Research Article

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Type Ib of pseudohypoparathyroidism is caused by a novel GNAS duplication associated with a loss-of-methylation limited to exons A and B (PHP1B).

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ABSTRACT

The hallmarks of pseudohypoparathyroidism type Ib (PHP1B) include resistance to parathyroid hormone (PTH), which causes hyperphosphatemia and hypocalcemia, as well as occasionally resistance to other hormones. A loss-of-methylation (LOM) at the differentially methylated GNAS exon A/B is a common feature among patients with this condition, since it lowers the production of the stimulatory G protein α-subunit (Gsα) from the maternal allele. In the proximal renal tubules, where the paternal GNAS allele has a negligible effect on the expression of this signaling protein, this results in little to no expression of Gsα, which causes PTH resistance. We now present the case of PHP1B patient with a de novo genomic GNAS duplication of about 88 kb linked to LOM limited to exon A/B.Comparative genomic hybridization (CGH), whole-genome sequencing (WGS), and multiplex ligation-dependent probe amplification (MLPA) all confirmed that the duplicated DNA fragment extends from GNAS exon AS1 (telomeric breakpoint) to a brief area between two imperfect repeats, directly upstream of LOC105372695 (centromeric breakpoint). Our unique duplication is significantly shorter than the duplications/triplications in that region of chromosome 20q13 that have been previously reported, and it has no effect on the methylation at exons AS and XL. It is likely that the detected genetic aberration interferes with a transcript's ability to create or sustain exon A/B methylation in cis, based on these and other studies. Our research expands on the molecular reasons of PHP1B and offers further understanding of the structural GNAS characteristics needed to preserve maternal Gsα expression and avoid PTH resistance. Copyright 2020 The American Society for Mineral Research (ASBMR) All rights reserved.

Keywords : PTH/Vit D/FGF23; CELL/TISSUE SIGNALING; ENDOCRINE PATHWAYS; PARATHYROID-RELATED DISORDERS; DISORDERS OF CALCIUM / PHOSPHATE METABOLISM; EPIGENETICS; GENETIC RESEARCH

INTRODUCTION

Maternal mutations affecting GNAS exons 1–13, which encode the α-subunit of the stimulatory G protein (Gsα), result in pseudohypoparathyroidism type Ia (PHP1A; MIM #103580).
In most tissues, Gsα is expressed in both directions. However, Gsα expression from the paternal allele is suppressed in a few tissues, such as brown adipocytes and proximal renal tubules, as well as some cells in the thyroid, pituitary, and certain areas of the central nervous system, through mechanisms that are still unknown.(1–5) Because inactivating mutations affect the maternal GNAS exons 1–13, little or no Gsα protein is produced in these organs, which explains hormone resistance at various Gsα-coupled receptors.(6–9) Apart from Gsα, other substitute first exons and their promoters result in the production of multiple other mRNA variations that are translated from the GNAS complex locus. These comprise the neuroendocrine secretory protein (NESP) transcript, which is derived from the maternal allele, and the XL and A/B transcripts, which are derived from the paternal allele. These three mRNAs are

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distinct due to their distinct first exons, but they are identical in their 30-regions because they splice onto GNAS exons 2–13 (Fig. 1A).The many variants of pseudohypoparathyroidism type Ib (PHP1B; MIM 603233) are produced by epigenetic problems at several differentially methylated regions (DMRs) within GNAS, as opposed to the genetic flaws that cause PHP1A through mutations impacting the GNAS exons encoding Gsα. Because all PHP1B variations share a loss-of-methylation (LOM) at the maternal GNAS exon A/B, this trait is very significant.

Because of resistance to parathyroid hormone (PTH) in the proximal renal tubules, individuals with PHP1A or PHP1B experience hypocalcemia and hyperphosphatemia. This results in insufficient synthesis of 1,25(OH)2 vitamin D and insufficient downregulation of the sodium-dependent phosphate co-transporters.(10,11) PHP1A and PHP1B both commonly exhibit resistance to other hormones that work through Gsα-coupled receptors, but PHP1A is more likely to exhibit shorter metacarpals and/or metatarsals, which are prominent characteristics of Albright's hereditary osteodystrophy (AHO).(6–9) The majority of family instances of autosomal dominant PHP1B (AD-PHP1B) result from a 3-kb deletion of STX16, if present on the maternal allele, which causes LOM at GNAS exon A/B alone.(12–16) Patients with other maternally inherited mutations also exhibit the same epigenetic changes as those caused by this recurrent STX16 deletion, specifically LOM restricted to exon A/B. These patients include STX16 deletions larger than 3 kb, deletions of GNAS exon NESP and the adjacent centromeric region, large inversions involving exon A/B and all of the exons encoding Gsα, and a few duplications/triplications involving the centromeric region of exon A/B.(19,20) These mutations cause the exon A/B promoter to express biallelicly, which lowers the production of Gsα (21 and consequently result in Gsα deficit in organs like the proximal renal tubules, where the maternal allele is the primary source of this signaling protein. Furthermore, the A/B transcript produces an amino-terminally shortened version of Gsα that can decrease agonist response by employing an initiator AUG found in GNAS exon 3.(22) Reduced Gsα expression and the A/B transcript's ability to produce an antagonist could be the two mechanisms causing PHP1B's hormonal resistance.

A large inherited duplication of the entire GNAS locus or inherited deletions comprising NESP and/or several AS exons that result in an apparent increase in methylation at the NESP DMR can cause loss of all maternal GNAS methylation imprints, in contrast to genetic mutations that cause LOM at GNAS exon A/B alone.(19, 23–25) The most common form of PHP1B, however, is sporadic (sporPHP1B), linked to gain-of-methylation (GOM) at exon NESP and loss-of-methylation (LOM) at the three maternal GNAS DMRs.(12, 25–29) The underlying molecular mechanisms of the epigenetic GNAS modifications resulting in sporadic PHP1B remain unclear, with the exception of cases attributed to paternal uniparental disomy involving chromosome 20q (patUPD20q).(30–35) We now report on a PHP1B patient who had a de novo genomic duplication that spanned approximately 88 kb and had a telomeric breakpoint at exon AS1. The patient had LOM at GNAS exon A/B alone and an apparent partial LOM at exon NESP. The duplication's location and extent may be used to inform theories about the mechanisms that produce LOM at GNAS exon A/B alone and, consequently, this disease.

PATIENTS AND APPROACHES

Results in a PHP1B sufferer and his relatives who are not affected We looked at a guy, now 30 years old (249/II-1), whose medical history was normal up until the tenth year of high school, when he started to experience extreme exhaustion that prevented him from attending classes for the entire day and from engaging in extracurricular activities. Following graduation, he spent a year studying in England. He experienced paresthesias and numbness in both forearms and both legs below the knees in the fall of that year. At first, this was assumed to be a secondary symptom of anxiousness. The patient was hospitalized for a second episode of acute exhaustion during the winter following a viral infection; physical examination results showed a positive Chvostek's sign.The results of laboratory tests conducted at the time showed low levels of 25-vitamin D (21 ng/mL), increased blood PTH (164 pg/mL), and an extremely low total serum calcium level (5.8 mg/dL). Three months after his symptoms improved with therapy with Alfacalcidol (1 μg daily) and Calcichew D3 (500 mg elemental calcium and 200 IU cholesterol), he made the decision to stop taking both drugs. When he returned to Boston two months later, one of the authors (JP) examined him and saw that he had a somewhat rounder face and mild bilateral shortening of the fourth metacarpals, but that his knuckles appeared normal. As a result, no radiographic tests of the hands were taken.A review of his test data at the time showed that his phosphate was 4.4 mg/dL, magnesium was 2.3 mg/dL, 25-vitamin D was 25 ng/mL, and 1,25(OH)2 vitamin D was 58 pg/mL. He also had low serum calcium (7.6 mg/dL) and increased PTH (202 pg/mL). With a free T4 (fT4) of 1.0 ng/dL, the TSH was measured at 3.6 μU/mL. The patient began receiving treatment with calcium supplements (2000 mg daily) and calcitriol (0.25 μg twice daily). The patient is a graduate student at a German institution at the moment.

A review of the family medical history did not turn up any evidence of hypocalcemia-related disorders. The Massachusetts General Hospital Institutional Review Board (MGH-IRB)-approved process was used to obtain written consent before drawing blood from the boy, his two brothers,

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and both parents for genomic DNA extraction.

Pyrosequence analysis of the STX16/GNAS region, methylation-sensitive multiplex ligation-dependent probe amplification, and multiplex ligation-dependent probe amplification Utilizing kit ME031 GNAS (MRC-Holland, Amsterdam, The Netherlands), multiplex ligation-dependent probe amplification (MLPA) and methylation-sensitive MLPA (MS-MLPA) were carried out in accordance with manufacturer's instructions (MRC Holland: Confi-Scanner 2.0 software (http://peak-scanner-software.software.informer.com/2.0/). The GNAS region was pyrosequenced according to the instructions(36), using the primers and PCR settings listed in Supplementary Table S1. dence in Copy Number Determination; https://www.mlpa.com/), as stated.(17,34) PCR results were analyzed at the Massachusetts General Hospital's CCIB DNA Core Facility (Cambridge, MA) using the ABI3730xl Genetic Analyzer and Peak Complete genome sequencing and comparative genomic hybridization As stated in reference (20), comparative genomic hybridization (CGH) was carried out; at the Broad Institute in Cambridge, Massachusetts, USA, whole-genome sequencing (WGS) was carried out with an average coverage of 30 x.

Examination of the GNAS region's duplication

GNAS-AS1-F (50 -CAATGTCTACTGACCTTCCACTTGT-30) and Cent249-3R (50 -ACACACAGCTTTGTCCTGAGTTT-30) are two primers that were created (Primer 3 software; https://primer3.org/) for PCR amplification of a 337-bp product in order to ascertain the orientation of the duplicated GNAS region and how the duplicated DNA fragments are fused together. Using QIAGEN Taq DNA polymerase (QIAGEN, Valencia, CA, USA), PCR was carried out in accordance with the manufacturer's instructions. The cycler program consisted of five minutes of denaturation at 94 degrees Celsius, thirty cycles at 94, 58, and 72 degrees Celsius, and a final elongation step at 72 degrees Celsius for ten minutes.

Four primers were created in order to evaluate the duplication's centromeric and telomeric breakpoints. Forward primer Cent249wt-F (50 -AATTTGATCAGTGGCATATCTGAAT-30) and reverse primer Dup249-R (50 -TAAGTGTTTCACTGTTCTGCAAGTC-30) were used (1149 bp) for amplification across the centromeric breakpoint; note that the resulting PCR product includes SNP rs13038557; cycler program: denaturation at 94 C for 5 min, followed by 35 cycles at 94 C for 30s, 58 C for 30s, and 72 C for 60s, with an additional elongation step at 72 C for 10 min.Forward primer GNAS-AS1-F and reverse primer AS1-249-2R (50 -CAAGACGGCTTAACCACTTGAG-30) were used for amplification across the telomeric breakpoint; the cycler program consisted of 5 minutes of denaturation at 94 degrees Celsius, 35 cycles of 30 seconds at 94 degrees Celsius, 30 seconds at 58 degrees Celsius, and 30 seconds at 72 degrees Celsius, followed by an additional elongation step at 72 degrees Celsius for 10 minutes. A multiplex PCR was conducted to ascertain whether the brothers or the parents carry the mutant allele. The forward primer GNAS-AS1-F was combined with the reverse primers Cent249-3R and AS1-249-2R to enable amplification across the 337 bp site where the two duplicated DNA fragments on the mutant allele are joined, as well as amplification of the wild-type allele (567 bp). ExoSap-IT (Affymetrix, Santa Clara, CA, USA) was used to purify all PCR products, and the Massachusetts General Hospital's DNA core facility (Boston, MA, USA) performed the sequencing.

RESULTS

Medical history, PTH-resistant hypocalcemia and hyperphosphatemia in the lab, and the absence of significant AHO characteristics all pointed to PHP1B as the cause of our patient 249/II-1's condition.

First, MLPA and MS-MLPA were used to investigate his genomic DNA in order to corroborate this preliminary diagnosis. These investigations showed that the GNAS exons NESP, AS4, and AS3 had copy numbers that were roughly 50% higher than normal, indicating that these regions of the genetic locus had been duplicated (Fig. 1B). No proof of a deletion involving those STX16 or GNAS was found.Exons A and B displayed full LOM, despite areas that are known to be connected to methylation alterations impacting the maternal GNAS allele (12–17, 23–25). The DMRs at GNAS exons XL and AS1, on the other hand, showed results that were identical to control DNA samples, indicating about 50% methylation. Nevertheless, assessing the exon NESP DMR revealed an apparent partial reduction in methylation to 35.3 ~ 1.3% (mean ~ SD for three distinct probes and two independent assays) (Fig. 1C).

Pyrosequencing was carried out for the four GNAS DMRs employing in order to validate the methylation alterations determined by MS-MLPA.prior known procedures (36); for primers for amplification and sequencing, see Supplementary Table S1. The DMRs at exons AS and XL revealed normal methylation (46.3% ~ 4.0% and 44.6% ~ 1.3%, respectively); these studies validated the decrease in methylation at the DMRs for exons A/B (4.6% ~ 1.1%) and NESP (34.8% ~ 2.5%) (Fig. 1D).

When combined, these results supported a duplication that included the maternal exons NESP, AS4, and AS3, but not the exons encoding Gsα, XL, A/B, or AS1. While LOM at GNAS exon A/B, as identified by pyrosequencing and MS-MLPA, demonstrated that patient 249/II-1 is impacted by PHP1B, the underlying genetic abnormality that gave rise to this epigenetic abnormality seemed to be complicated.

It appears that the mutation in our patient happened de novo because MLPA and MS-MLPA analysis of genomic DNA from both parents revealed no indication of a copy number variation and no methylation anomaly within the GNAS gene (data not shown).

We used a previously published custom array to do comparative genomic hybridization (CGH) analysis on the patient's DNA in order to validate and expand upon these results.(20) These investigations provided evidence for an approximately 88-kb duplication that starts at GNAS exon AS1 and terminates near the centromeric region of loc105372695 (NR_134566), where a 213-bp imperfect repeat sequence is seen (Fig. 2). In addition to identifying other chimeric nucleotide sequences that included fragments of the duplicated region, WGS validated this duplication. However, only parts of the duplicated region were present in these chimeric sequences; genomic sequences from outside the presumed genomic duplication were absent (see Fig. 2, middle panels).Additionally, the data indicated that the duplication had occurred in a head-to-tail orientation and had extended from the centromeric breakpoint at nucleotide chr20:58,762,279 near LOC105372695 to the telomeric breakpoint within exon AS1 at chr20:58,850,781 (build 38, hg38). The PCR products produced with primers GNAS-AS1-F and Cent249-3R (Fig. 3) were intended to amplify across the region where the duplicated genomic DNA fragments were suspected to be fused to each other. Nucleotide sequence analysis of these products supported the latter conclusion (see Fig. 2, lower panel).

Additionally, sequence analysis conducted at the telomeric and centromeric break points only found the wild-type nucleotide sequence, excluding the possibility of discovering any other genomic abnormalities, such as little deletions, inversions, or duplications. It should be noted that the single nucleotide polymorphism (SNP) rs13038557 had indicated C/T heterozygosity in patient 249/II-1's WGS; the genomic DNA of his mother and father exhibited T/T and C/T, respectively (data not shown). The PCR result that was amplified across the centromeric breakpoint when employing primers Cent249wt-F and Dup249-R also showed heterozygosity for this SNP, indicating that both alleles had been amplified at the centromeric breakpoint.Using primers GNAS-AS1-F and Dup249-R, which only yields a 928-bp amplicon when the mutant allele is present, PCR analysis of the patient's genomic DNA indicated homozygosity for T, ruling out the possibility that the duplication occurs on the paternal allele with C at rs13038557. We were able to produce the 567-bp amplicon, which is derived from the wild-type allele, for both parents and both brothers by using the forward primer GNAS-AS1-F in combination with the reverse primers Cent249-3R and AS1-249-2R. Only the patient's genomic DNA revealed two amplicons, measuring 337 bp and 567 bp, respectively, indicating the presence of the wild-type and the mutant allele (Supplementary Fig. S1). Nucleotide sequence analysis throughout the region where the duplicated regions of DNA are joined, when combined with MLPA, MS-MLPA, and pyrosequencing, verified that the duplication had happened de novo in the correct head-to-tail orientation on the maternal GNAS allele without additional nucleotide changes.Nucleotide sequence analysis throughout the region where the duplicated regions of DNA are joined, when combined with MLPA, MS-MLPA, and pyrosequencing, verified that the duplication had happened de novo in the correct head-to-tail orientation on the maternal GNAS allele without additional nucleotide changes.

DISCUSSION

Patient 249/II-1 was thought to be impacted by the sporadic PHP1B variation due to his severe hypocalcemia despite raised PTH levels and a negative family history for hypocalcemic disorders. This most common variation of the disease has gain-of-methylation at exon NESP and LOM at the three maternal GNAS DMRs, but no indication of a change in copy number at the GNAS locus.(12, 25–29) However, as shown by MS-MLPA and pyrosequence analysis of the GNAS region, the genomic DNA from our patient showed a complete LOM at GNAS exon A/B alone and an apparent incomplete reduction in methylation at exon NESP, in contrast to the broad epigenetic abnormalities encountered in the sporadic disease.

The modifications in epigenetics were linked to with a roughly 50% increase in copy numbers for exons AS4, AS3, and NESP (since AS2 was not assessed). The modest chromosomal duplication that was thought to exist within the GNAS gene was verified by It was not discovered in the parents' or siblings' DNA, according to CGH and later WGS analysis. There was no evidence of maternal mosaicism, at least not when examining DNA taken from the mother's peripheral blood cells. Exon A/B is typically methylated on the maternal allele, therefore the duplication must have happened de novo on the maternal allele. It is also unclear that further genetic changes outside of the duplicated region are the cause of the condition because WGS of 249/II-1 revealed no indication of additional genetic problems within the STX16/GNAS region.PTH resistance in patient 249/II-1 and other PHP1B instances is explained by the epigenetic change at exon A/B, which results in biallelic production of A/B transcripts and limits the availability of Gsα, potentially through competition with the exon.

This signaling protein is expressed through one promoter. As an alternative, Gsα function can be diminished by a competitive strategy that uses an amino-terminally shortened Gsα variation that hinders the biological activity of full-length

Gsα and is produced from a translational start point in GNAS exon 3.

Because there is one methylated paternal allele and two nonmethylated maternal alleles, the apparent partial LOM at exon NESP can be explained by the found maternal GNAS duplication. But the process that causes LOM at exon A/B is still open. Only three PHP1B cases—Patient 249/II-1—are linked to LOM at exon A/B alone, and both are brought on by a significant genomic duplication or triplication involving the maternal GNAS.

rather than maternal deletions within the STX16/GNAS allele (19, 20).area.(12–16) The duplication found in our patient is less than the poorly defined duplication reported by Perez-Nanclares and colleagues and far simpler than the complicated genomic change reported by Nakamura and colleagues (20).(19) Nonetheless, all three mutations result in identical epigenetic modifications, suggesting that comparable The methylation at exon A/B is disrupted by processes in both our patient and the previously documented cases of AD-PHP1B. Indeed, there's a chance that a cis-regulating factor that inhibits exon A/B methylation establishment or maintenance originates from the centromeric region of exon NESP or that it comprises the actual transcript from NESP. Mice that have a polyadenylation signal inserted into their mother Nesp exon 1 (Tint2 mice) exhibit hypomethylation at the exon 1A DMR(39), which is consistent with NESP involvement. encoded by one exon in humans and two exons in mice, with human exon A/B being comparable to mouse exon 1A.

In the AD-PHP1B kindred reported by Richard and colleagues (17), where exon NESP and its centromere are deleted without altering the AS exons, lack of methylation at the maternal exon A/B DMR alone is also seen. Thus, normal AS expression is expected if the deletion is on the paternal allele. Contrary to a previously discovered deletion involving AS exons 3 and 4, which was shown to decrease exon NESP methylation and to increase exon A/B methylation when inherited paternally, the 18.9-kb deletion from male carriers did not affect GNAS methylation, supporting this conclusion.(24) The maternal 18.9-kb deletion, which eliminates NESP expression, alters the epigenetic GNAS.are comparable to those found in Tint2 mice as a result.Therefore, it is plausible that a factor containing the maternal NESP exon contributes in cis to normal exon A/B methylation. This would account for the results observed in both Tint2 mice and patients with PHP1B variants caused by the STX16/GNAS duplication found in our patient or those previously reported, or by the 18.9-kb deletion that includes NESP,(17) or by a large inversion telomeric of exon XL, (18).(19,20) In conclusion, a PHP1B case revealed the identification of a unique 88,502-kb duplication involving the maternal STX16/GNAS region, which via an as-yet-unknown mechanism results in an isolated LOM at exon A/B. Our research broadens the range of genetic mutations that only affect exon A/B and result in LOM, which affects Gα expression or function and causes proximal tubular PTH resistance.

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