows:

5 µl of DNA (100ng) was used to perform the analysis. The hybridisation master mix was prepared (Table 8), using 1.5 µL of SALSA ProbeMix MLPA® Buffer and 1.5 µL MLPA® Buffer. The DNA was initially denatured for 5 minutes at 98^0C and cooled down to 25^0C. 3 µl of the hybridisation master mix was added to each sample and continued the thermocycler at 95^0C for 1 minute and then at 60^0C for 16-20 hours.

Table 8: MLPA Hybridization reaction

Hybridization master mix	Quantity
SALSA ProbeMix MLPA® Buffer	1.5 µL
MLPA® Buffer	1.5 µL
Vol. finale	3 µL

A ligase-65 master mix was prepared as showed in table x. 32 µL of the ligase-65 master mix to each reaction tube while the thermocycler at 54^0C. Thermocycler program was continued at 54^0C for 15 minutes followed by 5 minutes at 98^0C for the inactivation of the Ligase-65 enzyme and then colled to 20^0C.

Table 9: MLPA Ligation reaction

Ligase-65 mix	**QUANTITÀ**
Ligase-65 Buffer A	3 µL
Ligase-65 Buffer B	3 µL
Ligase-65	1 µL
H_2O	25 µL
Vol. Finale	32 µL

The SALSA PCR mix was prepared as showed in table x. 10 µL of SALSA PCR Primer mix was added to each tube containing the ligation reaction product at room temperature. The thermocycler program was continued: cycled 35 times at 95^0C for 30 seconds, 60^0C for 30 seconds, 72^0C for 1 minute.finally the samples were incubated at 72^0C for 20 minutes and then cooled down to 15^0C.

Table 10: MLPA PCR reaction

SALSA PCR mix	**QUANTITÀ**
SALSA PCR Primer mix	2 µL
SALSA polymerase	0.5 µL
H_2O	7.5 µL
Volume Finale	10 µL

IV.11 ESE finder analysis

Analysis of the splicing regulatory motifs within exon 3 was performed using the software ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi). The default thresholds were

considered to identify sites responsible for the 4 SRproteins ASF/SF2, SC35, SRp40, and SRp55.

IV.12 Gel image analysis

The RT-PCR and Western blotting gel images were analyzed using the freeware ImageJ1.46r (http://rsb.info.nih.gov/ij/ ; National Institutes of Health), and the bands were quantified by measuring pixel intensity and normalized to the corresponding β-actin band intensity.

V. RESULTS AND DISCUSSIONS

V.1 NOVEL MUTATIONS IN THE GH GENE *(GH1)* UNCOVER PUTATIVE SPLICING REGULATORY ELEMENTS

V.1.1 Background

The *GH1* gene is located on chromosome 17q23 within a cluster of five highly homologous genes, all consisting of five exons and four introns, including the placentally expressed growth hormone gene *GH2*, two chorionic somatomammotropin genes *CSH1* and *CSH2* and a pseudogene *CSHP1(7)*. When correctly spliced, *GH1* produces the 22kDa isoform which includes all the five exons with the complete biological activity of GH (5,10) . Despite the correct processing, even under normal conditions a small percentage of alternatively spliced isoforms are produced. The presence of an in-frame cryptic splice site within exon 3 gives rise to a transcript lacking the first 45bp of exon 3 and encodes a shorter active isoform of 20kDa (representing 5-10% of GH transcripts) (23). A 17.5kDa isoform (representing 0.1-5% of GH transcripts) is produced by the complete skipping of Exon 3, thus lacking the entire loop connecting helix1 and helix 2 in the tertiary structure of GH and generating a GH isoform with no biological activity (144). Trace amounts of the severely truncated isoforms of 11.3kDa and 7.4kDa, which are biologically inactive, have also been identified being generated by the skipping of exons 3 and 4 or 2 to 4, respectively (27). Multiple mechanisms have evolved to maintain the small amounts of these aberrantly spliced isoforms, especially that encoding the 17.5kDa protein. Since *GH1* has weak canonical splice sites, multiple cis acting splicing regulatory elements (splicing enhancers) are essential to maintain the correct exon 3 definition through the activation of the canonical intron 2 and 3 splice sites and the silencing of the cryptic sites. Two Exon Splicing Enhancers, ESE1, encompassing the first seven bases of exon 3 (from c.172 to c.178), and ESE2 (from c.190 to c.204), located 12 nt upstream of the cryptic splice site in exon3 (Fig. 1a), and an Intron Splicing Enhancer (ISE) within intron 3, have been well characterized (14,16,34,35,51).

Several mutations, both non-synonymous and synonymous, leading to aberrant splicing have been reported in IGHD patients within these enhancer motifs (14,16,34,35). The increased amount of the 17.5 kDa isoform exhibits a dominant negative effect both in tissue culture and transgenic mice experiments by disrupting the secretory pathway and trafficking of normal GH and other hormones, including adrenocorticotropic hormone (ACTH) (25,26).

We here report the identification of variations within *GH1* exon 3 in two sporadic IGHD patients and absent in a group of 205 normal stature controls and in the public databases. *In silico* analysis suggested that two of these variations affect exon 3 splicing as they are located within a putative exon splicing enhancer. *In vitro* mRNA and Western Blot analysis confirmed the deleterious effect of the single variations on splicing, suggesting the presence of further splicing regulatory elements within *GH1* exon 3.

V.1.2 Genetic analysis of the IGHD patients

A total of 103 sporadic IGHD patients with height ranging from -1.8 to -4.5 SDS were investigated for the presence of functionally relevant mutations in *GH1*, including coding regions and introns. Two patients harbored a combination of 2 variations within exon 3 (Table 11) that were not previously reported in public databases, including dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), the Exome Variant Server (http://evs.gs.washington.edu/EVS/), and 1000 genomes (http://www.1000genomes.org/). Patient 1 carried c.261C>T (p.Pro87Pro) and c.272A>T (p.Glu91Val), and patient 2 carried c.255G>A (p.Pro85Pro) and c.261C>T (p.Pro87Pro). A third patient (patient 3) (Table 11) carried the nonsynonymous c.246G>C determining the substitution p.Glu82Asp in exon 3. This variation was reported in the dbSNP (rs61762497) only in 1 individual and in the Exome

Variant Server in 2 out of 13000 individuals. All the above exon 3 variations were absent in a panel of 410 chromosomes sequenced from 205 normal stature Italian control individuals.
The analysis of the parents of patients 2 and 3 demonstrated that their variants were inherited from the unaffected fathers. Unfortunately, the parents of patient 1 did not give their consent to DNA analysis. By subcloning the patient's PCR products in a TA cloning vector system, we confirmed that in patient 1, as well as in patient 2, both variants were on the same allele. The alignment of *GH1* with the paralogous genes of the GH cluster suggested that all the 4 variants in exon 3 were generated by nonallelic gene conversion from the *GH2* gene, because the other 3 genes (*CS-5*, *CS-2*, and *CS-1*) have the same *GH1* sequence at these sites.

V.1.3 Detailed description of patients carrying the *GH1* variations

The variants identified in the three patients are reported in Table 11.

Case 1: This patient is a boy born at term with no perinatal complications. He came to our attention when he was 7.3 years old with a height of -2.7 SDS. He had normal stature parents: the father is -1.7 SDS and the mother -0.6 SDS. He was prepubertal. GHD was diagnosed based on low response to insulin tolerance test and clonidine provocative tests (8.9 ng/ml and 8.3 ng/ml, respectively). The IGF-I level was 130 ng/mL. The bone age was delayed by 3.8 years. GH therapy was initiated with a good clinical response reaching a height of -1.7 SDS after 6 years.

Case 2: This case is a male born from non-consanguineous parents by vaginal delivery after 41 weeks of gestation. At birth it was adequate for gestational age (birth weight 3330 g length 52 cm). Both parents presented normal height: father 174 cm (-0.2SDS) and mother, 155 cm (-1.3 SDS). He came to our attention at age of 13 presenting short stature with a height of -3.4 SDS. His growth rate in the year preceding the diagnosis was 5.0 cm/year (-1.5 SDS). At the diagnosis the pubertal stage were: PH 2, G2 and bilateral testicular volume 5. The bone age

was delayed: (11.6 years, TW2 method). GH secrection peaks after stimulus with arginine and clonidine were 3.9 ng/ml and 8.3 ng/ml respectively with IGF1 level of 65 ng/ml. He promptly started the recombinant human GH replacement therapy (0.22 mg/kg/week) and reached the height of -1.8 SDS after 4 years.

Case 3: This boy came for the first time to our attention for growth failure at the age of 16.5 years old. At diagnosis his stature was −2.7 SDS. He was born to non-consanguineous parents with normal pregnancy at 40 week gestation, adequate for gestational age (birth weight 2950 g). His father and mother presented normal stature (-0.5 SDS and -0.3 SDS, respectively). The pubertal stage were: Pubic Hair development 3, Genital development 2 and bilateral testicular volume 8 ml. The growth velocity was 4.5 cm/year (1.6 SDS, but low when corrected for pubertal stage), and x-ray revealed a bone age (TW2) of 14.2 years. Laboratory analysis revealed normal IGF1 level (180 ng/ml) and low level of circulating GH after two provocative tests (7.7 ng/ml with arginine and 3.7 with clonidine). The recombinant human GH replacement therapy (0.24 mg/Kg/week) was begun with a good response, as he reached the height of -0.7 SDS after 3 years.

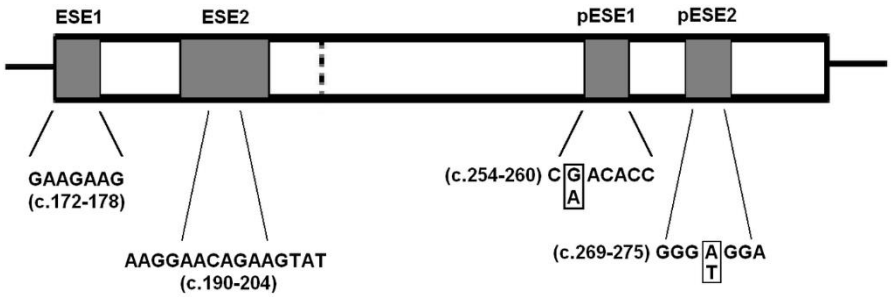
Table 11: Exon 3 variation identified in IGHD patients

Patient	Variation		Within a predicted ESE
	Nucleotide	Amino acid	
1	c.261C>T	p.Pro87Pro	No
	c.272A>T	p.Glu91Val	Yes
2	c.255G>A	p.Pro85Pro	Yes
	c.261C>T	p.Pro87Pro	No
3	c.246G>C	p.Glu82Asp	No

V.1.4 The exon 3 variations fall within predicted ESEs

Because several exon 3 mutations lead to missplicing of mRNA and production of increased amounts of the 17.5 kDa protein, we evaluated the possible involvement in the splicing regulation of the variants identified in our patients within this exon. None of them was included in the previously described ESEs (ESE1 and ESE2). An *in silico* analysis using ESE finder 3.0 (Figure 9, A and B) revealed the presence of 2 high scoring ESE motifs from c.254 to c.260 (CGACACC) and c.269 to c.275 (GGGAGGA). These 2 sequences include the variations c.255G>A and c.272A>T, respectively (Figure 9A). The position c.255 is located within an ESE motif recognized by the SR protein SRp40 and c.272 within a sequence recognized by SRp40 and SF2/ASF. Both putative ESE sequences showed an increased score compared with the threshold value (Figure 9B). By substituting the wild-type nucleotides with those found in the patients, namely c.255A and c.272T, the software predicted the complete loss of these 2 putative ESEs (Figure 9B).

A.



B.

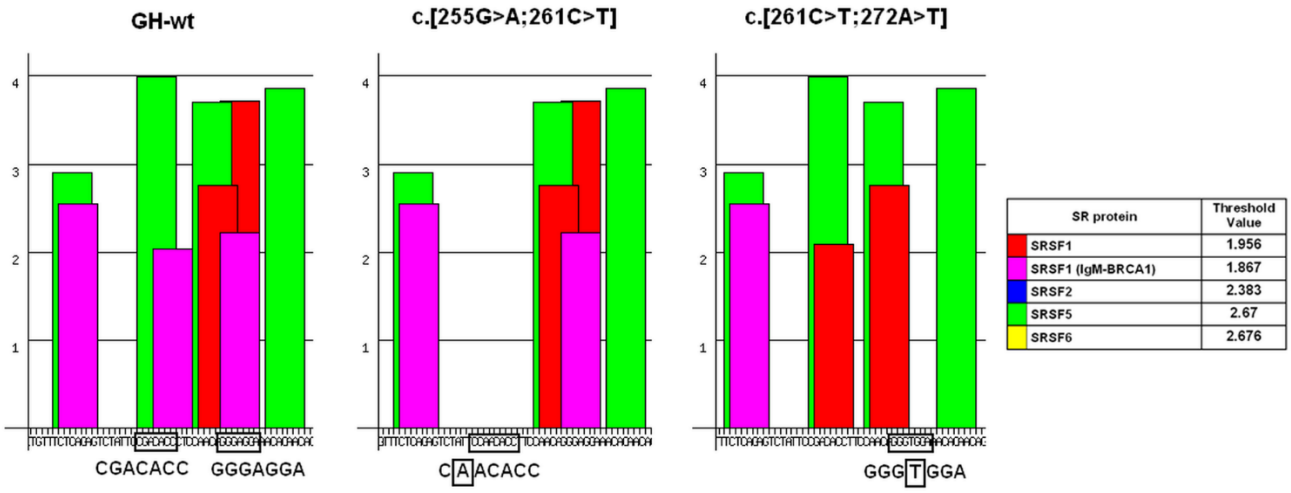


Figure 9: A, ESEs in exon 3. The 2 known ESEs (ESE1 and ESE2) are indicated. The cryptic splice site is at c.216 and is indicated with a dotted line. The newly identified putative ESEs are indicated as pESE1 and pESE2. The mutations identified in patient 1 and patient 2 in the pESEs are boxed. B, ESE finder analysis performed on the wild-type exon 3 and on the different exon 3 alleles identified in patients. The analysis using wild-type sequence revealed all the putative exon splicing enhancer sites within exon 3. ESE motifs with scores above the threshold for each SR protein are indicated in differently colored bars. The bar heights reflect the score of the motifs. Threshold values and color code for each of the different motifs are indicated on the right. The introduction of c.255G_A and c.272A_T variations caused the abolition of the 2 putative ESE elements at positions c.254–260 and c.269–275, respectively.

V.1.5 Variants c.255A and c.272T affect *GH1* splicing *in vitro*

To evaluate whether exon 3 variants actually had some effect on mRNA splicing in vitro, we transfected GH4C1 rat pituitary cells with an expression vector containing 1) the wild-type allele (wt-*GH1*), 2) the alleles carrying the single mutations, and 3) the alleles containing the mutations combined as they were in patients 1 and 2. The mRNA from transfected cells was reverse transcribed, and the resulting cDNA was amplified with primers (Figure 10A) specific for the human *GH1* that did not amplify the rat mRNA. The RT-PCR on the wt-*GH1* mRNA (Figure 10B, lane 2) yielded an intense band corresponding to the *GH1* full-length transcript (518 bp) producing the 22-kDa protein and faint bands corresponding to the 20-kDa isoform

(473 bp), the 17.5-kDa isoform (398 bp), and the 11.3-kDa isoform (233 bp). The average yield of the mRNA corresponding to the 17.5-kDa isoform over 4 independent experiments was 4.4±1% of the total mRNA yield with a ratio 17.5 kDa/22 kDa of about 1:20. The 246C construct (Figure 10B, lane 3) was also tested, although the c.246C variation was not predicted to influence splicing. This construct generated a band pattern similar to that observed in the wild-type accordingly to the in silico prediction. In contrast, 255A and 272T constructs (Figure 10B, lanes 4 and 6, respectively) produced a higher level of the exon 3-skipped mRNA with a ratio 17.5 kDa/22 kDa of about 1:2 and 1:2.4, respectively. In these 2 constructs, there was also an evident increased production of the exon 3–4-skipped transcript (233-bp band). Interestingly, the exon 3-skipped mRNA was not evident in the transcripts from the 261T construct, suggesting that this variant might strengthen the correct splicing. When the variants were combined on the same construct to reproduce the status of patients 1 and 2, the exon 3-skipped mRNA produced by the construct 255A/261T (patient 2) (Figure 10B, lane 7) was less abundant than that observed for 255A but still evident, with a 17.5 kDa/22 kDa ratio of 1:3.3. Conversely, the 261T/272T construct (patient 1) (Figure 10B, lane 8) showed a splicing pattern very similar to the wild type.

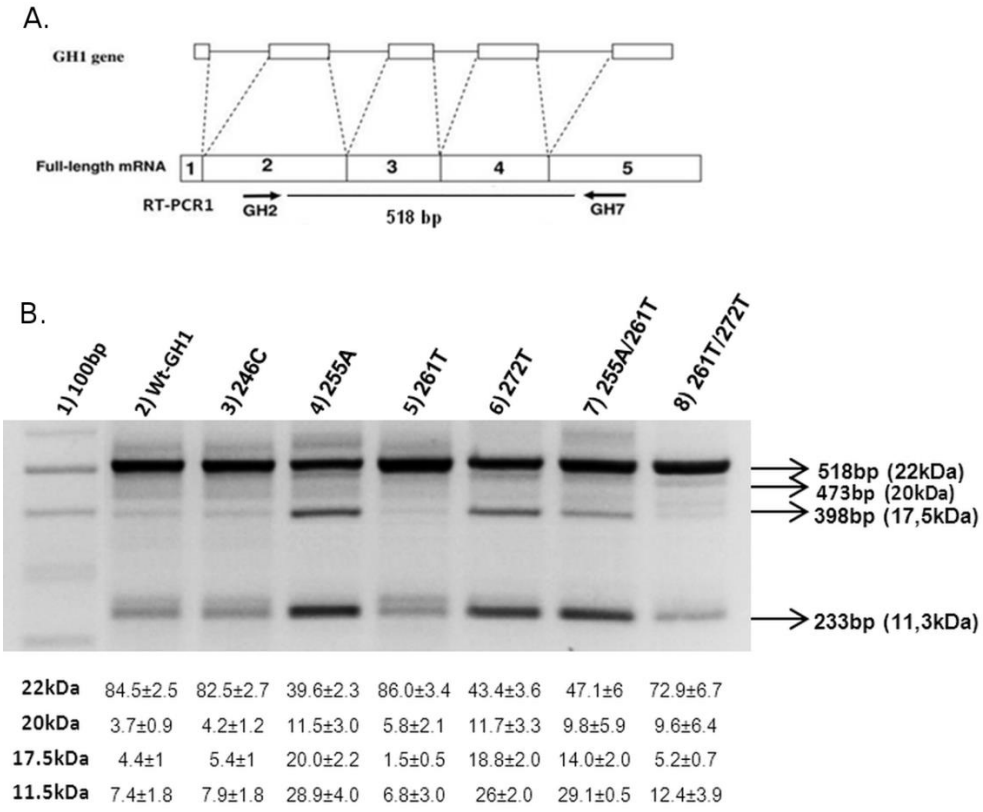


Figure 10: Scheme of *GH1*pre-mRNA splicing showing the full-length mRNA and the corresponding RT-PCR product. (b) RT-PCR performed on mRNA extracted from GH4C1 pituitary cells transfected with the wild type *GH1* (lane 2) or constructs carrying different variations (lane 3-8). The size of the bands are indicated on the right with the corresponding protein molecular weight of the different isoforms. Untransfected rat cDNA did not show any bands (data not shown). Each PCR product was eluted from the gel and characterized by sequencing. The relative band intensity (expressed as the percentage on the total yield) calculated as the mean ± SD over four different independent transfection experiments is reported for each construct below the corresponding lane.

We then investigated the consequences of the variants on protein synthesis (Figure 11). Western blot analysis confirmed that the transfected wt-*GH1* produced mostly the 22-kDa protein with only traces of the 20 and 17.5 kDa. A higher level of the 17.5-kDa product was produced by 255A, 272T, and by the 255A/261T constructs. Although the 261T/272T plasmid carrying the 2 variations detected in patient 1 showed a mRNA pattern similar to the wild type (Figure 10B, lane 8), it exhibited a band in correspondence of the 17.5-kDa protein more intense than the wild type (about 10% of the total GH proteins) (Figure 11, lane7). This band in the 261T construct was weak (as in the wt-*GH1*), but the 20-kDa isoform was more intense

than in all the other constructs. The corresponding 11.3-kDa protein isoform was not detectable by the antibody used for Western blot analysis.

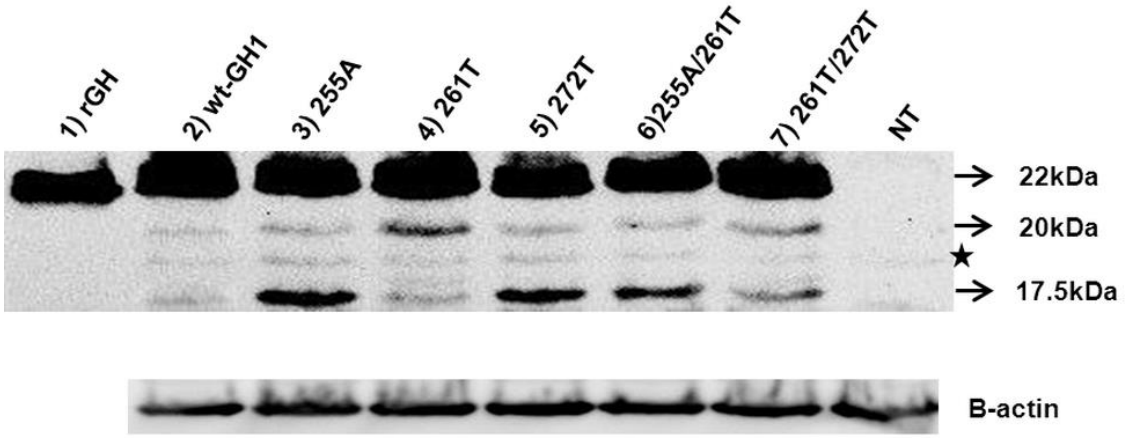


Figure 11: Western immunoblot analysis of the different GH isoforms encoded by the different splicing products. The CHO cells were transiently transfected with either wt-*GH1* (Lane 2) or constructs carrying different variations (Lane 3-7). Untransfected CHO cells.

V.1.6 Discussion

In the present study, *GH1* was sequenced in 103 IGHD patients with clinically variable phenotypes and no family history to search for mutations that might be 1) inherited from healthy parents (incomplete penetrance), 2) arisen de novo, or 3) biallelic (recessive inheritance). We identified 3 patients that carried variations in exon 3, and 2 of them carried 2 variants on the same allele. All the variants were likely generated by gene conversion, an event that is frequently associated to *GH1* sequence variability (36,145,146). An in silico analysis was performed using ESE finder 3.0, which has been designed to identify SR protein binding sites by generating a motif score that reflects the binding site strength. The sequences from c.254 to c.260 and from c.269 to c.275, including the positions c.255 and c.272 mutated in patient 2 and in patient 1, respectively, were predicted to represent ESE motifs recognized by theSRp40 and SF2/ASF proteins with a score above the threshold (Figure 9B). The same factors were predicted to recognize ESE2 with the same strength (51). It has been demonstrated that SF2/ASF activates exon 3 inclusion and that disruption of this motif causes increased exon

3 skipping (147). The substitution of the wild-type nucleotides c.255G and c.272A with the mutants c.255A and c.272T, respectively, was predicted to abolish the binding of these proteins in both putative ESEs (Figure 9). The RT-PCR analysis (Figure 10) confirmed the influence of c.255A and c.272T on splicing by increasing the exon 3-skipped isoform to18%–20% of the total GH mRNA (Figure 10B, lanes 4 and 6, respectively).

Mutations within ESE1 in *GH1* have been previously reported to cause either complete or partial exon 3 skipping and generation of increased amounts of the 20- and the 17.5-kDa isoforms at various concentrations (20%–37% and 35%–68%, respectively) (16,51). The clinical variability observed in patients carrying these mutations has been explained by variable amounts of the 17.5-kDa isoform consequent to a weakened exon 3 recognition. Hamid et al (148) reported a large pedigree with the c.172G>A splicing mutation, and they suggested that the ratio of 17.5 kDa/22 kDa transcripts in the lymphocytes correlated with the height SDS before GH replacement therapy. In this family, there were individuals with a height SDS more than -2 that inherited the mutation (incomplete penetrance). Another heterozygous missense mutation, c.200A>G, within ESE2 induces exon skipping in about 20% of the transcripts, giving rise to different phenotypes ranging from short stature to normal stature in the same large pedigrees (36).

It is thus conceivable that mutations associated with variable expressivity and incomplete penetrance might be responsible for at least some milder forms of IGHD. The effect determined by the here detected variants, c.255G>A and c.272 A>T, is comparable with the effect caused by most of the previously described mutations falling within ESE1 and ESE2 (16,36,148). However, when these 2 variants were combined with c.261T, as in patients 1 and 2, the effect on splicing was maintained, although weaker, only for the 255A/261T construct (patient 2) (Figure 10B, lane7). In contrast, the 261T/272T construct (patient 1) (Figure 10B, lanes 8) was very similar to the wild-type, although the Western blotting for the same construct showed a

slightly increased amount of the 17.5-kDa band (Figure 11). This discrepancy might be attributable to the different sensitivity of the 2 methods.

Thus, the c.261 variant, which is not included in the putative ESE sites, partially hides the negative effect of the c.272. It can be speculated that c.261 influences splicing by strengthening the affinity for other proteins involved in the correct splicing regulation.

It is worth considering that the 233-bp band corresponding to the bio inactive 11.3 kDa (exon 3–4-skipped isoform) is strongly increased in all mutant constructs, 261T and 261T/272T (Figure 10B). The relative total amount of the 2 alternatively spliced mRNA (exon 3 and exon 3–4 skipped) in the 255A, 272T, and 255A/ 261T constructs can be roughly estimated to represent nearly 50% of the total *GH1* mRNA in contrast to the wild-type, where these transcripts represent about 12% of the *GH1* transcripts.

The low serum GH level detected in vivo in the patients might be in part determined by the effect on splicing of these variants and in part by other yet unidentified genetic factors. Notably, patient 1 carried on the other allele the *GH1* promoter haplotype 1 (data not shown) that has been associated to IGHD and to a reduced luciferase activity *in vitro* (149).

The functional significance of the Glu82Asp variant carried by patient 3 is uncertain. From our experiments, it does not seem to influence splicing (Figure 10B, lane 3), and it is not predicted to have an effect on SR protein binding (data not shown). This variant is very rare, because it was reported in the dbSNP database (rs61762497) only in 1 individual of African ancestry, it is present in 2 individuals out of 13000 of the Exome Variant Server, and it was absent in our panel of 205 normal stature individuals. However, it is not predicted to exert a damaging effect on the protein function by the software Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). It might, thus, represent either a rare benign polymorphism, or alternatively it might contribute to a multigenic form of IGHD in this patient.

In conclusion, the analysis of *GH1* in individuals with sporadic IGHD led to the identification of 2 novel *GH1* exon 3 variations, c.255 G>A and c.272 A>T, included within 2 novel putative splicing regulatory elements that increase the aberrant splicing in vitro. When combined in cis, with c.261 C>T, as in the patients, their effect was reduced but still evident on the protein synthesis. It can be hypothesized that also a minimally increased amount of the 17.5-kDa protein might exert a dominant negative effect on the GH synthesis in vivo. The phenotype of patients 1 and 2 might be associated to these *GH1* splicing variations that by themselves only partially influence the amount of GH secretion but that might act in concert with other genetic variants.

V.2 NOVEL *GLI2* MUTATIONS IN PATIENTS WITH COMBINED PITUITARY HORMONE DEFICIENCY (CPHD)

V.2.1 Background

Combined Pituitary Hormone Deficiency (CPHD) is defined as the impaired production of at least two anterior pituitary hormones. It is usually characterized by growth hormone deficiency (GHD) and is associated with a wide range of phenotypes ranging from Short stature, Hypothyroidism, impaired sexual development and hypocortisolism. Congenital CPHD has an incidence of approximately 1:8000 births, mostly sporadic and 5-30% with familial cases (2,150,151).

Pituitary development is dependent upon a complex genetic cascade of transcription factors and signaling molecules acting as activators or repressors which dictate organ commitment, cell differentiation and cell proliferation (54,55). Mutations in several of these transcription factors, such as PIT1, PROP1, HESX1, LHX3, LHX4, OTX2 and SOX2, implicated in hypopituitarism with or without extra pituitary manifestations in mice and humans (73,152-155). However, screening of mutations in these transcription factors has identified aetiology of hypopituitarism in only a small percentage of patients.

During early steps of pituitary ontogenesis Sonic hedgehog (SHH) signaling pathway plays a crucial role in combination with several other signaling molecules in the development of pituitary gland. Shh is expressed in the ventral diencephalon and the oral ectoderm except primordial Rathke's Pouch (89). Three related zinc finger transcription factors GLI1, GLI2 and GLI3 acts as major effector protein downstream of the shh pathway, are expressed in the ventral dicencephalon and developing Rathke's pouch and mediate the activation of target genes (156). *GLI2* deficient mice showed defects in the pituitary development that have been included in the partial loss of anterior pituitary and complete loss of posterior pituitary (94,95).

Human GLI2 is a 1586 amino acid protein encoded by 13 exons on chromosome 2q14. The GLI superfamily proteins are characterized by a central DNA binding domain consisting of 5 consecutive zinc finger motifs in the pattern X3-Cys-X4-Cys-X12-His-X4-His-X3, where x represents any amino acid(157). In addition to the DNA binding domain, the GLI2 protein also contain an N-terminal repressor domain and C-terminal transcriptional activator domain (158,159). It has been shown that N-terminal repressor domain plays a central role in the pathogenic dominant negative activity resulting from mutations and constructs representing pathogenic human *GLI2* mutants with C-terminal deletions had undetectable transcriptional activity (96).

Heterozygous *GLI2* loss of function mutations were initially reported in patients with Holoprocensephaly (HPE), a condition of incomplete or failed forebrain separation, or HPE-like phenotypes with pituitary anomalies and postaxial polydactyly (96,97). Since then, several *GLI2* sequence variants were reported in patients with HPE characterized by a wide spectrum of phenotypes including craniofacial abnormalities, brancial arch anomalies, polydactyly and may or may not comprise pituitary defects (98-101). Franca et al (87,102) reported novel heterozygous frame shift or nonsense *GLI2* mutations and high frequency of non-synonymous *GLI2* variants in patients with congenital hypopituitarism without HPE and most of these patients presented with CPHD and an ectopic posterior pituitary lobe. More recently, several individuals with truncation mutations in *GLI2* were reported with the presence of typical pituitary anomalies, polydactyly and subtle facial features rather than HPE(103).

In all the patients so far identified with *GLI2* mutations, the pattern of inheritance was dominant with incomplete penetrance and variable phenotype. These previous studies conclude that patients with CPHD and an ectopic posterior with or without polydactyly, midline facial defects or HPE can be considered as candidates for *GLI2* study (87,102,103).

In the present study, we sequenced *GLI2* in a cohort of 136 patients with CPHD for the search of mutations and described the clinical findings of patients with possibly pathogenic mutations.

V.2.2 *GLI2* variants

Four CPHD patients were identified with heterozygous non-synonymous variations: two missense (p.Tyr575His and p.Ala593Val), one frameshift (p.Val1111Glyfs*19) and one nonsense (p.Arg1226*) (Figure 12). Among the 4 missense variants, 2 were located in the Zinc-finger domain and the one in the N-terminal repressor domain and the other in the C-terminal activator domain. Both the truncating variants were located in the C-terminal activation domain. Both the missense variants identified were not reported in any of the public databases used in this study (DbSNP, Exome variant Server and 1000 Genomes project consortium database). The patients with mutations in GLI2

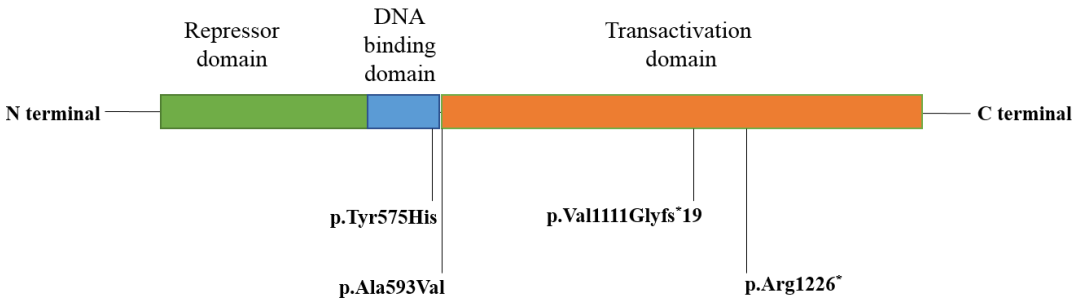


Figure 12: Schematic representation of *GLI2* with the variations identified among CPHD patients in this study

Table 12: Clinical characteristics of CPHD patients identified with *GLI2* mutations.

Patient ID	#1116	#1014	#1107	#836
Mutation	p.Tyr575His	p.Ala593Val	p.Val1111Glyfs*19	p.Arg1226*
Sex	M	F	F	M
Sporadic/ familial	Familial	Sporadic	Sporadic	Sporadic
Consanguinity Yes/No	No	No	No	No
Birth data	At term, AGA	At term, AGA	At term, AGA	At term, AGA
Age at diagnosis, yr	9.4	28	10.9	3
Height SDS at diagnosis	-2.2	-2.1	-1.6	-1.8
Bone age delay at diagnosis, yr	1.8	NA	NA	NA
GH TSH ACTH LH, FSH PRL	D D D PP NA	D D D D NA	D D D D NA	D D D PP NA
Pituitary and cerebral imaging	APH, EP	NA	APH, EP	APH, EP
Other Clinical Characteristics	Polydactyly, Craniofacial abnormalities, hypercholesterolemia	NA	Congenital Poly-malformative syndrome, mentalretardation, myopia	NA

AGA = appropriate for gestational age, NA= not available, D= deficiency of the evaluated pituitary axis, PP= prepubertal status at diagnosis, APH= anterior pituitary hypoplasia

V.2.3 *Insilico* analysis

The damaging effects of the missense variants were predicted by three publicly available algorithms; PolyPhen-2, SIFT and PROVEAN. PolyPhen-2 is a tool that predict the impact of an amino acid substitution on the structure and function of human protein(160), SIFT predicts whether an amino acid substitution affects protein function based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences(161) and PROVEAN predicts whether an amino acid substitution has an impact on the biological function of a protein grounded on the alignment-based score(162). Both the missense variants were predicted to be deleterious by PolyPhen and PROVEAN with very high scores while only p.Ala593Val was predicted deleterious by SIFT (Table 13).

Table 13: Insilico analysis of the novel variants identified in this study. X, predicted to be deleterious by respective site.

Patient	Variants		Exon	PolyPhen	PROVEAN	SIFT
1	p.Tyr575His	c.1723 T>C	11	X	X	X
2	p.Ala593Val	c.1778 C>T	11	X	X	-
3	p.Val1111Glyfs*19	c.3332delT	13			
4	p.Arg1226*	c.3676 C>T	13			

V.2.4 Discussion

Heterozygous Loss of mutations in Human *GLI2* were initially associated with HPE or HPE-like features charecterised by abnormal anterior pituitary formation and Hypopituitarism with inappropriately divided forebrain(97). More recently Franca et al (87,102) reported high frequency of *GLI2* variations in patients with congenital hypopituitarism without Holoprocencephaly. Most of these patients were presented with CPHD and an ectopic pituitary lobe.

The *GLI2* gene mutations were searched only in a limited number of previous studies with a considerable mutation detection rate. However *GLI2* is a large and highly polymorphic gene with several rare variations reported in the exome server database. This makes the evaluation of the pathogenicity of the reported variations difficult in the absence of functional studies. We thus decided to consider only variants with high evidence for pathogenicity, i.e. absent in the public databases and predicted to be "probably damaging" by at least two prediction software in addition to truncating and frameshift mutations. In the present study, we report 2 heterozygous missense mutations, one frameshift mutation and a nonsense mutation identified in CPHD patients. Two among these variants (p.Tyr575His and p.Ala593Val) fell within the zinc finger region of *GLI2*. Both the truncating variants, (p.Val1111Glyfs*19 and p.Arg1226*) were located in the C-terminal activator domain.

The DNA binding properties of the transcription factors are localised in the zinc finger domain which interact with the DNA at consensus binding sites. GLI2 is a C_2H_2 zinc finger protein that contain two canonical cysteine and histidine residues. The zinc finger motifs folds properly only when Zn^{2+} binds to the canonical residues and the folded motifs bind with the DNA where the unfolded zinc fingers do not bind with the target DNA. It is notable that both the missense variants reported in this study falls within the DNA binding region. Previous reports (96) indicate that all *GLI2* mutations leading to truncation are expected to lose the activation activity

regardless the location because of the distal location of the activation domain. The variants p.Val1111Glyfs*19 and p.Arg1226* identified in patients 5 and 6 respectively, are predicted to generate proteins lacking considerable portion of the C-terminal activation domain. Rather than simply acting as loss of function alleles, these mutations may exhibit dominant negative properties. It has been demonstrated by Roessler et al. (96) that the Gli2 carboxyl terminal domain had transcriptional activity and the constructs carrying C-terminal deletions exhibited undetectable transcriptional activity *in vitro*. More interestingly, these constructs when co-transfected with Wild-type GLI2 constructs revealed a strong dominant negative effect(96).

Variability in the phenotypic spectrum in our patients carrying *GLI2* variations is consistent with those previously reported in patients presented with CPHD and mutations in *GLI2* including pituitary hypoplasia, ectopic pituitary lobe, midline facial defects and polydactyly. Any of the patients in our study were presented with HPE or HPE-like features. However, the wide phenotypic spectrum, variability in expressivity may be the effect of other genetic or environmental factors.

The *GLI2* gene mutations were searched in large cohorts of CPHD patients only in a limited number of studies with a considerable mutation detection rate. However, considering the high polymorphism rate and the presence of rare variants of *GLI2* in the control population makes the evaluation of the pathogenicity difficult without functional studies.

V.3 SCREENING FOR *SHOX* MUTATIONS IN ISS PATIENTS

V.3.1 Background

The Short Stature Homeobox containing gene (*SHOX*), is located on the very tip of the short arm of both sex chromosomes X and Y within the telomeric part of pseudoautosomal region 1 (PAR1, Xp22.33 and Yp11.3) which comprises about 2.6 Mb.

Loss of function mutations of one *SHOX* allele (*SHOX* haploinsufficency) causes a wide spectrum of short stature phenotypes associated with Turner syndrome (TS), Leri-Weill dyschondrosteosis (LWD) and idiopathic short stature (ISS). Heterozygous *SHOX* defects have been identified in 50-90% of patients in LWD (117-120). Homozygous mutations or compound heterozygous mutations resulting in the complete loss of *SHOX* causes an extreme phenotype called Langer mesomelic dysplasia (LMD) in almost all the cases (122).

Heterozygous mutations in *SHOX* has been identified in 2-15% of idiopathic short stature (ISS) patients (106,109,110). The deletions which encompass *SHOX* gene itself or the regulatory enhancer regions accounts for 80% of all the *SHOX* mutations. The other gene defects are point mutations reported all over the gene, but predominantly in exon 3 and 4 encoding the homeodomain. Partial and complete *SHOX* duplications of varying sizes were also reported in the short stature patients (124,125).

In this study, a large cohort of ISS patients with mild and severe short stature has been screened for the *SHOX* defects.

V.3.2 Molecular analysis of *SHOX* defects in ISS patients

Commercial *SHOX* MLPA probes were used for the screening for deletions/duplication of the exons, the entire gene and the enhancers. In the absence of deletions, direct sequencing of *SHOX* was performed to identify point mutations (Figure 13).

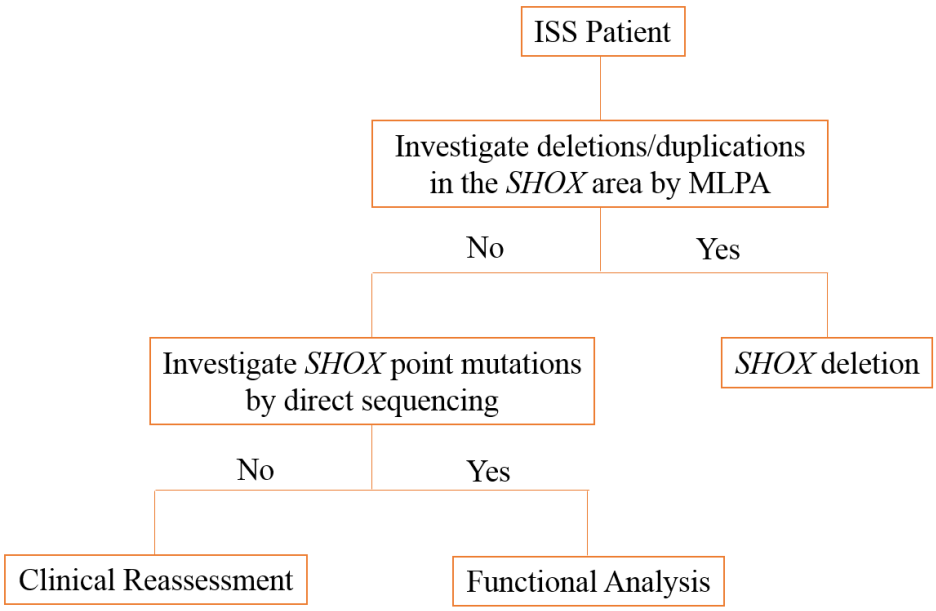


Figure 13: Flow chart detailing the strategy for the molecular investigation in ISS patients

Two hundred and fifty two idiopathic short stature individuals aged between 0.1 and 18 years were screened for *SHOX* gene mutations. A total of 20 patients (7.9%) were identified with *SHOX* defects. Among them 14 patients had deletions or duplications of *SHOX* gene or the regulatory regions, identified by MLPA. Six patients were identified with mutations in the *SHOX* coding region by direct sequencing. The mean height SDS of the total mutated patients was -2.07±0.7. The relatives of 13 patients (a total of 44 individuals) were also analyzed for the presence of the *SHOX* mutations identified in the probands while the relatives of the other six patients were not available for molecular analysis at the time of the compilation of this thesis.

V.3.3 Deletions/duplications

Fourteen patients were identified carrying deletions/duplications in different regions within the *SHOX* area (Figure 14). Ten patients showed deletions as revealed by the MLPA probe signals (Table 14). Among them, 6 index cases (43% of the total deletions) have been identified with deletions of probes 05645-L05099 and 05646-L24249 encompassing a *SHOX* enhancer CNE7

(Figure 15 C, D, E and Figure B, C, D). The size of this deletion was estimated as 47.5 kb by previous studies (163,164). One female patient was identified to be homozygous for this deletion (Figure 15 E).

Two patients had heterozygous deletions removing the entire *SHOX* gene and the flanking regulatory regions (Figure 15F and Figure 16A). One patient was identified with a large deletion (~816.5-1117 kb) removing all the downstream enhancers of *SHOX* (CNE4, CNE5, CNE9 and CNE7). A relatively small deletion (<130 kb) further downstream of all the known enhancers of *SHOX* was also identified in one patient (Figure 15 B). This deletion has never been reported previously.

Four patients were found to have duplications related to *SHOX* (Figure 17). Two patients had duplication of exon 2 of the *SHOX* gene. The complete duplication of the exon could alter the reading frame and introduce a premature stop codon leading to the production of a truncated protein. However, it is difficult to predict how it will affect the reading frame as it is difficult to understand the exact extension of the deletion by MLPA. It is also suggested that the partial tandem duplications within the gene may affect the gene expression by altering the distance between the coding part and the regulatory regions (125).

One patient carried a duplication involving the entire gene and its regulatory regions. This duplication has been previously reported in ISS patients. The remaining patient had a duplication encompassing two probes downstream of the known enhancer region. Interestingly, all the duplications identified were *de novo* which were absent in their healthy relatives, whereas all the deletions were inherited from parents.

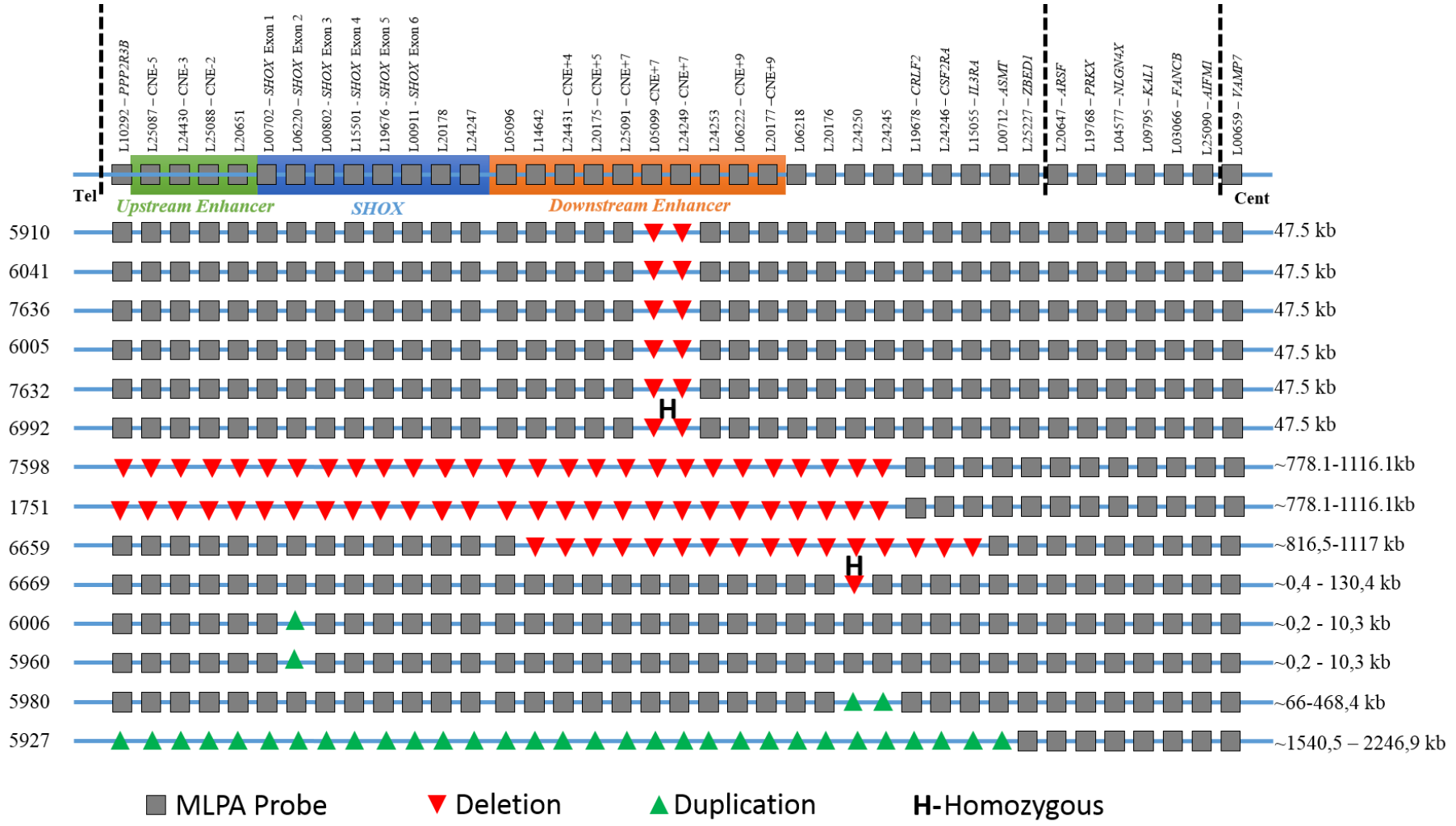


Figure 14: The schematic representation of the genomic locations and the approximate extension of the deletions or duplications of the SHOX area identified in ISS patients.

Table 14: Clinical characteristics of patients identified with deletions/duplications

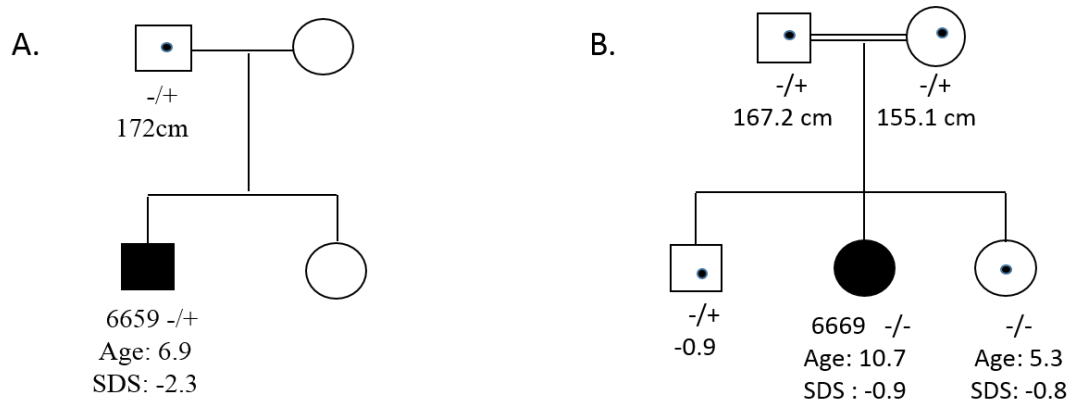
Patient ID	**Sex**	**Age**	**Height SDS**	***SHOX* Defect (MLPA Probes)**	**Extension**	**Location**	**Inheritance**	**Rappold Score**
5910	F	5.3	-1.9	Heterozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Paternal Transmission	2
6041	F	6.6	-1.9	Heterozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Maternal Transmission	6
7636	F	4.7	-1.6	Heterozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Nd	2
6005	F	16.4	-1.2	Heterozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Nd	-
7632	M	8.9	-2.1	Heterozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Nd	3
6992	F	27	-2.7	Homozygous deletion 05645-L05099 and 05646-L24249	47.5 kb	*SHOX* downstream Enhancer	Paternal Transmission	-
7598	M	14	-2.8	Heterozygous deletion 09333-L10292 to 14697-L24245	~778.1-1116.1kb	Whole gene	Maternal Transmission	-
1751	F	13.7	-3.7	Heterozygous deletion 09333-L10292 to 14697-L24245	~778.1-1116.1kb	Whole gene	Nd	-
6659	M	6.9	-2.3	Heterozygous deletion 13821-L14642 to 13597-L15055	~816,5-1117 kb	*SHOX* downstream Enhancer	Paternal Transmission	3
6669	F	10.7	-0.9	Homozygous deletion 09335-L24250	~0,4 - 130,4 kb	*SHOX* downstream area	Paternal and Maternal Transmission	16
6006	F	3.9	-2.2	Duplication 01146-L06220	~0,2 - 10,3 kb	*SHOX* Exon 2	*De Novo*	9
5960	M	10.9	-1	Duplication 01146-L06220	~0,2 - 10,3 kb	*SHOX* Exon 2	*De Novo*	-
5980	M	12.3	-2.1	Duplication 09335-L24250 and 14697-L24245	~66-468,4 kb	*SHOX* downstream area	*De Novo*	-
5927	M	11.3	-1.6	Duplication 09333-L10292 to 01153-L00712	~1540,5 – 2246,9 kb	*SHOX* Area	*De Novo*	-

V.3.4 Point Mutations

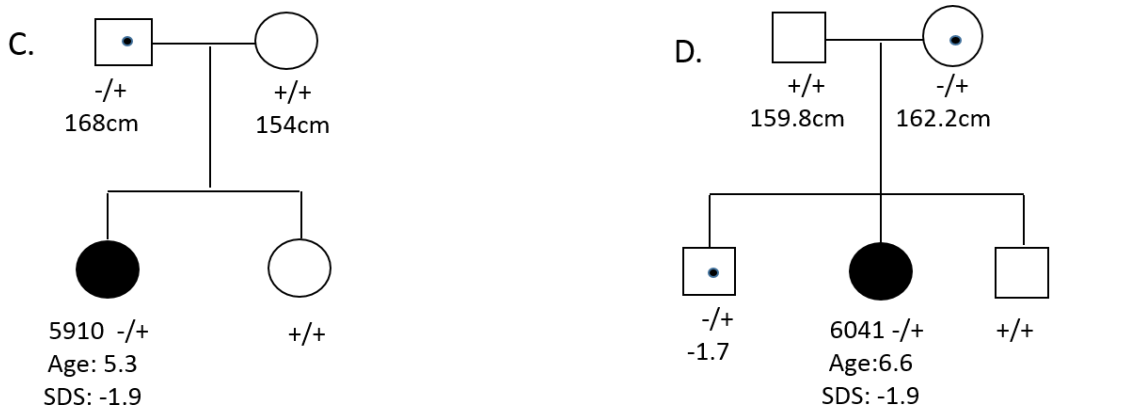
Six heterozygous variations (2%) were identified by direct sequencing (Table 15). Two patients had frame shift mutation (p.*226Argext*22) which change the stop codon into an arginine, resulting in the addition of 22 amino acid residues to the C-terminal of the protein. This mutation has been already reported in idiopathic short stature patients by previous studies (107,165). In one case the mother, was also short, was tested and identified with the same mutation (165). Two missense variations (p.Asp58Glu and Arg218His) and two 5'UTR variations (c.55C>T and c.-51G>A) were also identified. These variations were not present in the public databases of healthy individuals (DbSNP, Exome variant Server and 1000 Genomes project consortium database). The effect of these variations on *SHOX* is unknown. However, p.Asp58Glu has been reported in the *SHOX* mutation database in 35 index cases among a total of 5729 ISS patients screened, which makes it the most frequent *SHOX* point mutation reported till now. The mean height SDS among the patients with point mutations was -1.9±0.8.

Table 15: Point mutations identified in ISS patients

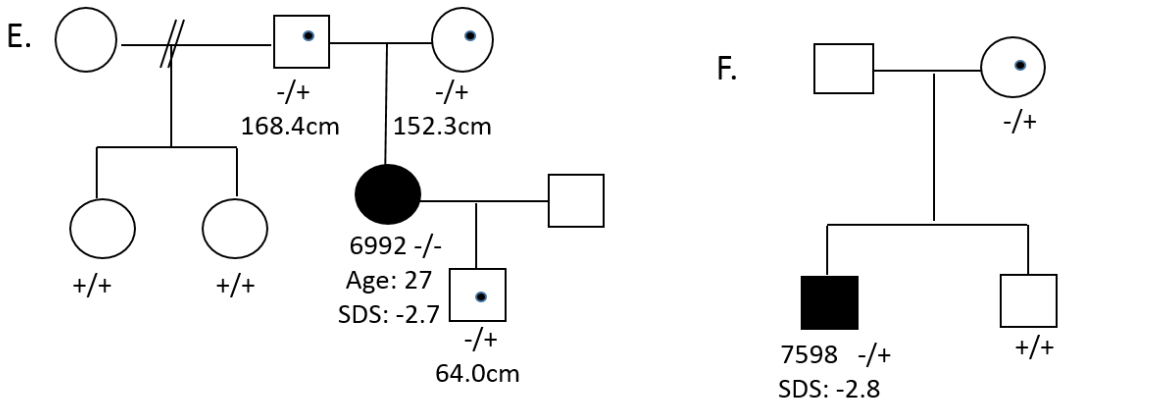
Patient ID	Sex	Age at visit	Height SDS	Variation	Location	Inheritance	Rappold Score
6011	F	4.4	-2.0	p.*226Argext*22	Exon 6B	Maternal Transmission	2
6158	F	8.5	-1.8	p.*226Argext*22	Exon 6B	Maternal Transmission	5
5894	M	9.7	-2.8	p.Asp58Glu	Exon 2	Paternal and Maternal Transmission	-
6148	F	9.5	-2.1	p.Arg218His	Exon 6B	Nd	0
6568	F	14.6	-1.8	c.-55C>T	5' UTR	Nd	-
7256	M	12.2	-2.9	c.-51G>A	5' UTR	Nd	2



Whole deletion of SHOX downstream Enhancer **Deletion within SHOX downstream area**



Deletion of SHOX downstream Enhancer **Deletion of SHOX downstream Enhancer**



Deletion of SHOX downstream Enhancer **Whole Deletion of the SHOX area**

Figure 15: Family pedigrees of the ISS probands carrying deletions. Relatives of these probands have been screend for *SHOX* defects. (-) and (+) represents mutated and wild type alleles respectively.

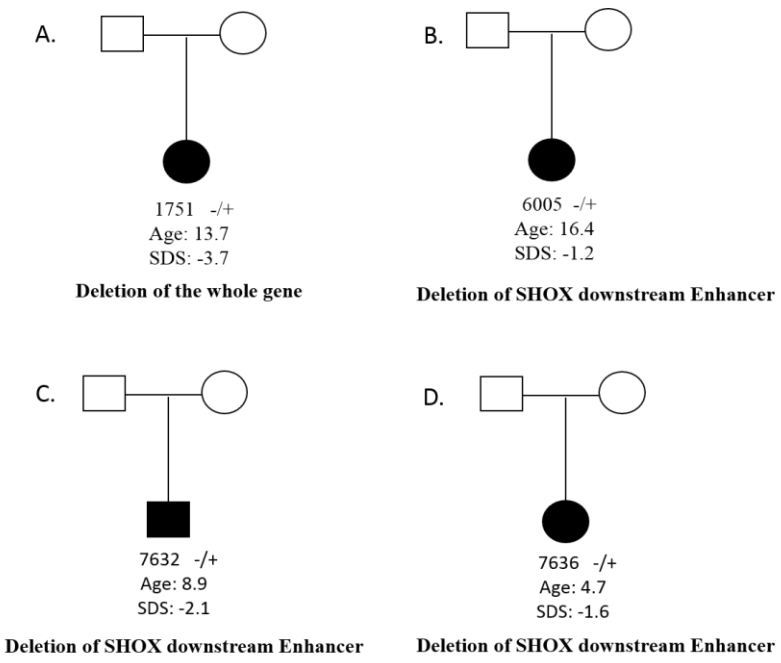


Figure 16: Family pedigrees of the ISS probands carrying deletions whose relatives were not available for the molecular analysis. (-) and (+) represents mutated and wild type alleles respectively.

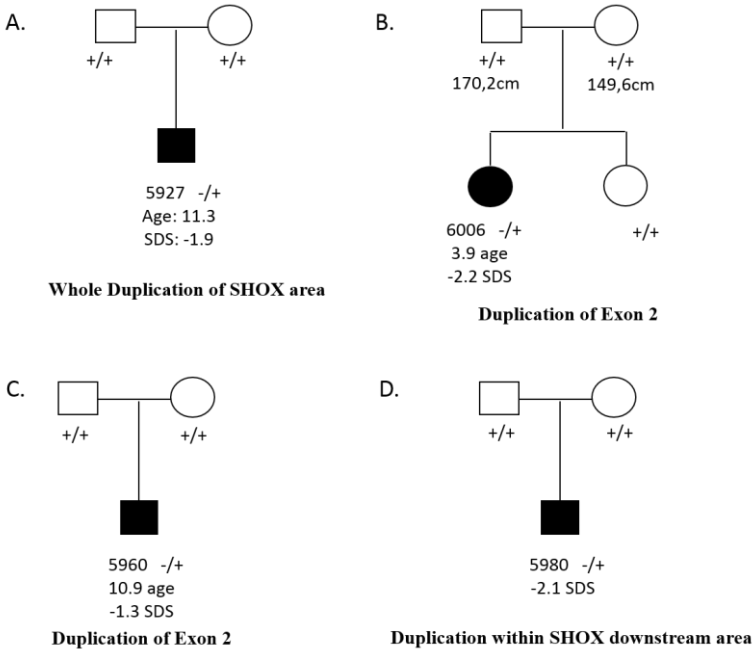
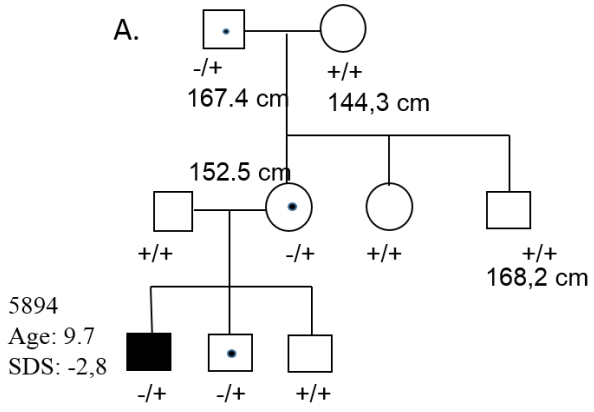
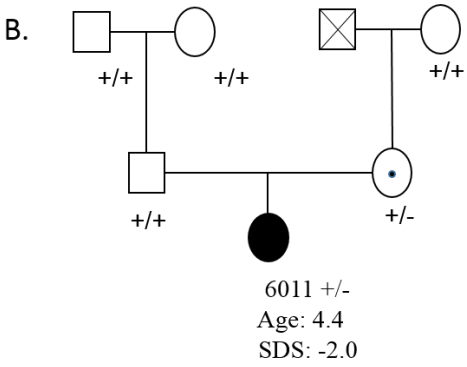


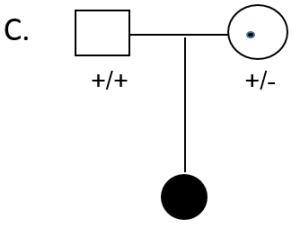
Figure 17: Family pedigrees of the ISS probands carrying duplications. All the duplication identified in this study are *de novo* mutations. (-) and (+) represents mutated and wild type alleles respectively.



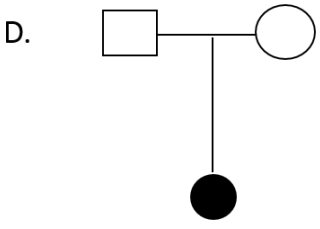
p.Asp58Glu



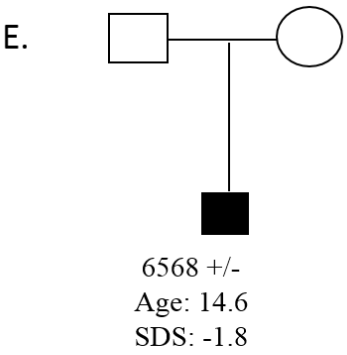
p.*226Argext*22



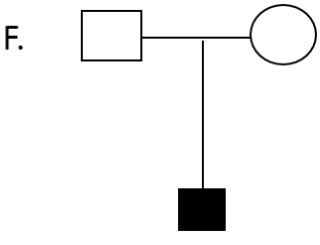
p.*226Argext*22



p.Arg218His



c.-55C>T



c.-51G>A

Figure 18: Family pedigrees of the ISS probands carrying point mutations. Relatives of 6148, 6568 and 7256 were not available for the *SHOX* screening. (-) and (+) represents mutated and wild type alleles respectively.

V.3.5 Discussion

SHOX haploinsufficency is the principal known genetic cause of short stature and in 70% of the patients with *SHOX* defects, the cause is a deletions that affect the *SHOX* gene or its regulatory regions. The high incidents of deletions and rare duplications are due to the high crossing over rate and to the presence of repeated sequences that mediate unequal chromosomal exchanges. Thus, the *SHOX* region of PAR1represents a hot spot for non-allelic homologous recombination between X and Y chromosomes, resulting in a high percentage of recombinant fractions.

In the present study, the *SHOX* region molecular defects were screened in 252 unrelated Idiopathic Short stature patients. Twenty mutations were identified in this study with a mutation frequency of 7.9%, which is consistent with the 2-15% reported in the literature.

A deletion of 47.5kb, previously described by Benito-Sanz et al., was identified in 5 index cases making it the most frequent *SHOX* defect in this study (Figure 14). This deletion is mapped downstream of *SHOX* and include a conserved regulatory elements CNE7. All the 47.5 kb deletions were identified at the heterozygous state except one case of homozygous deletion in a 27 years old female patient (#6992) with a height SDS of -2.7 (Figure 15 E). The MLPA analysis of 471 controls by Bunyan et al. (125) showed that 47.5kb downstream deletion is not present in the healthy individuals, though high frequency is described in different *SHOX* cohorts including the present study suggesting its involvement in the phenotypic effect. The effect of this deletion could also be due to the change in the structure of the *SHOX* downstream region and thereby changing the distance between the coding region and the other regulatory regions affecting their function.

In patient #6669 (figure 15B), a homozygous deletion was identified outside the classical enhancer region of *SHOX*. This deletion did not include known conserved regions downstream

of *SHOX*. This deleted region might be a part of a putative regulatory region further downstream of the known enhancers. In fact, recent studies have identified large deletions outside the known enhancer regions of *SHOX* (136,166) in patients with ISS, that overlap with the segments identified in patient #6669.

Two patients 6568 and 7256 were identified with variations in the 5' UTR of the exon 2 (c.-55C>T and c.-51G>A) (Figure 18 E, F) which were absent in the public databases. The variant c.-55C>T has been previously identified in ISS patients and reported in the SHOX mutation database. Whether the 5'UTR variations identified in this study affect the translational efficiency of *SHOX* are yet to be demonstrated by *in vitro* functional studies. A suitable model for the functional analysis could be luciferase reporter assay to understand how the variants affect the translational efficiency of the gene.

The patients identified with *SHOX* mutations showed high phenotypic variability and the penetrance appeared to be incomplete. Rappold et al. reported that the phenotypes of idiopathic short stature patients carrying *SHOX* gene mutation is not different from phenotypes of the patients without *SHOX* mutations. This makes the analysis of phenotype-genotype correlation difficult in patients with *SHOX* defects. Previous reports suggested that the phenotypic variability among the patients with deletions in the enhancer region cause a relatively milder phenotypes compared to the exonic or whole gene deletions (117). Consistent with these reports, the patients identified with the whole *SHOX* deletions showed severe short stature (-2.8 and -3.7 SDS) among all the patients. In the case of enhancer deletions, the size of the deletions did not have any effect on the phenotypes of the patients. In this cohort, the mean height SDS of the patients with deletions/duplications and the mean height SDS of the patients with point mutations did not differ.

The clinical data required for the Rappold score (111) were not available from all the patients screened in this study. However, the available Rappold score was available for 11 patients with *SHOX* mutations. Only two of them had scores above 7, which is the cut off value recommended for the *SHOX* screening. These findings highlights the limitations of Rappold scoring system for the selection of ISS patients for the molecular analysis of *SHOX*. Moreover, the use of body mass index in this system gives high score for obese patients. Thus, it is worth considering that the Rappold criteria are not distinctive for *SHOX* deficiency.

The family pedigree of #6992 (Figure 15E) and #5894 (Figure 18 A), with a 47.5 kb deletion and p.Asp58Glu respectively, well demonstrate the high rate of homologous recombination in the PAR1 region. In both families, carrier male did not transmit the mutation to all the daughters. The high recombination rate between the X and Y chromosomes during male meiosis could be accountable for this phenomenon.

The present study along with the recent studies by Bunyan et al.2012 and Tsuchiya et al.2013 identified deletions involving parts of the PAR1 region further downstream of the classical enhancers of *SHOX*. The effect of deletions involving this regions on the *SHOX* deficiency patients are becoming increasingly evident. The current MLPA kit used in all the diagnostic labs contains probes in all the exons and in the known regulatory regions upstream and downstream of *SHOX* that are more frequently deleted in short stature patients. The probes in the PAR1 region downstream of the classical enhancers are spaced relatively far from each other, the wider including a 338kb separation between probes L24245 and L19678. Alternative methods like a customized aCGH, specific for PAR1 would enable to map the alterations within these regions with a higher probe density and possibly to identify the deletions not detected by the current methods.

VI. CONCLUSION

Mutational screening of three genes (*GH1*, *GLI2* and *SHOX*) has been carried out in short stature associated to growth disorders with and without pituitary hormone deficiencies. This study confirms the involvement of multiple genes, both well established as well as not widely studied, in the growth disorders. New strategies are required for wide screening in large cohorts with a large number of genes. This will reduce the costly and laborious gene by gene analysis currently used in this field. The introduction of targeted next generation platforms that simultaneously allow the analysis of multiple genes in large number of patients will greatly improve the diagnosis of growth defects.

VII. REFERENCES

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VIII. APPENDICES

IX.1 APPENDIX 1: Primer sequences

Primer sequences for the amplification of *GH1*

Primer	**sequence 5' → 3'**	**Amplified Fragment**
Gh32 (f)	CCAGCAATGCTCAGGGAAAG	Entire *GH1*
Gh33 (r)	TGTCCCACCGGTTGGGCATGGCCAGGTAGCC	
Gh40 (f)	TTTGGGCACAATGTGTCCT	Exon 1
Gh13 (r)	CTGAGCTCCTTAGTCTCCTCCTC	
Gh14 (f)	GACTTTCCCCCGCTGGAAATAAG	Exon 2
Gh27 (r)	AGATGCGGAGCAGCTCTAGG	
Gh26 (f)	CTCAGAGTCTATTCCGACACCC	Exon 3
Gh15 (r)	GTGTTTCTCTAACACAGCTCTC	
IVS4 (f)	CCCACTGACTTTGAGAGCTG	Exon 4
IVS4 (r)	CATGTCCTTCCTGAAGCAGT	
Gh16.17 (f)	TCACACAACGATGACGCACT	Exon 5
Gh31 (r)	CCAGGGCCAGGAGAGGCACTGGGG	

Primer sequences for the amplification of *SHOX*

Primer	**sequence 5' → 3'**	**Amplified Fragment**
SHOX 2f	GAGACGCGCGCATCCACCA	Exon 2
SHOX 2r	GAGGCGCCGAACCCCAGGAG	
SHOX 3f	CACGTTGCGCAAAACCTC	Exon 3
SHOX 3r	CGTCCCTCACCCAACCTC	
SHOX 4-5f	AGTGCTTGGTTCAGCCTCAT	Exon 4 and Exon 5
SHOX 4-5r	TTTCTAAGGGCCAGCTGAGA	
SHOX 6af	AAGAGGCACGTTGGAGGTTT	Exon 6a
SHOX 6ar	CGGGGTTGAGTGCAGGAC	
SHOX 6bf	CTCCTCTTCCCGGGTTCAC	Exon 6b
SHOX 6br	GGTGGTGGGCACCTGTAA	

Primer sequences for the amplification of *GLI2*

Primer	sequence 5' → 3'	Amplified fragment
Gli2_1F	TGGGTTTGGGCTCAGTGT	Exon 1
Gli2_1R	CCTCTTCGCCCTCCATAAAC	
Gli2_2F	TGGCTGCTCTTGCTATGAAA	Exon 2
Gli2_2R	GCAGGAGATGTGGCTGAGG	
Gli2_3F	CATGTTGGTTTTGGGGTCTT	Exon 3
Gli2_3R	GACCAAGGCTGAGGAGTTGA	
Gli2_4F	CAGGTTCTGACGGCTTCTTT	Exon 4
Gli2_4R	TTGTCCCCAAAAGAAACAGC	
Gli2_5F	CCTTGCAGGCTCTTCCTATC	Exon 5
Gli2_5R	TCTTTCTCCTCGGGTCAAAA	
Gli2_6F	TGGGCAAGGTTCTCTCTGTC	Exon 6
Gli2_6R	CTTAGCATGAGCTGGCAGTG	
Gli2_7F	TGTGCGGAGAGATCCTAGAG	Exon 7
Gli2_7R	TTCACCACCAAGGGTACAGC	
Gli2_8F	TTCCCCACAGCACTTCGAT	Exon 8
Gli2_8R	TCCAGCCCCTTCTGTCTAGT	
Gli2_9F	GACAGCAGGGGGTGGTCT	Exon 9
Gli2_9R	CCACCTCCAAACATGATCC	
Gli2_10F	GGTTGGAGCAGAGCAGAGAA	Exon 10
Gli2_10R	GGCACCTGGCTATCTACTGG	
Gli2_11F	CGTGGGTAGCTTCAGGAGAA	Exon 11
Gli2_11R	GATATCGCTGTGCCCCTAGA	
Gli2_12F	GCCTGTGCAGGCCTAGAG	Exon 12
Gli2_12R	GTGGGTGCCAGCCTAGTTG	
Gli2_13.1F	GTGTTGCAAGCCCTCTTCTC	Exon 13
Gli2_13.1R	AGTGGCTGCCGCGTACTT	
Gli2_13.2F	AGCAGTACAGCCTGCGGGCCAAGT	Exon 13
Gli2_13.2R	CTCCATCGCCACGTTCTCGCT	
Gli2_13.3F	CTTCCACAGCACCCACAAC	Exon 13
Gli2_13.3R	CCTTGCGGACTGTAGCCC	
Gli2_13.4F	GCAGTGGAATGAGGTGAGCT	Exon 13
Gli2_13.4R	GATGGCTCTGCTGTGGGTAG	
Gli2_13.5F	CCCTCAGCAGACAGAAGTGG	Exon 13
Gli2_13.5R	GTACATGTGGATCTGGCCGT	
Gli2_13.6F	CAGTCAGGAAACAGCAGAGG	Exon 13
Gli2_13.6R	GGAAAAAGACAAGACAGCTGGA	

Primer sequences for site directed mutagenesis:

PRIMER	SEQUENCE	MUTATION
GH82Asp_for: GH82Asp_rev:	CCCTCTGTTTCTCAGACTCTATTCCGACACC GGTGTCGGAATAGAGTCTGAGAAACAGAGGG	p.Glu82Asp
GH85Pro_for: GH85Pro_rev:	CTCAGAGTCTATTCCAACACCCTCCAACAGGG CCCTGTTGGAGGGTGTTGGAATAGACTCTGAG	p.Pro85Pro
GH87Pro_for: GH87Pro_rev:	GTCTATTCCGACACCTTCCAACAGGGAGGAAAC GTTTCCTCCCTGTTGGAAGGTGTCGGAATAGAC	p.Pro87Pro
GH91Val_for: GH91Val_rev:	GACACCCTCCAACAGGGTGGAAACACAACAG TGTTGTGTTTCCACCCTGTTGGAGGGTGTC	p.Glu91Val
GH85+87for: GH85+87rev:	CTCAGAGTCTATTCCAACACCTTCCAACAGGGAGG CCTCCCTGTTGGAAGGTGTTGGAATAGACTCTGAG	p.Pro85Pro p.Pro87Pro
GH87+91for: GH87+91rev:	CTATTCCGACACCTTCCAACAGGGTGGAAACACAAC GTTGTGTTTCCACCCTGTTGGAAGGTGTCGGAATAG	p.Pro87Pro p.Glu91Val

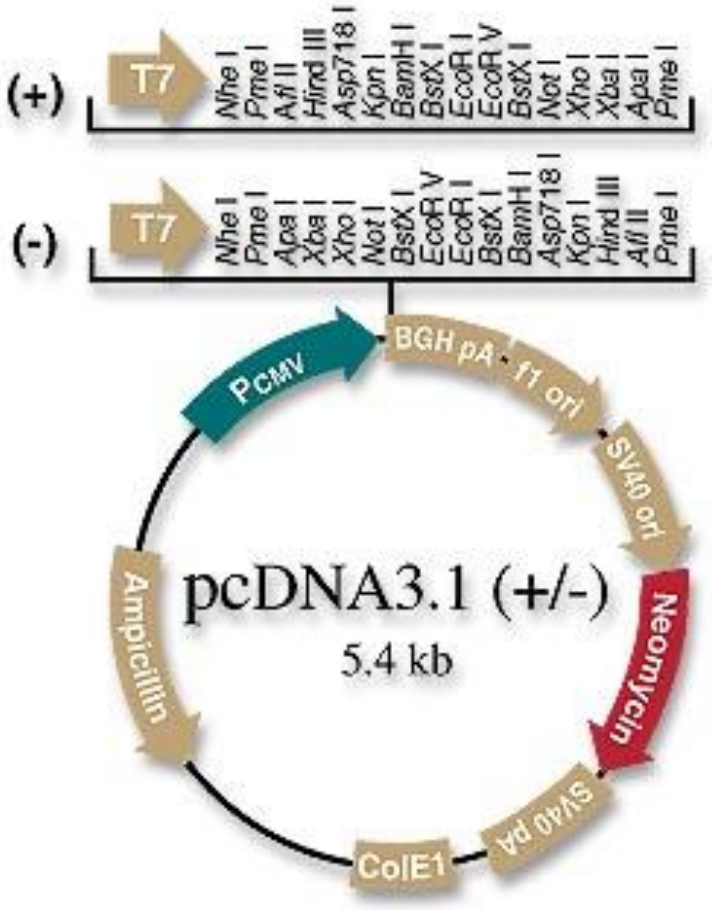
IX.2 APPENDIX 2: Probes used for the MLPA analysis of *SHOX*

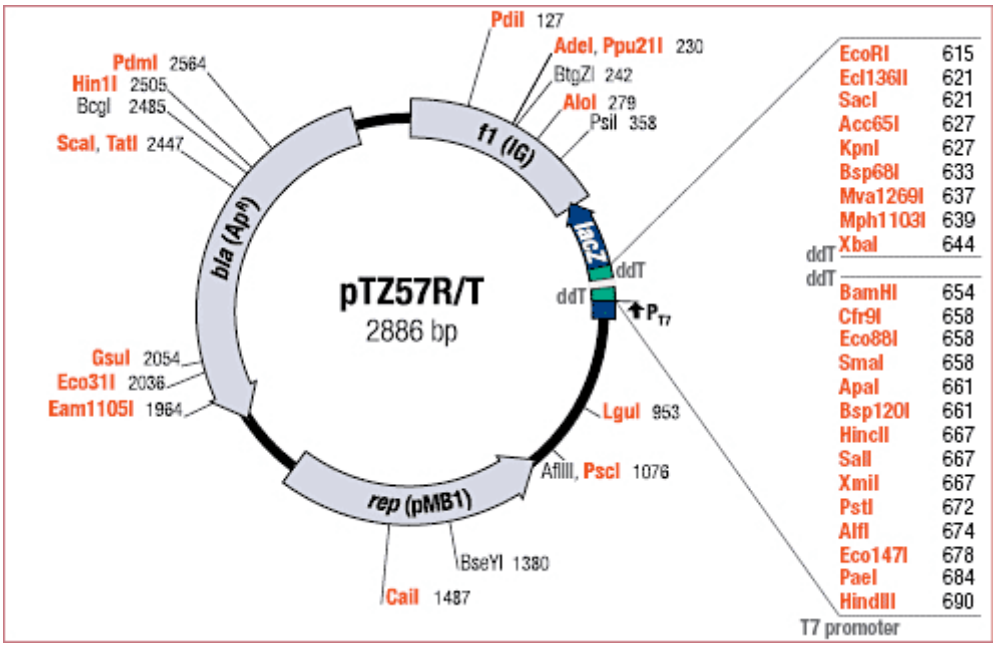
Length (nt)	SALSA MLPA probe	Gene Exon	Ligation site	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		p-telomere			227.0 kb
		---------- Start of PAR1 ----------			
211 ~	09333-L10292	*PPP2R3B gene*	PAR1	CGTCCGAGTTCC-ACTCGCGCTACA	91.6 kb
364 *	18889-L25087	SHOX AREA	CNE-5	GAAATGTTAACA-GCTCCCCGAGCT	61.7 kb
130 *	18885-L24430	SHOX AREA	CNE-3	ATGGCAGAGCAT-TTGTACCCCTGG	56.3 kb
427 *	18891-L25088	SHOX AREA	CNE-2	TACACCGTTATG-CGGATGCTCGTT	63.5 kb
266	01341-L20651	SHOX AREA LOC159015	*Upstream of SHOX*	GCCTGGAACAGA-ACTTCCGCGGGG	4.7 kb
		SHOX	**NM_000451.3**		
		start codon	*692-694 (Ex 2)*		
166	01145-L00702	Exon 1	99-100	TTTCTACTGCAA-ACAGAAATGGGA	6.7 kb
204	01146-L06220	Exon 2	920-921	ACCACGTAGACA-ATGACAAGGAGA	3.6 kb
245	01147-L00802	Exon 3	1032-1033	CGGGCAGACCAA-GCTGAAACAGAG	6.2 kb
300 #	01148-L15501	Exon 4	1198-1199	CAGAACCGGAGA-GCCAAGTGCCGC	0.2 kb
337	01149-L19676	Exon 5	1261-1262	ACAGCCAACCAC-CTAGACGCCTGC	3.5 kb
231	09337-L00911	Exon 6	1506-1507	AAGCAACAGCAA-GAATTCCAGCAT	6.4 kb
		stop codon	*1568-1570 (Ex 6)*		
226	09336-L20178	*SHOX* Intron 6	6.4 kb after Ex 6; 8 kb before Ex 7	TGGCTTCACGAG-TTCAGCCCATTG	6.4 kb
395	09338-L24247	*SHOX* Intron 6	1.4 kb before Ex 7	TCCCACATTCTT-GGAATCACAATG	56.9 kb
136	05642-L05096	SHOX AREA		GCAGCAGTGAAA-GTGAGCATTCCC	19.8 kb
154	13821-L14642	SHOX AREA		GATGGCTGATAA-TTACTCCGTATG	19.4 kb
172 *	18886-L24431	SHOX AREA	CNE+4	GCCTCCGATACA-GTTTACGGCTTC	37.4 kb
199	13296-L20175	SHOX AREA	CNE+5	GGAAAACCACGT-TCCTATCGATCC	29.6 kb
481 ^*	18893-L25091	SHOX AREA	ECR1 / CNE+7	CAGACCAGGTCT-CCTGTTTCATGT	28.1 kb
318 §^	05645-L05099	SHOX AREA		TGTTCCCACCGT-AAAACTCACTCC	8.4 kb
439 §^	05646-L24249	SHOX AREA		TGCATGTCTGCT-TTTTGAATGGCC	10.7 kb
466 §	13297-L24253	SHOX AREA		TACAGCAAATGA-TACGTATAAATT	6.3 kb
290 §	06291-L06222	SHOX AREA	ECS4/CNE+9	CTTGAAAGGGCA-GGAACTCTAATT	0.4 kb
185 §	06293-L20177	SHOX AREA	ECS4/CNE+9	TAATTGATGAGA-TGCAGAAGCCAG	15.4 kb
148	05648-L06218	Xp22-PAR1		TGGTGCTGAAAT-GAGGAAGCCCTG	48.7 kb
178	05649-L20176	Xp22-PAR1		TGAGGAGGTACC-TCAAAGCTAAAC	64.4 kb
445	09335-L24250	Xp22-PAR1		GAAATTCAGTTT-TAATAACACAGA	66.0 kb
379	14697-L24245	Xp22-PAR1		CTCTGGTGAGAT-GCCATCTAGAGA	338.0 kb
403	13911-L19678	*CRLF2 gene*	PAR1-Xp22PAR	GAATGCCAGCAA-ATACTCCAGGAC	73.9 kb
387	10251-L24246	*CSF2RA gene*	PAR1-Xp22PAR	GACAAGCCTTCT-GCTCTGTGAGTT	69.8 kb
142	13597-L15055	*IL3RA gene*	PAR1-Xp22PAR	TGCACAGATAAG-TTTGTCGTCTTT	280.7 kb
310 ~	01153-L00712	*ASMT gene*	PAR1-Xp22PAR	GACATCCCAGAA-GTGGTGTGGACG	706.4 kb
456	16858-L25227	*ZBED1 gene*	PAR1-Xp22PAR	TCGTCAAGAGCA-ACACGGAGCAGA	593.8 kb
		---------- End of PAR1 ----------			
254 ˣ	16846-L20647	*ARSF gene*	Xp22.33	CATCCATATAAT-TATGGGTTTGAC	536.9 kb
328 ˣ	16898-L19768	*PRKX gene*	Xp22.33	CGATTAGGAAAC-ATGAAGGTCAGT	2606.5 kb
283 ˣ	05587-L04577	*NLGN4X gene*	Xp22.31	GACGGCTTGGGT-GATGCACGAAAT	2311.9 kb
238 ˣ	06402-L09795	*KAL1 gene*	Xp22.31	GTTTCCTGAAGC-GTGTGCCCACAA	6304.2 kb
274 ˣ	03906-L03066	*FANCB gene*	Xp22.2	TCTCATCAGAAT-TCTCCCTATAAA	114321.4 kb
420 ˣ	00820-L25090	*AIFM1 gene*	Xq26.1	TATTGGTCTTGT-GGACAGTAGTTT	25732.3 kb
		----------Start of PAR2 ----------			
355 ∞¥	01156-L00659	*VAMP7 gene*	PAR2	TGTGGGAAAAGT-GTTTCCATTCTG	98 kb

* New in version G1 (from lot G1-0513 onwards).
x X-chromosome, outside PAR region. Gives half the signal in males as compared to females.
§ These five probes are in the putative SHOX- regulatory region (CNE9)
^ These 3 probes are within the recurrent ~47.5 kb which includes the ECR1 regulatory region.
A founder SHOX point mutation in the Spanish Gypsy population, c.508G>C (p.A170P) results in a strongly reducedprobe signal of the 300 nt exon 4 probe
~ This probe has been found to be duplicated in an apparently healthy individual in our quality tests.
∞This probe has been found to be deleted in an apparently healthy individual in our quality tests.
¥ The VAMP7 probe at 355 nt is located very close to the q-telomere of X and Y in the PAR2.
£ The 254 nt ARSF probe has been reported to have a variable copy number.
CNE = Conserved Non-coding DNA Element. ECR = Evolutionary Conserved Region. ECS = Evolutionary Conserved Sequence.

Source: MRC Holland

IX.3 APPENDIX 3: Plasmids





IX. PUBLICATIONS

1. **Babu D**, Mellone S, Fusco I, Petri A, Walker GE, Bellone S, Prodam F, Momigliano-Richiardi P, Bona G, Giordano M. Novel mutations in the GH gene (GH1) uncover putative splicing regulatory elements. Endocrinology 2014; 155:1786-1792

2. Prodam F, Savastio S, Genoni G, **Babu D**, Giordano M, Ricotti R, Aimaretti G, Bona G, Bellone S. Effects of growth hormone (GH) therapy withdrawal on glucose metabolism in not confirmed GH deficient adolescents at final height. PLoS One 2014; 9:e87157.

Novel Mutations in the GH Gene (*GH1*) Uncover Putative Splicing Regulatory Elements

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Mutations affecting exon 3 splicing are the main cause of autosomal dominant Isolated GH Deficiency II (IGHDII) by increasing the level of exon 3-skipped mRNA encoding the functionally inactive dominant-negative 17.5-kDa isoform. The exons and introns of the gene encoding GH (*GH1*) were screened for the presence of mutations in 103 sporadic isolated GH deficiency cases. Four different variations within exon 3 were identified in 3 patients. One carried c.261C>T (p.Pro87Pro) and c.272A>T (p.Glu91Val), the second c.255G>A (p.Pro85Pro) and c.261 C>T, and the third c.246G>C (p.Glu82Asp). All the variants were likely generated by gene conversion from an homologous gene in the *GH1* cluster. In silico analysis predicted that positions c.255 and c.272 were included within 2 putative novel exon splicing enhancers (ESEs). Their effect on splicing was confirmed in vitro. Constructs bearing these 2 variants induced consistently higher levels both of transcript and protein corresponding to the 17.5-kDa isoform. When c.255 and c.272 were combined in *cis* with the c.261 variant, as in our patients, their effect was weaker. In conclusion, we identified 2 variations, c.255G>A and c.272A>T, located in 2 novel putative exon splicing enhancers and affecting *GH1* splicing in vitro by increasing the production of alternatively spliced isoforms. The amount of aberrant isoforms is further regulated by the presence in *cis* of the c.261 variant. Thus, our results evidenced novel putative splicing regulatory elements within exon 3, confirming the crucial role of this exon in mRNA processing. **(*Endocrinology* 155: 1786–1792, 2014)**

The *GH1* gene is located on chromosome 17q23 within a cluster of 5 highly homologous genes, all consisting of 5 exons and 4 introns, including the placentally expressed *GH2*, 2 chorionic somatomammotropin genes *CSH1* and *CSH2*, and a pseudogene *CSHP1* (1). When correctly spliced, *GH1* produces the 22-kDa isoform that includes all the 5 exons with the complete biological activity of GH (2, 3). Despite the correct processing, even under normal conditions, a small percentage of alternatively spliced isoforms are produced. The presence of an in-frame cryptic splice site within exon 3 gives rise to a transcript lacking the first 45 bp of exon 3 and encodes a shorter active isoform of 20 kDa, representing 5%–10% of GH transcripts (4). A 17.5-kDa isoform (representing 0.1%–5% of GH transcripts) is produced by the complete skipping of exon 3, thus lacking the entire loop connecting helix 1 and helix 2 in the tertiary structure of GH and generating a GH isoform with no biological activity (5). Trace amounts of the severely truncated isoforms of 11.3 and 7.4 kDa, which are biologically inactive, have also been identified being generated by the skipping of exons 3 and 4 or 2 to 4, respectively (6). Multiple mechanisms have evolved to maintain the small amounts of these aberrantly spliced isoforms, especially that encoding the 17.5-kDa protein. Because *GH1* has weak canonical splice sites, multiple *cis*-acting splicing regulatory elements (splicing

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received December 18, 2013. Accepted March 4, 2014.
First Published Online March 17, 2014

Abbreviations: ASF/SF2, alternative splicing factor 1/pre-mRNA splicing factor SF2; ESE, exon splicing enhancer; IGHD, isolated GH deficiency; SR proteins, serine/arginine-rich proteins; SRp40, serine/arginine-rich splicing factor 5; TW2, Tanner-Whitehouse 2nd Edition.

doi: 10.1210/en.2013-2146

enhancers) are essential to maintain the correct exon 3 definition through the activation of the canonical intron 2 and 3 splice sites and silencing of the cryptic sites. Two exon splicing enhancers (ESEs), ESE1, encompassing the first 7 bases of exon 3 (from c.172 to c.178), and ESE2 (from c.190 to c.204), located 12 nt upstream of the cryptic splice site in exon 3 (Figure 1A), and an intron splicing enhancer (ISE) within intron 3, have been well characterized (7–11).

Several mutations leading to aberrant splicing have been reported in isolated GH deficiency (IGHD) patients within these enhancer motifs (7, 8, 10, 11). The increased amount of the 17.5-kDa isoform exhibits a dominant negative effect both in tissue culture and transgenic mice experiments by disrupting the secretory pathway and trafficking of normal GH and other hormones, including ACTH (12, 13).

We here report the identification of variations within *GH1* exon 3 in sporadic IGHD patients and absent in a group of 205 normal stature controls and in the public databases. In silico analysis suggested that 2 of these variations affect exon 3 splicing, because they are located within putative ESEs. In vitro mRNA and Western blot analysis confirmed the deleterious effect of the single variations on splicing, suggesting the presence of further splicing regulatory elements within *GH1* exon 3.

Subjects and Methods

Subjects

A total of 103 sporadic patients with IGHD and 205 normal stature individuals, all belonging to the Italian population, were included in the genetic analysis. The subjects were referred to the clinical centers because they had a height less than or equal to −2 SDS or a height less than or equal to −1.8 SDS in combination with a height velocity over 1 year less than −1.5 SDS using the criteria of Tanner-Whitehouse (14). Patients with a known postnatal cause of acquired hypopituitarism were excluded. Skeletal maturation was estimated as bone age (radius, ulna, and short bone) with the TW2 (Tanner-Whitehouse 2nd Edition) method by a pediatric endocrinologist (15). They were all evaluated for GH serum levels after 2 provocative tests (with arginine or clonidine or insulin or glucagon or with GHRH + arginine (16). Traditionally, a diagnosis of GHD is supported by GH peaks less than 10 ng/mL both after the 2 different stimuli (17), or less than 20 ng/mL after the double provocative test with GHRH + arginine. The GHD patients fulfill these criteria and had a mean (±SD) secretion peak of 4.4 ± 2.5 ng/mL after the classical stimuli (n = 78) or 9.4 ± 5.8 ng/mL after the test with GHRH + arginine (n = 25). None of the GHD patients was deficient for

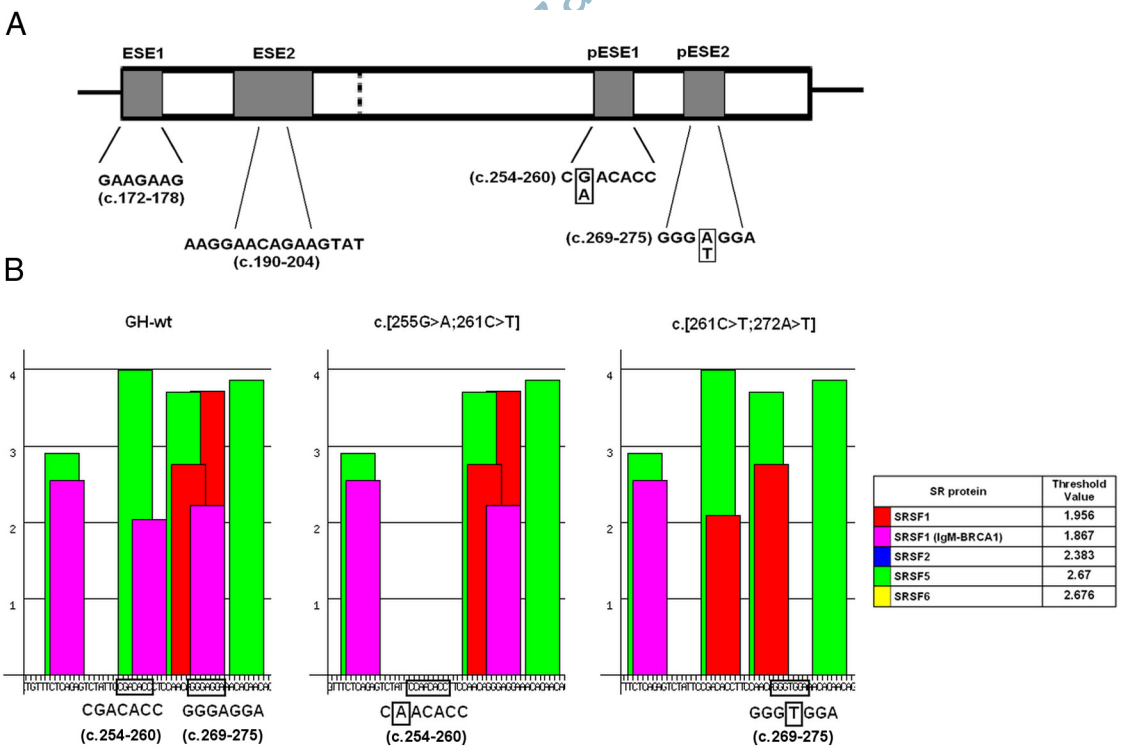

Figure 1. A, ESEs in exon 3. The 2 known ESEs (ESE1 and ESE2) are indicated. The cryptic splice site is at c.216 and is indicated with a dotted line. The newly identified putative ESEs are indicated as pESE1 and pESE2. The mutations identified in patient 1 and patient 2 in the pESEs are boxed. B, ESE finder analysis performed on the wild-type exon 3 and on the different exon 3 alleles identified in patients. The analysis using wild-type sequence revealed all the putative exon splicing enhancer sites within exon 3. ESE motifs with scores above the threshold for each SR protein are indicated in differently colored bars. The bar heights reflect the score of the motifs. Threshold values and color code for each of the different motifs are indicated in Table 1. The introduction of c.255G>A and c.272A>T variations caused the abolition of the 2 putative ESE elements at positions c.254–260 and c.269–275, respectively.

other pituitary hormones, and none had a documented family history of the disease or consanguineous parents. All the patients have been also screened for mutations in *GHRHR* (data not shown). Patients carrying mutations in this gene were not included in the IGHD cohort described here. Normal stature controls included University and Hospital staff, as well as medical students not tested for GH secretion levels. A written informed consent was obtained from the patient's parents, because they were all aged less than 18, and from the normal stature controls.

Detailed description of patients carrying the *GH1* variations

The variants identified in the three patients are reported in Table 1.

Case 1

This patient is a boy born at term with no perinatal complications. He came to our attention when he was 7.3 years old with a height of −2.7 SDS. He has normal stature parents: the father is −1.7 SDS and the mother −0.6 SDS. He was prepubertal. GHD was diagnosed based on low response to insulin tolerance test and clonidine provocative tests (8.9 and 8.6 ng/mL, respectively). The IGF-I level was 130 ng/mL. The bone age was delayed by 3.8 years. GH therapy was initiated with a good clinical response reaching a height of −1.7 SDS after 1.3 after 6 years.

Case 2

This case is a male born from nonconsanguineous parents by vaginal delivery after 41 weeks of gestation. At birth, he was adequate for gestational age (birth weight, 3330 g; length, 52 cm). Both parents presented normal height: father, 174 cm (−0.2 SDS) and mother, 155 cm (−1.3 SDS). He came to our attention at age 13 presenting short stature with a height of −3.4 SDS. His growth rate in the year preceding the diagnosis was 5.0 cm/y (−1.5 SDS). At the diagnosis, the pubertal stage was pubic hair development 2, genital development 2, and bilateral testicular volume 5. The bone age was delayed (11.6, TW2 method). GH secretion peaks after stimulus with arginine and clonidine were 3.9 and 8.3 ng/mL, respectively, with an IGF-I level of 65 ng/mL. He promptly started the recombinant human GH replacement therapy (0.22 mg/kg·wk) and reached the height of −1.8 SDS after 4 years.

Case 3

This boy came for the first time to our attention for growth failure at the age of 16.5 years. At diagnosis, his stature was −2.7 SDS. He was born to nonconsanguineous parents after a normal pregnancy at 40 weeks of gestation, adequate for gestational age (birth weight, 2950 g). His father and mother presented normal (−0.5 and −0.3 SDS, respectively). The pubertal stage was: pubic hair development 3, genital development 3, and bilateral testicular volume 8 mL. The growth velocity was 4.5 cm/y (1.6 SDS, 1.6 SDS, but low when corrected for pubertal stage), and x-ray revealed a bone age (TW2) of 14.2 years. Laboratory analysis revealed a normal IGF-I level (180 ng/mL) and low level of circulating GH after 2 provocative tests (7.7 ng/mL with arginine and 3.7 with clonididne). The recombinant human GH replacement therapy (0.24 mg/kg·wk) was begun with a good response, because he reached the height of −0.7 SDS after 3 years.

Table 1. Exon 3 Variations Detected in the IGHD Patients

Patient	Variation Nucleotide	Amino acid	Within a predicted ESE
1	c.261C>T	(p.Pro87Pro)	No
	c.272A>T	(p.Glu91Val)	Yes
2	c.255G>A	(p.Pro85Pro)	Yes
	c.261C>T	(p.Pro87Pro)	No
3	c.246G>C	(p.Glu82Asp)	No

Predicted ESE by the software ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

Screening of *GH1*

Genomic DNA was amplified by PCR using previously described primers (18) and a proofreading Taq polymerase (Finnzymes). The resulting 2.7-kb product, including the whole *GH1*, was used as template for a series of nested PCRs using internal primers for the proximal promoter, 5 exons, 4 introns, and the untranslated regions of the *GH1*. These primers are designed specifically for the *GH1* and do not amplify other genes in the cluster. PCR conditions and primer sequences are available upon request. The PCR products were visualized on a 2% agarose gel and purified using ExoSAP-IT enzymatic PCR clean up system (Affymetrix). The purified products were then sequenced with the Big Dye Terminator kit (Applied Biosystems) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The PCR products containing the variations in the heterozygous state were then cloned into the plasmid vector pTZ57R/T using the InsTAclone PCR cloning kit (Fermentas), and the 2 alleles were separately sequenced.

Site-directed mutagenesis

The 2.7-kb fragment containing *GH1* was inserted into the pcDNA 3.1(+) expression vector (Invitrogen). The constructs bearing the single variants, namely 246C, 255A, 261T, and 272T, and the variants combined as in the patients, namely 261T/272T (patient 1) and 255A/261T (patient 2), were generated from the GH(wt)-pcDNA 3.1 plasmid by the QuikChange Site-Directed Mutagenesis kit from Stratagene using mismatch complementary primers containing the desired mutations. PCR conditions and primer sequences are available upon request. DH5a competent cells were transformed with the different constructs and grown on Luria Broth/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using Maxiprep kit (QIAGEN). The desired mutation was confirmed by sequencing.

Cell culture, transfection, and isolation of RNA

The GH4C1 rat pituitary cell line was used for the transfection experiments. The stock culture was grown in Ham's F10 medium (Gibco-Life Technologies) supplemented with 15% horse serum, 2.5% fetal bovine serum, 100-U/mL penicillin, and 100-μg/mL streptomycin in a 5% CO_2. A day before transfection, 4×10^5 cells were seeded into each well of a 6-well tissue culture plate in 2.5-mL medium. The wells were previously treated with 1:10 diluted poly-L-lysine solution (Sigma-Aldrich) to allow the cells to completely attach to the plate. At 50%–70% confluency, cells were transfected with 2.5-μg DNA of the

wt-GH or the mutated constructs using the Trans IT-LT1 transfection reagent (Mirus Bio LLC). A green fluorescent protein control was used to test transfection efficiency. Forty-eight hours after transfection, total RNA was isolated and purified from the cells using the QIAGEN RNA mini kit (QIAGEN).

cDNA synthesis

cDNA was synthesized from 1.5 μg of RNA by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. The different transcripts produced by alternative splicing were analyzed using primers specific for *GH1* cDNA (Figure 2A). The RT-PCR was performed with primers GH2 (5′-CGTCTGCACCAGCTGGCCTTT-3′) and GH7 (5′-AAGCCACAGCTGCCCTCCACAGA-3′), which amplify part of exon 2, exon 3, exon 4, and part of exon 5, allowing detection of both exon 3- and exon 4-skipped products.

Western immunoblot analysis

CHO cells were transiently transfected with wt-GH and mutated constructs, as described above. After 48 hours, whole-cell lysates (WCL) were collected using the standard radio-immuno precipitation assay (RIPA) lysis buffer containing 0.1% sodium dodecyl sulfate (SDS). A total of 20 μg of WCL were separated on 15% SDS-PAGE gel and blotted on Immun-Blot PVDF membrane (Bio-Rad). Membranes were probed with a polyclonal rabbit antihuman GH antibody (Abnova) and detected with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Millipore). A polyclonal antiactin antibody (Sigma-Aldrich) was used to normalize the protein loading. Protein bands were visualized using enhanced chemiluminescence reagent (Thermo Scientific) with image capture performed using a charge-coupled device camera linked to ChemiDoc apparatus (Bio-Rad).

CHO cells were used instead of GH4C1 cells for protein analysis, because the GH4C1 showed many unspecific bands after Western blotting, likely due to cross-reactions with endogenous proteins.

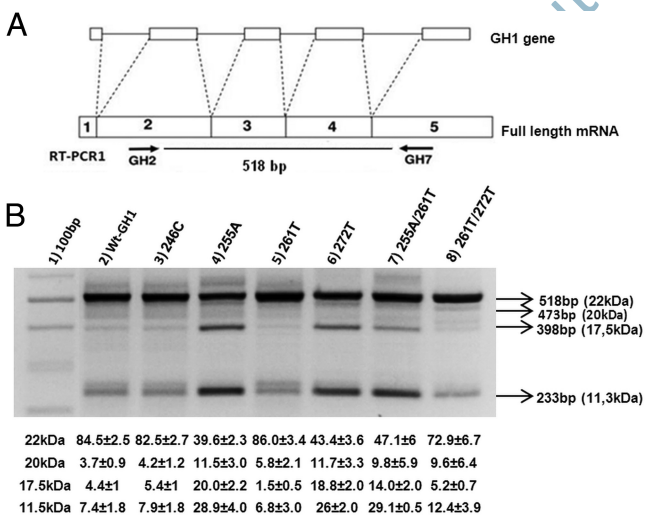

Figure 2. A, Scheme of GH1 pre-mRNA splicing showing the full-length mRNA and the corresponding RT-PCR product. B, RT-PCR performed on mRNA extracted from GH4C1 pituitary cells transfected with the wild-type *GH1* (lane 2) or constructs carrying different variations (lanes 3–8). The size of the bands are indicated on the right with the corresponding protein molecular weight of the different isoforms. Untransfected rat cDNA did not show any bands (data not shown). Each PCR product was eluted from the gel and characterized by sequencing. The relative band intensity (expressed as the percentage on the total yield) calculated as the mean ± SD over 4 different independent transfection experiments is reported for each construct below the corresponding lane.

ESE finder analysis

Analysis of the splicing regulatory motifs within exon 3 was performed using the software ESE finder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi). The default thresholds were considered to identify sites responsible for the 4 serine/arginine-rich (SR) proteins alternative splicing factor 1/pre-mRNA splicing factor SF2 (ASF/SF2), serine/arginine-rich splicing factor 3 (SC35), serine/arginine-rich splicing factor 5 (SRp40), and serine/arginine-rich splicing factor 6 (SRp55).

Gel image analysis

The RT-PCR and Western blotting gel images were analyzed using the freeware ImageJ1.46r (http://rsb.info.nih.gov/ij/; National Institutes of Health), and the bands were quantified by measuring pixel intensity and normalized to the corresponding β-actin band intensity.

Results

Genetic analysis of the IGHD patients

A total of 103 sporadic IGHD patients with height ranging from −1.8 to −4.5 SDS were investigated for the presence of functionally relevant mutations in *GH1*, including coding regions and introns. Two patients harbored a combination of 2 variations within exon 3 (Table 1) that were not previously reported in public databases, including dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), the Exome Variant Server (http://evs.gs.washington.edu/EVS/), and 1000 genomes (http://www.1000genomes.org/). Patient 1 carried c.261C>T (p.Pro87Pro) and c.272A>T (p.Glu91Val), and patient 2 carried c.255G>A (p.Pro85Pro) and c.261C>T (p.Pro87Pro).

A third patient (patient 3) (Table 1) carried the non-synonymous c.246G>C determining the substitution p.Glu82Asp in exon 3. This variation was reported in the dbSNP (rs61762497) only in 1 individual and in the Exome Variant Server in 2 out of 13 000 individuals.

All the above exon 3 variations were absent in a panel of 410 chromosomes sequenced from 205 normal stature Italian control individuals.

The analysis of the parents of patients 2 and 3 demonstrated that their variants were inherited from the unaffected fathers. Unfortunately, the parents of patient 1 did not give their consent to DNA analysis. By subcloning the patient's PCR products in a TA cloning vector system, we confirmed that in patient 1, as well as in patient 2, both

variants were on the same allele. The alignment of *GH1* with the paralogous genes of the GH cluster suggested that all the 4 variants in exon 3 were generated by nonallelic gene conversion from the *GH2* gene, because the other 3 genes (*CS-5*, *CS-2*, and *CS-1*) have the same *GH1* sequence at these sites.

The exon 3 variations fall within predicted ESEs

Because several exon 3 mutations lead to missplicing of mRNA and production of increased amounts of the 17.5-kDa protein, we evaluated the possible involvement in the splicing regulation of the variants identified in our patients within this exon. None of them was included in the previously described ESEs (ESE1 and ESE2). An in silico analysis using ESE finder 3.0 (Figure 1, A and B) revealed the presence of 2 high scoring ESE motifs from c.254 to c.260 (CGACACC) and c.269 to c.275 (GGGAGGA). These 2 sequences include the variations c.255G>A and c.272 A>T, respectively (Figure 1A). The position c.255 is located within an ESE motif recognized by the SR protein SRp40 and c.272 within a sequence recognized by SRp40 and SF2/ASF. Both putative ESE sequences showed an increased score compared with the threshold value (Figure 1B). By substituting the wild-type nucleotides with those found in the patients, namely c.255A and c.272T, the software predicted the complete loss of these 2 putative ESEs (Figure 1B).

Variants c.255A and c.272T affect *GH1* splicing in vitro

To evaluate whether exon 3 variants actually had some effect on mRNA splicing in vitro, we transfected GH4C1 rat pituitary cells with an expression vector containing 1) the wild-type allele (wt-*GH1*), 2) the alleles carrying the single mutations, and 3) the alleles containing the mutations combined as they were in patients 1 and 2. The mRNA from transfected cells was reverse transcribed, and the resulting cDNA was amplified with primers (Figure 2A) specific for the human *GH1* that did not amplify the rat mRNA. The RT-PCR on the wt-*GH1* mRNA (Figure 2B, lane 2) yielded an intense band corresponding to the *GH1* full-length transcript (518 bp) producing the 22-kDa protein and faint bands corresponding to the 20-kDa isoform (473 bp), the 17.5-kDa isoform (398 bp), and the 11.3-kDa isoform (233 bp). The average yield of the mRNA corresponding to the 17.5-kDa isoform over 4 independent experiments was 4.4 ± 1% of the total mRNA yield with a ratio 17.5 kDa/22 kDa of about 1:20. The 246C construct (Figure 2B, lane 3) was also tested, although the c.246C variation was not predicted to influence splicing. This construct generated a band pattern similar to that observed in the wild-type accordingly to the in silico prediction. In contrast, 255A and 272T constructs (Figure 2B, lanes 4 and 6, respectively) produced a higher level of the exon 3-skipped mRNA with a ratio 17.5 kDa/22 kDa of about 1:2 and 1:2.4, respectively. In these 2 constructs, there was also an evident increased production of the exon 3–4-skipped transcript (233-bp band). Interestingly, the exon 3-skipped mRNA was not evident in the transcripts from the 261T construct, suggesting that this variant might strengthen the correct splicing. When the variants were combined on the same construct to reproduce the status of patients 1 and 2, the exon 3-skipped mRNA produced by the construct 255A/261T (patient 2) (Figure 2B, lane 7) was less abundant than that observed for 255A but still evident, with a 17.5 kDa/22 kDa ratio of 1:3.3. Conversely, the 261T/272T construct (patient 1) (Figure 2B, lane 8) showed a splicing pattern very similar to the wild type.

We then investigated the consequences of the variants on protein synthesis (Figure 3). Western blot analysis confirmed that the transfected wt-*GH1*-produced mostly the 22-kDa protein with only traces of the 20 and 17.5 kDa. A higher level of the 17.5-kDa product was produced by 255A, 272T, and by the 255A/261T constructs. Although the 261T/272T plasmid carrying the 2 variations detected in patient 1 showed a mRNA pattern similar to the wild type (Figure 2B, lane 8), it exhibited a band in correspondence of the 17.5-kDa protein more intense than the wild type (about 10% of the total GH proteins) (Figure 3, lane 7). This band in the 261T construct was weak (as in the wt-*GH1*), but the 20-kDa isoform was more intense than in all the other constructs. The corresponding 11.3-kDa protein isoform was not detectable by the antibody used for Western blot analysis.

Discussion

In the present study, we sequenced *GH1* in 103 IGHD patients with clinically variable phenotypes and no family

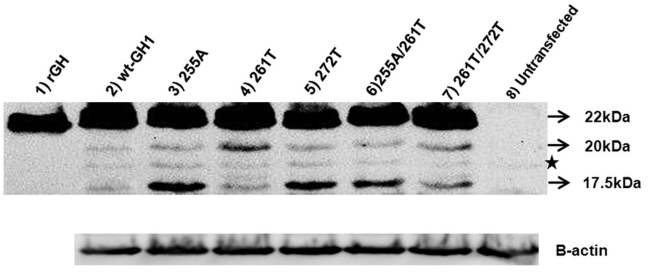

Figure 3. Western immunoblot analysis of the different GH isoforms encoded by the different splicing products. The CHO cells were transiently transfected with either wt-*GH1* (lane 2) or constructs carrying different variations (lanes 3–7). Untransfected CHO cells were used as a negative control (lane 8); *, nonspecific bands also present in the untransfected CHO cell lysate.

history to search for mutations that might be 1) inherited from healthy parents (incomplete penetrance), 2) arisen de novo, or 3) biallelic (recessive inheritance). We identified 3 patients that carried variations in exon 3, and 2 of them carried 2 variants on the same allele. All the variants were likely generated by gene conversion, an event that is frequently associated to *GH1* sequence variability (19–21). An in silico analysis was performed using ESE finder 3.0, which has been designed to identify SR protein binding sites by generating a motif score that reflects the binding site strength. The sequences from c.254 to c.260 and from c.269 to c.275, including the positions c.255 and c.272 mutated in patient 2 and in patient 1, respectively, were predicted to represent ESE motifs recognized by the SRp40 and SF2/ASF proteins with a score above the threshold (Figure 1B). The same factors were predicted to recognize ESE2 with the same strength (9). It has been demonstrated that SF2/ASF activates exon 3 inclusion and that disruption of this motif causes increased exon 3 skipping (22). The substitution of the wild-type nucleotides c.255G and c.272A with the mutants c.255A and c.272T, respectively, was predicted to abolish the binding of these proteins in both putative ESEs (Figure 1). The RT-PCR analysis (Figure 2) confirmed the influence of c.255A and c.272T on splicing by increasing the exon 3-skipped isoform to 18%–20% of the total GH mRNA (Figure 2B, lanes 4 and 6, respectively).

Mutations within ESE1 in *GH1* have been previously reported to cause either complete or partial exon 3 skipping and generation of increased amounts of the 20- and the 17.5-kDa isoforms at various concentrations (20%–37% and 35%–68%, respectively) (8, 9). The clinical variability observed in patients carrying these mutations has been explained by variable amounts of the 17.5-kDa isoform consequent to a weakened exon 3 recognition. Hamid et al (23) reported a large pedigree with the c.172G>A splicing mutation, and they suggested that the ratio of 17.5 kDa/22 kDa transcripts in the lymphocytes correlated with the height SDS before GH replacement therapy. In this family, there were individuals with a height SDS more than −2 that inherited the mutation (incomplete penetrance). Another heterozygous missense mutation, c.200A>G, within ESE2 induces exon skipping in about 20% of the transcripts, giving rise to different phenotypes ranging from short stature to normal stature in the same large pedigrees (19).

It is thus conceivable that mutations associated with variable expressivity and incomplete penetrance might be responsible for at least some milder forms of IGHD.

The effect determined by the here detected variants, c.255G>A and c.272 A>T, is comparable with the effect caused by most of the previously described mutations falling within ESE1 and ESE2 (8, 19, 23). However, when these 2 variants were combined with c.261T, as in patients 1 and 2, the effect on splicing was maintained, although weaker, only for the 255A/261T construct (patient 2) (Figure 2B, lane 7). In contrast, the 261T/272T construct (patient 1) (Figure 2B, lane 8) was very similar to the wild-type, although the Western blotting for the same construct showed a slightly increased amount of the 17.5-kDa band (Figure 3, lane 7). This discrepancy might be attributable to the different sensitivity of the two methods.

Thus, the c.261 variant, which is not included in the putative ESE sites, partially hides the negative effect of the c.272. It can be speculated that c.261 influences splicing by strengthening the affinity for other proteins involved in the correct splicing regulation.

It is worth considering that the 233-bp band corresponding to the bioinactive 11.3 kDa (exon 3–4-skipped isoform) is strongly increased in all mutant constructs, but 261T and 261T/272T (Figure 2B). The relative total amount of the 2 alternatively spliced mRNA (exon 3 and exon 3–4 skipped) in the 255A, 272T, and 255A/261T constructs can be roughly estimated to represent nearly 50% of the total *GH1* mRNA in contrast to the wild-type, where these transcripts represent about 12% of the *GH1* transcripts.

The low serum GH level detected in vivo in the patients might be in part determined by the effect on splicing of these variants and in part by other yet unidentified genetic factors. Notably, patient 1 carried on the other allele the *GH1* promoter haplotype 1 (data not shown) that has been associated to IGHD and to a reduced luciferase activity in vitro (24).

The functional significance of the Glu82Asp variant carried by patient 3 is uncertain. From our experiments, it does not seem to influence splicing (Figure 2B, lane 3), and it is not predicted to have an effect on SR protein binding (data not shown). This variant is very rare, because it was reported in the dbSNP database (rs61762497) only in 1 individual of African ancestry, it is present in 2 individuals out of 13 000 of the Exome Variant Server, and it was absent in our panel of 205 normal stature individuals. However, it is not predicted to exert a damaging effect on the protein function by the software Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). It might, thus, represent either a rare benign polymorphism, or alternatively it might contribute to a multigenic form of IGHD in this patient.

In conclusion, the analysis of *GH1* in individuals with sporadic IGHD led to the identification of 2 novel *GH1* exon 3 variations, c.255 G>A and c.272 A>T, included within 2 novel putative splicing regulatory elements that increase the aberrant splicing in vitro. When combined in

cis, with c.261 C>T, as in the patients, their effect was reduced but still evident on the protein synthesis. It can be hypothesized that also a minimally increased amount of the 17.5-kDa protein might exert a dominant negative effect on the GH synthesis in vivo. The phenotype of patients 1 and 2 might be associated to these *GH1* splicing variations that by themselves only partially influence the amount of GH secretion but that might act in concert with other genetic variants.

Acknowledgments

We thank the patients and parents that participated to this study.

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This work was supported by the Cariplo Foundation Grant Project 2009–2609.

Disclosure Summary: The authors have nothing to disclose.

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PLOS ONE

Effects of Growth Hormone (GH) Therapy Withdrawal on Glucose Metabolism in Not Confirmed GH Deficient Adolescents at Final Height

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Abstract

Context, objective: Growth hormone deficiency (GHD) is associated with insulin resistance and diabetes, in particular after treatment in children and adults with pre-existing metabolic risk factors. Our aims were. i) to evaluate the effect on glucose metabolism of rhGH treatment and withdrawal in not confirmed GHD adolescents at the achievement of adult height; ii) to investigate the impact of GH receptor gene genomic deletion of exon 3 (d3GHR).

Design, setting: We performed a longitudinal study (1 year) in a tertiary care center.

Methods: 23 GHD adolescent were followed in the last year of rhGH treatment (T0), 6 (T6) and 12 (T12) months after rhGH withdrawal with fasting and post-OGTT evaluations. 40 healthy adolescents were used as controls. HOMA-IR, HOMA%β, insulinogenic (INS) and disposition (DI) indexes were calculated. GHR genotypes were determined by multiplex PCR.

Results: In the group as a whole, fasting insulin ($p<0.05$), HOMA-IR ($p<0.05$), insulin and glucose levels during OGTT ($p<0.01$) progressively decreased from T0 to T12 becoming similar to controls. During rhGH, a compensatory insulin secretion with a stable DI was recorded, and, then, HOMAβ and INS decreased at T6 and T12 ($p<0.05$). By evaluating the GHR genotype, nDel GHD showed a decrease from T0 to T12 in HOMA-IR, HOMAβ, INS ($p<0.05$) and DI. Del GHD showed a gradual increase in DI ($p<0.05$) and INS with a stable HOMA-IR and higher HDL-cholesterol ($p<0.01$).

Conclusions: In not confirmed GHD adolescents the fasting deterioration in glucose homeostasis during rhGH is efficaciously coupled with a compensatory insulin secretion and activity at OGTT. The presence of at least one d3GHR allele is associated with lower glucose levels and higher HOMA-β and DI after rhGH withdrawal. Screening for the d3GHR in the pediatric age may help physicians to follow and phenotype GHD patients also by a metabolic point of view.

Citation: Prodam F, Savastio S, Genoni G, Babu D, Giordano M, et al. (2014) Effects of Growth Hormone (GH) Therapy Withdrawal on Glucose Metabolism in Not Confirmed GH Deficient Adolescents at Final Height. PLoS ONE 9(1): e87157. doi:10.1371/journal.pone.0087157

Editor: Marta Letizia Hribal, University of Catanzaro Magna Graecia, Italy

Received September 5, 2013; **Accepted** December 18, 2013; **Published** January 30, 2014

Funding: This study was supported by Regione Piemonte (grant n° 2827, 2008), Università del Piemonte Orientale "A. Avogadro", Ministero dell'Università e della Ricerca Scientifica (MIUR, grant n° 20082P8CCE, 2008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Growth hormone (GH) has pleiotropic functions in humans. GH/insulin-like growth factor-1 (IGF-I) axis is the main regulator of post-natal growth, but it has other main metabolic actions such as the regulation of body composition, muscle and bone metabolism. Furthermore, in the post-absorptive state, GH mainly acts on stimulating lipolysis and lipid oxidation in order to switch metabolism from glucose and protein to lipid utilization. At present, it is reported that GH administration is followed by lipolysis but also by insulin resistance and relatively sustained hyperglycemia [1–3]. GH-induced lipolysis appears as the most important determinant of GH anti-insulin actions, by inhibiting insulin-stimulated glucose uptake especially in muscles [4,5]. Whether the impairment in peripheral insulin sensitivity is mainly located in muscle and mostly due to higher disposable free fatty acids, GH is also able to reduce hepatic insulin sensitivity in healthy humans and to counterbalance the anti-lipolytic actions of hyperinsulinemia [1,6]. Some of these effects are direct actions, whereas others are IGF-I mediated [1].

Other mechanisms may be implicated on the metabolic effects of GH, as the interaction of GH with the insulin receptor [7,8] and the presence of several polymorphisms including the GH receptor (GHR) exon 3 deletion (d3GHR) which seems to play a role in glucose homeostasis in GHD subjects [9] and in general population [10,11].

Several studies in adults have determined the effects of GH replacement therapy on insulin sensitivity. Short-term rhGH

therapy deteriorates insulin sensitivity with an improvement on long-term in the majority but not in all studies. These conflicting data are probably due to differences in sample size, methods of evaluation of insulin sensitivity and doses of rhGH [1,12–15]. There are suggestions that rhGH therapy increases the risk of type 2 and/or secondary diabetes in GHD adults with pre-existing metabolic risk factors [16]. Similarly, in children data from registries show a slower increase in the incidence of diabetes due to GH treatment in those patients with pre-existing risk factors [17,18]. Despite this, a few data describe insulin secretion at final height in the "so called" transition phase. Evidence to date suggests an increase in insulin sensitivity after cessation of GH treatment [19–21], but more studies are needed to clarify. In particular, no attention has been paid on those subjects which were not reconfirmed as GHD after stopping therapy.

Euglycemic hyper-insulinemic clamp is considered as the "gold standard" for quantifying insulin sensitivity in vivo, but this method is not applicable on a great population in daily clinical practice. Insulinogenic index (INS) and disposition index (DI) are new indirect methods for measure beta-cell function, using oral glucose tolerance test (OGTT) -derived measures, and are early markers of inadequate beta-cell or peripheral compensation. They allow the investigation of a larger number of patients [22]. Recent data indicate INS and DI as the best predictors of future type 2 diabetes in adults [23–25].

In order to understand the metabolic effects of rhGH therapy in adolescents with unconfirmed GHD at the achievement of adult height, we evaluated glucose metabolism in the last year of rhGH replacement and in first year after rhGH discontinuation compared with a healthy counterpart. Because some polymorphisms of the GHR could have a role in glucose metabolism and insulin resistance, we also evaluated whether the d3GHR deletion has a role on glucose homoeostasis during and after rhGH withdrawal.

Methods

This was a single-center longitudinal study conducted at Division of Pediatrics, University of Piemonte Orientale (Novara, Italy). We consecutively recruited 35 GH-deficient (GHD) adolescents at the end of puberty and in the last year of rhGH therapy and 45 healthy age and sex matched adolescents. The recruitment was opened from September 2006 to December 2011 The study was approved by the Ethics Committee of Maggiore della Carità Hospital (Novara) and informed written consent was obtained from all subjects and their parents before study. Patients were eligible if they had an idiopathic isolated GHD at diagnosis, had completed puberty (Tanner stage 4 and 5), the adult height was almost achieved with a bone age similar to the chronologic age and growth velocity near to 2 cm/year, and GHD was not confirmed at retesting (GD, group 1). Patients with coexistent other chronic or endocrine diseases, syndromes, diabetes, tumors or drugs interfering with glucose metabolism were excluded. Healthy control adolescents (CS, group 2) were eligible if they were at the end of puberty, normal-weight, with no history of organic or psychiatric diseases in particular no neurological, endocrine, liver, and kidney abnormalities.

Study Design and Assays

GHD adolescents were evaluated at fasting for IGF-1, total- (T-c), HDL- (HDL-c) cholesterol, triglycerides (TG), C-peptide and with an OGTT (1.75 g of glucose solution per kg, maximum 75 gr) in the last year of rhGH therapy (T0), and at 6 (T6) and 12 months (T12) after therapy withdrawal. In the year after GH discontinuation the retesting was performed with the GHRH plus arginine test with the adoption of the transition cut-offs [26]. In healthy adolescents the OGTT was performed at baseline.

Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) were defined according to American Diabetes Association classifications as fasting plasma glucose of ≥100 to 125 mg/dl nmol/l, and as 2-h post-OGTT glucose of ≥140 to 199 mg/dl, respectively. Also the definition of diabetes was performed according to the criteria of the American Diabetes Association [27].

Blood samples during OGTT were drawn for the determination of glucose and insulin every 30 min from 0′ to 120′ min. The area under curve (AUC) for plasma glucose and insulin were calculated by the trapezoidal rule.

Insulin resistance was estimated, in the basal state, by use of the homeostasis model assessment (HOMA-IR) = fasting glucose × fasting insulin/22.5; beta-cell function at fasting was calculated using the formula of HOMA-β = (20 × fasting insulin)/(fasting glucose −3.5). Insulin sensitivity was calculated from the Matsuda [10,000/$\sqrt{}$(fasting glucose × fasting insulin) × (Gm×Im)] and QUICKI ($1/\log_{10}$ fasting insulin+$\log_{10}$ fasting glucose) indexes [28].

The area under the curve (AUC) for parameters after OGTT was calculated according to the trapezoidal rule. Delta glucose (ΔG_{30-0}) and insulin (ΔI_{30-0}) were evaluated as the change in glucose and insulin concentrations from 0 to 30 min. The stimulus for insulin secretion in the increment in plasma glucose as insulinogenic index was calculated as the ratio of the changes in insulin and glucose concentration from 0 to 30 min (INS). *B*eta-cell compensatory capacity was evaluated by the disposition index defined as the product of the Matsuda Index and INS (DI) [29]. In addition, each subgroup and all subjects together were divided into four groups according to the 2-h glucose levels,: 1) less than 100 mg/dL, 2) 100–119 mg/dL, 3) 120–139 mg/dL 4) above 140 mg/dL according to the risk to have a lower DI [30].

Plasma glucose levels (mg/dl; 1 mg/dl:0,05551 mMol/liter) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy). Insulin (μUI/ml; 1 μUI/ml = 7.175 pmol/l) was measured by chemiluminescent enzyme-labelled immunometric assay (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity: 2 μUI/ml. Intra- and inter-assay CV ranges: 2.5–8.3 and 4.4–8.6%.

HbA1c levels were measured by the high-performance liquid chromatography (HPLC), using a Variant machine (Biorad, Hercules, CA); intra- and inter-assay coefficients of variation are respectively lower than 0.6 and 1.6%. Linearity is excellent from 3.2% (11 mmol/mol) to 18.3% (177 mmol/mol).

T-c (mg/dl; 1 mg/dl: 0.0259 mMol/l), HDL-c (mg/dl; 1 mg/dl: 0.0259 mMol/l), TG (mg/dl; 1 mg/dl: 0.0113 mMol/l), and C-peptide (ng/ml) were evaluated using standardized methods in the hospital's chemistry laboratory. Plasma T-c concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer Diagnostics, Newbury, UK); coefficient of variation (CV) 1.9%. Plasma TG and HDL-c concentrations were measured by enzymatic determination (Advia 1650, Bayer Diagnostics, Newbury, UK); CV 1.7%. LDL-c was calculated by Friedwald mathematical for individuals with TG<150 mg/dl.

Serum IGF-I was measured by Liason automated chemiluminescence analyzer supplied by DiaSorin with a measurement range of 3–1500 ng/ml. Age and gender-reference ranges were used to calculate an IGF1 SDS for each patient.

Anthropometric Measurements

Height was measured by the Harpenden stadiometer and weight by using electronic scale. Body mass index (BMI) was calculated as body weight divided by squared height (kg/m^2). Height, weight and BMI were stratified according to Italian growth charts [31]. Height velocity (HV) was calculated from the difference of mean heights obtained from 2 consecutive visits, divided by time between visits, and adjusted to a 12-month interval. Waist circumference was measured with a soft tape, midway between the lowest rib margin and the iliac crest, in the standing position. Hip circumference was measured over the widest part of the gluteal region, and the waist-to-hip ratio was calculated. Systolic (SBP) and diastolic (DBP) blood pressure were measured three times at the left arms by using a standard mercury sphygmomanometer and the mean value was recorded and stratified according to paediatric percentiles of National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents [32].

DNA Extraction and Genetic Analysis

At the end of the clinical protocol, after T12, genomic DNA was isolated from peripheral blood leukocytes by standard methods. The d3GHR polymorphism was detected as described previously [33] based on a multiplex PCR assay with a combination of 3 primers (G1, G2 and G3) that specifically amplify the wild type (935 base pairs bp) and the deleted (532 bp) alleles. Amplification products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (Figure S1).

Subjects with at least one copy of the exon-3 deleted GHR (d3/d3 and fl/d3; Del) were grouped together for comparison with full-length homozygote subjects (fl/fl; nDel).

Statistical Analysis

Data are expressed as mean±SEM. For continuous variables, the variation between groups was compared by means on nonparametric Wilcoxon, Mann-Whitney U, or chi-square tests, where appropriate. Trends were assessed by nonparametric Friedman test. A correlation analysis was performed using the Pearson's correlation test with a logarithmic transformation when necessary.

Statistical significance was assumed for $p<0.05$. All statistical analyses were performed with SPSS for Windows version 17.0 (SPSS INC; Chicago, IL, USA).

Results

GHD before and after GH Discontinuation

Of the 35 GHD enrolled subjects, 23 performed two OGTTs and completed the study. Of the 45 CS, 5 subjects had discomfort during OGTT and were excluded.

Clinical characteristics of CS and GHD at T0 and T12 are reported in Table 1 and 2. GHD subjects had received GH replacement therapy for 7.3±2.0 years. The last dose of GH was 12.0±0.5 mg/week. The GH peak at the Arginine+GHRH test was of 72.0±5.6 ng/ml.

One GHD subject showed IFG at T0 and T6 and two subjects IGT at T0. No glucose alterations were found at T12. The distribution of post-OGTT 2hrs glucose levels at each time point was reported in Table 2. HbA1c levels were 5.3±0.1% (34.0±1.0 mmol/mol) in the last year of therapy. No differences in waist, waist/hip ratio, BMI, SBP, DBP, LDL-c and TG were shown in GHD among visits. T-c and HDL-c levels were lower at T6 than T0, and HDL-c levels also at T12 than T0 in GHD although the trend among visits was not significant (Table 2).

Table 1. Clinical parameters of growth hormone deficient (GHD, group 1) children at the end (T0) of rhGH therapy (T0), after 12 months rhGH withdrawal (T12) and of control subjects (CS, group 2).

	GHD (group 1) T0	GHD (group 1) T12	CS (group 2)
N°	23	23	40
M/F	12/11	12/11	22/18
IFG/IGT	1/2	0/0	0/0
Age (yr)	15.9±0.2	17.9±0.2	15.4±0.2
Weight (kg)	55.4±1.8	58.6±2.1	61.3±1.4
Centile weight	23±2.1	25±2.1	27±1.5
Height (cm)	163.5±1.7[a]	166.1±1.2[c]	173.7±1.2[a,c]
SDS height	−0.9±0.1[a]	−1±0.2[c]	1.1±0,1[a,c]
BMI (kg/m^2)	20.9±0.5	21.2±0.5	20.4±0.2
HV (cm/yr)	1.5±0.4	1.0±0.1	–
Waist (cm)	72.8±1.2[a]	77.4±1.4	79±0.8[a]
SBP (mmHg)	119.7±2.1[b]	119.4±3	124.4±1.3[b]
DBP (mmHg)	77.2±1.4	78.0±2.6	79.8±1.2

Abbreviations. BMI, body mass index; HV, height velocity; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; SBP: systolic blood pressure; DBP: diastolic blood pressure, SDS: standard deviation score.
Data are expressed as mean±SEM. a: $p<0.0001$ GHD T0 vs CS; b: $p<0.05$ GHD T0 vs CS; c: $p<0.0001$ GHD T12 vs CS.
doi:10.1371/journal.pone.0087157.t001

Basal glucose levels were similar among the 3 times. Fasting insulin ($p<0.05$), HOMA-IR ($p<0.05$) insulin and glucose levels at each time point after OGTT ($p<0.01$), ΔG_{0-30} ($p<0.05$) and ΔI_{0-30}, insulin mean and insulin AUC ($p<0.01$) progressively decreased from T0 to T12. Conversely, C-peptide ($p<0.0001$), Matsuda and QUICKI indexes increased from T0 to T12 ($p<0.05$). Despite higher insulin resistance during the last year of GH treatment, a compensatory insulin secretion was recorded: HOMA-β and INS were higher at T0, and progressively decreased at T6 and T12 ($p<0.05$), with a stable DI. Also IGF1 levels and IGF-I SDS decreased from T0 to T12 ($p<0.0001$) (Table 2).

A trend to decrease in DI across 2 h glucose groups (<100; 100–119; 120–139; >140 mg/dl) was observed and maintained at each time point in GHD subjects; T0 (8.7±1.4; 7.2±1.1; 5.2±1.4; 3.2±0.8, respectively; $p<0.01$; χ^2 12.156), T6 (7.5±1; 7.0±0.9; 5.6±0.1, respectively; $p<0.05$; χ^2 6.695) and T12 (8.7±1.4; 7.6±1.7; 3.6±1.3, respectively; $p<0.05$; χ^2 6.593).

HOMA-IR ($p<0.03$), HOMA-β ($p<0.002$), QUICKI ($p<0.03$), Matsuda indexes ($p<0.002$), fasting ($p<0.05$) and at 120 min glucose ($p<0.01$) were worse in GHD subjects at T0 than in CS. No differences in metabolic parameters were shown between CS and GHD at T12 (Table 1).

GHD before and after GH Discontinuation According to GHR Genotype

By analysing the GHD group as a whole, we observed a huge dispersion in INS and DI as mean±SD. Indeed, we addressed to understand whether d3GHR has a role, according to some literature suggestions (9,10). All the GHD subjects were analysed for the GHR genotype, meanwhile 5 out of 40 CS withdrawn the consent to perform this analysis.

Table 2. Glucometabolic parameters of growth hormone deficient (GHD, group 1) children at the end of rhGH therapy (T0) and 6 (T6) and 12 (T12) months after rhGH withdrawal.

	T0	T6	T12	$p^{for\ trend}$
Fasting glucose (mg/dl)	83.6±1.7	84.5±1.8	84.1±1.8	NS
30-min plasma glucose (mg/dl)	145.5±5.7	138.3±5.4	127.2±5.9	<0.05
2-h plasma glucose (mg/dl)	110.1±4.2	98.6±3	92.5±5	<0.01
AUC Glucose 0-120 min	11592.4±521	11273.4±381	10691.8±613	NS
Mean Glucose (mg/dl)	114.7±4.2	111.1±3	105.4±4.9	NS
ΔG_{30-0}	59.9±4.8	53.2±4.9	42.9±5.5	<0.05
Fasting insulin (μUI/ml)	10.5±1.1	8±0.7	7.7±0.6	<0.05
30-min plasma insulin (μUI/ml)	99.4±12	80.6±11.6	59.8±9	<0.01
2-h plasma insulin (μUI/ml)	59.6±6.8	40.9±5.4	34.2±4.8	<0.01
AUC Insulin 0–120 min	7875.6±915	6554.4±864	4882.1±591	<0.01
Mean Insulin (μUI/ml)	66±7.2	53.3±6.4	40.6±4.3	<0.01
ΔI_{30-0}	88.8±11.2	72.5±11	52.2±8.6	<0.01
HOMA-IR	2.2±0.2	1.7±0.1	1.6±0.1	<0.05
HOMA%β	205.2±23.8	148.5±15.9	148.3±16.2	<0.05
INS	1.6±0.2	1.3±0.1	1.2±0.2	<0.05
DI	6.8±0.7	7.2±0.6	7.9±1.0	NS
C-peptide (ng/ml)	0.21±0.05	0.19±0.04	0.36±0.05	P<0.01
T-c (mg/dl)	141.6±4.6	136.2±4.5	136.8±6.9	NS
HDL-c (mg/dl)	52.2±2.0	46.2±1.7	48.2±2.6	NS
LDL-c (mg/dl)	76.1±3.6	77.5±3.2	76.6±5.7	NS
TG (mg/dl)	60.7±4.1	62.7±6.2	59.2±5.3	NS
IGF-1 (ng/ml)	629.6±38	356.8±17	332.6±25	<0.0001
IGF-1 SDS	1.21±0.19	-0.01±0.06	-0.15±0.17	<0.0001

Abbreviations. DI, disposition index; INS, insulinogenic index; AUC, area under the curve; ΔG0-30: delta glucose;; ΔI0-30: delta insulin; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; T-c, total-cholesterol; TG, triglycerides. Data are expressed as mean±SEM. The significance among the three measures (T0, T6 and T12) was calculated by Friedman test.

doi:10.1371/journal.pone.0087157.t002

The frequencies of the tree genotypes, d3/d3, fl/d3 and fl/fl were respectively 9% [2], 35% [8], 56% [13] in GHD. Allele frequencies were not different from those observed in the 35 CS (Figure S1). Because frequency, the participants were therefore divided into two groups, those homozygote for the fl/fl alleles (Del) and those hetero- and homozygote for the deleted isoform combined (nDel).

Del and nDel GHD had similar anthropometric and metabolic parameters at T0 with exception of HDL-c which was higher in Del GHD (56.5±1.9 vs 49.1±3.0 mg/dl, p<0.01).

At T6, Del GHD showed lower glucose levels at baseline and after OGTT than nDel GHD (fasting glucose: 78.7±1.8 vs 87.5±2.9 mg/dl, p<0.01; T120 glucose: 90.7±4.6 vs 101.0±3.5 mg/dl, p<0.01; AUC 9703.0±476.6 vs 12316.7±447.7 mg/dl*h, p<0.01 and mean glucose 97.1±3.6 vs 118.5±2.8 mg/dl, p<0.0001). DI was higher in Del than nDel GHD (10.4±1.0 vs 5.4±0.4; p<0.01) without differences in INS, HOMA-IR and HOMA-β.

At T12, Del GHD showed lower glucose AUC (9449.0±552.4 vs 11771.0±1068.6 mg/dl*h; p<0.05) and ΔG_{30-0} (29.5±4.5 vs 53.0±9.0 mg/dl; p<0.05), and higher fasting insulin (9.0±0.8 vs 6.4±0.8 μUI/ml; p<0.05),HOMA-IR (1.8±0.1 vs 1.3±0.2; p<0.05) and C-peptide (0.39±0.09 vs 0.24±0.03 ng/ml; p<0.04) than nDel GHD. Moreover, Del GHD had INS (1.8±0.5 vs 0.7±0.1; p<0.01) and DI (10.7±1.8 vs 5.0±1.0; p<0.01) higher than nDel GHD. nDel GHD showed a progressively decrease from T0 to T12 in HOMA-IR, HOMA-β, INS (p<0.05) and DI (not significant) with stable C-peptide levels. Conversely, Del GHD showed a gradual increase in DI (p<0.05), INS (not significant), and C-peptide (0.20±0.08 vs 0.39±0.09; p<0.01) with a HOMA-IR decreasing from T0 to T6 with a slight increase to T12 (p<0.01 for trend) (Figure 1).

Del GHD presented stable higher HDL-c levels than nDel GHD at any time point, whereas T-c, LDL-c and TG remained similar. T-c levels decreased from T0 to T12 in nDel GHD (141.0±7.4 at T0; 137.2±7.5 mg/dl at T6, 133.5±8.4 mg/dl at T12; p<0.0001).

At T12, Del GHD presented lower fasting glucose (83.5±2.2 vs 88.0±2.2 mg/dl p<0.03), and higher fasting insulin (9.0±0.8 vs 6.8±0.6 μUI/ml; p<0.03), HOMA-β (167.7±20.1 vs 96.9±10.1; p<0.003), and DI (10.7±1.8 vs 9.1±1.0 p<0.05) than Del CS. Conversely, at T12 nDel GHD presented lower HOMA-IR (1.3±0.2 vs 1.8±0.1 p<0.03), INS (0.7±0.1 vs 1.2±0.1 p<0.04), and DI (5.0±1.0 vs 8.1±1.2 p<0.04) than nDel CS. Del and nDel CS had higher C-peptide levels with respect to their GHD counterpart (p<0.001).

Correlations

In GHD subjects during GH therapy, both INS and DI were correlated with GH weekly dose (r: −0.399; p<0.05 and r: −0.529; p<0.01, respectively). Correlation was maintained when

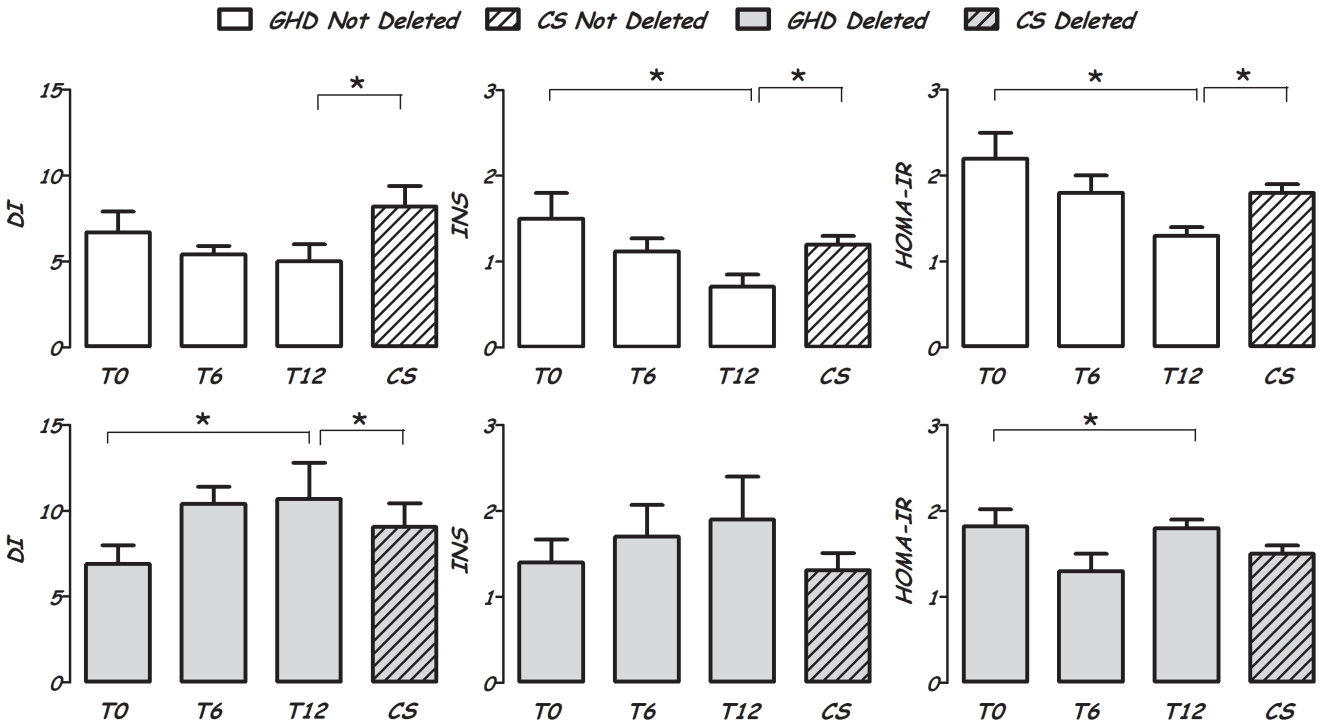

Figure 1. HOMA-IR, Insulinogenic (INS) and disposition (DI) index in GH deficient (GHD, group 1) and healthy (CS, group 2) adolescents with (Del, 27 subjects) and without (nDel, 31 subjects) the GH receptor (GHR) exon 3 deletion (d3GHR). GHD adolescents are evaluated in the last year of therapy (T0) and after six (T6) and twelve (T12) rhGH withdrawal. Data are expressed as mean±SEM. The significance among the three measures (T0, T6 and T12) was calculated by Friedman test. The significance between GHD and CS was calculated by Mann-Whitney U test. *$p<0.05$.
doi:10.1371/journal.pone.0087157.g001

correcting for IGF-1 (INS r: −0.392, $p<0.05$; DI r: −0.526, $p<0.01p<0.01$) or IGF-SDS (INS r: −0.398, $p<0.05$; DI r: −0.531, $p<0.01$) which also weights for age and gender. No correlations were recorded after GH discontinuation.

IGF-1 levels and IGF-SDS were correlated with HOMA-IR at T6 (r: 0.518 and r: 0.514, $p<0.01$) and T12 (r: 0.518 and r: 0.518, $p<0.01$), and also with QUICKY and Matsuda indexes at T6 (QUICKI, r: −0.495 and r: −0.476, $p<0.03$; Matsuda index, −0.443 and r: 0.302; $p<0.05$) and T12 (QUICKI, r: −0.611 and r: 0.–609, $p<0.02$; Matsuda index, −0.543 and −0.359; $p<0.05$).

Discussion

A large number of studies have addressed the metabolic consequences of adult GHD, in contrast studies investigating glucose and insulin metabolism at the end of GH therapy in not confirmed GHD adolescents during the transition phase are lacking. The lack of published data reflects the clinical practice of stopping the follow up in adolescents at the achievement of adult height and with not confirmed GHD at reevaluation. This practice makes a gap in the understanding of GH biology and of long-term safety of rhGH treatment. In the present study, we observed that after 1 year of rhGH withdrawal metabolic parameters are similar between not confirmed GHD and matched control subjects. Moreover, during GH treatment, higher insulin-resistance at fasting and lower insulin-sensitivity during OGTT are associated with higher HOMA-β and insulinogenic index and a stable DI in the group as a whole. The d3GHR deletion may have a role on the metabolic risk with a more pronounced reduction of HOMA-IR and compensatory insulin secretion in nDel GHD, and with lower glucose levels and a higher increase in DI and fasting C-peptide in Del GHD at the end of the treatment.

First of all, we showed that whether glucose impairment and/or insulin resistance occurred during the treatment with rhGH, these are transient and quickly restored also in not confirmed GHD adolescents, as already demonstrated in literature in adolescents with persistent GHD [19,34]. Although puberty is ongoing, insulin resistance at fasting and insulin sensitivity during OGTT progressively return to similar levels to age, puberty, and weight matched healthy adolescents. On the other hand, it is interesting that GHD treated adolescents have the well-known higher insulin resistance at fasting [35,36] but it is coupled with a compensatory insulin secretion both at fasting, measured as HOMA-β, and during OGTT, measured as INS with also a stable DI. Indexes of insulin sensitivity (Matsuda index) and early insulin responses to oral glucose (INS) that were derived from baseline and follow-up OGTTs did not appear to be significant predictors for the development of type 2 diabetes. In contrast, the baseline DI, which significantly assesses beta-cell function in the context of insulin sensitivity, predicts the risk of deteriorating glucose tolerance in youths [30]. Thus, insulin secretion can be truly evaluated only in relation to the degree of insulin sensitivity. Indeed, our data on DI suggest that whether the treatment with GH has a detrimental role on insulin resistance because it stimulates lipolysis, in youths pancreas maintains a beta-cell compensatory capacity and is able to answer to a relative hyperglycemia with a higher insulin secretion which counterbalances the GH inhibited insulin-stimulated glucose uptake in the muscles without impact on future type 2 diabetes risk in those healthy [4,5]. Similarly, although M values at euglycemic hyperinsulinemic clamp were higher after a short rhGH withdrawal, in GHD treated young adults M values

were similar than matched controls [36]. Also in small for gestational age GH treated adolescents, insulin sensitivity decreased but DI remained stable at puberty [37]. We could hypothesize that the preserved beta-cell function at early age protects from type 2 diabetes and that when DI progressively decreases with age and obesity a sustained glucose impairment arises more likely as in adults. A direct role of GH on insulin antagonism is supported by the negative correlation between GH weekly dose and INS and DI, suggesting that the pancreas compensation we observed is secondary to the peripheral detrimental action of GH.

Studies conducted in adults and children suggest that insulin resistance and glucose alteration are more frequent in GH treated subjects with risk factors like age, obesity, an adverse metabolic profile before therapy, syndromes or history of tumors [16]. Genomic profiles, like GHR polymorphisms could be inserted in this list. The d3GHR deletion is the most investigated polymorphism of GHR in both healthy subjects and patients with GHD or other diseases such as acromegaly or type 2 diabetes [38]. The d3GHR variant consisting of genomic exon 3 deletion has been linked with increased receptor activity due to an enhanced signal transduction. Its biological role on glucose metabolism is still controversial. Data derived by the Stockholm Diabetes Prevention Program suggest the homozygosity for the d3GHR allele as preventive of type 2 diabetes in adults. However, when other factors cause overt type 2 diabetes, the d3GHR allele confers a phenotype indicative of risk for metabolic disorders [10]. In Chinese obese children fasting insulin, HOMA-IR, and lipid profile were significantly lower in the homozygous and heterozygous d3GHR group than in the full-length GHR group [39]. Moreover, the presence of at least one d3GHR allele was associated with higher insulin secretion for a given degree of insulin sensitivity and with a higher disposition index in healthy normal weight children and adolescents during puberty in the COPENHAGEN puberty study [11].

All these data are in agreement with our observation that glucose levels are lower and DI, fasting C-peptide and nearly to significance INS, were higher in Del GHD adolescents after stopping therapy, suggesting that GHR exon 3 deletion gene polymorphism may play a role in modulating insulin secretion to a glucose challenge and peripheral insulin activity at least during periods of high endogenous GH secretion like puberty. Higher fasting C-peptide in subjects with at least one copy of the exon-3 deleted GHR than those with the full length one were also demonstrated in adults [10]. However, the changes are apparent after rhGH withdrawal in our population. This result is in line with that of Giavoli *et al.* who recorded a higher prevalence of impaired glucose tolerance after both 1 and 5 years of rhGH therapy in GHD adults positive for the d3GHR [9]. The enhanced activity of the d3GHR isoform may mediate the metabolic effects of rhGH on glucose homeostasis. We can speculate that a more pronounced GH activity has a strong lipolytic effect and insulin antagonism. As a consequence, a worse glucose homeostasis in adults and, more likely, a partial deterioration in peripheral insulin activity, reflected by DI, in adolescents become apparent. This hypothesis is fully in agreement with the fact that in healthy people when other factors cause overt type 2 diabetes, the d3GHR allele is not protective but confers a more impaired metabolic phenotype [10]. Because normal adolescents with d3GHR have higher DI during puberty and GHD unconfirmed subjects present an increase in DI after rhGH withdrawal, a threshold for the metabolic GH activity is suggested, in agreement with studies which failed to record alterations in glucose metabolism for treatments with rhGH very low doses [40].

GH plays an important role in the regulation of lipoprotein metabolism. In patients with d3GHR we also observed higher HDL-cholesterol levels during rhGH treatment and after rhGH withdrawal. These findings are in line with data in healthy and hypertensive adults [41] and support the idea that sequence variations in the GHR may have important effects on metabolic phenotype as a whole. Accordingly, other polymorphisms in the GHR have been shown to modify HDL cholesterol concentration also in hyperlipidaemic patients [42].

It has to note that our subjects progressively decreased IGF-I levels after rhGH withdrawal but remained in the middle part of the normal range. Higher IGF-I levels in the last year of rhGH treatment were due to the treatment and, in absence of a confirmed GHD during the transition phase, their levels quickly restored in the middle of normality. Interestingly, insulin resistance and sensitivity negatively correlated with them only after the withdrawal of the therapy. This is adequate with previous study that attest the U shape relation of IGF-I with the risk of diabetes is evident in a healthy population when confounders are removed [43].

There are limitations in the present study. First is that we did not perform an intravenous glucose tolerance test (IVGTT). The IVGTT is validated and more reproducible than the OGTT, because independent by confounding factors as the incretin effect is. However, the IVGTT only describes an experimental model and is far to be used in clinical practice. We decided to use the OGTT instead of IVGTT because OGTT describes the post-meal dynamics which is of interest for the GH physiology being GH a hormone that principally acts in the post-absorptive phase [1]. Moreover, we chose the OGTT-derived indices of insulin sensitivity and secretion because they correlate well with clamp measures in children [44,45], are easily calculated in epidemiological or intervention studies, and DI calculated by the OGTT has been demonstrated to predict diabetes development in adults [24]. Furthermore, the OGTT is the test that is classically performed in the clinical practice during rhGH treatment being simpler and cheaper than IVGTT. Moreover, we did not evaluate C-peptide during the test but only at fasting. The second limitation is that we were unable to demonstrate a gene dosage effect with respect to the d3GHR allele because the group of homozygous subjects was too small to analyse them separately. A study in a wider cohort could address this question. However, many published studies on the d3GHR also in wider populations [9–11] grouped together subjects bearing at least one copy of the exon-3 deleted GHR according to the hypothesis of the dominant model [46].

On the whole, in not confirmed GHD adolescents the fasting deterioration in glucose homeostasis is efficaciously coupled with a compensatory insulin secretion and activity for the degree of insulin-sensitivity at glucose challenge also in puberty. In adolescents during puberty, the presence of at least one d3GHR allele, despite a slightly higher fasting insulin resistance, is associated with lower glucose levels and higher HOMA-β, fasting C-peptide, HDL-cholesterol and DI at OGTT after rhGH withdrawal. Because a more pronounced activity of the d3GHR isoform, a major risk to develop an impairment in glucose homeostasis during the treatment could be hypothesized and future studies should address this question. OGTT should be routinely performed in clinical practice to better stratified GHD subjects during the treatment. Screening for the d3GHR in GHD treated subjects, also in childhood may help physicians to follow and phenotype patients during the treatment.

Supporting Information

Figure S1 Analysis of the GHR exon 3 polymorphism. a) Schematic representation of the multiplex PCR assay used to detect the GHR exon 3 polymorphism. One forward (G1) and two reverse primers (G2 and G3) were used. Primers G1 and G3 (this located within exon 3) are designed to detect the GHR-fl allele by the amplification of a 935 bp fragment (the 3248 bp fragment is non amplified under the conditions used); primers G1 and G2 allow the amplification of the GHR-d3 by producing a 532 bp fragment. b) Genotyping of the GHR exon 3 polymorphism. The presence of a 935 bp band indicates the genotypes homozygous for GHR-fl (#1,2,3,6,8,10,11,12) the presence of a band of 532 bp indicates the genotype homozygous for GHR-d3 (#4) and the presence of both the bands indicates the herozygotes (#5,7,9,13). A 100 bp ladder is used as a molecular weight marker. c) Distribution of the genotype in GHD and control (CS) subjects. Five out of 40 CS did not give the consent to perform the genetic analysis.
(TIF)

Acknowledgments

The authors wish to thank Caterina Balossini, Enza Giglione, Silvia Parlamento, and Stefania Moia for their technical assistance.

Author Contributions

Conceived and designed the experiments: FP GA GB MG SB. Performed the experiments: SS DB RR MG. Analyzed the data: FP SS GG MG SB. Contributed reagents/materials/analysis tools: DB RR MG. Wrote the paper: FP GA MG GB SB.

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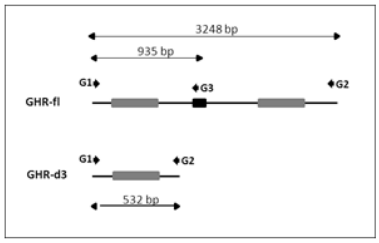
Supporting Information

a)



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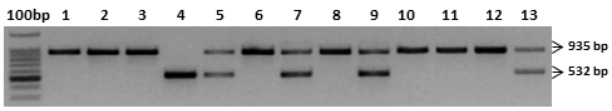

c)

	GHD (23 subjects)	CS (35 subjects)
fl/fl	2 (9%)	3 (8.5%)
d3/fl	8 (35%)	14 (40%)
d3/d3	13 (56%)	18 (61.5%)

Analysis of the GHR exon 3 polymorphism. a) Schematic representation of the multiplex PCR assay used to detect the GHR exon 3 polymorphism. One forward (G1) and two reverse primers (G2 and G3) were used. Primers G1 and G3 (this located within exon 3) are designed to detect the GHR-fl allele by the amplification of a 935 bp fragment (the 3248 bp fragment is non amplified under the conditions used); primers G1 and G2 allow the amplification of the GHR-d3 by producing a 532 bp fragment. b) Genotyping of the GHR exon 3 polymorphism. The presence of a 935 bp band indicates the genotypes homozygous for GHR-fl (#1,2,3,6,8,10,11,12) the presence of a band of 532 bp indicates the genotype homozygous for GHR-d3 (#4) and the presence of both the bands indicates the herozygotes (#5,7,9,13). A 100 bp ladder is used as a molecular weight marker. c) Distribution of the genotype in GHD and control (CS) subjects. Five out of 40 CS did not give the consent to perform the genetic analysis.