GH mutant (R77C) in a pedigree presenting with the delay of growth and pubertal development: structural analysis of the mutant and evaluation of the biological activity

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Abstract

A heterozygous missense mutation in the *GH-1* gene converting codon 77 from arginine (R) to cysteine (C), which was previously reported to have some GH antagonistic effect, was identified in a Syrian family. The index patient, a boy, was referred for assessment of his short stature (-2.5 SDS) at the age of 6 years. His mother and grandfather were also carrying the same mutation, but did not differ in adult height from the other unaffected family members. Hormonal examination in all affected subjects revealed increased basal GH, low IGF-I concentrations, and subnormal IGF-I response in generation test leading to the diagnosis of partial GH insensitivity. However, GH receptor gene (*GHR*) sequencing demonstrated no abnormalities. As other family members carrying the GH-R77C form showed similar alterations at the hormonal level, but presented with normal final height, no GH therapy was given to the boy, but he was followed through his pubertal development which was delayed. At the age of 20 years he reached his final height, which was normal within his parental target height. Functional characterization of the GH-R77C, assessed through activation of Jak2/Stat5 pathway, revealed no differences in the bioactivity between wild-type-GH (wt-GH) and GH-R77C. Detailed structural analysis indicated that the structure of GH-R77C, in terms of disulfide bond formation, is almost identical to that of the wt-GH despite the introduced mutation (Cys77). Previous studies from our group demonstrated a reduced capability of GH-R77C to induce *GHR/GHBP* gene transcription rate when compared with wt-GH. Therefore, reduced *GHR/GHBP* expression might well be the possible cause for the partial GH insensitivity found in our patients. In addition, this group of patients deserve further attention because they could represent a distinct clinical entity underlining that an altered GH peptide may also have a direct impact on *GHR/GHBP* gene expression causing partial GH insensitivity. This might be responsible for the delay of growth and pubertal development. Finally, we clearly demonstrate that GH-R77C is not invariably associated with short stature, but that great care needs to be taken in ascribing growth failure to various heterozygous mutations affecting the GH-IGF axis and that careful functional studies are mandatory.

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Introduction

Growth disorders and short stature are a common clinical problem. Although most cases are sporadic and result from environmental insults or developmental abnormalities, there are estimates of the frequency of genetic causes, including growth hormone deficiency (GHD), GH insensitivity (Laron syndrome), and/or reduced bioactivity of GH (Kowarski syndrome) ranging from 1 in 4000 to 1 in 10 000 live births (1–4).

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GH plays a major role in postnatal growth. The gene is mainly expressed in the pituitary gland as a protein, which is characterized by an arrangement of four antiparallel α -helices separated by connecting loops (5). GH receptor (GHR) exists as an inactive dimer at the cell surface, and its activation involves a hormone-induced relative rotation of subunits, bringing the Jak2 kinases in close proximity (6, 7). The kinases phosphorylate the receptor, crossphosphorylate themselves, and start signal transduction by phosphorylating downstream signal transduction molecules, including signal transducer and activator of transcription Stat5 (8, 9). Activated Stat5 is translocated to the nucleus where it transactivates a series of GH-responsive genes (10).

Takahashi *et al.* described severe short stature in a boy due to a single mutation converting codon 77 from arginine to cysteine (GH-R77C) (11, 12). However, the patient's father was of normal stature even though he was carrying the same heterozygous mutation. Recombinant GH therapy used in this study was not sufficiently effective in stimulating somatic growth of this patient. Structural analysis of GH-R77C mutant expressed and purified from *Escherichia coli* performed by Chihara *et al.* (12) indicated the presence of a disulfide bond between cysteine 77 and 53 or 165, presumably causing a conformational change of GH-R77C. Deladoey *et al.* studied the effect of GH-R77C on GHR/GH-binding protein (GHBP) gene transcription and clearly showed, when compared with wild-type-GH (wt-GH), a reduced capability of GH-R77C in stimulation of GHR/GHBP gene transcription (13). Our group has recently reported the detailed functional characterization of GH-R77C when compared with wt-GH describing very similar affinity of wt-GH and GH-R77C for the GHR and GHBP and almost identically regulated secretion of both peptides in AtT-20 cells (14). These data were complemented by cell proliferation results showing that GH-R77C does not exert any effect different from that of the wt-GH in terms of cell viability and/or proliferation in AtT-20 cells.

Here, we report a pedigree over three generations of a Syrian family where all affected subjects carry the same heterozygous GH-R77C mutation. Our patient presented with short stature (-2.5 SDS) at the age of 6 years caused by partial GH insensitivity. All other affected family members carrying the GH-R77C allele reached a normal final height within the parental target height after a reported delay of growth and pubertal development in childhood. In addition, we focus on detailed structural analysis of this GH-R77C mutant form expressed and purified from mammalian cells, comparing it with wt-GH.

Subject and methods

Patient and family

The patient, of Syrian (Kurd) ancestry, immigrated to Switzerland at the age of 6 years. He presented at this time with short stature (-2.5 s.d. score for age and sex) (15). The pedigree of his non-consanguineous family is presented in Fig. 1A. Standard auxological assessment was performed (16). He had no evidence of an organic disease, psychological deprivation, or any eating disorder, and renal and hepatic functions were normal. A standard pharmacological stimulation test (insulin-induced hypoglycemia) was performed (17). Auxological and laboratory data are presented in Table 1. Genetic analysis revealed a normal GHR gene (all the exons, exon/intron boundaries were sequenced), but a familial R77C heterozygous mutation of the GH-1 gene was found. By analyzing the hereditary distribution of the GH-1 gene defect throughout the pedigree, an autosomal dominant 'growth' disorder could be suggested. However, as the other genetically

Figure 1 (A) A three-generation pedigree of the patient's family is shown, with affected individuals indicated in open symbols containing smaller solid symbols. The adult height s.d. score is reported. DNA sequencing revealed that the affected subjects presented heterozygosity for the R77C transition mutation of the GH-1 gene. The R77C mutations was screened for, but was not detected, in 100 alleles from unrelated normal individuals. (B) Isoelectric focusing of growth hormone in serum from the patient III:3. The serum fractions were pooled separately and assayed for growth hormone immunoreactivity. The pH gradient formed during isoelectric focusing is indicated. The peaks for wild-type and mutant growth hormone are indicated by the open and solid arrows respectively.

affected family members were of normal adult stature and, at follow-up of this affected boy, the height velocity remained in the normal range for his family, no GH treatment was initiated; but he was followed through puberty, which was delayed allowing the clinical diagnosis of delay of growth and pubertal development at that time. Finally, at the age of 20 years he reached the final height of 167.2 cm (-1.9 s.d.s) in good health and his school performance was normal. Informed written consent was obtained from the patient, subjects examined, and from the parents of the patients enrolled in this study.

Isoelectric focusing

Isoelectric focusing was performed as described previously (18). Serum samples (200–300 μ l) were electrofocused in

Table 1 Auxological and laboratory data.

Age (years)	Basal values					ITT	Following IGF-I generation test					
	Height (cm) (SDS)	Weight (kg) (SDS)	Height velocity (cm/year) (SDS)	Bone age (year)	GH (ng/ml)		IGF-I (ng/ml) (SDS) (control: range)	IGF-BP3 (mg/l) (SDS) (control: range)	GHBP (pmol/l) (control: range)	Peak GH (ng/ml)	IGF-I (ng/ml) (control: range*)	GHBP (pmol/l)
III:1 18	157.8 (−1.2)	51 (−0.9)			6	389 (0.1) (163–584)	5.2 (0.2) (3.1–7.9)	3790 (534–5785)				
III:3 6	107.1 (−2.5)	15.9 (−2.3)		5.0	28	45 (−2.2) (52–297)	2.4 (−0.5) (1.3–5.6)					
6.5	109.8 (−2.6)	16.4 (−2.6)	5.4 (−1.3)		30	51 (−2.1) (57–316)	2.6 (−0.4) (1.4–6.1)					
7	112.0 (−2.3)	17.8 (−2.8)	4.4 (−3.3)	5.75	36	39 (−2.5) (57–316)	2.2 (−0.9) (1.4–6.1)	129 (320–3820)	253	124* (205–758)	724*	
16	152.1 (−3.3)	32.2 (−3.8)	6.1 (+1.8)	12.5	39	189 (−3.1) (1.7–6.2)	2.4 (−1.0) (1.7–6.2)	132 (320–3820)		317 (267–935)	1215	
20	167.2 (−1.9)	58.2 (−0.9)	0.5		35	127 (−2.7) (193–731)	4.8 (−0.8) (3.2–8.7)					
II:4 36	168.2 (−1.5)	68.0 (0.5)			4	180 (0.0) (109–284)	4.6 (−0.2) (3.4–6.7)	2341 (556–3944)				
II:3 32	156.1 (−1.4)	57.8 (0.3)			32	108 (−2.1) (115–307)	3.6 (−1.9) (3.5–7.6)	461 (750–4868)	261	189*/322 (201–873)	803*/2140	
I:1 64	167.7 (−1.7)	71 (0.4)			26	37 (−2.3) (49–200)	1.5 (−1.7) 1.4–3.2	316 (560–2559)	242	96*/254 (186–714)	741*/1896	

Two IGF-I generation tests which involve daily injection of (a: rhGH of 0.1 IU/kg per day and 0.2 IU/kg for 4 and 3 days; b: rhGH of 0.2 IU/kg per day for 7 days) were performed. *Indicates values obtained with test a), so the IGF-I control values stated are standardized to test a) as well. The normative data of the IGF-I generation test stated were obtained by the analysis of $n=6$ (males and females) of various age groups; <10 years; 10-17 years; 18-40 years; >40 years.

a buffer containing 1% hydroxypropyl methylcellulose and 4% ampholine (pH gradient, 3.5–8.0) at 200 V for 12 h and then at 500 V for 12 h. The fractions were collected and assayed for immunoreactive GH. Pooled serum samples from seven normal subjects were used as the control.

Cell culture and treatment

Chinese hamster ovary (CHO-K1) cells were a gift from Prof. U Wiesmann (Inselspital, Bern, Switzerland) and were cultured in Ham's F12 medium (Biochrom AG Seromed, Berlin, Germany) supplemented with 10% fetal calf serum, 100 U/l penicillin/streptomycin (Biochrom AG), and 2 mM L-glutamine (Gibco-BRL, Life Technologies).

Human embryonic kidney 293 cells stably expressing human GHR (293GHR) were grown in Dublecco's modified Eagle's medium–nutrient mix F12 (Gibco), supplemented with 10% FCS, 100 U/l penicillin/streptomycin, 2 mM L-glutamine, and 400 µg/ml Geneticin G418 (Promega Corp).

Expression vectors

Wt-GH was cloned in pcDNA3.1 (–)/Neo (Invitrogen) vector as described (19), and GH mutant studied (GH-R77C) was generated using site-directed mutagenesis (14).

Production of GH peptides

In order to produce GH variants, wt-GH and GH-R77C, stable clones were generated in CHO-K1 cells by transfection with FuGene 6 (Roche Diagnostics AG).

The concentration of the GH produced by the CHO cells during 3 days in Ham's F12 media (without FCS) was measured by the DSL-GH ELISA Kit (DSL, Webster, TX, USA). To confirm that the mutation R77C does not affect the affinity of the antibody used in the DSL-ELISA, additionally two different GH assays were performed on two samples of CHO supernatant and the results were compared (20).

Hormonal measurement

The serum GH, insulin-like growth factor-I (IGF-I), IGF-binding protein-3 (IGF-BP3), and GHBP were measured as described previously (14).

Luciferase reporter gene assay of Stat5 activation

293GHR cells were used to assay Stat5 activation as described previously (21, 22). Briefly, cells were transfected with a Stat5-responsive luciferase reporter gene construct (23, 24) and treated with either recombinant human GH (rhGH), wt-GH or GH-R77C (all at 50 ng/ml concentrations) for 6 h. Luciferase expression was then measured with a luminometer (Mediators PhL, Aureon Biosystems, Vienna, Austria).

Recombinant human GHBP (rhGHBP) was a gift from Terri Smith, Genetech Inc. (San Francisco, CA, USA).

Structure of GH-R77C

In order to compare and study the chemical structure of GH-R77C, wt-GH was extracted and purified from somatope cell material (Anti-Doping Laboratory, Cologne, Germany), and GH-R77C from the cell culture supernatant as described. The extracts were subjected to solid-phase extraction using pre-packed Oasis HLB C-18 cartridges (Waters, Milford, MA, USA) and subsequently hydrolyzed using sequencing grade porcine trypsin (Promega). The samples were applied to a capillary liquid chromatograph (Agilent Technologies, Waldbronn, Germany) interfaced to an LTQ-Orbitrap mass analyzer (Thermo Electron, Bremen, Germany). After gradient elution, full scan mass spectrometry was performed using the LTQ-Orbitrap at a resolution of 30 000 and product ion spectra were recorded employing the LTQ linear ion trap only. In addition, GH-R77C was derivatized using acrylamide under non-reducing conditions and subsequently trypsinized, and additional alkylation studies were performed and the disulfide bonds were structurally analyzed.

Results

Clinical diagnosis and follow-up

As this family entered Switzerland as refugees, no exact data on birth weight and length are available. However, the parents reported no abnormalities, as was his psychomotor development. As the clinical phenotype as well as the laboratory finding of rather high basal GH concentration, high peak GH level after provocation test, and low basal IGF-I concentration in this 7-year-old boy suggested partial GH insensitivity (Table 1), the *GHR* gene was sequenced and an IGF-I generation test was performed (25). This detailed assessment, however, presented a normal sequence of the *GHR*-gene (exons, exon/intron boundaries) but only a subnormal IGF-I (124 ng/ml) response after an IGF-I generation test (daily injection of rhGH of 0.1 IU/kg per day and 0.2 IU/kg for 4 and 3 days respectively), and therefore the possibility of a functionally altered GH affecting GHR binding/signaling was hypothesized. The sequencing of the *GH-1* gene revealed a heterozygous R77C transition mutation, which was previously reported to have some GH antagonistic effect (19, 20). Therefore, the whole family was clinically studied (his unaffected father and sister and affected mother as well as grandfather) in more detail (Fig. 1A, Table 1). Importantly, isoelectric focusing revealed the presence of an abnormal GH peak in addition to a normal peak (Fig. 1B: III:3) in the boy. In this family, the presence of GH-R77C correlated with the delay in growth and pubertal development (mother menarche at the age of 18 years) but did not present a clear phenotype in terms of

short stature/final height. In contrast, at the hormonal level, a slight GH insensitivity was hypothesized and therefore further studies were performed in order to define in more detail the effect of GH-R77C at the cellular level. In addition, at the age of 16 years the IGF-I generation test was repeated in the boy. However, the dose of rhGH was increased to 0.2 IU/kg for 7 days. The IGF-I response was significantly increased from a subnormal value (189 ng/ml) to an accepted normal value (317 ng/ml; Table 1). Importantly, under these circumstances GHBP increased as well (Table 1). Additionally, in affected subjects (II:3; I:1) two IGF-I generations tests were performed (a: daily injection of rhGH of 0.1 IU/kg per day and 0.2 IU/kg for 4 and 3 days respectively; b: daily injection of rhGH of 0.2 IU/kg per day for 7 days), confirming the results obtained in III:3 (Table 1).

Functional and structural characterization of GH-R77C

To investigate in more detail the bioactivity of GH-R77C, 293GHR cells stably expressing GHR were used. Furthermore, a Stat5-responsive luciferase reporter gene assay system (21, 22) performed in these cells, which requires all stages of the Jak2/Stat5 signaling pathway to be functional, was used to assay and quantify signal transduction activity of GH-R77C.

As depicted in Fig. 2, the effects of rhGH, wt-GH, and GH-R77C (all at 50 ng/ml concentrations) on activation of the Jak2/Stat5 pathway were compared and found not to be significantly different. Of note is that the western blots performed with the supernatant from CHO cells, containing either wt-GH or GH-R77C, confirmed that the sizes of both GH variants were the same at 22 kDa (data not shown).

As the additional cysteine at position 77 may affect disulfide bond formation and, therefore, change the structural conformation, GH-R77C was expressed and purified from mammalian cells (CHO cells) and the structural analysis of the mutant was performed and

Figure 2 Jak2/Stat5 signaling capacity of rhGH, wt-GH, and GH-R77C. Results are expressed as a fold induction relative to the basic activity of non-stimulated cells and represent the means $\pm$ s.d. of three independent experiments. NS, non-stimulated.

compared with wt-GH. After the derivatization of the introduced cysteine residue (C*) using acrylamide, the LC-ESI-MS/MS spectrum (Fig. 3A) of the triply charged molecule ($M + 3H$)³⁺ at m/z 967 of the peptide resulting from trypsinization of GH-R77C mutation confirmed the presence of R77C mutation.

Extracted ion chromatograms obtained from GH-R77C (Fig. 3B) by LC-ESI-high resolution/high accuracy mass spectrometry and ion trap tandem mass spectrometry illustrated the presence of a triply charged molecule (the inset in Fig. 3B) at m/z 967.5444 (apex) with an average molecular mass of 2898.6 kDa (theor: 2898.4) and extracted ion traces of abundant product ions (y_6 – y_8) resulting from the target peptide after collision-induced dissociation. Extracted ion chromatograms obtained from wt-GH (Fig. 3C) illustrated the absence of a triply charged molecule at m/z 967.5444 (retention time 29.2 min) and, thus, the lack of the mutant peptide in Fig. 3B. The extracted ion traces of the target peptide after collision-induced dissociation also confirm no R/C substitution in wt-GH.

Moreover, GH-R77C was derivatized using acrylamide under non-reducing conditions and subsequently trypsinized. The peptide comprising the modified position (Cys77) was found to be alkylated, indicating that the free sulfhydryl residue is not involved in other intramolecular disulfide bonds, although it has been reported that the alkylation of cysteines involved in disulfide bonds may occur to a very minor extent (26).

Discussion

A heterozygous missense mutation in the *GH-1* gene converting codon 77 from arginine (R) to cysteine (C) was identified in our patient. Apart from the affected patient, his mother and grandfather were also carrying the same mutation. However, they did not differ in terms of phenotype (final height) but in terms of laboratory values (GH insensitivity) from the other unaffected family members (Fig. 1A and Table 1). Isoelectric focusing confirmed the presence of both wt-GH and GH-R77C in the patient's serum (Fig. 1B). Our recently published data demonstrated very similar affinity of wt-GH and GH-R77C for the GHR and GHBP (14). Furthermore, comparing the association of either wt-GH or GH-R77C with different compartments within the secretory pathway, namely the endoplasmic reticulum, Golgi, and secretory vesicles clearly showed that both GH peptides pass through the very complex secretory machinery in an almost identical manner. Therefore, our clinical and experimental findings do not support the previously reported hypothesis that the presence of GH-R77C in the circulation correlates with the severe short stature (11, 12).

Further functional characterization of this mutant was pursued by bioassay. The efficiency of this assay focusing on the GH Jak2/Stat5 signaling pathway was assessed first by stimulation of the 293GHR cells with increasing

supporting the notion that the *GHR/GHBP* gene transcription was directly stimulated and depended, at least partially, on some pre-existing factors.

On the clinical and protein basis, GHBP was significantly decreased ($P < 0.001$) in the affected subjects compared with the normal controls. Further, as the GHR was sequenced and found to be normal, the reduced GHR/GHBP expression caused by the GH-R77C mutant might well be responsible for causing GH insensitivity. Additional functional studies focusing on the GHR are supporting this notion (14). However, this possible direct impact of GH-R77C at the GHR/GHBP level, also still unclear in its mechanism, is underlined by the normalization of IGF-I response when rhGH dose was increased from 0.1 IU/kg per day to 0.2 IU/kg during the period of 7 days as described previously (14).

It is well known that under normal physiological conditions, GHBP is bound to almost half of GH in human plasma (31, 32), acting as a reservoir of GH and thereby prolonging its half-life. GHBP is generated by proteolytic cleavage of GHR at the cell surface and modulation of GHR turnover/internalization might impact the level of GHR available for proteolysis. Therefore, one could hypothesize that the reduced *GHR/GHBP* gene transcription rate evoked by GH-R77C compared with the *wt*-GH may lead to a reduced amount of GHR, which are going to be produced and exported at the cell surface. Consequently, that might affect the level of GHBP generated by proteolytic cleavage of GHR and might explain the low levels of GHBP found in our patient carrying GH-R77C mutation.

In conclusion, we report further details on a heterozygous missense mutation, R77C in the GH molecule in a patient with growth retardation, and delayed pubertal development. No differences between *wt*-GH and GH-R77C were found by functional characterization of the GH-R77C through GHR binding, activation of Jak2/Stat5 pathway, and additional secretion studies together with cell proliferation when stably GHR transfected cells (293GHR) were used (14). In addition, the structural characterization of GH-R77C mutant reported in this study revealed no structural differences when compared with *wt*-GH. On the other hand, reduced capability of GH-R77C to directly induce *GHR/GHBP* gene transcription rate could indirectly affect the levels of GHBP in the circulation of our patient. In addition, this group of patients deserve further attention because they could represent a distinct clinical entity underlining that an altered GH peptide may cause partial GH insensitivity through direct impact on *GHR/GHBP* gene expression, leading to the delay of growth and pubertal development. Finally, GH-R77C is not invariably associated with short stature, although the serum IGF-I levels are low, the GH is elevated, and the GHBP levels are somewhat low, consistent with some degree of GH insensitivity, which is presumably compensated for by excess of GH production. Whether this is due to GHR transcription defects remains unclear.

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