Clinical and Biochemical Impact of the d3 Growth Hormone Receptor Genotype in Acromegaly

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Context: Lack of exon 3 of the GH receptor (d3-GHR) has been associated with increased responsiveness to GH therapy. By analogy, we hypothesized that patients with acromegaly bearing the d3-GHR genotype may have a more morbid clinical and biochemical picture.

Objective: Our objective was to determine whether the GHR genotype, by modifying tissue sensitivity to GH, influences the clinical/biochemical expression of acromegaly and its outcome after treatment.

Setting: The study was conducted at a specialized clinic at a tertiary care hospital.

Design, Patients, and Methods: We conducted a prospective genotype investigation and retrospective analysis and correlation with clinical, biochemical, and outcome data from a group of 148 patients. Samples from 175 healthy blood donors were used as controls. GHR genotyping was performed by real-time PCR.

Main Outcome Measures: We assessed prevalence of the three GHR genotypes (fl/fl, d3/d3, and d3/fl), associations between the genotypes, and baseline as well as post-therapeutic characteristics.

Results: Prevalence of the fl/fl, d3/d3, and d3/fl genotypes was 45, 22, and 32%, respectively, similar to what was found in the controls. Baseline characteristics were similar in carriers of the three genotypes. A positive correlation between IGF-I and log GH concentrations was significant only in homo- or heterozygous d3 carriers. Among d3-GHR carriers, diabetes, but no other comorbidities, was more prevalent (odds ratio = 2.02; 95% confidence interval = 0.96–4.2). d3-GHR carriers had significantly higher IGF-I concentrations after treatment. Multiple regression analysis revealed that the homo- or heterozygous lack of exon 3 was the strongest predictor of persistent biochemical activity (odds ratio = 1.29; 95% confidence interval = 0.65–2.58).

Conclusions: The absence of exon 3 of the GHR may be associated with a more morbid acromegalic clinical and biochemical picture and a lower chance of achieving IGF-I normalization after therapy. (***J Clin Endocrinol Metab*** **93: 3411–3415, 2008)**

The GH receptor (GHR) gene is located in the short arm of chromosome 5 (p13.1-p12) and contains nine coding exons (1, 2). The extracellular, ligand-binding domain of the receptor is encoded by exons 3–7, whereas exon 8 encodes the transmembrane portion and exons 9–10 the intracellular domain (1, 2). Several GHR isoforms have been identified; one of these isoforms lacks a 22-amino-acid sequence encoded by exon 3 (3, 4). The existence of exon 3-containing (fl-GHR) and exon 3-lacking (d3-GHR) isoforms was once believed to be due to alternative splicing of the immature mRNA (3, 4). It has recently been shown that the existence of these two isoforms is the consequence of an in-frame deletion derived from an intrachromo-

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Abbreviations: CI, Confidence interval; CV, coefficients of variation; d3-GHR, exon 3-lacking GHR; fl-GHR, exon 3-containing GHR; GHR, GH receptor; GH-V, placental GH; OR, odds ratio; SGA, small for gestational age.

somal recombination event between two similar primate-specific retroelements (DNA sequences derived from a retrovirus) that flank exon 3, and occurred late during primate evolution (5). During cell division, these flanking sequences may undergo recombination, which in turn results in a 2.7-kb genomic deletion that spans exon 3 (5).

In most studies that have looked into the frequency distribution of these GHR isoforms in healthy controls, approximately half of the subjects are homozygous for the fl-GHR; the d3-GHR polymorphism occurs in 30–40% in the heterozygous state and in 10–20% in the homozygous state (5–7). For many years, the functional significance of such a dimorphic expression remained a mystery because the *in vitro* binding affinities of both fl-GHR and d3-GHR for 22-kDa GH, 20-kDa GH, placental GH (GH-V), placental lactogen, ovine prolactin, and other ligands appeared to be the same (8, 9). More recently, transfection experiments in HEK 293 cells have shown that GH-induced signal transduction is significantly higher through d3-GHR homo- or heterodimers than through fl-GHR homodimers (6). Studies involving children born small for gestational age (SGA) (6, 10), children with idiopathic short stature (6), girls with Turner's syndrome (10) and children with severe GH deficiency (11) have suggested that the presence of the d3-GHR polymorphism confers a greater response to exogenous recombinant human GH administration. Nevertheless, other authors have failed to confirm this in SGA and isolated GH deficiency cohorts (12–14). Interestingly, a more recent study in children with isolated GH deficiency found that the d3-GHR genotype was associated with a greater initial growth velocity; yet, the final height was no different among carriers of the different genotypes (15).

The clinical spectrum of acromegaly ranges from a relatively asymptomatic condition with slowly evolving acral changes to a severe incapacitating disorder that includes headaches, arthropathy, nerve entrapment syndromes, diabetes, hypertension, and even cardiomyopathy (16). In most but not all patients, the clinical phenotype correlates with the degree of GH hypersecretion and the resulting excess in IGF-I generation (17). As with many other chronic diseases, genetic and environmental factors play a role in the variability of the acromegalic clinical phenotype. Thus, the present study was undertaken to determine whether the GHR genotype influences the clinical expression of acromegaly.

Patients and Methods

Patients and controls

The study population consisted of 152 patients followed at the Acromegaly Clinic of the Hospital de Especialidades, Centro Médico Nacional siglo XXI. This clinic has been following patients with acromegaly since 1998 and has a large database including periodical clinical, biochemical, and imaging information. Thus, all patients had a well established clinical and biochemical diagnosis and periodical follow-up information. All subjects had been treated (transsphenoidal surgery, radiation therapy, and pharmacotherapy) within 1–5 yr before the study; no newly diagnosed, untreated subjects were included, because they did not have follow-up information available. Diabetes mellitus and glucose intolerance were defined according to the World Health Organization (WHO) criteria (18). All patients signed an informed consent, and the study was approved by our ethics committee. The control population consisted of 175 samples obtained form healthy blood donors.

Hormone assays

Both GH and IGF-I values are expressed in mass units. To convert to SI units, multiply by 0.13 in the case of IGF-I and by 2 in the case of GH. GH was measured using a solid-phase, two-site chemiluminescent enzyme immunometric assay on an automated analyzer (Immulite; Diagnostic Products Corp., Los Angeles CA) with a detection limit of 0.01 ng/ml and intra- and interassay coefficients of variation (CV) of 6%; the International Reference Preparation used in the calibration of the GH assay was WHO second 95/574. IGF-I was separated from its binding proteins by means of an acid-ethanol extraction step, and the hormone levels were quantified in the extracted samples by a two-site immunoradiometric assay (Diagnostic Systems Laboratory, Webster, TX), with advertised intra- and interassay CV of 2.6 and 4.4%, respectively; the International Reference Preparation (IRP) used in the calibration of the IGF-I assay was WHO second 87/518. We established our own normative IGF-I data analyzing serum samples from healthy individuals (20 subjects for each gender and age group) and thus calculated the real intra- and interassay CVs as 9.6%. The resulting normal age- and gender-adjusted reference values are as follows: 20–25 yr, 215–590 ng/ml in men and 110–521 ng/ml in women; 26–30 yr, 120–480 ng/ml in men and 129–502 ng/ml in women; 31–40 yr, 100–470 ng/ml in men and 130–354 ng/ml in women; 41–50 yr, 100–300 ng/ml in men and women; 51–70 yr, 78–260 ng/ml in men and women. An IGF-I index was calculated by dividing the patient's result by the upper limit of normal for age and gender, and a value of 1.09 or greater was considered as abnormally elevated (17).

GHR genotyping

Genomic DNA was isolated from peripheral blood mononuclear cells using the QIAamp Blood Mini Kit (QIAGEN Gmbh, Manheim, Germany). GHR exon 3 genotyping was carried out on a LightCycler, real-time PCR equipment (Roche Diagnostics, Manheim, Germany). The oligonucleotides G1, G2, and G3 described previously by Pantel *et al.* (5) were used as primers (GenBank accession numbers AF155912 and AF210633). Differentiation between exon 3-containing and exon 3-lacking isoforms was achieved by analyzing the corresponding sequence-specific melting curves. The PCR products were visualized on 1% agarose gel electrophoresis for size confirmation. Ten random samples (five patients and five controls) were sequenced using the Dye Terminator Cycle Sequencing, GenomeLab Methods Development Kit and an automatic Beckman Coulter CEQ.8800 Genetic Analysis System (Beckman Coulter Inc., Fullerton CA).

Statistical analysis

Data are presented as means ± SD in the case of continuous variables, whereas proportions and frequencies were used for categorical variables. Differences in categorical variables among the three genotype groups were analyzed by χ^2 test, and a two-tailed, unpaired, Student's *t* test was used for continuous variables. Odds ratios (OR) were calculated to estimate association strengths between the different variables and outcomes. A stepwise, multiple regression analysis was performed to explore which patient characteristic (including genotype) is independently associated with persistent acromegalic activity upon long-term follow-up. Statistical software package consisted of Stata, version 8. A P value of <0.05 was considered statistically significant.

Results

The amplified products were visualized as 935- and 535-bp bands, corresponding to the fl-GHR and d3-GHR isoforms, respectively, on agarose gel electrophoresis. The nature of the 10

TABLE 1. Baseline characteristics

	fl/fl	d3/d3	fl/d3	*P*
n (total 148)	68	32	48	
Genotype proportion (%)	45	22	32	
Age (yr)	45 ± 13 (21–81)	47 ± 11 (27–78)	48 ± 12 (24–71)	NS
Disease duration (yr)	12 ± 7 (1–34)	13 ± 8 (2–40)	12 ± 7 (3–35)	NS
Females (%)	57	69	64	NS
Macroadenoma (%)	66	78	73	NS
GH (ng/ml)	26 ± 44.3 (1.1–322)	24.2 ± 24.3 (1.4–100)	41.6 ± 128 (1.1–849)	0.1
IGF-I (ng/ml)	617 ± 151 (311–1339)	615 ± 150 (264–948)	678 ± 202 (397–1613)	0.2
IGF-I index	1.92 ± 0.47 (1.2–3.96)	2.04 ± 0.59 (1.02–3.22)	2.24 ± 0.73 (1.4–5.3)	0.5
Proportion with:				
Diabetes (%)	28	44	47	0.04
Hypertension (%)	32	22	42	NS
Neuropathy (%)	62	56	62	NS
Sleep apnea (%)	62	62	55	NS
Arthralgia (%)	62	72	79	NS

Values are expressed as means ± SD (range). NS, Not significant.

randomly selected products was successfully corroborated by direct sequencing. The distribution of the three GHR genotypes in patients with acromegaly was similar to that found in healthy controls. Among the 152 patients with acromegaly, 45, 22, and 32% harbored the fl/fl, d3/d3, and d3/fl GHR genotypes, respectively. The proportion of the 175 healthy controls with the three different genotypes was 53% for fl/fl, 17% for d3/d3, and 30% for d3/fl. Of the initial 152 patients, four had to be excluded because of incomplete biochemical follow-up information. As shown in Table 1, baseline characteristics such as age, female to male ratio, proportion of patients with macroadenomas, and estimated duration of disease before diagnosis were similar in carriers of the three genotypes.

No difference in baseline GH or IGF-I concentrations at diagnosis was found among patients with the different genotypes (Table 1). The positive correlation between baseline IGF-I levels and log GH concentrations was statistically significant only in carriers of at least one d3-GHR allele (d3-GHR: r = 0.21, $P = 0.05$; fl-GHR: r = 0.19, $P = 0.11$) (Fig. 1). The proportion of patients having clinical evidence of hypertension, neuropathy, sleep apnea, or arthropathy was no different in subjects harboring the three different genotypes (Table 1). Diabetes mellitus, however, was significantly more prevalent among carriers of the d3-GHR genotype in either the homo- or heterozygous state (Table 1). When homo- and heterozygous d3-GHR carriers were grouped together and analyzed against fl-GHR homozygous, the former group had a higher relative risk of being diabetic [OR = 2.02; 95% confidence interval (CI) = 0.96–4.2).

Treatment modality did not differ among the three groups. The majority of patients underwent transsphenoidal surgery as initial treatment of the disease; only 8–10% of patients were treated primarily with somatostatin analogs. External-beam radiation therapy was used in 5.8, 12.5, and 9.3% of patients carrying the fl/fl, fl/d3, and d3/d3 genotypes, respectively; the mean time elapsing between radiation and the final IGF-I was 4 yr (range, 0.7–10 yr) and did not differ among groups. The proportion of patients requiring multiple forms of therapy (surgery, radiotherapy, and pharmacological therapy) was also similar between carriers of the three genotypes; however, there was a trend, albeit not statistically significant, for patients homozygous for the fl-GHR genotype to require triple therapy less often (OR = 0.57; 95% CI = 0.28–1.15).

Post-therapeutic IGF-I concentrations were higher in d3-

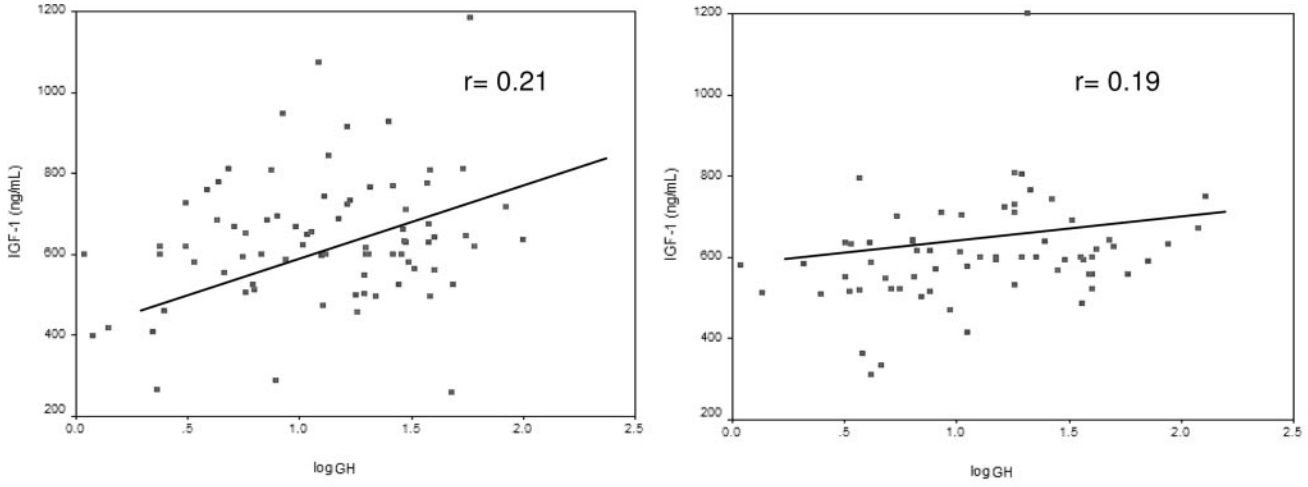

FIG. 1. Correlation between pretherapeutic IGF-I levels and log GH concentrations. A, d3-GHR carriers (r = 0.21; $P = 0.05$); B, fl-GHR carriers (r = 0.19; $P = 0.11$).

TABLE 2. Post-therapeutic biochemical outcome among carriers of the three different GHR genotypes

	fl/fl (n = 68)	d3/d3 (n = 32)	fl/d3 (n = 48)	*P*
GH (ng/ml)	6.8 ± 24.5 (0.05–188)	8 ± 15 (0.1–80)	6 ± 15.8 (0.16–104)	0.5
IGF-I (ng/ml)	367 ± 204 (52–832)	497 ± 212 (45–955)	391 ± 193 (59–850)	0.008
IGF-I index	1.31 ± 0.73 (0.16–3.4)	1.96 ± 1.14 (0.14–5.3)	1.5 ± 1.05 (0.2–5.3)	0.002
% with IGF-I index >1.09	54.4	77.4	55	0.023
OR for IGF-I index >1.09 (95% CI)	0.67 (0.33–1.38)	2.38 (1.07–8.3)	0.76 (0.36–1.62)	

Values are expressed as means ± SD (range).

GHR carriers than in subjects with the homozygous fl-GHR genotype (Table 2). Upon their last biochemical follow-up, a higher proportion of patients with the homozygous d3-GHR genotype (77%) had an elevated age- and gender-adjusted IGF-I level (expressed as an IGF-I index > 1.09) (Table 2). A stepwise, multiple regression analysis including variables such as age at diagnosis, gender, baseline GH and IGF-I levels, tumor size, and GHR genotype revealed that the lack of exon 3 in either the homo- or heterozygous state was the strongest predictor of a persistently elevated IGF-I upon last biochemical follow-up (OR = 1.29; 95% CI = 0.65–2.58).

Discussion

Exon 3 of the GHR encodes a 21-amino-acid sequence that is replaced by a single Asp in the d3-GHR isoform (1, 2). This portion of the molecule is relatively flexible because it does not participate in an ordered crystal structure (19). The absence of exon 3 eliminates a potential N-linked glycosylation consensus sequence located at the exon 3-exon 4 boundary (19). Yet, exon 3 is not necessary for binding as shown by recombinant protein ligands in both naked and glycosylated receptor forms (8, 20). Both receptor isoforms bind equally well to 22-kDa GH and GH-V, whereas they both bind poorly to 20-kDa GH (8, 20). Thus, any functional or biological difference between the two receptor isoforms must come from differences in ligand-induced dimerization, signal transduction, or receptor recycling. Recent transfection experiments in HEK 293 cells have shown that GH-induced signal transduction [signal transducer and activator of transcription 5 (STAT5) activation] is significantly higher through d3-GHR homo- or heterodimers than through fl-GHR homodimers (6). In this study, the difference in STAT5 activation in transfected cells carrying at least one d3-GHR allele and cells homozygous for the fl-GHR was particularly evident at high GH concentrations (3–50 ng/ml) (6).

The distribution of the GHR isoforms has been rather consistent both in healthy controls and in the studied populations (be it children with short stature or patients with acromegaly) (5–7). It is fair to mention that other authors have found the technique we used to amplify the GHR isoforms somewhat inaccurate (around 20%) (7, 21); yet, in every single case and control, we ran the PCR products on agarose gel electrophoresis, and the bands were always neatly visualized. Approximately 50% are fl-GHR homozygous, 35% are d3-GHR heterozygous, and 15% d3-GHR homozygous. In the basal healthy state, carrying either isoform of the GHR is not associated with a greater stature or with any other distinctive phenotypic characteristic. However, short children who are carriers of the d3-GHR isoform respond better to exogenous GH treatment in terms of growth velocity than those homozygous for the fl-GHR (6, 10, 11). This has been documented in children born SGA as well as in subjects with idiopathic short stature, Turner's syndrome, and severe GH deficiency (6, 10, 11). However, two other reports in children born SGA (12, 14) and with isolated GH deficiency (13) did not find any difference in growth rate responsiveness among the three different GHR genotypes. Although the GHR genotype is not considered when estimating height prediction in children treated with exogenous GH, it has been suggested that carriers of the d3-GHR genotype may require lower dosages.

Acromegaly represents a state of GH excess with a rather variable phenotype that does not solely depend on the degree of hypersomatotropinemia. The earlier-mentioned observations in children treated with exogenous GH prompted us to postulate that patients with acromegaly carrying the d3-GHR genotype may in some way have a more morbid clinical picture. Before us, Schmid *et al.* (22) have found in a small cohort of acromegalic patients that the relationship between GH and IGF-I is stronger in carriers of the d3-GHR genotype. These authors did not find any difference in clinical characteristics in acromegalic patients carrying the three different GHR genotypes. In our study, the well-established correlation between IGF-I levels and the log GH concentrations is confirmed only in subjects harboring at least one d3-GHR allele; although the difference between d3 carriers and non-d3 carriers was not very large, the correlation was statistically significant only in the former group. We have previously reported a diabetes prevalence of 32% within our ethnogenetically homogeneous acromegalic population (17). Interestingly, d3-GHR carriers had a prevalence of diabetes between 44 and 47%. Furthermore, d3-GHR carriers were at higher risk of being diabetic than subjects homozygous for the fl-GHR genotype. More striking were the results of the multivariate analysis whereby the absence of exon 3 in either the homo- or heterozygous state was associated with a greater probability of not achieving a normal IGF-I after either surgical or medical therapy. The retrospective nature of our study and therefore the lack of control regarding the type of therapy oblige us to consider our outcome data with great caution. Although in healthy people, the normal negative feedback control of GH secretion should compensate for any differences in GH sensitivity at the target tissue level, this adaptive mechanism is clearly mitigated in acromegaly (23). Thus, at the supraphysiological GH concentrations found in acromegaly, differences in signaling

through either isoform of the GHR may contribute to the variable clinical phenotype seen in this complex disease.

Having a more sensitive GHR may partially explain some unusual acromegaly cases with only minor elevations of serum GH but clearly abnormal IGF-I levels (17). Similarly, differences in GHR genotype could be responsible for some of the postoperative discordances in GH and IGF-I levels (24). Should our findings be corroborated in other (preferably prospective) studies, then the knowledge of the GHR genotype could be used in deciding optimal therapy for the individual patient. Finally, it is not known whether GH antagonists such as pegvisomant are more effective at preventing functional dimerization of the GHR in either the d3- or the fl-GHR genotypes (25). If there is indeed a difference, GHR genotype knowledge may help us decide what patients are better candidates for this costly treatment (25). In conclusion, in patients with acromegaly, the lack of exon 3 of the GHR may be associated with a more morbid clinical and biochemical picture and a lower chance of achieving IGF-I normalization with therapy.

Acknowledgments

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