Autosomal Dominant Pseudohypoparathyroidism Type Ib: A Novel Inherited Deletion Ablating *STX16* Causes Loss of Imprinting at the A/B DMR

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Context: Pseudohypoparathyroidism type Ib (PHP-Ib) is a rare imprinting disorder characterized by end-organ resistance to PTH and, frequently, to thyroid-stimulating hormone. PHP-Ib familial form, with an autosomal dominant pattern of transmission (autosomal dominant pseudohypoparathyroidism type Ib [AD-PHP-Ib]), is typically characterized by an isolated loss of methylation at the guanine nucleotide-binding protein α-stimulating activity polypeptide 1 A/B differentially methylated region (DMR), secondary to genetic deletions disrupting the upstream imprinting control region in the syntaxin-16 (STX16) locus. However, deletions described up to now failed to account some cases of patients with a methylation defect limited to the A/B DMR; thus, it is expected the existence of other still unknown rearrangements, undetectable with conventional molecular diagnostic methods.

Objective: We investigated a PHP-Ib patient with a methylation defect limited to the A/B DMR and no known STX16 deletions to find the underlying primary genetic defect.

Patient and Methods: A PHP-Ib patient (hypocalcaemia, hyperphosphataemia, raised serum PTH levels, no vitamin D deficiency) and his unaffected relatives were investigated by methylation specific-multiplex ligand-dependent probe amplification to search for novel pathogenetic defects affecting the guanine nucleotide-binding protein α-stimulating activity polypeptide 1 and STX16 loci.

Results: We report the clinical, biochemical, and molecular analysis of an AD-PHP-Ib patient with a novel STX16 deletion overlapping with previously identified STX16 deletions but that, unlike these genetic defects associated with AD-PHP-Ib, goes unnoticed with commonly used first-level diagnostic techniques.

Conclusions: Our work highlights the importance of performing accurate investigations in PHP-Ib patients with methylation defects to allow precise genetic counseling because, in case of deletions, the segregation ratio is about 50% and the disease phenotype is transmitted in an autosomal dominant fashion via the mother. **(*J Clin Endocrinol Metab* 99: E724–E728, 2014)**

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Abbreviations: AD-PHP-Ib, autosomal dominant pseudohypoparathyroidism type Ib; AHO, Albright hereditary osteodystrophy; DMR, differentially methylated region; fT3, free T_3; fT4, free T_4; GNAS, guanine nucleotide-binding protein α-stimulating activity polypeptide 1; Gsα, α-subunit of the heterotrimeric stimulatory G protein; ICR, imprinting control region; LoM, loss of methylation; MS-MLPA, methylation specific-multiplex ligand-dependent probe amplification; PHP, pseudohypoparathyroidism; STX16, syntaxin-16.

Pseudohypoparathyroidism (PHP) type Ib (Mendelian Inheritance in Man number 603233) is a rare imprinting disorder characterized by end-organ resistance to PTH and, frequently, to TSH as well, without physical features of Albright hereditary osteodystrophy (AHO), such as short stature, rounded face, brachydactyly, ectopic ossifications, and mental retardation, which are instead typical of PHP-Ia (Mendelian Inheritance in Man number 103580). However, during the last decade, a subset of patients with PHP-Ib and variable degrees of AHO have been detected by independent groups, suggesting a molecular overlap between PHP-Ia and PHP-Ib (1, 2).

PHP-Ib has been associated with methylation defects in the guanine nucleotide-binding protein α-stimulating activity polypeptide 1 (GNAS) imprinted cluster (Mendelian Inheritance in Man number 139320), a complex locus that generates five transcripts using alternative first exons. The α-subunit of the heterotrimeric stimulatory G protein (Gsα) is the best characterized GNAS locus product (Figure 1A) (3–6).

Unlike other GNAS products, Gsα promoter is not differentially methylated, and Gsα expression occurs from both alleles in most tissues, albeit in some tissues, including proximal renal tubules, pituitary, gonads, and thyroid, this gene is transcribed predominantly from the maternal allele (7–9). Gsα tissue-specific imprinting is assumed to be controlled by the upstream A/B differentially methylated region (DMR), which likely has cis-acting, methylation-sensitive, and tissue-specific negative regulatory elements for the Gsα promoter because methylation defects affecting the maternal A/B DMR lead to biallelic expression of the A/B transcript and the loss of Gsα expression in renal proximal tubules but have little effect in tissues in which Gsα is biallelically expressed (4, 5, 6, 10, 12). The A/B DMR epigenetic status is maintained by an imprinting

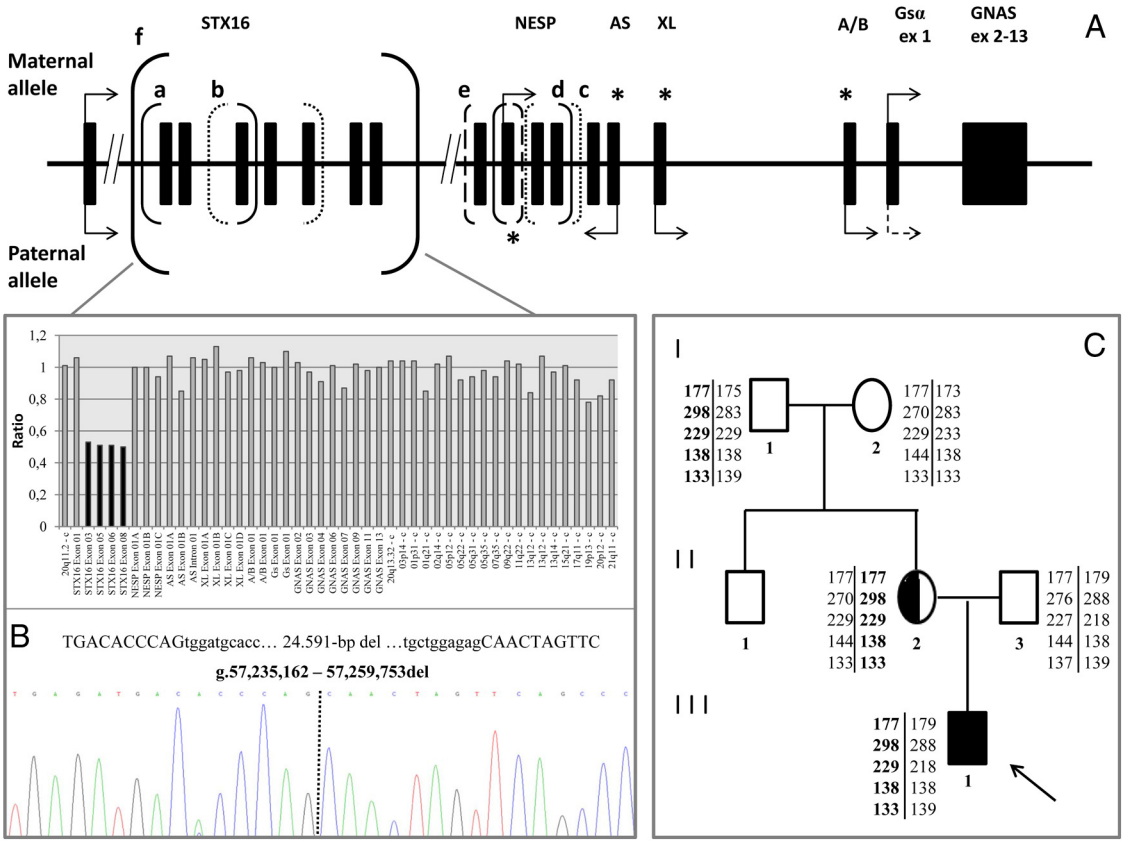

Figure 1. A, Schematic graph of general organization and imprinting at GNAS and STX16 loci. The diagram is not drawn to scale. Exons are indicated as black rectangles, the four DMRs are highlighted with an asterisk on the methylated allele, and the allelic origin of transcription are indicated as arrows on the paternal or the maternal allele. *STX16* and *NESP-AS* deletions are surrounded by brackets: a, Ref. 18; b, Ref. 16; c, Ref. 19; d, Ref. 17; e, Ref. 11; f, the novel deletion identified in the present paper. B, Upper panel. This figure shows a summary chart of the data analysis for copy number determination performed by MS-MLPA. On the x-axis MLPA probes are shown according to their chromosome location, while on the y-axis the final probe ratio, or ploidy status, is shown. Probes specific to *STX16* exons 3–8 (black bars) give a 35%–50% reduced signal during capillary electrophoresis, suggesting the presence of a heterozygous deletion. Lower panel, Wild-type nucleotide sequence and chromatogram of the boundaries of the *STX16* deletion (g.57 235 162–57 259 753del) obtained by Sanger sequencing of the long range PCR. C, Patient's pedigree chart. ◐, Female carrier; ■, affected male; ○, wild-type female; □, wild-type male; →, index. The haplotype of the patient and his relatives is shown near the symbol. The analysis of the grandparents' DNA confirmed that the rearrangement arose on the paternal allele of the mother, as highlighted in bold.

Table 1. Table Resuming Laboratory Findings in the Patient, His Mother, and Maternal Grandparents

	PTH	TSH	FT3	FT4	25-OH-VitD	Ca	P
Index	654.2	3.8	3.5	9.1	29.1	4.7	9.5
Mother	32.9	2.34	2.9	8.9	28.1	8.7	3.1
Grandfather	43.6	1.95	3.09	1.22	22.3	9.7	3.1
Grandmother	63.5	3.53	2.99	1.07	14.1	9.2	3.1

Abbreviations: Ca, calcium; 25-OH-VitD, 25hydroxyvitamin D; P, phosphorus. Normal values are as follows: PTH, 10–65 pg/mL; TSH, 0.3–4 μIU; FT3, 2.3–4.2 pg/mL; FT4, 7.6–14.6 pg/mL or 0.9–2.3 ng/dL; 25-OH-VitD, > 30 ng/mL; Ca, 8.1–10.5 mg/dL; and P, 2.5–4.7 mg/dL.

control region (ICR) located in the upstream syntaxin-16 gene (*STX16*; Mendelian Inheritance in Man number 603666).

PHP-Ib is often sporadic, but it may occasionally present as familial, with an autosomal dominant and maternally inherited pattern of transmission (autosomal dominant pseudohypoparathyroidism type Ib [AD-PHP-Ib]) (13). No clinical differences have been observed between the sporadic and the familial form (14). Sporadic PHP-Ib cases show broad GNAS imprinting abnormalities involving multiple DMRs, with no known underlying genetic lesion (15). AD-PHP-Ib cases are typically caused by an isolated loss of methylation (LoM) at the A/B DMR, secondary to genetic deletions disrupting the STX16 ICR or, less frequently, by the loss of all the maternal GNAS imprints due to deletions removing the NESP55 DMR (11, 16–19) (Figure 1A).

A 3-kb deletion removing *STX16* exons 4–6 is the most frequent genetic defect found in the AD-PHP-Ib form (16), whereas a 4.4-kb deletion overlapping the smaller one and removing *STX16* exons 2–4 has been detected in few familial cases, confirming that the *STX16* gene contains an important regulatory element involved in PHP-Ib pathogenesis (18). Overall, the finding that most patients share a common sized deletion confirms that this chromosomal rearrangement arises through a specific mechanism involving the DNA sequence.

However, routine molecular and cytogenetic diagnostic methods (ie, Sanger sequencing and routine or high resolution karyotyping) fail to detect these deletions in a minority of PHP-Ib patients carrying methylation defects limited to the A/B DMR, suggesting the existence of other unknown genetic rearrangements within the *STX16* gene or elsewhere in the genome (1).

In the present study, we report the clinical, biochemical, and molecular analysis of a PHP-Ib patient with a novel STX16 locus deletion of about 24.6 kb. This new deletion overlaps with previously identified STX16 deletions (16, 18), but, unlike classical genetic defects associated with AD-PHP-Ib, it is undetectable with commonly used molecular diagnostic methods. Our work highlights the importance of performing accurate investigations in apparently sporadic PHP-Ib patients with GNAS methylation defects to give appropriate genetic counseling to familial cases.

Patients and Methods

The proband, a 21-year-old Italian male, was diagnosed as affected by pseudohypoparathyroidism at the age of 13 years, after he was hospitalized for transient loss of consciousness and brief tetanic crisis. Laboratory findings showed a phospho-calcium metabolism disorder (calcium 4.7 mg/dL, normal range 9–10.5 mg/dL; phosphatemia 9.5 mg/dL, normal range 2.8–4.5 mg/dL) consistent with the diagnosis of hypocalcemic tetanus, whose correction led to a significant clinical improvement. The brain computed tomography scan showed the presence of rough calcifications at the level of the basal ganglia and the corticomedullary junction. Hypocalcemia, hyperphosphatemia, and raised serum PTH levels (PTH 654.2 pg/mL, normal range 10–65 pg/mL) in the absence of vitamin D deficiency were suggestive of pseudohypoparathyroidism. No other endocrine disorder was evident at the clinical examination, and the thyroid function was normal [TSH 3.8 μIU, normal range 0.3–4 μIU; free T_3 (fT3) 3.5 pg/mL, normal range 2.3–4.2 pg/mL; free T_4 (fT4) 9.1 pg/mL, normal range 7.6–14.6 pg/mL].

His past clinical history was unremarkable: he did not show signs of AHO and there was no familial history of phospho-calcium metabolism disorders. In particular, after the discovery of a maternally inherited STX16 deletion in the proband, his mother and grandparents were investigated, and laboratory findings confirmed the absence of endocrine defects (Table 1). Informed consent for genetic and epigenetic studies was obtained from the patient and his relatives.

GNAS DMRs methylation status was assessed both by combined bisulfite restriction analysis and methylation specific-multiplex ligand-dependent probe amplification (MS-MLPA) (Supplemental Figures 1A and 2, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The presence of *STX16* gene deletions was investigated by multiplex PCR and MS-MLPA (Supplemental Figure 1, B and C, and Supplemental Figure 2). The novel *STX16* deletion detected in the patient was further confirmed and characterized by Southern blot analysis, semiquantitative PCR, and long-range PCR of the STX16 locus (Figure 1B, lower panel, and Supplemental Figure 3). The inheritance pattern of the deleted allele was confirmed by the segregation analysis of polymorphic microsatellite markers located on the long arm of chromosome 20. An expanded Materials and Methods section describing molecular biology techniques is provided as Supplemental Data.

Results and Discussion

PHP-Ib is caused by the disruption of long-range imprinting control regions regulating GNAS cluster methylation status and is often sporadic, but it may occasionally present as familial, with an autosomal dominant pattern of transmission (AD-PHP-Ib). Hormonal resistance develops only after maternal inheritance of the disease, whereas paternal inheritance of the same defect is not associated with endocrine abnormalities (2). No phenotypic differences have been observed between the sporadic and the familial form (14).

About 10 years ago, the AD-PHP-Ib form was linked to a region on 20q13.3, and Bastepe et al (16) isolated the first causative genetic defect, a 3-kb at STX16 locus, that is the predicted chromosomal location for the ICR controlling the A/B DMR imprinting. During the last decade, different research units reported few other causative STX16 and GNAS loci deletions, demonstrating the need to investigate for these less common genetic defects associated with PHP-Ib (11, 17–19).

Nevertheless, deletions described up to now do not account for a subset of PHP-Ib patients carrying methylation defects limited to the A/B DMR, suggesting the existence of additional, still-unknown rearrangements, undetectable by the diagnostic techniques routinely used for PHP-Ib. Indeed, these genetic defects are undetectable by Sanger sequencing and often below the detection threshold of routine cytogenetic analysis because they may affect segments smaller than band resolution. Such rearrangements can be recurrent, with a common size and fixed break points, or nonrecurrent, with different sizes and distinct break points for each event. A powerful tool for evaluating such subtle genetic abnormalities in PHP patients is MS-MLPA, a multiplex PCR method that allows, at the same time, a targeted investigation of deletions/duplications affecting GNAS and STX16 loci and o the methylation status of GNAS DMRs.

In the present study, we extensively investigated a patient showing a typical PHP-Ib phenotype and an isolated imprinting defect at GNAS A/B DMR but no evidence of classical *STX16* gene deletions.

Methylation analysis of GNAS DMRs by the combined bisulfite restriction analysis demonstrated LoM limited to the A/B DMR and an emimethylated status at NESP, AS, and XL DMRs (Supplemental Figure 1A), but multiplex PCR failed to detect known 3-kb and 4.4-kb *STX16* deletions in this patient (Supplemental Figure 1, B and C). Subsequent MS-MLPA analysis confirmed LoM at the A/B DMR associated with the presence of an extended loss of diploidy affecting the STX16 locus, which pointed to for the presence of a large deletion removing *STX16* exons 3–8 (Figure 1B, upper panel).

To confirm this finding, we performed a Southern blot analysis of patient's genomic DNA, together with his relatives' DNA. Southern blot confirmed a 50% signal reduction in the index compared with a wild-type control. Molecular analysis of available unaffected relatives, parents, and maternal grandparents confirmed that the mutation arose de novo on the paternal allele of the unaffected mother, thus demonstrating the maternal inheritance of the defect in the index (Figure 1C and Supplemental Figure 2). Moreover, MS-MLPA showed that his mother carried the same STX16 deletion without LoM at the A/B DMR. Segregation analysis of polymorphic microsatellite markers located on the long arm of chromosome 20 finally established that the deletion was located on the paternal allele. This finding is in accordance with the observation that individuals carrying paternally derived *STX16* mutations are clinically normal, obligate carriers.

As a first step toward the delimitation of this novel STX16 deletion, we made a semiquantitative amplification of specific sequences in the region spanning from STX16 and NESP loci and within *STX16* intron 1 (Supplemental Figure 3). These experiments allowed the raw localization of upper, between chromosome positions *57 229 525* and *57 236 686*, and lower break points, between *57 257 525* and *57 290 623*.

Finally, to locate break points at the nucleotide level, we performed long-range PCRs using primers flanking the deleted sequence followed by direct sequencing, revealing a 24.591-bp deletion (g.57 235 162–57 259 753del) (Figure 1B, lower panel). Based on these data, the deletion, starting in intron 1, removes *STX16* exons from 2 to 8, overlapping with previously described rearrangements (14, 16). In particular, this nonrecurrent deletion shares a common genomic region of overlap with previously identified 3-kb and 4.4-kb STX16 deletions, which encompasses the critical region containing the putative cis-acting ICR necessary for the maintenance of the methylation imprint at the exon A/B.

In conclusion, in the present work, we have identified and refined the boundaries of a novel *STX16* gene deletion, resulting in a switch from a maternal to a paternal epigenotype at the exon A/B DMR, as a cause of AD-PHP-Ib. Our findings further confirm the parent-of-origin-specific transmission of the disease, the possibility that mothers carrying disease-causing deletions may be clinically unaffected when the genetic defect is on the paternal allele and the existence of control elements within the STX16 region crucial for keeping across generations imprinting at the A/B DMR. Moreover, our work stresses the impor-

tance of performing additional investigations in PHP-Ib patients with apparently sporadic methylation defects to allow precise genetic counseling; as in the case of submicroscopic structural mutations, the disease phenotype is transmitted in an autosomal dominant fashion via the mother.

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