Constitutional delay of growth and puberty is not commonly associated with mutations in the acid labile subunit gene

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Abstract
Objectives: Constitutional delay of growth and puberty (CDGP) is a common clinical condition that may be inherited as an autosomal dominant, recessive or X-linked trait. However, single-gene defects underlying CDGP have not yet been identified. A small number of children (to date 10) with modest growth failure and in the majority delayed puberty, a phenotype similar to that of CDGP, have been reported to carry mutations in the IGF acid labile subunit (IGFALS) gene which encodes the ALS, a part of the ternary complex carrying IGF-I in the circulation. The aim of our study was to screen a well-characterised CDGP cohort exhibiting a range of growth retardation and pubertal delay for pathogenic sequence variants in IGFALS.

Design and methods: We used denaturing high performance liquid chromatography (dHPLC) to screen for IGFALS mutations in DNA samples from 90 children (80 males) with CDGP of predominantly White European origin. DNA fragments generating abnormal waveforms were directly sequenced.

Results: No IGFALS mutation was identified in the coding sequences or exon–intron boundaries in our CDGP cohort. One abnormal waveform pattern in dHPLC in 15 children with CDGP was found to represent a recognised synonymous single-nucleotide polymorphism of the coding transcript in the second exon in residue 210 of IGFALS.

Conclusions: IGFALS sequence variants are unlikely to be a common association with pubertal delay in children with CDGP.

Introduction
Constitutional delay of growth and puberty (CDGP) is a common reason for referral to a paediatric endocrinologist and it is estimated that genetic factors account for 50–80% of the variability in pubertal delay (1, 2). CDGP is a complex trait with variable degrees of growth retardation and pubertal delay, and is likely to be related to a number of genes (3). Nevertheless, in pedigree analysis of CDGP children, transmission may follow an apparently autosomal dominant pattern with variable penetrance, as well as autosomal recessive and X-linked patterns of inheritance. This implies that single-gene defects may play a part in some cases of CDGP (1).

Mutations in gonadotrophin-releasing hormone (GnRH), GnRH receptor (GnRHR), Kallmann syndrome (KAL1), fibroblast growth factor receptor 1 (FGFR1) and G protein-coupled receptor 54 (GPR54) can all cause hypogonadotropic hypogonadism with markedly delayed or absent puberty. However, no pathogenic gene mutations have been clearly associated with the CDGP phenotype that is characterised by a slowdown in height velocity and tempo of pubertal development. Of the single genes investigated for association with CDGP, polymorphic markers in the genes for GnRH and GnRH-R show only weak association (3). Common polymorphisms in the leptin and leptin receptor genes are not associated with CDGP, although a specific leptin genotype does influence the CDGP phenotype: homozygotes for the long allele for the CT repeat in the 3’ UTR have a greater body mass index (BMI) SDS than heterozygotes or short allele homozygotes (4). Furthermore, there is no association between a polymorphic adenine deletion in the cocaine and amphetamine-regulated transcript (CART) gene, which modulates the action of leptin on GnRH secretion and CDGP (PE Clayton, unpublished observations).

The growth hormone (GH) – insulin-like growth factor-I (IGF-I) axis influences the onset and progression of puberty (5, 6) and therefore has a role in its temporal regulation. Thus, a genetic abnormality in this axis could be associated with the CDGP phenotype (7). The acid labile subunit (ALS) is a GH-dependent peptide that binds to IGF-I and IGF-binding protein-3 (IGFBP-3) forming a stable ‘ternary’ complex that carries IGF-I around in the circulation. A recent report noted that a frameshift mutation in the ALS gene (IGFALS) in nucleotide
positions 1334 through to 1338 in exon 2 led to a complete absence of circulating ALS in a child with severe pubertal delay but only a modest degree of growth failure (8). Another study reported a point mutation in IGFALS at nucleotide position 1318 in a child presenting with short stature and relatively mild pubertal delay (9, 10). Three other children, two with pubertal delay and one with significant short stature, have been reported in a family with novel compound heterozygous mutations in exon 2 of IGFALS (11). There are also recent reports of IGFALS mutations in three males with idiopathic short stature (12) and in two other males with short stature and osteoporosis (13). In most of these cases, the pubertal development and the pubertal growth spurt were delayed, a pattern consistent with that occurring in children with CDGP (Table 1). We hypothesised that pathogenic sequence variants in IGFALS could be present in children with CDGP and have investigated a cohort of children with CDGP to determine their frequency.

Subjects and methods

The study was approved by the local research ethics committee. We recruited 90 children (80 males) with CDGP from among those attending Paediatric Endocrinology outpatient clinics. The diagnosis of CDGP was established by a paediatric endocrinologist and case notes were reviewed for confirmation. The following inclusion criteria were used (3, 4): a) evidence of delayed puberty – lack of breast development (Tanner stage 2) by the age of 13 years in boys (1S.D. criteria), b) evidence of slowdown of 2 S.D. below mean) – lack of breast development in girls ≥13 years or testicular volume <4 ml by the age of 14 years (3, 4). Individual growth charts were reviewed to confirm that each child with CDGP demonstrated a slowdown in height gain and subsequent catch up with progression of puberty. Height and BMI were converted to SDS using British 1990 normative data (14, 15). Bone age was measured by the Greulich–Pyle method and bone age delay noted as the difference between skeletal and chronological ages. Target height was calculated for each individual and parent-adjusted height was calculated as (height SDS – parental target height SDS).

The IGFALS gene is located on chromosome 16 and consists of two exons. PCR amplification of the coding sequences and exon–intron boundaries was undertaken using PCR Express thermal cycler (Hybaid) on buccal DNA extracted by standard procedures. The following forward (f) and reverse (r) primer pairs were designed:

- f 5’-acgaggggttaaagagc–3’, r 5’-agactggccacccctactgaggt–3’ (exon 1)
- f 5’-gagcctagctcttctgccac-3’, r 5’-cctgtcagacggttggttg-3’ (exon 2, fragment 1)
- f 5’-ctggcctagagaactctgtgtg-3’, r 5’-tggctgcaagctcctct-3’ (exon 2, fragment 2)
- f 5’-gagcaacgcttgctcctac-3’, r 5’-ctcaggtgcaggtgtg-3’ (exon 2, fragment 3)
- f 5’-ggtggtctagacgctct-3’, r 5’-aagctccgagtagttgtgt-3’ (exon 2, fragment 4)
- f 5’-ctgagctctgccaaac-3’, r 5’-ctcgcttccaccccac-3’ (exon 2, fragment 5).

Table 1 Auxological characteristics and age at pubertal onset in cases reported with insulin-like growth factor acid labile subunit (IGFALS) mutations.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Reference</th>
<th>Patient number</th>
<th>Sex</th>
<th>Height SDS at presentation</th>
<th>Bone age delay (years)</th>
<th>Near final height SDS</th>
<th>Age at onset of puberty (years)</th>
<th>Pubertal delay*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Domene et al. (8)</td>
<td>I</td>
<td>M</td>
<td>-2.0</td>
<td>2</td>
<td>0.9</td>
<td>&gt;14.6</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Hwa et al. (9)</td>
<td>II</td>
<td>M</td>
<td>-2.9</td>
<td>2</td>
<td>-2.1</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Domene et al. (11)</td>
<td>III1</td>
<td>M</td>
<td>-2.0</td>
<td>2.1</td>
<td>-0.5</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Domene et al. (11)</td>
<td>III2</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-0.5</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Domene et al. (11)</td>
<td>III3</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-1.0</td>
<td>Menarche 13</td>
<td>No delay</td>
</tr>
<tr>
<td>6</td>
<td>Campos-Barros et al. (12)</td>
<td>IV1</td>
<td>M</td>
<td>-2.2</td>
<td>1</td>
<td>-</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Campos-Barros et al. (12)</td>
<td>IV2</td>
<td>M</td>
<td>-3.8</td>
<td>1</td>
<td>-</td>
<td>12</td>
<td>No delay</td>
</tr>
<tr>
<td>8</td>
<td>Campos-Barros et al. (12)</td>
<td>IV3</td>
<td>M</td>
<td>-3.0</td>
<td>3</td>
<td>-</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>Duyvenvoorde van et al. (13)</td>
<td>V1</td>
<td>M</td>
<td>-4.2</td>
<td>0</td>
<td>-4.2</td>
<td>14.6</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>Duyvenvoorde van et al. (13)</td>
<td>V2</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-4.3</td>
<td>Not known</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

M, male; F, female.

*Pubertal delay: 1 = pubertal delay by relaxed (1 SD) criteria and 2 = pubertal delay by strict criteria (2 SD) (3, 4).
for cystic fibrosis mutations, not identified as CDGP and sequenced to confirm the absence of any known variants. Heteroduplexed amplicons were passed through denaturing high performance liquid chromatography (dHPLC; WAVE nucleic acid fragment analysis system, Transgenomic Inc., Glasgow, UK) columns at partially denaturing temperatures (range 55–68 °C) with individualised acetonitrile gradients and triethylammonium concentrations that optimised u.v. light absorbance for each amplicon. U.v. absorbance was plotted as a function of retention time taken to elute from a hydrophobic bead cartridge and analysed with Navigator software, version 1.6.2 (Glasgow, UK).

DNA was sequenced in those (n = 9) with abnormal dHPLC patterns with an ABI 3730 capillary sequencer using Big Dye Terminator cycle sequencer, version 3.1 (Applied Biosystems, Foster City, CA, USA) and analysed by Sequence Scanner, version 1.0 (Applied Biosystems, Foster City, CA, USA). Primers used for PCR amplification were used for sequencing reactions.

Results

Clinical phenotype

The patient characteristics of 90 children with CDGP at the time of their clinical presentation are given in Tables 2 and 3. Eighty-two children were of White European origin, seven originated from the Indian subcontinent and one was of mixed ethnicity. Forty-seven children (53%) with CDGP had severe pubertal delay. The anthropometric phenotype and bone age delay were wide ranging. Nevertheless, most children with CDGP were short and thin, particularly so in those with severe pubertal delay (Table 3).

dHPLC and DNA sequencing

Of the 90 subjects, amplicons were heteroduplexed in 87 for exon 1, 90 for fragment 1 (exon 2), 90 for fragment 2, 84 for fragment 3, 88 for fragment 4 and 90 for fragment 5. In those subjects where heteroduplexing was unsuccessful, DNA was directly sequenced and found to be identical to that of published sequences. In dHPLC, nine waveform types were identified as abnormal in comparison to normal controls. DNA sequences in these patients were identical to that of published control sequences (www.ensembl.org). One of the nine abnormal waveform types (Fig. 1) was found in 15 children with CDGP. This represented a recognised synonymous T > C single-nucleotide polymorphism (SNP) at residue 210 of the coding transcript in the first fragment of exon 2. In our cohort, the frequency of the variant (minor) allele for this SNP was 16%, similar to the frequency (22%) reported in Caucasian populations (www.ensembl.org). There was no difference between minor and major alleles for height SDS (mean (s.d.) −2.3 (0.7) vs −2.6 (0.5), P = 0.2), BMI SDS (−0.5 (1.4) vs −0.4 (1.3), P = 0.7), bone age delay (−2.2 (1.0) vs −2.4 (0.8), P = 0.7) or history of parental pubertal delay (64% vs 71%, P = 0.7).

Discussion

We have not identified pathogenic sequence alterations within exons 1 and 2 of the IGFALS gene in a large cohort of CDGP children with both moderate and severe pubertal delay. Routine screening for IGFALS mutations in children with CDGP is therefore unlikely to be useful in clinical practice.

ALS is an important component of the GH-IGF system and ALS-deficient mice have been noted to have post-natal growth retardation (16). However, these mice did not show features of pubertal delay. In contrast, in humans, IGFALS mutations have been reported in several children with short stature associated with delayed puberty (8–13) (Table 1). The clinical phenotype appears to be similar to that of children with CDGP (4). The diagnosis of CDGP is based on the presence of pubertal delay usually with short stature, delayed bone age, and often with a family history of pubertal delay and the absence of other disorders. Detailed investigations are usually not undertaken. However, in those with IGFALS mutations, full assessment of the GH-IGF-I axis had been undertaken; serum IGF-I and IGFBP-3 concentrations were very low and circulating ALS levels were undetectable.

The first child reported with an IGFALS mutation (8) was prepubertal at 14.6 years, indicating severe delay, and was short (height SDS −2.05) and thin (weight SDS −2.34) with a bone age delay exceeding 2 years. There is

Table 3 Patient characteristics in 90 children with constitutional delay of growth and puberty (CDGP).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (s.d.)</th>
<th>Moderate pubertal delay (N = 43)</th>
<th>Severe pubertal delay (N = 47)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height SDS</td>
<td>−2.3 (0.7)</td>
<td>−2.0 (0.6)</td>
<td>−2.6 (0.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Parent-adjusted height SDS</td>
<td>−1.8 (0.7)</td>
<td>−1.5 (0.5)</td>
<td>−2.0 (0.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>−0.6 (1.5)</td>
<td>−0.3 (1.3)</td>
<td>−0.9 (1.7)</td>
<td>0.167</td>
</tr>
<tr>
<td>Weight SDS</td>
<td>−1.7 (1.3)</td>
<td>−1.5 (0.9)</td>
<td>−1.9 (1.5)</td>
<td>0.096</td>
</tr>
<tr>
<td>Bone age delay (years)</td>
<td>−2.3 (1.0)</td>
<td>−1.9 (1.0)</td>
<td>−2.6 (1.0)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

P values are for differences in variables between those with moderate and severe pubertal delay.

www.eje-online.org
Figure 1 Abnormal ‘bifid’ peak waveform is highlighted in an individual with a synonymous SNP. Other normal waveforms are provided for comparison.

no record of parental pubertal delay as this child was adopted at an early age. GH levels on provocative testing were normal but serum IGF-I (−5.3 SDS) and IGFBP-3 (−9.7 SDS) concentrations were very low and ALS was undetectable. He reached a final adult height of 0.9 SDS below the mean at age 19 years. DNA sequencing revealed a homozygous deletion in one of five consecutive guanines at positions 1334 through to 1338 in exon 2 in IGFALS, resulting in the substitution of a lysine for a glutamic acid at codon 35 and the appearance of an early stop codon at amino acid position 120.

The second reported child (9) with a mutation in IGFALS was also short (height SDS −2.9) but not thin (BMI 75th centile). His parents were consanguineous but there was no record of parental pubertal delay. The child’s GH levels on provocative testing were normal but the serum IGF-I (−5.8 SDS), IGFBP-3 (−7.2 SDS) and ALS (<0.4 μg/ml) levels were extremely low. Although the authors considered the timing of his puberty to be normal, he had most likely mild-to-moderate pubertal delay (genital stage 2 at age 13 years, bone age delay of 2.3 years at age 12.3 years). His last reported height was −2.1 SDS at age 15.3 years, which was normal for his parental target centiles (father −2 SDS, mother −2.8 SDS). The child had learning disabilities, mild speech disorder, mild tooth abnormalities and exophthalmos, features not present in any child in our UK CDGP cohort. IGFALS sequencing revealed a homozygous substitution at nucleotide 1318 of the pre-peptide, which altered the codon encoding aspartic acid (GAC) to asparagine (AAC) at residue 440.

The third child reported, a 17-year-old boy with a heterozygous IGFALS mutation had the phenotype of short stature (height SDS −2.0), normal weight (56 kg at 15.3 years, weight SDS −0.3) and severe pubertal delay (Tanner stage 1 at age 16.1 years, onset of puberty 16.9 years), similar to CDGP (11). His father and mother were relatively tall with height measurements of +1.5 SDS and −0.9 SDS respectively. His GH response was adequate but his serum IGF-I (−9.6 SDS) and IGFBP-3 (−4.0 SDS) levels were very low and his ALS levels were undetectable. He had a compound heterozygous mutation in exon 2 in IGFALS. One was a T>C substitution in position 1618 resulting in the loss of a highly conserved cysteine residue at position 540 in the C-terminal domain of a leucine-rich repeat region. The other mutation was a duplication between codon positions 583 and 591 resulting in three extra amino acid residues between positions 195 and 197. Both mutations resulted in new enzymatic restriction sites, thereby making restriction fragment length polymorphism (RFLP) analysis possible in the siblings. RFLP analysis showed the presence of similar compound heterozygous mutations in the brother and sister. The brother had mild growth delay but demonstrated a catch-up growth spurt similar to individuals with CDGP and achieved a near final height of −0.5 SDS at 19.6 years. His serum IGF-I (−10.2 SDS) and IGFBP-3 (−4.0 SDS) were low and his ALS levels were undetectable, similar to his brother. The sister had a normally timed menarche but was relatively short (height −1.0 SDS at 15.3 years of age). Like her brothers, her serum IGF-I (−7.2 SDS) and IGFBP-3 (−7.4 SDS) levels were low and serum ALS was undetectable.

Recently, IGFALS mutations have been identified in three unrelated males with the phenotype of idiopathic short stature (height −2.2 to −3.8 SDS) (12). All individuals had low serum IGF-I and IGFBP-3 levels and serum ALS levels were undetectable. One individual had delayed puberty by the 2 S.D. criteria (3, 4) with the onset of puberty at age 15 years, while another had delayed puberty by the 1 S.D. criteria with the onset of puberty noted at age 13 years. In another report, IGFALS homozygous mutations were found in two brothers of consanguineous parents with short stature, microcephaly and osteoporosis (13). Puberty was significantly delayed (Tanner stage 2 at age 14.6 years) in one of the brothers.

On the account of the phenotypic similarities, it would be reasonable to consider that IGFALS mutations might be found in those with CDGP. We used dHPLC as a reliable and accurate high throughput screening tool for exonic and exon–intron boundary mutations (17, 18), in a relatively large cohort of children with CDGPs but did not find any pathogenic variants. This study did not include the assessment of intronic or promoter regions, and it is therefore possible that mutations may be present in these regions. However, our intention was to quantify the risk of having a mutation in the coding sequence of the IGFALS gene, in keeping with the reports on the 10 cases to date. The absence of mutations in our well-characterised cohort indicates that IGFALS mutations are not a common occurrence in CDGP. However, such variants should nonetheless be considered, in particular if the short stature-delayed puberty phenotype co-exists with clinical markers atypical of CDGP, such as learning disabilities, evidence of dysmorphism or a history of frequent fractures (8–13).

ALS is considered to play a role in stabilising the ternary complex of IGF-I and its binding proteins, chiefly IGFBP-3, in the circulation (8, 16). Reduced or absent levels of ALS might therefore contribute to growth failure through a relative reduction in circulating IGF-I concentrations. Free IGF-I concentrations in one case have been found to be higher than levels found in GH deficiency or congenital GH insensitivity but lower than...
the level in normal pooled sera (9). In addition, low levels of free and bioactive IGF-I have been reported in the first child reported with an IGFALS mutation (8, 19).

The phenotype of patients with IGFALS mutations includes modest growth retardation with a final height within parental target, a variable effect on pubertal timing but overall a shift to a delayed pubertal onset and in some low bone density (8, 9). This slowdown in growth and late pubertal maturation resembles that in some low bone density (8, 9). This slowdown in timing but overall a shift to a delayed pubertal onset and within parental target, a variable effect on pubertal includes modest growth retardation with a final height

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References


