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## **GPR101 Mutations are not a frequent cause of Congenital Isolated Growth Hormone Deficiency**

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### **Abstract**

**Objective:** Patients with Xq26.3 microduplication present early-childhood gigantism due to marked growth hormone (GH) hypersecretion from mixed GH-PRL adenomas and hyperplasia. The microduplication includes *GPR101* which is upregulated in patients' tumor tissue. The *GPR101* gene codes for an orphan G protein coupled receptor that is normally highly expressed in the hypothalamus. Our aim was to determine whether *GPR101* loss of function mutations or deletions could be involved in patients with congenital isolated GH deficiency (GHD).

**Design and methods:** Taking advantage of the cohort of patients from the GENHYPOPIT network, we studied 41 patients with unexplained isolated GHD. All patients had Sanger sequencing of the *GPR101* gene and array comparative genome hybridization (aCGH) to look for deletions. Functional studies (cell culture with GH secretion measurements, cAMP response) were performed.

**Results:** One novel *GPR101* variant, c.589 G>T (p.V197L), was seen in the heterozygous state in a patient with isolated GHD. *In silico* analysis suggested that this variant could be deleterious.

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Functional studies did not show any significant difference in comparison with wild type for GH secretion and cAMP response. No truncating, frameshift, or small insertion-deletion (indel) *GPR101* mutations were seen in the 41 patients. No deletion or other copy number variation at chromosome Xq26.3 was found on aCGH.

**Conclusion:** We found a novel *GPR101* variant of unknown significance, in a patient with isolated GH deficiency. Our study did not identify *GPR101* abnormalities as a frequent cause of GH deficiency.

### Keywords

hypopituitarism; gigantism; X-LAG syndrome; growth hormone deficiency; congenital hypopituitarism

### Introduction

Isolated GH deficiency (IGHD) is the most common pituitary hormone deficiency. The incidence of congenital IGHD is estimated to be 1/4000–10000, and as up to 30% are familial, this suggests that genetic causes are likely to be frequent [1, 2]. Genetic forms of IGHD are classified into 4 different types, based on the mode of inheritance (autosomal dominant or recessive) and the severity of the phenotypes [2, 3]. Mutations of *GHI*, or the genes encoding the GHRH receptor or ghrelin are the most frequent etiologies of genetic forms of IGHD [1]. Of note, GHD can also be the initial clinical manifestation of a wider phenotype of combined pituitary hormone deficiencies; in those cases, mutations of several genes encoding transcription factors (*POU1F1*, *PROPI*, *LHX3*, *LHX4*) have been described [4, 5]. Altogether, however, the genetic etiologies of <20% of GHD cases are known currently and the cause of the majority of supposedly genetic GHD therefore remains undetermined [1]. Recently, we reported a microduplication on chromosome Xq26.3 in patients with early childhood-onset gigantism [6]. Patients with this condition -termed X-linked acrogigantism (X-LAG) syndrome- present with mixed GH-PRL adenomas with or without hyperplasia that leads to marked GH and IGF-1 hypersecretion [7]. This is usually accompanied by hyperprolactinemia of variable severity. The clinical profile is one of generally normal-sized infants born following unremarkable pregnancies in which dramatically accelerated height and weight gain occurs at a median age of 12 months [6, 7]. The usual presentation is sporadic, but Xq26.3 microduplications also have been found in two familial isolated pituitary adenoma (FIPA) kindreds [6]. The microduplicated region contains four protein-coding genes, among which only *GPR101* is upregulated in tumor tissue from X-LAG patients. The *GPR101* gene codes for an orphan G protein coupled receptor (GPCR) that is expressed in the hypothalamus [6]. Overexpression studies of a putative activating *GPR101* variant in GH3 cells led to increased GH secretion; this *GPR101* sequence variant also occurs in a subgroup of patients with acromegaly, usually in tumor tissue [6, 8].

Given the phenotypes involving *GPR101* in X-LAG syndrome and acromegaly, we wanted to study whether deletions or loss-of-function mutations of *GPR101* could explain cases of congenital isolated GHD of unknown etiology. Taking advantage of the international GENHYPOPIT network [9], aimed at identifying new aetiologies of congenital

hypopituitarism, we looked for mutations of *GPR101* and deletions on chromosome Xq26.3 in a group of patients with congenital isolated GHD.

## Methods

### Subjects

The GENHYPOPIT network was launched as a multicenter study involving both French and international pediatric and adult endocrinology centers. The precise characteristics of this network have been previously reported[9]. After written informed consent was given, blood samples were collected from patients and, whenever possible, first-degree relatives. Informed written consent was obtained from the parents, caretakers or guardians on behalf of the minor/children enrolled in the study. The study was approved by the Ethics committees of the University of Aix-Marseille II (France) and the Centre Hospitalier Universitaire de Liege (Belgium).

### Endocrine and imaging studies

Hormonal studies and intracranial imaging were performed in all patients at each referring medical center. Structural malformations on magnetic resonance imaging (MRI) were systematically sought and recorded. Patients with a known postnatal cause of acquired hypopituitarism were excluded. Complete GH deficiency was defined by subnormal response (<10 mUI/L) to at least 1 provocative test: insulin test (0.05 U/kg), GHRH infusion test or arginine-insulin test. Partial GH deficiency was defined by a response between 10 and 20 mUI/L to the same provocative tests. In adults, gonadotrope deficiency was defined by low plasma testosterone with non-elevated gonadotropin levels in men, amenorrhea with low plasma estradiol and low or normal gonadotropins in non-menopausal women, and a lack of increased gonadotropins in postmenopausal women. The diagnosis of thyrotrope deficiency was based on low free T4 level with normal or diminished TSH. Corticotrope deficiency was defined by subnormal response of cortisol to an insulin tolerance test (peak < 550 nmol/liter), associated with basal ACTH levels less than 5 ng/ml at 08:00 h.

### GPR101 sequencing

All patients underwent Sanger sequencing of the *GPR101* gene using peripheral blood leukocyte DNA. The sequences of the primers for *GPR101* are given as supplemental data. Samples underwent PCR (conditions available on request) and were sequenced on an ABI 3130XL (Applied Biosystems). Genetic sequences and variants were called against the human *GPR101* reference sequence (NM\_054021.1, hg19 NCBI build 37). *GHI* sequencing was performed in all patients, and no mutation was identified. *LHX4*, *LHX3* and *HESX1* sequencing was performed in patients with extra-pituitary anomaly, and no mutation was identified.

### Array comparative genome hybridization (aCGH)

Patients' peripheral blood leukocytes DNA underwent aCGH as previously described [6]. Briefly, aCGH analysis used an 8×60K (G4827A-031746; Agilent Technologies, Santa Clara, CA, USA). The arrays were scanned with a G2565CA microarray scanner (Agilent Technologies, Santa Clara, CA, USA) and the images were extracted and analyzed with

CytoGenomics software v2.0 (Agilent Technologies, Santa Clara, CA, USA). An ADM-2 algorithm (cut-off 6.0), followed by a filter to select regions with three or more adjacent probes and a minimum average  $\log_2$  ratio  $\pm 0.25$ , was used to detect copy number changes. The quality of each experiment (log ratio spread) was assessed with CytoGenomics software v2.0. Genomic positions were based on the UCSC February 2009 human reference sequence (hg19 NCBI build 37 reference sequence assembly). Filtering of copy number changes was carried out using the BENCHlab CNV software (Cartagenia, Leuven, Belgium).

### In silico analysis

Previously reported *GPR101* variants were identified via Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: <http://exac.broadinstitute.org>) [date accessed (September, 2015)]. Predict SNP (<http://loschmidt.chemi.muni.cz/predictsnp>) was used to appraise the potential pathogenicity of novel allelic variants. Alignment of amino acid sequences for *homo sapiens*, *mus musculus*, *rattus norvegicus*, *bos taurus*, *chimpanzee*, *equus caballus* and *canis familiaris* was performed with the Clustal omega program accessible via UniProtKB (<http://www.uniprot.org>).

### In vitro characterization studies

The effect of a novel p.V197L *GPR101* variant was studied in vitro in the rat somatomammotroph GH3 cell line [6]. For all studies, effects were calculated as the average of two experiments performed in triplicate. Statistical comparisons were performed using a one-way ANOVA as compared to mock/vehicle. Briefly, GH3 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Gemini Bio-Products), and 1% antibiotic-antimycotic (Life Technologies) in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. Cells were seeded in 12-well plates at a density of  $2 \times 10^5$  cells/well. After 24 h, cells were starved with DMEM without serum for 16 h and then transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol, using Opti-MEM I Reduced Serum Medium (Life Technologies) and 1  $\mu$ g of each vector, alone or in combination. The human wild-type *GPR101* (NM\_054021.1) coding sequence cloned into the pCMV-XL5 vector was purchased from Origene (SC120214). The p.V197L variant was introduced into the human *GPR101*wt template using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The empty pCMV-XL5 vector was used as a negative control. 24 h after transfection supernatants were collected and GH secretion was measured using the Rat/Mouse Growth Hormone ELISA kit (EZRMGH-45K, EMD Millipore). GH secretion values (expressed in ng/ml) were normalized on protein content. For cAMP pathway activation studies, GH3 cells were seeded in 12-well plates at a density of  $2 \times 10^5$  cells/well. After 24 h, cells were starved with DMEM without serum for 16 h and then transfected with Lipofectamine 2000 (Life Technologies), using Opti-MEM I Reduced Serum Medium (31985-070, Life Technologies), 1  $\mu$ g of each *GPR101* vector (human wild-type *GPR101* and p.V197L *GPR101*), 800 ng of pGL4.29[luc2P/CRE/Hygro] vector containing a cAMP response element (CRE) that drives the transcription of the luciferase reporter gene (Promega), and 40 ng of the Renilla vector (pRL-SV40, Promega). The empty pCMV-XL5 vector was used as negative control. 24h after transfection, a subset of cells was treated with 10  $\mu$ M forskolin (Sigma-Aldrich) for 1 h and then lysed. Firefly and Renilla luciferase

activities were measured consecutively in the same sample using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. Ratios of Firefly vs. Renilla luminescence signals, serving as a measure for reporter activity normalized for transfection efficiency, were measured using a FLUOstar Omega microplate reader (BMG LABTECH).

## Results

### Cohort characteristics

Forty-one patients, 27 males and 14 females, with a median age of 12.9 years (min, 2.4 ; max, 44) at last follow-up, were included in this study. Thirty-seven patients (90.2%) presented with complete GH deficiency, whereas the remaining 4 had partial GH deficiency. The median age at diagnosis of GH deficiency was 5 years (min, neonatal ; max, 44). Only one patient was diagnosed as an adult. Fourteen patients (34.1%) had at least one other family member with GH deficiency.

On pituitary MRI, 17 patients (41.4%) had pituitary hypoplasia whereas one (2.4%) had enlargement suggestive of pituitary hyperplasia. Fourteen patients (34.1%) had stalk interruption syndrome, with an ectopic posterior lobe and a thin or non-visualized pituitary stalk. Five patients also had other disorders such as eye anomalies such as strabismus (n=1) or cataract (n=1), diabetes insipidus (n=1), Chiari syndrome (n=1) or cleft palate (n=1).

### Genetic analyses

One previously undescribed variant c.589 G>T (p.V197L) was seen in the heterozygous state in one patient and was further studied *in vitro* (see below). This caucasian female patient was aged 5 when complete GH deficiency was diagnosed (GH <0.3 ng/ml after one provocative stimulation test; IGF1 57 ng/ml (Normal value, 99–254)). She had no other pituitary hormone deficiency. Pituitary MRI was normal. Sequencing of *GHI* did not reveal any anomaly. No clinical or genomic data was available for her parents.

No deletions or other copy number variants were found at chromosome Xq26.3 on aCGH. On sequencing, no truncating, frameshift, or small insertion-deletion (indel) *GPR101* mutations were seen in the 43 analyzed patients. A number of previously described common missense variants in *GPR101* were seen: c.370G>T (p.V124L), c.712G>A (p.V238I), c.878C>T (p.T293I), and c.1127T>C (p.L376P). A c.166T>C synonymous variant was observed in homozygosis.

### In silico analysis

The p.V197L variant was not identified as a known polymorphism in ExAC. The Predict SNP platform identified p.V197L as a deleterious variant with PolyPhen2, MAPP and SNAP algorithms. Alignment of sequences identified the valine in position 197 as a highly conserved amino-acid in all species studied (Figure 1).

### In vitro characterization

The effect of the c.589 G>T (p.V197L) change was studied further in GH3 cells. As shown in Figure 1, this variant was associated with a not statistically significant decrease in GH secretion as compared with wild-type GPR101. As compared with mock transfection, both wild-type GPR101 and the p.V197L change led to a statistically significant increase in CRE activation; while activation was less with p.V197L than wild-type GPR101, the difference was not statistically significant (both with and without forskolin; Figure 2).

### Discussion and conclusions

Microduplications on chromosome Xq26.3 including GPR101 lead to X-LAG syndrome, a dramatic pediatric-onset gigantism disorder associated with mixed GH-PRL secreting pituitary macroadenomas/hyperplasia that usually occur in the first year of life [6]. X-LAG syndrome represents about 10% of pituitary gigantism cases and differs significantly from other genetic (e.g. *AIP*-related) and unexplained forms of pituitary gigantism [10, 11]. We also reported a *GPR101* change (p.E308D) at an apparently increased frequency in patients with acromegaly [6, 8]. In a relevant *in vitro* model, the rat somatotrope GH3 cell, p.E308D led to increases in GH secretion as compared with wild-type GPR101. These data provide evidence for the involvement of GPR101 in the somatotrope axis. However, the precise roles of GPR101 in the pituitary and the brain remain to be studied, even if studies in endometrial models suggest that GnRH(1–5) may act via GPR101 to modulate epidermal growth factor receptor activity [12, 13]. Given the phenotype of marked GH hypersecretion associated with duplications involving *GPR101*, we were interested in studying the opposite hormonal condition, namely patients with GHD. Also as we previously reported, analysis of public databases have noted cases with short stature that had genomic deletions including chromosome Xq26.3; however most of those cases had large deletions spanning many more genes beyond this specific region<sup>6</sup>. In fact we show in the current study that no deletions of *GPR101* and the surrounding genomic region were seen on aCGH. *GPR101* sequencing showed that truncating or other clearly deleterious mutations were not found in our cohort. Taken together, these results suggest that inactivation or deletion of *GPR101* is not a frequent cause of unexplained congenital GHD.

In the current study we noted a previously undescribed missense variant in *GPR101*, p.V197L, present in heterozygosis in one patient. When expressed in GH3 cells, this variant did not lead to a statistically significant change in GH secretion or cAMP activity in comparison with wild-type *GPR101*. There are nevertheless several strands of indirect evidence for a deleterious effect of this variant: three prediction algorithms suggested that this variant was pathogenic and the amino acid at position 197 is highly conserved across species. It is the first amino acid of the fifth transmembrane helix of GPR101 suggesting that changing this amino-acid could lead to conformational changes of the protein and altered function, as previously shown for mutations of transcription factors involved in corticotroph deficiency, for instance [14]. Finally, this variant has never been described in online databases such as ExAC. These prediction models, however are difficult to interpret if family segregation data are missing; moreover, some studies indicated that 30% of disease-causing genetic variations cited in the literature based on prediction models were actually

polymorphisms or misinterpreted variants [15]. Family segregation would have given major information on the pathogenicity of this variant ; these data were however not accessible. It is thus highly uncertain whether this *GPR101* variant could be responsible for the phenotype of our patients. While highly expressed in the hypothalamus and striatum, the physiological role of GPR101 remains largely obscure and the manner by which it may influence the somatotrope axis is unknown [16–18]. Moreover, it is well recognized that functional studies performed in heterologous cells, an artificial *in vitro* system, at pharmacological doses that do not necessarily reflect biological processes, may not provide a complete view of the activity of genetic variants (e.g. in functional studies performed with variants of LIM transcription factors [19, 20]). On balance, *GPR101* variants in patients with growth disorders should be re-analyzed as more evidence of the function of GPR101 becomes apparent.

The rarity of identified *GPR101* anomalies in our cohort may be due to a number of reasons. We may not have selected an ideal group to study, as we chose a group that was generally representative of the GHD population, including one third of familial cases. Moreover, our cohort was mainly including males, whereas X-LAG was mainly observed in females. It may be that by focusing on more severe GHD a greater number of pathological variants could be identified. Concentrating the screening activities on groups with pituitary hypoplasia and other brain malformations could provide useful information on a potential role for this receptor in GHD. The fact that the patient with the p.V197L variant had pure GH deficiency is evidence for considering that further studies should examine a larger number of patients with a pure isolated GHD phenotype. Finally, some of our patients were presenting with extra-pituitary anomaly and isolated GH deficiency: even if the phenotype of patients with GPR101 mutations in X-LAG syndrome was pure pituitary hypersecretion, we decided to screen them as the precise roles of GPR101 during fetal development remains unknown, and the phenotype of GPR101 deficiency might have been more complex than anticipated.

To conclude, this study is the first to analyze the potential involvement of *GPR101* deletions and single nucleotide variants in congenital GHD. We did not find an unequivocal link between *GPR101* loss of function and GHD. Of note, a rare novel p.V197L *GPR101* variant was found in a patient with isolated GHD: its clinical significance remains unknown. This suggests that with growing understanding of the role of GPR101 in somatotrope axis function, the contribution of functional *GPR101* variants to GHD and pituitary development should be further explored in a larger number of patients.

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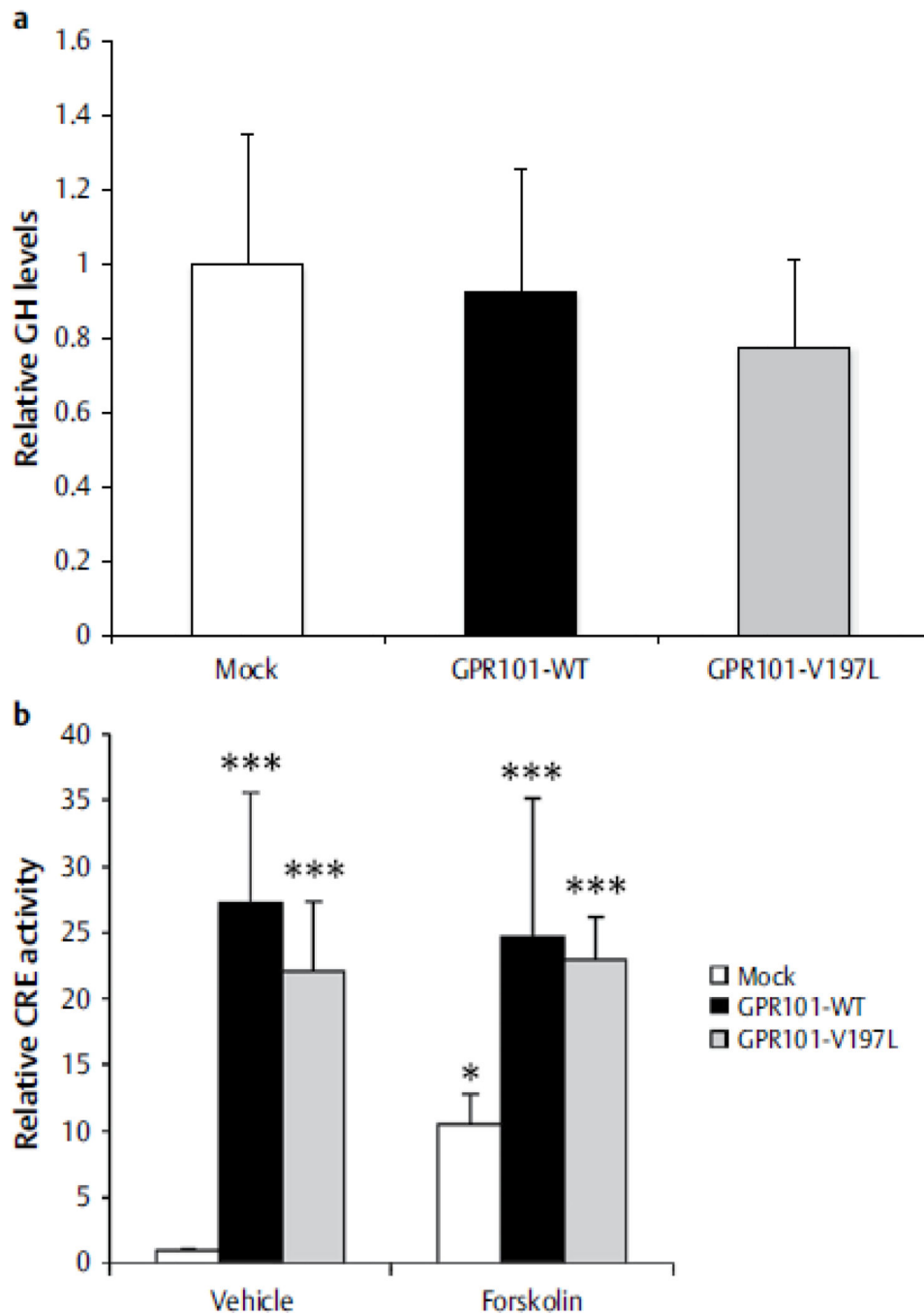


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D4ABK4_RAT	181	FCSMIWGDSPAYTVVSVV	SFLVIPLGVMIACYSVV	FGAARRQALLYKAKSHRFQVRVKD	240
GP101_MOUSE	181	FCSMIWGASPAYTVVSVV	SFLVIPLGVMIACYSVV	FGAARRQALLYKAKSHRLEVRVED	240
GP101_HUMAN	181	LCSMIWGASPSYTI	VSFIVIVPLIVMIACYSVV	FCAARRQHALLYNVKRHSLEVRVKD	240
F1MDI5_BOVIN	181	LCSMIWGASPSYTI	VSFIVIVPLIVMIACYSVV	FCAARRQHALLYNVKSHSLEVRKAD	240
H2QZ65_PANTR	181	LCSMIWGASPSYTI	VSFIVIVPLIVMIACYSVV	FCAARRQHALLYNVKRHSLEVRVKD	240
J9NSD2_CANFA	181	LCSMIWGASPSYTI	VSFIIIPLVVMIACYSVV	FCAARRQHALLYNVKSHSLEVRKAD	240
F6Z943_HORSE	163	LCSMIWGASPSYTI	VSFIIIPLVVMIACYSVV	FCAARRQHALLYNVKSHSLEVRKAD	222

**Figure 1.**

Sequence alignment of *GPR101* in several species (extracted from UniProtKB). RAT, rattus norvegicus; MOUSE, mus musculus; BOVIN, bos taurus; PANTR, chimpanzee; CANFA, canis familiaris; HORSE, equus caballus. Black box, Valine in position 197.



**Figure 2.**

Panel A. A numerical decrease in GH secretion was seen in GH3 cells expressing the GPR101 p.V197L variant as compared with wild-type GPR101. The difference was not statistically significant. Panel B. There was a significant increase in CRE activity with the p.V197L GPR101 variant relative to mock transfection, however this difference was not statistically significant as compared with wild type GPR101. Results are expressed as mean  $\pm$  SD of 2 experiments done in triplicate, one-way ANOVA compared to mock/vehicle. \*