A mosaic de novo duplication of 17q21–25 is associated with GH insensitivity, disturbed in vitro CD28-mediated signaling, and decreased STAT5B, PI3K, and NF-κB activation

D Mul1,*,†, S Wu2,3,4,*, R A de Paus5, W Oostdijk1, A C Lankester1, H A van Duyvenvoorde1,6, C A L Ruivenkamp6, M Losekoot6, M J D van Tol1, F De Luca3,4, E van de Vosse5 and J M Wit1

1Department of Pediatrics, J6S, Leiden University Medical Center, PO Box 9600, 2300RC Leiden, The Netherlands, 2School of Medicine, First Affiliated Hospital, Xi’an Jiaotong University, Xi’an, Shaanxi 710061, People’s Republic of China, 3Section of Endocrinology and Diabetes and 4Department of Pediatrics, St. Christopher’s Hospital for Children, Drexel University College of Medicine, Philadelphia, Pennsylvania, USA, Departments of 5Infectious Diseases and 6Clinical Genetics, Leiden University Medical Center, PO Box 9600, 2300RC Leiden, The Netherlands

(Correspondence should be addressed to W Oostdijk; Email: w.oostdijk@lumc.nl)

*(D Mul and S Wu contributed equally to this work)
†(D Mul is now at Haga Teaching Hospital, Juliana Children’s Hospital, The Hague, The Netherlands)

Abstract

Objective: The established causes of GH insensitivity include defects of the GH receptor and STAT5B. The latter condition is also characterized by severe immunodeficiency. A recent case with short stature, GH resistance, and immunodeficiency due to an IkB mutation suggests that the NF-κB pathway may interact with STAT5B signaling.

Design: Here, we present a case of a short child with several congenital anomalies as well as GH insensitivity and mild immunodeficiency associated with a mosaic de novo duplication of chromosome 17q21–25, suggesting that overexpression of one of the duplicated genes may be implicated in GH resistance.

Methods and results: In vitro studies on blood lymphocytes showed disturbed signaling of the CD28 pathway, involving NF-κB and related proteins. Functional studies on cultured skin fibroblasts revealed that NF-κB activation, PI3K activity, and STAT5 phosphorylation in response to GH were suppressed, while the sensitivity to GH in terms of MAPK phosphorylation was increased. An in silico analysis of the duplicated genes showed that MAP3K3 and PRKCA are associated with the NF-κB pathway. Baseline MAP3K3 expression in T-cell blasts (TCBs) was normal, but PRKCA expression in TCBs and fibroblasts was significantly higher than that in control cells.

Conclusions: We conclude that the 17q21–25 duplication is associated with GH insensitivity and disturbed STAT5B, PI3K, and NF-κB signaling, possibly due to PRKCA mRNA overexpression.

European Journal of Endocrinology 166 743–752

Introduction

The differential diagnosis of proportional short stature associated with low serum insulin-like growth factor 1 (IGF1) and a normal or high GH peak in a provocation test includes a GH1 mutation leading to a bioinactive GH molecule (1, 2) and four well-established causes of GH insensitivity: i) mutations in the GH receptor gene (GHR), affecting the extracellular, transmembrane, or intracellular domains leading to the GH insensitivity syndrome (3, 4); ii) mutations of STAT5B, the major component of the GH signaling pathway (3, 4, 5); iii) IGFALS defects (6); and iv) IGF1 defects (7, 8, 9, 10). In addition, it has been shown that activation of the mitogen-activated protein kinase (MAPK) pathway, as in Noonan syndrome, is associated with partial GH insensitivity (11).

In vitro studies have shown that there are three major pathways of GH signaling. The JAK2–STAT5B pathway is most important for GH signaling in terms of growth and is also essential in interleukin 2 (IL2)-mediated T-cell signaling, as proven by the severe short stature due to GH insensitivity in combination with immunodeficiency in all patients except one (12) with homozygous STAT5B defects (3, 4, 5, 13, 14). The second pathway is the RAS–MAPK signaling pathway. Activation of this pathway is associated with short stature; activating mutations of PTPN11 and variants of other genes in this pathway lead to Noonan syndrome and related syndromes (11). The third signal transduction pathway of GH is the phosphoinositide-3 kinase (PI3K) pathway (for review, see (15)).

Besides these three pathways, which also interact with each other, signaling proteins may also be
involved. For example, the PI3K and STAT5 pathways induce NF-κB nuclear translocation and DNA binding after phosphorylation and subsequent degradation of the inhibitory protein IκB (16). Recently, we reported on a short patient with an IκBα mutation who showed GH insensitivity and immunodeficiency (16).

In this paper, we report on a short girl with severe GH insensitivity, multiple congenital anomalies, and mild immunodeficiency, who has a mosaic de novo duplication of chromosome 17q21−25 containing 223 genes. Similar de novo distal 17q duplications have been reported in several cases (17, 18, 19, 20, 21, 22), two of whom were mosaics (17, 18, 19, 20). All these reported patients (except one with a relatively small duplication (21)) were short. Other clinical features include learning disability, high-arched palate, and abnormalities of hands and feet, resembling Ellis–van Creveld syndrome.

We investigated the STAT5, RAS–MAPK, PI3K, and NF-κB pathways in vitro in T-cell blasts (TCBs) and skin fibroblasts and performed an in silico analysis of the duplicated genes in relation to the clinical and experimental findings. We hypothesized that in this patient, dysregulation of the NF-κB pathway may explain the GH insensitivity and the mild immunodeficiency, while the extremely increased GH secretion may be caused by the combination of GH resistance and GH1 gene duplication.

**Patients and methods**

The female patient was first presented at the pediatric endocrine outpatient clinic at 5 years of age for progressive growth failure. She was known to have a number of congenital anomalies (loose skin in the neck and abdomen, narrow forehead, depressed nasal bridge, long philtrum, split uvula, sacral dimple, rhizomelic shortening of all extremities, postaxial polydactyly of hands and feet, bilateral pes equinovarus, and extreme hypermobility of the large joints), recurrent infections, and developmental delay. Parents gave informed consent for all laboratory and genetic investigations.

**Genetic investigations**

Shortly after birth, an interstitial duplication of chromosome 17q21−25 was detected by conventional karyotyping in 87% of peripheral lymphocytes. In the remaining cells, a normal karyotype was seen. The 17q duplication was also observed in fibroblasts from skin and cartilage. The duplication was further characterized by the Affymetrix GeneChip Human Mapping 262K NspI.

To identify genes in the 17q duplicated region that are associated with endocrine and immunological/hematological abnormalities, we first performed a gene prioritization (source: Cartagenia, Leuven, Belgium) (23). Secondly, we screened for genes in the duplicated region that either encode proteins involved in one of the known GH signaling pathways or proteins that interact with the NF-κB pathway (String 8.3; http://string-db.org/).

The duplication of the GH1 gene was investigated by multiplex ligation-dependent probe amplification (MLPA) using the P216-A2 GH deficiency SALSA MLPA kit according to the manufacturer’s instructions (MRC Holland, Amsterdam, The Netherlands). The kit contains probes for GH1 exons 1, 3, 4, and 5. MLPA of GHR and STAT5B was performed using the P262 GH insensitivity SALSA MLPA kit according to the manufacturer’s instructions (MRC Holland). MLPA of IGFALS was carried out using the P217-B1 IGF1R kit (MRC Holland). Exons 2−5 of the GH1 gene, including flanking intron sequences, GHR, STAT5B, IGF1, and IGFALS were sequenced according to the standard procedures.

**Endocrine studies**

GH secretion was assessed using a clonidine test (0.15 mg/m² per os (p.o.)). Plasma GH was determined using time-resolved immunofluoro metric assay (IFMA) (Wallace/PE, Turku, Finland), using the WHO 80/505 as a standard (1 mg = 2.6 IU), and expressed as milliunits per liter. The results were converted to microgram per liter based on the most recent GH standard (1 μg/l = 3 mU/l, WHO IS 98/574) (24). Plasma IGF1 and IGFBP3 were measured using Immulite 2500 immunoanalyser of Siemens Healthcare Diagnostics (Deerfield, IL, USA) and expressed as SDS based on the national age references (25). An IGF1 generation test was performed with increasing GH dosages (0.7, 1.4 and 2.8 mg/m² body surface per day s.c. for 7 days), separated by washout periods of at least 3 weeks, as described previously (8).

**Studies on blood lymphocytes**

**Immunoglobulins and lymphocyte subpopulations**

Serum immunoglobulins were measured by nephelometry. IgA and IgG anti-transglutaminase antibodies were determined by ELISA as described previously (26). Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll separation. Lymphocyte subpopulations in blood were analyzed by flow cytometry (FACS Calibur; BD Biosciences, Oxford, UK) using Moabs anti-CD45, -CD14, -CD33, and -GPA for defining the lymphocyte: anti-CD3, -CD4, -CD8, -CD45RA, and -CD45RO for determining naïve and memory/effector cells in the CD4+ and CD8+ T-cell subsets; anti-CD3, -CD56, and -CD16 for determining NK cells; and anti-CD19, -CD20, and -CD27 for determining naïve and memory B cells. Data were analyzed using Cell Quest Software (BD Biosciences).

**Innate immunity**

After stimulation assays of whole blood of the patient and controls with lipopoly saccharide (LPS) and IFNγ or various other Toll-like receptor (TLR) stimuli, production of cytokines (IL10,
tumour necrosis factor α (TNFα), IL12p40, IL1β, IL6, and IL8) was measured in the supernatants by ELISA (BioSource/Invitrogen, Breda, The Netherlands).

**PBMC cultures** PBMCs were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Lonza, Vervier, Belgium) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM Gluta-MAX (Invitrogen). TCBs were generated by stimulating PBMCs with 800 ng/ml phytohemagglutinin (Abbott Murex, Dartford, UK) in the presence of 30 U/ml IL2 (Proleukin, Chiron, Emeryville, CA, USA). TCBs were allowed to proliferate for at least 14 days before use.

**STAT5 signal transduction pathway** TCBs were washed three times and cultured for 18 h in medium without IL2. Thereafter, 2 × 10^5 cells were stimulated in 200 μl culture medium for 0–90 min with or without 100 U/ml IL2, 10 ng/ml IL7, or 10 ng/ml IL15 (R&D Systems, Abingdon, UK). TCBs were fixed with 4% paraformaldehyde (Sigma) and permeabilized with 90% methanol (Merck). Subsequently, the cells were blocked with 10% normal goat serum (Sanquin, Amsterdam, The Netherlands) in PBS, 2% BSA (Sigma), stained with the PE-conjugated antibody pY694-STAT5 (BD Biosciences), and washed twice before analyzing on a FACS Calibur (BD Biosciences).

**CD28 signal transduction pathway** One of the duplicated genes in the 17q21–25 region (MAP3K3) is part of the GH signaling pathways and the CD28 signal transduction pathway. To investigate the latter pathway, TCBs and PBMCs were stimulated with anti-CD2/CD28, anti-CD3/CD28, IL2, or a combination of anti-CD2/CD28 or anti-CD3/CD28 with IL2, and proliferation was measured. TCBs or PBMCs (1 × 10^5) were stimulated in 200 μl culture medium in a 96-well microtiter plate. The cells were stimulated with 2 μg/ml anti-CD2 (CLB-T11.1/1 and CLB-T11.2/1 (Sanquin)) or 1 μg/ml anti-CD3 (UMC Pharmacy, Leiden, The Netherlands) and 2 μg/ml anti-CD28 (CLB-CD28/1; Sanquin) in the presence of 100 U/ml IL2. After 72 h incubation, 100 μl medium was removed from each well and 25 μl RPMI medium containing 0.5 μCi 3H-thymidine (PerkinElmer, Waltham, MA, USA) was added. After 16 h of incubation, the cells were harvested and 3H incorporation was determined using a liquid scintillation counter (Wallac, PerkinElmer). To further investigate at which level the CD28 signal transduction pathway was interrupted, we determined CD28 expression by FACS in TCBs from the patient and three controls in unstimulated cells as well as in IL2, IGF1, and IL2 plus IGF1-stimulated cells.

**MAP3K3 and PRKCA transcription** MAP3K3 and PRKCA transscripts were quantified in TCBs from the patients and controls 4 days after restimulation by quantitative RT-PCR. TCBs were stimulated for 24 h with IL2 or IGF1, after 4 days of prestimulation with anti-CD2/anti-CD28. RNA was isolated using Trizol (Invitrogen) and cDNA generated using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s conditions. Quantitative PCR was performed using Taqman gold and SYBRgreen I (Roche) in a MyiQ Thermal Cycler (Bio-Rad). Primers and conditions are available on request.

**IFNγ production by CD2/CD28-activated cells** PBMCs were stimulated with anti-CD2/CD28, and the percentages of activated cells were compared by analyzing the number of IFNγ-producing cells in response to IL12, IL18, or a combination of IL12 and IL18. PBMCs (2 × 10^5) were cultured in 200 μl culture medium in a 96-well microtiter plate and stimulated with or without 2 μg/ml anti-CD2 and 2 μg/ml anti-CD28, 100 U/ml IL2, 1 ng/ml IL12 (R&D Systems), and 50 ng/ml IL18 (MBL, Nagoya, Japan). The cells were incubated with cytokines for 32 h and for an additional 11 h in the presence of Golgiplug (BD Biosciences). The cells were stained with anti-CD3-FITC (BD Biosciences) in PBS–2% BSA. Subsequently, the cells were fixed with paraformaldehyde and permeabilized with Saponin (Sigma). The cells were blocked with 10% FCS in PBS and stained with anti-IFNγ-APC (BD Biosciences). Before analyzing on a FACS Calibur, the cells were washed twice. The number of IFNγ-producing cells was calculated for the CD3-negative and -positive fraction as percentage of total cells.

**Fibroblast studies** A skin biopsy was taken shortly after birth and fibroblasts were cultured under standard conditions. The fibroblasts were stored in liquid nitrogen, and 7 years later the fibroblasts were thawed and used for functional studies. Fibroblasts from two healthy controls aged 9.3 and 4 years stored in liquid nitrogen were used for comparison.

**NF-κB transcription factor assay** NF-κB p65 DNA binding activity was determined by ELISA (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer’s instructions. A specific double-stranded DNA sequence containing the NF-κB p65 response element was immobilized onto the bottom of wells of a 96-well plate. Fibroblasts were treated with graded GH for 24 h and then nuclear extracts were collected. Nuclear extracts containing NF-κB p65 were added to the plate and incubated overnight at 4 °C without agitation. NF-κB p65 was detected by addition of specific primary antibody directed against NF-κB p65 (Cayman Chemicals). A secondary antibody conjugated to HRP (Cayman Chemicals) was added to provide a sensitive colorimetric readout at 450 nm. Data are expressed as OD450/μg nuclear extract and represent three separate experiments.
**PI3 kinase assay** PI3 kinase activities were determined by an in vitro kinase assay (SuperArray Bioscience Corporation, Frederick, MD, USA). Cells were seeded into 96-well plates and incubated with graded GH for 24 h. Cells were then fixed with 4% formaldehyde for 20 min at room temperature to preserve phosphorylation. Two primary antibodies were included in the kit. One antibody recognizes only the activated (phosphorylated) form of the specific target protein (phosphoprotein-specific antibody), while another recognizes the specific target protein regardless of its activation state (pan-protein-specific antibody). Following incubation with primary and secondary antibodies, the amount of bound antibody in each well was determined using a developing solution and an ELISA plate reader. The absorbance readings were then normalized to relative cell number as determined by a cell staining solution. The relative extent of target protein phosphorylation was determined by normalizing the absorbance reading of the phosphoprotein-specific antibody to the pan-protein-specific antibody for the same experimental condition. Experiments were performed three times and data were expressed as mean ± S.E.M.

**Western blot** Whole cell lysates were solubilized with 1% SDS sample buffer and electrophoresed on a 4–15% SDS–PAGE gel (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane and were probed with the following primary antibodies: rabbit polyclonal antibodies against p-STAT5 (Cell Signaling Technology, Inc., Danvers, MA, USA), goat polyclonal antibody against STAT5 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2; T202/Y204; Cell Signaling Technology, Inc.), anti-ERK1/2 (Cell Signaling Technology, Inc.), and rabbit polyclonal antibody against β-actin (Sigma–Aldrich). The blots were developed using an HRP-conjugated polyclonal goat antirabbit or donkey antigoat IgG antibody and ECL system (Amersham). The intensity of the bands on Western blots was analyzed using Image J Software (NCBI, Bethesda, MD, USA). The protein size was confirmed by molecular weight standards (Invitrogen).

**Statistical analysis** All data of the fibroblast experiments are expressed as the mean ± S.E.M. Statistical significance was determined by t-test or ANOVA.

**Results**

**Case report**

At birth, the patient’s weight was 3.43 kg (−0.45 SDS) and head circumference was 34.9 cm (0.1 SDS) (27). In the first 5 years of life, she suffered from several medical conditions, including strabismus (exotropy), dissociated vertical deviation, and anisometry. She went through recurrent episodes of viral and bacterial infections, including herpes zoster, pneumonia, and otitis media, whose frequency diminished after starting prophylactic antibiotics. Surgical interventions were carried out to correct cervical instability, club feet, and scoliosis.

At the first presentation to the pediatric endocrine clinic at 5.5 years, height was 93.2 cm (−4.7 SDS), body mass index 17.0 kg/m² (+0.9 SDS), and head circumference 48.3 cm (−1.5 SDS) (28). Sitting height/height ratio was 0.61 (+5.0 SDS) (29). Retrospective collection of previous growth data showed a progressive growth retardation (Fig. 1). At 6.45 years, she was operated on for scoliosis, and shortly thereafter, high-dose GH treatment was started (1.4 mg/m² body surface per day). Height SDS increased from −4.4 to −3.65 in the first year and stabilized in the following 6 months (Fig. 1), consistent with partial GH insensitivity. Sitting height/height ratio SDS were +5.6, +4.9, and +4.5 before and after 12 and 18 months of GH treatment respectively.

**Endocrine studies**

The GH peak after clonidine was 38.3 μg/l (115 mU/l), with a baseline value of 10.2 μg/l (30.5 mU/l) and a minimum of 2.4 μg/l (7.3 mU/l). Initial baseline IGF1 level was 4.6 nmol/l (−3.1 SDS) and IGFBP3 was 2.4 mg/l (−0.9 SDS).

The results of the IGF1 generation test are shown in Table 1. Although IGF1 appeared to slightly increase in the lowest GH dose, this increase did not reach 1 S.D. (the response criterion in this test). The intermediate dose (equivalent with 50 μg/kg per day) did not result in any increase in IGF1 and IGFBP3, and only the highest dose produced a substantial rise of IGF1. Still, the stimulated value was below average for age.
Serum IGF1 increased from $-3.6$ SDS before high-dose GH treatment to $-0.2$, $-0.6$, and $+0.1$ SDS after 3, 12, and 18 months respectively, and IGFBP3 increased from $-2.6$ to $+0.5$, $-0.3$, and $+0.3$ SDS.

**Genetic investigations**

A precise assessment of the size of the 17q duplication was performed with SNP array analysis. The duplication is $25.81$ Mb and contains 223 genes (Supplementary Table 1, see section on supplementary data given at the end of this article).

Twelve genes in the duplicated region were associated with endocrine abnormalities and 45 with immunological/hematological abnormalities. The nine genes associated with both are PRKAR1A, SSTR2, GIP, EPX, POLG2, COL1A1, PRKCA, ACE, and GH1. The only gene encoding a protein in one of the established endocrine abnormalities and 45 with immunological/hematological abnormalities. The nine genes associated with both are PRKAR1A, SSTR2, GIP, EPX, POLG2, COL1A1, PRKCA, ACE, and GH1. The only gene encoding a protein in one of the established GH signaling pathways is MAP3K3. Using String 8.3 Software, only proteins encoded by MAP3K3, NGFR, and PRKCA interact with the NF-kB pathway. A literature search suggested that involvement of NGFR in GH insensitivity would be unlikely, so MAP3K3 and PRKCA were considered as candidate genes to explain the clinical presentation of this girl.

Sequences of GH1, GHR, STAT5B, IGFALS, and IGF1 were normal, as well as the MLPA for GHR, STAT5B, and IGFALS. MLPA for GH1 confirmed that the GH1 gene was duplicated.

**Studies on blood lymphocytes**

**Immunoglobulins and lymphocyte subpopulations**

IGM was $0.54$ g/l (normal: $0.24–1.8$ g/l), IgA $0.22$ g/l (normal: $0.55–2.2$ g/l), total IgG $3.89$ g/l (normal: $5.2–13.4$ g/l), and subclasses showed a decreased IgG1 level ($2.59$ g/l, normal: $3.7–10.0$ g/l). IgA and IgG anti-transglutaminase antibodies were absent. Lymphocyte subpopulations showed normal counts of T, B, and NK cells and normal numbers of naive and memory/effector cells within the CD4$^+$ and CD8$^+$ T-cell subsets and the B-cell compartment (data not shown).

**Innate immunity**

In the stimulation assays of whole blood with LPS and IFNγ or various other TLR stimuli, the IL10, TNFα, IL12p40, IL1β, IL6, and IL8 production by the patient’s cells was approximately six times higher than in controls. This was explained by an approximately six times higher number of cells in blood from the patient compared with controls (data not shown).

**STAT5 signal transduction pathway**

The effect of stimulation of TCBs with IL2, IL7, or IL15 on STAT5 phosphorylation is shown in Fig. 2A. STAT5 phosphorylation in TCBs from the patient was similar to that in TCBs from controls in response to each of the stimuli.

**CD28 signal transduction pathway**

No difference could be detected in CD28 expression by unstimulated TCBs of the patient or controls and IL2 and/or IGF1 did not affect CD28 expression (data not shown). TCBs of the patient did not respond to either CD2/CD28 or CD3/CD28 stimulation alone (Fig. 2B), or to CD2/CD28 stimulation plus low (1 and 10 U) concentrations of IL2 (data not shown), suggesting that the CD28 response pathway is affected. However, high concentrations of IL2 (100 U; Fig. 2B) yielded a significantly enhanced response. In contrast, PBMCs of the patient proliferated well in response to CD2/CD28 or CD3/CD28 with or without IL2 (Fig. 2B), which may be explained by the presence of antigen-presenting cells among PBMCs that may provide alternative co-stimulation.

**IFNγ production by CD2/CD28-activated cells**

The percentage of CD2/CD28-activated cells producing IFNγ after stimulation of PBMCs was greatly reduced in the patient compared with the controls (Fig. 2C). This holds for CD3$^+$ T cells as well as for CD3 (presumably NK) cells. Additional stimulation with IL12 or IL18 hardly increased the percentage of IFNγ-producing cells in both the cell populations, while combined addition of IL12 and IL18 did greatly increase the number of IFNγ-producing cells in CD3$^+$ cells, but only slightly in CD3$^-$ cells (T cells; Fig. 2C).

**MAP3K3 and PRKCA transcription**

Quantitative RT-PCR analysis of MAP3K3 transcripts in unstimulated cells showed no difference between patient and controls. As it is unknown which stimuli regulate MAP3K3 or PRKCA transcription, we hypothesized that IL2 or IGF1 might be able to have such an effect. Four days after prestimulation, TCBs were stimulated with IL2 or IGF1.

---

**Table 1** Insulin-like growth factor 1 (IGF1) generation test.  

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After GH stimulation (0.7 mg/m² per day)</th>
<th>Baseline</th>
<th>After GH stimulation (1.4 mg/m² per day)</th>
<th>Baseline</th>
<th>After GH stimulation (2.8 mg/m² per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1 (nmol/l)b</td>
<td>7.8</td>
<td>10.4</td>
<td>5.3</td>
<td>5.0</td>
<td>4.7</td>
<td>11.5</td>
</tr>
<tr>
<td>IGFSDS</td>
<td>−2.0</td>
<td>−1.3</td>
<td>−3.0</td>
<td>−3.2</td>
<td>−3.5</td>
<td>−1.1</td>
</tr>
<tr>
<td>IGFBP3 (mg/l)</td>
<td>2.0</td>
<td>2.8</td>
<td>2.1</td>
<td>2.1</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>IGFBP3 SDS</td>
<td>−1.7</td>
<td>−0.5</td>
<td>−1.6</td>
<td>−1.6</td>
<td>−2.6</td>
<td>−1.4</td>
</tr>
</tbody>
</table>

*aGH was administered for 7 days; a washout period of 3 weeks separated the GH stimulation periods.
*bFor IGF1 concentration in nanogram per milliliter, multiply by 7.649."
In two controls, graded concentrations of GH induced a dose–response effect on NF-κB DNA binding activity, while higher concentrations of GH significantly induced NF-κB DNA binding activity (Fig. 4A). In the patient’s fibroblasts, however, none of the concentrations of GH used in this experiment induced NF-κB DNA binding activity. Similar to what was observed for NF-κB activation, patient’s fibroblasts also failed to respond to GH regarding PI3K activity. In contrast, the higher GH concentration(s) induced a significant increase in PI3K activity in control fibroblasts (Fig. 4B).

**GH-induced STAT5 and MAPK phosphorylation in fibroblasts** Since STAT5 is well known to be involved in GH-mediated signaling pathways, we assessed STAT5 phosphorylation in response to GH in fibroblasts. The addition of GH to the culture medium of control fibroblasts for 24 h induced STAT5 phosphorylation (assessed by Western blotting, Fig. 5A and B), but no such induction was observed in the patient’s fibroblasts cultured with GH. The two higher concentrations of GH caused a similar induction in MAPK phosphorylation in patient’s fibroblasts as in controls, but on the lowest GH concentration, only the patient’s fibroblasts responded (patient vs controls, \( P < 0.01 \), suggesting an increased sensitivity to GH for this pathway (Fig. 5C and D).

**PRKCA expression** In unstimulated conditions, the relative PRKCA mRNA expression normalized for β-actin was clearly elevated in patient’s fibroblasts (Fig. 6), confirming the data in lymphocytes (Fig. 3B).

**Discussion**

This is the first case with a mosaic de novo duplication of chromosome 17q21–25 in which a detailed characterization of the duplication by SNP array analysis and an in-depth study of GH and CD28 signaling were performed. As in previously described cases (17, 18, 19, 20, 21, 22), this patient has short stature. Our patient, like previously described patients, has a disproportionate dwarfism because of rhizomelic shortening of the extremities, which usually would not be consistent with a disorder of the GH–IGF1 axis. However, the increased GH secretion may be explained by the GH insensitivity only, the GH secretion in this case is so high that we speculate it is caused by the combination of GH insensitivity and overexpression of the duplicated GH1 gene. Confirmation of this speculation awaits further studies on GH secretion in other cases of GH1 duplication. At present, no data on GH secretion have been reported on the three cases with...
overlapping duplications (5–22.7 Mb) in the International Standards for Cytogenomic Arrays database, nor on the four patients with relatively large duplications containing GH1 as part of unbalanced translocation in the Ecaruca database. In the Decipher database, no cases of GH1 duplication have been reported.

In our search for a common cause for a disturbance of GH signaling and the mild immunodeficiency, we first focused on the JAK2–STAT5 signaling pathway. In the in vitro studies on the patient’s TCBs, we did not find evidence for a decreased STAT5 phosphorylation in response to IL2, IL7, or IL15, but in the patient’s fibroblasts, the addition of GH did not result in STAT5 phosphorylation. This might suggest a cell- or ligand-specific abnormality in STAT5 signaling.

We then investigated the functionality of the CD28 signaling pathway, in which NF-κB and related proteins play an important role (30). These proteins also play a role in the two other GH signal transduction pathways (the RAS–MAPK and PI3K pathways). We observed that anti-CD2/CD28-induced proliferation was strongly diminished in cultured TCBs and that the percentage of CD2/CD28-activated T cells producing IFNγ was greatly reduced in cultures of the patient’s PBMCs compared with the controls. The experiments in cultured patient’s fibroblasts showed that GH did not induce NF-κB DNA binding activity, in contrast to control fibroblasts. These results suggest that the NF-κB signal transduction pathway is disturbed in both cell types. The degree of GH insensitivity in the present patient is similar to that reported by Wu et al., but the immune problems in the patient with the IκBα mutation were far more severe (16, 31). Our patient had a mild hypogammaglobulinemia and recurrent infections responding to antibiotics, suggesting that the observed impairment of immunity is mainly limited to the humoral immune response.

The next step was to screen the list of duplicated genes for genes associated with endocrine or immunological disorders and for genes associated with the 17q21–25 duplication and disturbed GH signaling (749).
NF-κB pathway. In our first analysis, one of these genes, **MAP3K3** (MAPK kinase kinase 3, also known as **MEKK3**) came out as the only gene that encoded a protein involved in CD28 signaling (through NF-κB and its associated proteins) as well as in GH signaling (RAS–MAPK and PI3K pathways). **MAP3K3** is involved in T-cell activation through activating IKK (IκB kinase), which is essential for the stimulation of the transcription factor NF-κB (32). Proximal signaling components of NF-κB stimulation in T cells include the T-cell receptor (TCR)/CD3 complex and CD28 (30). We therefore postulated that **MAP3K3** overexpression might lead to partial disruption of both pathways. However, our hypothesis was not supported by the observation that the number of **MAP3K3** transcripts was not different in the patient’s TCBs compared with controls, although the response to IL2 was higher than that in control cells and the sensitivity of MAPK phosphorylation in response to GH in the patient’s fibroblasts was increased.

A later in silico analysis showed that there is another gene in the duplicated region, **PRKCA**, encoding protein kinase Cα (PKCα), which is involved in the NF-κB pathway and various other signaling pathways. PKCα is one of the classical (cPKC) Ca2+-dependent members of the PKC family that regulate a wide variety of biological events within the cell, regulating multiple biological processes, including cell proliferation, apoptosis, differentiation, migration, and adhesion (for recent reviews, see (33, 34, 35)). Its expression in our patient’s TCBs and skin fibroblasts was higher compared with control cells, and it is therefore tempting to speculate that elevated expression of **PRKCA** may be involved in the GH insensitivity and possibly other clinical and laboratory features of this syndrome. However, defining biological roles for specific PKC isoforms is complex because they have ubiquitous expression patterns, which results in a level of functional redundancy between the isoforms.

Another gene in the duplicated region that may be associated with the clinical features is **PRKAR1A**, encoding a critical component of type I PKA, the main mediator of cAMP signaling in mammals. A recent paper showed that **PRKAR1A** haploinsufficiency is associated with the activation of proinflammatory pathways in osteoblast progenitors (36) so that one can speculate that **PRKAR1A** duplication might lead to decreased PKA activity. Obviously, we acknowledge that we cannot

---

**Figure 5** Dose–response effect of GH on STAT5 and MAPK phosphorylation in fibroblasts. Fibroblasts were cultured in the absence or presence of 1–10–100 ng/ml GH. At the end of the 24 h culture period, fibroblasts were harvested, lysed, electrophoresed, and immunoblotted for p-STAT5, total STAT5 and P-MAPK and total MAPK. The intensity of the bands on Western blots was analyzed using Image J (software from NCBI). Results are expressed as ratio of p-STAT5 to total STAT5 and ratio of p-P42/44 to MAPK (mean±S.E.M.). (A) A representative blot for p-STAT5 from three independent experiments is presented. (B) Densitometry of ratio of p-STAT to total STAT from three experiments. (C) A representative blot for p-P42/44 from three independent experiments is presented. (D) Densitometry of ratio of p-P42/44 to total MAPK from three experiments. *P<0.05 and **P<0.01 vs untreated fibroblasts.

**Figure 6** Relative **PRKCA** mRNA expression in the patient’s fibroblasts and controls. The relative **PRKCA** expression levels of mRNA were normalized for β-actin in the same samples. Results are expressed as fold changes compared with control 1. Experiments were performed in triplicate and data were expressed as mean±S.E.M. *P<0.05 vs controls 1 and 2.
exclude that the clinical features may be associated with other genes in the duplicated region, about which less
information on interactions with endocrine and immune function is available in the literature.

Based on the available literature and our present findings, GH insensitivity might, in this case, be caused by
a combination of disturbed STAT5B, NF-kB, and PI3K signaling and increased MAPK signaling. We
speculate that PKCα overexpression and/or the increased activity of MAPK may alter GH signaling at
the level of the GHR or JAK2. Interestingly, overexpression of PKCα has been associated with decreased
tyrosine phosphorylation and increased serine phosphorylation at the insulin receptor, and it causes
internalization of the insulin receptor (37). Furthermore, PKCα has been implicated in the activation of
NF-κB downstream of TCR/CD28-induced T-cell activation (38), and it can also play an inhibitory role
during the regulation of the cell cycle (33). With respect to PI3K signaling, PKCα, even at physiological concen-
trations, serves as an endogenous negative feedback inhibitor of insulin signaling through IRS1, PI3K, PKB,
and PKCα to the glucose transport system in both skeletal muscles and adipocytes (39), which is in line with
the decreased PI3K phosphorylation in response to GH in the patient’s fibroblasts. Finally, PKCα increases
phosphorylation of Raf-1 kinase, which leads to the activation of the ERK–MAPK cascade (40), which is
consistent with the increased sensitivity to GH of MAPK phosphorylation. As mentioned before, activation of
this cascade has been associated with GH insensitivity (11).

In conclusion, we speculate that the growth failure, GH insensitivity, and immunodeficiency of this patient
in a mosaic de novo duplication of chromosome 17q21–25 may be due to dysregulation of the three
classical GHR signaling pathways (STAT5B, MAPK, and PI3K) and NF-κB signaling, possibly related to PKCα
mRNA overexpression.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-11-0774.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

1 Besson A, Salemi S, Deladoey J, Vuissos JM, Eble A, Billigmaier M, Burgi S, Honegger U, Fluck C & Mullis PE. Short stature caused by
2 Rodriguez S, Gaunt TR & Day IN. Molecular genetics of human growth hormone, insulin-like growth factors and their pathways in
3 Rosenfeld RG, Belgorosky A, Camacho-Hubner C, Savage MO, Wit JM & Hwa V. Defects in growth hormone receptor signaling.
Trends in Endocrinology and Metabolism 2007 18 134–141. (doi:10.1016/j.tem.2007.03.004)
4 Savage MO, Burren CP & Rosenfeld RG. The continuum of growth hormone–IGF-I axis defects causing short stature: diagnostic and
5 Hwa V, Nadeau K, Wit JM & Rosenfeld RG. STAT5b deficiency: lessons from STAT5b gene mutations. Best Practice and Research.
6 Domene HM, Hwa V, Argente J, Wit JM, Camacho-Hubner C, Jasper HG, Poro J, van Duyvenvoorde HA, Yakar S, Fofanova-
Gambetti OV & Rosenfeld RG. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences.
Hormone Research 2009 72 129–141. (doi:10.1159/000232486)
7 Woods KA, Camacho-Hubner C, Savage MO & Clark AJ. Intratertiary growth retardation and postnatal growth failure associated
M199610313351805)
Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role
10 van Duyvenvoorde HA, van Setten PA, Walenkamp MJ, van Doorn J, Koenig J, Gauguin L, Oostdijk W, Ruivenkamp KA,
Clinical and biochemical characteristics of a male patient with a novel homozygous STAT5B mutation. Journal of Clinical
11 Binder G. Noonan syndrome, the Ras–MAPK signalling pathway and short stature. Hormone Research 2009 71 (Suppl 2) 64–70. (doi:11.1506/000912439)
Kruitblof MF, van Dissel J, Janssen R, Wit JM & Romijn JA. Clinical and biochemical characteristics of a male
Rosenfeld RG. Growth hormone insensitivity associated with a STAT5B mutation. New England Journal of Medicine 2003 349
Characterization of immunodeficiency in a patient with growth hormone insensitivity secondary to a novel STAT5b gene


39 Leitges M, Plomann M, Standaert ML, Bandyopadhyay G, Sajan MP, Kanoh Y & Farese RV. Knockout of PKC alpha enhances insulin signaling through PI3K. *Molecular Endocrinology* 2002 16 847–858. (doi:10.1210/me.16.4.847)