Phenotypic variation in constitutional delay of growth and puberty: relationship to specific leptin and leptin receptor gene polymorphisms

Indraneel Banerjee1, Julie A Trueman2, Catherine M Hall1, David A Price1, Leena Patel1, Andrew J Whatmore2, Joel N Hirschhorn3, Andrew P Read4, Mark R Palmert5 and Peter E Clayton1,2

1Department of Paediatric Endocrinology, Royal Manchester Children’s Hospital, UK, 2 Endocrine Science Research Group, University of Manchester, UK, 3Department of Genetics, Harvard Medical School, Boston, USA, 4Academic Unit of Medical Genetics, University of Manchester, UK and 5Division of Paediatric Endocrinology and Metabolism, Rainbow Babies and Children’s Hospital, Department of Paediatrics and Genetics, Case School of Medicine, Cleveland, USA

(Correspondence should be addressed to P E Clayton; Email: peter.clayton@manchester.ac.uk)

Abstract

Objectives: Constitutional delay of growth and puberty (CDGP) is a variant of normal pubertal timing and progress, often with dominant inheritance. It is likely that one or more genes will be associated with CDGP. Possible candidates are the leptin (L) and the leptin receptor (LR) genes, as the leptin axis links nutritional status to pubertal development. This study has assessed whether a) L or LR gene polymorphisms were associated with CDGP and b) the CDGP phenotype was influenced by these polymorphisms.

Design: Case–control and transmission disequilibrium tests were used to test genetic association of L and LR polymorphisms with CDGP.

Methods: We genotyped L (3’CTTT repeat) and LR polymorphisms (Gln > Arg substitution, exon 6) in 81 CDGP children and 94 controls in the UK and 88 CDGP children from the US and assessed the effect of genotype on their anthropometric characteristics.

Results: There was no association of these L or LR gene polymorphisms with CDGP. There was no difference in height or bone age delay within L or LR genotypes. However, UK CDGP children homozygous for the L short allele were heavier than heterozygotes and long allele homozygotes, with a similar trend in the US cohort. UK CDGP children with severe pubertal delay, who were thin, had significantly greater bone age delay and an increased frequency of parental pubertal delay than other groups and were less likely to be L short allele homozygotes.

Conclusions: There was no association of specific L or LR polymorphisms with CDGP, but L short allele carriage influenced the phenotype within CDGP.

European Journal of Endocrinology 155 121–126

Introduction

Constitutional delay of growth and puberty (CDGP) is a variant of normal pubertal development without a defined endocrine abnormality. In clinical practice, it is most frequently encountered in boys (1). CDGP is accompanied by a reduced growth velocity in adolescence. The condition may also be present in early childhood with slow growth, delayed bone age and no endocrine abnormality–constitutional delay in growth.

Calculated measures of heritability suggest that 50–80% of the variance in pubertal onset may be genetically controlled (2). A number of families of children with CDGP show dominant inheritance patterns, but no genes have yet been identified that are associated with CDGP. Although dominant inheritance exists in a significant proportion, the timing and regulation of puberty is more likely to be a complex, multigenic trait. It is therefore possible that a number of genes act in collaboration to facilitate puberty and polymorphisms in such genes may be responsible for CDGP.

Leptin (L), a 167 amino acid secreted by adipocytes, has been proposed to contribute to hypothalamic–pituitary–gonadal function (3). Mice with an L gene deletion are infertile and L treatment results in reversal of infertility (4–6). In prepubertal rats, L treatment reduces the starvation-induced delay in sexual maturation (7). Adult humans with L and leptin receptor (LR) gene mutations do not enter puberty and have hypogonadotropic hypogonadism (8, 9). In normal boys, serum L rises and then falls in puberty (10) unlike girls where L levels rise through puberty. This suggests that L has a permissive effect in facilitating puberty and plays a role in the tempo of its progression.
As CDGP is a variation of the onset and timing of puberty and L has a role in puberty, it is possible that polymorphisms of L and LR genes may contribute to CDGP. This study has addressed two questions:

1. Is there genetic association between CDGP and polymorphisms within L or LR genes?
2. Does carriage of a specific L or LR allele influence any aspect of the CDGP phenotype?

Subjects and methods

Approval for the study was obtained from the Salford and Trafford and the University of Manchester Research Ethics Committees. Eighty-one cases (70 males) (<18 years) of White European origin attending paediatric endocrine outpatient clinics in Manchester, UK and their parents were recruited with consent. The children were initially examined by a paediatric endocrinologist, who established the diagnosis of CDGP. Case notes were reviewed to confirm the diagnosis. The inclusion criteria comprised of a) evidence of delayed puberty—lack of breast development (Tanner stage 2) by the age of 12 years in girls and testicular volume <4 ml by the age of 13 years in boys (1 s.d. criteria) with or without height below that predicted by genetic potential and reduced height velocity (2), b) evidence of growth slow down in longitudinal measurements of height as plotted on growth charts (11) and c) absence of other causes of delayed puberty on history, examination and investigations. Children with delayed puberty were further subdivided into: a) moderate pubertal delay (1–2 s.d. below mean) and b) severe pubertal delay (<2 s.d. below mean)—lack of breast development in girls ≥13 years, or testicular volume <4 ml in boys ≥14 years. Serial bone age assessments usually indicated a delay >1 year (4 of the 81 patients had bone age delay <1 year). Individual growth charts were reviewed to confirm that each child with CDGP demonstrated a slow down in height gain and subsequent catch up with development and progression of puberty.

Height and body mass index (BMI) were measured either at the time of presentation to the paediatric endocrine clinic in the adolescent years, prior to a course of sex-steroid treatment, or at the nadir of pubertal delay or in the immediate postpubertal period and SDS were calculated using Centers for Disease Control and Prevention normal ranges (14).

It is recognised that the diagnosis of CDGP is associated with both moderate and severe pubertal delay (2, 13). The influence of gene polymorphisms is more likely in the severe group; in our analysis, we have therefore examined not only the whole group, but also the subgroup of severe pubertal delay.

The control group included 94 White European children (36 males) between the ages of 8 and 18 years from a previous cross-sectional study in the UK (10). These children had normal growth and development. The prepubertal children (N=29) had age appropriate auxology and therefore were unlikely to show delayed onset of puberty. The pubertal children (N=65) were at Tanner pubertal stages 2–5, appropriate for age.

DNA was obtained from buccal cells of UK children with CDGP and their parents and from peripheral blood in the US subjects and extracted by standard methods (15). The polymorphism chosen from the L gene was a tetrancleotide (CTTT)_n variable number tandem repeat (VNTR) polymorphism situated in the 3′ flanking region of the L gene, 157 bp downstream from the end of exon 3 (16). The VNTR has 15 alleles distributed in two class sizes—allele 1 (short 121–145 bp) and allele 2 (long 197–225 bp), and has been investigated in the context of adult obesity (16). The polymorphism examined in the LR gene was the single nucleotide polymorphism (SNP) in exon 197–225 bp), and has been investigated in the context of adult obesity (16). The polymorphism examined in the LR gene was the single nucleotide polymorphism (SNP) in exon 6 (17–19). Here, an adenine to guanine (CAG→CGG) base substitution at position 668 leads to a change of Gln (allele Q) to Arg (allele R) in the extracellular domain of the receptor (Gln223Arg). This changes the amino acid charge from neutral to positive and may therefore have functional consequences.

To genotype the L gene, primers were selected from previously published data for PCR amplification (16). Products were size separated by gel electrophoresis, and classified as short or long alleles. For the LR gene, the fragment containing the Gln223Arg SNP was amplified using PCR (20) and then subjected to restriction enzyme digest, MspI, which recognises the sequence C/CGG created by the presence of the CAG→CGG polymorphism.

We used denaturing HPLC (DHPLC) to screen all the L exons for novel SNPs (WAVE™, Transgenomic, UK) in 30 randomly selected UK CDGP patients and 30 controls. DHPLC was also used to screen all LR exons for SNPs in 16 US CDGP subjects. The altered mobility down the acetonitrile column was monitored and analysed by the WAVEMAKER™ Version 4.1.25 software.

The L and LR genotype frequencies were tested for Hardy–Weinberg equilibrium. Genetic association was tested by i) a case-control design comparing allele
Results

Genetic association

L and LR genotypes for cases and controls were in Hardy–Weinberg equilibrium. There were no differences in L (L allele 1, 45 vs 45%, \( P = 1.0 \)) or LR (LR allele Q, 55 vs 59%, \( P = 0.7 \)) allele frequencies between all UK CDGP subjects (severe + moderate delay) and controls (Table 1). Analysis was also undertaken in severe UK CDGP vs controls, in which normal pubertal timing was documented (\( n = 65 \)). There were no differences in either L (L allele 1, 47 vs 42%, \( P = 0.4 \)) or LR (LR allele Q, 55 vs 59%, \( P = 0.7 \)) allele frequencies between cases and controls.

In TDT analysis in UK CDGP children (severe + moderate), 72 parents were heterozygous for the L gene polymorphism, of whom 36 parents transmitted allele 1 and 36 transmitted allele 2 (\( P = 1.0 \)). Of the 79 heterozygous parents carrying the LR gene polymorphism in the same group, 40 parents transmitted allele Q, whilst 39 transmitted the allele R (\( P = 0.9 \)). In the subgroup of UK children with severe CDGP, 28 parents were heterozygous for the L gene polymorphism, of whom 16 transmitted allele 1 (\( P = 0.3 \)), whilst 39 parents were heterozygous for the LR gene polymorphism, of whom 20 transmitted allele Q (\( P = 0.8 \)).

<table>
<thead>
<tr>
<th>Severe + moderate CDGP</th>
<th>Severe CDGP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cases</strong> (( n = 81 ))</td>
<td><strong>Controls</strong> (( n = 94 ))</td>
</tr>
<tr>
<td>Allele 1 (%)</td>
<td>45</td>
</tr>
<tr>
<td>Allele 2 (%)</td>
<td>55</td>
</tr>
<tr>
<td>Allele Q (%)</td>
<td>55</td>
</tr>
<tr>
<td>Allele R (%)</td>
<td>45</td>
</tr>
</tbody>
</table>

No novel SNPs were identified by DPHLC in the L gene among UK CDGP cases or controls or in LR among the US subjects.

CDGP phenotype

**Anthropometric characteristics.** The age range at presentation of children in the UK CDGP cohort was 10–17 years. The mean age at assessment, height SDS and bone age delay was similar across L and LR genotypes for the whole cohort and for the severe CDGP subgroup (Table 2).

The mean (s.d.) BMI SDS in UK children with severe CDGP was lower than in controls (CDGP vs controls \( = -0.6 \) (1.6) vs 0.3 (0.9), \( P < 0.001 \)). When comparing BMI SDS for individual L and LR genotypes (Table 3), all genotypes except L allele 1 and LR allele R homozygotes had a lower BMI SDS in cases compared to controls.

In the whole UK CDGP group, there was a trend to increased BMI SDS in those homozygous for L allele 1 (L allele 1 vs control \( 1.1 \) vs 1.4, \( P = 0.06 \)) but not for LR genotypes (LR allele Q vs control \( = 0.4 \) vs 1.3, \( P = 0.6 \)). Combining L heterozygotes with allele 2 homozygotes in this group (BMI SDS \( = 0.6 \) (1.3)), there was a significant BMI difference from L allele 1 homozygotes (BMI SDS 0.1 (1.5), \( P = 0.02 \)). These differences in BMI SDS between L genotypes were more prominent in the severe CDGP subgroup (Table 3).

These observations were also tested in the whole US CDGP cohort. There were no significant differences between L genotypes (BMI SDS, L allele 1 vs control \( = -0.1 \) (0.9) vs –0.6 (1.1), \( P = 0.4 \)) but there was a trend for L allele 1 homozygotes to be heavier than the other combined genotypes (BMI SDS, L allele 1 vs control \( = -0.1 \) (0.9) vs –0.6 (1.2), \( P = 0.1 \)). This trend was also seen in the US children with severe CDGP (BMI SDS, L allele 1 vs control \( = -0.1 \) (0.9) vs –0.6 (1.2), \( P = 0.1 \)).

**CDGP severity.** Children with CDGP exhibit a significant range of values within their anthropometric parameters: this is particularly so for BMI SDS (mean \( = -0.4 \) (range \( = -3.8 \) to +2.7)). Thus, the characteristics of CDGP with BMI SDS \( < 0 \) and \( \geq 0 \) have been examined in those with moderate and severe pubertal delay in UK subjects. Bone age delay was significantly greater in those with severe
CDGP who were thinner than those in the other categories (mean (s.d.) bone age delay in severe CDGP, thin vs severe CDGP, not thin = −2.8 (0.8) vs −1.9 (1.0), P = 0.002) (Fig. 1). In addition, a history of parental delay was more frequent in those with severe CDGP who were thin (80 vs 50%, P = 0.03). In severe CDGP, L allele 1 was significantly less frequent in thin than not thin children (40% vs 60%, P = 0.01). Factors influencing bone age were assessed in univariate ANOVA in moderate and severe CDGP children. In our model, bone age delay was the dependent variable while BMI SDS was an independent variable, with L and LR genotype and history of parental pubertal delay as independent factors. When controlling for other factors, bone age delay had independent significant association with BMI SDS in both the whole UK CDGP cohort and the severe subgroup (Table 4). Although L genotype per se was not significant in its main effects, the interaction of L genotype and BMI SDS significantly influenced bone age delay: for a given BMI SDS, bone age delay was less if the patient was an L allele 1 homozygote.

Discussion

In this study, we have found no significant association of specific L and LR gene polymorphisms with CDGP but we have observed that homozygosity for the L 3’CTTT repeat allele is associated with an increased BMI SDS in CDGP children.

This study has addressed an important issue, namely the genetic regulation of puberty, using CDGP as a model of an extreme variant of pubertal timing. Despite likely genetic association, the identity of genes regulating timing of puberty has not been determined. A recent study indicated that genes for gonadotrophin-releasing hormone (GnRH) and its receptor (GnRHR), although pivotal regulators of the onset of puberty, were not associated with CDGP (13), raising the possibility that mechanisms that trigger puberty may not be crucial to the pathogenesis of CDGP. An alternative explanation for CDGP is therefore a delay in the overall tempo of growth associated with late onset of puberty. Thus, the L and LR genes were chosen as attractive candidates that may determine the tempo of growth and hence puberty. L signals through the LR to regulate appetite and

### Table 2 Mean (s.d.) age (years), height SDS and bone age delay (years) in the UK cohort with severe CDGP for L and LR genotypes.

<table>
<thead>
<tr>
<th>L genotypes</th>
<th>Cases (n=47)</th>
<th>P-value*</th>
<th>LR genotypes</th>
<th>Cases (n=46)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>11</td>
<td>14.9 (0.7)</td>
<td>0.6</td>
<td>AA</td>
<td>−14.9 (1.3)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>15.2 (1.1)</td>
<td></td>
<td>AG</td>
<td>−15.4 (1.0)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>15.1 (1.2)</td>
<td></td>
<td>GG</td>
<td>−14.8 (0.8)</td>
</tr>
<tr>
<td>Height SDS</td>
<td>11</td>
<td>−2.6 (0.5)</td>
<td>0.9</td>
<td>AA</td>
<td>−2.6 (0.8)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>−2.6 (0.8)</td>
<td></td>
<td>AG</td>
<td>−2.6 (0.7)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>−2.5 (0.7)</td>
<td></td>
<td>GG</td>
<td>−2.4 (0.6)</td>
</tr>
<tr>
<td>Bone age delay (year)</td>
<td>11</td>
<td>−2.4 (1.3)</td>
<td>0.6</td>
<td>AA</td>
<td>−2.5 (1.0)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>−2.6 (0.6)</td>
<td></td>
<td>AG</td>
<td>−2.6 (0.9)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>−2.5 (1.2)</td>
<td></td>
<td>GG</td>
<td>−2.5 (1.4)</td>
</tr>
</tbody>
</table>

*Indicates P-value from ANOVA within genotypes.
thereby control body adiposity content with serum L concentrations being positively correlated to BMI (10, 22). In rodents and humans, L is clearly significant in pubertal development and progression (5–9), most likely having a permissive rather than a triggering effect on puberty (23). Thus, the L axis links nutritional status and body adiposity to the facilitation of puberty. We hypothesised that L or LR gene polymorphisms may be implicated in altering the tempo of puberty and therefore investigated whether such polymorphisms would influence the CDGP phenotype.

We chose to examine the VNTR L polymorphism as the number of repeats can potentially affect mRNA stability and thereby lead to altered translation efficiency. We also examined the Gln>$Arg substitution in exon 6 of the LR gene that results in an alteration of charge from neutral to positive, and lies within one of the putative binding sites for L (18). This polymorphism may cause a conformational change in the binding site motif, thereby altering signalling properties. This SNP has been reported in several studies in adult populations to be variably related to BMI (19).

This study is strengthened by the replication of findings in two independent cohorts. In both UK and US CDGP cohorts, the frequencies of L and LR alleles were similar, akin to that in other populations (20, 24, 25). In the UK cohort, L allele 1 homozygotes were heavier than those with the other L genotypes, with this trend also seen in the US cohort, although not achieving significance. This may be related to the time at which anthropometric data were obtained. In the UK cohort, height and weight measurements were taken either from the lowest point in the growth trajectory, or the time-point preceding treatment with sex steroids. In the US cohort, height and weight measurements were taken at the pubertal nadir or just after puberty. The latter measurements would be influenced by the extent of the growth spurt and other exogenous factors affecting weight. This may mask the L genotype effect seen in the UK children assessed in puberty.

UK CDGP children exhibited a wide range of BMI SDS. As a group, they were thinner than controls, but 37% did have a BMI SDS ≥ 0. In those with severe CDGP, BMI SDS influenced the extent of bone age delay with the thin child with severe CDGP having the most severe bone age delay compared to not thin children with severe CDGP or those with moderate delay (Fig. 1). It is the thin child with severe CDGP who was least likely to be an L allele 1 homozygote. We did not find that being homozygous for the L allele 1 influenced BMI SDS in our control population, but this genotype has been associated with increased BMI in adult populations (16).

The interaction between BMI SDS and L genotype was also revealed by univariate analysis (Table 4). BMI SDS was the primary determinant of the extent of bone age delay in CDGP, but the interaction between BMI and L genotype was also significant. LR genotype had no influence on the model.

In conclusion, this study has shown no direct association of specific L or LR polymorphisms with CDGP. However, L genotype has been found to influence the phenotype of CDGP; those homozygous for the L 3’CTTT short repeat have a higher BMI SDS than those carrying the other L genotypes. In keeping with this, those with severe CDGP and the most severe bone age delay who are thin are least likely to be L short allele homozygotes. Thus, the L and LR genes may not be primary candidates to control tempo, but the L gene does appear to modulate the relationship between nutritional and pubertal development.

Acknowledgements

This work was funded by the Wellcome Trust and NIH grants K23RR15544, RO1HD048960 (MRP), RR002172 (Children’s Hospital GCRC) and RR00080 (University Hospitals of Cleveland/Case Western Reserve University GCRC).

References

7 Cheung CC, Thornton JE, Kuiper JL, Weigle DS, Clifton DK & Steiner RA. Leptin is a metabolic gate for the onset of puberty in the female rat. Endocrinology 1997 138 855–858.
23 Gill MS, Hall CM, Tillmann T & Clayton PE. Constitutional delay in growth and puberty (CDGP) is associated with hypoleptinaemia. Clinical Endocrinology 1999 50 721–726.

Received 2 November 2005
Accepted 12 April 2006