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Case Report

A novel synonymous variant in exon 1 of *GNAS* gene results in a cryptic splice site and causes pseudohypoparathyroidism type 1A and pseudo-pseudohypoparathyroidism in a French family

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ABSTRACT

Introduction: Pseudohypoparathyroidism type 1A (PHP1A) and pseudopseudohypoparathyroidism (PPHP) (Inactivating PTH/PTHrP Signaling Disorders type 2, IPPSD2) are two rare autosomal disorders caused by loss-of-function mutations on either maternal or paternal allele, respectively, in the imprinted *GNAS* gene, which encodes the α subunit of the ubiquitously-expressed stimulatory G protein (*G α s*).

Case presentation: We investigated a synonymous *GNAS* variant NM_001077488.2: c.108C>A / p.(Val36=) identified in a family presenting with IPPSD2 phenotype. *In silico* splicing prediction algorithms were in favor of a deleterious effect of this variant, by creating a new donor splicing site. The *GNAS* expression studies in blood suggested haploinsufficiency and showed an alternate splice product demonstrating the unmasking of a cryptic site, leading to a 34 base pairs deletion and the creation of a probable unstable RNA.

We present the first familial case of IPPSD2 caused by a pathogenic synonymous variant in *GNAS* gene.

1. Introduction

Pseudohypoparathyroidism type 1A (PHP1A, MIM#103580) is characterized by physical features known as Albright's Hereditary Osteodystrophy (AHO): early-onset obesity, short stature, round-shaped face, heterotopic ossifications, type E brachymetacarpus, a variable degree of intellectual disability, associated with an end-organ resistance to parathormone (PTH), characterized by high serum PTH, hypocalcemia and hyperphosphatemia and to other hormones, such as thyroid stimulating hormone (TSH) and gonadotropin (Albright et al., 1942). Pseudo-pseudohypoparathyroidism (PPHP, MIM#612463) is a related disorder, characterized by AHO and intrauterine growth retardation without hormonal resistance (Kottler, 2015; Richard et al., 2013). The nomenclature proposed by EuroPHP network classifies both disorders as Inactivating PTH/PTHrP Signaling Disorders type 2 (IPPSD2) associated with *GNAS* loss of function mutations (Thiele et al., 2016).

GNAS imprinted gene encodes the α subunit of the ubiquitously-expressed G protein (*G α s*), which plays an essential role in a multitude of physiological processes (Weinstein et al., 2001). Maternally inherited

loss of function mutations lead to PHP1A, whereas paternally inherited loss of function mutations lead to PPHP. Therefore, the two phenotypes usually coexist in the same family, but never in the same sibship (Puzhko et al., 2011; Hayward et al., 1998). The absence of hormonal resistance in PPHP is explained by the predominant expression of *GNAS* from the maternal allele in the renal proximal tubules, parathyroid, thyroid, pituitary gland and ovaries (Mantovani et al., 2002; Yu et al., 1998).

In the last years, a growing number of synonymous variants affecting the transcripts' splicing have been implicated in different diseases (Hunt et al., 2014; Sauna and Kimchi-Sarfaty, 2011). Few splicing variants in *GNAS* have already been described in PHP1A or PPHP. To the best of our knowledge, no synonymous splicing variation has been described in *GNAS* (Nakamura et al., 2011; Ham et al., 2015; Lemos and Thakker, 2015; Thiele et al., 2015; Wu et al., 2014).

Herein, we describe a novel synonymous variant in exon 1 of the *GNAS* gene which results in a cryptic splice site and causes both PHP1A and PPHP in a French family.

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2. Patients and methods

2.1. Patients

We report on a French family including 2 patients, III.3 presenting PHP1A phenotype and IV.4 presenting PPHP phenotype (Fig. 1A). All patients who benefit from genetic analyses gave their written informed consent, as required by the national bioethics laws and the Declaration of Helsinki.

2.2. GNAS sequencing

Genomic DNA was isolated from peripheral blood following the routine procedure used in the laboratory. *GNAS* exons 1–13 and intron/exon boundaries were sequenced (reference sequence NM_001077488.2) and interpreted following the routine protocol in our molecular genetics laboratory and National Reference Center for Rare Calcium and Phosphate Metabolism Disorders, as previously submitted to publication (Snanoudj et al., 2020). In addition, S_{PI}P and Alamut Visual version 2.11.0 (Interactive Biosoftware, Rouen, France), which

regroups SpliceSiteFinder, MaxEntScan, NNSplice and Gene Splicer splicing-prediction algorithms were used (Raphaël et al., 2020; Tang et al., 2016).

2.3. GNAS expression analysis

Total RNA was extracted from peripheral blood collected on PAX-gene® Blood RNA tube using Maxwell® RSC Simply RNA Blood Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s protocol. cDNA was obtained by reverse-transcription polymerase chain reaction (RT-PCR) using SuperScript™ II Reverse Transcriptase kit and a oligo(dT)₁₈ primer (Thermo Fisher Scientific).

2.3.1. Quantification

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to assess the expression of *GNAS* in cDNA obtained by RT-PCR using the oligo(dT)₁₈ primer in the proband and in a healthy, sex and aged-paired control. The qRT-PCR was performed using a SYBR Green PCR Kit (Thermo Fisher Scientific) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Primers spanned the coding

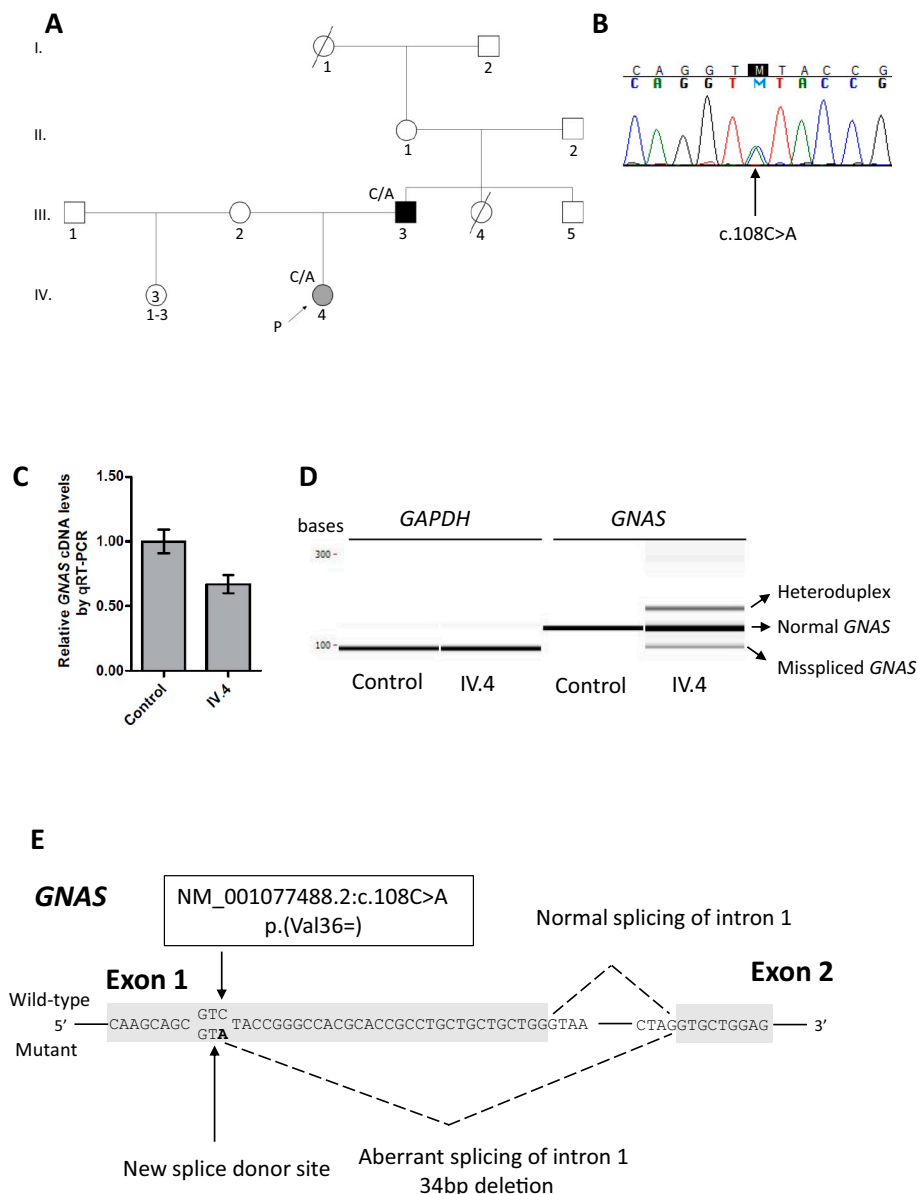


Fig. 1. Rare synonymous germline mutation in *GNAS* segregates with PHP1A/PPHP and causes aberrant RNA splicing. **A.** Pedigree tree of the family, consistent with the autosomal dominant mode of inheritance with parental imprinting of PHP1A/PPHP. The black square and the grey circle represent the affected member with confirmed PHP1A when maternally inherited and PPHP when paternally inherited respectively. The genotypes are provided when known. The arrow indicates the proband. **B.** Chromatogram from DNA sequencing of a nucleotide change from C to A in the proband (IV.4). **C.** Relative *GNAS* expression level measured by qRT-PCR using oligo(dT)₁₈, normalized to *GAPDH* expression level in the proband (IV.4) and a healthy control. **D.** Capillary electrophoresis performed on the cDNA obtained by RT-PCR using *GNAS* gene specific primer. On the first two lanes: *GAPDH* PCR product in the healthy control and in the proband, the third lane: *GNAS* PCR product in the control, on the last lane: 3 *GNAS* PCR products in the proband, corresponding to the wild-type allele, the mutant, shorter allele and the heteroduplex (a mixture of wild-type and mutant allele) product. **E.** Schematic representation of the splicing effect of the variant NM_001077488.2:c.108C>A on the splicing of intron 1 of *GNAS* (downward arrow). Transcript analysis uncovered the use of the new cryptic splicing donor consensus dinucleotide “GT” (upward arrow) that is adjacent to the substitution (bold characters). This aberrant splicing led to the deletion of 34 exonic nucleotides in the exon 1 of *GNAS*.

region of exons 1 and 2. Relative quantification for each target was calculated by the $2^{-\Delta\Delta CT}$ method with *GAPDH* gene for normalization (Barnes et al., 2008; Yu et al., 2009).

2.3.2. Transcript study and sequencing

RT-PCR using a gene-specific primer localized in the exon 5 was realized in order to identify the mutant allele. Then, PCR using primers localized in exons 1 and 2 was performed. A capillary electrophoresis analysis with LabChip®GX reagents and material (Caliper Life Sciences, Perkin Elmer Inc., Hopkinton, MA, USA) following the manufacturer's protocol allowed to isolate and characterize the PCR products before sequencing. PCR products were sequenced using Big-Dye Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific) and an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems).

Sequences of all primers are available in Supplementary Table 1.

3. Results

3.1. Clinical report

The proband is a 21-years old female (IV.4, Fig. 1A), presenting with short stature (height = 1.41 m or -3.6 SD, weight = 49 kg, BMI = 24.64), a round-shaped face, type E brachymetacarp and micromely. Her medical history included intrauterine growth retardation: she was born at 38⁺⁴ gestational weeks (GW, weeks^{+days}) with a length of 38.5 cm (-6 SD), a weight of 1500 g (-4.6 SD), without calcium or phosphate neonatal disturbance. She was treated with growth hormone between 4 and 7 years old and presented learning difficulties. She had no history of obesity and her serum biochemical parameters were within the normal range: calcium = 2.47 mmol/L (N: 2.15–2.50 mmol/L), phosphate = 0.74 mmol/L (N: 0.81–1.45 mmol/L), PTH = 28 ng/L (N: 15–65 ng/L, second generation assay), 25-hydroxyvitamin D (25-OH D) = 49.9 nmol/L (N: 75–200 nmol/L).

There was no familial consanguinity. There was no remarkable familial medical history on her mother's side. On the paternal side of the family, her father (III.3), aunt (III.4), grand-mother (II.1) and great grand-mother (I.1) presented a small stature. Her father's height was 1.43 m (-4.6 SD). He also presented brachymetacarp and a thyroid dysfunction. His serum PTH was high = 405 ng/L (N: 15–65 ng/L), with serum calcium at the lower limit of the normal = 2.17 mmol/L (N: 2.15–2.50 mmol/L), serum phosphate = 1.07 mmol/L (N: 0.81–1.45 mmol/L), in spite of a normal 25-hydroxyvitamin D (25-OH D) level = 170.3 nmol/L (N: 75–200 nmol/L), in favor of PHP1A phenotype. Her paternal aunt (III.4) also presented obesity and joint anomalies. She died of a sudden cardiac arrest during infancy.

3.2. GNAS sequencing

A heterozygous variation Chr20(GRCh37):g.57466889C>A, NM_001077488.2:c.108C>A p.(Val36=) localized in the exon 1 of *GNAS* gene, absent from international databases (GnomAD, LOVD, ClinVar) and from our local database, was identified in IV.4. Multiple splicing prediction tools suggested the creation of a new alternative cryptic donor site 34 base pairs (bp) upstream the original one (Supplementary Fig. 1). This variation was inherited from her father (III.3).

3.3. GNAS expression analysis

Expression quantification using qRT-PCR in blood extracted cDNA showed a reduction of *GNAS* expression by 35% in comparison with control (Fig. 1C).

However, this RT-PCR performed with the oligo(dT)₁₈ primer did not allow identifying the mutant allele. A second RT-PCR using a gene-specific primer for the reverse transcription, located in the exon 5 followed by PCR using primers localized in the exons 1 and 2 allowed to amplify the expected PCR product and 2 additional products in smaller

quantities, a shorter one and a longer one, respectively corresponding to the wild-type, the mutant allele and a heteroduplex (a mixture of wild-type and mutant allele) product (Fig. 1D and Supplementary Fig. 2). The sequencing of these cDNA PCR products showed a 34 bp deletion in the mutant allele (Fig. 1E).

Altogether, these results demonstrate that this synonymous *GNAS* variation is responsible for a splicing defect leading to the premature splicing of the first exon and to a probably unstable RNA or truncated protein. The protein prediction tools suggested a frameshift deletion leading to the appearance of a premature stop-codon (Supplementary Fig. 3).

4. Discussion

To the best of our knowledge, we describe the first IPPSD2 family presenting a pathogenic synonymous variant in the *GNAS* gene. This observation illustrates the need to provide adequate clinical information and gene-specific expertise to appropriately curate genetic variations.

In the era of massive parallel sequencing (MPS), generalizing the use of wider genetic analyses such as whole exome sequencing, laboratory geneticists are increasingly confronted with genetic variation in genes which they do not particularly know. Furthermore, owing to the huge amount of data, with thousands of genetic variations in only one individual's DNA, the bioinformatic trimming of data is incontestably necessary. Guidelines have previously been published to filter genetic variations and guide the interpretation of genetic data. They rely on different variations' characteristics, such as allelic frequency in population and pathogenicity prediction algorithms. Most guidelines give an important pathogenicity burden to nonsense and splice sites variations (*i.e.* +1, +2 or -2 , -1) whereas synonymous variations may usually be considered as benign.

However, *in silico* predictions, mainly based on the DNA sequence alignment in different species, are not always reliable and missense or synonymous variations may affect splicing. Thus, *in vivo* evaluations, whenever possible, remain most of the time necessary to question the biological consequences of genetic variations (Wai et al., 2020).

In this report, RNA studies suggest that the NM_001077488.2:c.108C>A variation creates a new splicing donor site, leading to an abnormal splicing between the exons 1 and 2 of *GNAS* with a 34 bp deletion in the *GNAS* transcript. As the number of deleted base pairs is not a multiple of 3, it may induce frameshift and premature stop codon. As we observed that the mutant RNA is significantly under-represented in comparison with the wild-type RNA, especially when using an oligo (dT)₁₈ primer for the reverse transcription, it may also reflect an instability of the mutant RNA and decay, resulting in a haploinsufficiency of *GNAS* gene product. In order to further assess the impact of this variation, *in vitro* models with minigene assays or *in vivo* animal models may be carried out.

In conclusion, this observation supports the increasing interest in the identification of splicing variants, particularly those predicted as synonymous, which are often systematically categorized as benign on this sole element (Sauna and Kimchi-Sarfaty, 2011). Splice-prediction algorithms could be added to the bioinformatic pipelines for MPS data in routine analysis to improve the detection of such variations (Raphaël et al., 2020; Wai et al., 2020).

CRedit authorship contribution statement

Design and oversight of the entire study: RN and AA.

Collection of DNA samples and of clinical informations: NG, RG, GM and MA.

Analysis and interpretation of NGS data: MA and BC, RT-PCR and nucleotide sequence analysis: AA, RA, GN, RN.

Drafting manuscript: AA. Revising manuscript content: RN, MA, NG, GN, RA, CB, GM. Approving final version of the manuscript: RN, MA.

Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bonr.2021.101073>.

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