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Transcriptional response of peripheral blood mononuclear cells to recombinant human growth hormone in a routine four-days IGF-I generation test[☆,☆☆]

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ABSTRACT

Background: There are very few laboratory markers which reflect the biological sensitivity of children to recombinant human growth hormone (rhGH) treatment. Genome-wide transcriptional changes in peripheral blood mononuclear cells (PBMC) have been widely used as functional readout for different pharmacological stimuli.

Objective: To characterize transcriptional changes in PBMC induced by rhGH during a routine short-term IGF-I generation test (IGFGT) in children with growth disorders.

Materials and methods: Blood was obtained for IGF-I determination and RNA-preparation from PBMC of 12 children before and after 4 days treatment with 30 μg rhGH/kg body weight/day s.c. Transcriptional changes were assessed by cDNA-microarrays in the first six children. Selected genes were validated in all 12 cases by RT-qPCR.

Results: Serum IGF-I rose in all patients except one ($p<0.0001$), confirming biological response to rhGH. Unsupervised microarray data analysis in the first six children revealed 313 transcripts with abundant transcriptional changes but considerable inter-individual variability of response patterns. Many patients showed a large cluster of up-regulated genes, including EGR1, EGR2, FOS and to a lesser extent STAT2 and 5b. Exemplarily, EGR1, EGR2 and FOS data were independently reproduced by RT-qPCR. Gene ontology analysis revealed that pathways involved in cell proliferation and immune functions were significantly over represented.

Conclusion: The IGFGT is a suitable method for measuring reproducible and biologically conclusive transcriptional changes in PBMC. As our unsupervised data analysis strategy exposed a considerable inter-individual variability of response profiles a search for molecules of diagnostic and even prognostic value needs to be based on large long-term studies.

1. Introduction

The IGF-I generation test (IGFGT) measures the increase of the serum IGF-I concentration following treatment with recombinant human growth hormone (rhGH). It is an easily accessible functional biological readout of the responsiveness of the GH–GHR system [1]. Accordingly, patients insensitive to rhGH (Laron Syndrome) can be detected by a missing IGF-I increase in response to rhGH [2,3]. Normal IGF-I generation in response to rhGH can be observed in patients with low baseline IGF-I due to so called bioinactive growth hormone (Kowarski syndrome, [4,5]). Moreover, the IGFGT has been used as a parameter in prediction models of rhGH therapy. This, however, often yielded conflicting results (reviewed in [4–6]). One reason may be the lack of comparable test protocols, another might be the fact that only the single parameter IGF-I was used as readout likely not well reflecting the complexity of individual growth regulation. This is underlined by studies showing a wide variability of IGF-I levels during rhGH therapy with only limited correlation of individual growth responses with IGF-I levels [7,8]. Therefore, other factors such as gene polymorphisms may alter individual responsiveness to exogenous and endogenous GH. A well-documented genetic factor is the exon 3 minus polymorphism of the GHR gene (d3-GHR) which was found to enhance GH action in vitro [9] and in vivo [10–12]. Other groups, however, yielded contradictory results [13,14].

It is important to stress that not every short statured child does have an underlying disorder in the GH-system. Savage and co-authors

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recently suggested that defects of the GH/IGF-I axis might represent a wide functional continuum of GH secretion and GH sensitivity [6] and the transition from sick to healthy is fluent. Therefore, additional laboratory markers reflecting the individual GH responsiveness are of high interest for understanding the biology, the differential diagnosis and eventually the prognosis of children with short stature in the context of rhGH therapy.

Peripheral blood mononuclear cells (PBMCs) express the GHR and the IGF-IR [15] and are able to increase IGF-I production in vitro [16]. PBMC are easily accessible during routine blood sampling as part of a short-term four day IGFGT in children without the need of additional venipuncture. Therefore, we hooked up a cDNA-microarray-based genome-wide search for transcriptional markers of GH action in PBMC to our routine four-day IGFGT protocol. To date, there are only two other published studies on microarray analysis of GH action in PBMC, however those studies had different designs. The first using a long-term protocol in six children [17] and the second studying a cohort of ten adult women [18]. We here present evidence for the suitability of assessing short-term transcriptional changes in PBMC in response to rhGH in children as part of a simple four day IGFGT protocol. This offers an experimental portal to identify novel laboratory markers of rhGH.

2. Materials and methods

The study was approved by the ethical review board of the Christian-Albrechts University (CAU) of Kiel, Germany. Informed consent was obtained from all patients and/or their caretakers.

2.1. Patients

In total, we investigated 12 patients (6 males, 6 females). Diagnoses included growth hormone deficiency due to panhypopituitarism following Craniopharyngeoma (N = 1), growth hormone deficiency due to neurosecretory dysfunction (NSD, N = 7), so called qualitative growth hormone deficiency (Kowarski syndrome, N = 2), small for gestational age (SGA, N = 1), and Turner's syndrome (TS, N = 1). Diagnosis of neurosecretory dysfunction was made in short children without underlying diseases, with low normal response to GH stimulation tests but a pathological overnight GH secretion according to Bierich et al. [19]. All patients fulfilled the auxological and endocrine criteria for treatment with rhGH and were naïve to GH treatment. One patient with resistance to GH who received IGF-I treatment was additionally analyzed for control purposes. Patient characteristics at the time of diagnosis are summarized in Table 1.

2.2. IGF-I generation test

As part of our routine clinical workup before the start of treatment, all patients underwent an IGFGT. Blood samples were drawn before the first rhGH dose and at the first morning after 4 days of rhGH treatment (30 μg/kg body weight/day). The control patient with resistance to GH received recombinant IGF-I instead of rhGH for 4 days. All samples were obtained between 8 and 10 a.m. after an overnight fast to reduce circadian influences on gene expression. Additionally, auxological parameters (height, weight, and knemometry rate of the lower limbs [20]) were determined. IGF-I and IGFBP3 were measured by a standard radioimmunoassay (Mediagnost, Reutlingen, Germany). In addition, we obtained 5–10 ml of whole blood (EDTA tube) at both of the time points for PBMC isolation and a complete blood count.

2.3. PBMC isolation

Blood samples were processed immediately. PBMC were isolated using Ficoll (Biochrom AG, Berlin, Germany) gradient centrifugation. The viability of the PBMCs was analyzed by using 1:10 dilution of Turks solution (Merck, Darmstadt, Germany). PBMCs were resuspended in RPMI 1640 medium (Gibco, Karlsruhe, Germany) with 10% DMSO, 10% heat-inactivated FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) and frozen down in liquid nitrogen until use.

2.4. RNA isolation and amplification

RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Potential DNA contamination was removed using DNA-free™ kit (Ambion, Austin, USA). The quality of the RNA samples was measured in a 2100 Bioanalyser (Agilent Technologies, Agilent, Palo Alto, USA) using the Eukaryote Total RNA Nano assay. Total RNA for microarrays was amplified according to the MessageAmp II aRNA kit protocol (Ambion, Austin, USA) according to the manufacturer's protocol.

2.5. RNA labeling and microarray hybridization

PBMC-RNA derived from six GH-treated patients (P1–P6) and the IGF-I-treated patient was analyzed by microarrays. Spotted complimentary

Table 1
Patient characteristics.

	Patient no.	Clinical diagnosis	Age (years)	Height (cm)	H-SDS	PAH-SDS	HV-SDS	IGF-I[a] (centile)	AIT[b]	IHT[b]	NP[c]
Dataset 1	1	Panhypopituitarism	10 8/12	138.6	−1.1	−1.81	−3.17	<0.1	<0.5	n.d.	n.d.
	2	Kowarski-Syndrome	9 6/12	127.1	−2.1	−2.68	−0.92	0.1	28.1	12	3822
	3	Neurosecretory dysfunction	9 4/12	124.4	−2.43	−1.41	−1.09	4	34.3	19	1387
	4	Turner Syndrome	12 3/12	138.7	−2.39	−4.42	−1.01	16	29.1	n.d.	n.d.
	5	Neurosecretory dysfunction	7 8/12	112.8	−3.4	−2.5	−1.4	18	8.6	11.8	773
	6	Neurosecretory dysfunction, JCA	14 9/12	155.4	−1.84	−4.99	−0.71	<0.1	8.2	36	209
	IGF-I	GHIS	9 7/12	115.5	−3.21	−3.25	−0.75	<0.1	29.4	n.d.	2280
Dataset 2	7	Neurosecretory dysfunction	13 8/12	141.8	−3.14	−3.01	−0.28	<0.1	17.4	9.1	1688
	8	Kowarski-Syndrome	10 2/12	130	−2.02	−3.74	−1.23	<0.1	22.5	18.1	3114
	9	Neurosecretory dysfunction, neuroblastoma	10 2/12	126.2	−2.67	−2	−0.77	2	n.d.	n.d.	1872
	10	Neurosecretory dysfunction	8 2/12	116.3	−3.14	−2.28	−1.56	8	52.8	33.2	1895
	11	Neurosecretory dysfunction	10 7/12	128.7	−2.78	−2.84	−1.63	3	15.1	13.4	445
	12	SGA	9 7/12	125	−2.66	−2.97	−1.06	91	19.5	27.7	n.d.

Abbreviations: GH insensitivity syndrome, SGA: small for gestational age, JCA: juvenile chronic arthritis, H-SDS: height SDS, HV: height velocity, n.d.: not determined. AIT: max. GH increment in the arginine infusion test performed with 0.5 g arginine hydrochloride/kg body weight; IHT: max. GH increment in the insulin-hypoglycaemia test (0.1 IE insulin/kg body weight), NP: profile of spontaneous overnight GH secretion over a period of at least 6 h. All GH values in AIT, IHT and NP were measured with the IRP 66/217 standard.

[a] Measured at diagnosis.

[b] Values are given in ng/ml. GH increment > 10 ng/ml excludes hypopituitary GHD.

[c] Values are given as mean GH secretion in ng⁎min⁎ml^{-1}, normal range 2000–6800.