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“A. Avogadro”

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IDENTIFICATION OF GENETIC VARIATIONS INVOLVED IN ISOLATED GROWTH HORMONE DEFICIENCY (IGHD)

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1. INTRODUCTION

1. INTRODUCTION

In the four years of my PhD I was involved in a project concerning the search for genetics variations contributing to Isolated Growth Hormone Deficiency (IGHD). All the patients, most of which sporadic, recruited in the last ten years by the laboratory of Human Genetics and the Unit of Pediatrics were analysed for the presence of causal variations in candidates genes (i.e. the GH1 and GHRHR genes).

As high penetrance mutations have been detected only in one familial case we hypothesized that IGHD in sporadic cases has a multifactorial origin.

The here presented work shows the results obtained from the screening of the GH1 gene and from the association study performed on candidate region (i.e. the GH1 gene promoter). All the identified variations have been investigated from the functional point of view.

1.1 HUMAN GROWTH HORMONE (hGH)

Growth Hormone (GH), the main regulator of longitudinal growth in mammals [1] is a pleiotropic cytokine efficiently synthesized by the somatotropic cells of the anterior pituitary. It promotes the postnatal growth of skeletal and soft tissue, as well as acting as an important regulator of carbohydrate, lipid, nitrogen and mineral metabolism [2, 3]. It also stimulates differentiation and mitogenesis in a variety of cell types in different tissues [4], it is important in the maintenance of the immune system [5], heart development [6] and can act on the brain to modulate emotion, stress response and behavior [7].

The Growth hormone GH is secreted in a pulsatile manner under the concerted regulation of the hypothalamic hormones growth hormone releasing hormone (GHRH), which is a positive regulator, and somatostatin (SS), which is a negative regulator [8]. Once released into the plasma GH binds to growth hormone binding protein (GHBP), which is a proteolytic fragment of the GH receptor. The primary site of GH action is the liver, where it stimulates insulin-like growth factor-I (IGF-I) production. Many of the effects of GH are

mediated through the actions of this peptide, which acts through its own receptor to enhance the proliferation and maturation of many tissues, including bone, cartilage and skeletal muscle. GH also has direct effects on a number of tissues, including bone and the epiphyseal growth-plate [9].

Responsiveness to GH in target cells is primarily dependent upon the expression of the GH receptor (GHR) [10]. GHR is a single membrane-spanning cell surface protein member of the Class I cytokine receptor superfamily [11]. Like other members of the family, GHR lacks intrinsic kinase activity and signal transduction is mediated by Janus Kinase 2 (JAK2), a cytoplasmic tyrosine kinase that associates to the so-called box 1 in the membrane proximal region of the GHR cytoplasmic domain [12, 13]. A single GH molecule contains two GH receptor (GHR)-binding sites and these bind two GHR molecules sequentially, binding first at site 1 and then at site 2, inducing receptor dimerization and hence activation [14, 15]. Dimerization is believed to be necessary for the signal transduction which is associated with the intracellular tyrosine kinase JAK-2. Interaction of the dimerized GHR with JAK-2 leads to phosphorylation of downstream signal transduction molecules, induction of signal transducers and activators of transcription (STAT proteins). Activated STAT5 is translocated to the nucleus where it transactivates a series of GH-responsive genes [16]: Mitogen-Activated Protein Kinases (MAPK), Insulin Receptor Substrate 1 (IRS-1), Focal Adhesion Kinase (FAK), Protein Kinase C (PKC), Ras-like GTPases and the Signal Transducer and Activator of Transcription (STATs) family of transcription factors [17]. The contribution of each of these pathways to the physiological actions of GH remains unclear, as many of them are also activated by several additional growth factors and cytokines and in many cases, the data has been obtained only from in vitro studies.

1.2 hGH-1 GENE

The mature human GH is a single chain peptide, made up of 191-amino acids (22 kDa). X-ray crystallographic studies have shown that GH comprises a core of four antiparallel a-helices separated by connecting loops, arranged in an up-up-down-down fashion and stabilised by two intramolecular disulphide linkages (Cys53–Cys165, Cys182–

Cys189) [18]. GH is encoded by the GH-1 gene, located on the long arm of chromosome 17 (17q22-24). It includes 5 exons and 4 introns for a total of 1600 bp, within a 66 Kb cluster comprehending five highly homologous (92–98%) genes: (*hGH-1*)-(*hCSHP-1*)-(*hCSH-1*)-(*hGH-2*)-(*hCSH-2*) [19, 20] (Fig.1). Despite this high degree of sequence homology, each of these genes is expressed selectively in a tissue-specific fashion: the *hGH-1* gene exclusively in somatotrope cells of the anterior pituitary gland, and the *hCSH-1, hCSH-2* and *hGH-2* genes in the placental syncytiotrophoblast cells, under the control of a downstream tissuespecific enhancer [21]. While *hCSHP-1* was initially classified as a non-expressed pseudogene its expression in human placenta was subsequently demonstrated.

In non-primates, GH is encoded by a single gene. Evolution of the GH gene cluster has taken place over the last 15 million years and has proceeded via a process of duplication and divergence [22, 23]. The first event was thought to be the duplication of a single ancestral GH gene to generate pre-GH and pre-CSH genes, perhaps about 15 million years ago [24] followed by the duplication of the newly created gene pair to yield GH1, CSH1, GH2 and CSH2. Finally, the CSH1 gene was duplicated to form two genes, one of which (CSHP1) became inactivated through mutation. The GH gene cluster contains some 48 Alu sequences in both orientations within 70 kb genomic DNA [23]. Some of these repeats are thought to have mediated the recombination events responsible for the gene duplication events by unequal exchange between homologous yet nonallelic Alu sequences [24]. On the other hand, some of the Alu sequences have been duplicated along with their associated genes during the duplication process. One consequence of the evolutionary history of the GH gene cluster is that multiple sequence homologies and internal repetitions occur within it.

All five genes show the same transcriptional orientation and are separated by intergenic regions of 6 to 13 kb in length. They share a very similar structure, with five exons interrupted at identical positions by short introns, 256 bp, 209 bp, 93 bp and 253 bp in length in the case of GH1 [24-27]. Exon 1 of the GH1 gene contains 60 bp of 5’ untranslated sequence and the first nucleotide of codon -23 corresponding to the start of the 26-amino acid leader sequence. Exon 2 encodes the rest of the leader peptide and the first 31 amino acids of mature GH. Exons 3–5 encode amino acids 32–71, 72–126 and 127–191, respectively. Exon 5 also encodes 112 bp 3’ untranslated sequence culminating in the polyadenylation site. An Alu repetitive sequence element is present 100 bp 3’ to the GH1

polyadenylation site. Although the five related genes are highly homologous throughout their 5' flanking and coding regions, they diverge in their 3' flanking regions.

The GH1 and GH2 genes differ with respect to their mRNA splicing patterns. As noted above, in 9% of GH1 transcripts, exon 2 is spliced to an alternative acceptor splice site 45 bp into exon 3 to generate a 20 kDa isoform instead of the normal 22 kDa protein [28-30]. The GH2 gene is not alternatively spliced in this fashion [31]. A third 17.5 kDa variant, which lacks the 40 amino acids encoded by exon 3 of GH1, has also been reported [32].

The CSH1 and CSH2 loci encode proteins of identical sequence and are 93% homologous to the GH1 sequence at the DNA level. The CSH gene sequences compared to the CSHP1 pseudogene contains 25 nucleotide substitutions within its "exons" plus a G→A transition in the obligate +1 position of the donor splice site of intron 2 which partially inactivates its expression [27, 33].

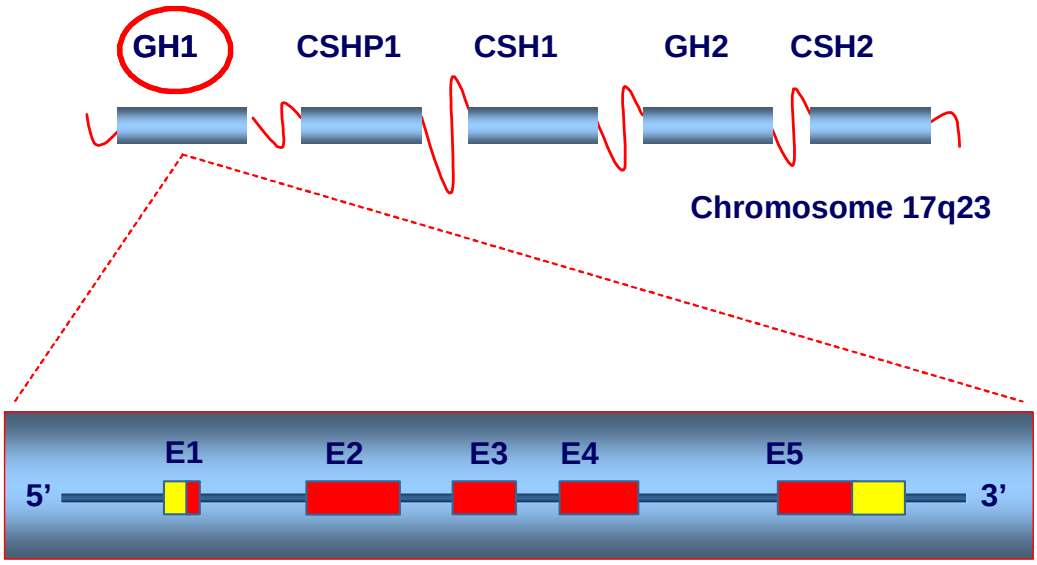
The sequencing of cDNA and genomic clones from a range of different vertebrates has allowed the determination of the amino acid sequences of the corresponding GH proteins as well as the exon/intron structure of the genes encoding them. By pinpointing evolutionarily conserved regions in the GH molecule, the methodical comparison of coding sequences can aid in the identification of functionally significant residues [34]. Such knowledge will be invaluable for the assessment of the likely authenticity of missense mutations found in the GH1 gene and critical for studies of the relationship between structure and function of the GH molecule. The elucidation of the structures of GH genes from several vertebrate species has revealed a high degree of conservation in the 5' and 3' non-coding regions [35] but exon number [36] and intron size [37] vary between species.

The level of expression of the GH1 gene in the pituitary is quite high, as judged by the proportion (3%) of pituitaryderived cDNA clones corresponding to GH1 [23]. There is, however, some evidence for expression of the GH1 gene in extra-pituitary tissues such as the hypothalamus [38], the thymus [39] and neutrophils [40]. Intriguingly, GH gene transcription has been observed at the morula stage in the mouse [41], raising the possibility that GH has an important role in early embryonic development. Cell culture experiments using either rat or human cells have provided evidence that glucocorticoid hormone [42], retinoic acid [43] and GHRH up-regulate GH1 gene expression whereas thyroid hormone [44], insulin-like growth factor [45] and insulin [46] down-regulate it. Glucocorticoids also exert their effects post-transcriptionally by enhancing the stability of GH1 mRNA [47].

The regulation of *hGH-1* gene transcription is considered to be exerted by specific interactions of tissue-specific and other more ubiquitous regulatory proteins (*trans*-factors) which bind specific DNA sequences (*cis*-elements) located mainly within the immediate 5'-flanking promoter region. Different types of *cis–trans* coupling are thought to allow both tissue-specific expression and also hormonal induction or inhibition of gene transcription.

Various cis-acting regulatory sequences have now been identified in the proximal GH1 gene promoter which are responsible for these inductive or repressive effects on GH1 gene expression [48, 49]. Basal expression is controlled by several general tissue factors that can act both positively and negatively. These include NF-1 (binding site at−286 to−274) [50], Sp1 (−136 to−127) [51], Zn-15 (−110 to−95) [52], Vitamin D receptor (−60 to −46 and −37 to −31) [53] and CREB, a protein that interacts with cAMP-responsive elements (−187 to−183 and−99 to−95) [54]. The pituitary restricted expression is mainly controlled by the pituitary specific factor Pit-1 [55], a POU homeodomain protein binding to two highly conserved elements in the GH1 proximal promoter located between −87/−72 (proximal Pit-1 binding site) and −127/−107 (distal Pit-1 binding site). Several studies demonstrated that Pit-1 binding sites are critical for GH1 promoter activity both by cell free transcription experiments [56] and transient transfection into tissue cultured cells [57]. However studies of human GH expression in transgenic mice demonstrated that binding of Pit-1 to the two Pit-1 sites in the GH1 promoter are not sufficient for high level tissue-specific GH expression in vivo [58]. Three further Pit-1 binding sites in a locus control region (LCR) located between 14.5 kb and 32 kb upstream the GH1 gene are necessary to confer high level somatotropic-specific GH expression [59]. The LCR contains multiple DNase I hypersensitive sites and is required for the activation of the genes of the GH gene cluster in both pituitary and placenta [60].

Fig. 1. GH1 cluster, located on the long arm of chromosome 17 (17q22-24).



1.3 ISOLATED GROWTH HORMONE DEFICIENCY (IGHD).

Isolated growth hormone deficiency (IGHD) refers to conditions of GH deficiency that are associated with childhood growth failure due to lack of growth hormone (GH) action, and not necessarily associated with other pituitary hormone deficiencies or with an organic lesion other than a congenital structural abnormality. The incidence of IGHD is estimated to be 1/3,480–1/10,000 live births [61]. Although most cases are sporadic and are believed to result from environmental insults or developmental abnormalities, 3–30% of the cases reported have an affected first-degree relative, suggesting that a significant proportion of the cases with GHD are in fact familial [62]. While the majority of IGHD cases are sporadic and thought to be caused by in utero, natal or postnatal hypothalamic or pituitary insults, anatomic abnormalities are found in only 12% of such patients examined by magnetic resonance imaging (MRI) [63]. In the majority of children with IGHD no cause can be identified; this group is often referred to as idiopathic GH deficiency or idiopathic IGHD. Clinical features associated with idiopathic IGHD are variable, and include breech position at birth, neonatal or early onset hypoglycemia, prolonged or severe

neonatal jaundice, micropenis, and single central incisor [64, 65]. Recent MRI observations in children with idiopathic GHD suggested a positive relationship between the volume of the adenohypophysis and secretory GH capacity [66]. However, within this group of idiopathic cases, there is increasing recognition of associated genetic defects. General clinical findings in patients with IGHD may include frontal bossing, immature facies for chronological age, poor development of the nasal bridge, delayed secondary dentition, and a high-pitched voice [67]. In infants closure of the anterior fontanel may be delayed and male infants may present with micropenis. Puberty may be delayed until the late teens, but eventual sexual maturation and fertility are usually normal.

Mutations in the GH1 gene have been detected in about 12.5% of familial and 10% of sporadic IGHD and include deletions of the entire gene and nonsense mutations in the most severe forms, and splicing mutations in the milder forms [68]. These mutations cause different types of IGHD, which has been classified on the basis of clinical characteristics, including inheritance patterns and GH secretion, into three types (tab.1).

IGHD type 1, characterized by autosomal recessive transmission, is further separated into sub-types 1a and b. **IGHD type 1A** is the most severe form of IGHD. Patients with IGHD type 1A typically present early and profound growth failure [60], neonatal hypoglycemia, prolonged jaundice associated with severe postnatal growth retardation and characteristic facies that can be apparent by 6 months of age [69,70]. Patients with IGHD type 1A lack detectable serum GH, or extremely low levels on provocation testing. They show a strong initial response to therapy with GH, but often develop anti-GH neutralizing antibodies with continued exogenous hGH treatment, resulting in a markedly decreased final height as an adult. The characteristic facial appearance of GHD with mid-facial hypoplasia, delayed dentition and frontal bossing is well recognized. The disorder is inherited in an autosomal recessive manner and all reported families to date are consanguineous. The majority of patients with type IA isolated GHD have large deletions (6/7– 45 kb) within the GH-1 gene [64]. However, microdeletions such as that of a single base pair at codon 10 leading to an altered reading frame with premature termination of translation and an ensuing truncated protein have also been described. The exact prevalence of this disorder is unclear. Sporadic cases may go unrecognized, and possibly account for the apparent low incidence of this disorder. A prevalence of 9–38% for GH-1 gene deletions in markedly short (height less than −4 standard deviations) individuals has been suggested [65]. There is marked heterogeneity in the phenotype of these patients, in addition to considerable variability in antibody formation and response to hGH treatment, even within families with the same

deletions. Recombinant IGF-1 has also been used, particularly in patients with a poor initial response to hGH treatment and formation of high antibody titres. With improvements in recombinant technology, purer forms of GH can now be produced that alleviate the problem of antibody formation to some extent.

IGHD type 1B is also associated with a prenatal onset of GHD, but the phenotype is milder than that of IGHD type 1a and mid-facial hypoplasia and a microphallus are not common features of the phenotype. Serum GH levels after provocative stimuli are low, but detectable. Affected individuals usually respond well to GH therapy and do not develop neutralizing antibodies. The condition is inherited as an autosomal recessive trait. Children present with marked short stature and a poor growth velocity, and the condition is characteristically associated with a good response to exogenous hGH treatment with no formation of GH antibodies [60].

IGHD type IB is due to either homozygous splice site mutations within the GH-1 gene or mutations within the gene encoding the GHRH receptor (GHRHR) [66,67]. The human GHRHR gene spans approximately 15 kb, maps to chromosome 7p15, and encodes a protein containing 423 aa. The receptor is a G-protein-coupled receptor characterized by seven transmembrane domains with a–high binding affinity for GHRH. GHRHR is also required for proliferation of somatotrophs and therefore plays an important role in anterior pituitary development.

IGHD type 2 is characterized by short stature due to dominant-negative mutations of the human growth hormone gene (*GH1*). These individuals show variably reduced plasma GH levels and growth rates but usually respond positively to exogenous GH treatment [67]. IGHDII is most commonly caused by mutations within the first 6 bp of intervening sequences 3 (5'IVS-3), which lead to the production of two alternatively spliced GH molecules, 20 kDa and 17·5 kDa hGH. This product is due to the skipping and the subsequent loss of exon 3 (E3), producing a human GH (hGH) variant which lacks aa 32–71, representing the entire loop that connects helix 1 and helix 2 in the tertiary structure of hGH [18]. The loss of exon 3 deletes the linker domain between the first two helices of GH, disrupting an internal disulfide bridge, and thereby overall protein structure. Trace amounts of severely truncated 11.3- and 7.4-kDa isoforms, encoded by transcripts that skip exons 3–4 or exons 2–4, have also been detected in normal human pituitary [19]. Skipping of exon 3 caused by GH-1 gene alterations other than those at the donor splice site in 5'IVS-3 has also been reported in other patients with IGHD II. These include mutations in exon splice enhancer (ESE) [ESE1 in E3] (E3 +1G->T: ESE1m1; E3 + 5A→G: ESE1m2)

and within suggested intron splice enhancer (ISE) (IVS-3+28 G->A: ISEm; IVS-3del+28–45: ISEm2) sequences. Such mutations lie within purine-rich sequences and cause increased levels of E3-skipped transcripts, suggesting that the usage of the normal splicing elements (ESE1 at the 5' end of exon 3 as well as ISE in intron 3) may be disrupted [68]. The first seven nucleotides in E3 (ESE1) are crucial for the splicing of GH mRNA such that some nonsense mutations might cause skipping of one or even more exons during mRNA splicing in the nucleus. This phenomenon is called nonsense-mediated altered splicing; its underlying mechanisms are still unknown. In addition to the abovedescribed splice site mutations that result in the production of GH product lacking aa 32–71 , three other mutations within the GH-1 gene (missense mutations) are reported to be responsible for IGHDII, namely the substitution of leucine for proline, histidine for arginine, and phenylalanine for valine at aa positions 89 (P89L), 183 (R183H), and 110 (V110F), respectively [69,70]. At the functional level, the 17.5-kDa variant exhibits a dominant-negative effect on the secretion of the 22-kDa isoforms in both tissue cultures and transgenic animals. The 17.5-kDa variant is initially retained in the endoplasmic reticulum, disrupts the Golgi apparatus, impairs both GH and other hormonal trafficking [71], and partially reduces the stability of the 22-kDa isoform [72]. Furthermore, transgenic mice overexpressing the 17.5-kDa variant exhibit a defect in the maturation of GH secretory vesicles and anterior pituitary gland hypoplasia due to a loss of the majority of somatotropes. Trace amounts of the 17.5-kDa variant, however, are found present at the mRNA in normal pituitaries [74].

From a clinical point of view, severe short stature [<-4.5 sd score (SDS)] is not present in all affected individuals, indicating that in some forms growth failure in IGHD II is less severe than one might expect. It has been hypothesized that children with splice site mutations may be younger and shorter at diagnosis than their counterparts with missense mutations[75]. Furthermore, more recent in vitro and animal data suggest that both a quantitative and qualitative difference in phenotype may result from variable splice site mutations, causing differing degrees of E3 skipping. To summarize, the variable phenotype of autosomal dominant GH deficiency (GHD) may reflect a threshold and a dosedependent effect of the amount of 17.5-kDa relative to 22-kDa hGH. Specifically, this has a variable impact on pituitary size and onset and severity of GHD, and, unexpectedly, the most severe, rapid onset forms of GHD might be subsequently associated with the evolution of other pituitary hormone deficiencies.

IGHD type 3 has an X-linked mode of inheritance with a highly variable phenotype. Affected individuals in some kindreds have an associated X-linked agammaglobulinemia. This suggests that a contiguous gene deletion disorder of Xq21.3 + q22 may occur in some cases. Interestingly, other cases of IGHD have been found to have an interstitial deletion of Xp22.3 or duplication of Xq13.3 + q21.2, suggesting that multiple loci may cause IGHD III [61].

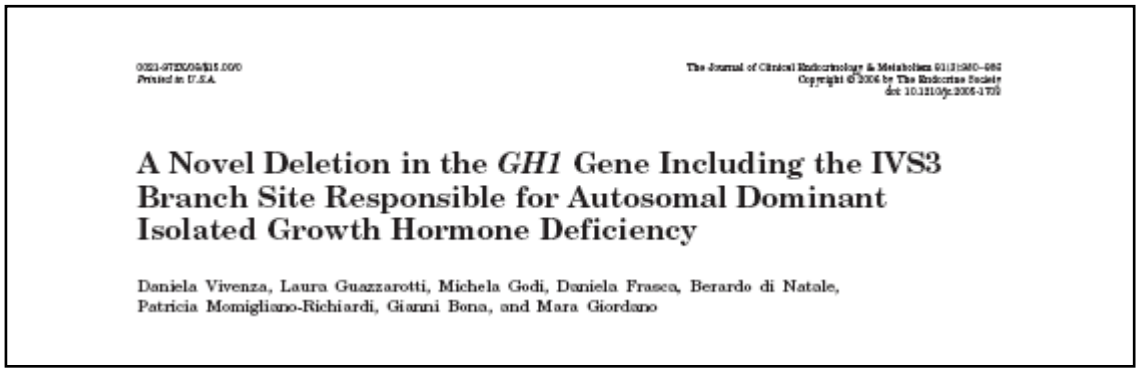
The clinical classification system for IGHD may have limited future utility since there is increasing recognition of identifiable causes for IGHD. About 5–30% of patients with IGHD are found to have affected first-degree relatives, suggesting genetic causes [62]. As described already, defects in the gene for growth hormone releasing hormone receptor (GHRH-R) or in the GH1 gene have been identified in an increasing number of individuals with IGHD.

Table 1. Classification of IGHD.

IGHD	Associated gene defects	Reported inheritance patterns	GH levels	Phenotype
Type 1a	*GH1* mutations	Autosomal recessive	Absent	Severe short stature
	Deletions, microdeletions, nonsense mutations			Neutralizing anti-GH antibodies often develop during GH replacement therapy
Type 1b	*GH1* mutations	Autosomal recessive	Low	Height ≥3 SD below the mean
	GHRHR mutations			
	Splice site mutations			
Type 2	*GH1* mutations	Autsomal dominant	Low	Short stature
	Splice site mutations, intron and exon splice enhancer, missense mutations			At least one affected parent
				Variable phenotype
				Good response to GH therapy
Type 3	None to-date	X-linked recessive	Low	Associated with X-linked aggamaglobulinemia in some individuals

2. RESULTS

2.1 PAPER 1.



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A Novel Deletion in the *GH1* Gene Including the IVS3 Branch Site Responsible for Autosomal Dominant Isolated Growth Hormone Deficiency

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A 2-yr-old child and her mother affected by IGHD with severe growth failure at diagnosis (-5.8 and -6.9 SD score, respectively) and low response to GH secretion stimuli were investigated for the presence of GH1 mutations. Sequencing of the GH1 gene revealed the presence of a heterozygous 22-bp deletion in IVS3. This mutation encompasses the branch point sequence (BPS) located between -20 and -25 upstream of the intron 3/exon 4 junction, generating at least three aberrant isoforms.

To test weather this mutation could affect RNA processing, lymphocytes RNA has been extracted from the two patients and a transcript analysis was performed on cDNA.

The splicing pattern showed by the proband and her mother revealed two principal mRNA species approximately in equal amount, namely the full-length mRNA, encoded by the normal allele, and an aberrant splicing product with the skipping of exon 3 encoded by the mutant allele.

To assess whether the skipping of exon 3 was the only aberrant product generated by the del+56–77 mutation, we also performed an *in vitro* analysis with the transcripts of the mutated allele. To this purpose, we transfected GH4C1 rat pituitary cells with an expression vector containing either the normal GH1 allele (wt-GH1) or the GH1 allele with the IVS3 del+56–77 mutation (del-GH1). In addition to the product corresponding to exon 3 skipped mRNA, we detected two other aberrantly spliced isoforms, in experimental conditions that excluded the exon 3 skipped mRNA from the possible targets. One isoform, showed complete skipping of exon 4 and encodes for a putative mature peptide lacking amino acids 72–126. The other was an mRNA devoid of the first 86 bases of exon 4, and it causes a frameshift leading to a putative protein differing from the wild type from amino

acid 72 and prematurely truncated at residue 77. The presence of this isoform could be attributable to the use of a cryptic acceptor splice site at position 85–86 of exon 4, whose recognition might be mediated by the presence of a putative cryptic BPS (CGCCAAC), matching six of the seven bases of the consensus sequence, located between nt 78 and 84 of exon 4.

The mutation described in this paper is different from other intronic mutations detected in IGHD II families because it leaves intact the splice junctions and the splicing enhancers (namely ISE and ESE, respectively), whereas it completely removes the BPS. The two aberrant splicing events involving exon 4 are the direct consequence of the missing BPS, as demonstrated by a mutagenized construct lacking exclusively the 7 bp of the BPS consensus. Conversely, the high content of the exon 3 skipped isoform remains to be explained. It was recently demonstrated that the overall size of IVS3 is crucial for exon 3 inclusion in the mRNA. Deletions of 12–14 bp might be thus sufficient to increase exon 3 skipping. We can thus speculate that the main effect of the 22-bp deletion (i.e. the increased exon 3 skipping with production of the IGHD II-specific pathological amount of the 17.5-kDa isoform) is the consequence of the remarkably decreased size of IVS3 that overwhelms the effect caused by the BPS deletion.

In conclusion, despite the absence of the canonical BPS, the exon 3 skipped is the principal transcript and the cause of IGHD II in our patients. Thus, the new mutation described, i.e. the 22-bp deletion, has a greater phenotypic influence than the BPS mutation itself. It is unlikely that the two isoforms with an aberrant splicing of exon 4 also contribute to the IGHD II phenotype because they are presumably poorly expressed. In fact he content of this abnormal mRNA lacking exon 4, or a part of it, was likely very low in the patients' lymphocytes compared with the normal-sized and to the exon 3 skipped mRNA and could therefore be visualized only under specific experimental conditions. One of the reasons for the low content, at least for the prematurely truncated isoform, might be its rapid degradation owing to nonsense-mediated mRNA decay.

*2.2 **PAPER 2.***

A Functional Common Polymorphism in the Vitamin D-Responsive Element of the *GH1* Promoter Contributes to Isolated Growth Hormone Deficiency

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Because causal mutations have been detected in a minority of IGHD patients, we tested the hypothesis that low penetrance genetic variations with a quantitative effect on GH1 transcription might contribute to IGHD. We thus performed a case-control study between GH1 polymorphisms and IGHD in the Italian population. We included 118 sporadic IGHD patients showing a "non-severe" phenotype (height -4SDS/-1SDS and partial GH deficiency) and two control groups, normal stature (N=200) and short stature individuals with normal GH secretion (N=113).

We identified a variation in the promoter of GH1 gene, within the vitamin D receptor binding element (VDRE), showing a significant positive association with our IGHD patients.

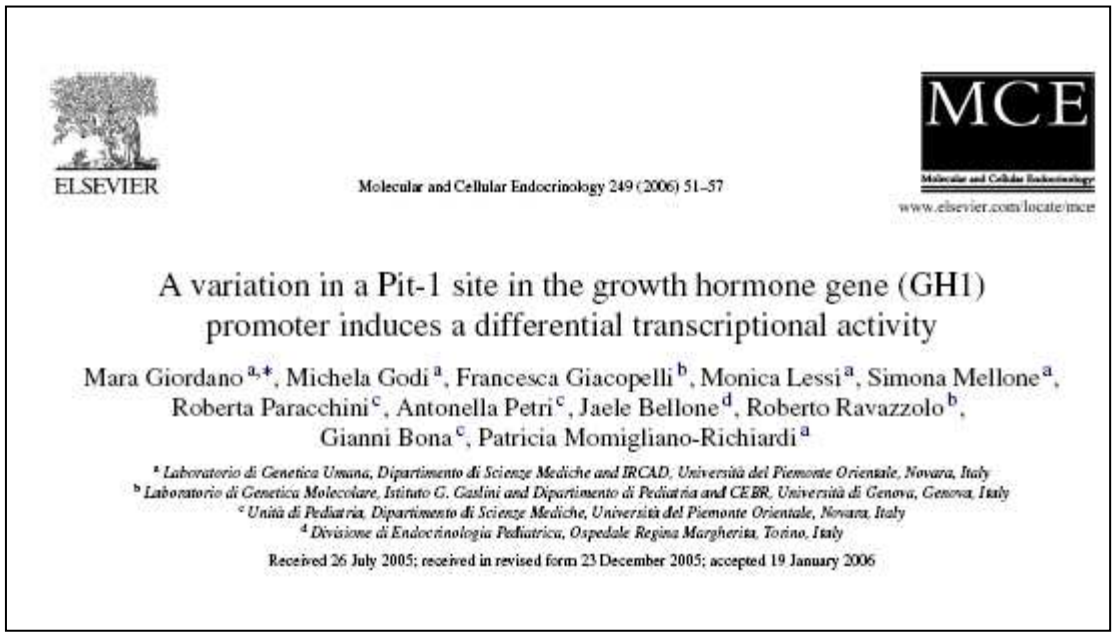
The functional role of this polymorphism was thus assessed through an *in vitro* analysis, after transfection in the mammary adenocarcinoma MCF7 cell line, a human lineage expressing both GH and VDR and largely utilised as a model to study GH1 gene expression. At first we tested the functional relevance of this variation through its capacity of modulating the expression of a luciferase-reporter gene, subsequently confirmed by EMSA (Electrophoretic Mobility Shift Assay) experiments.

This work allowed to get new insight into the multifactorial nature of IGHD. Since the associated allele localized within Gh1 promoter has a high binding affinity for the vitamin

D receptor and VDR is expressed in GH producing pituitary cells, the gene encoding the VDR is an interesting further candidate gene for genetic studies of stature.

The human 1,25-dihydroxyvitamin D3 receptor (hVDR) is a member of the superfamily of ligand-inducible transcription factors, which include the steroid and nuclear receptors [76]. It plays an important role not only in skeletal metabolism, but also in other metabolic pathways, such as those involved in osteoarthritis and in the immune response. Many allelic variants within this gene have been detected in the human population [77]. Their expression associates with different pathologies, such as decreased bone density, propensity to hyperparathyroidism, resistance to vitamin D therapy, susceptibility to infections, autoimmune diseases, and cancer [76].

*2.3 **PAPER 3.***



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A variation in a Pit-1 site in the growth hormone gene (GH1) promoter induces a differential transcriptional activity

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The association study on the GH1 SNPs showed that the promoter variation -75A/G was not associated to IGHD. However it falls within the proximal binding site for the transcription factor Pit-1. In the present study we demonstrated the potential functional role of this variation.

Pituitary transcription factor-1 (Pit-1), a member of the POU domain factor family (Pit-1, Oct-1, and Unc-86), plays a key role in cell differentiation during organogenesis of the anterior pituitary in mammals [55] and as a transcriptional activator for pituitary gene transcription (i.e. transcription of the genes for growth hormone, prolactin, and Pit-1 itself) [56,57]. Pit-1 contains two protein domains, namely Pou-specific (POUs) and POU-homeo (POUh), both necessary for DNAbinding. They are located within GH1 proximal promoter located between −87/−72 (proximal Pit-1 binding site) and −127/−107 (distal Pit-1 binding site) [55]. It generally binds as a dimer to its cognate recognition elements. Each of these elements consists of a core motif including a higher affinity 5’ consensus sequence, ATGNATA followed at the 3’ by an adjacent lower affinity A/T rich sequence. One Pit-1 monomer binds to the 5’ higher affinity sequence and the second monomer binds cooperatively to the 3’ lower affinity region [55]. The −75A/G polymorphism is located at the 3’ end of the lower affinity region. Several studies demonstrated that Pit-1 binding sites

are critical for GH1 promoter activity both by cell free transcription experiments and transient transfection into tissue cultured cells. However binding of Pit-1 to this two sites in the GH1 promoter are not sufficient for high level tissue-specific GH expression in vivo [57]. Three further Pit-1 binding sites in a locus control region (LCR) located between 14.5 kb and 32 kb upstream the GH1 gene are necessary to confer high level somatotropic-specific GH expression [60].

In order to test the functional role of the -75A/G variation, luciferase-reporter assay were performed through transient transfections in the rat pituitary cell line GH4C1. The binding affinity of Pit-1 peptide to its binding site in the presence of either allele A or allele G was further tested by EMSA. As the−75G promoter variation displayed a relevant effect *in vitro*, we investigated the possibility that it exerts a similar effect *in vivo* through an association study between the polymorphism and isolated growth hormone deficiency (IGHD). We genotyped one hundred and forty-five IGHD patients and 168 normal stature controls were for the −75A/G polymorphism. Our results showed that the allele and genotype frequencies were not significantly different between patients and controls.

In spite of the lack of association with IGHD, we can not exclude a potential effect of the variation on the level of GH secretion *in vivo*. Since GH secretion is regulated by the balanced interaction of several factors the effect of the −75G variation *in vivo* might be hidden by the overall effect of the complex regulatory GH secretion network. Moreover, we tested selected populations of individuals with short stature in which we cannot exclude the presence of other unknown genetic variations with a stronger effect on Growth Hormone production.

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A Novel Deletion in the *GH1* Gene Including the IVS3 Branch Site Responsible for Autosomal Dominant Isolated Growth Hormone Deficiency

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Context: The majority of mutations responsible for isolated GH type II deficiency (IGHD II) lead to dominant negative deleteriously increased levels of the *GH1* exon 3 skipped transcripts.

Objective: The aim of this study was the characterization of the molecular defect causing a familial case of IGHD II.

Patients: A 2-yr-old child and her mother with severe growth failure at diagnosis (−5.8 and −6.9 SD score, respectively) and IGHD were investigated for the presence of *GH1* mutations.

Results: We identified a novel 22-bp deletion in IVS3 (IVS3 del+56–77) removing the putative branch point sequence (BPS). Analysis of patients' lymphocyte mRNA showed an excess exon 3 skipping. The mutated allele transfected into rat pituitary cells produced four differently spliced products: the exon 3 skipped mRNA as the main product and lower amounts of the full-length cDNA and of two novel mRNA aberrant isoforms, one with the first 86 bases of exon 4 deleted and the other lacking the entire exon 4. A mutagenized construct lacking exclusively the 7 bp of the BPS only generated the exon 4 skipped and the full-length isoforms. The presence of the full-length transcript in the absence of the canonical BPS points to an alternative BPS in IVS3.

Conclusion: The IVS3 del+56–77 mutation, causing IGHD II in this family, has two separate effects on mRNA processing: 1) exon 3 skipping, analogous to most described cases of IGHD II, an effect likely caused by the reduction in size of the IVS3, and 2) partial or total exon 4 skipping, as a result of the removal of the BPS. (*J Clin Endocrinol Metab* 91: 980–986, 2006)

APPROXIMATELY 75% OF the circulating GH is a 191-amino-acid peptide with a molecular mass of 22 kDa. Alternative splicing of the original transcript gives rise to minor amounts of several smaller isoforms. The most represented of these alternative spliced transcripts lacks the first 45 bp of exon 3 through activation of an in-frame cryptic splice site and produces a 20-kDa peptide missing amino acids 32–46 (1). Complete skipping of exon 3, accounting for 1–5% of the total *GH1* transcripts, results in a 17.5-kDa form lacking amino acids 32–71. Two isoforms lacking exons 3–4 and exons 2–4 and encoding 11.3- and 7.4-kDa peptides have also been detected (2).

Mutations in the *GH1* gene have been identified both in type I (autosomal recessive) and type II (autosomal dominant) familial isolated GH deficiency (IGHD) (2, 3). IGHD II is caused mainly by mutations affecting GH mRNA splicing, falling within the first six bases of the IVS3 5′ donor splice site (4–8) or disrupting splicing enhancer elements. The latter includes two mutations in an exon 3 splicing enhancer, ESEm1 + 1G→T (9) and ESEm2 + 5A→G (10), and two mutations in an IVS3 splicing enhancer, ISEm1(IVS3 + 28 G→A) and ISEm2(IVS3Δ28–45) (11, 12). All the reported splicing mutations lead to deleteriously increased levels of the exon 3 skipped transcripts encoding the 17.5-kDa isoform. This isoform lacks the protein linker domain between helix 1 and helix 2 of the mature GH and a cysteine residue, Cys^{53}, involved in the interaction between helix 1 and helix 4 (13). Thus, without the amino acids encoded by the third exon, the molecule cannot fold normally. This altered GH structure exhibits a dominant negative effect on secretion of the 22-kDa isoform. The 17.5-kDa isoform is initially retained in the endoplasmic reticulum, disrupts the Golgi apparatus, impairs both GH and other hormonal trafficking (14), and decreases the intracellular stability of the wild-type GH (15). Moreover, the 17.5-kDa isoform causes a dose-dependent disruption of GH secretory vesicles when expressed in GC cells and transgenic mice (16).

In addition to the above cited splicing mutations, three missense mutations, V110F, P89L, and R183H, are reported (8, 17, 18).

We here describe a novel splicing mutation in an IGHD II family, a 22-bp deletion in the IVS3 of the *GH1* gene encompassing the branch point sequence (BPS) (11). This mutation generates at least three aberrant isoforms: the more abundant exon 3 skipped isoform and two minor isoforms lacking the first 86 nucleotides of exon 4 and the entire exon 4, respectively.

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Abbreviations: BPS, Branch point sequence; IGHD, isolated GH deficiency; SDS, SD score.

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Subjects and Methods

Subjects

The proband, a 2-yr-old girl, came to our observation for severe growth failure at the age of 7 months. She was born by spontaneous delivery after a normal pregnancy at 36 wk gestation with a weight of 2720 g (25th percentile) and a length of 44 cm (3rd percentile). Her phenotype had the IGHD characteristic features: slow but harmonious body development with regular segment proportions, prominent frontal bones, and saddle nose. After biochemical evaluations that excluded organic diseases, two stimulation tests for GH secretion study were performed showing a complete GH deficiency (immunoradiometric assay; DiaSorin, Stillwater, MN) confirmed by the IGF-I dosage (chemiluminescent enzyme-labeled immunometric assay; Medical System, Los Angeles, CA) (Table 1). Analysis of TSH (electrochemiluminescence immunoassay; Roche, Indianapolis, IN) and cortisol (RIA; Adaltis, Montreal, Canada) excluded other anterior pituitary hormone deficiencies (Table 1). The magnetic resonance imaging of the pituitary region performed with narrow scanning and gadolinium injection reported a normal anatomy of the region with the sagittal T1 images showing a mild hypoplasia of the pituitary gland with a height at −2.0 SD score (SDS) for age (21). Therapy with human recombinant GH, started at the age of 9 months at the dose of 0.033 mg/kg·d, yielded a very good response; the growth velocity reached 20 cm/yr (Table 1).

The proband's mother, now 39 yr old, arrived for medical observation for severe growth failure at the age of 4.6 yr. She was born after a normal pregnancy and delivery, but no information is available about her weight and length at birth. Her clinical and hormonal characteristics are shown in Table 1. She was treated, in a noncontinuous way, with human pituitary-derived GH until the age of 16 yr with a final height of 146 cm, which was her target height (mother's height, 148 cm; father's height, 160 cm). After GH suspension at the age of 16, she developed severe obesity. A new test for hypophyseal hormones at the age of 38 confirmed complete GH deficiency, whereas the other pituitary hormones were in the normal range (Table 1). She has now resumed exogenous GH therapy, and her weight is decreasing. The magnetic resonance of the pituitary region, not previously performed, was done with narrow scanning and gadolinium injection, showing a normal anatomy and size of the adenohypophysis for age and sex (−0.5 SDS) (22). A written informed consent was obtained from the proband's mother for herself and her daughter and from all the tested family members.

PCR amplification and sequencing of the GH1 gene

Genomic DNA was amplified by PCR using primers flanking the *GH1* gene and a proofreading *Taq* polymerase (Finnzymes, Espoo, Finland). The resulting 2.7-kb product was used as template for a series of nested PCRs using internal primers for the proximal promoter, the five exons, the four introns, and the untranslated regions of the *GH1* gene. PCR conditions and primer sequences are available upon request.

The resulting PCR products were visualized on a 2% agarose gel, purified from the gel using a Perfectprep Gel Cleanup system (Eppendorf, Hamburg, Germany) and used as template for sequencing in both senses with the Big-dye terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer.

The PCR product containing the IVS3 deletion in the heterozygous state was then cloned by the pMOS*Blue* T-vector kit (Amersham Biosciences, Little Chalfont, UK) following the manufacturer's instructions, and the two alleles were separately sequenced.

Synthesis and amplification of GH cDNA

Total RNA was extracted from lymphocytes by RNAwiz (Ambion, Austin, TX). cDNA was obtained by RT-PCR using the RETROscript Kit (Ambion) and amplified with three different couples of primers designed to amplify different parts of the wild-type cDNA (Fig. 1).

The products were extracted from a 2% agarose gel and directly sequenced.

Plasmid preparation and cell transfection

The 2700-bp PCR product encompassing the entire *GH1*, obtained from the proband's genomic DNA, was cloned into the pMOS plasmid (pMOSBlue T-vector kit; Amersham). Two clones containing the wild-type and the mutated allele, respectively, were selected by sequencing. A fragment of 2 kb containing the whole *GH1* sequence was released by digestion with *Bam*HI, purified from gel by the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany), and inserted into the pcDNA3.1(+) expression vector (Invitrogen, Chatsworth, CA) previously digested with *Bam*HI.

The *in vitro* mutagenized mut$\Delta 7$ was generated from the wild-type *GH1*-carrying plasmid by the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using two mismatch complementary oligonucleotides.

DH101F' competent cells were then transformed with the different constructs and grown on LB/ampicillin media.

Plasmid DNA was extracted (Plasmid Midiprep; QIAGEN), sequenced, and used for transient transfection of the rat pituitary GH4C1 cell line (American Type Culture Collection, Rockville, MD). Cells were grown to approximately 80% confluence in 35-mm dishes and transfected with 2 μg of each construct using the Fugene 6 transfection

TABLE 1. Clinical and hormonal data of the two IGHD II patients

	Age (yr)	HSDS	BMI SDS	GH peak (ng/ml)	IGF-I (ng/ml)	IGF-I SDS (ng/ml)	Free T_4 (pg/ml)	TSH (μU/ml)	Cortisol (ng/ml)
Child									
h	0.16	−3.4	0.0						
h	0.42	−4.9	−0.7						
A	0.58	−5.8	−0.8	0.4[a]/0.4[b]	<15	−5.0	11.5	2.92	75.5
B	1.6	−2.9	−2.1	NA	38.9	−2.5	12.4	5.9	80.2
Mother									
h	2.0	−5.0							
A	4.6	−6.9	−0.4	3.0[c]/3.6[c]	NA	NA	NA	NA	105.4
B	5.6	−5.6	−1.2	NA	NA	NA	NA	NA	NA
C	16	−3.0	4.3	NA	NA	NA	14.5	4.2	NA
D	38	−2.7	6.7	0.5[d]	50	−4.0	15.4	1.5	148.2
E	39	−2.7	5.7	NA	164	−1.0	16.2	1.3	NA

NA, Not available; BMI SDS, body mass index SD score calculated from BMI Rolland-Cachera charts in pediatric age (19) and from BMI charts from American National Health and Nutrition Survey data in adulthood age (20); HSDS, height SD score; h, historical data; A, diagnosis; B, after 1 yr of GH treatment; C, stop of GH; D, restart of GH; E, after 1 yr of GH restart. Normal ranges: peak GH above 10 μg/ml; IGF-I 55–327 (0–1 yr), 51–303 (1–2 yr), 109–284 (36–40 yr); free T_4 11–20 (0–1 yr), 9–17 (1–6 yr), 9.3–17 (>18 yr); TSH 1.36–8.8 (0–1 yr), 0.85–6.5 (1–6 yr), 0.27–4.2 (>18 yr); cortisol 50–250 (early morning).

[a] Arginine stimulation test.
[b] Dexamethazone stimulation test.
[c] Insulin stimulation test.
[d] GHRH + arginine stimulation test.

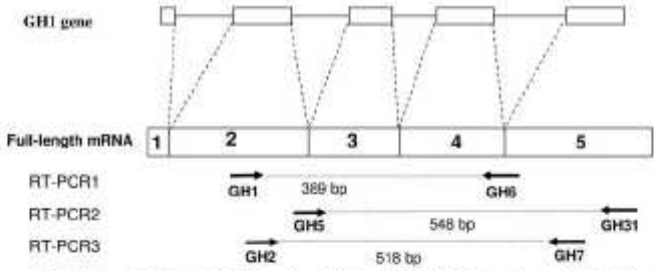

FIG. 1. Scheme of *GH1* pre-mRNA splicing showing the full-length mRNA and the RT-PCR products. RT-PCR1 performed with primers GH1 (5′-TTCCCAACCATTCCCTTATC-3′) and GH6 (5′-TCTTCCAGCCTCCCCATCAGC-3) amplifies part of exon 2, exon 3, and exon 4 allowing detection of exon 3 skipped products. RT-PCR2 performed with primers GH5 (5′-TACCAGGAGTTTGAAGAAGC-3′) and GH31 (5′-TCCAGGGC-CAGGAGAGGCACTGGGG-3′) amplifies exon 3, exon 4, and exon 5 allowing detection of exon 4 skipped products. RT-PCR3 performed with primers GH2 (5′-CGTCTGCACCAGCTGGCCTTT-3′) and GH7 (5′-AAGCCACAGCTGCCCTCCACAGA-3′) amplifies part of exon 2, exon 3, exon 4, and part of exon 5 allowing detection of both exon 3 and exon 4 skipped products.

reagent (Roche), following the manufacturer's instructions. Total RNA was purified from rat cells 48 h after transfection by RNeasy Mini Kit (QIAGEN) and reverse transcribed.

Results

Sequencing of the GH1 gene

Sequencing of the *GH1* gene in the proband of an IGHD II family revealed the presence of a heterozygous 22-bp deletion in IVS3 (Fig. 2). The deletion is flanked by a stretch of five Cs (nt 53–57 of the IVS3) at the 5′ side and of three Cs (nt 75–77) at the 3′ side. Because three of these Cs are retained in the deleted allele, it is not possible to exactly define the limits of the deletion. We arbitrarily named the mutation as IVS3 del+56–77. The deletion includes the IVS3 BPS, which extends from nt 67 to nt 73 of IVS3 (between −25 and −20 from the 3′ splice site) (11). The same deletion was detected in the proband's mother (subject II-2, Fig. 3A), whereas it was absent in all the tested unaffected family members.

To identify the origin of the deletion, all the family members were typed for previously described promoter polymorphisms (23, 24), and *GH1* haplotype transmission was deduced from family segregation (Fig. 3A). The 22-bp deletion was carried by a *GH1* haplotype (−278T/−75A/−57G/−6G/+3G) that subject II-2 inherited from her father (I-1). The same haplotype was transmitted without the deletion from I-1 to II-3, II-5, and II-6. Thus, IVS3 del+56–77 is a *de novo* mutation in II-2.

Analysis of GH1 transcripts in lymphocytes

Ectopic transcript analysis was performed on cDNA from lymphocyte mRNA of all the family members. Two couples of primers were specifically designed to detect an aberrant splicing involving either exon 3 (RT-PCR1) or exon 4 (RT-PCR2), immediately 5′ and 3′ of the intron with the deletion (Fig. 1).

After amplification with RT-PCR1 primers (Fig. 3B), a band of 389 bp corresponding to the wild-type full-length cDNA was detected in all the individuals. In addition, the affected subjects III-1 and II-2 showed a smaller band of 269 bp with approximately the same intensity of the full-length band. This 269-bp band was scarcely detectable in the normal-height members and corresponded to the 17.5-kDa isoform lacking the entire exon 3. A faint 344-bp band, corresponding to the 20-kDa isoform, was visible in all the subjects.

Most of the previously reported BPS mutations in humans lead to either the partial or total skipping of the exon that follows the mutated intron (25–28) or to the retention of the intron carrying the mutated BPS (29). The presence of aberrant splicing products involving exon 4 was therefore investigated by RT-PCR2 (Fig. 3C). A unique band of 548 bp, corresponding to the full-length transcript, was detected both in the affected and unaffected family members.

Thus, the information deriving from lymphocyte mRNA

FIG. 2. Sequence of GH1 IVS3 and of part of exons 3 and 4. The intron splicing enhancer (ISE) and the BPS are in *bold*. The putative cryptic splice site is *boxed*. In IVS3 and exon 4, the cryptic BPSs are *underlined*.

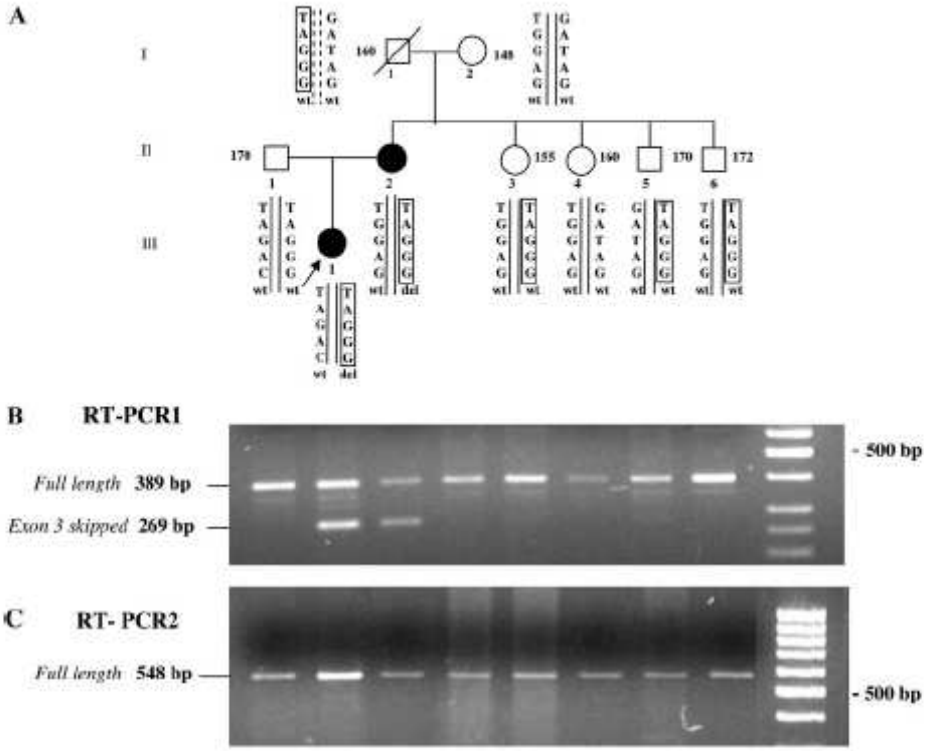

FIG. 3. A, Pedigree of the IGHD II family. *Black symbols* indicate affected individuals. For each member are indicated the stature (expressed in centimeters; only in unaffected individuals), the GH1 promoter haplotypes resulting, from top to bottom, from the allelic combinations of single-nucleotide polymorphisms −278G/T, −75A/G, −57T/G, −6A/G, +3G/C, and the IVS3 sequence (wt, wild-type; del, IVS3 del+56–77 mutation). From family segregation, it was possible to reconstruct both *GH1* haplotypes (indicated with a *broken line*) for the deceased individual I-1. The haplotype in which the *de novo* mutation occurred is *boxed*. The proband is indicated by an *arrow*. B, RT-PCR1 performed on lymphocyte mRNA; C, RT-PCR2 performed on lymphocyte mRNA. The gel lanes are positioned under the corresponding individual. All the visible bands were extracted from the gel and sequenced.

analysis was that the IVS3 deletion induces the production of a remarkable amount of mRNA lacking exon 3.

In vitro expression of the GH1 allele harboring the IVS3 del+56–77 mutation

To assess whether the skipping of exon 3 was the only aberrant product generated by the del+56–77 mutation, we analyzed the transcripts of the mutated allele in pituitary cells *in vitro*. To this purpose, we transfected GH4C1 rat pituitary cells with an expression vector containing either the normal *GH1* allele (wt-GH1) or the *GH1* allele with the IVS3 del+56–77 mutation (del-GH1). Because the two constructs were independently transfected, this analysis also allowed us to discriminate the products of the mutant and of the wild-type allele, which were coamplified in the lymphocyte cDNA.

The mRNAs from transfected cells were reverse transcribed and amplified with RT-PCR1 and RT-PCR2 primers. These are specific for the human *GH1* and did not amplify the rat mRNA from untransfected GH4C1 cells (Fig. 4, lane 2).

RT-PCR1 on the wt-GH1 mRNA yielded three bands corresponding to three *GH1* transcripts (Fig. 4A, lane 3) also detected in the lymphocytes of normal subjects, *i.e.* the main 22-kDa full-length transcript (389 bp), the 20-kDa isoform (344 bp), and the 17.5-kDa isoform (269 bp). On the contrary, the del-GH1 construct generated exclusively one intense band corresponding to the exon 3 skipped (17.5-kDa) transcript (Fig. 4A, lane 4).

When amplified with RT-PCR2 primers (Fig. 4B), which do not anneal to the exon 3 skipped product, the cDNA from cells transfected with wt-GH1 only contained the full-length transcript (lane 3), whereas del-GH1 generated three differently spliced mRNAs (lane 4) corresponding to 1) the full-length mRNA (548 bp), 2) a 462-bp transcript in which the first 86 bp of the exon 4 were deleted, and 3) a 383-bp transcript in which the 165 bp of exon 4 were absent. Thus, in

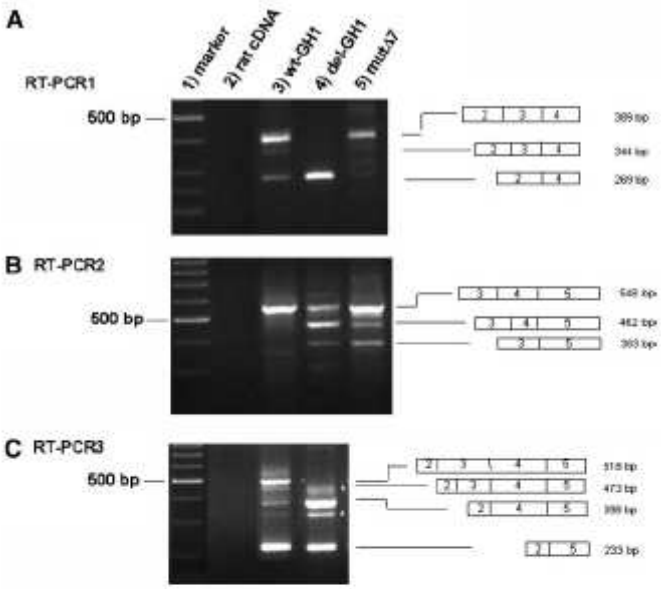

FIG. 4. RT-PCR performed on the mRNA extracted from GH4C1 pituitary cells: untransfected (rat cDNA; lane 2), transfected with the wild-type *GH1* gene (wt-GH1; lane 3), transfected with the *GH1* allele harboring the IVS3 del+56–77 mutation (del-GH1; lane 4), and transfected with the mutagenized *GH1* gene harboring the 7-bp BPS deletion (mutΔ7; lane 5). The RT-PCR was performed with the three couples of primers of RT-PCR1 (A), RT-PCR2 (B), and RT-PCR3 (C). Each PCR product was eluted from the gel and characterized by sequencing. The result is outlined on the *right* of each band. *, Heteroduplex molecules formed by mispairing of the 398- and 233-bp fragment.

pituitary cells transfected with the deleted allele, it was possible to detect two novel aberrantly spliced isoforms involving exon 4 that were not visible in the patients' lymphocytes and in pituitary cells transfected with the wild-type allele. Moreover, the RT-PCR2 primers showed that the mutant allele also produces a small amount of the full-length mRNA.

To investigate which of the above described alternatively spliced fragments was the direct consequence of the BPS deletion, the wt-GH1 plasmid was mutagenized to obtain a sequence lacking exclusively the 7 bp of the BPS consensus (mutΔ7). The cDNA produced by mutΔ7 showed only the aberrant exon 4 skipped fragments as expected for a BPS mutation (Fig. 4, A and B). As observed for del-GH1, the mutΔ7 construct also produced a visible amount of the full-length cDNA.

A third set of primers (RT-PCR3) located within exons 1–2 and exon 5 was designed to amplify all the isoforms in the same reaction (Fig. 1). Because we did not perform a quantitative PCR, we can only roughly estimate the relative amount of the different isoforms, with the limitation that smaller fragments are in any case preferentially amplified. Four fragments were visible on a 2% agarose gel in correspondence of the deleted allele (Fig. 4C, lane 4). The two bands of higher intensity corresponded to the 17.5-kDa isoform (398 bp) and to a smaller transcript lacking both exons 3 and 4 (233 bp), encoding the normal 11.3-kDa isoform (2). The latter is detectable only under the RT-PCR3 conditions (Fig. 1) and was visible with a similar intensity also among the wt-GH1 transcripts (Fig. 4C, lane 3). The two fainter bands corresponded to heteroduplex molecules formed by mispairing of the 398- and 233-bp fragments. The full-length cDNA and the transcripts with aberrant exon 4 skipping were not visible in this PCR. The wt allele (Fig. 4C, lane 3) yielded, besides the 233-bp fragment, a strong band corresponding to the full-length cDNA and two fainter bands corresponding to the 17.5- and 20-kDa isoforms.

Discussion

Mutations in the *GH1* splicing elements that result in an increased level of exon 3 skipped transcripts encoding the 17.5-kDa isoform are a common cause of autosomal dominant type II IGHD and severe growth failure (4–12).

Here we report a new mutation, consisting of a 22-bp deletion in the IVS3 (IVS3 del+56–77) causing IGHD II in a child and her mother. The deletion completely removes the reported BPS (AGCCAAT) (11) located between −20 and −25 upstream of the intron 3/exon 4 junction (Fig. 2) matching five of the seven residues of the weakly defined mammalian BPS ($Y_{81}NY_{100}T_{87}R_{81}A_{100}Y_{94}$, where Y represents pyrimidine, R represents purine, and N represents any base) (30). The A at position −21 (underlined) likely represents the highly conserved adenine involved in the lariat formation with the donor splice site.

Lymphocyte mRNA analysis showed that this mutation induced a splicing pattern analogous to that observed in most cases of IGHD II; the proband and her mother showed two principal mRNA species approximately in equal amount, namely the full-length mRNA (encoded by the normal allele) and an aberrant splicing product with the skipping of exon 3 (encoded by the mutant allele) (Fig. 3B).

Accordingly, the clinical phenotype determined by the 22-bp IVS3 deletion correlated with that observed in other IGHD II patients harboring splice site mutations. The phe-

notype of IGHD II patients is heterogeneous. On average, patients carrying splice site mutations show earlier age of onset of the growth failure that is progressive and more severe than patients carrying missense mutations (8, 31, 32). However, phenotype variability is present also among affected members of the same family (8, 31, 33) showing that the GH-dependent growth during childhood is individually different and modified by several factors, most of them still unknown. Both our patients showed an early postnatal onset of the growth failure that was progressive and severe. Patient III-1 showed an arrest of growth at the age of 4 months and was diagnosed at 7 months (−5.8 SDS); her mother was diagnosed at 4.6 yr (−6.9 SDS) and showed a height of −5 SDS at the age of 2 yr (no information is available at an earlier age). The clinical phenotype was somewhat less severe in the mother; GH secretion after provocative test, completely absent in the daughter, was severely but not completely reduced in the mother at the childhood age diagnosis, and the size of the pituitary gland was normal in the mother at the adult age, whereas it was at the lower limit size in the child. When retested in adulthood, the mother had no GH response to the strong GHRH plus arginine stimulation test, suggesting an exhausted function of the somatotropic cells along the years. Conversely, she did not develop other pituitary hormone deficiencies as described in some adult IGHD II patients (32).

The mutation described here is different from other intronic mutations detected in IGHD II families (4–12) because it leaves intact the splice junctions and the enhancers, whereas it completely removes the BPS. On the basis of what has been observed in other human diseases (25–29), we predicted that a mutation involving the BPS would induce the total or partial skipping of exon 4. However, lymphocyte mRNA analysis as well as analysis on the mRNA extracted from rat pituitary cells transfected with del-GH1 revealed that the major transcript was the exon 3 skipped. Two aberrantly spliced isoforms expected for a BPS mutation were detectable exclusively in experimental conditions where the exon 3 skipped mRNA was excluded from the possible targets (Fig. 4B, lane 4). The first isoform, lacking the entire exon 4, encodes for a putative mature peptide depleted of amino acids 72–126. The other was an mRNA devoid of the first 86 bases of exon 4, and it causes a frameshift leading to a putative protein differing from the wild type from amino acid 72 and prematurely truncated at residue 77. It likely results from the use of a cryptic acceptor splice site at position 85–86 of exon 4. The recognition of this splice site might be mediated by the presence of a putative cryptic BPS (CGC-CAAC), matching six of the seven bases of the consensus sequence, located between nt 78 and 84 of exon 4 (Fig. 2).

The content of the abnormal mRNA lacking exon 4, or a part of it, is likely very low in the patients' lymphocytes compared with the normal-sized and to the exon 3 skipped mRNA and can therefore be visualized only under specific experimental conditions. One of the reasons for the low content, at least for the prematurely truncated isoform, might be its rapid degradation owing to nonsense-mediated mRNA decay (34).

The two aberrant splicing events involving exon 4 are the direct consequence of the missing BPS, as demonstrated by the mutagenized construct lacking exclusively the 7 bp of the BPS consensus (Fig. 4B, lane 5). Conversely, the high content of the exon 3 skipped isoform remains to be explained. In a recent report, Ryther and colleagues (35) demonstrated that the overall size of IVS3 is crucial for exon 3 inclusion in the mRNA and showed that deletions of 12–14 bp are sufficient to increase exon 3 skipping. They suggested that this is because the decreased IVS3 size rather than the deletion of specific sequences. We can thus speculate that the main effect of the 22-bp deletion, *i.e.* the increased exon 3 skipping with production of the IGHD II-specific pathological amount of the 17.5-kDa isoform, is the consequence of the remarkably decreased size of IVS3 that overwhelms the effect caused by the BPS deletion.

However, in the deletion mutants reported by Ryther *et al.* (35), the IVS3 BPS was unaltered and mediated the lariat formation with the IVS2 donor splice site. In our case, it is not clear how the two mutants lacking the IVS3 branch site (namely the del+56–77 allele and the mutagenized mutΔ7) can correctly process the IVS3 splicing necessary for the exon 3 skipped and the full-length mRNA (36). A similar situation was reported for other disease-causing BPS mutations where the mutated allele also produced the wild-type isoform in addition to the isoform with an aberrant splicing (29, 37, 38). An obvious explanation for these observations is the use of an alternative BPS elsewhere in the IVS3 efficiently replacing the deleted one. Two sequences located between −40/−46 and −61/−67 from the GH1-IVS3 acceptor splice site in the deleted allele, within a distance compatible with a BPS function (39), match the consensus more than others. However, the del-GH1 plasmid mutagenized at the highly conserved A of either or both of these two cryptic BPSs yielded the same splicing pattern (data not shown) as the nonmutagenized construct (Fig. 4). It is thus conceivable that other sequences with less homology to the consensus might act as a cryptic BPS. In fact, although several reported BPS mutations cause severe phenotypes, it has also been demonstrated that a mutated BPS can be replaced by a cryptic BPS with no resemblance to the consensus branch site, apart from the A residue necessary for the lariat formation (40).

In conclusion, despite the absence of the canonical BPS, the exon 3 skipped is the principal transcript and the cause of IGHD II in our patients. Thus, the context in which the BPS mutation is located, *i.e.* the 22-bp deletion, has a greater phenotypic influence than the BPS mutation itself. It is unlikely that the two isoforms with an aberrant splicing of exon 4 also contribute to the IGHD II phenotype because they are presumably poorly expressed.

Acknowledgments

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A Functional Common Polymorphism in the Vitamin D-Responsive Element of the *GH1* Promoter Contributes to Isolated Growth Hormone Deficiency

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Context: Causal mutations have been detected only in a minority of isolated GH deficiency (IGHD) patients. Idiopathic IGHD might be the result of the interaction between several low-penetrance genetic factors and the environment.

Objective: The aim of this study was to test the contribution to IGHD of genetic variations in the *GH1* gene regulatory regions.

Design and Patients: A case-control association study was performed including 118 sporadic IGHD patients with a nonsevere phenotype (height −4/−1 SD score and partial GH deficiency) and two control groups, normal stature (n = 200) and short-stature individuals with normal GH secretion (n = 113). Seven single-nucleotide polymorphisms in the *GH1* promoter, one in the IVS4 region, and two in the locus control region were analyzed.

Results: The −57T allele within the vitamin D-responsive element showed a positive significant association when comparing patients with normal ($P = 0.006$) or short stature ($P = 0.0011$) controls. The genotype −57TT showed an odds ratio of 2.93 (1.44–5.99) and 2.99 (1.42–6.31), respectively. The functional relevance of the −57 variation was demonstrated by the luciferase assay in the presence of vitamin D. The vitamin D-induced inhibition of luciferase activity was significantly ($P = 0.012$) stronger for the promoter haplotype carrying the associated variation −57T [haplotype #1 (hp#1)] with respect to hp#2, bearing −57G. Replacement of the T with a G at −57 on hp#1 abolished the repression, demonstrating that the T at position −57 is necessary to determine the greater vitamin D-induced inhibitory effect of hp#1. EMSA experiments showed a different band-shift pattern of the T and G sequences.

Conclusion: The common −57G→T polymorphism contributes to IGHD susceptibility, indicating that it may have a multifactorial etiology. (*J Clin Endocrinol Metab* 93: 1005–1012, 2008)

AQ: A

High-penetrance mutations in the *GH1* gene have been found in severe and in familial forms of isolated GH deficiency (IGHD) (1, 2). However, most IGHD subjects present with a nonsevere GH deficiency (GHD) (3), no family history, and no deleterious mutations in the *GH1* coding sequence or in other genes known to be involved in GH production (*e.g. GHRHR*) (4). The pathogenic mechanisms for this idiopathic IGHD have not as yet been clarified. In the present paper, we considered the hypothesis that IGHD, especially when excluding extreme phenotypes, can be determined by the interaction of several low-

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Abbreviations: CI, Confidence interval; GHD, GH deficiency; hp#1, haplotype #1; IGHD, isolated GHD; LCR, locus control region; LD, linkage disequilibrium; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; NF-1, nuclear factor-1; OR, odds ratio; SDS, SD score; SNP, single-nucleotide polymorphism; VDR, vitamin D receptor; VDRE, VDR-binding element; VDRE cons, VDRE consensus sequence; VDRE mut, VDRE mutant sequence.

<zjs;> • <zjss;10,11>
<zshorttitle>A Functional Common Polymorphism in the Vitamin D-Responsive Element of the GH1 Promoter Contributes to Isolated Growth Hormone Deficiency

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penetrance genetic factors and the environment rather than by the presence of a major-effect mutation, thus behaving as a multifactorial trait.

GH secretion is dependent on a complex network of intracellular and extracellular signals that regulate the hormone synthesis and release. The regulation of *GH1* gene expression has been characterized in detail, and both ubiquitous and pituitary-specific *cis*/*trans* elements have been identified in the *GH1* proximal promoter (Fig. 1). Transcriptional factors controlling the *GH1* basal expression include nuclear factor-1 (NF-1) (5), specific protein-1 Sp1 (6), Zn-15 (7), vitamin D receptor, (8), and cAMP response element-binding protein CREB, a protein that interacts with cAMP-responsive elements (9). The pituitary-restricted *GH1* expression is mainly controlled by the pituitary-specific factor Pit-1 (10), a POU-homeodomain protein, which binds to two highly conserved elements in the *GH1* proximal promoter. Three additional Pit-1 binding sites in a locus control region (LCR) located 14.5 kb upstream the *GH1* gene are necessary to confer high-level somatotropic-specific GH expression (11).

The *GH1* proximal promoter is highly polymorphic, with 15 single-nucleotide polymorphisms (SNPs) in the 500 bp upstream of the transcription initiation site (12, 13), giving rise to at least 40 different haplotypic combinations (14).

Functional studies suggest that the *GH1* promoter polymorphisms might influence the circulating GH level through their effect on transcription regulation. It has been shown that the different promoter haplotypes induce a 12-fold range of expression level in a reporter gene assay (14). We recently demonstrated that the *GH1* promoter variation −75A→G, within the proximal Pit-1 binding site, influences the transcription *in vitro*, although it is not associated with a pathologically decreased GH secretion *in vivo* (15).

The involvement of *GH1* polymorphisms has been investigated and found in several pathological conditions, including breast and colorectal cancer (16–20), accelerated bone loss (21), hypertension and stroke (22). Conversely, the only reported association study between *GH1* polymorphisms and IGHD was performed in a small cohort of Japanese patients with mild GH deficiency in which the A allele of the intronic SNP IVS4+90A→T was significantly increased with respect to individuals with normal GH secretion (23).

Here we report the first systematic association study with *GH1* SNPs in a Caucasoid population of idiopathic sporadic IGHD patients and matched controls. The selected cases presented with a nonsevere form of IGHD. A significant association with IGHD of a polymorphism within the vitamin D receptor (VDR)-binding element (VDRE) was detected. Functional analysis demonstrated that this variation has a significant influence on the transcriptional activity of the *GH1* promoter.

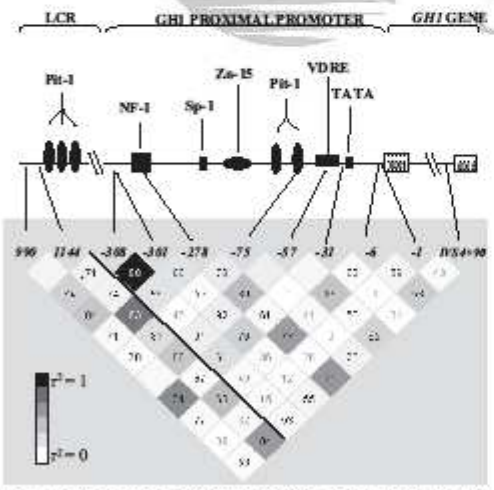

FIG. 1. Location and pairwise LD values of the SNPs in the *GH1* gene and the 14.5-kb upstream LCR tested for association with IGHD. The promoter SNPs are numbered considering +1 the first transcribed nucleotide and the IVS4 SNP is the IVS4 90th nucleotide (according to the sequence with accession number M13438, http://www.ncbi.nlm.nih.gov/). The LCR SNPs are numbered according to the sequence AF010280. The position of the binding sites for transcriptional factors is shown. Reported LD values were calculated in normal-stature controls. D′ values are shown in the *boxes*. D′ values = 100 are indicated as *empty boxes*. The r^2 value is indicated by the *box color intensity*.

Subjects and Methods

Subjects

A total of 118 sporadic patients with IGHD, 113 short-stature individuals with normal GH secretion (normal short), and 200 normal-stature individuals, all belonging to the Italian population, were included in the genetic association analysis. The IGHD patients and normal-stature controls in part overlap with those included in a previous work (15).

The short-stature subjects were referred to the clinical centers because they had a height less than or equal to −2 SD score (SDS) (24) and/or a height velocity over 1 yr of less than −1.5 SDS. Patients with a known postnatal cause of acquired hypopituitarism were excluded. Skeletal maturation was estimated as bone age (radius, ulna, and short bone) (25) by an auxologist. The clinical, auxological, and radiological characteristics of the included subjects are shown in Table 1. They were all evaluated for GH serum level either after two consecutive classical provocative tests (with arginine or clonidine or insulin) or after one double stimulus with GHRH plus arginine (26). Traditionally, a diagnosis of GHD is supported by GH peaks less than 10 ng/ml after both consecutive stimuli (27) or less than 20 ng/ml after the double provocative test (26). On this basis, 118 subjects were diagnosed as GHD and 113 as short-stature individuals with normal GH secretion (normal short). The short stature in the latter was classified as familial short stature (n = 31), idiopathic short stature (n = 73), and constitutional delay of growth (n = 9). The GHD patients had a mean secretion peak of 4.2 ± 2.2 ng/ml after the single stimuli (n = 103) or 9.2 ± 5.7 ng/ml after the double provocative test (n = 15). Normal short individuals displayed a GH peak of more than 10 ng/ml after stimulus with either arginine or clonidine or insulin or more than 20 ng/ml after the GHRH+arginine test. A possible miscategorization of patients with a borderline phenotype cannot be excluded.

None of the GHD patients was deficient for other pituitary hormones, had a documented family history of the disease or consanguineous parents, or carried deleterious mutations in the *GH1* and *GHRHR* genes.

Fifty-six IGHD patients underwent pituitary magnetic resonance imaging, and 33 of them had an abnormal finding (most of the images revealed pituitary hypoplasia and some ectopic posterior pituitary). None of the normal short underwent MRI, because of lack of any clinical indication.

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TABLE 1. Clinical characteristics of the patients at the time of diagnosis

	Males			Females		
	IGHD (n = 82)	Short stature (n = 62)	P	IGHD (n = 36)	Short stature (n = 51)	P
Prepubertal (n)	67	34	0.009	31	31	0.02
Pubertal (n)	15	28		5	20	
Mean age ± SD (yr)	8.2 ± 4.2	10.2 ± 3.5	0.006	7.4 ± 3.0	9.6 ± 3.4	0.003
Mean Δ bone age[a] SDS ± SD	−1.5 ± 1.2	−1.2 ± 1.3	0.2	−1.4 ± 1.0	−0.9 ± 1.1	0.06
Mean height SDS ± SD (range)	−2.4 ± 0.9 (−4.3 to −1.0)	−2.3 ± 0.6 (−3.2 to −1.0)	0.3	−2.5 ± 1.5 (−6.0 to −1.0)	−2.4 ± 0.8 (−4.1 to −0.7)	0.5
Height SDS ≤ −4 (n)	3	0	0.25	5	1	0.08
Height SDS ≤ −2 (n)	50	45	0.20	17	38	0.02
Mean target corrected height[b] SDS ± SD	−1.7 ± 1.2	−1.0 ± 0.8	0.001	−1.2 ± 1.0	−0.9 ± 0.9	0.2
Mean growth velocity[c] SDS ± SD	−2.03 ± 2.1	−2.3 ± 1.1	0.3	−1.7 ± 2.0	−2.8 ± 1.2	0.002

[a] Difference between the chronological age and the bone age at diagnosis.

[b] Target height calculated from the parents' stature.

[c] Over 1 yr.

The normal-stature control individuals included university and hospital staff and medical students and were not tested for GH secretion levels.

A written informed consent was obtained from the patients' parents and from the normal-stature controls.

PCR amplification and sequencing

A 2.7-kb specific *GH1* fragment was amplified from genomic DNA with primers 5′-CCAGCAATGCTCAGGGAAAG-3′ (forward) and 5′-TGTCCCACCGGTTGGGCATGGCCAGGTAGCC-3′ (reverse) and used as template for a nested PCR with primers 5′-TTAAACATGCGGGGAGGAA-3′ (forward) and 5′-GCCCCCGTCCATCTACAGGT-3′ (reverse) that amplify the *GH1* gene from −397 to +148 (considering +1 the transcription initiation; sequence M13438, http://www.ncbi.nlm.nih.gov/).

The LCR was amplified with primers 5′-TCAATATTTTCTGGGGTACAGG-3′ (forward) and 5′-CTAGGCCTCGGACCTGATA-3′ (reverse) from nucleotides 913-1593 of the sequence AF010280 (28).

The promoter and LCR fragments were directly sequenced using an automated ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA).

The IVS4 fragment was amplified with primers 5′-CCCACTGACTTTGAGAGCTG-3′ and 5′-CATGTCCTTCCTGAAGCAGT-3′ from the 2.7-kb amplicon and the IVS4+90A→T polymorphism was genotyped by denaturing HPLC heteroduplex analysis on a Transgenomic HPLC Instrument (29) at a column temperature of 59.9 C with a 58–68% gradient of buffer B (25% acetonitrile, 01 M triethylamine acetate supplied by Transgenomic, Omaha, NE).

SNPs 990G→A and 1144A→C in the LCR were genotyped by the SNaPshot method (Applied Biosystems) following the manufacturers' instructions using the internal primer 5′-ATTTCTGAGATTTTAGC-3′ and 5′GCACACGTGTTTGTGGGGGG3′, respectively. The reactions were visualized on the automated ABI PRISM 3100 sequencer (Applied Biosystems).

Plasmid construction and transfection

GH1 promoter haplotypes #1 and #2 (hp#1 and #2) were cloned into the luciferase reporter vector pGL3 basic (Promega, Madison, WI) as previously described (15). The construct bearing hp#1 was used as the template into which the T at −57 was substituted with a G (hp#1-MUT1) and the G at −278 was substituted with a T (hp#1-MUT2) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

The haplotype activity was evaluated by transfection in the MCF-7 human breast adenocarcinoma cell line (American Type Culture Collection, Rockville, MD). A total of 1.5×10^5 cells grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin were seeded into six-well tissue culture plates in 2.0 ml medium and allowed to attach overnight. Transient transfections were carried out using 1 μg of each construct with Fugene 6 (Roche Diagnostics, Indianapolis, IN). Twenty-four hours after transfection, the cells were washed with PBS and cultured in serum-free medium supplemented with 500 nM 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (Sigma-Aldrich, St. Louis, MO) or an equal volume of vehicle (ethanol). After 48 h, the cells were lysed with the buffer of the Luciferase Assay System (Promega). The luciferase activities were measured using a luminometer (Anthos Lucy1; BioTek, Winooski, VT) and normalized with respect to protein concentration (Bradford assay; Bio-Rad, Hercules, CA).

EMSA

Nuclear extracts were prepared from MCF7 cells grown for 48 h in serum-free medium containing 500 nM 1,25$(OH)_2D_3$ using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium).

Double-stranded oligonucleotides were labeled with γ-[^{32}P]ATP using a T4 polynucleotide kinase (Promega) and purified on a Microspin G-25 column. Five micrograms of nuclear extract were incubated 30 min in binding buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 2 μg poly(dIdC), 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride] with 15 fmol of the ^{32}P-labeled probes. The reaction mixtures were run on a 5% nondenaturing polyacrylamide gel (19:1 acrylamide to bisacrylamide) in a 0.25× Tris-boric acid running buffer at 100 V for 2.5 h.

Statistical analysis

The association analysis was performed by binary logistic regression adjusted for sex. The strength of the association was evaluated by the odds ratio (OR) with 95% confidence intervals (CI). Statistical significance was assessed by the likelihood ratio test. When not specified, the reported *P* values were not corrected for the number of comparisons. Analyses were carried out using SAS version 8.01 and STATA version 8.

Pairwise linkage disequilibria (LD) between SNPs were calculated by D′ and r^2 using the Haploview program version 3.2. The same software was used to estimate the haplotype structures and their frequencies from unphased genotype data.

The *t* test was used to compare the distribution of age, height SDS, bone age SDS, target corrected height SDS, and mean growth velocity SDS between IGHD and normal short.

The transfection experiment data are represented as the mean ± SD. All values are expressed as a percentage of the hp#1 mean value. The Mann-Whitney *U* test was used to compare the relative luciferase activity between two groups.

Results

Association between *GH1* SNPs and IGHD

The *GH1* region including the proximal promoter, the 5′-untranslated region, and exon 1 were sequenced in 118 unrelated

TABLE 2. Comparison of genotype frequencies of associated *GH1* SNPs in IGHD patients and normal-stature and normal short controls

SNP	Genotype	IGHD (n = 118) n (%)	Normal stature (n = 200) n (%)	$P_{overall}$[a]	OR[a] (95% CI)	Normal short (n = 113) n (%)	OR[b] (95% CI)	$P_{overall}$[b]
−278T→G	TT	19 (16.1)	47 (23.5)		1 (reference)	25 (22.1)	1 (reference)	
	GT	49 (41.5)	100 (50.0)		1.13 (0.58–2.20)	51 (45.1)	1.27 (0.62–2.61)	
	GG	50 (42.4)	53 (26.5)		2.15 (1.08–4.30)	37 (32.7)	1.70 (0.81–3.56)	
				0.03				0.34
−57G→T	GG	38 (32.2)	77 (38.5)		1 (reference)	54 (47.8)	1 (reference)	
	GT	49 (41.5)	102 (51.0)		0.98 (0.57–1.68)	43 (38.1)	1.80 (0.98 to −3.27)	
	TT	31 (26.3)	21 (10.5)		2.93 (1.44–5.99)	16 (14.1)	2.99 (1.42–6.31)	
				0.004				0.010
IVS4+90A→T	AA	18 (15.2)	44 (22.0)		1 (reference)	26 (23.0)	1 (reference)	
	AT	52 (44.1)	106 (53.0)		0.96 (0.49 to −1.89	48 (42.5)	1.54 (0.75–3.18)	
	TT	48 (40.7)	50 (25.0)		1.99 (0.98 to −4.06)	39 (34.5)	1.69 (0.80 to −3.54)	
				0.02				0.36

[a] IGHD vs. normal stature.

[b] IGHD vs. normal short.

short-stature individuals with IGHD, 200 normal-stature individuals, and 113 short-stature individuals with normal GH secretion (normal short). Fourteen SNPs were detected, namely −308G→T (rs1811081), −301G→T (rs2011732), −278T→G (rs2005171), −168G→T (rs2727338), −75A→G (rs11568828), −57G→T (rs2005172), −31delG (rs41299067), −6A→G (rs6171), −1A→T→C (rs695), +3C→G (rs6175), +16A→G (rs282699), +25A→C (rs6172), +59A→C (rs6173), and Thr3Ala (rs2001345), corresponding to those already described in this region (12–14, 16, 30). All the individuals were also genotyped for the IVS4 polymorphism IVS4+90A→T (rs2665802).

Only SNPs with minor allele frequency higher than 2% were considered (Fig. 1). Their pairwise LD values are shown in Fig. 1. Because an almost perfect LD was observed between polymorphisms at −308 and −301, ($D' = 0.98$; $r^2 = 0.9$), only one of them, namely −308G→T, was tested for association.

Allele frequencies of the eight selected SNPs were compared between IGHD, normal-stature, and normal short individuals. Four alleles (−278G, −57T, −1A, IVS4+90T) showed a nominally significant positive association with IGHD when comparing patients with normal-stature individuals. Of these, only the association with the −57T allele remained significant when P values were corrected for the number of analyzed SNPs (n = 8; corrected $P = 0.048$). The association with −57T was confirmed (corrected $P = 0.008$) when the patients were compared with normal short individuals, which represent an independent pediatric control group matched for stature (Table 1).

Genotype frequencies were consistent with those expected from Hardy-Weinberg equilibrium for all the tested SNPs in the three panels. The overall genotype distribution was significantly different between IGHD and normal-stature controls for −278T→G, −57G→T, and IVS4+90A→T (Table 2). An OR value higher than 1 was observed only for the homozygous genotypes −278GG (OR = 2.15), −57TT (OR = 2.93), and IVS4+90TT (OR = 1.99), suggesting a recessive effect of the associated variations. Only the association with −57 was confirmed when comparing the IGHD genotype distribution with that of normal short controls (Table 2). T2

When considering the haplotypic combinations of the −278T→G, −57G→T and IVS4+90A→T SNPs, the GTT combination (hp#1; Table 3) was the commonest in the patients (42.9%) and the second most common in both control groups (29.4 and 26.5%). Notably, this haplotype was more significantly associated with IGHD than the allele −57T when comparing the patients both with normal-stature ($P = 6.5 \times 10^{-4}$) and with normal short ($P = 3.6 \times 10^{-4}$) controls. The homozygous diplotype #1/1 was present in 22.0% IGHD *vs.* 6.5% normal stature ($P = 9.0 \times 10^{-5}$; OR = 4.07; 95% CI = 1.90–8.80) and 8.8% normal short individuals ($P = 9.8 \times 10^{-5}$; OR = 2.91; 95% CI = 1.26–6.86). T3

TABLE 3. Comparison of haplotype frequencies in IGHD patients and normal-stature and normal short controls

Haplotype no.	−278T→G	−57G→T	IVS4+90A→T	IGHD (n = 118)	Normal stature (n = 200)	P	OR[a] (95% CI)	Normal short (n = 113)	P	OR[b] (95% CI)
1	G	T	T	0.429	0.294	6.5×10^{-4}	1.81 (1.28–2.57)	0.265	3.6×10^{-4}	2.07(1.37to−3.12)
2	T	G	A	0.320	0.392			0.369		
3	G	G	T	0.156	0.150			0.214		
4	T	G	T	0.041	0.061			0.068		
5	G	T	A	0.034	0.035			0.056		
6	G	G	A	0.012	0.037			0.017		
7	T	T	A	0.007	0.021			0.0		
8	T	T	T	0.001	0.011			0.010		

Only statistically significant P and OR values are indicated.

[a] IGHD vs. normal stature.

[b] IGHD vs. normal short.

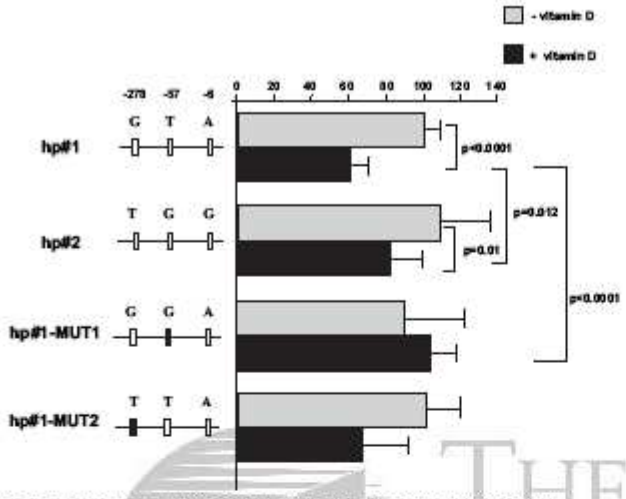

FIG. 2. Reporter gene assay of pGL3-hp#1 and pGL3hp#2 constructs and of pGL3-hp#1 mutagenized at position −57 (hp#1-MUT1) or −278 (hp#1-MUT2) transfected into MCF7 cells, performed in the presence of $1,25(OH)_2D_3$ (*black histograms*) or ethanol (*gray histograms*). On the *left* are reported the positions at which the promoter haplotypes differ. The mutagenized nucleotide on hp#1-MUT1 and hp#1-MUT2 is indicated as a *filled rectangle*. The transcriptional activity of the reporter constructs is normalized to that of hp#1 in the absence of $1,25(OH)_2D_3$. Figures are the mean ± SD of the normalized activity from four experiments done in triplicate.

Association between LCR SNPs and IGHD

Previous reports show that LD in the *GH1* gene extends to the LCR located 14.5 kb upstream to *GH1* (Fig. 1) (14, 20). To test whether the association with the −57G→T SNP was secondary to an association with a causal variation in the LCR, we screened 50 IGHD patients, including 15 hp#1/1 homozygotes, and 20 normal-stature controls for sequence variations in the LCR between nucleotides 911 and 1593 including the three Pit-1 binding sites. We detected three common SNPs already described in this region, namely 990G→A, 1144A→C, and 1194C→T (14), and two rare variations (1497C→T in one patient and 1498G→C in one control), none of which fall within Pit-1 sites. The polymorphisms at positions 1144 and 1194 were in perfect LD, as described (14, 20). All the IGHD patients and the two control panels were thus genotyped for 990G→A and 1144A→C. No association with IGHD was detected, either considering allele or genotype frequencies or their haplotypic combinations. LD values of the two LCR with the *GH1* SNPs are shown in Fig. 1. A strong LD was detected between the *GH1* hp#1 and the LCR haplotype 990G, 1144A ($D' = 0.95$; $r^2 = 0.56$). The inclusion of the LCR SNPs did not increase the strength of the association with hp#1 or the #1/1 diplotype.

Functional analysis of the *GH1* promoter haplotypes

The functional relevance of the associated haplotype was tested through its capacity of modulating the expression of a reporter gene (luciferase) after transfection in the mammary adenocarcinoma MCF7 cell line. This is a human lineage expressing both GH and VDR and largely used as a model to study *GH1* gene expression (8, 31). Two pGL3-based plasmids were constructed harboring either the IGHD-associated hp#1 or the hp#2 haplotypes. These haplotypes differ at positions −278, −57, and −6 (Fig. 2). The polymorphism at −278 lies in a NF-1 target element needed for transactivation (5), whereas the −57 polymorphism lies in a sequence bound by the VDR and involved in the vitamin D-dependent repression of GH expression (8). The VDR binds to its DNA cognate site after activation by its ligand, the metabolite of vitamin D, $1,25(OH)_2D_3$. Thus, we analyzed the transcription activity of the two constructs both in the presence and absence of $1,25(OH)_2D_3$. Vitamin D treatment repressed the activity of both *GH1* promoters (Fig. 2), confirming the direct involvement of activated VDR in the control of *GH1* gene expression. However, the promoter carrying the IGHD-associated hp#1 was significantly ($P = 0.012$) and consistently more repressed than hp#2. Because the difference was observed only in the presence of $1,25(OH)_2D_3$, we hypothesized that this was due to the SNP −57 within the VDRE. To validate this hypothesis, hp#1 was mutagenized by replacing the −57T with a G (hp#1-MUT1), and the activity of this *in vitro* created haplotype, which differed from hp#1 only at position −57, was compared with that of hp#1 (Fig. 2). As a control, the same comparison was done with hp#1 mutagenized only at position −278 by replacing the G with a T (hp#1-MUT2). In the absence of $1,25(OH)_2D_3$, both mutagenized promoters showed an activity comparable to that of hp#1. Conversely, in the presence of $1,25(OH)_2D_3$, hp#1-MUT1 was no longer inhibited, whereas hp#1-MUT2 did not differ from hp#1.

These results clearly demonstrate that the T at position −57 is necessary for the vitamin D-induced reduction of the transcriptional activity in the hp#1 context and consequently is responsible for the greater, vitamin D-induced inhibitory effect of this haplotype.

EMSA

To determine whether the SNP at −57 affects the binding of VDR to the *GH1* VDRE site, we performed EMSA using vitamin D-treated MCF7 nuclear extracts and probes corresponding to the *GH1* VDRE sequence containing either the T or the G at position −57 (−57T and −57G oligos, respectively; Fig. 3). As controls, we used a high-affinity VDRE consensus sequence (VDRE cons) and a related sequence modified at conserved positions [VDRE mutant sequence (VDRE mut)].

Three complexes (C1–3) were observed with the VDRE cons probe (Fig. 3, lane 2). C1 and C2 are likely nonspecific complexes. In fact, C2 bound both the VDRE cons and the VDRE mut probes (Fig. 3, lanes 1 and 2), and both complexes were similarly titrated by the VDRE cons and VDRE mut competitors

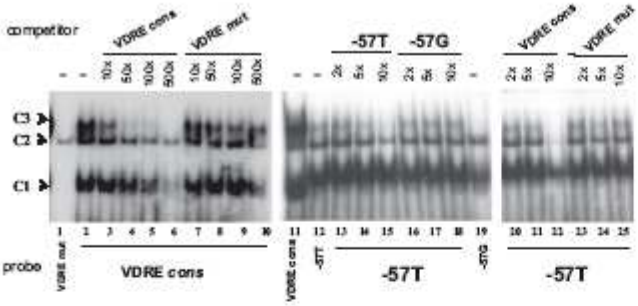

FIG. 3. EMSA performed with nuclear extract from MCF7 cells grown in the presence of vitamin D. Labeled probes used for the EMSA are indicated in the *lower* part of the figure. They include the two allelic sequences spanning position −57 (−57T, 5′-AGGTGGGGTCAACAGTGGGA-3′, and −57G, 5′-AGGTGGGGCAACAGTGGGA-3′) and two control sequences, namely a consensus VDR binding sequence (VDRE cons oligo: 5′-AGCTTCAGGTCAAGGAGGTCAGAGAGC-3′) and a VDRE mutant oligonucleotide (VDRE mut oligo: 5′-AGCTTCAGAACAAGGAGAACAGAGAGC-3′). For competition experiments, the radiolabeled probes were incubated with increasing amounts of the unlabeled probes. The cold oligos used as competitors and their molar excess with respect to the labeled probes are indicated in the upper part of the figure. The complexes formed by the labeled probes are indicated as C1, C2, and C3. Results suggest that VDR is likely contained in the more slowly migrating C3 complex. In fact, the C3 complex was formed in the presence of the VDRE cons probe (lane 2) but not of VDRE mut (lane 1) and binding to the VDRE cons probe was efficiently competed by VDRE cons (lanes 3–6) but not by VDRE mut (lanes 7–10). When the nuclear extract was incubated with the two allelic sequences at position −57, the C3 complex was formed in the presence of −57T (lane 12) but not of −57G (lane 19). When binding to the −57T probe was challenged with competitor DNAs, the C3 complex was efficiently competed by the −57T (lanes 13–15) and the VDRE cons (lanes 20–22) oligos but not by −57G (lanes 16–18) and VDRE mut (lanes 23–25).

only at a high molar excess (Fig. 3, lanes 3–6 and 7–10). In contrast, complex C3 was specifically competed by VDRE cons but not by VDRE mut cold oligos (Fig. 3, lanes 3–6 and 7–10), indicating that this shifted band results from specific binding of the VDR present in the MCF7 cell extract. Three complexes with the same electrophoretic mobility as C1–C3 were observed with the −57T probe (Fig. 3, lane 12). Notably, the slower migrating C3-like complex was not detectable with the −57G probe (Fig. 3, lane 19). The binding properties of these complexes were characterized by competition. Binding of the C3 complex to the −57T probe was efficiently competed by the −57T (lanes 13–15) but not by the −57G cold oligo (lanes 16–18), even in the presence of a 200-fold excess of −57G competitor (data not shown). In contrast, C1 and C2 complexes were not titrated by either competitor DNA, suggesting that they are either nonspecific binding complexes or complexes with a low affinity for the *GH1* VDRE sequence (see below). To confirm that the C3 complex contains VDR activity, we challenged its binding to the −57T probe by VDRE cons or VDRE mut competitor DNAs (Fig. 3, lanes 20–25). The C3 complex was efficiently and specifically competed by the VDRE cons DNA, demonstrating that the VDRE cons and the −57T oligos bind the same proteins in this complex. However, the VDRE cons binding affinity was higher than that of the −57T sequence as indicated by the different molar excess of the VDRE cons competitor needed to abolish the C3 complex formed by the VDRE cons and −57T probes (Fig 3, lanes 4 and 22, respectively). In addition, it must be noted that the binding of complex C2 to the −57T probe was competed by a relatively low amount of the high-affinity VDRE cons oligo (lane 22), indicating that this complex might also contain VDR, but in a form binding the *GH1* VDRE element with a lower affinity (*e.g.* as a heterodimer complexed with other factors).

Discussion

Causal mutations have been detected in a minority of IGHD patients (32). We tested the hypothesis that low-penetrance genetic variations with a quantitative effect on *GH1* transcription might contribute to IGHD. We thus performed an association study between *GH1* polymorphisms and IGHD in the Italian population. The included IGHD patients were all sporadic, and most of them presented with a partial GHD.

We detected a positive association with IGHD of the −57T allele and of two alleles, namely −278G and IVS4+90T, in strong LD with it. Contrary to our results, a significant association was detected in the Japanese population with the IVS4+90 allele A, that the authors described to be in complete LD with the alleles −278T and −57G (23). Thus, in the Italian and Japanese population, the association with IGHD concerned different haplotypes, corresponding to our hp#1 and #2, respectively (Table 3). The discrepant results obtained in the Japanese population could be driven by another causal variation, in LD with the IVS4+90 allele A, which arose independently in the Japanese population. However, it must also be considered that the Japanese patient cohort was small (43 patients) and was recruited with less stringent inclusion criteria than ours.

Our results, both from the association analysis and from functional experiments, point to a primary involvement of the −57T sequence in the VDRE for the low GH production of the IGHD patients.

The VDRE in the human *GH1* promoter acts as a negative regulator of *GH1* transcription (8). It is an imperfect direct repeat [GGG(T/G)CAACAGTGGGA] separated by three bases that binds to a homodimeric VDR complex (8). The −57 SNP corresponds to position 4 of the 5′ half-site, which is occupied by a T in the majority of the VDREs in different human gene promoters (33). This highly conserved base forms a hydrogen bond with Glu42 in the activated VDR, as indicated by crystallographic studies of the VDR complexed to the VDRE in the mouse osteopontin promoter (that has a 5′ half-site very similar to that of the human *GH1*) (34). The determining role of position −57 in the *GH1* promoter VDRE function is demonstrated by our data showing the higher vitamin D-induced inhibition of the transcriptional activity in the presence of the T *vs.* the G sequence in this site (Fig. 2). Actually, the substitution by site-specific mutagenesis of the T with a G at position −57 on the hp#1 context completely abolished the vitamin D-induced inhibitory

response of this haplotype (Fig. 2). The functional relevance of the −57 sequence was also indicated by EMSA (Fig. 3) showing that the T and the G alleles had a different protein-binding affinity.

According to functional data, the −57T allele was significantly associated with IGHD when compared both with normal-stature and with normal short controls. Individuals homozygous for the −57T allele had an almost 3-fold increased risk of IGHD (Table 2). The risk was somewhat increased when we further considered homozygosity for all the alleles that are in LD with −57T (diplotype #1/1). The apparently stronger effect of the haplotypic combination has two possible explanations.1) The hp#1 is in LD with another causal variation. However, this putative variant must lie outside the *GH1* region because no further associated variation was detected when sequencing the promoter and the entire gene (introns and exons) in all the patients (data not shown). Moreover, a possible causal variation was not found within the Pit-1 binding sites in the LCR when sequencing patients carrying diplotype #1/1. Although no new variation was detected in this region, the presence of other functional polymorphisms between the LCR and the *GH1* gene cannot be excluded. 2) The associated variations interactively contribute to IGHD susceptibility. A hint that this might be the case comes from the experiments of luciferase induction showing that the presence of −57T seems to be crucial for the vitamin D-induced inhibition only in the context of hp#1 (Fig. 2). In fact, hp#2, naturally carrying −57G, is anyway able to induce inhibition, although at a significantly lower level than hp#1 (Fig. 3), indicating that the flanking sequences contribute to increase the *per se* low binding affinity of −57G.

In conclusion, we have identified a common polymorphism in the *GH1* promoter contributing to the IGHD phenotype, thus supporting the hypothesis that in most sporadic patients, GHD has a multifactorial etiology. Because the associated allele has a high binding affinity for the VDR and VDR is expressed in GH-producing pituitary cells (35), the gene encoding the VDR is an obvious additional candidate.

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A variation in a Pit-1 site in the growth hormone gene (GH1) promoter induces a differential transcriptional activity

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Abstract

The proximal promoter of the human growth hormone gene (GH1) is highly polymorphic. We tested if promoter haplotypes differing at possibly functional sites, namely −278T/G (in the NF1 binding site), −75A/G (in the proximal Pit-1 binding site) and −57G/T (in the VDR binding site), induced a different luciferase activity when transfected in a rat pituitary cell line. The presence of a G instead of an A at position −75 induced a more than two-fold reduced activity ($p < 0.0001$). In accordance with this findings the electrophoretic mobility shift assay demonstrated a reduced affinity of the −75G for the pituitary transcription factor Pit-1. Despite the strong effect of this polymorphism in vitro, the −75G variation was not associated to an impairment of the GH secretion in vivo.

Keywords: Polymorphism; Growth hormone gene; Pit-1; Luciferase assay

1. Introduction

The human growth hormone (GH) gene (GH1) is efficiently synthesized by the somatotropic cells of the anterior pituitary. The regulation of GH1 transcription has been characterized in detail and both ubiquitous and pituitary-specific *cis*/*trans* elements have been identified in the GH1 proximal promoter. Basal expression is controlled by several general tissue factors that can act both positively and negatively. These include NF-1 (binding site at −286 to −274; Courtois et al., 1990), Sp1 (−136 to −127; Lemaigre et al., 1989), Zn-15 (−110 to −95; Lipkin et al., 1993), Vitamin D receptor (−60 to −46 and −37 to −31; Seoane et al., 2002) and CREB, a protein that interacts with cAMP-responsive elements (−187 to −183 and −99 to −95; Shepard et al., 1994). The pituitary restricted expression is mainly controlled by the pituitary specific factor Pit-1 (Ingraham et al., 1988), a POU homeodomain protein binding to two highly conserved elements in the GH1 proximal promoter located between −87/−72 (proximal Pit-1 binding site) and −127/−107 (distal Pit-1 binding site). Several studies demonstrated that Pit-1 binding sites are critical for GH1 promoter activity both by cell free transcription experiments (Bodner and Karin, 1987) and transient transfection into tissue cultured cells (Lefevre et al., 1987). However studies of human GH expression in transgenic mice demonstrated that binding of Pit-1 to the two Pit-1 sites in the GH1 promoter are not sufficient for high level tissue-specific GH expression in vivo (Jones et al., 1995). Three further Pit-1 binding sites in a locus control region (LCR) located 14.5 kb upstream the GH1 gene are necessary to confer high level somatotropic-specific GH expression (Shewchuk et al., 2002).

The GH1 proximal promoter is characterized by an extremely high level of polymorphism, with 15 single nucleotide polymorphisms (SNPs) occurring in the 500 bp upstream the transcription initiation site (Giordano et al., 1997; Wagner et al., 1997). These polymorphisms give rise to at least 40 different haplotypic combinations (Horan et al., 2003) displaying a great variability in their in vitro transcriptional activity, with a 10-fold differ-

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ence between the highest and the lowest efficiency haplotype. Accordingly, it was evaluated that polymorphism in the GH1 promoter contributes for at least 3.3% of the total variation in adult height.

The aim of the present study was to explore the involvement in GH expression of three GH1 promoter polymorphisms located within binding sites for transcriptional factors, namely positions −278 (in the NF1 binding site), −75 (in the proximal Pit-1 binding site) and −57 (in the VDR binding site).

2. Materials and methods

2.1. PCR amplification and sequencing of the GH1 proximal promoter

The entire GH1 gene of three previously sequenced individuals, which were heterozygous for the GH1 promoter haplotypes, was first amplified from genomic DNA with primers GH32 (5′-CCAGCAATGCTCAGGGAAG-3′; from −608 to −589) and GH33 (5′-TGTCCCACCGGTTGGGCATG-GCAGGTAGCC-3′; from nt 2091 to nt 2061) (numbering relative to the transcription initiation site at +1; GenBank accession M13438) that amplify a 2.7 kb specific GH1 fragment and do not amplify the other homologous genes of the GH cluster. This amplicon was used as template for a nested PCR with primers GH7 (forward; 5′-AATAGGTACCCCAGCAATGCT-CAGGGAAA-3′; from −608 to −589) and primer GH8 (reverse; 5′-CATCAAGCTTCGCTAGGTGAGCTGTCCACA-3′ from +40 to +59) that included the proximal promoter. A non-template restriction endonuclease recognition sequence was added to the 5′ end of both primers (underlined above): KpnI (GH7) and HindIII (GH8). The PCR reactions were performed using a proofreading Taq polymerase (DyNAzyme™ DNA polymerase, Finnzymes).

The −75 genotype was determined by sequencing with the Big-dye terminator cycle sequencing reaction kit (Applied Biosystems) and an ABI 3100 automated sequencer.

2.2. Construction of luciferase reporter gene expression vectors

The PCR products, containing the two promoter haplotypes from each individual, were cloned into the pMOS plasmid (pMOSBlue T-vector kit, Amersham) and analyzed by DNA sequencing to check for polymerase-induced errors. The promoter fragments were released from p-MOS by double digestion with KpnI and HindIII and purified from gel with Qiaquick PCR purification kit (Qiagen). The purified fragments were then inserted into the Luciferase reporter vector pGL3 basic (Promega), digested with the same endonucleases and gel purified.

Transformed JM109 competent cells were grown on LB/ampicillin media. Plasmid DNA was extracted (Plasmid Midiprep, Qiagen) and the insert sequenced.

2.3. Site directed mutagenesis

The pGL3 plasmid bearing haplotype #a (pGL3-GH1hap#a) was used as the template into which the A at −75 was substituted with a G by site directed mutagenesis. A PCR reaction was carried out using primer GH9 (forward; 5′-AGTGGCCCCATGCATAAATGTGCACAGAAACAG-3′; from −63 to −96 in the GH1 promoter; the position −75 to mutagenize A → G is in bold) and primer GH8 (reverse; see above). Both primers were designed to include a unique restriction fragment site on the template, a NsiI restriction site in the upstream primer and a HindIII site in the downstream primer respectively (both sites are underlined in the primer sequence). Amplification was performed using 50ng of the template plasmid in a final volume of 50 μl containing 20pm of each primer, 200 μM of each dNTPs and 1 U of the proofreading Taq polymerase (DyNAzyme™ DNA polymerase, Finnzymes). The PCR product and the PGL3-GH1-hap#a were digested with NsiI and HindIII. The mutagenized −75G PCR fragment was substituted to the wild-type −75A in the template.

2.4. Cell culture and transfection

The transcriptional activity of the different GH1 promoter haplotypes was evaluated by transfection experiments after insertion of the appropriate sequences in the promoterless luciferase expression plasmid pGL3-Basic (Promega). Since a human pituitary cell line is not available, we used the rat pituitary GH4C1 cell line (ATCC) cultured in Ham's F-10 (Gibco, BRL) supplemented with 15% horse serum and 2.5% calf serum in a humidified 5% CO_2 incubator at 37 °C. The day before transfection 10^5 cells were seeded into six-well tissue culture plates in 1.5 ml medium and grown to ~80% confluence. Transient transfections were carried out using the polyethylenimine (PEI) cationic polymer, as previously described (Patrone et al., 1997). Cells were co-transfected with 2 μg of the luciferase reporter constructs and 200 ng of the control plasmid pRL-CMV (Promega) expressing the Renilla luciferase gene. A transfection mixture containing the two plasmids in 75 μl of serum-free medium with 7.5 μl of 10 mM PEI (Sigma–Aldrich) was prepared for each construct, incubated at room temperature for 15 min and added to the cells inside each corresponding well in a total amount of 750 μl of fresh complete medium. After an incubation of 3 h at 37 °C in 5% CO_2, the PEI containing solution was substituted with 2 ml of complete medium. After further 48 h incubation at 37 °C, cells were lysed with the lysis buffer provided with the Dual-luciferase Reporter Assay System (Promega). Firefly and Renilla reniformis luciferase activities were sequentially determined in the same sample of cell lysate with the Dual luciferase kit assay on a luminometer microplate. The activity of Renilla luciferase, expressed from the pRL-CMV plasmid provided an internal control to monitor transfection efficiency. The firefly luciferase activity was thus normalized on the basis of Renilla luciferase activity.

2.5. Extract preparation and electrophoretic mobility shift assay

Preparation of the nuclear extract from the GH4C1 cell line was performed according to Dignam et al. (1983). The EMSA oligonucleotides (corresponding to nt −93 to −62 in the GH1 promoter) including the position −75 [−75A: 5′-GCCCCATGCATAAATGTACACAGAAACAGG-3′ and −75G 5′-GCCCCATGCATAAATGTGCACAGAAACAGG-3′] were synthesized in both sense and antisense orientation as single-stranded molecules. The complementary oligonucleotides were first incubated at 85 °C in the annealing buffer and then gradually cooled to 45 °C to generate double stranded DNA molecules. Two double stranded commercial oligonucleotides (Geneka Biotechnology) were used as controls: a Pit-1 wild type oligonucleotide corresponding to a Pit-1 consensus binding sequence (5′-CCTGATTATATATATATTCATGAA-3′) as positive control (Pit-1 consensus oligo) and a Pit-1 mutant oligonucleotide (5′-CCTGATGCGGTATCTGGATCATGAA-3′) as negative control (Mut-oligo). The 5′-ends of the double stranded oligonucleotides were labelled with [γ^{32}P]ATP using a T4 polynucleotide kinase (Promega) and used as probes in EMSA experiments.

GH4C1 cell nuclear extract (1.5–4.0 μg of protein) was incubated 20 min at room temperature with 15 fmol of the ^{32}P labelled probes in 10 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol DTT, 50 mM KCl, 1 μg of poly(dI-dC), 10% glycerol and 1× protease inhibitor cocktail (Roche Molecular Biochemicals). For supershift experiments 2 μl of an anti-Pit-1 rabbit polyclonal antibody (Geneka Biotechnology) and 2 μl of the rabbit polyclonal antibody anti-EGR2 (Santa Cruz Biotechnology) were added to the reaction mixture and incubated 5 min in ice. Then the labelled probe was added and incubated for additional 20 min at room temperature.

For the competition studies a 50-, 100- and 250-fold excess of unlabelled over the radiolabelled oligonucleotide, were added to the binding reaction.

The reaction mixtures were then subjected to electrophoresis on a 5% non-denaturing polyacrylamide gel (19:1 acrylamide:bisacrylamide), and electrophoresed in a 0.25× tris–glycine buffer at 150 V for 1.5 h. The gel was dried on Whatman 3 mm paper and subjected to autoradiography with Kodak Biomax film.

2.6. Subjects

One hundred and forty-five sporadic Italian patients with isolated, hypophyseal GH deficiency (IGHD), 43 short stature individuals with normal GH

secretion (normal short) and 168 normal stature individuals were included in the genetic association analysis. None of the IGHD patients carried mutations in the GH1 and GHRHR genes. GH secretion was determined after either two consecutive provocative tests with arginine or clonidine or insuline (113 IGHD and 18 normal short individuals) or one double stimulus with GHRH + arginine (32 IGHD and 25 normal short). A diagnosis of IGHD was given when the GH peak was lower than 10 ng/ml after both the single stimuli or lower than 20 ng/ml after the double provocative test (Ghigo et al., 1996). The normal stature individuals were not tested for GH secretion levels.

2.7. Statistical analysis

The transfection experiment data were represented as the mean ± standard deviation of the mean (S.D.). All the values are expressed as a percentage of the haplotype #a mean value. The *t*-test was used to compare the relative luciferase activity between two groups. Allele and genotype frequencies were analyzed with the χ^2 test; correlation between GH secretion peaks and genotypes at −75 was evaluated by the Mann–Whitney test.

3. Results

3.1. Luciferase in vitro assay

In order to test the functional role of the three polymorphic variations at −278, −75 and −57 falling in the binding site of NF1, Pit-1 and VDR respectively, different GH1 proximal promoter haplotypic combinations (Fig. 1) were tested for their ability to induce luciferase synthesis in vitro. In previous studies haplotype (hp) #a was found to be the most frequent both in the Italian (Giordano et al., 2001) and in the British (Horan et al., 2003) populations and was thus considered as the wild type reference haplotype.

Three haplotypes (#b, d and e) differing from hp#a in at least one of the three potentially relevant positions (Fig. 1) were selected from a panel of previously sequenced samples. Promoter fragments containing the selected hps #a, #b, #d and #e were cloned and fused to luciferase reporter constructs, which were then transiently transfected into the rat pituitary cell line GH4C1. Each construct was tested in triplicate in at least three independent transfection experiments.

The highest activity was exhibited by hp#d, differing from hp#a at position −278, which directed luciferase synthesis to a 116% higher level than hp#a (Fig. 2a). This difference had a borderline statistical significance ($p = 0.05$) not holding the Bonferroni correction for the number of comparisons ($N = 6$). Hp#b, differing from hp#a at positions −278 and −57, induced a luciferase activity identical to that of hp#a. Conversely, hp#e showed a significantly decreased promoter activity with respect to all the other tested fragments (Fig. 2a). The luciferase expression directed by hp#e was 42% that of hp#a ($p < 0.0001$).

Considering the three positions included in the transcriptional binding sites (−278, −75, −57), hp#e differs from the other three for a G instead of an A at −75. This finding suggested that the presence of a G at −75 in the binding site of Pit-1 might be responsible for the decreased activity of hp#e. However, hp#e also differed from all the other tested haplotypes at position −308 and from hp#a at position −57. To test whether −75G was the only variation directly responsible for the observed decreased activity of hp#e, the reference hp#a was mutagenized at position −75 by replacing the A with a G. This in vitro created haplotype (hp#*muta*), which differed from hp#a only at −75, induced a 45% luciferase activity respect to that induced by hp#a, similarly to what observed for hp#e (Fig. 2b). Thus −75G is the polymorphism responsible for the decreased activity of hp#e in our experimental conditions.

3.2. EMSA

The capacity of −75G to decrease the affinity of Pit-1 to its binding site was tested by an electrophoretic mobility shift assay.

Rat pituitary cells (GH4C1) were used as a source of nuclear protein. The test was performed using 30mer labelled oligonucleotides corresponding to the promoter region from −93 to −62 containing either A or G at position −75 (−75A and −75G oligos, respectively). Two commercial oligonucleotides corresponding to a Pit-1 consensus sequence (*Pit-1* oligo) and to the same sequence mutagenized for the Pit-1 site (*Mut* oligo) served as positive and negative controls, respectively.

Two shifted complexes (indicated with arrows in Fig. 3a) were observed with the Pit-1 consensus and the −75A labelled probes (Fig. 3, lanes 1 and 2, respectively) while only one shifted band, corresponding to the complex 2, was detectable with the −75G sequence (Fig. 3a, lane 3). A faint band with the same

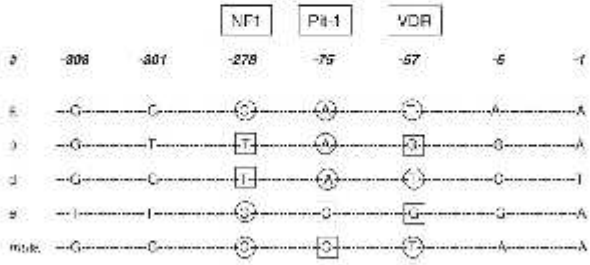

Fig. 1. Proximal promoter haplotypes used in the luciferase assay. The polymorphic positions are indicated from −308 to −1. The nucleotide at polymorphic positions in the NF-1, Pit-1 and VDR binding sites is marked with a circle if corresponding to that present in haplotype #a or with a square if differing. Haplotypes #a and #e are naturally occurring haplotypes and correspond respectively to hps #1 and #32 described by Horan et al. (2003). Haplotypes #b and #d are newly identified haplotypes. Haplotype #*muta* was obtained from haplotype #a by site directed mutagenesis of position −75.

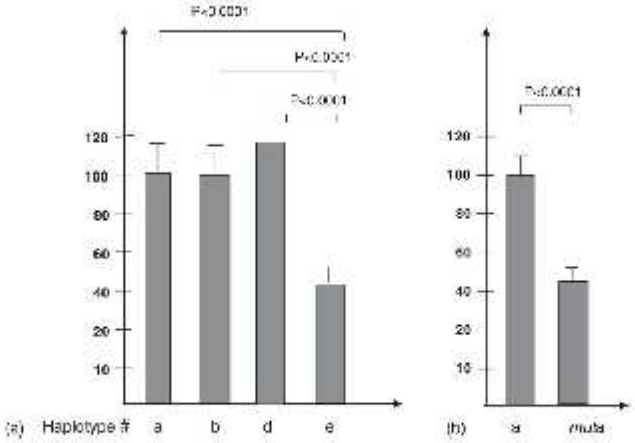

Fig. 2. Comparison of the GH promoter activity among haplotype promoter constructs #a, #b, #d and #e (a) and between haplotype #a and #mut a (b). The results are expressed relative to that of haplotype #a. Bars show the mean and S.D. of at least three separate experiments each performed in triplicate. Significant p values are shown.

electrophoretic mobility as complex 2 was present with the *mut* oligo.

To verify whether the retarded complexes included the transcription factor Pit-1, a mobility shift experiment was performed in the presence of the specific anti-Pit1 antibody (Fig. 3b). An intense supershifted band was detected in correspondence of the −75A probe, while only a weak supershift was present with the −75G probe. The supershift was not induced by a non-specific antibody (Fig. 3b, lanes 2 and 3). When the −75A and −75G oligos were used as cold competitors at increasing molar excess, the −75A oligo showed an evident higher affinity (Fig. 3c) being able to compete a significant part of binding to the labelled probe at the lowest (50×) competitor amount.

3.3. Correlation between GH secretion and the −75A/G variation in vivo

As the −75G promoter variation displayed a relevant effect in vitro, we investigated the possibility that it exerts a similar effect in vivo through an association study between the polymorphism and isolated growth hormone deficiency (IGHD). One hundred and forty-five IGHD patients and 168 normal stature controls

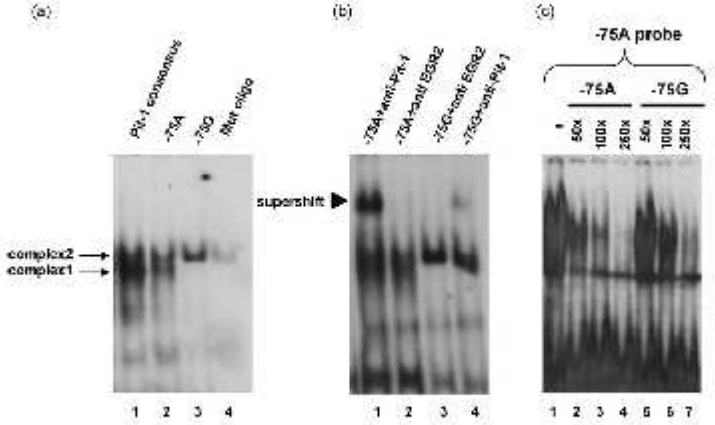

Fig. 3. The electrophoretic mobility shift assay (EMSA) in presence of GH4C1 nuclear extract. (a) Two different complexes (1 and 2) were seen with the −75A and consensus probes; the −75G and the Mut oligos displayed only the shifted complex 2. (b) A supershift was induced by an anti-Pit-1 antibody. A non-specific antibody (anti-EGR2) was used as a negative control. (c) Competition with a 50-, 100- and 250-fold excess of unlabelled −75A and −75G over the −75A radiolabelled oligonucleotide.

Table 1
Association studies

Genotype at −75	Genotype frequency		Mean ± S.D. of GH peaks (ng/ml)		
	IGHD (N = 145)	Normal stature (N = 168)	IGHD (N = 113)[a]	Normal short A[b] (N = 18)	Normal short B[c] (N = 25)
AA	0.731 (106)	0.726 (122)	5.1 ± 2.2 (86)	12.3 ± 4.7 (12)	46.1 ± 18.6 (18)
AG	0.262 (38)	0.244 (41)	5.4 ± 2.5 (26)	13.8 ± 8.8 (6)	61.2 ± 26.5 (5)
GG	0.007 (1)	0.03 (5)	2.2 (1)		45.0 ± 7.0 (2)

The numbers in parentheses indicate the numbers of subjects studied.
[a] For the calculation of GH peak means we considered only the 113 IGHD patients, out of 145, that were diagnosed by two stimuli with arginine or clonidine or insuline.
[b] GH peaks determined after two stimuli with arginine or clonidine or insuline.
[c] GH peaks determined after one double stimulus with GHRH + arginine.

were genotyped for the −75A/G polymorphism: the allele and genotype frequencies were not significantly different between patients and controls (frequency of the −75G allele: 13.8% in patients versus 15.1% in controls; Table 1).

The lack of association with the disease does not exclude a potential effect of the variation on the level of GH secretion in vivo. We evaluated the mean of GH secretion peaks after stimulation in patients with severe growth failure caused by IGHD and in normal short individuals with no defect in GH secretion. The mean level of the GH peaks was not significantly different between AA homozygous and AG heterozygous individuals in both IGHD and normal short patients (Table 1). The GG homozygous individuals were too few to perform any statistical analysis.

4. Discussion

In the present study we investigated the potential functional role of GH1 promoter polymorphisms falling within three transcriptional factor binding sites: −278 T/G in the NF1 binding site, −75 A/G in the proximal Pit-1 binding site and −57 G/T in the VDR binding site. Promoter haplotypes that differed for at least one of these positions from the reference hp#a (Fig. 1) were compared in transient transfection experiments by the in vitro luciferase assay. Haplotypes differing at positions −278 and −57 did not induce a significantly different luciferase activity. We therefore conclude that these two variations do not influence the level of GH transcription, at least in our experimental conditions.

Conversely, the presence of a G instead of an A in the Pit-1 recognition site at position −75 induced a more than two-fold reduced luciferase activity (Fig. 2a). This result was displayed both by the naturally occurring hp#e and by an in vitro mutagenized sequence obtained from hp#a by replacing the A with a G at position −75. Thus the different activity between hps #a and #e was totally dependent upon the genetic variation at position −75.

The reduction of the GH transcriptional activity displayed by the −75G sequence was explained by the EMSA results showing that: (1) the G probe formed only one of the two shifted complexes formed both by the A and by the Pit-1 consensus probes (Fig. 3a); (2) a supershifted band complexed with an anti-Pit1 antibody (Fig. 3b) was strongly evident with the −75A probe while it was faint with the −75G probe in the same experimental conditions. Thus a strong supershift induced by the anti-Pit-1 antibody was associated to the presence of complex 1; (3) a higher amount of the G than of the A cold oligo was required to compete binding of the labelled A probe. These results suggest that: (1) the largest amount of the Pit1 protein is included in complex 1; (2) the G probe does not bind Pit-1 in its "complex 1" form while it binds a small amount of Pit-1 in its "complex 2" form; (3) the G probe has a lower affinity than the A probe for the A binding molecule(s); (4) the G probe mainly binds uncharacterized molecule(s) present in complex 2. The latter might represent a complex, not sensitive to the base change at −75, formed by the interaction between Pit-1 and other co-factors involved in the synergistic activation of the GH gene transcription (e.g. the retinoid X receptor, the retinoid acid receptor and the thyroid hormone receptor; Schaufele et al., 1992; Chang et al., 1996; Palomino et al., 1998).

Pit-1 contains two protein domains, namely Pou-specific (POU_s) and POU-homeo (POU_h), both necessary for DNA binding. It generally binds as a dimer to its cognate recognition elements. Each of these elements consists of a core motif including a higher affinity 5′ consensus sequence, ATGNATA followed at the 3′ by an adjacent lower affinity A/T rich sequence. One Pit-1 monomer binds to the 5′ higher affinity sequence and the second monomer binds cooperatively to the 3′ lower affinity region (Elsholtz et al., 1990; Ingraham et al., 1990). The −75A/G polymorphism is located at the 3′ end of the lower affinity region. The presence of a G instead of an A in the AT/rich region could destabilize the PIT-1 binding to DNA as a dimer, which is normally provided by this region after one monomer has bound to the high affinity region (Holloway et al., 1995). Thus, the reduced binding of the −75G sequence to Pit-1 might be addressed to modifications of the Pit-1 molecule conformation induced by the base change. In turn, the decreased binding of the Pit-1 dimer to its site in the GH1 proximal promoter is likely responsible for the reduction of the transcriptional activity observed in transfection experiments.

The first 200 bp of the proximal promoter of the GH gene encompassing the two Pit-1 binding sites are highly conserved among mammals. Remarkably, the position corresponding to the human −75 is always occupied by an A in the 12 species analyzed (Krawczak et al., 1999). Thanks to this high homology, crystallographic data on the rat Pit-1 bound to its proximal

binding site on rGH promoter (Scully et al., 2000) allow us to further speculate about the functional relevance of the −75A → G change. The A nucleotide at the position homologous to the human −75 in the rGH promoter contacts the Asn 51 residue of the POU_H domain in the DNA major groove. This aminoacid is responsible together with other three residues for the specificity of the protein–DNA interaction in the AT/rich region. Indeed, it would be very interesting to evaluate the effect induced by the −75A → G change on the crystallographic structure of Pit-1 bound DNA.

The biological relevance of the interaction between Pit-1 and the target promoter sequences for pituitary hormone production is well documented by the observation that genetic mutations in the Pit-1 gene, leading to loss of Pit-1 activity, cause combined pituitary hormone (GH, PRL and TSH) deficiency in animals and humans (Cohen and Radovick, 2002). In addition, a mutation in the proximal Pit-1 binding site in the promoter of the GHRHR gene, markedly reducing Pit-1 binding, was reported in an isolated growth hormone deficiency (IGHD) compound heterozygous patient (Salvatori et al., 2002). We therefore tested the association of the −75G in the GH1 promoter with a reduced GH synthesis. Our results demonstrate that the −75 polymorphism is not involved in IGHD. Moreover the presence of a G at position −75 had no detectable effect on GH serum levels after provocative tests in vivo. Unfortunately, we do not have sufficient data on the GH serum levels of individuals carrying two −75 G alleles as the GG genotype is rare in our population (the estimated homozygous frequency is about 2%) and serological data on the five homozygous normal stature control individuals are not available.

Thus the strong effect of −75G seen in vitro on GH transcription is not visible in vivo on GH secretion. However, since GH secretion is regulated by the balanced interaction of many factors (Giustina and Veldhuis, 1998) the effect of the −75G variation in vivo might be hidden by the overall effect of the complex regulatory GH secretion network. Moreover, we tested selected populations of individuals with short stature in which we cannot exclude the presence of unknown genetic variations with a stronger effect on GH production.

Acknowledgments

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FURTHER PAPERS

During these four years of PhD I published three further papers concerning Cri du Chat Syndrome and Mowat Wilson Syndrome, in collaboration with Professor Paola Cerruti-Mainardi. These papers were not included into my thesis.

- Cerruti Mainardi P, Garavelli L, Pastore G, Virdis R, Pedori S, **Godi M**, Provera S, Rauch A, Zweier C, Castronovo C, Zollino M, Banchini G, Bernasconi S, Neri G. "Mowat–Wilson syndrome and mutation of the Zinc Finger Homeo Box 1B gene: a new syndrome probably under-diagnosed". Ital J Pediatr 2005;31:116-125.

- Garavelli L, Cerruti Mainardi P, Virdis R, Pedori S, Pastore G, **Godi M**, Provera S, Rauch A, Zweier C, Zollino M, Banchini G, Longo N, Mowat D, Neri G, Bernasconi S. "Genitourinary anomalies in Mowat-Wilson syndrome with deletion/mutation in the Zinc Finger Homeo Box 1B gene (ZFHX1B). Report of three Italian cases with hypospadias and review". Horm Res. 2005;63(4):187-192.

- Cerruti Mainardi P, Pastore G, Castronovo C, **Godi M**, Guala A, Tamiazzo S, Provera S, Pierluigi M, Dagna Bricarelli F. "The natural history of Cri du Chat Syndrome. A report from the Italian Register". Eur J Med Genet. 49 (2006) 363-83.

ORIGINAL ARTICLE
ARTICOLO ORIGINALE

Mowat-Wilson syndrome and mutation of the Zinc Finger Homeo Box 1B gene: a new syndrome probably under-diagnosed

Sindrome di Mowat-Wilson e mutazione del gene Zinc Finger Homeo Box 1B: una nuova sindrome probabilmente sotto-diagnosticata

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Summary

Objectives. The aim of this study is to increase the awareness of a probably under-diagnosed syndrome. Clinical recognition of the syndrome in infants and children with and without HSCR is important for the selection of patients for cytogenetic and molecular analyses.
Methods. The clinical features of four Italian patients are reported. FISH and mutational analyses were performed. Evaluation of psychomotor development was performed using the Denver Developmental Screening Test II (DDTS II).
Results. All four patients presented with mutations of the *ZFHX1B* gene; patients 1,3 and 4 showed novel mutations, never previously described. The clinical features of our patients were compared with those of the literature. The most frequent malformations were HSCR (62.8%), congenital heart diseases (50.7%), agenesis of the corpus callosum (45.6%). Hypospadias were present in 46.2% of the patients. Seizures were very frequent (75.0%).
Conclusions. Mowat-Wilson syndrome is well recognisable, like classical genetic syndromes, for the distinct facial phenotype, the associated malformations, seizures, microcephaly and severe mental retardation. The confirmation of the diagnosis by the presence of mutations in the *ZFHX1B* gene is important for genetic counselling since all patients thus far reported have been sporadic findings, even if the possibility of gonadal mosaicism cannot be excluded.

Key words

Mowat-Wilson syndrome • *ZFHX1B* gene • HSCR • Mental retardation

Parole chiave

Sindrome di Mowat-Wilson • gene ZFHX1B • HSCR • Ritardo mentale

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Riassunto

Obiettivi. *Lo scopo di questo studio è di diffondere le conoscenze su questa nuova sindrome probabilmente sotto-diagnosticata. Il sospetto clinico in età neonatale e nelle età successive in bambini con o senza HSCR è importante per selezionare i pazienti da sottoporre alle analisi citogenetiche e molecolari.*
Metodi. *Vengono riferiti i dati clinici relativi a quattro pazienti italiani ai quali sono state effettuate la FISH e l'analisi mutazionale. Lo sviluppo psicomotorio è stato valutato con il Denver Developmental Screening Test II (DDTS II).*
Risultati. *In tutti e quattro i pazienti italiani sono state identificate mutazioni del gene ZFHX1B. I pazienti 1, 3 e 4 presentavano nuove mutazioni, non precedentemente descritte. Le caratteristiche cliniche dei nostri pazienti sono state confrontate con quelle dei pazienti della letteratura. Le malformazioni più frequenti sono risultate la HSCR (62,8%), le cardiopatie congenite (50,7%), l'agenesia del corpo calloso (45,6%). L'ipospadia era presente nel 46,2%. Molto frequenti risultavano le crisi convulsive (75,0%).*
Conclusioni. *La sindrome di Mowat-Wilson è ben riconoscibile clinicamente come le sindromi genetiche classiche per il caratteristico fenotipo facciale, le malformazioni associate, le convulsioni, la microcefalia e il grave ritardo mentale. La conferma della diagnosi, mediante la dimostrazione di una mutazione del gene ZFHX1B, è importante per la consulenza genetica in quanto tutti i pazienti fino ad ora segnalati sono casi sporadici, anche se la possibilità di mosaicismo gonadico non può essere esclusa.*

Introduction

In 1998 Mowat et al. [1] delineated a new syndrome in six patients with Hirschsprung disease (HSCR), microcephaly, mental retardation and characteristic facial features, and identified a locus on chromosome 2q21-q23. In 2001 Wakamatsu et al. [2] identified mutations in the ZFHX1B gene in a patient with a translocation t(2;3)(q22;q22) and in three other patients. Independently Cacheux et al. [3] identified mutations in the ZFHX1B gene in two patients of Kääriäinen et al. [4], one of them with a translocation t(2;11)(q22.2;q21), and in three patients of Mowat et al. [1]. All these patients presented "syndromic HSCR". Subsequently Yamada et al. [5] reported ZFHX1B mutation in patients with complex developmental disorder, and Amiel et al. [6] in patients with syndromic HSCR. Zweier et al. in 2002 found the ZFHX1B mutation in four patients with typical facial gestalt, two with and two without HSCR [7]. The authors concluded that HSCR was not an obligatory symptom but one of the multiple variable anomalies of the syndrome, for which they proposed the name of "Mowat-Wilson Syndrome" (MWS). Garavelli et al. [8] reported the first Italian patient with *ZFHX1B* mutation and confirmed the clinical peculiarity of the MWS. Cerruti Mainardi et al. [9] reported a detailed description of two new Italian patients and a review of the literature. So far 70 patients with MWS mutation/deletion/translocation of the *ZFHX1B* gene have been referred [1-17], of whom a new Italian patient recently described by Garavelli et al. [18], with particular attention to the genitourinary anomalies in this syndrome. However it seems likely that the syndrome is under-diagnosed, particularly in patients without HSCR. Therefore we think it is useful to report the clinical picture of the four Italian patients with MWS and mutations in the *ZFHX1B* gene, three with and one without HSCR. The clinical recognition is essential in the selection of patients for cytogenetic and molecular analysis.

Clinical reports

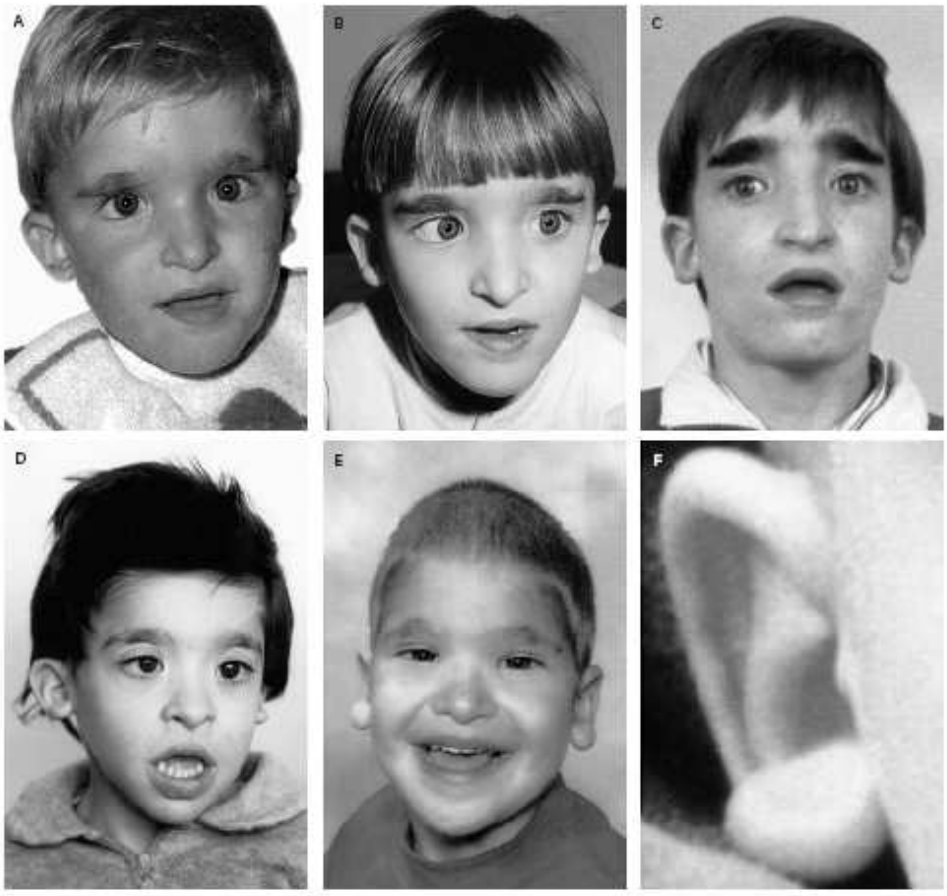
Patient 1

The boy was the first of two children of healthy, unrelated parents. The sister was healthy. The mother's sister had Noonan syndrome. Prenatal ultrasound at 15 weeks gestation showed bilateral mild dilatation of the renal pelvis. There were poor fetal movements. He was born at 40 weeks gestation by vaginal delivery with a birth weight of 3750 g (75th centile), length of 51 cm (50th) and head circumference of 34 cm (25th-50th). APGAR scores were 9 and 10 at 1 and 5 minutes, respectively. At two days of age the baby underwent surgery for HSCR. He had hypotonia and global developmental delay with involuntary nodding of the head and exaggerated reaction to acoustic stimulus. At 17 months he developed recurrent seizures (partial complex epilepsy), which responded to anticonvulsants. At 18 months his head circumference was 46 cm (10th centile), height 83.5 cm (50th-75th) and weight 10.160 kg (10th); at 3 years and 3 months his head circumference was 47 cm (3rd), height 96 cm (50th-75th) and weight 14,500 g (25th-50th). He had fine blond hair, high forehead, frontal bossing, prominent supraorbital ridges, deep set eyes, small nose with a bulbous tip, mouth held open, "M-shaped" upper lip, pointed chin, posterior angulation of ears, uplifted ear lobes (Fig. 1 F), fetal finger pads, deep palmar and plantar creases, long halluces, penile hypospadias and undescended right testis. Dermatoglyphics showed 8/10 ulnar loops on the fingertips. Lactate, ammonium, QFQ banded karyotype at 650 band resolution, molecular analysis for fragile X syndrome were normal. Skeletal survey showed wormian bones, hypoplasia of the distal phalanges of the fingers and hypoplasia of the middle and distal phalanges of the 2nd, 3rd, 4th, 5th toes and long and hypertrophic first ray of the feet. Cranial CT demonstrated enlarged subarachnoid spaces in the frontal region, and MRI of the brain showed bilateral mild symmetrical accentuation of the apex of temporal horns. Abdominal ultrasounds were normal. As the clinical picture was typical of Mowat-Wilson syndrome, the *ZFHX1B* mutational analysis was performed.

Patient 2

This boy was the first child of healthy, unrelated parents. The sister was healthy. He was born at 41 weeks gestation with a birth weight of 3200 g (10th-25th centile), a length of 48 cm (10th centile), and a head circumference of 33 cm (3rd-10th centile). HSCR was diagnosed on day 2, colostomy was carried out on day 3 and closed at 8 months. He had severe constipation since the first months of life. High degree vescicoureteral reflux was clinically observed. At 14 months, he was diagnosed with hypotonia and global developmental delay. He developed seizures at 16 months, confirmed by EEG, and sodium valproate was started. On examination at 5 years of age, the boy had several minor facial anomalies, fine hair, high forehead, frontal bossing, broad nasal bridge, medially flaring thick eyebrows, downward slanting palpebral fissures, hypertelorism, epicanthus, deep set blue eyes, small nose with prominent columella, open mouth, triangular jaw, prominent chin, posterior low set ears with uplifted lobes (Fig. 1 A, B). He also had high arched palate, penoscrotal hypospadias with penis recurvation (surgical treatment at 4 years 5/12), right cryptorchidism with non-palpable testis, pes planus with calcaneovalgus, mild pectus excavatum, long tapered fingers, single transverse palmar creases, microcephaly (48.5 cm, < 3rd centile) and severe psychomotor retardation. The Denver Test [19] showed that he "grasped" at 15 months, walked alone at 3 years and still had not developed speech at 5 years of age. The child was affectionate and smiling. A cardiac systolic murmur was present. ECG was normal, echocardiography showed mild fibrosis of the anterior tricuspid leaflet with slight valvular insufficiency. Seizures remained frequent despite treatment. MRI of

Fig. 1. Patient 2 at age 2 years (A), 5 years (B) and 13 years and 8 months (C). (D) Patient 3 at age 3 years and 5 months. (E) Patient 4 at age 2 years. (F) Patient 1: uplifted ear lobe.

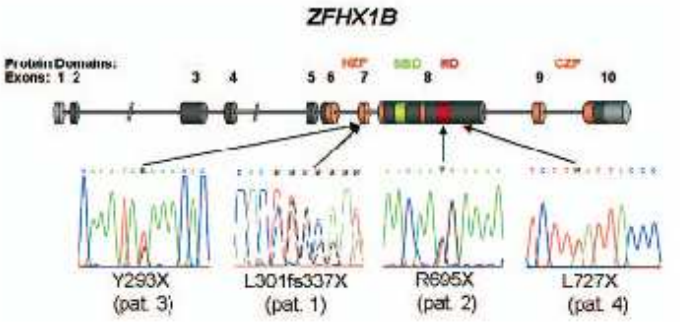


the brain showed marked hypoplasia of the corpus callosum with narrow lateral ventricles. Ocular fundus examination showed a grey pigmentary halo in the right optic papilla whereas the left eye was normal. The audiometric examination was normal. Standard karyotype was normal, and FRAXA mutation, Angelman syndrome, and metabolic diseases were excluded. On the last examination at 13 years and 8 months weight was 29 kg (< 3rd), height 138 cm (< 3rd) (target > 10th), and head circumference 50 cm (< 3rd). Long face, horizontal palpebral fissures, less evident epicanthic folds were observed. The other facial anomalies were unchanged (Fig. 1 C). Thinness was present. Pubertal development was advanced. No seizures had occurred since the age of 6 years; the EEG showed non-specific anomalies. The severe psychomotor and mental retardation persisted: he was able to run and climb stairs at age 6 years and to drink alone at 8 years; he was toilet trained with help at 8 years and still had no speech. As the clinical picture was typical of the Mowat-Wilson syndrome, *ZFHX1B* mutational analysis was performed.

Patient 3

The girl was the first child of healthy, unrelated parents. A younger brother was healthy. She was born at 41 weeks gestation with a birth weight of 3,700 g (75th), length 51 cm (75th), head circumference 32 cm (3rd). Cyanosis and cardiac systolic murmur were present at birth, and the echocardiogram showed the tetralogy of Fallot, with absence of the pulmonary valve. Dysmorphism was observed but the karyotype was normal. At 20 days she presented with vomiting and ab-

Fig. 2. Scheme of the *ZFHX1B* exons and corresponding protein structure adapted from Zweier et al. [7], showing the position of the mutations presented. NZF, N-terminal zinc finger cluster; CZF, C-terminal zinc finger cluster; SBD, Smad binding domain; HD, homeodomain-like segment.



Tab. I. Mowat-Wilson syndrome. Clinical characteristics on 70 patients.

Clinical characteristics	MWS patients	% of total	Present study			
			1	2	3	4
Sex	M/F 46/24		M	M	F	M
Facial phenotype						
Sparse/fine hair	10/14*	71.4	+	+	+	+
High, broad forehead/frontal bossing	9/12	75.0	+	+	+	–
Medially flaring thick eyebrows	38/38	100.0	+	+	+	+
Hypertelorism	46/49	98.0	+	+	+	–
Strabismus	20/28	71.4	–	–	+	–
Deep set eyes	37/38	97.4	+	+	+	–
Epicanthus	9/15	60.0	–	+	–	+
Downward slanting palpebral fissures	11/17	64.7	–	+	+	–
Broad nasal bridge/saddle nose	45/46	97.8	–	+	+	+
Small nose, rounded nasal tip/prominent columella	29/31	93.5	+	+	+	–
Open mouth	25/29	86.2	+	+	+	+
Prominent pointed chin/triangular jaw	42/42	100.0	+	+	+	+
Posterior low set ears with uplifted lobes	38/38	100.0	+	+	+	+
Other clinical features						
HSCR	44/70	62.8	+	+	+	–
Seizures	51/68	75.0	+	+	+	–
Congenital heart disease	35/69	50.7	–	–	+	–
Agenesis/hypoplasia of corpus callosum	26/57	45.6	–	+	+	–
Renal anomalies	11/50	22.0	+	+	–	–
Hypospadias	18/39 (24°)	46.2	+	+	°	+
Cryptorchidism	17/40 (24°)	42.5	+	+	°	–
Cleft submucous palate/high arched palate	11/34	32.4	–	+	+	–
Dermatoglyphics anomalies	8/10	80.0	+	+	+	°
Tapered fingers	16/19	84.2	+	+	+	–
Pes planus with calcaneovalgus	12/17	70.6	+	+	+	–
Mental retardation	70/70	100.0	+	+	+	+
Microcephaly (≤ 3rd centile) (last follow-up)	59/69	85.5	–	+	+	–
Hypotonia	29/31	93.5	+	+	+	+
Short stature (< 3rd centile) (last follow-up)	18/40	45.0	–	+	–	–
Happy, affectionate personality	28/29	96.6	+	+	+	+
Sporadic	70/70	100.0	+	+	+	+

1, patient 1; 2, patient 2; 3, patient 3; 4, patient 4; *: number of the patients for whom the data were available; °: not relevant; °: not available

Tab. II. Mowat-Wilson syndrome. Clinical data on 70 patients and mutation/deletion/translocation of the *ZFHX1B* gene. *(continues)*

Reference	Patient	Sex	Age (A)	Gestation (G)	Birth Weight (g), C	Birth Length (cm), C	Birth CC (cm), C	Micro-cephaly (L)	Facial Features (FF)
[1, 3, 4]	1	M	3	42	3950 75th	55 97th	35.5 50th	3rd	+
	2	M	4 1/2	*	3700 75th	50 50th	33 3rd-10th	3rd	+
	3	F	3	*	4820 >97th	52 90th-97th	34 25th-50th	<3rd	+
	4	M	3 1/2	*	3400 50th	49.5 25th	33.5 10th	< 3rd	+
	5	M	9	33	1880 25th	43 25th	30.5 25th	< 3rd	+
	6	F	8	38	3380 75th	52 90th-97th	34.5 75th	3rd	+
[10]	7	M	2 1/2	37	4025 90th	*	*	< 3rd	+
[2, 5, 12]°	8	F	9	40	3000 25th	*	31.5 < 3rd	+	+
	9	F	20	41	3045 10th-25th	*	33 10th	+	+
	10	M	25	38	2820 25th	*	31.5 3rd	+	+
	11	M	25	39	3200 25th-50th	*	31 < 3rd	+	+
	12	M	28	42	3570 25th-50th	*	*	+	+
	13	M	30	40	3950 90th	55 97th	33 3rd-10th	+	+
	14	M	26	40	3410 50th	50 25th-50th	33 3rd-10th	+	+
	15	M	27	39	3260 50th	48 10th-25th	33 10th	+	+
	16	F	6	36	2690 50th	46.5 25th-50th	30.5 3rd-10th	+	+
	17	M	3	38	2960 25th	47 10th	32 3rd	+	+
[6]	18	M	*	*	*	*	*	+	+
	19	M	*	*	*	*	*	+	+
	20	F	*	*	*	*	*	+	+
	21	F	*	*	*	*	*	+	*
	22	M	*	*	*	*	*	+	+
	23	F	*	*	*	*	*	*	+
	24	F	*	*	*	*	*	+	+
	25	F	*	*	*	*	*	+	+
[7]	26	M	1 1mo	40	3940 90th	54 90th	?	10th	+
	27	M	6 8mo	40	3600 50th	54 90th	?	< 3rd	+
	28	M	6 10mo	40	2670 10th	47 3rd	32 < 3rd	<3rd	+
	29	M	1 6mo	37	2910 40th	49 50th	32.8 25th	25th-50th	+
[13]	30	M	*	*	*	*	*	3rd	+
	31	M	*	*	*	*	*	< 3rd	+
	32	M	*	*	*	*	*	3rd	+
	33	M	*	*	*	*	*	3rd	+
	34	F	*	*	*	*	*	<< 3rd	+
	35	F	*	*	*	*	*	10th-25th	+
	36	M	*	*	*	*	*	<< 3rd	+
	37	M	*	*	*	*	*	3rd	+
	38	F	*	*	*	*	*	25th	+
	39	M	*	*	*	*	*	3rd	+
	40	M	*	*	*	*	*	25th	+
	41	M	*	*	*	*	*	< 3rd	+
	42	M	*	*	*	*	*	< 3rd	+
	43	F	*	*	*	*	*	< 3rd	+
	44	M	*	*	*	*	*	<< 3rd	+

HSCR	Seizures	Heart Defects	Callosum agenesis	Genital anomalies	Renal anomalies	W	MR	Mutation	Exon mutated
+	+	–	+	Hypospadias Bifid scrotum	+	–	+	del2(q22-q23)	All
+	+	–	–	–	+	–	+	920delA	8
+	+	–	–	?	–	–	+	594delC	6
+	+	+	–	Cryptorchidism	–	–	+	1421insA	8
+	–	–	+	Hypospadias	–	6	+	Translocation	int 2
+	+	+	–	?	–	?	+	1216delAC	8
+	+	+	?	–	–	?	+	del2(q22-q23)	All
+	+	+	–	?	?	–	+	Deletion	All
+	+	+	+	?	?	3 6mo	+	R549X	8
+	+	–	?	?	?	8	+	R695X	8
+	+	+	–	?	?	5 3mo	+	1173delAACA	8
?	+	–	–	?	?	6	+	R695X	8
?	+	–	–	Cryptorchidism	?	4	+	R695X	8
?	+	–	–	?	?	2 6mo	+	R695X	8
–	+	–	–	?	?	2	+	760insCA	6
–	–	–	+	?	?	4 8mo	+	272delC	3
?	–	–	HCC	?	?	–	+	2178delTT	8
+	?	–	+	Hypospadias	?	?	+	935delC	8
+	+	–	+	Cryptorchidism	VUR	?	+	1805delA	8
+	+	+	–	?	–	?	+	Deletion	All
+	+	+	+	?	?	?	+	Deletion	All
+	+	+	+	Hypospadias Cryptorchidism	?	?	+	Deletion	All
+	–	+	–	?	Hydronephrosis	?	+	2453insT	8
+	+	+	+	?	?	?	+	Deletion	All
?	+	–	+	?	–	?	+	L562X	8
+	–	–	–	Cryptorchidism	+	–	+	3567insCC	10
+	+	+	?	–	–	6 6mo	+	1892delA	8
–	+	+	+	Cryptorchidism	–	5	+	553insTG	5
–	–	+	–	Hypospadias Cryptorchidism	–	–	+	S852X	8
+	–	+	–	Hypospadias	VUR Hydronephrosis	?	+	S856X	8
+	+	–	?	Hypospadias	?	?	+	615-616delTG	6
+	–	+	–	Hypospadias	–	?	+	851-852delGC	7
+	–	–	+	Cryptorchidism Webbed penis	VUR	?	+	2661delC	8
–	+	–	?	?	?	?	+	3350delC	10
–	+	–	–	?	?	?	+	2967-85delGT	9
+	+	+	–	–	–	?	+	219insC	3
–	+	–	?	Webbed penis	–	?	+	1366insT	6
–	+	–	+	?	–	?	+	S142X	5
–	+	+	?	Hypospadias Cryptorchidism	VUR	?	+	R695X	8
+	+	–	?	–	?	?	+	Q461X	8
–	+	+	?	Cryptorchidism Hooded prepuce	Bilateral PUJ	?	+	R548X	8
+	–	+	+	Hypospadias	–	?	+	2335delA	6
–	+	+	–	?	–	?	+	1666delAAGA	8
–	–	–	–	–	?	?	+	IVS2+1G-A	int 2

Tab. II. Mowat-Wilson syndrome. Clinical data on 70 patients and mutation/deletion/translocation of the *ZFHX1B* gene. *(follows)*

Reference	Patient	Sex	Age (A)	Gestation (G)	Birth Weight (g), C	Birth Length (cm), C	Birth CC (cm), C	Micro-cephaly (L)	Facial Features (FF)
[14, 16]	45	F	10	*	50th	*	< 3rd	+	+
	46	M	9	34	2260 50th-90th	45 10th-50th	29.5 10th-50th	+	+
	47	M	5 wks	*	60th	5th	20th	+	+
	48	M	13		90th	90th	*	–	+
[15]	49	F	*	*	*	*	*	+	+
[16]	50	F	1 4mo	36	2700 50th	51 97th	33 50th-75th	+	+
[17]	51	M	5	*	*	*	*	+	+
	52	F	11	*	*	*	*	+	+
	53	M	10	*	*	*	*	+	+
	54	M	7	*	*	*	*	+	+
	55	M	28	*	*	*	*	–	+
	56	M	4	*	*	*	*	+	+
	57	M	6	*	*	*	*	+	+
	58	M	2	*	*	*	*	–	+
	59	F	3	*	*	*	*	+	+
	60	F	3	41	3320 25th-50th	*	31.8 3rd	< 3rd	+
	61	M	3	38	3338 50th-75th	*	33 10th-25th	<3rd	+
	62	M	18	40	2720 3rd	*	33 3rd-10th	<3rd	+
	63	M	17	40	3140 25th	*	32 < 3rd	< 3rd	+
	64	F	3	36	1968 3rd-10th	*	27.2 < 3rd	<3rd	+
	65	F	1	38	2848 25th	*	32.2 10th-25th	<3rd	+
	66	F	26	41	3200 25th	*	32 3rd	< 3rd	+
Present study	67	M	1 6mo	40	3750 75th	51 50th	34 25th	10th	+
	68	M	13 8mo	41	3200 10th-25th	48 10th	33 3rd-10th	<3rd	+
	69	F	3 5mo	41	3700 75th	51 75th	32 3rd	<< 3rd	+
	70	M	2 1mo	40	3580 50th-75th	49 10th-25th	34.5 25th-50th	–	+

F: female; M: male; +, presence of clinical sign; –, absence of clinical sign; *: not available; !: not relevant; A: years; G: weeks; CC: cranial circumference (cm); C: centiles; L: centile at last follow-up; FF: facial features as described by Mowat et al. [1]; ↑: constipation; HCC: hypoplasia of corpus callosum; VUR: vesico-ureteric reflux; PUJ: pelvi-ureteric junction obstruction; W: walking age (years); MR: mental retardation; ‡: updated data provided by Ishihara et al. [17].

dominal distension. HSCR was diagnosed and a colostomy was carried out on day 22. At 3 months surgery for Fallot's tetralogy was performed; agenesis of the pulmonary valve and severe dilation of pulmonary branches were present. At 11 months the colostomy was closed. At 1 year she developed recurrent seizures. The EEG showed slow widespread anomalies of the frontotemporal bilateral derivations, and phenobarbital therapy was started. Ultrasound of the brain showed hypoplasia of the corpus callosum. During the first year of life hypotonia and psychomotor retardation were observed. At the last examination at 3 years and 5 months weight was 11.300 kg (< 3rd), length 93 cm (25th), head circumference 43 cm (< 3rd). She had fine hair, high forehead, frontal bossing, prominent supraorbital ridges, medially flaring eyebrows, hypertelorism, deep set eyes, small nose with prominent columella, short philtrum, open mouth, "M-shaped" upper lip, prominent chin, posterior low set ears with uplifted lobes (Fig. 1 D). She also presented high arched palate, long tapered fingers, single incomplete transverse palmar creases, and pes planus with calcaneovalgus. Seizures were recurrent. Cranial MRI confirmed corpus callosum hypoplasia. The Denver test evidenced that she "sat head steady" at 1 year, did not sit without support, "grasped" at 2 years and 6 months, "turned to voice" at 2 years and had no speech. As the clinical picture was typical of the Mowat-Wil-

HSCR	Seizures	Heart Defects	Callosum agenesis	Genital anomalies	Renal anomalies	W	MR	Mutation	Exon mutated
–	+	+	–	‡	–	6	+	Deletion	5Mb
–	+	–	+	Hypospadias Cryptorchidism	–	9	+	Deletion	700kb
+	+	+	–	Cryptorchidism Microphallus	–	–	+	Deletion	11Mb
+	+	+	+	Hypospadias	–	2 6mo	+	Deletion	300kb
+	*	*	+	‡	*	*	+	604X	8
–	+	–	HCC	‡	+	*	+	R545X	8
–	–	–	HCC	–	–	–	+	390fs430X	8
+	+	+	–	‡	–	3	+	465fs467X	8
*	+	+	+	Cryptorchidism	–	6	+	286fs293X	7
+	+	–	*	Cryptorchidism	–	5 2mo	+	R502X	7
–	+	–	*	–	–	2	+	Q497X	8
+	–	+	*	Hypospadias	–	–	+	R545X	8
+	+	+	*	Hypospadias	–	–	+	R695X	8
+	+	+	HCC	Hypospadias Cryptorchidism	–	–	+	R695X	8
+	+	+	–	Septum of vagina	–	–	+	R695X	8
+	+	–	–	‡	–	3 4mo	+	Deletion	1-2
+	–	–	HCC	–	–	–	+	Deletion	All
*	+	+	–	–	–	7	+	Deletion	All
+	+	+	–	–	–	–	+	Deletion	All
+	–	+	+	‡	–	–	+	Deletion	1-4
*	–	+	–	‡	–	–	+	Deletion	All
+	+	–	–	‡	–	4 5mo	+	Deletion	3-10
+	+	–	–	Hypospadias Cryptorchidism	+	–	+	901delC	7
+	+	–	+	Hypospadias Cryptorchidism	VUR	3	+	R695X	8
+	+	+	+	‡	–	–	+	Y295X	7
–	–	–	–	Hypospadias	–	1 11mo	+	L727X	8

son syndrome, the *ZFHX1B* mutational analysis was performed.

PATIENT 4

He was born at 40 weeks gestation after an uneventful pregnancy, with a weight of 3580 g (50th-75th), a length of 49 cm (25th) and a head circumference of 34.5 cm (25th-50th centile). The family history was unremarkable. He has a healthy brother. APGAR scores were 7 and 9 at 1 and 5 minutes, respectively. He had hypotonia and delayed milestones: he sat without support at 12 months, walked at 23 months and could not yet speak at 25 months. He had repetitive stereotyped hand movements. He is an affectionate, happy boy. At 6 months of age he had surgical correction of penoscrotal hypospadias and chorda. At 25 months of age, his height was 85.7 cm (50th centile), weight 11.6 kg (10th centile), head circumference 46 cm (10th centile). He had dolichocephaly, sparse hair, sparse eyebrows in the middle part, medial flared eyebrows, epicanthic folds, up-slanting palpebral fissures, "M-shaped" upper lip, short philtrum, pointed chin, thickened helix and uplifted ear lobes, supernumerary nipples, and shawl scrotum (Fig. 1 E). He did not have constipation. QFQ banded karyotype at 550 band resolution, FISH telomeres, cholesterol, 7-dehydrocholesterol, thyroid function, testosteron, DHEA-S, Delta-4 Androstendion, and lactate were all normal. Brain CT, EEG, echocardiog-

raphy, X-Rays of the spine and abdominal ultrasounds were all normal. As the clinical picture was typical of the Mowat-Wilson syndrome, the *ZFHX1B* mutational analysis was performed.

Methods and results

FISH analysis for exclusion of whole gene deletion was performed as previously described [14], with normal results in all patients. Sequencing of the complete coding sequence of the *ZFHX1B* gene [7] showed a de novo heterozygous exon 7 frameshift mutation nt901delC in patient 1 [8]. The analysis in patient 2 [9] showed a de novo heterozygous exon 8 stop mutation nt2083C/T (R695X) and a de novo heterozygous exon 7 stop mutation nt879T/G (Y293X) in patient 3 [9]. The analysis in patient 4 [18] showed a de novo heterozygous exon 8 stop mutation nt2180T/A (L727X) (Fig. 2).
Evaluation of psychomotor development was performed using the Denver Developmental Screening Test II (DDTS II) [19]. The clinical and molecular data of our four patients are summarized in Tables I and II, and compared with those of patients reported in the literature, presenting with mutation, deletion or translocation.

Discussion

The Mowat-Wilson syndrome is a well defined clinical entity recognisable like classical genetic syndromes, that finds its confirmation in the *ZFHX1B* gene mutation. The clinical features of the four Italian patients are very typical and, when recognised, they are enough to suggest the diagnosis. Particularly important is the facial "gestalt" [20], characterised by medially flaring thick eyebrows, deep set eyes, hypertelorism, strabismus, downward slanting palpebral fissures, small nose with prominent columella, open mouth, prominent pointed chin, low set ears with uplifted lobes. Not all features are specific, but their combination is very typical (Fig. 1 A-F). The "M" shape of the upper lip, centrally full, laterally thin with prominent philtral pillars is frequent in childhood [21] and present in our four patients (Fig. 1 A, B, D, E). In children with blue irides, dark patches of iris pigment are present and suggest a heterochromia of the irides [21] (Fig. 1 A-C). The facial features change with age: the face becomes long with prognathism and the palpebral fissures horizontal, the epicanthal folds become less evident, the nasal profile "aquiline" [9 13 21] (Fig. 1 C).
HSCR, when present, is a strong cross reference marker, particularly in the neonatal age, but it is not constant (3/4 our patients). It is noteworthy that as the number of patients described rises, the percentage of patients with HSCR decreases: 70.0% of 30 patients [14], 63.8% of 47 patients [9], 62.8% of 70 patients in present study (Tab. I). It is obvious that this number will decrease again as the diagnosis on patients without HSCR rises (bias of ascertainment). Also the male preponderance of HSCR in general populations (4:1) [22] can cause a bias of ascertainment and explain the male excess in MWS [21]. In fact the male/female ratio decreases from 2.13/1 on 47 patients (M/F 32/15) [9] to 1.92/1 on 70 patients (M/F 46/24) (Tab. I). The manifestation of HSCR is not influenced by deletion size [14], and *ZFHX1B* knockout mice do not exhibit HSCR [23]. Other frequent clinical anomalies are congenital heart defect (50.7% of the total), present only in one patient out of the four here reported, but this girl had tetralogy of Fallot with absent pulmonary valve. A rare anomaly, pulmonary artery stenosis, was identified in one patient [17]. Agenesis of the corpus callosum (45.6%) was present in 2 of the 4 patients. Hypospadias (46.2%) was present in all three male patients. The presence of genitourinary anomalies, in particular hypospadias, contributes to the diagnosis in patients with typical facial dysmorphism and mental retardation [18]. Moreover midline defects such as vaginal septum (one patient) [17] and piloric stenosis, have been reported (7 patients) [6 12 13 17]. Other minor malformations (dermatoglyphics anomalies 80.0%, tapered fingers 84.2%, pes planus 70.6%) were frequent, but not invariably reported. Seizures are very frequent (75.0%), and were present in 3 of our patients. Repeated vomiting attacks suggestive of epilepsy were observed in five cases [17]. Mental retardation, usually severe, was constant. Our four patients presented hypotonia, severe psychomotor and mental retardation and had no speech. A happy affectionate personality was reported in 96.6% of patients.
Clinical features in MWS suggest that the *ZFHX1B* gene, coding for Smad interacting protein-1 (*SIP1*), is involved in neural cells and neural crest development (enteric nervous system and craniofacial mesectoderm) as well as in midline development (corpus callosum agenesis, genitourinary anomalies and pyloric stenosis) [6 7 17]. Since patients 2 and 3 showed the same severe phenotype as patients with large 2q22 deletions, we pointed out that MWS is not a contiguous gene syndrome, and that truncating mutations of one allele of the *ZFHX1B* gene resulted in this complex phenotype [9]. Then the haploinsufficiency is likely the underlying mechanism [6 14]. Two studies about genotype-phenotype analysis [14 17] have shown that phenotype spectrum was similar in patients with deletion and in patients with truncating mutations, except for patients with very large deletions. In particular, one patient with the 11Mb deletion presented seizures in the neonatal age and marked hypoplasia of the big toes; he died in early infancy [14]. On the other hand, an atypical phenotype with late adult onset, severe constipation and mild mental retardation in the absence of specific facial anomalies, seizures, and other malformations caused by non-truncating mutations, was described in a patient with a 3bp in frame deletion [24]. Patient 1, 3 and 4 described here showed a novel heterozygous stop mutation. Patient 2 presented with the R695X mutation already reported in other 8 patients [2 5 13 17]. The comparison of the clinical data concerning the associated malformations (HSCR,

congenital heart defect, agenesis of the corpus callosum) demonstrates some phenotypic variability resulting from a single mutation in the Mowat-Wilson syndrome [9]. The origin of the deleted chromosome is paternal in all 8 patients examined thus far [2 6 14 17].

In conclusion, we wish to highlight the importance of the clinical detection of this syndrome in infants and children with typical facial gestalt and mental retardation, with and without HSCR. The diagnosis is important for the families, who are grateful for the information provided, despite the prognosis. The differential diagnosis with other types of syndromic HSCR such as the Goldberg-Shprintzen syndrome is important for genetic counselling since this syndrome is most likely autosomal recessive, whereas all of the patients with MWS so far reported have been sporadic cases. Nevertheless the possibility of gonadal mosaicism cannot be excluded and two cases have been reported (recurrence risk ~ 2%) (D.R. Mowat's personal communication and A. Rauch's personal communication). The confirmation of the diagnosis based on the presence of a mutation, deletion or translocation of the *ZFHX1B* gene will allow other patients to be identified and our knowledge on this syndrome, which is probably under-diagnosed, to increase.

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Original article

The natural history of Cri du Chat Syndrome. A report from the Italian Register

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Abstract

The aim of this report is to provide an update on the natural history of the Cri du Chat Syndrome by means of the Italian Register (I.R.). Two hundred twenty patients were diagnosed by standard cytogenetic methods and 112 of these were also characterised by molecular-cytogenetic investigation (FISH). FISH analysis showed interstitial deletions, short terminal deletions and other rare rearrangements not previously correctly diagnosed by standard cytogenetics. The diagnosis was made in the first month of life in 42% and within first year in 82% of cases. The remaining 18% were diagnosed at an age ranging from 13 months to 47 years. At the last follow-up, patient age ranged from 8 months to 61 years. Mortality, already low, has decreased over time as it is lower between 1984–2002 compared to 1965–1983. Mortality was higher in patients with unbalanced translocations resulting in 5p deletions. Our data confirm that the cat-like cry and peculiar timbre of voice are the most typical signs of the syndrome, not only at birth but also later and these are the only signs which might suggest the diagnosis in patients with small deletions and mild clinical picture. A cytogenetic and clinical variability must be underlined. Cardiac, cerebral, renal and gastrointestinal malformations were more frequent in the patients with unbalanced translocations resulting in 5p deletions. Sucking and feeding difficulties and respiratory infections are frequent in the first months or years of life. Intubation difficulties linked to larynx anomalies must be considered. Psychomotor development is delayed in all patients but there is a variability related to deletion size and type as well as other genetic and environmental factors. However, the results showed an

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improvement in the acquisition of the development skills and progress in social introduction which should encourage caregivers and parents to work together in carrying out the rehabilitative and educational interventions.

Keywords: Cri du Chat syndrome; Cri du Chat syndrome natural history; 5p deletion; FISH; Mental retardation

1. Introduction

The Cri du Chat Syndrome (CdCS), first described by Lejeune et al. [30] in 1963, is a chromosomal disorder resulting from the deletion of the short arm of chromosome 5. The size of the deletion ranges from the entire short arm to the region 5p15.3 (5–40 Mb) [39,47]. Hallmark clinical features include a high-pitched cat-like cry, microcephaly, a distinct facial phenotype and severe psychomotor and mental retardation. The incidence is low, ranging from 1:15,000 [27] to 1:50,000 [37] live-born infants, but it is probably the most common autosomal deletion syndrome in humans [46]. Following the description of the syndrome, several clinical and cytogenetic studies in patients were reported [6,7,20,21,35–38,51]. After these initial reports, few studies have been published, principally because of the rarity of the syndrome and most of these were on isolated cases presenting clinical or cytogenetic peculiarities.

Molecular-cytogenetic analysis by fluorescent in situ hybridisation (FISH) has renewed the interest in this syndrome and allowed a molecular and phenotypic map of 5p to be defined [12,13,25,26,39]. Two genes, Semaphorine F (SEMAF) [48] and δ-catenine (CTNND2) [34], mapped to the "critical regions", and are potentially involved in cerebral development and thus their deletion may be associated with mental retardation. Recently the telomerase reverse transcriptase (hTERT) gene has been localised in 5p15.33 and its deletion might contribute to the phenotypic changes in CdCS children [55].

A clinical and molecular characterisation of 80 Italian patients in order to perform a phenotype-genotype correlation revealed clinical and cytogenetic variability. The identification of phenotype subsets associated with specific size and type of deletion is of diagnostic and prognostic relevance and allows a more personalised evaluation of each patient [10]. Early data on psychomotor development in institutionalised patients were discouraging [3,37]. Studies on home-reared patients who underwent early educational treatments demonstrate a better prognosis [5,15,17,50]. A specific psychomotor development chart on the Italian patients [9] and growth charts by a multi-centre international study [33] have been elaborated. A review on CdCS was also published [11]. FISH analysis with BAC clones in a patient without typical CdCS features, permitted a correlation of cat-like cry and mild mental retardation with a deletion in 5p15.31 at 8.5 Mb from the short arm telomere [45]. A recent genotype-phenotype relationship study using array comparative genome hybridisation enabled a refinement of the critical regions and confirmed the increase of mental retardation with the deletion size and type [56]. Another study by quantitative PCR allowed a further narrowing of the critical region for the cat-like cry and the characterisation of three candidate genes [53].

The aim of this paper is to further report on the natural history of CdCS in a large series of patients from the hospital-based Italian Register (I.R.) of the syndrome. The I.R., set up in the 80s (PCM), currently collects information on over 220 patients. Up to date clinical data in-

creases our knowledge of this syndrome and may be helpful in implementing guidelines to better assist the patients.

2. Material and methods

2.1. Patients and methods

The data of 220 patients of the CdCS I. R. up to June 2002 were analysed. The data included information provided by cytogenetic laboratories, genetic counselling services and paediatric units and the Italian Cri du Chat Children's Association. The information was collected by means of an ad hoc form that includes demographic and anthropometric data at birth and at later follow-up, clinical features of childhood and adult age, major and minor malformations and other medical problems, the institute where the diagnosis was made and cytogenetic reports. Moreover laboratory analyses, clinical documents and photos were available. Not all questionnaires provided information of all sections; therefore the total number of patients for which data were recorded is specified in each section of the results (text and tables). The patients were selected on the basis of clinical suspicion, confirmed by cytogenetic examination. They were included in the I.R. even if their personal and clinical data were incomplete because the inclusion criterion was a cytogenetic diagnosis. Of 137 patients in active follow-up, 123 were followed by the same clinician (PCM) and 14 by other doctors.

Two hundred twenty patients were diagnosed by standard cytogenetic methods and 112 of these were also characterised by FISH, 85 using phage probes [10] and 27 using YAC probes, previously mapped to 5p [32,41]. YAC DNAs were labelled by nick-translation with biotin-16 dUTP (Boehringer-Mannheim). FISH was performed as described by Lichter and Cremer [31]. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (200 ng/ml) and analysed and photographed by Power Gene 8860 (PSI, UK).

The patients with unbalanced translocations resulting in 5p deletions were studied separately from those with isolated deletions with regard to familial and neonatal data, survival, malformations, medical problems and psychomotor development in order to verify the greater severity reported in a previous study [51].

Clinical data were available for 185 patients: 159 with an isolated deletion (150 with a terminal deletion, one with a terminal deletion resulting from a paternal inversion, one with a terminal deletion with paternal mosaicism and seven with interstitial deletions), all showing typical CdCS facial features, and 26 patients with an unbalanced translocation resulting in a 5p deletion. The latter were excluded from the evaluation of the facial dysmorphism because of the possible phenotypic effect of the associated partial trisomy of another chromosome. Likewise, three patients with mosaicism that were previously reported [40] and one patient with a ring chromosome were excluded from the clinical analysis because of the misleading effect of these complex rearrangements on the phenotypes.

Evaluation of psychomotor development was performed using the Denver Developmental Screening Test II (DDTS II) [24]. The detailed method was previously reported [9]. All clinical, genetic and developmental data were collected in a database and statistical analysis was performed using the χ^2 test with Yates' correction.

Informed consent was obtained for all the individuals and their parents. Publication of the material included in this work has been authorised according to the terms of the Italian privacy law 196/03.

3. Results

3.1. Age and sex distribution

The distribution of 220 patients for age, year of birth, sex and vital status is reported in Table 1. Sex ratio M:F at diagnosis was 0.82. The sex ratio decreased from 0.89 for patients diagnosed in the period 1965–1983 (period I) to 0.75 in the period 1984–2002 (period II). The age ranged from 8 months to 61 years at the last follow-up or at death. One hundred thirty-three patients out of 137 in active follow-up were home-reared, four were institutionalised. The number of patients in our Register born in period I was 99 compared to an expected number of 274 (based on an estimated incidence of 1:50,000 live births) while in period II it was 109 (expected value = 205) ($P < 0.05$).

3.2. Cytogenetic and molecular-cytogenetic analyses

The results of cytogenetic and molecular-cytogenetic analyses performed in 220 patients are summarised in Table 2. All 220 patients are deleted for at least the CdCS critical region in 5p15.2 [39]. The size of deletion ranged from 5p15.2 to 5p11 (Fig. 1).

3.3. Age of the diagnosis

The precise age at diagnosis was available for 193 patients and was within the first month of life in 82 (42.5%) (including one prenatal diagnosis for advanced maternal age), before three months of age in 114 (59.1%) and between the fourth and 12th month in 44 (22.8%). In total, 158 patients (81.9%) were diagnosed in the first year of life, 35 (18.1%) at an age ranging from 13 months to 47 years. In the period 1984–2002 the percentage of diagnosis in the first month of life was higher than in the period 1965–1983 ($P < 0.05$) (Table 3).

Table 1
I.R. for CdCS. Age grouping, period of birth, sex distribution and vital status for 220 patients as at 2002

Age grouping (years)	Year of birth	Total	%	M	F	Dead	Patients in active follow-up
0–4	2002–1998	17	7.7	8	9	1	16
5–9	1997–1993	35	15.9	13	22	0	34
10–14	1992–1988	38	17.3	17	21	1	33
15–19	1987–1983	28	12.8	15	13	1	17
20–24	1982–1978	24	10.9	12	12	2	11
25–29	1977–1973	39	17.7	12	27	2	18
30–34	1972–1968	18	8.2	10	8	5	2
35–39	1967–1963	17	7.7	9	8	1	4
40–49	1962–1953	2	0.9	2	0	1	1
50–59	1952–1943	0	/	0	0	0	0
60–69	1942–1931	1	0.5	0	1	0	1
Data not available	/	1	0.5	1	0	0	0
Totals		220	100.0	99	121	14	137

Table 2
I.R. for CdCS. Cytogenetic and molecular-cytogenetic analyses for 220 patients

Chromosomal rearrangements	Total patients	%
Terminal deletions [a]	180	81.8
De novo interstitial deletions [b]	7	3.2
Familial translocations [c]	17	7.7
De novo translocations [c]	10	4.5
Terminal deletions from a paternal inversion	1	0.5
Terminal deletions from a paternal mosaicism	1	0.5
Mosaicism	3	1.4
Ring chromosome	1	0.5
Total	220	100.0

[a] For 45 patients data are not available for both parents.
[b] Seven out of 112 patients analysed by FISH.
[c] Patients with unbalanced translocations resulting in 5p deletions.

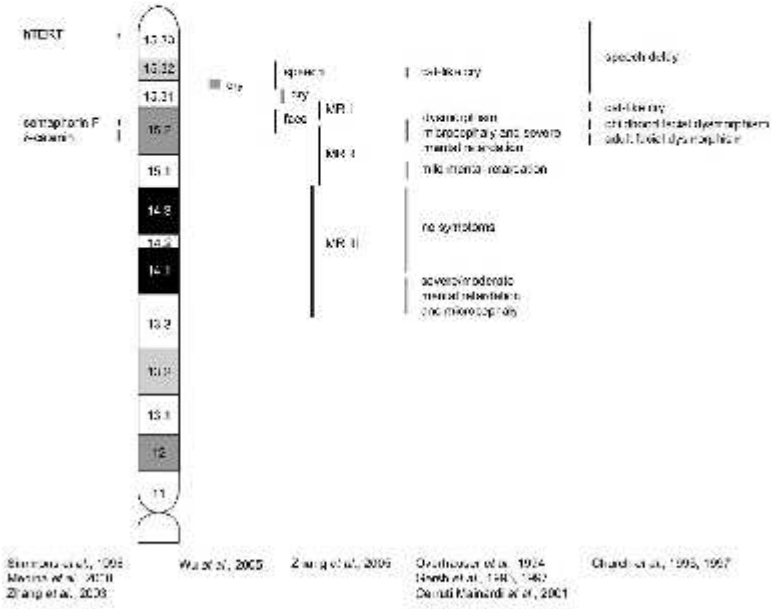

Fig. 1. Phenotypic map of 5p for the critical regions for the cry and for other signs of CdCS. MR, mental retardation, increasing in severity from MR I to MR III. Vertical grey lines in p15.1, p14 and p13 refer to clinical symptoms reported in individual families with interstitial deletion. Chromosome bands are reported according to ISCN (1995) [28].

Table 3
I.R. for CdCS. Age of diagnosis for 193 patients [a] in the period 1965–2002

Age of diagnosis	Number of patients	%	Periods of diagnosis			
			1965–1983	%	1984–2002	%
In the first month	82	42.5	28	32.9	54	50.0[b]
2–12 months	76	39.4	36	42.4	40	37.0
13 months-5 years	26	13.5	17	20.0	9	8.3[b]
Over 6 years	9	4.7	4	4.7	5	4.6
Total	193	100.0	85	100.0	108	100.0

[a] Patients with precise diagnosis age available (156 with terminal deletions, seven with interstitial deletions, 26 with unbalanced translocations resulting in 5p deletions, three with mosaicism and one with ring chromosome).
[b] $P < 0.05$.

3.4. Family and neonatal data

In the period 1965–1983 the mean maternal and paternal age at birth, available for 46 patients with isolated deletions, was respectively 27 years and 7/12 (standard deviation (S.D.) = ± 5 years and 2/12) and 32 years and 6/12 (S.D. = ± 5 years and 10/12). In the period 1984–2002 the age for the 74 patients with isolated deletions was respectively 29 years and 7/12 (S.D. = ± 5 years and 2/12) and 33 years and 11/12 (S.D. = ± 7 years and 2/12). The mean gestational age for 126 patients with isolated deletions was 38 weeks and 5 days (S.D. = ± 2 weeks and 1 day). The mean birth weight, for 151 patients with isolated deletions was 2614 g (S.D. = ± 483 g), the mean length was 46.9 cm (S.D. = ± 3.5 cm) in 92 patients with isolated deletions and the mean head circumference at birth was 31.8 cm (S.D. = ± 2.1 cm) in 86 patients with deletions. There were no significant differences for the 27 patients with unbalanced translocations resulting in 5p deletions.

3.5. Survival

The death of 14 patients out of 220 has been reported (6.4%, seven males and seven females) (Table 4). Nine patients had a terminal deletion and five had an unbalanced translocation resulting in a 5p deletion (three familial, two de novo). Mortality was higher in patients with unbalanced translocations resulting in 5p deletions (5/27 = 18.5%) than in those with terminal deletions (9/189 = 4.8%) ($P < 0.05$). Eleven out of 106 patients (10.4%) diagnosed in the period 1965–1983 died compared to only three out of the 114 (2.6%) diagnosed in the period 1984–2002 ($P < 0.05$).

3.6. Clinical features I and II

Table 5 reports the facial dysmorphism and other typical features observed at diagnosis in 159 patients with isolated deletions, for which the data were available. Included are follow-up data in 49 out of 159 patients. Changes in clinical features with age are reported in Table 6.

Table 4
I.R. for CdCS. Causes of death of 14 patients

ID number	Age at death	Causes of death	Karyotype
38	1 day (1975)	Respiratory insufficiency	46,XX,del(5p→pter)
86	2 months (1986)		46,XX,del(5)(p13→pter)
45	1 day (1968)	Congenital cardiopathy	46,XXdel(5)(p12→pter)
47	1 year (1975)		46,XX,–5,+der(5)t(1;5p) fam
49	4 days (1979)		46,XY,–5,+der(5)t(5p;X) de novo
87	4 days (1982)		46,XX,–5,+der(5)t(5;8)(p11;p11) mat
91	1 day (1991)		46,XX,–5,+der(5)t(5;16)(p15.1;q24) pat
4	14 months (1965)	Respiratory infection	45,XY,–5,+der(5)t(5p;15) de novo
12	5 months (1970)		46,XY,del(5p→pter)
63	16 years (1984)	Intestinal occlusion	46,XY,del(5p→pter)
27	29 years (1983)	Hepatitis	46,XY,del(5)(p13→pter)
64	14 years (1984)	Convulsions, tachycardia, cardiopathy	46,XY,del(5p→pter)
185	3 months (1999)	Sudden death	46,XY,del(5)(p14→pter)
15	6 years (1976)	Unknown	46,XX,del(5p→pter)

ID: identification.

Table 5
I.R. for CdCS. Clinical features (I)

	159 patients [a] at diagnosis	%	49 patients [b] at diagnosis	%	49 patients [b] > 15 years	%
Facial features						
Round face	96/115	83.5	29/31	93.5	1/48	2.1
Prominent metopic bossing	58/82	70.7	16/21	76.2	12/41	29.3
Broad nasal bridge	102/117	87.2	31/35	88.6	28/43	65.1
Lateral downward slanting palpebral fissures	70/123	56.9	21/38	55.3	14/47	29.8
Hypertelorism	105/129	81.4	34/41	82.9	29/46	63.0
Epicanthal folds	119/132	90.2	39/43	90.7	27/48	56.2
Strabismus divergent/ convergent	48/101	47.5	18/34	52.9	21/47	44.7
Short philtrum	52/86	60.5	15/23	65.2	43/49	87.8
Down turned corners of the mouth	81/100	81.0	31/32	96.9	27/42	64.3
Low-set ears	81/116	69.8	21/30	70.0	15/46	32.6
Microretrognathia	119/123	96.7	37/38	97.4	33/46	71.7
Other clinical features						
Typical cry/acute voice	141/147	95.9	46/48	95.8	30/45	66.7
High arched palate	62/74	83.8	13/14	92.9	23/40	57.5
Short neck	41/73	56.2	7/15	46.7	10/39	25.6
Transverse flexion creases	103/112	92.0	38/40	95.0	38/40	95.0
Hypoplasia thenar eminence	42/57	73.7	10/14	71.4	26.35	74.3
Small pelvis	31/42	73.8	4/7	57.1	13/24	54.2
Diastasis recti	43/56	76.8	5/8	62.5	20/27	74.1
Hypotonia	78/108	72.2	34/35	97.1	1/40	2.5

[a] 159 patients with isolated deletions (152 with terminal deletions and seven with interstitial deletions) for which the data were available.
[b] Longitudinal study on 49 out of 159 patients (47 with terminal deletions and two with interstitial deletions) for which it has been possible to evaluate the persistence of the clinical features in time.

Table 6
I.R. for CdCS. Clinical features (II)

	50 patients[a] > 15 years	%
Facial features		
Thin/long face	34/48	70.8
Asymmetric face	16/45	35.6
Supra-orbital arch prominent	13.42	31.0
Long/coarse nose	18/39	46.2
Full lower lip	19/42	45.2
Dental malocclusion	36.48	75.0
Other clinical features		
Miopia	5/34	14.7
Short metacarpals	38/46	82.6
Short metatarsals	30/40	75.0
Scoliosis	20/47	42.6
Muscle hypotrophia	6/37	16.2
Hypertonia/hipereflexia	28/38	73.7
Pes planus	27/43	62.8
Premature greying	14/46	30.4
Normal sexual development	46/48	95.8
Hyperactivity	18/47	38.3

[a] Adolescent and adult patients (48 with terminal deletions and two with interstitial deletions) for whom the data were available. These patients include the 49 patients of Table 5.

3.7. Clinical features III

A malformation was present in 185 patients (159 with isolated deletions and 26 with unbalanced translocations resulting in 5p deletions) (Table 7). Both maximal and minimal percentages were always higher in patients with unbalanced translocations resulting in 5p deletions than in those with an isolated deletion; for the minimal percentage the difference was significant for cardiac, cerebral and renal anomalies ($P < 0.05$). The frequency of cardiac, cerebral, renal and gastrointestinal anomalies observed in all patients, independently of the type of deletion, was 19% in the period 1965–1983 and 66% in the period 1984–2002 ($P < 0.01$).

3.8. Medical problems

Data about medical problems, surgical operations and hospitalisation are reported in Table 8. One hundred fifty-nine patients with isolated deletions, for which data were available, were evaluated. There were no significant differences for 26 patients with unbalanced translocations resulting in 5p deletions.

3.9. Psychomotor development

The evaluation of the psychomotor development (Denver Test II [24,42]) was performed separately in 103 patients with an isolated deletion and in 13 with an unbalanced translocation. The results for 103 patients with isolated deletions are reported in Fig. 2. The youngest reported age of independent walking was 15 months, the median age was 3 years and all children learned to walk. Forty-four patients out of 103 (42.7%) were able to form sentences, the first at 18 months, 25% at 4.5 years, the median age was 5.5 years and 86.4% by 10 years of

Table 7
LR. for CdCS. Clinical features (III) [a]

	Isolated deletion [b]	% max [c]	% min [d]	Malformations	Translocation [b]	% max [c]	% min [d]	Malformations
Congenital heart disease	29/81	35.8	18.2 [e]	IVD 14, IVD + IAD 1, IVD + aortic valve stenosis 1, PDA 6, IAD 3, IAD + pulmonary valve stenosis 1, Tetralogy of Fallot 1, aortic valve stenosis 1, not specified 1.	11/19	57.9	42.3	PDA 3, IAD 3, IVD 1, IVD + pulmonary valve stenosis 1, not specified 3.
Neurological abnormalities	17/57	29.8	10.7 [e]	H/ACC 5, ACC + scarce white matter myelination 1, ACC + brainstem hypoplasia 1; ACC + cerebellar hypoplasia 1, cerebral atrophy 5, cerebral + cerebellar atrophy 1, cerebral atrophy + hydrocephalus 1, cerebellar atrophy 1, periventricular leukomalacia 1	8/13	61.6	30.8	H/ACC 3, ACC + cerebellar anomalies 1, cerebellar atrophy 1, cerebellar atrophy + hydrocephalus 1, herniated cerebellum + meningocele + micropolygyria of the frontal lobes1, cerebral arteriovenous abnormality 1.
Renal	9/49	18.4	5.7 [e]	unilateral kidney 2, unilateral kidney + pelvic and renal ectasia 1, renal ectopia 2, horseshoe kidney 2, renal hypoplasia 1, hydronephrosis 1.	5/13	38.5	19.2	renal hypoplasia 2, hydronephrosis 2, renal ectopia 1
Gastrointestinal	6/28	21.4	3.8	congenital megacolon 3, anteriody placed anus 3.	1/4	25.0	3.8	anteriorly placed anus 1.
Genital	20/159	/	12.6	cryptorchidism 14, external genitalia hypoplasia 3, phimosis 2, hypospadias 1.	4/26	/	15.4	cryptorchidism 3, cryptorchidism + external genitalia hypoplasia 1.
Feet	83/159	/	52.2	pes planus 30, pes planusvalgus 25, pes planusvarus 5, pes planus + overlapping toes 4, syndactyly +pes planus/ valgus/ varus/clubfoot/rocker-bottom 10, clubfoot 3, syndactyly 2, camptodactyly 2, short hallux 1, sandal sign 1.	16/26	/	61.5	pes planus 4, pes planusvalgus 5, pes planusvalgus +hammer hallux 1, clubfoot 3, clubfoot+hammer hallux 1, syndactyly 1, syndactyly + short hallux 1.
Hands	31/159	/	19.5	clinodactyly V finger 21, camptodactyly 3, syndactyly 2, preaxial polydactyly right thumb 1, ulnar polydactyly1, thumb laxity 1, trident hand 1, fingers ovelapping 1.	7/26	/	26.9	clinodactyly V finger 4, absent hand with five rough fingers 1, clubhand 1, thumb in hyperextension 1.

(continued)

Table 7 *(continued)*

	Isolated deletion [b]	% max [c]	% min [d]	Malformations	Translo-cation [b]	% max [c]	% min [d]	Malformations
Muscoloskeletal	40/159	/	25.2	inguinal hernia 17, joint hypermobility 9, joint hypemobility + hiatal hernia 1, joint hypemobility + tibia and knee vari 1, tibia vara/knee varus/valgus 7, tibia vara/knee varus/valgus + umbilical hernia 1, hip dysplasia 2, hip valgus 1, hiatal hernia 1.	9/26	/	34.6	inguinal hernia 4, inguinal hernia +joint hypermobility 1, joint hypermobilityn 2, hip dysplasia 2
Others	32/159	/	20.1	preauricular tag/fistula 13, preauricular tag/fistula + bifid uvula 1, preauricular tag/fistula + cutaneous aplasia 1, bifid uvula 4, angioma 3, angioma +hemihypertrophy 1, angioma + epitrochlear and suprapatellar fovea 1, microphtalmia 3, bilateral choanal atresia 2, polyotia 1, auditory canal stenosis 1, cleft lip 1.	5/26	/	19.2	preauricular tag/fistula 3, bifid uvula 1, hypodontia 1.

IVD: interventricular defect, IAD: interatrial defect, PDA: patent ductus arteriosus, H/ACC: hypoplasia/agenesis of corpus callosum.

[a] 185 patients for which the data were available, 159 with isolated deletions (152 with terminal deletions + 7 with interstitial deletions) and 26 with unbalanced translocations resulting in 5p deletions.

[b] The denominator refers to the number of patients in whom clinical manifestations and instrumental examinations were referred to.

[c] The patients without referred clinical manifestations and instrumental examinations were not considered (e.g. neurological abnormalities: 17/57 = 29.8% (%max)).

[d] The patients without referred clinical manifestations and instrumental examinations were considered negative or normal (e.g. neurological abnormalities: 17/159 = 10.7% (%min)).

[e] $P < 0.05$.

Table 8
I.R. for CdCS. Medical problems [a]

	Frequency	%
Neonatal [b]		
- Asphyxia/cianosis	32	26.9
- Feeding difficulties	52	43.7
Anesthesiological [b]	1	0.8
Respiratory infections	83	52.2
Other infections [b]		
- Otitis	18	15.1
- Gastrointestinal	9	7.6
- Urinary	4	3.4
Orthopedic		
- Pes planus/valgus/varus/equinus/clubfoot	75	47.2
- Scoliosis	34	21.4
Dental		
- Malocclusion	61	38.4
- Caries	7	4.4
- Not specified	19	11.9
Ocular		
- Strabismus	57	35.8
- Myopia	13	8.2
- Astigmatism	7	4.4
- Retinopathy	7	4.4
- Blindness	4	2.5
- Cataract	2	1.3
Gastrointestinal [b]		
- Constipation	28	23.5
- GER/regurgitation/esophagitis/vomit	15	12.6
Seizures	25	15.7
Allergies [b]		
- Cutaneous	4	3.4
- Food allergy	3	2.5
- Respiratory	3	2.5
Other clinical problems		
Hospitalisations [b]		
- Surgery operation	56	47.1
- Respiratory infection	9	7.7
- Gastrointestinal infection	5	4.2
Surgery operations [b]		
- Inguinal hernia	9	7.7
- Cryptorchidism	8	6.7
- Congenital heart defects	7	5.9
- Strabismus	4	3.4
- Other	28	23.5

GER: gastroesophageal reflux.
[a] Data available for 159 patients with isolated deletions (152 with terminal deletions and seven with interstitial deletions).
[b] Data available for 119 out of 159 patients.

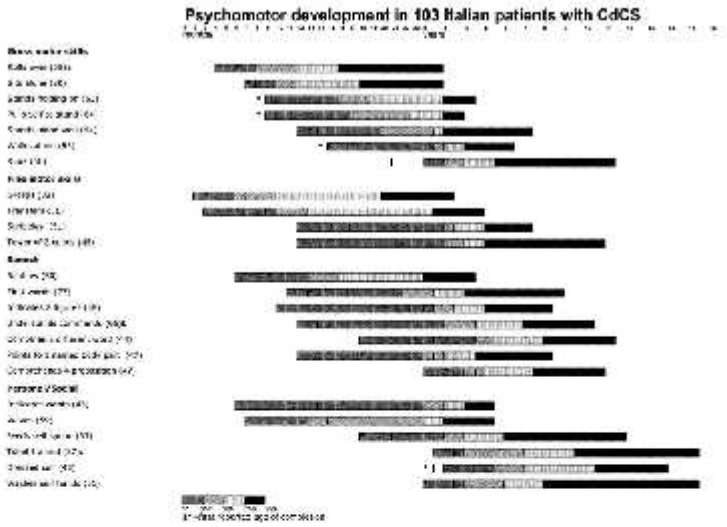

Fig. 2. | = 90th of normal American population (Denver Developmental Screening Test II) [24]. * = 90th of the normal Italian population for the available milestones [42]. [a] Number of informative cases in brackets. [b, c] Skills not screened for on the DDST II [24], see in Cerruti Mainardi et al. [9].

age. With regard to autonomy, the youngest age by which a child learned to eat by himself was 18 months, the median age was 4 years. The youngest age for dressing oneself was 3 years, median age was 7 years. The number of patients with an unbalanced translocation who achieved the milestones below the 25th centile was lower compared to those with isolated deletions for 17/24 skills but the difference never reached statistical significance. If the size of the sample were twice as big, statistical significance would be reached for "sits alone" and "walks alone" ($P < 0.05$).

The correlation between the beginning of physiotherapy and the achievement of developmental skills was evaluated in 95 patients with an isolated deletion. Most patients started therapy before the age of one year: ≤ 1 year (68/95 = 71.6%), 1–3 years (21/95 = 22.1%), > 3 years (6/95 = 6.3%). Of 95 patients, 28 (29.5%) were born in the period 1965–1983 and 67 (70.5%) in the period 1984–2002. Only 14 out of the 28 patients (50.0%) born in the period 1964–1983 started physiotherapy before one year, while 54 of 67 patients (80.6%) born in the period 1984–2002 ($P < 0.01$) did so. Patients who attained the skills below the 25th centile (e.g. a child who rolled over before 8 months, achieved the milestone below the 25th centile (Fig. 2) were more frequent in the group who started the therapy ≤ 1 year of life than in the group who started the therapy > 1 year for only 11 of 24 skills and the difference never reached statistical significance.

4. Discussion

This study on 220 Italian patients enabled us to confirm and to expand the literature data on this rare disease. Cytogenetic and molecular-cytogenetic analyses showed a large variability of the deletions with breakpoints ranging from 5p15.2 to 5p11 (Fig. 1). This study confirmed the high percentage of terminal deletions (81.8%; about 80% in Niebuhr [37] data). A lower percentage (77.7%) was present in 112 out of 220 patients studied with FISH analysis, which enabled the identification of five interstitial deletions, one de novo unbalanced translocation and one mosaicism not correctly diagnosed by standard cytogenetics. Therefore the importance of FISH for a correct diagnosis of 5p deletions must be underlined, as it allows the breakpoint to be established with greater precision, and this is useful for a more personalised evaluation of the patient [10,32].

The number of patients included in the I.R. is lower than that expected because our I.R is hospital-based and not population-based. However, it should be pointed out that for the period 1984–2002 the number is much closer to the expected value (53.2%) than in the period 1965–1983 (36.1%) and this difference is significant. This may be partly due to diffusion of knowledge about the syndrome, home-rearing and to the Italian Cri du Chat Children's Association.

In most cases, the diagnosis was made in the first year of life (81.9%) but only 9.1% within the first 3 months, while for 18.1% (35 patients) the diagnosis was made at an age ranging from 13 months to 47 years. As foreseeable, in the period 1984–2002 the number of diagnoses made in the first month of life was higher than in the period 1965–1983 ($P < 0.05$). Nevertheless, even in the more recent period, still 14 patients out of 35 were diagnosed after the first year of life. In two cases a 5p deletion failed to be identified at prenatal diagnosis for advanced maternal age. In these last cases, it is possible that the chromosomal anomalies were not identified because of a small terminal deletion in one case (breakpoint in 5p15.1) and a familial unbalanced translocation resulting in 5p deletion in the other, which "apparently" did not modify the morphology of the involved chromosomes.

Of the 35 patients diagnosed after the first year of life, 10 were born shortly after the syndrome had been described, four were institutionalised (three born in the period 1965–1983 (period I), one in the period 1984–2002 (period II)), five had an unbalanced translocation resulting in 5p deletion (four I, one II), five a small terminal deletion in 5p15 (two I, three II), two an interstitial deletion (II), two had other associated pathologies (two II: one a severe prematurity, one a West syndrome), for three, preliminary karyotype results appeared to be normal (two I, one II). For four other patients (II) there is not enough data to explain the delay in diagnosis. These observations show the importance of knowing the clinical features at birth and their changes in time in order to perform karyotype analysis more often and, in doubtful cases perform FISH analysis. Early diagnosis is important for a correct evaluation of medical problems (e.g. possible intubation difficulties in the early months of life because of larynx anomalies) and for genetic counselling on the reproductive risk, more particularly to identify patients with a deletion caused by familial rearrangements which have a higher recurrence risk [8,22]. Moreover, late diagnosis prevents the early access to adequate information and psychological support, increasing the bewilderment of the family.

Mean maternal age at birth (period I) overlapped with data reported by Niebuhr [37] and that of the Italian general population (27 years) of the same period. In the second period maternal age at birth increased, like that of Italian general population (29 years) reflecting the present trend to conceive later. The neonatal parameters (gestational age, weight, length and head

circumference) are similar to those reported by Niebuhr [37]. Mortality, already quite low in previous studies, has decreased in time: 9.67% in 1978 [37], 8.75% in 1983 [51], and 6.36% in the present study. In the series from Niebuhr [37] 75% of deaths occurred in the first month and 90% in the first year of life. In the present study this decreased to 35.7% ($P < 0.05$) and 64.3%, respectively (almost statistically significant). Mortality in patients with unbalanced translocations resulting in 5p deletions was higher than in those with isolated deletions ($P < 0.05$) as already observed by Wilkins [51]. The number of deaths in period I (10.4%) was four times greater than that in period II (2.6%) ($P < 0.05$). This reduction can probably be ascribed to improvements in neonatal and paediatric care.

The examination of clinical features confirms that the cat-like cry represents the most typical sign of the syndrome, not only at birth and in the first years of life, but also later. The timbre of the voice (shrill, sometimes hoarse) remains abnormal in most adolescents and adults [5,7,30,37,43,49]. Kjaer and Niebuhr [29] recently suggested that the developmental connection between the malformations in the rhombencephalic cranial base-brainstem region and the laryngeal region responsible for the cry in CdC patients is related to the course of migration of the neurons to the larynx. The main facial features are not specific, but their combination produces a well recognisable facial gestalt [37,52] (Figs. 3 and 4). A longitudinal study carried out in 49 patients showed that the round face generally disappeared, prominent metopic bossing became less evident, palpebral fissures frequently became horizontal. The other features persisted in most patients and the short philtrum became more evident. Changes of facial dysmorphism in adolescent and adult age have been described [3,7,20,35,49]; the present study indicates the presence of a narrow face, long coarse nose, full lower lip, dental malocclusion (open bite), short metacarpals and metatarsals. However, the phenotype remains recognisable in most patients (Figs. 3 and 4). The muscle hypotonia, present in infancy, is replaced by hypertonia, evidenced by a hyperactive patellar reflex [7,37]. Premature greying was already seen after 15 years, normal sexual development was observed in most patients. Hyperactivity was present in 66.7% of patients from 10 to 15 years and in 38.3% of patients over 15 years; both percentages were lower than those reported in previous studies: 80% in Dykens and Clarke [23] (age range 2–40 years, mean age 12 years), 90% in Cornish et al. [16] (age range 4–16 years, mean age 7.6 years), likely because of different age range of patients considered. This finding can also be due to the decrease of hyperactivity with age, as already suggested [19], and it is of prognostic relevance. However, precocious educational interventions can improve the behaviour of CdC children [50] considering that hyperactivity is the most striking problem in CdCS [5,23,50]. Hyperactivity and distractibility are considered specific characteristics of this syndrome in comparison to others as Prader–Willi and Smith–Magenis syndrome [14].

The clinical features are present in most patients who had a deletion involving the critical region in 5p15.2 [39], but there is a variability of frequency and expression related to size and type of the deletion [10]. The facial dysmorphism is particularly mild in patients with a deletion in 5p15.1 and in 5p15.2 (Figs. 3 and 4). Moreover, the patients with interstitial deletions which do not include the critical regions for the cry [39] or the language [12,13], have not the typical cry or have a better development of the language, respectively [10, 12, 13. 39, 53, 56]. In the patients with unbalanced translocations resulting in 5p deletions (not evaluated in the examination of the facial features) the phenotype may be influenced by the partial trisomy of the other chromosome involved [8,39,51], and a different phenotype can be present in the patients with more rare rearrangements. Two patients, one with an interstitial deletion and one

Fig. 3. (A) Patient 103 at age 10 months, (B) 7 years, (C) 14 years. Diagnosis at 5 months, del(5)(p14.1). (D) Patient 111 at age 2 years, (E) 4 years, (F) 9 years. Diagnosis at 2 months, del(5)(p15.1). (G) Patient 104 at age 1 year, (H) 10 years, (I) 28 years. Diagnosis at 13 years, del(5)(p15.2). All the patients show terminal deletions.

with a small terminal deletion neither of which included the critical region in 5p15.2 [39], had mild facial dysmorphism (not typical of CdCS) and mild mental retardation. The patient with a small terminal deletion had the typical cry because he lost the more distal cat-like cry critical region. These two patients were not included in the present study and they confirm that not all the 5p deletions can be considered CdCS [1,2,10,12,13,18,25,26,39,45,53,56]. This variability must be kept in mind for a precocious diagnosis and in order to permit a more precise prognostic evaluation.

As regards major malformations, many patients, in particular the oldest ones, did not undergo instrumental investigations. Available data shows that congenital heart diseases were the most frequent, followed by cerebral, renal and gastrointestinal anomalies. The most prevalent cardiac defects were ventricular and atrial septal defects and patent ductus arteriosus. There was one case of tetralogy of Fallot. In addition to microcephaly, brain anomalies such as hy-

Fig. 4. (A) Patient 186 at age 4 months (B, C) 14 years. Diagnosis at 4 years, del(5)(p15.2). (D) Patient 69 at age 2 years, (E, F) 23 years. Diagnosis at 14 months, del(5)(p15.2). (G) Patient 110 at age 2 years and 8 months, (H, I) 20 years. Diagnosis at birth, del(5)(p14.1). (J) Patient 71 at age 7 years, (K, L) 25 years. Diagnosis at birth, del(5)(p14.1). All the patients show terminal deletions.

poplasia or agenesis of the corpus callosum, cerebral atrophy and cerebellar hypoplasia or atrophy were recorded. Renal anomalies included renal agenesis or hypoplasia, renal ectopia, horseshoe kidney and hydronephrosis. Three cases of congenital megacolon were reported. Anomalies of foot, hand and muscle-skeletal, were frequent. Among the other malformations preauricular tags or fistulae were not rare. In a previous study Wilkins et al. [51] found a higher frequency of malformations in patients with unbalanced translocations resulting in 5p deletions than in those with a simple deletion; our study confirms this for cardiac, cerebral and renal anomalies ($P < 0.05$). The evaluation of the frequency of cardiac, cerebral, renal and gastrointestinal malformations in the period 1965–1983 (19.0%) and 1984–2002 (66.0%), without distinction between patients with isolated deletions and those with unbalanced translocations resulting in 5p deletions, showed a significant increase in the second period ($P < 0.01$), probably due to the more frequent and detailed instrumental investigations in recent years.

A previous collaborative study [33] about growth confirmed the existence of pre- and postnatal growth retardation of weight, height and head circumference. Difficulties in feeding, frequently reported during the first years of life, can be the cause of the low weight. But reduced weight in adolescent and adult patients may also be explained by constitutional factors related to the syndrome [7,38]. Microcephaly was not present in all patients at birth. In our previous study [10] microcephaly at birth was shown to be correlated to deletion size, therefore this data is of prognostic relevance. Almost all patients became microcephalic with age, but the most severe microcephaly was found in patients with largest deletions. Specific growth charts for CdCS [33] are useful for a correct evaluation of development compared with a population of CdCS children and to avoid unnecessary interventions.

In 159 patients with isolated deletions, the most frequent medical problems in neonatal age were asphyxia/cyanosis and difficulties in feeding, usually resolved in regular neonataly ward and only occasionally requiring admission to a neonatal intensive care unit. Feeding difficulties may persist during the first months or year of life. There were difficulties (but also few attempts) in breast-feeding. In recent years the number of newborns receiving breast milk is increasing, helping to establish a good mother-child relationship. A correct communication of the diagnosis is important as a psychological support to the families, as is information about physiotheraphy which should be started right from the first weeks of life (to improve suction and swallowing). Even though genetic counselling indicates that the risk of recurrence is no higher than that of the general population, some couples do not wish additional children because of the heavy psychological impact.

In neonatal age and in the first months of life it is important to highlight the risk of the anaesthetic problems (difficulties in intubation), linked to larynx and epiglottis abnormalities. This problem was previously reported for four patients [4,54]: a newborn and three patients aged 7 weeks, 33 months and 48 months, respectively (one died, three survived). Two patients in this study aged 1 month and 3 months needed a tracheotomy after intubation. At an older age a considerable number of our patients have undergone total anaesthesia for surgery without problems. Surgery was usually for congenital heart defects, inguinal hernia, cryptorchidism and strabismus. Respiratory infections (bronchitis and bronco-pneumonia) were frequent (52.2%) but only in the first years of life. Neither clinical nor serological evidence of a higher sensibility to infections are reported [44]. All compulsory and recommended vaccinations are advised.

Gastroesophageal reflux and vomiting are frequently reported in the first year of life, and constipation is often present later. Examinations for ocular and orthopaedic problems are re-

commended. Strabismus is frequent and usually divergent, and has been surgically treated with success in four patients. Seizures are rare at all ages even if more frequent in this study (15.7%) than in the Niebuhr [37] series (2.7%) ($P < 0.05$). Dental malocclusion (open bite) was present in 36.8% of children and adolescents; dental and orthodontic treatments are possible. Hospitalisations were most frequently for surgery, respiratory and gastrointestinal infections. A higher frequency of hospitalisation for acute illness (respiratory infections) and surgery, noticed by Wilkins et al. [51] in patients with unbalanced translocations resulting in 5p deletions was not observed in this study.

Psychomotor delay was present in all patients. Nevertheless our data confirm a better prognosis in home-reared patients [5,9,15,17,50]. The comparison between patients with isolated deletions and those with unbalanced translocations resulting in 5p deletions showed that the latter achieved developmental skills later, confirming the report by Wilkins et al. [50], even though the difference did not reach statistical significance because of the small sample size. Patients with isolated deletions achieved developmental milestones earlier than observed by Wilkins et al. [50], (36% of patients walked alone at age 3 [50] and 50% in the present study) and all children learned to walk unlike previous studies [37,50]. Language delay was severe in most patients, but 27% managed to form sentences before age 10 in Wilkins et al. [50], compared to 86% in the present study ($P < 0.01$).

With regard to the importance of early physiotherapy, Wilkins et al. [50], reported that most patients started the therapy above age 3, while in the present study most patients, born mostly in the period 1984–2002, started the therapy within the first year. Starting physiotherapy early certainly contributed to the better results, even though there was no significant difference between the two groups in the timing of achievement of skills. This finding shows the existence of variability also for psychomotor development. In our previous genotype-phenotype study in patients with deletions [10], we found that the severity of psychomotor retardation was related to the size of the deletion, as noticed by others [18,29,51]. In the present study we found a more severe clinical picture in patients with unbalanced translocations resulting in 5p deletions. Recently Zhang et al. [56] in a large study using array CGH analysis confirmed a correlation between mental retardation and size and type of deletion. Therefore several genetic and environmental factors can influence the psychomotor development. However, our results showed an improvement in comparison with the past. In addition to the factors previously considered (home-rearing, early starting of physiotherapy), early education, the use of information technology and sport (Fig. 4), have certainly contributed to this result, also improving social insertion. The collected data shows a less pessimistic picture than in the past which ought to encourage caregivers and parents to work together in order to improve the quality of life of children and their families.

Electronic database information Online Mendelian Inheritance in man (OMIM): http://www.ncbi.nlm.nih.gov/Omim.

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