An immunologically anomalous but considerably bioactive GH produced by a novel GH1 mutation (p.D116E)

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Abstract

Context: Although GH values measured by an immunoassay usually reflect GH bioactivities, discrepancy exists between immunoactivity and bioactivity in a rare condition known as ‘bioinactive GH’. 
Objective: To report an immunologically anomalous but considerably bioactive GH.
Methods: We performed mutational and functional analyses of GH1 in a 7-year-old Japanese boy with short stature (−3.0 S.D.) in whom serum GH values measured with a Tosoh immunoassay kit were all undetectable in three provocation tests, whereas urine GH value measured with a Hitachi immunosassay kit was within the normal range. Serum IGF-1 was at a low-normal range, and IGF-binding protein-3 was below the normal range.
Results: Mutation analysis showed a missense GH produced by a novel GH1 mutation (p.D116E) of paternal origin and a frameshift mutation (p.Q68fsX106) of maternal origin. Genotype–phenotype correlations in this family and in vitro functional studies indicated that the p.D116E-GH was immeasurable with the Tosoh kit but was measurable, though maybe not precise, with a Daiichi kit, and had a reduced in vivo bioactivity. The p.Q68fsX106 yielded no GH protein.
Conclusions: The results suggest that the p.D116E affects the GH epitope primarily recognized by the Tosoh kit but not by the Hitachi or the Daiichi kits, thereby producing an immunologically anomalous but considerably bioactive GH. The presence of such a hormone discordant for immunoactivity and bioactivity should be kept in mind, to allow for an appropriate assessment of endocrine data.

Introduction

GH measurement by an immunoassay is indispensable for the diagnosis of GH deficiency. Indeed, GH provocation tests are almost invariably performed in children with short stature (1), and measured serum GH values usually reflect GH bioactivities. However, in a rare condition known as ‘bioinactive GH’, discrepancy exists between measured GH values and GH bioactivities (2–4). Thus, this condition is associated with low insulin-like growth factor-1 (IGF-1) values, short stature, and good responses to GH therapy, in the presence of apparently normal to mildly elevated serum GH values.

Here, we report an immunologically anomalous but considerably bioactive GH identified in a patient with short stature.

Patient and methods

Case report

This Japanese boy was born to non-consanguineous parents at 39 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 50.0 cm (+0.6 S.D.) and his weight was 2.97 kg (+0.2 S.D.).

At 7 years and 1 month of age, he was referred to us because of proportionate short stature (Fig. 1). Endocrine and auxological data are summarized in Tables 1 and 2. Notably, serum GH values measured with a Tosoh immunoenzymometric assay kit (Tosoh, Tokyo, Japan) were all undetectable during insulin, clonidine, and GH-releasing hormone provocation tests, whereas urine GH value measured with a Hitachi chemiluminescence enzyme immunosassay kit (Hitachi Chemical) was within the normal range. Serum IGF-1 value was at a low-normal range, and IGF-binding protein-3 (IGFBP-3) was below the normal range. Other pituitary hormones and thyroid hormones were normal. Since these endocrine and auxological data satisfied the criteria for GH therapy in Japan (the criteria in children aged ≥5 years: height, below −2.5 S.D.; peak GH value, below 6.0 ng/ml at least in two provocation tests; and serum IGF-1 value, below 200 ng/ml) (5), recombinant human GH therapy (0.175 mg/kg per week) was started at 7 years and...
3 months after consultation with the parents, but the responsiveness to this therapy was not so remarkable (Fig. 1 and Table 2).

Clinical data of the family members are summarized in Table 2. Since endocrine data of the sister and the mother were examined after the investigations of the patient, basal GH values were measured with the Tosoh kit and a Daiichi IRMA kit (Radio Isotope, Tokyo, Japan; endocrine data were not available in the father). In addition, the Daiichi kit was also applied to measure the basal GH in stocked serum samples of the patient, although the serum samples during the provocation tests at 7 years and 1 month of age were not preserved. Notably, the basal GH values of the patient and the sister were obviously different between the two kits, and the GH values in the sister did not show a simple 1:2 ratio between the two kits. The sister and the father had low but normal heights, and the sister had normal endocrine data. The mother had normal clinical findings.

**Mutation analysis**

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining written informed consent, leukocyte genomic DNA samples of this patient, the sister, and the parents were amplified by PCR for the coding exons 1–5 and their flanking splice sites of GH1, and the PCR products were subjected to direct sequencing on a CEQ 8000 autoséquencer (Beckman Coulter, Fullerton, CA, USA). To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA cloning kit (Invitrogen), and wildtype (WT) and mutant (MT) alleles were sequenced separately. The primers used are shown in Table 3, and the primer positions are depicted in Fig. 2A.

**Expression analysis**

WT-GH1 from a normal subject and MT-GH1 from this patient were PCR-amplified with primers GH-1F and GH-5R (Fig. 2A; Table 3) using genomic DNA samples, and the PCR products were subcloned into pCR2.1 plasmid using the TOPO TA cloning kit. Then, GH1 gene fragments were cleaved from the plasmid DNA with EcoR1 and ligated to the EcoR1 site of an expression vector pRK5. The expression vectors (8 μg) were transiently transfected to SDR-P-1D5 cells obtained from the GH-deficient spontaneous dwarf rat (6).

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**Table 1** Endocrine studies at 7 years of age.

<table>
<thead>
<tr>
<th>Stimulus (dosage)</th>
<th>Patient</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Peak</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH (ng/ml)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(Clonidine 0.1 mg/m²)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(GHRH 1 μg/kg)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>LH (mIU/ml) 100</td>
<td>&lt;0.2</td>
<td>5.0</td>
</tr>
<tr>
<td>FSH (mIU/ml) 100</td>
<td>2.5</td>
<td>19.2</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>26.1</td>
<td>179</td>
</tr>
<tr>
<td>TSH (μU/ml)</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Free T4 (ng/dl)</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Free T3 (pg/ml)</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>17</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Reference values indicate the normal ranges in age-matched Japanese boys (26, 27). Blood sampling during the provocation tests: 0, 15, 30, 60, 90, and 120 mins. IGF-1, insulin-like growth factor-1; T₄, thyroxine; T₃, tri-iodothyronine; and GHRH, growth hormone releasing hormone.

*Measured with a Tosoh immunoassay kit.

*Measured with a Hitachi immunoassay kit.
using Gene Pulser Electroporation System (Bio-Rad Laboratories). The transfected cells were incubated for 48 h in a plate with a diameter of 10 cm, and GH in the culture media was measured with the Tosoh and the Daiichi kits. This analysis was performed for three independent experiments. Furthermore, western blotting was performed for the culture media using Rabbit polyclonal GH antibodies (Abs) and anti-Rabbit IgG conjugated with alkaline phosphatase (Promega).

Bioassay

A cell proliferation bioassay was performed for WT-GH and MT-GH, using mouse pro-B cell lymphoma cells that express GH receptor (Ba/F3–hGHR cells) (7). The detailed protocol has been reported previously (8). In brief, WT-GH and MT-GH were prepared in solutions at concentrations of 5, 10, and 20 ng/ml that were determined with the Daiichi kit. Each GH solution of 25 μl was added to 200 μl of Ba/F3–hGHR cell suspension (1×10^5 cells/ml), and the mixture was incubated for 48 h at 37°C. At the end of the incubation, a colorimetric end point was obtained by an eluted stain bioassay (9), and a bioactive response was determined with a kinetic microplate reader (Molecular Devices, Menlo Park, CA, USA) using optical densities at the test wavelength of 550 nm and a reference wavelength of 650 nm to correct for differential scattering. The experiments were performed in quadruplicate. Statistical significance was examined by Student’s t-test.

Protein modeling analysis

The protein conformation was analyzed by Esy-Pred3D (10).

Results

Mutation analysis

Two novel mutations were identified in the patient, a 2 bp deletion at exon 3 (c.280–281delCA) that is predicted to cause a frameshift at the 68th codon for glutamine and resultant termination at the 106th codon (p.Q68fsX106) and a missense mutation at exon 4 (c.426C>0) that is predicted to result in a substitution of aspartic acid with glutamic acid at the 116th codon of GH produced by a novel GH1 mutation (p.D116E; Fig. 2B). The father and the sister were heterozygous for the p.D116E, and the mother was heterozygous for the p.Q68fsX106 mutation (Fig. 2C).

Functional studies

Expression analysis showed that the p.D116E-GH in the three different culture media was immeasurable with the Tosoh kit but was clearly measurable with the Daiichi kit, and that the p.Q68fsX106-GH was...
undetectable by both of the kits (Table 4). Western blot analysis delineated a 22 kDa band for the p.D116E-GH as well as for the WT-GH (Fig. 3A), and a similar band intensity was identified when 3 ng of the p.D116E-GH measured with the Daiichi kit (13 μl of culture media of experiment 3 in Table 4) and 5 ng of WT-GH were utilized. For the p.Q68fsX106-GH, no band was identified for the same amount of culture media (13 μl). Bioassay revealed that the bioactivity was similar between the WT-GH and the p.D116E-GH (P = 0.069, 0.066, and 0.127 at GH concentrations of 5, 10, and 20 ng/ml based on the Daiichi kit respectively; Fig. 3B). Protein-modeling analysis indicated a normal conformation of the p.D116E-GH (Fig. 3C).

**Discussion**

This patient had apparently complete GH deficiency and two novel compound GH1 mutations (p.D116E and p.Q68fsX106). However, his growth pattern including normal birth length, the relatively mild postnatal growth failure, and the poor response to GH therapy is not typical for congenital GH deficiency (11, 12), and the serum GH and urine IGF-1 and IGFBP-3 values indicate a hidden GH activity. Consistent with this, the p.D116E-GH was immeasurable with the Tosoh kit but was measurable with the Daiichi kit, and had an apparently normal in vitro biological function. In this regard, the three kits employed in this study utilize two monoclonal Abs for GH, one against an epitope within the 22 kDa GH-specific residues (32–46 amino acids) and the other against an epitope specific to each kit. The Daiichi kit detects an epitope at the N-terminal region, while the epitope specifically recognized by the Tosoh and Daiichi kits is unknown. Thus, while the p.Q68fsX106 appears to be an amorphic mutation that is incapable of producing GH probably because of nonsense-mediated mRNA decay (13), it is likely that the p.D116E affects the GH epitope primarily recognized by the Tosoh kit but not by the Hitachi or the Daiichi kit, thereby producing a possible immunologically anomalous but biologically active GH. This notion would also explain why the basal GH values measured with the Tosoh kit were obviously lower than those measured with the Daiichi kit in the patient and the sister with p.D116E.

It remains to be determined, however, whether the p.D116E-GH has a normal biological function in vivo. Although the in vitro bioassay indicated an apparently normal function for the p.D116E-GH, it is known that

![Figure 2](https://www.eje-online.org)

**Table 4** GH values in the culture media (ng/ml).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>p.D116E</th>
<th>p.Q68fsX106</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;0.1</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.1</td>
<td>107</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.1</td>
<td>232</td>
</tr>
</tbody>
</table>

![Figure 3](https://www.eje-online.org)

![Figure 3A](https://www.eje-online.org)

![Figure 3B](https://www.eje-online.org)

![Figure 3C](https://www.eje-online.org)
the results obtained with artificially constructed cell lines do not necessarily reflect the in vivo biological effects of hGH MTs (8, 14). Indeed, the difference in the GH value between the two kits in the sister and the relationship between the GH value and the band intensity in the western blotting may imply that the p.D116E-GH was not measured precisely even with the Daiichi kit, so that a relatively large amount of the p.D116E-GH was probably utilized in the in vitro bioassay, compensating for a possible hypofunction of the p.D116E-GH. Furthermore, since the previously described p.D116A-GH harboring a missense mutation within the GH receptor-binding site 2 has a 5.7-fold lower affinity to the GH receptor than the WT-GH (15), this would argue for a functional importance of the D116 residue and implicate a similar functional alteration of the p.D116E-GH. In addition, although GH1 missense mutations reported to date are relatively rare (16), GH missense MTs, including those within or near the GH receptor binding site 2, frequently have a reduced or altered biological activity (2, 4, 17–21).

In this regard, comparison of clinical data between the patient with functional hemizygosity for the p.D116E and the mother with functional hemizygosity for the WT GH1 would suggest that the p.D116E-GH has a reduced, though not abolished, in vivo bioactivity (Table 2). In support of this, most individuals with heterozygous GH1 deletions have normal stature (22) as observed in the mother, while this patient had short stature. It may also be possible that the p.D116E-GH is less secreted from the pituitary into the circulation when compared with an intact GH protein, although the clinical findings of the father and the sister heterozygous for the p.D116E would argue against the possibility that the p.D116E-GH exerts an obvious dominant negative effect (Table 2). However, since short stature is a highly heterogeneous phenotype subject to multiple genetic and environmental factors (23, 24), some factors other than the GH1 mutations may be involved in the development of short stature in this patient. In addition, there may be an ascertainment bias, because GH-related studies are almost exclusively performed in individuals with short stature. Further studies will permit to clarify the in vivo biological function of the p.D116E-GH and its relevance to the development of short stature.

Such an immunologically anomalous and biologically active hormone has been reported previously. It is known that the common LH variant (V-LH) with two completely linked Trp8Arg and Ile15Thr substitutions in the LHβ-subunit is immunologically undetectable when a MAB recognizing an epitope present in the intact LH α/β dimer is utilized, but is measurable when two monoclonal Abs recognizing specific sites in the LHβ subunit are utilized (25). Notably, the V-LH appears to have somewhat weaker bioactivity than the WT-LH, and is often associated with the primary ovarian dysfunction in the Japanese population (25). Nevertheless, elevated LH values characteristic of primary ovarian dysfunction cannot be identified without applying the method using two monoclonal Abs, although FSH values are definitely increased. Thus, when a discrepancy is present between values of a specific hormone and other biochemical data or clinical findings, it is recommended to measure the specific hormone with a different kit, to avoid the misdiagnosis of hormone deficiency.

In summary, we identified an immunologically anomalous but considerably bioactive GH produced by p.D116E mutation. Indeed, such abnormalities along the GH/IGF-1 axis may also be identified by performing GH-related endocrine studies in children with short stature. The presence of such an apparently immeasurable but bioactive hormone, as well as a measurable but bioinactive hormone, should be kept in mind, to allow for an appropriate assessment of endocrine data.

Declaration of interest
The authors declare no conflict of interest.

Funding
This research was supported by Grants for Child Health and Development and Research on Children and Families from the Ministry of Health, Labor, and Welfare.

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Received 19 May 2009
Accepted 19 May 2009