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THE GROWTH HORMONE RECEPTOR (GHR) POLYMORPHISM IN GROWTH-RETARDED CHILDREN WITH CUSHING DISEASE: LACK OF ASSOCIATION WITH GROWTH AND MEASURES OF THE SOMATOTROPIC AXIS

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Abstract

Objective—Pediatric Cushing disease (CD) often presents with short stature but we have observed significant inter-individual variability in the growth delay caused by endogenous hypercortisolism. Glucocorticoids cause growth retardation by affecting the growth hormone (GH) – insulin-like growth factor-1 (IGF 1) somatotrophic axis, but also other, GH-independent sites. Recently, the GH receptor (GHR) gene was found to have a common polymorphism (P) that leads to a deletion (d3) or retention of exon 3. In this study, we tested the hypothesis that the GH receptor polymorphism (GHR-P) maybe one of the significant variants that determine the degree of growth delay among patients with CD.

Design and methods—GHR genotyping was performed on 56 children with newly diagnosed CD (24 females, 32 males, mean age of 12.9±3.3 years) who were followed at our institution between the years 1997–2007. Correlation analysis included genotype, measures of growth and the somatotrophic axis, and anthropometrics.

Results—Within the group, 31 (12 girls, 19 boys) expressed the full length GHR allele, 10 (4 girls, 6 boys) were d3-GHR homozygotes and 15 (7 girls, 8 boys) were d3-GHR heterozygotes. No significant differences were found between the GHR genotypes and patient's height and or growth velocity, or any other measures that we evaluated.

Conclusions—The presence of a well-studied and common GHR polymorphism does not appear to be responsible for the variability of growth delay observed in patients with Cushing disease.

Keywords

Cushing syndrome; cortisol; pituitary gland; growth hormone receptor; genetics

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Introduction

Growth retardation is an almost universally present feature of pediatric Cushing's disease (CD) [1]. On average, growth slows down 0.2 to 5.1 years before diagnosis is made [2,3]. The classic dual feature of height standard deviation score (SDS) values below, and body mass index (BMI) consistently above the mean is diagnostic of exposure to endogenous hypercortisolemia [3]. Nevertheless, like many other effects on growth, there is significant inter-individual variability on the degree with which patients with CD respond to excess cortisol with growth delay. The mechanism for growth failure in CD is complex [4]. Glucocorticoids affect multiple elements of the growth hormone (GH) - insulin growth factor-1 (IGF-1) somatotrophic axis, including reductions in GH secretion and in the bioactivity of IGF-1 (although plasma IGF-1 levels are usually within the normal range) [4,5]. Glucocorticoids also have significant effects on non-GH-related sites. In a previous study, we have shown that all patients with CD have GH deficiency that is restored within the first year after surgery and cure of their Cushing syndrome [5]. Since then, we do not routinely screen patients with CD for GH deficiency but continued to observe variability in their growth delay. To date, it remains unclear what the variants that determine growth in patients with CD and GH deficiency are.

Growth hormone acts through binding to the growth hormone receptor (GHR)[6]. The human GHR gene has a common polymorphism that consists of either deletion (d3) or retention of exon 3 [7]. One half of Europeans have at least one allele encoding the d3GHR isoform which is positively associated with GH responsiveness [8]. The effect of this common polymorphism is still unclear; the binding of GH to its receptor appears not to differ between the two isoforms [8]. A few studies found that children with short stature with both normal or low GH levels and who had at least one allele coding for the d3GHR isoform grew better in response to GH therapy than those patients with the full length genotype [8–11]. However, some studies have failed to confirm such an association [12–17]. Several other studies showed a temporarily enhanced response to GH in patients with d3GHR allele, but no differences in the final height [18,19]. Furthermore, in a single study higher IGF-1 levels were noted in response to GH therapy among children with idiopathic short stature carrying the d3GHR allele [20]. The birth weight of small for gestational age (SGA) children who are d3GHR homozygotes was reported to be much lower than children without the deletion [16,19,21], while SGA children with the d3GHR allele appeared to have a higher rate of postnatal catch up growth [21,22]. In a more recent study, GHR polymorphisms showed no effect on the regulation of GH-IGF1/BP3 axis (nocturnal GH, IGF-1 and IGFBP-3 levels) and on insulin sensitivity in prepubertal LBW children [23].

As stated above, we have observed a variable effect on growth of CD; all these patients were GH-deficient but also had variable IGF-1 values [5]. In this study, we examined the hypothesis that at least part of this variability is due to the presence (or absence) of the d3GHR allele.

Subjects & Methods

Patients

Fifty-six patients-24 females, 32 males, ages 5.9–17.4 years- mean age 12.9 ± 3.3 newly diagnosed with isolated CD, were studied retrospectively to evaluate growth response according to GHR genotypes. Patients were treated at a single institution, the National Institutes of Health (NIH), between the years 1997–2007 and studied under an Institutional Review Board-approved protocol (97CH0076). Written informed consent was obtained from all the patients' guardians and assent from patients that were older than 8 years.

The inclusion criteria were: age (equal to or under 18 years), diagnosis of CD by standard criteria and no other abnormalities (i.e. patients with genetic conditions were excluded); all patients were studied before surgical therapy. Demographic and clinical data were recorded at the time of diagnosis. Height and body mass index (BMI) were transformed to Z-score using Height-Weight (HTWT) calculator (<https://web.emmes.com/study/ped/resources/htwtcalc.htm>, <http://www.kidsnutrition.org/bodycomp/bmiz2.html>). Delta height was calculated as the difference between the measurements of height before surgery and one year later (target height). Growth velocity was calculated in cm/year and using the Flanders' growth charts converted it to percentile according to age and sex.

Serum GH and IGF-1 were measured by commercial assays (Chemiluminescence immunoassay on Siemens Immulite 2500 analyzer). The standard deviation of IGF1 values was calculated from the log normal distribution by age provided by manufacture's data (Siemens Medical Solution Diagnostics- usa.siemens.com).

GHR genotyping

We obtained DNA from peripheral blood leukocytes by standard methods following the manufacture's instructions (DNeasy blood and tissue kit, Qiagen Inc). To determine the genotype at GHR exon 3, we designed a PCR assay, using primers G1, G2, and G3 [24] as follows: initial step of denaturation of 2 min at 94°C, followed by 16 cycles consisting of 15 sec at 95°C, 40 sec at 68°C (with -0.5°C in each cycle), and 1 min and 30 sec at 72°C, followed by 25 cycles consisting of 15 sec at 95°C, 40 sec at 60°C and 1 min and 30 sec at 72°C, followed by final extension at 72°C for 7 min. We analyzed the amplification products by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. The full length (fl) allele (GHRfl) is represented by a 935-bp fragment and the d3 allele (d3GHR) by a 532-bp fragment. When homozygous d3/d3 genotype was detected (a single band corresponding to 532 bp), a new PCR amplification using only G₁ and G₃ was carried to rule out the possibility for preferential amplification of the shorter product.

Statistical analysis

Results are expressed as percentages and data are given in mean $\pm$ SD. Differences for the variables evaluated for the 3 groups of genotypes were calculated using ANOVA parametric tests. P-Values < 0.05 were considered significant.

Results

We studied 56 patients newly diagnosed with CD. The data and demographics are provided in Table 1.

GHR genotyping

The multiplex assay of the GHR exon 3 locus yielded the expected fragments of 935 base pairs (indicating the presence of full-length GHRfl) and 532 base pairs (d3GHR), which accurately detected the three possible genotypes- fl/fl, d3/fl and d3/d3. The calculated genotype frequencies for GHRfl, d3/fl and d3GHR were 55%, 27% and 18%, respectively, which were not different from the expected based on the Hardy –Weinberg equilibrium ($P > 0.1$).

GHR genotype-phenotype correlation

No statistically significant differences were observed for age and sex among the three GHR genotypes. Clinical and anthropometric parameters for the 56 patients are shown (Table 1). There was also no significant statistical difference between the three genotypes when we

checked the duration of symptoms before diagnosis (Table 1). Z score Height and Z score BMI did not differ statistically among the three different GHR genotypes (Figure 1). Z-height score means were -1.21 ± 1.23 for the wild type vs. -0.93 ± 0.64 for homozygote presence of d3 allele and -0.97 ± 1.31 for heterozygote and -0.95 ± 1.07 for the homo- and the heterozygote put together (Table 1, Figure 1). We also checked delta heights and found no statistical difference between the three GHR genotypes- means of 6.80 ± 4.67 for the wild type vs. 4.58 ± 4.67 and 6.43 ± 4.78 for the heterozygous and homozygous respectively (Table 1). No statistical differences were found between the GHR genotypes and growth velocity percentile - the means growth velocity percentile were 71.26 ± 36.34 for the wild type, 79.33 ± 34.15 for the homozygote and 73.75 ± 37.12 for the heterozygote presence of the d3 allele (Table 1). Z-score BMI means were 1.94 ± 0.83 for the wild type, 1.98 ± 0.98 for the homozygous state, 1.97 ± 0.93 for the heterozygous state and 1.97 ± 0.93 for the two latter combined (Table 1, Figure 1). Serum IGF-1 levels (standards deviation score, SDS calculated from log normal distribution age matched control values) were -0.49 ± 1.87 for the wild type vs. -1.24 ± 1.40 for the heterozygous state, 0.33 ± 1.90 for the homozygous state and -0.59 ± 1.77 for the homo- or heterozygote presence of d3 allele (Table 1, Figure 1). These parameters were not significant even when we excluded children with Tanner's stage IV and V. There was also no significant statistical difference between the three genotypes when we checked these parameters in each sex separately.

Discussion

Growth in pediatric patients with CD is affected by glucocorticoids acting at several sites of the GH- IGF1 somatotrophic axis [4], as well as other, non-GH-dependent sites. GH binds the GHR in target tissues and this is followed by activation of the JAK-STAT pathway which subsequently increases the expression of IGF1 in the liver and elsewhere; other GH-dependent effects follow in a tissue-specific manner [8]. GHR consists of extracellular, transmembranal and cytoplasmic domains; exons 3–7 encode its extra cellular domain. The described polymorphism of the human GHR gene leads to expression of two different isoforms that contain either deletion or retention of exon 3, its effect on growth and in response to GH therapy is still not clear and even controversial [7,8].

In the present study, we studied the possibility that the d3GHR allele is a modifier of the variability observed in growth retardation of children with CD. None of the patients was frankly diabetic, although the degree of glucose intolerance was not studied. Also, these patients have not been tested for GH deficiency, because this is not part of our protocol, after we showed that GH deficiency (by stimulation testing and sampling) is a universal feature of patients with CD [5].

Although we confirmed the previously identified variability in both growth and measures of the somatotrophic axis [5], we found that there were no significant differences in Z-score heights, delta heights, growth velocity, Z-score BMI, and serum IGF-1 levels between patients with full-length GHR and those who were either hetero- or homozygotes for d3GHR. One limitation of our study is the lack of information to enable us to calculate deviation of the patient's height SDS in comparison to their target height SDS. It should be noted that in order to avoid inaccuracy in amplifying the GHRfl allele we performed a second PCR using only G1 and G3 primers when a sample was homozygous for the d3GHR, as it was suggested in the most recent studies eliminating the possible artifacts that may have not been avoided in the earlier investigations [13,14,17]. The second PCR led to GHRfl amplification and consequently to a heterozygous genotype (d3/fl) assignment in a total of 37% of the patients assigned as homozygous (d3/d3) after the first PCR with all three primers (G1, G2 and G3).

The frequency of the d3GHR allele among children with CD has not been reported before. Our study identified similar allele distribution to the one found by the majority of other studies on different categories of short children: idiopathic short stature, small for gestational age, GHD, and Turner syndrome [8,10,11,14,19]. GH responsiveness in a large multinational cohort of SGA children with short stature is related to the exon 3 GHR polymorphism [19]. Higher frequency of the d3GHR allele in our CD children is seen only when compared to the study of 107 children with isolated GHD reported by Blum et al ($p=0.005$) [15]; and the study of 100 GHD adults by Adetunji et al [25]. Less frequent d3GHR allele is also seen in two control groups of healthy individuals with normal growth [19,25]. Notably, all these study report lower frequency of the d3GHR allele. As discussed above, it is possible that the observed lower frequency reflects the competitive basis of the PCR amplification approach used and correcting step with only G1 and G3 primers on the samples identified as homozygous for the d3GHR may be needed.

To date, most of the studies that explored the significance of the GHR polymorphism on growth focused primarily on the response to GH therapy. Several studies found that children with at least one allele of d3GHR grew better in response to GH therapy [8–11]. However, other publications failed to confirm this association ([12–17]. With regards to IGF-1, SGA children treated with GH the GHR allele had no effect on serum levels [14]. Studies in adults have evaluated the association of the polymorphism with acromegaly and with adult GHD [6,26–30]. One study was performed on patients with acromegaly at the time of diagnosis and showed that patients who carried the d3GHR allele required lower levels of GH to have similar increases in IGF-1 levels and to yield comparable clinical expression of the disease [27]. The difference in the results between the latter study and ours can be explained by the fact that the GHR polymorphism may have an important effect in high exogenous or endogenous GH states but not in conditions associated with low GH levels, such as CD. As in our study, in prepubertal LBW children, endogenous nocturnal GH and total IGF-1 levels were not influenced by the type of GHR sequence present [23]. Because the present study aimed at exploring the significance of the GHR polymorphism on growth retardation at the time of diagnosis of CD, we cannot rule out a possible effect on the response to GH therapy in these patients; however, at present we have no such data in this cohort.

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Figure 1. Height and BMI Z scores and IGF-1 levels SDS in patients with CD according to GHR genotypes; each point represents a single patient. Horizontal lines indicate mean values.

Table 1Clinical characteristics of patients with CD grouped by the *GHR* genotype

GHR genotype	fl/fl	d3/fl	d3/d3	d3/fl+d3/d3
N(%)	31(55%)	15(27%)	10(18%)	25(45%)
Gender (male/female)	19/12	8/7	6/4	14/11
Age (years)	12.62±3.25	14.17±3.58	12.25±2.52	13.40±3.28
Duration of symptoms (months)	25.71±14.43	28±14.45	29.8±20.33	28.83±16.88
Pubertal stage	2.20±1.50	3.50±1.73	2.90±1.19	3.22±1.50
Height- z score	-1.21±1.23	-0.97±1.31	-0.93±0.64	-0.95±1.07
Delta height (cm)	6.80±4.67	4.58±4.67	6.43±4.78	5.38±4.63
Growth velocity percentile	71.26±36.34	73.75±37.12	79.33±34.15	76.14±34.15
BMI- z score	1.94±0.83	1.97±0.93	1.98±0.98	1.97±0.93
IGF1 (SDS)	-0.49±1.87	-1.24±1.40	0.33±1.90	-0.59±1.77