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Exon 3-Deleted/Full-Length Growth Hormone Receptor Polymorphism Genotype Frequencies in Spanish Short Small-for-Gestational-Age (SGA) Children and Adolescents (n = 247) and in an Adult Control Population (n = 289) Show Increased *fl/fl* in Short SGA

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Context: A polymorphism in the human GH receptor gene (*d3/fl*-GHR) resulting in genomic deletion of exon 3 has been associated with the degree of height increase in response to GH therapy.

Objective: The objective of the study was to evaluate the frequencies of *d3/fl*-GHR polymorphism genotypes in control and short small-for-gestational-age (SGA) populations.

Design: An adult control population with heights normally distributed (ACPNH) between −2 and +2 SD score (SDS) and a short non-GH-deficient SGA child population were selected.

Setting: Thirty Spanish hospitals participated in the selection of the short non-GH-deficient SGA children in the setting of a controlled, randomized trial, and one of these hospitals selected the ACPNH.

Controls and Patients: Two hundred eighty-nine adult subjects of both sexes constituted the ACPNH and 247 children and adolescents of both sexes the short SGA patients.

Main Outcome Measures: Heights and weights were recorded in the ACPNH, and auxologic and biochemical data were recorded at each hospital for the SGA patients; *d3/fl*-GHR genotypes were determined and data analyzed in a single hospital.

Results: In short SGA patients, *d3/fl*-GHR genotype frequencies were significantly different from those in ACPNH, with a higher frequency of *fl/fl* genotype ($P < 0.0001$). In ACPNH, a trend toward diminished *d3/d3* genotype frequency was observed in the shortest height group (height ≤−1 SDS and ≥−2 SDS, n = 60).

Conclusions: Our data showed significant differences in the frequency distribution of the *d3/fl*-GHR genotypes between a normally distributed adult height population and short SGA children, with the biologically less active *fl/fl* genotype being almost twice as frequent in SGA patients. These data suggest that the *d3/fl*-GHR polymorphism might be considered among the factors that contribute to the phenotypic expression of growth. **(*J Clin Endocrinol Metab* 91: 5038–5043, 2006)**

A POLYMORPHISM IN the human GH receptor gene (*d3/fl*-GHR) resulting in genomic deletion of exon 3 has been reported (1, 2) and recently associated with the degree of height increase in response to GH therapy in short French children born small for gestational age (SGA) or with idiopathic short stature (3), German Turner syndrome patients (4), and Brazilian GH-deficient children (5), although other studies found no such association (6, 7).

The frequency of this polymorphism has been reported in several control and patient cohorts; however, neither its association with adult height in a control population with heights normally distributed (ACPNH) between −2 SD score (SDS) and +2 SDS nor the comparison of frequencies between short non-GH-deficient SGA children and ACPNH have been described.

The aims of our work were to evaluate the frequency of *d3/fl*-GHR polymorphism in a Spanish ACPNH composed of 289 subjects of both sexes and in a large population of short SGA Spanish children and adolescents (n = 247). Relative *d3/fl*-GHR genotype frequencies and associations with height-SDS were evaluated in each population, and genotype frequencies were compared.

Subjects and Methods

Adult control height population

The adult control population was composed of 289 Caucasian subjects (127 men, 162 women; age range 25–50 yr) from the Barcelona area, voluntarily recruited from the medical, nursing, and laboratory staff of

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Abbreviations: ACPNH, Adult control population with heights normally distributed; BMI, body mass index; *d3/fl* , heterozygous genotype; *fl*, full-length allele; GHR, GH receptor; HWE, Hardy-Weinberg equilibrium; IGFBP, IGF binding protein; PGV, previous growth velocity; SDS, SD score; SGA, small for gestational age.

our hospital and parents of children who attended our pediatric outpatient endocrine clinic. Subjects had to fulfill the following criteria: Iberian Peninsula (except Basque) family origin and no family history of pathologic short stature. None had received therapy with GH or any other anabolic agent. A single subject per family was included.

Height and weight were recorded in our clinic and the corresponding SDS were calculated as follows: recorded value (centimeters) minus mean sex-matched control value (centimeters) and the result divided by the corresponding SD. Body mass index (BMI) and BMI-SDS were also calculated. Because a secular growth acceleration was recently reported by us in a Spanish adult height population, SDS for subjects over 30 yr were calculated with the Spanish reference data obtained 20 yr ago (8) and height SDS of subjects under 30 yr with the data obtained in 2004 (9). Heights for these individuals were normally distributed between −2 and +2 SDS (mean −0.016): 32 women and 28 men between −2.000 and −1.010; 97 women and 73 men between −1.000 and +0.910, and 33 women and 26 men between +1.010 and +1.980.

Short SGA population

The short SGA population consisted of 247 Spanish Caucasian subjects (122 boys and 125 girls) (Table 1) included in a 2-yr prospective and controlled trial. Thirty hospitals participated, and patients were recruited from September 2001 to December 2002. Inclusion criteria were: gestational age greater than 35 wk; birth weight and/or birth length less than −2 SD (10); age over 3 yr; height less than −2 SD (9); never having been treated with GH or other anabolic agents; normal thyroid, kidney, gastrointestinal, pulmonary, and liver function; GH response peak greater than 10 ng/ml; and normal karyotype in girls. Exclusion criteria were: neonatal brain injury, chromosome disorders, malformation syndromes, chronic diseases, and steroid therapy.

Height and weight were recorded by their physicians at inclusion and the corresponding height SDS were calculated (9) (Table 1). Growth velocities during the 6–12 months before inclusion (PGV) were also recorded, and the corresponding SDS were calculated using age-, sex- and pubertal stage-matched control values recently reported in a Spanish longitudinal study (11) (Table 1). One hundred eighty-seven were prepubertal (95 boys, 92 girls), and puberty (Tanner II or III) had begun in the other 60 (27 boys, 33 girls). Paternal and maternal heights were also measured by the physician at the time of inclusion in the study in 237 SGA children and adolescents (117 boys, 120 girls) (Table 1). The corresponding target height was calculated according to the same criteria used in the normal control population. BMI and BMI-SDS were calculated (Table 1).

Serum IGF-I and IGF binding protein (IGFBP)-3 were measured at entry into the study in 85 boys and 69 girls and expressed as SDS according to age- and sex-matched controls and the ratio IGF-I to IGFBP-3 (nanograms per milligram) calculated (Table 1).

Genotyping

Genomic DNA was obtained from peripheral blood. Amplification of a 3248-bp segment containing the GHRwt-GHRd3 polymorphisms reported by Stallings-Mann *et al.* (12) for the exon 3-surrounding region of the GHR gene was carried out. DNA was amplified by PCR using a multiplex strategy described by Pantel *et al.* (2) with modifications. Briefly, 200 ng genomic DNA was added to a 50-μl reaction mixture of 1.5 mM $MgCl_2$, 0.5 mM each dNTP, 0.2 μM of each primer, and 0.5 U Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). The G_1, G_2, and G_3 primers are described in GenBank accession no. AF 155912. Cycling conditions were as follows: initial step of denaturation of 30 sec at 98 C, followed by 40 cycles consisting of 98 C, 10 sec; 60 C, 30 sec; 72 C, 1 min 30 sec, followed by a final extension step of 7 min. Amplification products were analyzed by electrophoresis (90 V, 15 min at room temperature of 25 C) on premade 48-well 1.2% agarose gel containing ethidium bromide (ready-to-run agarose gel; Amersham Biosciences, San Francisco, CA).

When homozygous *d3/d3* genotype was detected (a single band corresponding to 532 bp) and/or when a band potentially corresponding to the 935-bp product was mildly amplified, a new PCR amplification using only G_1 and G_3 was carried out from DNA, in the same conditions, followed by analysis by electrophoresis to reveal the 935-bp product if mildly amplified in the multiplex reaction. In our experience, this second PCR led to the full-length allele (*fl*) amplification and consequently to a heterozygous genotype (*d3/fl*) assignment in a total of 20% of homozygous *d3/d3* in the first PCR.

Hormone measurements

Serum GH was measured in all short SGA children in sera from stimulation tests in each hospital laboratory by commercial assays. Serum IGF-I and IGFBP-3 were measured in a central laboratory by commercial RIA assays (Nichols Institute, San Juan Capistrano, CA).

Ethics

This work was approved by the Ethics Committee of Vall d'Hebron Hospital for the adult control population and the corresponding committees of each participating hospital for SGA patients and parents. Written informed consent was obtained for each subject older than 12 yr of age, and informed consent was also obtained from parents or legal guardians for all patients regardless of age.

Statistical analysis

Results were expressed as percentage and as mean ± SD. Standardized height in the control population was analyzed for normal distribution by the Kolmogorov-Smirnov test ($\chi^2 = 2.882$; $P = 0.4733$). Hardy-Weinberg equilibrium (HWE) was calculated according to standard procedures using χ^2 analysis. Differences for *d3/fl*-GHR genotype frequencies between short SGA patients and controls and between height-SDS groups in the control population were analyzed by the χ^2 test. Differences between *d3/fl*-GHR genotypes for anthropometric parameters in short SGA patients were assessed using the ANOVA Fisher's protected least significant difference test. The Statview 4.5 program (Abacus Concepts, Inc., Berkeley, CA) was used.

Results

Adult control population with normal height distribution

Similar frequencies for the three *d3/fl*-GHR genotypes were found in both sexes (data not shown), the entire pop-

TABLE 1. Anthropometric, hormonal, and parent height data in the 247 non-GH-deficient short SGA children and adolescents at genotype evaluation (mean ± SD)

	Boys (n = 122)	Girls (n = 125)
Age	9.3 ± 3.5	8.6 ± 3.0
Height-SDS	−3.24 ± 0.74	−3.29 ± 0.74
PGV (cm/yr)	4.8 ± 1.3	4.7 ± 1.1
PGV-SDS	−1.18 ± 1.11	−1.66 ± 1.35
BMI-SDS	−0.80 ± 1.13	−0.77 ± 0.79
IGF-I-SDS	−1.06 ± 1.33 (n = 85)	−0.62 ± 1.34 (n = 69)
IGFBP3-SDS	+0.60 ± 0.92 (n = 85)	+0.74 ± 1.04 (n = 69)
IGF-I to IGFBP3 (ng/mg)	53.6 ± 23.9 (n = 85)	62.3 ± 30.4 (n = 69)
Paternal height-SDS	−1.44 ± 1.18 (n = 117)	−1.37 ± 1.03 (n = 120)
Maternal height-SDS	−1.10 ± 1.06 (n = 117)	−1.20 ± 1.10 (n = 120)
Target height-SDS	−1.35 ± 0.87 (n = 117)	−1.22 ± 0.83 (n = 120)

TABLE 2. *d3/fl*-GHR genotype frequencies in ACPNH according to their height SDS values and in short SGA patients

	Adult control population				SGA patients
Height-SDS	≤ +2 and > +1	≤ +1 and > −1	≤ −1 and ≥ −2	≤ +2 and ≥ −2	−3.26 ± 0.74
Number of subjects	59	170	60	289	247
Frequency *d3/d3* (n)	15.3% (9)	18.2% (31)	6.7% (4)	15.2% (44)	11.3% (28)
Frequency *d3/fl* (n)	59.3% (35)	53.5% (91)	68.3% (41)	57.8% (167)	44.5% (110)
Frequency *fl/fl* (n)	25.4% (15)	28.3% (48)	25.0% (15)	27.0% (78)	44.2% (109)
P (χ^2 test)					$P < 0.0001$ *vs.* adults ≤ +2 and ≥ −2
d3 allele frequency	0.40	0.45	0.41	0.44	0.34
fl allele frequency	0.60	0.55	0.59	0.56	0.66
HWE (χ^2)	10.28	2.054	11.088	8.535	0.033
(*P* value)	($P < 0.01$)	($0.5 > P > 0.1$)	($P < 0.001$)	($P < 0.01$)	($P > 0.5$)

ulation (n = 289), the tallest group (height ≤ +2 SDS and > +1 SDS, n = 59), and the intermediate height group (height ≤ +1 SDS and > −1 SDS, n = 170) (Table 2). However, in the shortest group (height ≤ −1 SDS and ≥ −2 SDS, n = 60), the frequency of *d3/d3* genotype was lower and did not reach statistical significance (Table 2). Allele frequencies were similar in the three groups. Genotype frequencies reached HWE in the intermediate height group but not the tallest and shortest height groups (Table 2). Mean values of adult height-SDS were higher (although not statistically significant) in the *d3/d3* genotype group: +0.03 ± 0.85 SDS for the *d3/d3*, −0.09 ± 1.06 SDS for the *d3/fl*, and −0.10 ± 1.04 SDS for the *fl/fl* genotypes.

Short non-GH-deficient SGA population

Similar *d3/fl*-GHR genotype frequencies were found in both sexes and were significantly different from those in ACPNH, with a higher frequency of *fl/fl* genotype ($P < 0.0001$) (Table 2). Genotype frequencies were in perfect HWE (Table 2).

No significant differences were found for mean values of birth weight-SDS, birth length-SDS, height-SDS, BMI-SDS, PGV (in centimeters and SDS), IGF-I-SDS, IGFBP-3-SDS, and IGF-I to IGFBP-3 ratio at inclusion into the study according to the three genotypes (data not shown). However, a statistically significant difference was found for target height-SDS ($P = 0.04$) and paternal height-SDS ($P = 0.03$), with these mean values being lower in subjects with *d3/d3* genotype and higher in subjects with *fl/fl* genotype. A similar trend, although not statistically significant, was observed for maternal height-SDS (Fig. 1).

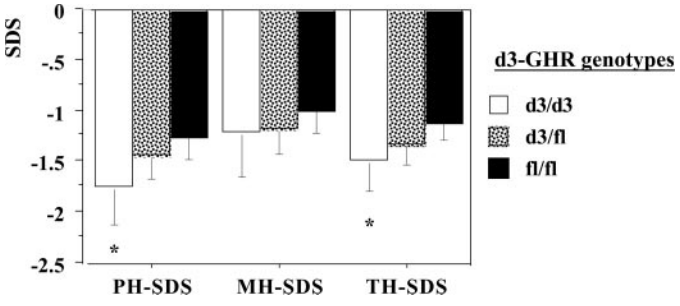

FIG. 1. Paternal (PH-SDS), maternal (MH-SDS), and target (TH-SDS) heights in SDS in short non-GH-deficient SGA patients according to patient's d3-GHR genotypes. *d3/d3 < fl/fl ($P = 0.03$ for PH-SDS and $P = 0.04$ for TH-SDS).

Comparison of d3-GHR genotype frequencies with previous reports

Previously reported *d3/fl*-GHR genotype frequencies in control populations were limited to adult control populations described by Pantel *et al.* (2), Dos Santos *et al.* (3), and Binder *et al.* (4); however, apart from mentioning that the population had normal heights (3), no precise data were given for association with heights. Although no statistical analysis can be made at this point (Table 3), data comparison appeared to reveal similar frequencies of heterozygous genotypes, except for our shortest height control group, whereas homozygous *d3/d3* genotype frequency varied considerably with the lowest frequencies in our shortest height ACPNH and the short SGA group. In addition, the *fl/fl* homozygous genotype frequency was the lowest in our entire control population.

Discussion

Skeletal growth and attainment of adult height are multifactorial phenomena in which genetic disposition, nutrition, homeostasis, hormones, and growth factors interact. Among these factors, GHR plays an important role, and loss-of-function mutations in the GHR gene lead to growth delay during infancy, childhood, and adolescence and short stature in adulthood (13–15). Expression of exon 3-retaining and -excluding GHR mRNA isoforms was first shown to be tissue and cell specific (1) and subsequently to correspond to an individually inherited pattern (2), with the exon 3-deleted allele resulting from an ancestral homologous recombination between two retroelements flanking exon 3. Although the exon 3-deleted isoform has been shown not to alter human GH binding (16, 17), the crystal structure of this region has not yet been modeled (18). Possible functional implications of GHR exon 3-deleted allele expression have only been described *in vitro* in transfected HEK293 cells, with the *d3*-GHR isoform showing increased response to GH with respect to the *fl* isoform (3). Several clinical studies have related the *d3/fl*-GHR polymorphism to the degree of response to GH therapy in GH-deficient (5), short GH nondeficiency (3), SGA (3), and Turner syndrome patients (4). Nevertheless, a recent study in GH-deficient patients did not seem to detect a different growth response to GH therapy as being related to several GHR gene polymorphisms, including the *d3* deleted allele (6). Furthermore, our study in a subset of short non-GH-deficient SGA patients failed to demonstrate an effect of the *d3/fl*-GHR genotypes on the magnitude of growth re-

TABLE 3. *d3/fl*-GHR genotype frequencies in control and patient studies

Studies	Type of subjects	No. of subjects	d3-GHR genotypes (%)		
			d3/d3	*d3/fl*	*fl/fl*
Pantel *et al.* (2)	Unrelated control individuals	150	9	33	58
Dos Santos *et al.* (3)	SGA and ISS (cohort 1)	76	21	31	47
Dos Santos *et al.* (3)	SGA and ISS (cohort 2)	96	8	39	52
Dos Santos *et al.* (3)	Control adults of normal height[a]	283	NR	NR	NR
Jorge *et al.* (5)	GHD	75	12	41	47
Binder *et al.* (4)	Controls[a]	62	15	40	45
Binder *et al.* (4)	Turner syndrome	53	20	30	50
Binder *et al.* (4)	SGA	60	14	38	48
Present study	Controls (height SDS between −2 and + 2 SDS)	289	15.2	57.8	27.0
Present study	Controls (height SDS ≤ +2 and > +1 SDS)	59	15.3	59.3	25.4
Present study	Controls (height SDS ≤ +1 and > −1 SDS)	170	18.2	53.5	28.3
Present study	Controls (height SDS ≤ −1 and ≥ −2 SDS)	60	6.7	68.3	25.0
Present study	SGA[b]	247	11.3	44.5	44.2

NR, Not reported; ISS, idiopathic short stature; GHD, GH deficiency.
[a] Height SDS not reported.
[b] *d3/fl*-GHR genotype frequencies significantly different from controls (−2 to +2 SDS) ($P < 0.0001$).

sponse to 2 yr of GH therapy, possibly because the GH dose was higher than that previously reported in other studies (7).

Because adult height distribution in normally growing populations of both sexes presents a wide range of variation (23 cm in women and 27 cm in men) (9, 11), and differences between populations with different ethnic backgrounds have been described (19–21), the question arises as to whether the *d3/fl*-GHR polymorphism would contribute as a factor, among others, to explain these differences.

The adult height population evaluated by us included a considerable number of subjects of both sexes with heights normally distributed within normal range, between −2 and +2 SDS. Although no statistically significant differences in mean height-SDS values among the three d3-GHR genotypes could be found, a trend toward the lowest values in the *fl/fl* genotype and the highest in the *d3/d3* genotype was observed. The frequency of *d3/d3* genotype was similar in the tallest and intermediate height groups (height-SDS values ≤ +2 and > +1 and ≤ +1 and > −1, respectively) and almost twice as frequent in both as in the shortest height group (height-SDS values ≤ −1 and ≥ +2), although differences did not reach statistical significance. By contrast, the frequency of the *fl/fl* genotype was similar in all three height SDS groups. Whether the lower *d3/d3* genotype frequency observed in the shortest height group could contribute to explaining, in part, the shortest adult height observed in it will require further studies.

Differences between normal height control populations and short SGA patients were not detected in French (3) or German (4) studies; however, no description of height range distribution in controls was presented. Our data show that *d3/fl*-GHR genotype frequencies in short SGA children differ statistically and significantly from those in the adult control population. The *fl/fl* genotype is approximately twice as frequent in short SGA children as the normal adult height control population and *d3/d3* genotype less frequent. These data suggest that, in addition to the multiple factors that may contribute to short stature in SGA children with normal GH response to acute stimuli, the *d3/fl*-GHR polymorphism might be considered an additional one because a higher frequency of the biologically less active genotype (*fl/fl*) was observed in this population in our study. If a different responsiveness to GH according to the *d3/fl*-GHR genotypes is confirmed in human target cells for GH, this would mean that homozygous *fl*-GHR subjects would be less responsive to GH than those homozygous *d3*-GHR. In the context of adequate-for-gestational-age individuals with normal pituitary secretion, this would probably have no major or measurable effect on growth; however, it could be that in short SGA children with normal GH secretion according to acute GH secretion stimulation tests, the homozygous *fl*-GHR subjects found it more difficult to recover the intrauterine growth delay, which could explain why they are more frequent among the short SGA population.

Mean values of birth weight-SDS, birth length-SDS, height-SDS, and spontaneous growth velocity-SDS observed in short SGA children with normal response to an acute GH stimulus were similar in the three *d3/fl*-GHR genotypes. These data suggest that, although *d3/fl*-GHR genotype may contribute to the height differences observed between adequate-for-gestational-age normally growing children and short SGA children, as discussed above, they do not contribute to explaining differences occurring during fetal and postnatal growth in short SGA children.

Target and paternal heights of short SGA children showed significant differences according to their offsprings' *d3/fl*-GHR genotypes, with *d3/d3* having the shortest paternal and target height-SDS and *fl/fl* the tallest; a similar trend was observed for maternal height-SDS, although differences were not statistically significant. Could the lowest target height in children with the *d3/d3* genotype explain why they were unable to recover growth delay more efficiently?

We found a certain percentage of inaccuracy in genotype assignment with the multiplex competitive PCR described by Pantel *et al.* (2), with the homozygous *d3/d3* and heterozygous *d3/fl* genotypes being reassigned after a second PCR for *fl* allele amplification. Inaccuracy of the technique of Pantel *et al.* (2) in amplifying the *fl* allele has also been recently reported by Horan *et al.* (22), who compared this technique with a real-time quantitative PCR technique and described a 30% descent in homozygous *d3/d3* genotypes by the quantitative PCR technique. DNAs from our control and SGA

populations were processed consecutively with the same criteria. Whether differences between the present and published studies could correspond to genetic differences among populations or technical differences remains to be clarified. Genotype frequencies were in HWE in SGA children and the intermediate height group of the ACPNH but were not so in the extremes of the ACPNH. ACPNH was recruited from the Barcelona area, whereas SGA children stemmed from the whole country. Eighty-four of the 247 SGA children originated from Catalonia and their *d3/fl*-GHR genotypes presented identical frequencies and HWE to the remaining 163 SGA children (data not shown), thus confirming comparison adequacy with the ACPNH. However, even though Spain did not receive significant immigration flows for centuries until recent years and because we did not perform a genomic control analysis of the studied populations, we cannot rule out the risk of stratification (23, 24), which, in our control and patient populations, could explain in part the differences observed in *d3/fl*-GHR genotype frequencies between them.

In summary, our data showed significant differences in the frequency distribution of the *d3/fl*-GHR polymorphism genotypes between a normally distributed adult height population and short SGA children, with the biologically less active genotype (*fl/fl*) being increased in SGA patients. In addition, SGA patients with the homozygous *d3/d3* genotype presented the lowest paternal and target heights, whereas those with the homozygous *fl/fl* presented the highest. These data suggest that the *d3/fl*-GHR polymorphism might be considered among the factors that contribute to the phenotypic expression of growth, although a differential response to GH according to the *d3/fl* alleles carried in human target cells, such as chondrocytes obtained early postmortem or peripheral blood lymphocytes, and any molecular mechanisms involved remain to be demonstrated.

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