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Screening for GPR101 defects in pediatric pituitary corticotropinomas

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Abstract

Cushing 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. We investigated the orphan G protein-coupled receptor *GPR101*, a gene known to be involved in somatotropinomas, for its possible involvement in corticotropinomas.

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Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

We performed *GPR101* sequencing, expression analyses by RT-qPCR and immunostaining, and functional studies (cell proliferation, pituitary hormones secretion, and cAMP measurement) in a series of patients with sporadic CD secondary to ACTH-secreting adenomas in whom we had peripheral and tumor DNA (N=36).

No increased GPR101 expression was observed in tumors compared to 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 seen.

GPR101 is not overexpressed in ACTH-secreting tumors compared to NPs. A 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 terms

GPR101; Cushing disease; ACTH-secreting adenomas

Introduction

Cushing disease (CD) affects approximately 70% of patients with endogenous Cushing syndrome (CS) (Tritos and 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 (Reincke, et al. 2015; Stratakis, et al. 2010). Hence, there remains a great need to identify other genetic alterations.

We recently found that the *GPR101* gene is involved in the pathogenesis of pituitary growth hormone (GH)-secreting adenomas (Beckers, et al. 2015; Trivellin, et al. 2014). *GPR101* encodes an orphan G protein-coupled receptor (GPCR) that is highly expressed in the hypothalamus, where it may play a role in the hypothalamic control of energy homeostasis and pituitary hormone secretion (Bates, et al. 2006; Lee, et al. 2001; Nilaweera, et al. 2007; Nilaweera, et al. 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 (X-LAG) syndrome caused by *GPR101* duplication, whereas it is expressed at low levels in normal pituitary tissue and in non-*GPR101* duplicated somatotropinomas (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 GPR101 can strongly activate the cAMP pathway (Bates et al. 2006; Trivellin et al. 2014), 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 furthermore report a case associated with a very rare missense variant (p.G31S).

Materials & 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, Valencia, CA, USA) 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: ACTGAGCCTGCAACCTGTCT; GPR101_1AR: TCCACTGACACCACGACAAT; GPR101_1BF: TTAGCCTCACCCACCTGTTC; GPR101_1BR: CTTCCTTCCTTGGCCTTCAG; GPR101_1CF: CAGCATGAAGGTGAGGTCAA; GPR101_1CR: CCCAGGGATAGCACATAGGA; GPR101_1DF: GTGCTACCAGTGCAAAGCTG; GPR101_1DR: TGAATTGTGGGTCCATTGAA. DNA sequencing was performed using the BigDye 3.1 Termination Chemistry (Applied Biosystems, Foster City, CA, USA) on a Genetic Sequencer ABI3500XL apparatus (Applied Biosystems). Sequences were visualized and aligned to the corresponding wild-type (WT) reference sequence using the SeqMan Pro software (DNAStar, Madison, WI, USA). All variants have been annotated according to Human Genome Variation Society (HGVS) recommendations (www.hgvs.org/mutnomen). The NM_054021.1 reference sequence was used to annotate *GPR101* variants.

In silico analysis

All *GPR101* variants were studied *in silico* and the allele frequencies observed in our patient population compared against public databases, including the 1000 Genomes Project (Abecasis, et al. 2012); the Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA (URL: http://evs.gs.washington.edu/EVS/) [date (February, 2016) accessed]; and the Exome Aggregation Consortium (ExAC), Cambridge, MA, USA (URL: http://exac.broadinstitute.org) [date (February, 2016) accessed]. *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-μm-

thick sections were stained with haematoxylin-eosin (H&E) 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, Carpinteria, CA, USA; 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, St. Louis, MO, USA) 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, Carlsbad, CA, USA) 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, Allendale, NJ, USA) 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 was 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 20 µl reactions containing 20 ng of cDNA, 10 µl TaqMan Gene Expression Master Mix (Applied Biosystems, catalog number 4369016) and 1 µl 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 using the $\Delta\Delta C_t$ method. Data analysis was performed using the 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, Santa Clara, CA, USA), 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, Grand Island, NY, USA) 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% CO_2.

Cell proliferation assay

GH3 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 24 h, cells were starved with DMEM without serum for 16 h and then transfected with Lipofectamine 2000 (11668030, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, using Opti-MEM I Reduced Serum Medium (31985-070, Gibco) and 125 ng of each vector (human WT GPR101, p.G31S GPR101), alone or in combination. The empty pCMV-XL5 vector was used as negative control.

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

cAMP reporter assay

GH3 and AtT-20 cells were seeded in 12-well plates at a density of 2×10^5 cells/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 (CRE) that drives the transcription of the luciferase reporter gene (Promega, Madison, WI, USA), 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 μM forskolin (F6886, Sigma-Aldrich, St. Louis, MO, USA) 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 previously described (Trivellin et al. 2014).

Hormone assays

GH3 and AtT-20 cells were seeded in 12-well plates and then transfected as reported in the previous section, using 1 μg of each GPR101 vector. At 24 h after transfection, supernatants were collected and hormone concentration measured. For GH3 cells, GH secretion was measured using the Rat/Mouse Growth Hormone ELISA kit (EZRMGH-45K, EMD Millipore, Atlanta, GA, USA) as previously described (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 manufacturer's protocol.

Statistical analysis

Statistical analysis was performed with StatsDirect software (Addison-Wesley-Longman, Cambridge, UK). Data are presented as the mean ± standard deviation (SD) 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 Chi-square 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 $P < 0.05$.

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 what 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 (Figure 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. While some *in silico* programs reported it as benign, others classified it as possibly damaging (Table 1). These patients had classic CD without atypical features (Supplemental Table 1). *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 three normal human pituitaries (NPs) collected at autopsy. Each NP was divided into the anterior and posterior lobe. None of these samples harbored *GPR101* mutations. We observed a trend towards higher *GPR101* expression in the tumors compared to NPs, with this being particularly evident for three samples (Figure 2A), but it did not reach statistical significance ($P = 0.14$, Figure 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 $R = 0.08$, $P = 0.83$, Figure 2C). ACTH expression in four available tumors was also investigated at the protein level by immunostaining (Figure 3). ACTH protein expression corresponded well with *POMC* mRNA levels shown in Figure 2C. ACTH expression levels in the patient harboring the p.G31S *GPR101* variant were intermediate between those of the other three analyzed tumors; the patient with the homozygous p.T293I variant was unremarkable (Figures 3 and 4). GPR101 staining was performed in the same specimens but resulted in non-specific 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 line (Figures 5A and 5B). Moreover, overexpression of the WT GPR101 construct did not increase cell proliferation in either cell line, 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 hormone was observed compared to cells transfected with mock control, neither for WT nor for mutated p.G31S *GPR101* (Figures 5C and 5D). Activation of the cAMP pathway was measured with a reporter assay in AtT-20 and GH3 cells upon p.G31S over-expression. In both cell lines a significant increase in cAMP levels was observed compared to mock control ($P < 0.001$); however, this increase was not different from what was seen with the WT GPR101 construct (Figures 5E and 5F). In basal conditions, the increase in cAMP activation caused by GPR101 overexpression in AtT-20 cells (Figure 5E) was lower than that seen in GH3 cells ($P < 0.001$, Figure 5F). The additional stimulation of the cAMP pathway with forskolin showed a synergistic effect only in AtT-20 cells (Figures 5E and 5F).

Discussion

GPR101 has been previously shown to be expressed in hypothalamic neurons expressing proopiomelanocortin (POMC), the precursor of ACTH (Bagnol 2010; Nilaweera et al. 2007). 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 over-expressed in ACTH-secreting tumors and the EGFR-mediated pathway is essential for POMC synthesis (Fukuoka, et al. 2011; Theodoropoulou, et al. 2004).

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. Since nothing is known regarding the expression of the receptor in these tumors we first measured its expression levels in 10 corticotropinomas and compared them to three normal pituitary samples. We did not observe different expression levels of GPR101 between tumors and normal pituitary tissues (Figures 2A and 2B). Moreover, no correlation with POMC expression levels was observed (Figure 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 (Figure 1). Both variants have been reported in public databases and in the literature (Castinetti, et al. 2016; Trivellin et al. 2014). While 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 (Figure 5). We were able to see a significant increase in cAMP pathway activation, compared to controls but this was not significantly different from that elicited by the WT GPR101 construct (Figures 5E and 5F). It is also interesting to note that in basal conditions the increase in cAMP pathway activation caused by GPR101 overexpression in AtT-20 cells (Figure 5E) was significantly lower than that seen in GH3 cells [Figure 5F and (Trivellin et al. 2014)]. Only in the presence of a potent stimulator of the cAMP pathway was GPR101 able to exert in 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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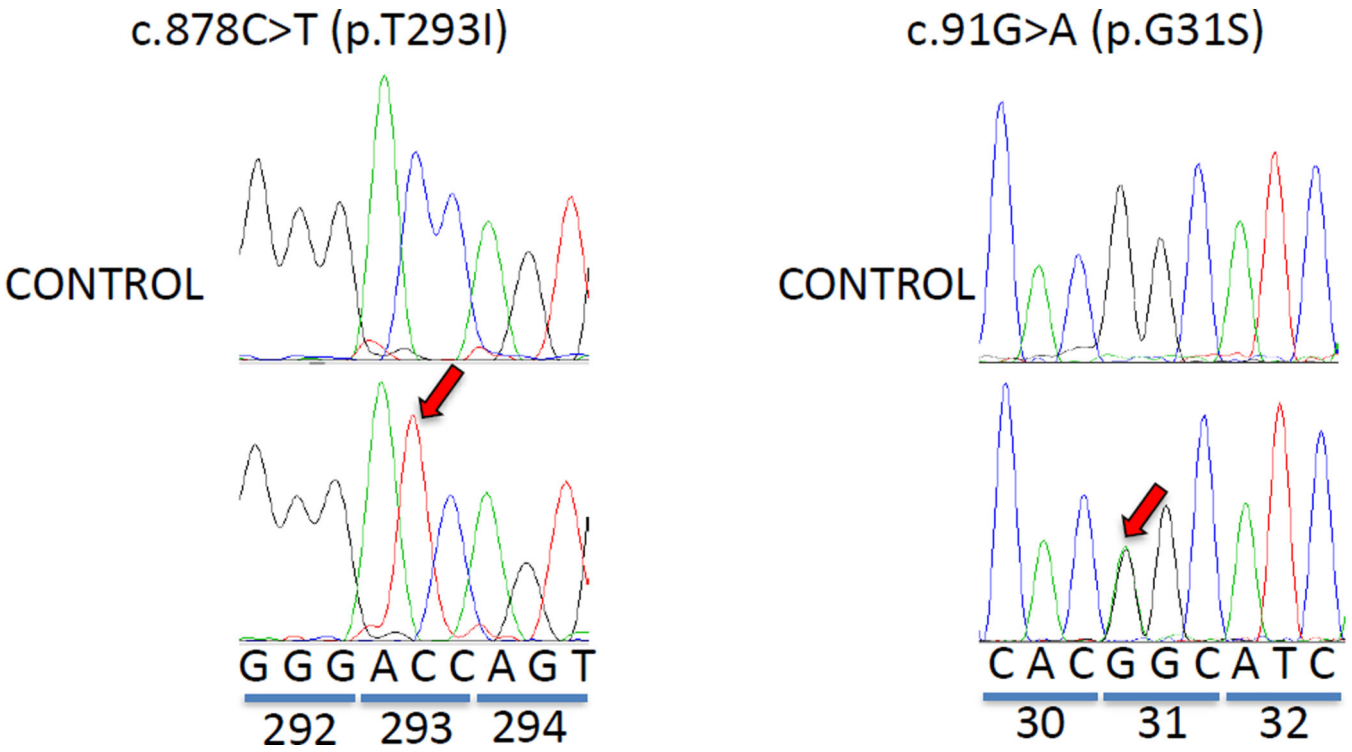

Figure 1. DNA sequence chromatograms showing the position of both *GPR101* variants

The homozygous p.T293I variant is shown on the left side, while 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.

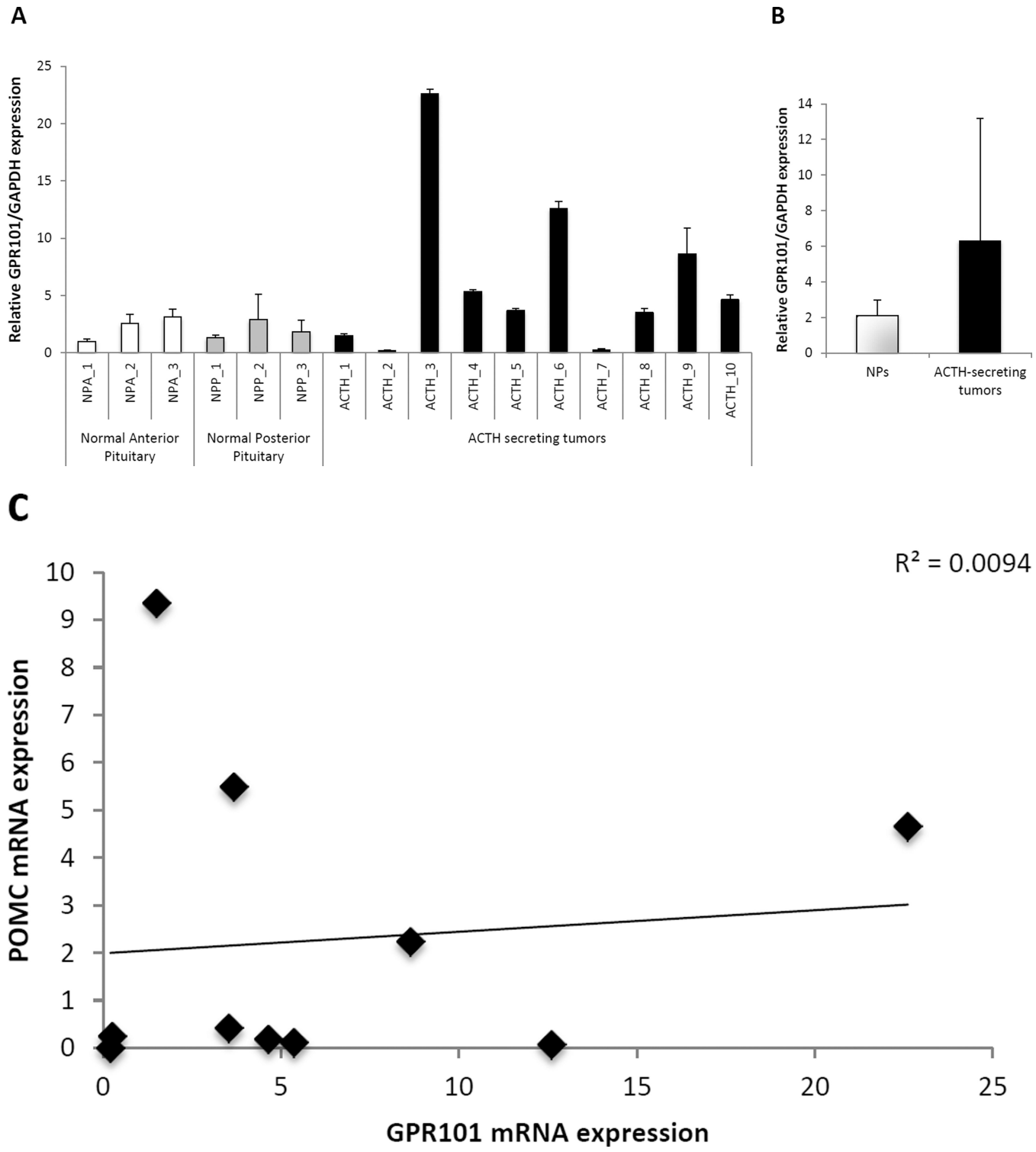

Figure 2. *GPR101* and *POMC* mRNA expression in corticotropinomas

None of the analyzed samples harbor *GPR101* mutations. A) and B) A trend towards higher *GPR101* expression in ACTH-secreting tumors can be seen, but it does not reach statistical significance (P = 0.14). C) No correlation between *GPR101* and *POMC* expression levels

was observed in ACTH-secreting tumors (Spearman's R = 0.08, P = 0.83). Data are expressed as mean ± SD. *, P<0.05. NPA: anterior lobe of normal pituitary; NPP: posterior lobe of normal pituitary; ACTH: ACTH-secreting tumor.

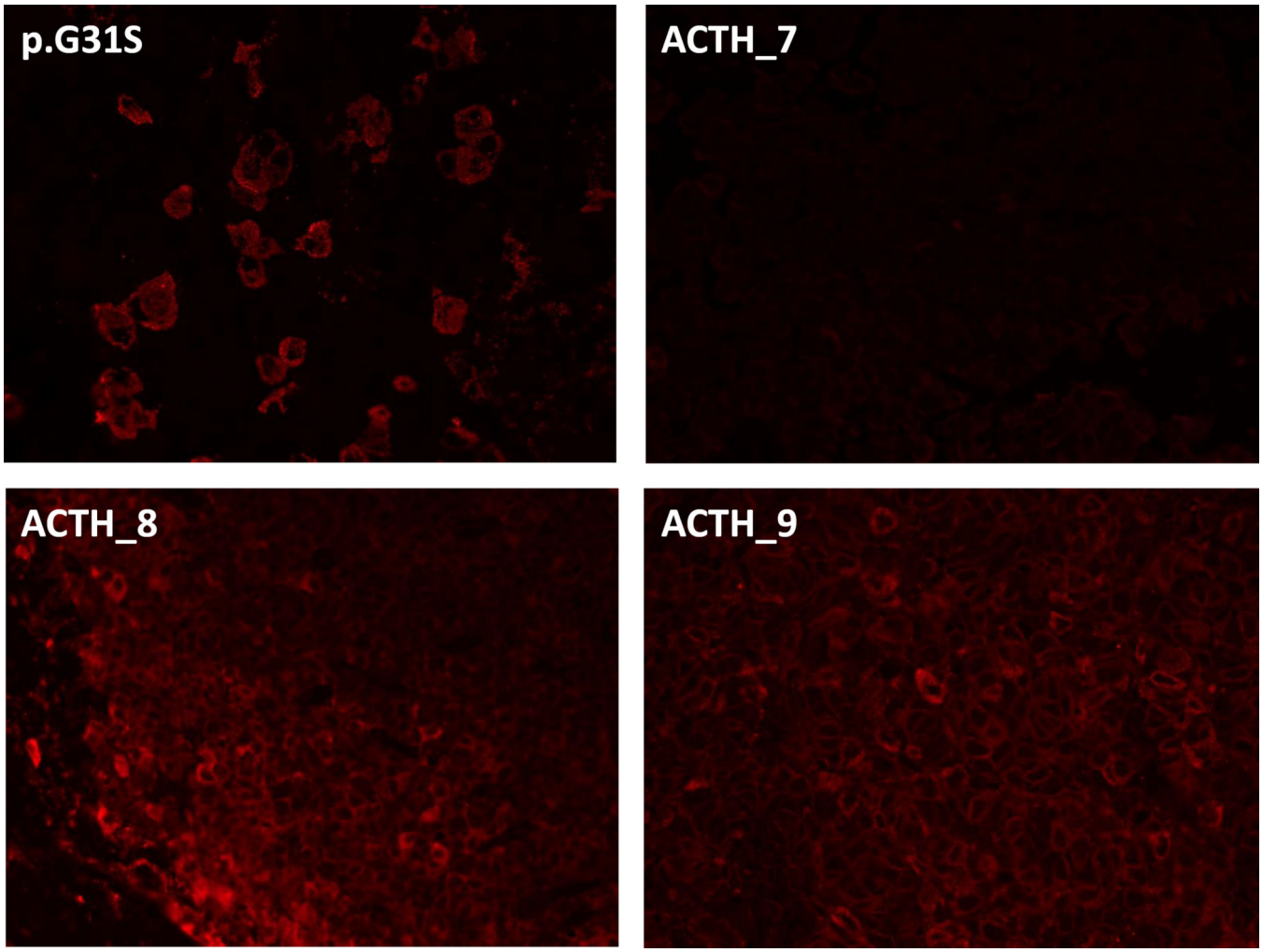

Figure 3. ACTH immunostaining in corticotroph tumors of four patients with CD

ACTH protein expression (red) of cases ACTH_7, 8, and 9 reflects the *POMC* mRNA levels shown in Figure 2C. The tumor of the patient harboring the p.G31S *GPR101* variant shows some ACTH-positive sparse cells, as also shown in Figure 4D.

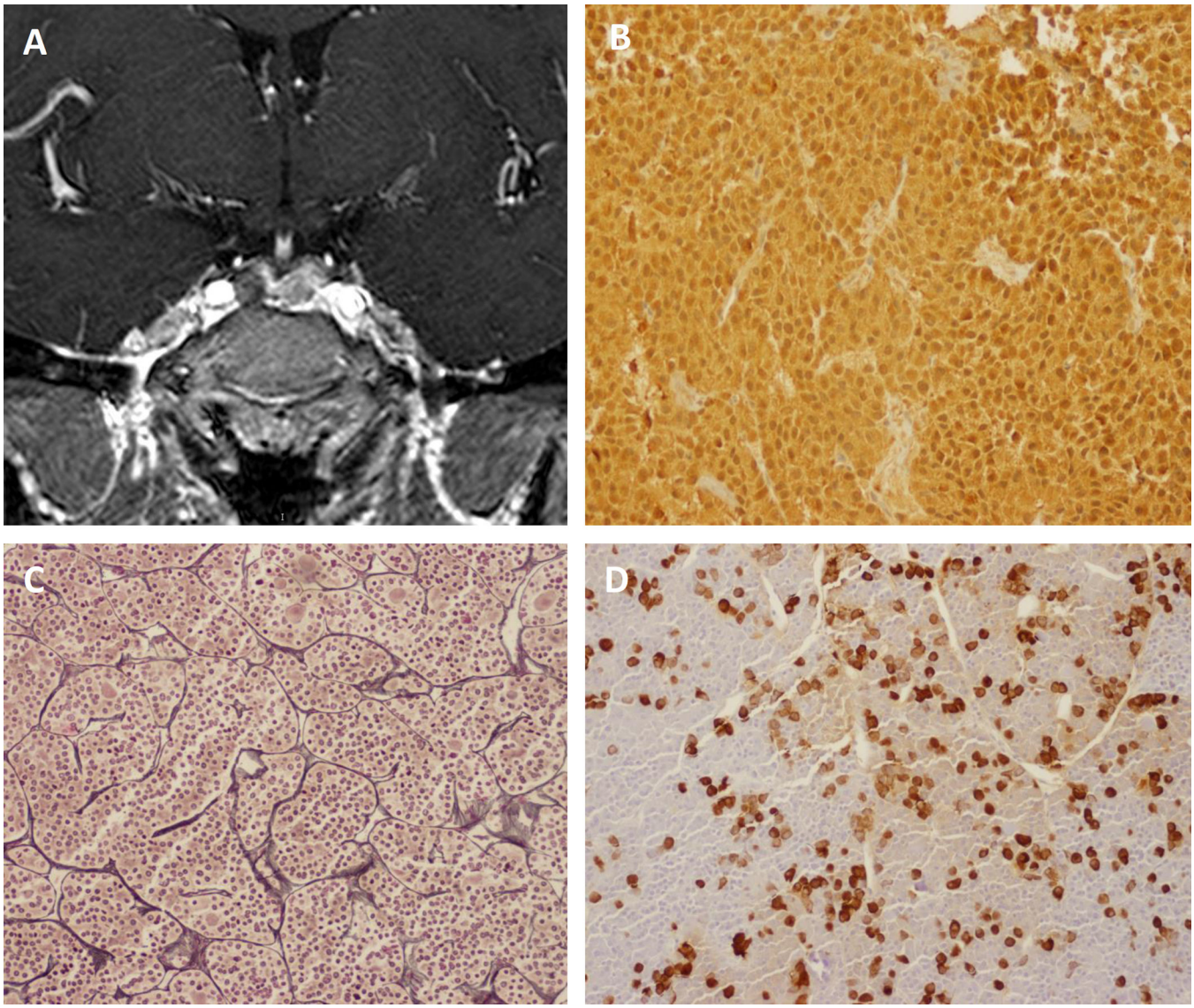

Figure 4. Imaging and histopathological findings for patients with *GPR101* variants

A) and B) Patient with the p.T293I *GPR101* variant in homozygosity: A) There is a round space-occupying lesion in the right half of the pituitary showing decrease enhancement with respect to normal pituitary tissue. This abnormality represents the microadenoma which abuts the intracavernous segment of the right internal carotid artery. Bony defect inferior to the adenoma is the result of a prior surgery. B) ACTH staining of the microadenoma showing diffuse and strong ACTH expression. C) and D): Patient with the heterozygous p.G31S *GPR101* variant: C) Reticulin staining showing disruption of reticulin network and expanded hyperplastic acini. D) ACTH staining showing an expression pattern typical of normal anterior pituitary. All three images were taken at 10× magnification.

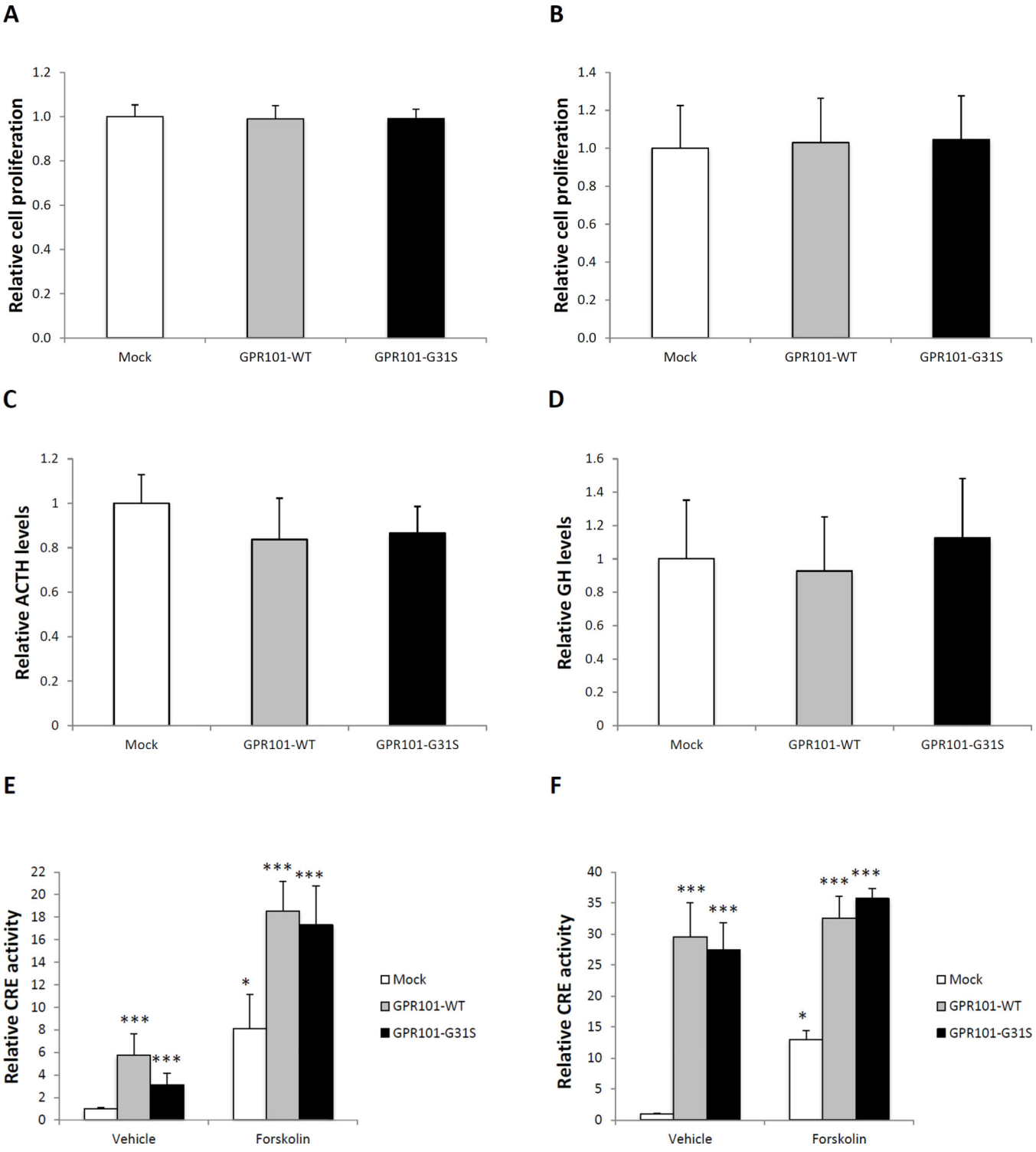

Figure 5. *In vitro* functional studies in pituitary tumor cell lines

An MTT assay was performed in AtT-20 (A) and GH3 cells (B). No effect of the WT GPR101 nor of the p.G31S variant was observed on cell proliferation. Mouse ACTH (C) and rat GH (D) secretion was measured with specific ELISA assays from AtT-20 and GH3 cell

supernatants, respectively. No effect of the WT GPR101 nor of the p.G31S variant was observed on the secretion of both hormones. cAMP pathway activation was measured in AtT-20 (E) and in GH3 (F) cells upon GPR101 overexpression. A significant increase in cAMP levels was observed in both cell lines compared to control but this increase was not different between WT and mutated GPR101. Data are expressed as mean ± SD of 2–3 independent experiments each performed in triplicate. *, $P<0.05$; ***, $P<0.001$.

Table 1

Germline nucleotide changes identified in *GPR101* in patients with CD (excluding the commons SNPs p.V124L and p.L376P).

DNA change	Protein change	SNP id	Patient MAF	Control MAF - EVS	Control MAF - ExAC	Control MAF – 1000 genomes	P-value	PolyPhen-2	SIFT	MutationTaster	PON-P2
c.91G>A	p.G31S	rs138068185	1.754	0.0284	0.1015	n.o.	< 0.05 n.s. n.a.	Possibly damaging (0.48)	Deleterious (0)	Polymorphism (0.746)	Neutral (0.076)
c.878C>T	p.T293I	rs73566014	3.509	6.5038	2.341	7.4580	n.s n.s. n.s.	Benign (0)	Tolerated (0.78)	Polymorphism (1)	Neutral (0.014)

The allelic frequencies identified in patients and in controls and the *in silico* predictions are shown. MAF frequencies are expressed in %. P-values were calculated for the comparison of the allelic frequency of eachvariant in patients versus controls (EVS, ExAC, and 1000 genomes, respectively). The number in parenthesis for PolyPhen-2 and SIFT represents score value, for MutationTaster represents p-value, and for PON-P2 represents probability of pathogenicity. MAF: minor allele frequency; n.s.: not significant; n.o.: not observed.; n.a.: not applicable.