Abstract

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

Aim: We aimed to determine whether high-mobility group A2 (HMGA2) and cyclin-dependent protein kinase 6 (CDK6) are involved in the pathogenicity of IGHD.

Methods: We directly sequenced coding regions and exon-intron boundaries of the genes HMGA2 and CDK6 in 105 Caucasian IGHD patients from the Dutch HYPOPIT study. In addition, we developed a new probe set of multiplex ligation-dependent probe amplification for both genes in order to detect copy number variations.

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

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

Introduction

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

The reported prevalence of IGHD ranges from 1 in 3480 to 1 in 10 000 live births (3). Five to thirty percent of cases have first-degree relatives with short stature (4), suggesting a genetic etiology. Even though mutations in GH1 and GHRHR have been found as a genetic cause of IGHD (reviewed (1)), the vast majority of patients do not carry mutations in these two genes.

This suggests that other genes are involved and the identification of these genes is important to elucidate the pathogenesis of this complex condition.

In recent years, genome-wide association (GWA) studies have revealed genes and biological pathways that were not previously known to be involved in human growth. In 2007, the first GWA study by Weedon et al. (5) identified high-mobility group A2 (HMGA2) as a new gene associated with childhood and adult height in the general population. Then, in 2008, three separate studies together reported 44 SNPs that were associated with height variability within the normal population (6–8). Only four of these loci were identified in all three studies, namely HMGA2, cyclin-dependent protein kinase 6 (CDK6), HHIP, and ZBTB38 (Fig. 1), and they have been confirmed as truly associated in meta-analyses (9, 10). Based on the published data, we focused our research on the first two of these genes.
progression through the G1 phase in cells that continue proliferating (24). Cdk4-null mice combine dwarfism phenotypes with infertility and resistance to human GHRH (25). Since it has been suggested that CDK6 compensates for loss of CDK4 and vice versa (26), mutations in CDK6 might also affect growth.

Based on the results of GWA studies and these other published data, we selected the candidate genes HMGA2 and CDK6 for mutation screening and copy number variation analysis in 105 Dutch patients with IGHD.

**Subjects and methods**

**Study subjects**

DNA samples were collected from 105 patients with IGHD who had participated in the Dutch HYPOPIT study, which investigates the genetic causes of GH deficiency (27). These patients had been recruited from the Endocrinology Departments at six university and two non-university hospitals and had been registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003. IGHD was defined as a peak GH response <20 mU/l to arginine or clonidine test, or <30 mU/l combined with serum IGF1 < −2 SDS and normal serum levels of other pituitary hormones. Exclusion criteria were as follows: GH deficiency of known cause, such as a brain tumor, brain surgery, brain radiation, and known syndromes. Written informed consent was obtained from all participating patients and their parents or legal guardian(s).

**Sequencing**

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

In all patients, HMGA2 (NM_001259.6) and CDK6 (NM_001259.6) coding exons and exon–intron boundaries were PCR amplified using Qiagen reagents of 5 units/μl Taq DNA Polymerase, 10× PCR buffer, 5× Q-Solution, 10 mM dNTPs, 25 mM MgCl₂, and primers (sequences available on request). Mixtures were incubated at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s and 60 °C for 1 min and 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min. The electrophoretic separation, sizing, and quantification of PCR products were performed using LabChip GX (Caliper Life Sciences, Hopkinton, USA) microfluidics technology. The amplified products were purified using illustra GFX 96 PCR purification kit (GE Healthcare). Sequencing was performed using Big Dye Terminator reaction kit (Applied Biosystems). After purification with Dyex 96 kit (Qiagen), the products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems).

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

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

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

Multiplex ligation-dependent probe amplification

Copy number analysis was carried out using multiplex ligation-dependent probe amplification (MLPA) combined synthetic probes and the P200 kit with control sequences (MRC Holland, Amsterdam, The Netherlands). We designed a home-made probe set in order to detect deletions or duplication in all coding exons in HMG2 (NM_003483.4) and CDK6 (NM_001259.6), plus the 3’-UTR region of HMG2 known to be regulated by miRNAs. The homemade probe set contained products ranging from 98 to 166 bp in size with a minimum product size difference of 4 bp, sufficient to be resolved by capillary electrophoresis (sequences available on request). The assay was performed according to the manufacturer’s instructions and analyzed using Gene Marker software (SoftGenetics LLC, State College, PA, USA).

Functional analysis of the HMGA2 mutant

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

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

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

Statistical analysis

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

Results

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

Mutation screening

We directly sequenced the complete coding region and intron–exon boundaries of HMG2 and CDK6 in all 105 patients. In one patient, we identified a yet unknown heterozygous deletion in HMG2. This was confirmed by the subcloning of the PCR product from the affected patient and the detection of two different fragments, the smaller fragment containing a 20 bp deletion 9 bp before the start of exon 4 (c.250-29_-9del) (Fig. 2A). The deletion was detectable by electrophoresis of the PCR product from the patient’s genomic DNA, showing a characteristic pattern of four bands (Fig. 2B). Taking advantage of this fact, we analyzed the PCR product of exon 4 in 94 healthy Dutch controls. The presence of only one wild-type band indicated that this

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

<table>
<thead>
<tr>
<th></th>
<th>IGHD (n=69)</th>
<th>pIGHD (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>47/22</td>
<td>25/11</td>
</tr>
<tr>
<td>Age (year)</td>
<td>20 (16 to 24)</td>
<td>21 (16 to 24)</td>
</tr>
<tr>
<td>BW SDS</td>
<td>−0.3 (−0.9 to 0.3)</td>
<td>−0.4 (−0.9 to 0.4)</td>
</tr>
<tr>
<td>BL SDS</td>
<td>−0.6 (−1.7 to 0.2)</td>
<td>−1.4 (−2.2 to −0.4)</td>
</tr>
<tr>
<td>Gestational age (w)</td>
<td>40 (38 to 40)</td>
<td>40 (38 to 40)</td>
</tr>
<tr>
<td>HSIDS at start of GH treatment</td>
<td>−3.4 (−3.9 to −2.6)</td>
<td>−3.0 (−3.5 to −2.5)</td>
</tr>
<tr>
<td>GH peak during arginine test (mU/l)</td>
<td>6 (4 to 10) (normal &gt;20)</td>
<td>16 (9 to 19) (normal &gt;20)</td>
</tr>
<tr>
<td>GH peak during clonidine test (mU/l)</td>
<td>8 (4 to 12) (normal &gt;20)</td>
<td>16 (11 to 18) (normal &gt;20)</td>
</tr>
<tr>
<td>IGFBP-3 SDS</td>
<td>−3.4 (−4.8 to −1.8)</td>
<td>−2.5 (−3.7 to −1.5)</td>
</tr>
<tr>
<td>IGFBP-3 SDS</td>
<td>−5.0 (−8.1 to −3.6)</td>
<td>−5.9 (−9.5 to −1.0)</td>
</tr>
<tr>
<td>MRI abnormalities</td>
<td>22 pts: normal 13 pts: normal (4 triad)²</td>
<td>15 pts: abnormal (1 triad)²</td>
</tr>
<tr>
<td>FDR with GHD</td>
<td>15% 3%</td>
<td></td>
</tr>
<tr>
<td>Microenephos</td>
<td>13% 6%</td>
<td></td>
</tr>
<tr>
<td>Neonatal jaundice²</td>
<td>20% 22%</td>
<td></td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>12% 6%</td>
<td></td>
</tr>
</tbody>
</table>

pIGHD, partial IGHD; BW, birth weight; BL, birth length; FDR, first-degree relatives; HSIDS, height SDS.

²Triad = hypoplastic anterior pituitary, ectopic posterior pituitary and interrupted/invisible stalk.

²Prolonged neonatal jaundice: more than 3 weeks.
deletion was absent in normal controls. The same analysis of genomic DNA from the phenotypically normal mother and two siblings of the patient also showed only the single wild-type band (Fig. 2B). Genetic material from the father with normal height of 185 cm was not available.

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

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

**Copy number analysis**

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

**Clinical features of the patient with \textit{HMGA2} c.250-29_9del deletion**

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

**Functional studies**

We performed functional studies in order to assess whether the 20 bp deletion in \textit{HMGA2} (c.250-29_9del) is pathogenic or not. For this purpose, we used the total RNA derived from the patient and control fibroblasts. In the first place, the presence of the 20 bp deletion was confirmed in the genomic DNA derived from the fibroblasts and the size and sequence of the major full-length \textit{HMGA2} transcript (330 bp) were detected in both control and patient’s samples (data not shown). Relative gene expression levels, determined...
from an average of triplicate real-time PCR experiments from fibroblast cDNA. did not show any changes in the expression of the major HMGA2 transcript (HMGA2α) in the patient compared with the control. Expression levels of five additional splice variants (HMGA2β–f) were measured; only the isoforms HMGA2d and HMGA2e showed a decreased expression in the patient compared with the control (Fig. 2C); the change in isoform (d) was statistically significant \((P < 0.02)\). We also measured the expression of IGF2BP2, the only transcription target gene of HMGA2 known to date, and found similar levels for the control and the patient (Fig. 2D).

**Discussion**

We performed sequencing and copy number variation analysis in two new candidate genes, HMGA2 and CDK6, in 105 Dutch IGHD patients. Our main finding was the identification of a new 20 bp intronic deletion \((c.250-29\_9\text{-del})\) in HMGA2. This deletion was identified in one patient with classical IGHD and MRI abnormalities, from the cohort of 69 patients with classical IGHD, of whom 35 had MRI abnormalities. This deletion was absent in the mother and siblings of the patient, in the normal population screened in this study as well as in the 1000 Genomes database, where 629 individuals (release November 2010) have been sequenced.

HMGA2 is an architectural transcription factor with three DNA-binding domains (AT-hooks) and a C-terminal acidic tail (Fig. 2A). Exons 1–3 encode the three AT-hooks, exon 5 encodes the C-terminal tail. These two motifs are separated by a spacer encoded by exon 4. The function of the C-terminal tail is not yet completely understood, but it could be involved in protein–protein interactions or transcription activity enhancement \((29, 30)\). Hauke et al. \((11, 12)\) have described the complex alternative splicing pattern of HMGA2, in which the five additional splice variants (β–f) differ from the major isoform (α) due to the replacement of exons 4 and 5 by sequences derived from the long intron 3. The 20 bp deletion that we found in our patient is localized 9 bp before the start of exon 4, which could affect this complex alternative splicing pattern.

Studies on *Hmga2*-null mice with pygmy-phenotype provided the first target gene of HMGA2: IGF2BP2 \((13, 14)\), a post-transcriptional regulator of IGF2. In order to evaluate the possible deleterious effect of the newly found 20 bp deletion, we performed real-time RT-PCR of fibroblast RNA and analyzed the expression pattern

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Position/rs number</th>
<th>Alleles</th>
<th>IGHD</th>
<th>Control</th>
<th>refSNP(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGA2</td>
<td>1</td>
<td>c.111 + 63C &gt; G</td>
<td>C/G</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.111 + 85G &gt; C</td>
<td>G/C</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>rs73115423</td>
<td>T/A</td>
<td>2.38%</td>
<td>2.75%</td>
<td>1.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs3834468</td>
<td>~G</td>
<td>8%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>c.250-29_9\text{-del}20</td>
<td>0.48%</td>
<td>0%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs57800850</td>
<td>T/C</td>
<td>0.48%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>CDK6</td>
<td>2</td>
<td>–</td>
<td>C/T</td>
<td>0.48%</td>
<td>1.10%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>rs35654944</td>
<td>C/T</td>
<td>0.95%</td>
<td>2.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>C/T</td>
<td>5.7%</td>
<td>5.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>rs3731373</td>
<td>T/C</td>
<td>26.2%</td>
<td>28.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>rs42043</td>
<td>G/A</td>
<td>25.7%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>rs3731387</td>
<td>TGTAT/-</td>
<td>25.7%</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

MAF, minor allele frequency; NA, not available. 

\(^a\)MAF only shown if European population data were available at time of the study. The minor allele is indicated in bold.
of the different HMGA2 splice variants as well as the IGF2BP2 gene, comparing the results between the affected patient and a normal control. Our initial hypothesis was that the expression of the major full-length transcript (a) would be decreased, whereas expression of one or more of the other isoforms could be increased. However, in our experiments, the expression levels of the full-length transcript (a) were similar for the patient and control: this could be explained by the fact that the normal allele is still present in the patient. Surprisingly, two of the other five isoforms (d and e) showed decreased expression (Fig. 2C). Currently, the function of these isoforms is unknown, but we cannot exclude the possibility that these low expression levels contribute to the patient’s phenotype.

Although we expected to find decreased expression of HMGA2(a) and IGF2BP2, we did not find any changes during our experiments (Fig. 2D). One possible hypothesis for this is that the expression of these genes is only essential during embryogenesis (13) and could be less important in adult fibroblasts. It has been demonstrated that HMGA genes show high expression during human embryogenesis and fetal development and low expression levels in adult tissues (31, 32). In mice, Hmg2a is found to be preferentially expressed by stem cells, showing a progressive decline in expression with age (33). Even though our patient was born with normal weight and height, the deficiency of an embryonic factor could cause an abnormal phenotype later in life. The deregulation and possibly decreased expression of HMGA2 during the development of specific somatotroph cells in the pituitary might lead to low GH production after birth, contributing to the IGHD phenotype.

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

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

In conclusion, this study provides the first report of a deletion in the HMGA2 gene that might be a rare cause of IGHD. We suggest that this gene is investigated in patients with a classical IGHD phenotype, in whom mutations in the classical candidate genes GH1, GHRHR, have been previously excluded. At a molecular level, further research should be performed to better understand the possible involvement of this gene in the classical GH–IGF1 axis and to investigate how genetic alterations in HMGA2 can affect human growth.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

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Acknowledgements

We acknowledge Roel Brekelmans (MRC Holland) for help in developing the MLPA HMGA2 and CDK6-specific kit and Hannie Douben for technical help with the MLPA analysis. We acknowledge Dr Jose Carlos Moreno and Dr Edith Friesema for helpful discussions.

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