ORIGINAL ARTICLE

The impact of the d3-growth hormone receptor (d3-GHR) polymorphism on the therapeutic effect of growth hormone replacement in children with idiopathic growth hormone deficiency in Poland

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Abstract

OBJECTIVES: The human growth hormone receptor (GHR) exon 3 deletion (d3) polymorphism has been reported to be associated with the responsiveness to growth hormone (GH) therapy. This study aimed to: (a) assess the frequency of this polymorphism in a group of Polish children with idiopathic growth hormone deficiency (IGHD) and (b) analyze their response to GH therapy.
METHODS: The study group consisted of 67 prepubertal children with IGHD. The control group was composed of 150 Caucasian newborns from whom umbilical cord blood samples were drawn. A genotype analysis was performed using the PCR multiplex technique in search for the existence or deletion of exon 3 of the GHR gene.
RESULTS: In the study group the following genotype distribution was observed: fl/fl-GHR 64.2%; fl/d3-GHR 29.9%; d3/d3-GHR 5.9%. The total percentage of patients with d3-GHR polymorphism was 35.8% and 64.2% patients had a fl/fl-GHR. No significant differences were noted in growth rate SD before introducing therapy and growth rate after one year of recombinant human GH therapy in patients with individual genotypes. In the control group the genotype distribution was: fl/fl-GHR 63.3%; fl/d3-GHR 29.9%; d3/d3-GHR 6.8%.
CONCLUSION: No differences were observed in genotype distribution between the study and the control group. Patients with IGHD did not differ among each other depending on their genotype (fl/fl-GHR or fl/d3-GHR) in terms of growth velocity before introducing therapy or growth rate after one year of recombinant human GH therapy.

Abbreviations:

BMI	- body mass index
d3	- exon 3 deletion
d3-GHR	- lack of exon 3 in the gene for growth hormone receptor
fl/d3-GHR	- heterozygous for lack of exon 3 in the gene for growth hormone receptor
fl/fl-GHR	- full-length growth hormone receptor
GH	- growth hormone
GHD	- growth hormone deficiency
GHR	- growth hormone receptor
IGFs	- insulin-like growth factor
IGFBP	- insulin growth factor binding protein
IGHD	- idiopathic growth hormone deficiency
ISS	- idiopathic short stature
rhGH	- recombinant human growth hormone
SGA	- small for gestational age
SD	- standard deviation
SDS	- standard deviation scores

INTRODUCTION

Children with endogenous growth hormone deficiency (GHD) are diagnosed mostly with idiopathic growth hormone deficiency (IGHD) (Shalet *et al.* 1998; Oczkowska 2009; Rosenfeld 1997). Presently, the only treatment option for these patients is hormone substitution. Recombinant human GH (rhGH) causes identical effects to endogenous GH regarding bone growth and mineralization, as well as the metabolic processes within the organism, which universally allows normalizing height during childhood, achieving adult height within population range and stabilizing metabolic disorders. However, it is important to note, that despite several decades of experience in the fields of GH treatment, standards of GHD patients' qualification and recommended rhGH dosage, growth response in children is characterized by a high individual variability (Wit 2002; Drake 2001; Jorge *et al.* 2006). The final growth effect of rhGH therapy is influenced by a number of factors. The noteworthy include: family genetic growth potential, birth weight, chronological age, height and weight at the beginning of the treatment, bone age delay, weekly rhGH dose and the maximal endogenous GH secretion measured in diagnostic stimulation tests (Ranke *et al.* 1999; Ranke *et al.* 2000; Blethen *et al.* 1993; Schönau *et al.* 2001). Based on these criteria, growth is predicted in only 61% of patients after the first year of treatment and in an even smaller percentage in the following years – subsequently: 40%; 37%; 30% and in around 40% patients after the definitive end of therapy (Ranke *et al.* 1999).

Genetic factors possibly influencing the growth effect of rhGH therapy remain vague. In 2004, Dos Santos *et al.* (2004) published the results of a study on the response to rhGH treatment in children with low birth weight (small for gestational age, or SGA) and in children with idiopathic short stature (ISS) depending on a polymorphism of the growth hormone receptor

(GHR) – an existence or the lack of exon 3 in the gene for GHR (d3-GHR). Authors proved that homozygotes and heterozygotes for d3-GHR responded better to hormonal substitution than a group of patients with full-length (fl)-GHR (from 1.7 to 2 times higher yearly growth velocity). Moreover, a positive correlation between an increased rhGH dosage and the growth rate of children with exon 3 deletion of the GHR gene was noticed. What is more, it was noted that over half of Europeans are heterozygotes and homozygotes with reference to the lack of exon 3 in the GHR gene (Dos Santos *et al.* 2004). The GHR gene is located on the short arm of the fifth chromosome in the 5p13-p12 region. It is composed of nine coding exons (exons 2–10) and several additional exons in the 5' untranslated region (Godowski *et al.* 1989). Exon 2 encodes the signal peptide, exons 3–7 – the extracellular domain, exon 8 – the transmembrane domain, and exons 9 and 10 – the cytoplasmic domain (Godowski *et al.* 1989; Pantel *et al.* 2000; Pilecka *et al.* 2006). There are two isoforms of GHR in humans, generated by retention or exclusion of exon 3 during splicing: a full-length isoform (fl-GHR) and an isoform that lacks exon 3 (d3-GHR) (Pantel *et al.* 2000). That way, from one gene more than one mRNA molecule (one missing 22 amino acids and a full-length one) can be created, resulting in GHR protein variation. However, studies by Pantel *et al.* (2003) supported the hypothesis that the d3-GHR isoform is transcribed from a GHR allele carrying a genomic deletion of exon 3, rather than by alternative splicing.

The significance of this polymorphism is still unclear, but most likely the d3-GHR isoform is specific for the human species as studies on mice revealed only one isoform in their genome – the fl-GHR (Pantel *et al.* 2000). A hypothesis that the d3-GHR polymorphism might have an amplifying effect on the efficacy of rhGH therapy in children with IGHD could be based on a worse growth response to GH in some of the children that cannot be explained by otherwise objective causes.

The aims of our study were to determine the influence of d3-GHR polymorphism on the results of rhGH therapy in Polish IGHD children with the fl/fl-GHR, d3/d3-GHR, fl/d3-GHR genotypes. In addition, we evaluated the frequency of the exon 3 deletion (d3) GHR polymorphism in IGHD compared to a control group. We have also assessed the potential differences in height, bone age delay, growth velocity and maximal GH secretion in the stimulation test prior to beginning of rhGH treatment in patients with GHD. Patients were divided into two groups depending on their genotype: combined fl/fl-GHR and d3/fl-GHR and a separate group d3/d3-GHR.

The aims of our study were 1) to compare prevalence of GHR polymorphism (fl/fl-GHR, d3/d3-GHR, fl/d3-GHR) in children with IGHD to prevalence in newborns from general population and 2) to assess the impact of genotype on the response to GH replacement therapy in children with IGHD.

SUBJECTS AND METHODS

The study group consisted of 67 short stature prepubertal Caucasian children (46 boys and 21 girls) with idiopathic and isolated GHD. The patients were recruited during routine diagnostics of short stature in the Department of Paediatrics, Endocrinology, Diabetology, Metabolic Diseases and Cardiology of the Developmental Age, Pomeranian University of Medicine, Szczecin. The diagnosis was confirmed by repeated GH maximal secretion stimulation test, whereby patients scored a value under 10 ng/ml, twice. At the same time, all the children qualified to the study had to present no abnormalities in other endocrine glands, especially in the thyroid gland. The children did not suffer from any other pituitary disorders. Children carrying other diseases that might impair growth (e.g. osteo- or chondrodysplasia, chromosome disorders, chronic digestive tract diseases including parasites and coeliac disease, chronic conditions of the cardiopulmonary system and kidney diseases) were also excluded.

Study group patients had undergone thorough auxological assessment: height measurement prior to beginning the therapy and after 12 months of its duration, weight, body mass index (BMI), yearly growth velocity assessment (based on at least two measures taken six months apart). To measure the height, a Harpenden stadiometer was used. The same instrument was used at the beginning and during the rhGH therapy. The measurements were taken in the Frankfurt plane. Family and auxological history was also taken into account (weight and height measurements of the patients' and their parents). Pituitary GH secretion capability was assessed based on two GH stimulation tests (either the insulin tolerance test, the L-dopa test or the clonidine test). Bone age was assessed by comparing dominant hand carpal radiographs with the Greulich and Pyle atlas (Greulich &Pyle 1959). Height and growth velocity standard deviation scores (SDS) were calculated based on population norms (Palczewska & Niedźwiedzka , 1999). All of the study group children were treated with rhGH in a substitute dose (0.03 mg/kg/day s.c.). The assessed parameters were measured before the beginning of therapy and after a year of treatment.

Control group consisted of 150 cord blood samples acquired from general population, term born, with normal birth weight, newborn babies born in Szczecin, Poland. Peripheral blood samples from GHD patients used in DNA tests were the residue after routine diagnostics procedures. Genomic DNA was extracted from blood leucocytes by using 200 μl peripheral blood or cord blood on the QIAamp DNA Mini Kit (Cat. No 51306, Qiagen GmbH, Hilden, Germany). To determine genotypes at GHR exon 3, we used simple multiplex PCR-assay as described in Pantel *et al.* (2000) however we reduced number of cycles from 35 to 26 to remove heteroduplexes.

All the DNA analyses were performed in the Department of Laboratory Diagnostics and Molecular Medicine.

The approval of the Pomeranian Medical University Bioethics Committee was acquired (No. BN-001/26/06). The patients' parents or legal guardians signed a written agreement for their children's participation.

Statistics: Statistical analysis was performed using the Shapiro-Wilk, the F Snedecor, t-Student, the Cocharan-Cox and the U-Mann-Whitney tests. We assumed a significance level of p=0.05.

RESULTS

In the study group the following genotype distribution was observed: 43 homozygotes without an exon 3 deletion with an fl/fl-GHR genotype (64.2%); 20 fl/d3-GHR heterozygotes with exon 3 deletion in one allele (29.9%); and 4 d3/d3-GHR homozygotes (5.9%) with exon 3 deletion in both alleles. Genotypes in the control group were as follows: 93 fl/fl-GHR (63.3%); 44 fl/d3-GHR (63.3%); and 10 d3/d3-GHR (6.8%).

In the study group, the total number of patients with a d3-GHR polymorphism (homo- and heterozygotes) was 24 (35.8%), while 43 patients (64.2%) had a full-length GHR gene. In the control group, the number of newborns with a d3-GHR polymorphism (homo- and heterozygotes) was 54 (36.7%) and the number with a full-length GHR gene was 93 (63.3%). Genotype distribution in both groups is illustrated in Table 1. The summary of the demographic, auxological and clinical characteristics of the study group qualified to growth hormone therapy is shown in Table 2.

In the GHD group, the majority of patients were boys – 46 (68.7%), compared to 21 girls (31.3%). This gender disproportion was particularly apparent in the fl/fl-GHR genotype group. There were no significant difference in growth velocity SDS prior to, or subsequent to, rhGH therapy when comparing patients with different genotypes. Prior to therapy, growth velocity SDS for fl/fl-GHR vs. fl/d3-GHR+d3/d3-GHR was −3.2 SDS and −3.2 SDS, respectively (p>0.05). After 12 months of GH therapy, the patients' growth veloc-

Tab. 1. Genotype distribution of the growth hormone receptor (GHR) in Polish children comparing the study (children with isolated growth hormone deficiency) and control groups, where fl is full-length and d3 is the exon 3 deletion.

GENOTYPE	STUDY GROUP (n=67) %	CONTROL GROUP (n= 150) %
fl/fl–GHR	64.27	63.3
fl/d3-GHR	29.9	29.9
d3/d3-GHR	5.9	6.8
fl/d3-GHR+ d3/d3-GHR	35.8	36.7

Tab. 2. Summary of the demographic, auxological and clinical characteristics of the study group prior.

Number of children with GHD	67 (46 boys + 21 girls)
Mean age (years)	9.0
Mean height (cm)	118.3
Mean height SDS	−3.2
Mean growth rate (cm/year) prior introducing to growth hormone therapy	4.4
Maximum GH secretion in a stimulation test(ng/l)	5.4
Maximum GH secretion SDS	2.6
Mean father's height (cm)	171.3
Mean mother's height (cm)	159.5
rhGH dosage (mg/kg/day)	0.030–0.031
Mean birth weight (g)	3025
Mean gestational age at birth (weeks)	39

ity were as follows: 9.3 cm/year (SDS +2.3) for the fl/fl-GHR genotype, and 9.3 cm/year (SDS +2.31) for the fl/d3-GHR+ d3/d3-GHR polymorphism. Furthermore, no significant differences were noted in terms of bone age delay, gender, maximal GH secretion in stimulation tests, parents' heights, birth weight and gestational age, across the genotypes (Table 3).

DISCUSSION

This study showed that the distribution of individual genotypes in a group of Polish children inhabiting the West Pomeranian region is similar to the distribution in the wider European population as described first by Pantel *et al.* (2000) and then by other researchers (Dos Santos *et al.* 2004; Blum 2006; Pilotta *et al.* 2006; Tauber *et al.* 2007; Meyer *et al.* 2007; Klaauw *et al.* 2008). In 2001, Bas *et al.* (2001) analyzed the GHR exon 3 deletion genotype distribution in a large sample of adult general population in Turkey. The results were also comparable with the European population. Moreover, the genotypic distribution amongst Brazilian children does not differ from the European population, as shown by Jorge *et al.* (2006). In contrast, the study from 2014 in the Iranian population, showed a significantly lower incidence of fl/fl-GHR genotype as compared to the genotype with exon 3 deletion in one or both alleles (31.4% vs 68.7%) (Palizban 2014). However, studies assessing the existence of the aforementioned GHR polymorphism in the Asian population present a distinct distribution of genotypes compared to the Caucasian race. In the Japanese, Chinese and Korean populations the fl/fl-GHR genotype occurs more frequently (Ito 2005; Qiu 2007; Ko 2009). Previous research has found no connection between the genotype distribution (d3/d3-GHR, d3/fl-GHR, fl/fl-GHR) and the etiology of short stature.

Only one previous study in 2006 by Audi *et al.* (2006) has observed significant differences. In this study, the fl/fl-GHR genotype occurs almost two times more often in patients which are short for gestational age (SGA) compared to the control group of healthy adults (Audi *et al.* 2006).

We observed that the full-length genotype (fl/fl-GHR) was seen more frequently in boys, although others have not seen such a correlation with gender in patients with GHD, SGA or Turner syndrome (TS) (Jorge *et al.* 2006; Audi *et al.* 2006; Carrascosa *et al.* 2008 b; Raz *et al.* 2008). As in most papers describing patients with GHD, in our study the parents' height, auxological birth parameters, predicted adult height, spontaneous growth velocity, and bone age delay, did not differ statistically between individual groups depending on their genotype (Blum 2006; Pilotta *et al.* 2006; Graaff *et al.*

Tab. 3. A comparison of selected biochemical and auxological parameters between people with full-length growth hormone receptor (GHR) (fl/fl genotype) and those that lack exon 3 (d3-GHR)(d3/fl+d3/d3 genotype).

	Genotype fl/fl-GHR	Genotype d3/fl-GHR + d3/d3-GHR
Mean age (years)	8.7	9.6
Mean height (cm)	117.1	120.5
Growth velocity prior to introducing rhGH therapy (cm/yr)	4.7	3.7
Growth rate after one year of rhGH therapy (cm/yr)	9.3	9.3
Growth rate SDS after one year of rhGH therapy (cm/yr)	2.31	2.30
Mean mother's height (cm)	159.3	159.7
Mean father's height (cm)	171.3	171.2
Bone age delay before rhGH treatment (months)	-27.3	-24.9
Maximum GH secretion in a stimulation test (ng/l)	5.6	5.1
Gestational age	38.6	39.5
Birth weight (g)	2913.7	3224.2

2008). However, there is information suggesting that GHR isoforms are associated with birth weight and placental weight, that is, that the fl/fl-GHR isoform positively correlated with birth weight and placental weight (Padidela *et al.* 2012). Similarly, Sorensen *et al.*(2010) described the correlation of d3/d3-GHR genotype with a lower birth weight and length compared to the other isoforms. Jensen *et al.* (2007), on the other hand, observed a positive correlation between the d3-GHR allele and spontaneous postpartum growth rate. Interestingly, they observed an inverse effect on prenatal development in a group of d3-GHR carriers with SGA.

The search for a connection between the GHR exon 3 deletion polymorphism and growth effect during rhGH therapy is most interesting. Dos Santos (2004) and colleagues showed a statistically significant correlation between the growth effect of rhGH therapy and the GHR gene exon 3 deletion in one or two alleles in two cohorts: children with SGA and ISS treated with 44–48 µg/kg/day of rhGH, and children with ISG and ISS treated with 30 µg/kg/day of rhGH . This initiated further research for the cause of the varied therapeutic effect of rhGH in short-statured patients. This results of this study were confirmed in another study conducted in Germany with Turner syndrome (TS) patients treated with 38 µg/kg/day of rhGH and in a study with Brazilian GHD patients treated with 33 µg/kg/day of rhGH (Jorge *et al.* 2006; Binder 2006).

Another study by Raz *et al.* (2008) also noted a statistically significant correlation between growth velocity during the first and second year of therapy and d3/d3-GHR genotype, compared to fl/fl-GHR genotype in patients with GHD. He did not, however, observe such a correlation when comparing the fl/fl-GHR group to the fl/d3-GHR including d3/d3-GHR groups. The study in the Asian population Zheng (2015) assessing body height and growth rate, showed significantly better effect of rhGH therapy in patients with genotype d3/fl GHR + d3/d3 GHR compared with fl/fl GHR. However, it should be noted, that the author made the assessment after just 6 months of treatment. The results acquired by Ko *et al.* show that the GHR-exon 3 polymorphism might have a positive impact on the effectiveness of short-term rhGH therapy of Korean children with ISS, although studies published in later years were not overly optimistic (Ko *et al.* 2009).

A positive correlation has not been found between the GHR gene exon 3 deletion genotype and the efficacy of rhGH therapy in children with IGHD (Blum 2006; Pilotta *et al.* 2006; Graaff *et al.* 2008; Marchisotti *et al.* 2009). Studies by Binder *et al.* (2006) (60 children with SGA treated with 56 µg/kg/day of rhGH) Carrascosa *et al.* (2006) (86 patients with SGA treated with 66 µg/kg/day of rhGH), and by Audi *et al.* (2008) (219 children with SGA treated with 66 µg/kg/day of rhGH) all report no correlation between the d3-GHR gene and rhGH efficacy on growth velocity. It was assumed that these results were linked to a masking effect of high doses of rhGH on the positive growth effect of fl/d3 and d3/d3 genotypes. However, a later study on a group of Spanish children with SGA treated with lower doses (32 µg/kg/day) also found no correlation between d3-GHR and rhGH efficacy (Carrascosa *et al.* 2008a).

All of the above studies were included in a meta-analysis titled "Association between GHR isoforms and baseline height and growth response to rhGH treatment" published in 2009 (Wassenaar *et al.* 2009). This analysis examined 15 studies with 18 study groups and concluded that the d3-GHR genotype is related to a higher baseline height in children with IGHD that is not observed in other study groups without GH deficiency. Moreover, the d3-GHR genotype correlates positively with growth rate during the first year of rhGH treatment, with an improvement in growth by over 0.5 cm in the first year. The link between d3-GHR and rhGH efficacy is more prominent in patients receiving lower rhGH doses and in older patients (Wassenaar *et al.* 2009).

The first study in Poland shows that the d3-GHR polymorphism does not alter the effect of standard dose (30 µg/kg/day) rhGH therapy on children with GHD. We observed the growth rate of patients with exon 3 GHR deletion, in one or both alleles, after the first year of treatment, was no different to the growth rate of patients without this deletion. Although, a positive correlation has been observed between the d3-GHR genotype and the growth rate in therapy using supraphysiological doses administered to patients with SGA, ISS or TS (Dos Santos *et al.* 2004; Tauber *et al.* 2007; Binder 2006), our results are comparable to studies where the GHD study group was treated with standard doses of rhGH (Blum 2006; Pilotta *et al.* 2006; Graaff *et al.* 2008; Marchisotti *et al.* 2009).

Studies suggest that the d3-GHR genotype is not related to a specific physiological growth variant, and is not a primary cause for short stature. However, a genotype-determined variable GHR activity might be masked by compensating endogenous GH secretion in these patients. This, in turn, might mask the effect of the d3-GHR polymorphism on spontaneous growth. (Wassenaar *et al.* 2009; Bougneres 2010). Despite the confirmed polymorphism in exon 3 of the GHR gene and the prevalence of this shortened form in the Caucasian race, and many studies trying to describe a hypothetical connection between this gene and the growth response to rhGH therapy, the potential benefit of having a d3-GHR isoform remains undiscovered. Although there is some evidence to correlate d3-GHR genotype to rhGH therapeutic efficacy, it seems too minor to have any practical implications

Studies in 2013 have implicated circulating components of the GH-insulin-like growth factor (IGFs)-insulin growth factor binding protein (IGFBP) system with GHR-exon 3 genotype in normal and ISS children (Ballerini *et al.* 2013).

Recent study from 2015 also shows different results, most of which do not indicate any relationship between

the presence of isoforms d3GHR and better response to treatment with rhGH (Hellgren *et al.* 2015; Seung Yang & Hwang 2015). Wei *et al.* (2015) described although a positive correlation between the rate of growth and genotype d3GHR in children with severe growth hormone deficiency, but the study group consisted only of 26 Chinese children in prepubertal age, treated for one year.

Major part of research studies on the GHR polymorphism d3 that were published in the last decade are excluding it as a main factor determining the final heigh. However, it should be considered as one of many elements that affect the therapeutic response rhGH therapy. This is confirmed by the study Jung *et al.* (2015), which involved 101 children with GHD treated with rhGH. The authors used the reactivity index (IOR) as an objective measure of response to treatment with rhGH. They prooved that people with genotype d3 GHR had higher IOR compared to patients with genotype without deletion of exon 3. The results of this study indicate that genetic analysis may be used as starting point for individualized treatment of GHD and evaluation of the genetic differences can serve as a prognostic marker for response to rhGH treatment.

In conclusion, our study is the first detailed analysis on the distribution of the exon 3 deleted/ full-length growth hormone receptor polymorphism in the Polish population. The isoform distribution of the d3-GHR compared to the fl-GHR reflects previous studies on the wider Caucasian race. The variability seen in rhGH therapeutic outcomes does not appear to be related to the d3-GHR polymorphism and requires further investigation to improve reproducible outcomes for IGHD patients.

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