Screening for GPR101 defects in pediatric pituitary corticotropinomas

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Abstract

Cushing’s disease (CD) in children is caused by adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas. Germline or somatic mutations in genes such as MEN1, CDKIs, AIP, and USP8 have been identified in pediatric CD, but the genetic defects in a significant percentage of cases are still unknown. In this study, we investigated the orphan G-protein-coupled receptor GPR101, a gene known to be involved in somatotropinomas, for its possible involvement in corticotropinomas. We performed GPR101 sequencing, expression analyses by RT-qPCR and immunostaining, and functional studies (cell proliferation, pituitary hormone secretion, and cAMP measurement) in a series of patients with sporadic CD secondary to ACTH-secreting adenomas in whom we extracted DNA from peripheral blood and pituitary tumor samples (n = 36). No increased GPR101 expression was observed in tumors compared with normal pituitary (NP) tissues, nor did we find a correlation between GPR101 and ACTH expression levels. Sequence analysis revealed a very rare germline heterozygous GPR101 variant (p.G31S) in one patient with CD. Overexpression of the p.G31S variant did not lead to increased growth and proliferation, although modest effects on cAMP signaling were observed. GPR101 is not overexpressed in ACTH-secreting tumors compared with NPs. In conclusion, rare germline GPR101 variant was found in one patient with CD, but in vitro studies did not support a consistent pathogenic effect. GPR101 is unlikely to be involved in the pathogenesis of CD.

Key Words
- GPR101
- Cushing’s disease
- ACTH-secreting adenomas

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Introduction

Cushing's disease (CD) affects approximately 70% of patients with endogenous Cushing's syndrome (Tritos & Biller 2014). CD is caused by adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas (corticotropinomas) (Newell-Price 2009). Although germline mutations in genes such as MEN1, CDKIs, and AIP, and somatic mutations in USP8 have been identified in patients with CD, for many corticotropinomas the molecular pathways involved in their pathogenesis remain unknown (Stratakis et al. 2010, Reincke et al. 2015).

We have recently found that the GPR101 gene is involved in the pathogenesis of pituitary growth hormone (GH)-secreting adenomas (Trivellin et al. 2014, Beckers et al. 2015). GPR101 encodes an orphan G protein-coupled receptor that is highly expressed in the hypothalamus, where it may play a role in the hypothalamic control of energy homeostasis and pituitary hormone secretion (Lee et al. 2001, Bates et al. 2006, Nilaweera et al. 2007, 2008, Regard et al. 2008, Trivellin et al. 2014). GPR101 is also highly expressed in the GH-secreting pituitary tumors of patients with X-linked acrogigantism caused by GPR101 duplication, whereas it is expressed at lower levels in normal pituitary (NP) tissue and in non-GPR101 duplicated somatomatotropinomas (Trivellin et al. 2014). The mechanism(s) by which GPR101 might increase pituitary hormones secretion remain unclear at present, although the possible involvement of GHRH has been shown (Beckers et al. 2015, Daly et al. 2016). Previous studies from our and other groups support the idea that GPR101 can strongly activate the cAMP pathway (Trivellin et al. 2014, Bates et al. 2006), whose mitogenic effects in pituitary cells are well established (Peverelli et al. 2014).

In this study, we investigated the possible involvement of GPR101 in CD by performing GPR101 sequencing, expression analyses, and functional studies in a series of sporadic ACTH-secreting adenomas. We further report a case associated with a very rare missense variant (p.G31S).

Subjects and methods

Subjects

A series of 36 patients with CD corroborated by biochemical testing and pathology positive for ACTH staining in pituitary adenomas were analyzed. These patients were selected because of the availability of tumor and peripheral DNA samples. Testing for germline mutations/deletions in genes associated with pituitary adenomas (AIP, MEN1) was performed and no defects were observed. Patients harboring a USP8 mutation at the tumor DNA level were also excluded from this analysis (data not shown). The Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Review Board approved this study, and informed consent was obtained from all the patients.

Sequencing analysis

DNA was extracted from peripheral blood and pituitary tumor samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocols. The whole coding region of GPR101 was PCR amplified and directly sequenced. The following primers were used: GPR101_1AF: ACTGAGCCCTGCAACCTGTCT; GPR101_1AR: TCCACTGACACCAGACAAT; GPR101_1BF: TTAGCCTCACCACACTGTTC; GPR101_1BR: CTTCCTCCTGGCCCTTACG; GPR101_1CF: CAGCATGAAGGTGAGGTCAA; GPR101_1CR: CCCAGGGATAGCACATAGGA; GPR101_1DF: GTGCTACCAGTGCAAAGCTG; GPR101_1DR: GPR101_1DF: GTGCTACCAGTGCAAAGCTG; GPR101_1DR: TGAATTGTGGG TCCATTGAA. DNA sequencing was performed using the BigDye 3.1 termination chemistry (Applied Biosystems) on a Genetic Sequencer ABI 3500XL apparatus (Applied Biosystems). Sequences were visualized and aligned to the corresponding wild-type (WT) reference sequence using SeqMan Pro software (DNASTAR, Madison, Wisconsin, USA). All variants have been annotated according to Human Genome Variation Society recommendations (www.hgvs.org/mutnomen). The NM_054021.1 reference sequence was used to annotate GPR101 variants.

Figure 1

DNA sequence chromatograms showing the position of two GPR101 variants found in our patients. The homozygous p.T293I variant is shown on the left side, whereas the heterozygous p.G31S variant on the right side. The location of each nucleotide change is indicated by a red arrow. Below each chromatogram, the WT nucleotide sequence and the corresponding codon numbers are reported.
In silico analysis

All GPR101 variants were studied in silico, and the allele frequencies observed in our patient population were compared against public databases, including the 1000 Genomes Project (Auton et al. 2015); the Exome Variant Server, NHLBI GO Exome Sequencing Project, Seattle, Washington, USA (http://evs.gs.washington.edu/EVS/) (accessed February 2016); and the Exome Aggregation Consortium, Cambridge, MA, USA (http://exac.broadinstitute.org) (accessed February 2016). In silico predictions were performed with PON-P2 (Niroula et al. 2015) and Alamut version 2.3 (Interactive Biosoftware, Rouen, France) software packages.

Tissue collection and expression studies

Pituitary tumor tissue was collected at surgery. Whenever possible, tissue slices were snap-frozen in dry ice; the remainder was fixed in formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin–eosin and reticulin for light microscopy. The avidin–biotin peroxidase complex technique was used to stain for ACTH using an anti-ACTH rabbit polyclonal antibody (Dako; catalog no. A0571) at a working dilution of 1:1000. Some samples were also stained in immunofluorescence for GPR101 (rabbit anti-GPR101, dilution 1:500; SAB4503289, Sigma-Aldrich) and ACTH (rabbit anti-ACTH, dilution 1:400, Dr A F Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA, USA). Immunofluorescence staining for GPR101 was performed using a Tyramide signal amplification kit (T20922, Invitrogen) with a horseradish peroxidase-goat anti-rabbit IgG and Alexa fluor 488 tyramide, according to the manufacturer’s instructions. The sections were mounted in Mowiol and visualized under a Leica AF6000 microscope (Leica) at 63× magnification with fixed time of exposure for all samples. The same linear adjustments for brightness, contrast, and color balance have been applied with Adobe Photoshop CS6 to each entire image.

GPR101 and POMC mRNA expression levels were measured by RT-qPCR with TaqMan assay IDs Hs00369662_s1 and Hs01596743_m1, respectively (Applied Biosystems) and normalized on GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression (TaqMan assay ID Hs99999905_m1, Applied Biosystems). TaqMan assays were performed according to the manufacturer’s protocol (Applied Biosystems). Briefly, experiments were prepared...
in 96-microwell plates and consisted of 20µL reactions containing 20ng of cDNA, 10µL TaqMan Gene Expression Master Mix (Applied Biosystems, catalog number 4369016), and 1µL each of GPR101 and GAPDH assay mixes. All reactions were performed in triplicate and were run on a ViiA 7 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were 95°C, 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Relative gene expression data were analyzed by ΔΔCt method. Data analysis was performed using ViiA 7 software (Applied Biosystems).

Plasmids

The human GPR101 WT (NM_054021.1) coding sequence cloned into the pCMV-XL5 vector was purchased from Origene (SC120214, Origene, Rockville, MD, USA). The p.G31S variant was introduced into the human GPR101 WT template using the QuikChange Lightning Site-directed Mutagenesis Kit (210518-5, Agilent Technologies), following the manufacturer’s protocol. The following mutagenic primers were used: GPR101-G31S_F: GAGCGGATGATGCTGTGGGCCAGGCTG; GPR101-G31S_R: CAGCCTGGCCCACAGCATCATCCGCTC.

Cell culture

The rat pituitary somatomammotroph GH3 cell line and the mouse pituitary corticotroph AtT-20 cell line were grown in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, pyruvate, no glutamine; 10313, Gibco) supplemented with 10% fetal bovine serum (100-106, Gemini Bio Products, West Sacramento, CA, USA) and 1% antibiotic-antimycotic (15240-062, Gibco) in a humidified atmosphere at 37°C with 5% CO₂.

Cell proliferation assay

GH3 cells were seeded into 96-well plates at a density of 2×10⁴ cells per well. After 24h, cells were starved with DMEM without serum for 16h and then transfected with Lipofectamine 2000 (11668030, Invitrogen) according to the manufacturer’s protocol, using Opti-MEM I Reduced Serum Medium (31985-070, Gibco) and 125ng of each vector (human WT GPR101, p.G31S GPR101), alone or in combination. The empty pCMV-XL5 vector was used as a negative control.

AtT-20 cells were plated and transfected following the same protocol, but were not starved, because we
observed that starvation significantly impacts cell viability. After 24 h transfection, cell viability and cellular proliferation were assessed for both cell lines with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based assay (Vybrant MTT Cell Proliferation Assay Kit, Invitrogen) as described previously (Trivellin et al. 2014).

**cAMP reporter assay**

GH3 and AtT-20 cells were seeded in 12-well plates at a density of $2 \times 10^5$ cells per well. After 24 h, GH3 cells only were starved with DMEM without serum for 16 h, and then both cell lines were transfected with Lipofectamine 2000 according to the manufacturer’s protocol, using Opti-MEM I Reduced Serum Medium, 1 μg of each GPR101 vector (human WT GPR101, p.G31S GPR101), 800 ng of pGL4.29[luc2P/CRE/Hygro] vector containing a cAMP response element 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 a negative control. At 24 h after transfection, a subset of cells was treated with 10μM forskolin (F6886, 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 (E1910, Promega) as described previously (Trivellin et al. 2014).

**Hormone assays**

GH3 and AtT-20 cells were seeded in 12-well plates and then transfected as reported in the ‘cAMP reporter assay’ section, using 1 μg of each GPR101 vector. At 24 h after transfection, supernatants were collected and hormone concentration was measured. For GH3 cells, GH secretion was measured using the rat/mouse Growth Hormone ELISA Kit (EZRMGH-45K, EMD Millipore) as described previously (Trivellin et al. 2014). For AtT-20 cells, ACTH secretion was measured using the ACTH (rat, mouse) Chemiluminescent EIA KIT (CEK-001-21, Phoenix Pharmaceuticals, Belmont, CA, USA) following the manufacturer’s protocol.

**Statistical analyses**

Statistical analysis was performed using StatsDirect software (Addison-Wesley-Longman, Cambridge, UK). Data are presented as the mean±s.d. of two to five independent experiments, each performed at least in triplicate. Comparisons were calculated using a two-tailed Student’s t-test for unpaired data and the Kruskal–Wallis test followed by the Conover–Inman test, as appropriate. A χ²-test was used to compare the allelic frequencies of the variants in patients and controls. Spearman’s correlation coefficient by rank was used to compare GPR101 and POMC mRNA expression levels. The data were considered to be significant when $P<0.05$. 

**Figure 3**

ACTH immunostaining in corticotroph tumors of four patients with CD. ACTH protein expression (red) of cases ACTH_7, 8, and 9 reflects the POMC mRNA levels shown in Fig. 2C. The tumor of the patient harboring the p.G31S GPR101 variant shows some ACTH-positive sparse cells, as also shown in Fig. 4D.
Results

GPR101 sequencing analysis in ACTH-secreting tumors

Germline DNA from 36 patients with CD (58% females, mean age at diagnosis: 14.4±8.13 years old) was sequenced for GPR101; 32 of these patients have been included in our previous series of pediatric patients with CD (Stratakis et al. 2010, Trivellin et al. 2014). Two common SNPs (p.V124L and p.L376P) were detected in several patients’ samples with frequencies comparable to those observed in control subjects, similarly to what we previously reported (Trivellin et al. 2014), and a less common SNP (p.T293I, 5% allele frequency in controls) was observed in one patient in the homozygote state (Fig. 1). In silico analysis was not supportive for pathogenic function (Table 1). A very rare variant (p.G31S) was detected in another patient in the heterozygote state. Although some in silico programs reported it as benign, others classified it as possibly damaging (Table 1). These patients had classic CD without atypical features (Supplementary Table 1, see section on supplementary data given at the end of this article). GPR101 sequencing was also performed successfully at the somatic level in 33 ACTH-secreting tumors; no de novo somatic variants were reported in GPR101.

GPR101–ACTH expression in ACTH-secreting tumors

We performed RT-qPCR for GPR101 in 10 corticotropinomas and 3 NPs collected at autopsy. Each NP was divided into the anterior and posterior lobes. None of these samples harbored GPR101 mutations. We observed a trend toward higher GPR101 expression in the tumors compared with NPs, with this being particularly evident for three samples (Fig. 2A), but it did not reach statistical significance (P=0.14, Fig. 2B).

We then measured POMC mRNA expression in the tumor samples to determine if there was a correlation with GPR101 mRNA levels. We found no correlation between GPR101 and POMC expression levels (Spearman’s R=0.08, P=0.83, Fig. 2C). ACTH expression in four available tumors was also investigated at the protein level by immunostaining (Fig. 3). ACTH protein expression corresponded well with POMC mRNA levels as shown in Fig. 2C. ACTH expression levels in the patient harboring the p.G31S GPR101 variant were intermediate between those of the other three analyzed tumors; in the patient with the homozygous p.T293I variant it was unremarkable (Figs 3 and 4). GPR101 staining was performed in the same specimens but resulted in nonspecific signal that could be a consequence of tissue collection/preparation (data not shown). We had successful double labeling with two antibodies raised in rabbit (including GPR101), but it just did not work with these tumor samples.

In vitro functional studies for GPR101 p.G31S variant

Based on the in silico results and on the very low allele frequency with which the p.G31S variant is reported in
the population (making it more likely to be pathogenic), we performed in vitro functional studies. An MTT assay was performed in AtT-20 and GH3 cells, but no effect of the variant was observed on cell proliferation in neither cell lines (Fig. 5A and B). Moreover, overexpression of the WT GPR101 construct did not increase cell proliferation in either cell lines, as previously observed (Trivellin et al. 2014). ACTH and GH secretion from, respectively, AtT-20 and GH3 cells was also measured after overexpression of the p.G31S variant. No increased secretion of either hormones was observed compared with cells transfected with mock control, neither for WT nor for mutated p.G31S GPR101 (Fig. 5C and D). Activation of the cAMP pathway was measured with a reporter assay in AtT-20 and GH3 cells upon p.G31S overexpression. In both cell lines, a significant increase in cAMP levels was observed compared with control (P<0.001); however, this increase was not different from what was seen with the WT GPR101 construct (Fig. 5E and F). In basal conditions, the increase in cAMP activation caused by GPR101 overexpression in AtT-20 cells (Fig. 5E) was lower than that seen in GH3 cells (P < 0.001, Fig. 5F). The additional stimulation of the cAMP pathway with forskolin showed a synergistic effect only in AtT-20 cells (Fig. 5E and F).

Discussion

GPR101 has been previously shown to be expressed in hypothalamic neurons expressing proopiomelanocortin (POMC), the precursor of ACTH (Nilaweera et al. 2007, Bagnol 2010). Moreover, GPR101 was shown to mediate the phosphorylation of epidermal growth factor receptor (EGFR), leading to enhanced cellular migration (Cho-Clark et al. 2014) and invasion (Cho-Clark et al. 2015). EGFR is frequently overexpressed in ACTH-secreting tumors, and the EGFR-mediated pathway is essential for POMC synthesis (Theodoropoulou et al. 2004, Fukuoka et al. 2011). A GPR101 variant, p.E308D, previously observed in some patients with GH-secreting adenomas (Trivellin et al. 2014) was also recently reported in two patients with ACTH-secreting adenomas (Lecoq et al. 2016).

Based on the possible link between GPR101 and the physiology of ACTH-secreting cells, we decided...
to study if GPR101 plays a role in the pathogenesis of corticotropinomas. As nothing is known regarding the expression of the receptor in these tumors, we first measured its expression levels in 10 corticotropinomas and compared them with three NP samples. We did not observe different expression levels of GPR101 between tumors and NP tissues (Fig. 2A and B). Moreover, no correlation with POMC expression levels was observed (Fig. 2C). We then sequenced a series of patients with CD to look for possible GPR101 germline and somatic mutations. In addition to two very common SNPs, two other missense variants (p.G31S and p.T293I) were observed at the germline level (Fig. 1). Both variants have been reported in public databases and in the literature (Trivellin et al. 2014, Castinetti et al. 2016). Although p.T293I is a relatively common variant with a minor allele frequency (MAF) of about 6% (an average calculated from three public databases, Table 1), p.G31S is a very rare variant, with a MAF of about 0.06%, and was predicted in silico to be possibly damaging. However, functional in vitro studies of this variant did not show an increase in hormone secretion nor in cell proliferation (Fig. 5). We were able to see a significant increase in cAMP pathway activation, compared with controls, but this was not significantly different from that elicited by the WT GPR101 construct (Fig. 5E and F). It is also interesting to note that in basal conditions, the increase in the cAMP pathway activation caused by GPR101 overexpression in AtT-20 cells (Fig. 5E) was significantly lower than that seen in GH3 cells (Fig. 5F; Trivellin et al. 2014). Only in the presence of a potent stimulator of the cAMP pathway, GPR101 was able to exert on the AtT-20 cells an effect of similar magnitude to that observed in GH3 cells. These findings suggest that GPR101 may activate the cAMP pathway at different magnitudes in different hormone-secreting cell types. The lower activation of the cAMP pathway in corticotrophs might be in line with some studies, indicating that this pathway does not play a significant pathogenetic role in corticotropinomas (reviewed in (Bertagna 2011)). Therefore, it might be possible that GPR101 activates different intracellular signaling pathways in corticotroph cells than in somatotrophs. It would be interesting to investigate this aspect in future studies, in particular in relation to the p.G31S variant.

In conclusion, in this study we investigated pediatric patients with CD for GPR101 defects but found little to support that this gene might be involved in the pathogenesis of corticotropinomas. The finding of a rare, potentially functional GPR101 variant in one patient with CD was interesting, but in vitro studies did not support a tumor-inducing role for this sequence change.


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