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A novel deletion involving *GNAS* exon 1 causes PHP1A and further refines the region required for normal methylation at exon A/B

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Abstract

GNAS exons 1–13 encode the biallelically expressed alpha-subunit of the stimulatory G protein (G α s). Additional transcripts derived from this locus use alternative first exons that undergo parent-specific methylation, thus allowing transcription only from the non-modified allele. Pseudohypoparathyroidism type Ia (PHP1A) is characterized by Albright's Hereditary Osteodystrophy (AHO) and resistance to multiple hormones; this disorder is caused by maternal inactivating mutations involving G α s exons. In contrast, pseudohypoparathyroidism type Ib (PHP1B) is characterized mostly by resistance to PTH and often mild TSH resistance, usually without AHO features. The autosomal dominant variant of PHP1B (AD-PHP1B) is caused by maternal deletions in *GNAS* or *STX16* that reduce G α s expression through loss-of-methylation at *GNAS* exon A/B alone or at multiple differentially methylated regions (DMR). Several large maternal deletions involve not only *GNAS* exons 1–13, but also one or several *GNAS* DMRs, thus causing PHP1A combined with apparent *GNAS* epigenetic changes that are indistinguishable from those observed in PHP1B. Some of these deletions include a large CpG island extending from exon A/B to the intron between *GNAS* exons 1 and 2, but there is no evidence for parent-specific exon 1 methylation. We now describe a family in which the female proband and her daughter presented with hypocalcemia, elevated PTH levels, shortened metacarpals, and obesity, but without obvious neurocognitive abnormalities. A maternally inherited 2015-bp deletion that includes *GNAS* exon 1 was identified thereby establishing the diagnosis of PHP1A. The centromeric deletion breakpoint is located 178 bp upstream of exon 1, yet no methylation changes were observed at exon A/B. This novel deletion therefore refines further the region between exon A/B and exon 1 that is critical for establishing or maintaining normal methylation at *GNAS* exon A/B. 1.

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1. Introduction

Several related disorders, including pseudohypoparathyroidism type Ia and type Ib (PHP1A and PHP1B) as well as pseudopseudohypoparathyroidism (PPHP), are caused by genetic or epigenetic abnormalities involving different loci within the telomeric region of chromosome 20q [1–4].

PHP1A is caused by inactivating mutations affecting the maternal *GNAS* exons 1–13 that encode the alpha-subunit of the stimulatory G protein (G α s). When located on the paternal allele, the same or similar *GNAS* mutations cause PPHP (Fig. 1). Heterozygous *GNAS* mutations on either parental allele lead to G α s haploin sufficiency, but hormonal resistance resulting in biochemical abnormalities occurs only if the genetic defect resides on the maternal allele. This effect specific for maternal *GNAS* mutations is due to reduced or absent G α s expression from the paternal allele in select tissues.

In contrast to PHP1A, hormonal resistance encountered in the different forms of PHP1B is not caused by mutations that impair or abolish G α s function, but instead by epigenetic changes at the maternal *GNAS* locus that reduce G α s expression from this parental allele. For example, some of the autosomal dominant forms of PHP1B (AD-PHP1B) are associated with loss-of-methylation (LOM) at *GNAS* exons at A/B, AS, and XL [4–6], which are caused by deletions affecting maternal *GNAS* exons NESP and/or AS3–4 (see Fig. 1). The most frequent form of AD-PHP1B, in which LOM is restricted to *GNAS* exon A/B alone, is caused by maternal deletions comprising *STX16*, the gene encoding syntaxin 16; another variant is due to a maternal deletion of *GNAS* exon NESP and the adjacent centromeric intron [7–11].

However, most PHP1B patients are affected by a sporadic form of the disease (sporPHP1B). Few of these cases are caused by paternal uniparental isodisomy involving chromosome 20q (patUPD20q) [12–14], but the majority of sporPHP1B patients remains undefined at the molecular level. These isolated cases typically display similarly broad *GNAS* methylation changes as those observed in patients with maternal NESP and/or AS3–4 deletions, namely a loss of all maternal methylation imprints, as well as biallelic methylation at exon NESP. Unlike the inherited forms of PHP1B, epigenetic *GNAS* changes in the sporadic variant can be incomplete [15–18] and it appears plausible that at least some of these sporPHP1B patients lack an as-of-yet unknown, *trans* acting factor that is required for maintaining or establishing all of the maternal *GNAS* methylation imprints.

Epigenetic changes resulting in the biallelic expression of A/B transcripts that reduce G α s expression were furthermore observed in the affected members of an AD-PHP1B family with a large inversion involving *GNAS* exon A/B and all exons encoding G α s [19]. This unusual mutation, which resembles some of the findings in PHP1B patients with duplications or triplications involving large regions centromeric of exon A/B [20,21], causes clinical and biochemical abnormalities similar to those encountered in the presence of other maternal deletions [5,6, 22].

Heterozygous inactivating mutations involving the maternal *GNAS* exons 1–13, i.e. those exons encoding G α s, lead to pseudohypoparathyroidism type Ia (PHP1A), typically a more

severe disease variant in which affected individuals display not only resistance towards different hormones, but also several developmental abnormalities [1,4,23,24]. These include brachydactyly, short stature, early-onset obesity, various degrees of neurocognitive impairment, and other as-yet incompletely defined abnormalities, i.e. clinical findings that are now referred to as Albrights Hereditary Osteodystrophy (AHO).

In some tissues, such as the proximal renal tubules, the thyroid, and several other tissues, *Gαs* is predominantly expressed from the maternal *GNAS* allele [2,25–29]. Consequently inactivating *Gαs* mutations or *STX16*/*GNAS* deletions involving this parental allele dramatically reduce or abolish *Gαs* expression in these tissues, thereby causing symptomatic hypocalcemia and hyperphosphatemia because of PTH-resistance in the proximal renal tubule. In addition, resistance occurs towards other hormones that mediate their actions through *Gαs*-coupled receptors.

Small deletions involving only one or few *GNAS* exons encoding *Gαs* are rare. Such mutations can extend from the region between exons XL and A/B to intron 5 (chr20:57,449,970-57,480,400; all sequence data are based on GRCh37/Hg19) [30], from exon A/B to the middle of the intron between exons E1 and E2 [31], or comprise the entire *GNAS* locus [32–34] (see Fig. 1). The patients with either of these deletions were diagnosed with PHP1A because of characteristic AHO features and laboratory changes consistent with this diagnosis. Maternal deletions involving exon A/B alone would lead to an apparent loss-of-methylation at this DMR, while the other DMRs reveal no abnormalities; in contrast to these predictions, larger deletions show apparent methylation changes at all DMRs. The only deletion comprising a small portion of *GNAS* exon 1 (E1) was reported for a small family in which several members are affected by PHP1A [35]; however, no epigenetic findings were reported since parent-specific methylation of the *GNAS* locus had not yet been established at the time [36–40].

Based on genome-wide maps (www.Genome.UCSC.edu), one large CpG island extends from the region centromeric of *GNAS* exon A/B to the intron between exons E1 and E2. However, unlike the DMR at exon A/B, the E1 exon does not undergo parent-specific methylation [36–39]. Methylation of the maternal allele downstream of exon A/B extends at least to nucleotide 57,465,295 of chromosome 20q [41], which is consistent with data reported for the mouse *Gnas* locus [42]. We have now identified a novel 2015-bp deletion comprising the entire E1 exon and the surrounding intronic region in a mother and her daughter, who are both affected by PHP1A, yet show no *GNAS* methylation changes. These findings indicate that the methylated portion of the exon A/B DMR does not extend into the promoter up-stream of exon E1, which is consistent with biallelic expression of *Gαs* in most tissues.

2. Patients and methods

2.1. Patients and healthy family members

All biochemical measurements had been performed at the Department of Endocrinology and Metabolism of Ankara Training and Research Hospital, Ankara, Turkey. Genomic DNA was extracted from peripheral blood leukocytes using standard methods. Analyses were

performed with informed consent from each adult family member and with maternal consent for the two children using forms approved by the Institutional Review Board of the Massachusetts General Hospital.

2.2. Multiplex ligation-dependent probe amplification (MLPA) of STX16 and GNAS

MLPA was performed using kit ME031 GNAS (MRC-Holland, Amsterdam, The Netherlands) following the manufacturer's instructions (<https://www.mlpa.com/>), as described [13,22]. PCR products were examined using the ABI3730xl Genetic Analyzer at the DNA Core Facility of the Massachusetts General Hospital and Peak Scanner 2.0 software (<http://peak-scanner-software.software.informer.com/2.0/>).

2.3. GNAS methylation analysis

Epigenetic *GNAS* changes were assessed by MS-MLPA, as previously described [13,22].

2.4. Single nucleotide polymorphisms (SNPs) and definition of the GNAS exon 1 deletion

The SNPs rs55830103 and rs12481574 were analyzed as described [13,22]. Primers for amplification across the exon 1 deletion: forward primer (a): 5'-ccaccatcgctgtgtagtgt-3'; reverse primer (b): 5'-tgcacaaaaggtgttgaaa-3'. The same primers were used for nucleotide sequence analysis of the PCR amplicon.

3. Results

The proband is a 36-year old female (192/I-2), born at term as the second child of nonconsanguineous parents; birth weight 4500 g. At the age of 29, she presented with muscle weakness and numbness in her fingers after giving birth to her first child (192/II-1) and laboratory studies revealed hypocalcemia, hyperphosphatemia and high PTH levels (data not available). Additionally, she displayed AHO features including round face, short stature, short third and fourth metacarpals (Archibald's sign) and a short fourth metatarsal of left foot, as well as obesity (BMI: 33.3). Moreover, due to her rapid weight loss four months after delivery additional laboratory studies were performed that revealed postpartum thyroiditis and after few months she developed hypothyroidism with high TSH levels 22 μ IU/L (normal range: 0.4–4.5) and elevated anti-thyroid peroxidase (TPO) antibody titers (>1000, normal: 0–9). Based on these findings, she was diagnosed with pseudohypoparathyroidism and autoimmune hypothyroidism, and treatment with levothyroxine, calcitriol, and calcium was started. Her complaints disappeared over time.

Her most recent laboratory findings during treatment showed a normal calcium level (9.10mg/dl; normal range: 8.5–10.5), yet a phosphate level at the upper end of normal (4.50 mg/dl; adult normal range: 2.5–4.5) and a significantly elevated PTH level of 120 pg/ml (normal range: 15–65). The patient was euthyroid while receiving levothyroxine (Fig. 2A). Radiographic studies at the age of 35 showed shortening of several metacarpals (Suppl. Fig. 1).

Her daughter (192/II-1) was born at 36 weeks of gestation; birth weight was 3600 g. Because of an elevated TSH (12 μ IU/L, normal: 0.4–4.5) at 8 months of age, she was

diagnosed with hypothyroidism and treatment with levothyroxine was initiated. During a routine visit to her pediatrician in 2015, at the age of 6 years, no symptoms of hypocalcemia were noted, but physical examination revealed short third and fourth metacarpals, round face, and obesity; cognitive function is apparently normal. Because of these clinical findings and her mother's diagnosis of PHP, calcium, phosphate, and PTH were measured. These studies revealed a much reduced total calcium level (6.96 mg/dl), an elevated blood phosphate level (7.82 mg/dl), and a considerably elevated PTH level (639 pg/ml); the 25OHD level was not measured. Treatment with calcitriol at 0.25 mcg bid was commenced. When laboratory studies were repeated two years later (Fig. 2A), she had normal total calcium (9.0 mg/dL; normal: 8.5–10.5) and improved blood phosphate levels (5.8 mg/dL; normal: 2.5–4.5), but the PTH level remained significantly elevated (650 pg/ml; normal: 15–65); the ALP level was elevated (226 U/L; normal: <141) and the 25(OH) vitamin D level was low (13.2 µg/L; recommended: >30),

The proband's mother (192/0-1) revealed, at the age of 63 years, normal calcium and phosphate levels (9.48 and 4.27 mg/dl, respectively), yet an elevated PTH level (153 pg/ml) associated with a low 25OH vitamin D level (10.5 µg/L) and Hashimoto's hypothyroidism with elevated anti-TPO titers (data not shown); the proband's 34 year-old sister (192/I-3) and the proband's 10 year-old son (192/II-2) are both unaffected and revealed no laboratory or radiographic abnormalities.

PHP1A was considered as the underlying disorder because of short metacarpals; however, patients affected by either PHP1A or PHP1B can present with foreshortened metacarpals and/or -tarsals. The diagnosis of PHP1B was therefore considered as well, particularly because the proband had shown normal neurocognitive development. The 3-kb deletion in *STX16* as the most frequent cause of AD-PHP1B was excluded and analysis of five microsatellite markers, 907-rep2, 261P9-CA1, 806-CA-80085, 543J19-TTA, and D20S171 [7,22], revealed no evidence for discordance between the proband 192/I-2 and her daughter 192/II-1, thus making a large chromosomal deletion unlikely (data not shown).

Next, we performed MLPA and MS-MLPA to search for deletions and/or epigenetic *GNAS* changes. For the proband and her daughter, these studies provided evidence for a heterozygous deletion restricted to *GNAS* exon 1, yet normal methylation at all four DMRs within this complex locus (Fig. 2B). To define the extent of the suspected deletion, we investigated several SNPs that are located centromeric and telomeric of exon 1 (Fig. 2C). Two of these SNPs, rs55830103 and rs12481574, were heterozygous for the proband or her daughter, thus delimiting the deletion. PCR using primers flanking these SNPs (Fig. 3A) amplified a product of approximately 1100 bp from genomic DNA of the proband and her daughter (Fig. 3B). The same deletion was furthermore identified in DNA of the proband's mother (192/0-1), but not in her unaffected son (192/II-2), her unaffected sister (192/I-3), and her unaffected husband (192/I-1).

Nucleotide sequence analysis of the amplicons from 192/I-2 and 192/II-1, using the primers used in the PCR, revealed that 2015-bp are deleted between nucleotides 57,466,247 and 57,468,263 on chromosome 20q (Fig. 3C). The centromeric breakpoint of the deletion resides 178 nucleotides upstream of *GNAS* exon 1.

4. Discussion

Deletions involving small regions affecting those *GNAS* exons that encode *Gαs* are rarely described as a cause of pseudohypoparathyroidism; in fact, besides the 2015-bp deletion presented in this report, only one other deletion, which removes portions of exon 1, has been identified in a family with multiple members affected by PHP1A [35]. However, numerous point mutations have been reported for this exon (reviewed in [34]), the majority of which are nonsense mutations. These findings in PHP1A patients strongly suggest that alternative translational start sites in other *GNAS* exons do not provide a functional *Gαs*.

The region comprising exon A/B and exon 1 is located within a single large CpG island extending from the region centromeric of *GNAS* exon A/B to intron 1 (www.Genome.UCSC.edu). However, while exon A/B and flanking intronic regions are differentially methylated, the region comprising *GNAS* exon 1 is not known to undergo parent-specific methylation [36–39,41]. In fact, Rochtus et al. have shown that methylation of the maternal *GNAS* allele extends at least to nucleotide 57,465,295, which is 654 nucleotides down-stream of exon A/B [41]; this finding is consistent with data reported for the mouse *Gnas* locus [42].

The novel deletion involving *GNAS* exon 1 led, in the proband (192/I-2) and her daughter (192/II-1), to a phenotype consistent with PHP1A indicating that fully functional *Gαs* is not made from the maternal *GNAS* allele. The proband's mother (192/O-1), who carries the same deletion, did not show evidence for brachydactyly, yet revealed a significant elevation in PTH despite normal serum calcium levels. Her 25OHD level was well below the recommended range, consistent with vitamin D deficiency which could potentially explain her secondary hyperparathyroidism. However, her serum phosphate level was at the upper end of the adult normal range, making PTH-resistance more plausible. Despite the uncertainties regarding the diagnosis for 192/O-1, she has clearly passed the *GNAS* exon 1 deletion to her daughter 192/I-2 and granddaughter 192/II-1; maternal inheritance of the mutation thus provided a reasonable explanation for the laboratory and radiographic findings in both.

The identified *GNAS* exon 1 deletion caused no abnormalities in parent-specific methylation at exon A/B, indicating that the mutation does not lead to epigenetic changes at CpG residues much further telomeric than nucleotide 57,465,295, i.e. the 3'-end of the most telomeric probe (chr20:57,464,928-57,465,295) that was shown to detect differential methylation at *GNAS* exon A/B [41]. Thus, 952 nucleotides between chr20:57,465,295 and chr20:57,466,247 (beginning of the *GNAS* deletion identified herein) could potentially play a role in establishing or maintaining parent-specific methylation at *GNAS* exon A/B (Fig. 3). However, genome-wide assessment of methylation (UCSC Genome Browser) has shown that intermediate methylation (namely methylation of only one parental allele) does not appear extend beyond nucleotide 57,465,500, indicating that some of the 952 nucleotides do not undergo epigenetic changes.

It is now widely recognized that there can be considerable clinical and laboratory overlap between PHP1A and PHP1B [22,24,40,43–51]. Furthermore, some patients with PHP1A can

show apparent *GNAS* methylation changes, normally indicative of PHP1B, if a maternal deletion involves one or several *GNAS* DMRs [30–34]. It may therefore be necessary to screen patients, who are clinically and biochemically classified as PHP1A, for deletions involving *GNAS* exons 1–13, as was done by Garin et al. [31]. In fact, defining the genetic defect in family 192 would have been difficult without MLPA, since direct nucleotide sequence analysis would have most likely failed to identify any abnormality, especially since exon 1 is more difficult to investigate due to high GC content. Moreover, primers for the sequence analysis of this exon would most likely reside within the deleted region, thus leading to the PCR amplification of the intact allele only.

In summary, we described a family in which two females are affected by PHP1A, which is most likely caused by a maternally inherited, novel 2015-bp deletion involving *GNAS* exon 1 and adjacent intronic sequences. Although the deletion extends towards exon A/B, no methylation changes were observed at this *GNAS* DMR, indicating that the deleted region is not involved in establishing or maintaining methylation at this site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic depiction of the region extending from the syntaxin 16 (*STX16*) gene to the *GNAS* complex locus located about 250 kb further telomeric. Maternal (Mat.) and paternal (Pat.) alleles are shown; boxes represent *STX16* and the different *GNAS* exons. Several alternatively spliced transcripts are derived from *GNAS*: *Gsα* transcript, exons 1–13; A/B transcript, exon A/B spliced onto exons 2–13; XLas s transcript, exon XL spliced onto exons 2–13; NESP55 transcript, exon NESP spliced onto exons 2–13. Note that the NESP exon comprises an in-frame termination codon and the portion of the NESP55 mRNA derived from exons 2–13 is therefore located in the 3′-non-coding region. A non-coding antisense mRNA is derived from antisense exons 1–5 (numbers in italics). Note that *Gsα* is expressed in most tissues from both parental alleles; however, in proximal renal tubules, thyroid, pituitary, and most likely other tissues/cells *Gsα* expression from the paternal *GNAS* allele is reduced or absent. The regions comprising mutations that cause of either PPHP (mutations affecting the paternal allele) or PHP1A (mutations affecting the maternal allele), or the different mutations or epigenetic changes that cause PHP1B are indicated by brackets; note that most sporadic PHP1B variants have not yet been defined at the molecular level, but affected patients usually show a complete loss of all maternal *GNAS* methylation imprints. The locations of these differentially methylated regions are indicated by **.

Fig. 2.

Laboratory, genetic, and epigenetic findings in a family in which several members are affected by pseudohypoparathyroidism. Panel A: Pedigree of the investigated family 192, which is consistent with an autosomal dominant mode of inheritance; arrow, proband; circles, females; squares, males; black symbols, affected; white symbols, unaffected; grey symbol, carrier status not clearly defined. Age and laboratory findings when investigated for this study. Methylation status at *GNAS* exons A/B, XL, AS, and NESP, as determined by MSMLPA, is provided. Analysis of two SNPs (rs55830103 and rs12481574) for the proband, her husband and her daughter; -, not available or not determined; nl, normal. Panel B: MLPA of the *STX16-GNAS* region for the proband (results obtained for her daughter were essentially identical; not shown). Note that two or more probes were available for *STX16* and *GNAS* exons NESP, AS, XL, A/B, and exon 1; only one probe for other *GNAS* exons. Panel C: Schematic depiction of the region extending from *STX16* to *GNAS* and location of the two investigated SNPs.

Fig. 3.

Identification and location of the *GNAS* exon 1 deletion. Panel A: Schematic depiction of the region comprising *GNAS* exon A/B and exons 1–13; locations of the two investigated SNPs (vertical arrows) and the primers for PCR amplification (horizontal arrows) are indicated. Panel B: PCR amplification (primers a and b) across the deletion involving *GNAS* exon 1 using genomic DNA from the available family members. Note that an amplicon of approximately 1100 bp was observed for 192 family members I-2, II-1, and O-1. Panel C: Nucleotide sequence analysis of the PCR product shown in panel B. In the affected family members, a 2015-bp deletion was identified that extends from 178 bp upstream to 1342 bp

downstream of exon 1 (495 bp in size). The purple dashed line depicts the extend of monoallelic methylation as determined through genome-wide analyses (UCSC Genome Browser); the red horizontal bar indicates the location of the most telomeric position for monoallelic DNA methylation [41]. Based on these findings it plausible that additional portions of the 952 bp (dashed black line) undergo differential methylation between exons A/B and 1. The breakpoints of the identified 2015-bp deletion are indicated (GRCh37/Hg19). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)