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Analysis of growth hormone receptor gene expression in tall and short stature children

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Abstract

Background: The majority of children who present for evaluation of tall stature fall under the diagnosis of constitutional tall stature (CTS).
Methods: To investigate mechanisms of tall stature, we evaluated serum IGF-I values and the expression of the *GHR* gene in the peripheral blood cells of 46 subjects with normal height, 38 with tall stature and 30 healthy children with short stature.
Results: Our results showed significantly lower IGF-I levels in children with short stature (-0.57 ± 0.18 SDS) compared to control children (0.056 ± 0.19 SDS; $p < 0.0001$) and to subjects with tall stature (0.594 ± 0.17; $p = 0.00067$). Furthermore, we found significantly higher *GHR* gene expression levels in tall children (321.84 ± 90.04 agGHR/5×10^5agGAPDH) compared with other groups of subjects (short children: 30.13 ± 7.5 agGHR/5×10^5agGAPDH, $p < 0.0001$; controls: $86.81\text{ag} \pm 19.5$ GHR/5×10^5agGAPDH, $p = 0.035$). The *GHR* gene expression level in short children was significantly lower compared with control subjects ($p = 0.0068$).
Conclusions: Significantly higher *GHR* gene expression levels in tall subjects suggests a sensitization of the GHR-IGF system leading to overgrowth in CTS.

Keywords: GH; GHBP; *GHR* gene; growth; IGF-I; tall children.

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Introduction

Growth disorders are the most common referrals to pediatric endocrinologists, with short stature being the commonest cause and tall stature more unusual. Being tall is in fact usually perceived as an advantage and is associated with several benefits, even as an advantage in mate selection [1, 2].

Indeed, in the majority of cases, tall stature is to be considered a benign condition and reassurance is all that is needed; in most cases, no specific aetiology is found, and subjects are diagnosed with constitutional or idiopathic tall stature. In some selected cases, however, tall stature can be a sign of an underlying disorder, thus worthy of further investigations and treatment. In addition, excessive height could also have negative psychological and social consequences, especially in girls, with most referrals for tall stature occurring for female subjects [3].

Stature is an extremely heritable trait, controlled by genetic and environmental factors. Growth hormone (GH), GH-binding proteins (GHBPs) and insulin-like growth factor-I (IGF-I) are among the key molecules involved in human growth. GH is considered to be the most important postnatal growth regulator, whereas GHBP reflects the number of cellular receptors for GH, and IGF-I is the major GH-dependent peripheral mediator. Studies of 24-h GH secretion in tall children have found heterogeneity, with some children having high GH secretion while others display reduced levels of GH; excessive GH secretion, thus, cannot be considered a universal cause of tall stature, as children with low GH levels can still present increased growth velocity and increased final height. Moreover, constitutional tall stature (CTS) children with higher GH secretion have been shown to display significantly higher levels of IGF-I, while children with reduced GH levels display normal IGF-I concentrations [4]. Responsiveness to GH stimulation is also increased. Such findings indicate that constitutionally tall children could belong to two different groups, one with hypersecretion of GH and the other with hypersensitivity to GH [5].

Another possibility, as we have previously shown, is that the elevated serum IGF-II observed in the tall but not in the normal children might underpin the increased growth velocity in the former [6]. In these subjects, the

IGFs/IGFBPs molar ratio was significantly higher compared to normal height children, thus allowing for an increased availability of free IGF to the target tissues [6].

In almost all cases, familiarity for CTS is present and at least one of the subject's parents is tall and has followed a similar growth pattern, thus suggesting a strong genetic component [7].

In fact, human stature is considered a highly inheritable polygenic trait, with as many as 180 single-nucleotide polymorphisms (SNPs) found to be associated with height, a large number of which are involved in the determination of tall stature. Polymorphisms of genes such as *FBN1* [7] and *HMGA2* [8] are associated with increased height; the –202 promoter polymorphism in the *IGFBP3* gene has been found to correlate with circulating IGFBP-3 levels and height variation also within tall subjects [9, 10]. However, in spite of the large number of loci identified, such SNPs only explain about 10% of the height variance in the study populations [11].

The full mechanisms underlying CTS are yet to be identified, and many factors and variables that could contribute to increased final height should be taken into consideration.

For this reason, we evaluated *GHR* gene expression to verify whether an alteration of this parameter could confirm the abnormal sensitivity to GH in CTS subjects compared to normal and short subjects.

Subjects and methods

In this study, we enrolled 46 children of Caucasian origin and normal height [between the 25th and 75th percentiles according to Tanner charts (age: 10.57 ± 0.42 years; height: –0.24 ± 0.12 SDS; BMI: –0.58 ± 0.17)], 38 healthy children of tall stature (age: 11.19 ± 0.52 years; height: 2.76 ± 0.11 SDS; BMI: 0.31 ± 0.16) and 30 healthy children of short stature (age: 11.9 ± 0.58 years; height: –2.25 ± 0.22 SDS; BMI: –0.86 ± 0.18). No chronic diseases, including endocrinological and systemic conditions, chromosome and skeletal disorders, Turner syndrome or dysmorphic syndromes were observed. The study was conducted according to the Declaration of Helsinki and that the Institutional Ethical Committee approved the study.

IGF-I assay

The serum IGF-I concentration was measured by an automatic assay that utilizes a solid-phase, enzyme-labeled chemiluminescent immunometric (Immulite 2000 IGF-I-DPC, Los Angeles, CA, USA, and Immulite Analyzer). The intra-assay coefficients of variation were 3.9%–2.4% for a quality control range of 77–1358 ng/mL.

Based on the molecular weight of IGF-I (7650 Da) and IGFBP-3 (31670 Da), we calculated the molar ratio of IGF-I/IGFBP-3 by multiplying the ratio by 4.14 (31670/7650).

GHR gene expression

Peripheral blood mononuclear cells (PBMC) of subjects were separated by Ficoll density gradient centrifugation using a standard procedure (centrifugation at 1800 rpm for 30 min at room temperature followed by recovery of the PBMC ring at the interface).

For real-time *GHR* gene expression analysis, total RNA was isolated from PBMC using RNAeasy mini-columns (Qiagen, Hilden, Germany). Reverse transcriptase PCR (RT-PCR) was carried out with the SuperScript First-Strand Synthesis System. An RNA/primer mixture containing total RNA, oligo dT (50 ng/μL), 10 mM dNTP mix and DEPC water was prepared. The samples were incubated at 65 °C for 5 min and then chilled on ice for at least 1 min. A master reaction mixture for each sample, containing 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT and RNAase OUT was prepared. The reaction mixture was then added to the RNA/primer mixture; the samples were mixed briefly and kept at room temperature for 2 min. Fifty units of SuperScript II RT were added to each tube; then, the samples were mixed and incubated at 25 °C for 10 min. The tubes were incubated at 42 °C for 50 min, heat-inactivated at 70 °C for 15 min and chilled on ice. First-strand cDNA was stored at –20 °C until use for real-time PCR. Quantization of GHR mRNA expression was determined by quantitative real-time RT-PCR (Real-Time PCR 3500-Applied Biosystems, Waltham, MA, USA) and assays on demand were used (Hs00174872 + mL Applied Biosystems, Waltham, MA, USA). Normalization and validation of the data were carried out using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control. The GHR and GAPDH probes were labeled with a fluorescent reporter (FAM). In detail, a 25-μL volume reaction mixture containing 1.25 μL of assay, 12.5 μL of master mix, 10.25 μL of H_2O and 1 μL of cDNA was treated under the following conditions: 95 °C for 10 min, 95 °C for 45 s, 60 °C for 1 min, for 40 cycles. Quantitative real-time PCR data were calculated by a standard curve and expressed as agGHR/5X10^5 agGAPDH.

GHBP evaluation

Serum levels of GHBP were measured by a commercially available ELISA (DSL-10-48100 ACTIVE hGHBP Elisa-Webster, TX, USA). The minimum detectable concentration was 1.69 pmol/L. The intra- and inter-assay coefficients of variation were 5.59%–4.78% and 8.36%–5.11%, for a quality control range of 20.25–198.24 pmol/L and 19.99–195.78 pmol/L, respectively.

IGFBP-3 assay

Serum levels of IGFBP-3 were measured by a commercially available ELISA (Thermo-scientific EHIGFBP3 ELISA-KIT, MA USA). The minimum detectable concentration was 80 pg/mL. The intra- and inter-assay coefficients of variation were <10% and <12%, respectively.

Statistical analysis

Data were expressed as mean ± SEM. Statistical differences between groups of different subjects were determined using a one-way ANOVA test when data followed a normal distribution and the non-parametric

Kruskall-Wallis test when the data were not normally distributed. If a statistical significance was found, an adequate post-test identified which group differed from which. Correlations were analyzed using Spearman's rank correlation test. A value of $p < 0.05$ was considered statistically significant.

Results

Our results showed significantly lower IGF-I levels in children with short stature (–0.57 ± 0.18 SDS) compared with control children (0.056 ± 0.19 SDS; $p < 0.0001$) (Figure 1). The IGF-I values were significantly higher in subjects with tall stature (0.594 ± 0.17) than in children with short stature ($p = 0.00067$). The IGF-I values were also higher in tall stature children than in controls, but the data did not reach statistical significance. On the contrary, IGFBP-3 (tall stature children: 71.39 ± 3.25 ng/mL; short stature children: 72.32 ± 5.95 ng/mL; controls: 69.68 ± 3.31 ng/mL) and the IGF/IGFBP-3 molar ratio (tall stature children: 22.93 ± 3.67; short stature children: 20.56 ± 2.81; controls: 21.69 ± 2.05) values were not significantly different among the three groups.

Furthermore, we found significantly higher *GHR* gene expression levels in tall children (321.84 ± 90.04 agGHR/5 × 10^5agGAPDH) compared with the other groups of subjects (short children: 30.13 ± 7.5 agGHR/5 × 10^5agGAPDH, $p < 0.0001$; controls: 86.81ag ± 19.5 GHR/5 × 10^5agGAPDH, $p = 0.035$). *GHR* gene expression level in short children was significantly lower compared with control subjects ($p = 0.0068$) (Figure 2).

No significantly different values of GHBP (tall stature children: 8.47 ± 0.54 ng/mL; short stature children: 10.69 ± 1.8 ng/mL; controls: 10.43 ± 0.97 ng/mL) were found among the three groups.

Finally, we observed significant correlations between height and IGF-I only in short children ($p = 0.0006$). On the contrary, we found a significant correlation between IGF-I and BMI in control children ($p = 0.04$).

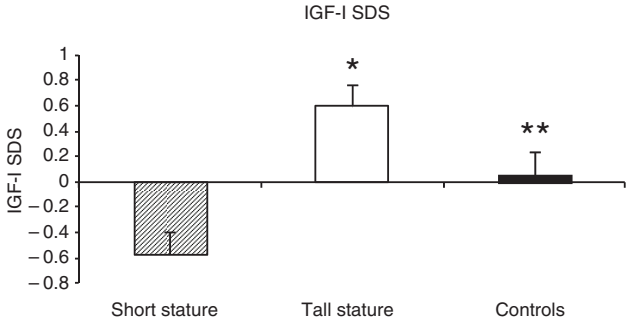

Figure 1: Serum value of IGF-I in controls, in tall stature children and in short stature children.
**p=0.00067, short children vs. controls. *p<0.0001, tall children vs. controls.

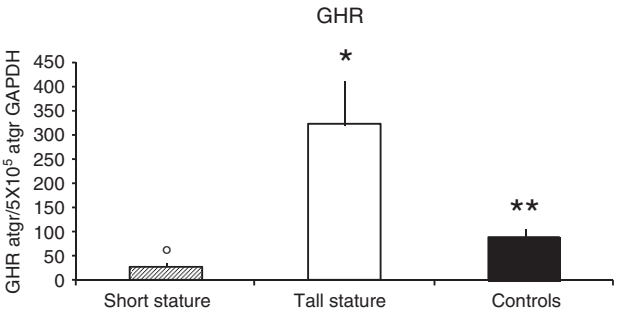

Figure 2: *GHR* gene expression values in controls, in tall stature children and in short stature children expressed as agGHR/5X10^5agGAPDH.
**p = 0.00067, short children vs. controls. *p < 0.0001, tall children vs. controls. ° p = 0.00014, tall children vs. short children.

Discussion

In our study, we examined three groups: normal height children, tall and short subjects. They showed comparable chronological age but significantly different height and BMI values. The basal levels of serum IGF-I were significantly lower in short children compared with normal height children and significantly higher in tall children compared to short children.

To investigate other mechanisms on the basis of the low/high IGF-I secretion in short and tall stature, respectively, we evaluated the expression of the *GHR* gene in peripheral blood cells obtained from children with different statures.

We found significantly higher *GHR* gene expression levels in tall children compared with the other two groups of subjects and significantly lower *GHR* gene expression level in short children compared with control subjects.

Our data thus support a previous hypothesis that a variation in GH sensitivity could be involved in the mechanisms of short and tall stature and reduced or increased IGF-I levels of these subjects.

Several clinical observations indicate that there is a considerable inter-individual variation in GH responsiveness, even in healthy subjects. The molecular mechanisms behind differences in GH responsiveness could include variations at the level of GHR. Molecular studies described at least 180 distinct loci associated with height, and SNP genotypes at these loci, such as the IGFBP –202 SNP, associated not only with height but also with serum

IGFBP-3 levels in tall stature [9], and the *HMGA2* gene SNP [11] explained only approximately 10% of the variation of height in the population. Furthermore, mutations in the *GHR* gene have been found in fewer than 5% of idiopathic short stature patients, suggesting that other molecules involved in GHR signalling might be altered and cause short stature [11]. There are many reports on structural defects in the *GHR* gene in patients with GH insensitivity, but much less is known about variation in *GHR* gene expression in patients with reduced GH sensitivity [12]. For example, *GH* gene expression is 1.8-fold reduced and *GHR* gene expression is 8-fold reduced in adult Pygmies in comparison with sympatric adult Bantu, and this reduction is not associated with sequence variants of the *GHR* gene. The marked decrease of *GHR* expression in Pygmies is associated with reduced serum levels of IGF-I and GHBP levels [13]. In our cases, no significantly different values of GHBP were found among the three groups, even if the GHBP level in tall stature was higher than in short stature children and controls. Therefore, as in other physiological and pathological conditions, we found that GHBP level and GHR function are not closely correlated [14, 15]. GHBP has tissue-specific properties, in terms of GHR regulation and its cleavage to GHBP. It is possible that, in our patients after therapy, these mechanisms aim at increasing GHR availability on the cell's surface, improving GH action [16].

In this study, we found no correlation between *GHR* gene expression in peripheral blood cells and the other parameters analyzed, but we could hypothesize a different result at the local level, such as in bone tissue where GH plays a direct peripheral role.

We found a positive correlation between IGF-I and height in the short but not in the tall children. A possible explanation might be that local IGF-I production, which is not mirrored by the circulating production, is the main cause for overgrowth in these children.

In conclusion, tall stature could be due to hypersensitivity to GH. The significantly higher *GHR* gene expression levels in tall subjects compared with short children and controls suggest a sensitization of the GHR-IGF system leading to overgrowth in constitutionally tall children/adolescents.

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