Clinical Research Article

High-throughput Molecular Analysis of Pseudohypoparathyroidism 1b Patients Reveals Novel Genetic and Epigenetic Defects

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Abbreviations: aCGH, comparative genomic hybridization using a highly customized array; AHO, Albright hereditary osteodystrophy; AS, antisense; CNV, copy number variation; DMR, differentially methylated region; GOM, gain of methylation; Gsα, G protein Gs; HLL, high, high, and low methylation; HNL, high, normal, and low methylation; ICR, imprinting control region; iPPSD, inactivating PTH/PTHrP signaling disorder; LCL, lymphoblastoid cell line; LOM, loss of methylation; NNL, normal, normal, and low methylation; patUPD, paternal uniparental disomy; PHP1, pseudohypoparathyroidism type 1; SNP, single nucleotide polymorphism.

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Abstract

Context: Patients with pseudohypoparathyroidism type 1b (PHP1b) show disordered imprinting of the maternal GNAS allele or paternal uniparental disomy (UPD). Genetic deletions in STX16 or in upstream exons of GNAS are present in many familial but not sporadic cases.

Objective: Characterization of epigenetic and genetic defects in patients with PHP1b.

Design and Patients: DNA from 84 subjects, including 26 subjects with sporadic PHP1b, 27 affected subjects and 17 unaffected and/or obligate gene carriers from 12 PHP1b families, 11 healthy individuals, and 3 subjects with PHP1a was subjected to quantitative pyrosequencing of GNAS differentially methylated regions (DMRs), microarray analysis, and microsatellite haplotype analysis.

Setting: Academic medical center.

Main Outcome Measurements: Molecular pathology of PHP1b.

Results: Healthy subjects, unaffected family members and obligate carriers of paternal PHP1b alleles, and subjects with PHP1a showed normal methylation of all DMRs. All PHP1b subjects showed loss of methylation (LOM) at the exon A/B DMR. Affected members of 9 PHP1b kindreds showed LOM only at the exon A/B DMR, which was associated with a
Pseudohypoparathyroidism type 1 (PHP1; also termed inactivating PTH/PTHrP signaling disorder, iPPSD (1)) is a classical disorder of hormone resistance in which the inability of PTH to activate endocrine signaling processes in target cells leads to a state of functional hypoparathyroidism (2, 3). PTH resistance in PHP1 is due to reduced expression or function of the alpha subunit of the heterotrimeric (α, β, and γ) G protein-Gs (Gα) that couples the PTHR1 receptor as well as other heptahelical receptors to stimulation of adenylyl cyclase (4, 5). Gα is encoded by GNAS, a complex gene that contains at least 3 alternative first exons as well as an antisense (AS) transcript. Transcription from promoters associated with these alternative first exons is controlled by reciprocal imprinting, a mechanism that suppresses expression from an allele based on parent-of-origin-dependent methylation of cytosines in CpG sequences located with differentially methylated regions (DMRs) (6, 7). Transcription of NESP from the paternal GNAS allele is suppressed by methylation of CpG dinucleotides that are present within the NESP DMR (GNAS-NESP:TSS-DMR), whereas transcription of XL, AS, and exon A/B (termed exon IA in mice) from the maternal GNAS allele is inhibited by methylation of the corresponding DMRs (GNAS-AS1:TSS-DMR, GNAS-XL:Ex1-DMR, and GNAS A/B:TSS-DMR) (Fig. 1) on that allele (8). In addition, a novel fifth DMR, termed GNAS-AS2:TSS-DMR (9) and that appears to consist of 2 subdomains (10), has been identified telomeric of GNAS-AS1:TSS-DMR. By contrast, there does not appear to be a DMR that controls transcription of exon 1 for Gα. Nevertheless, although most tissues express Gα from both alleles, Gα expression is suppressed from the paternal allele in certain tissues and cells. In certain cell types, including those in the proximal renal tubule, pituitary somatotrophs, thyroid follicular cells, gonads, and regions of the hypothalamus, cell type-specific suppression of the paternal allele leads to a state in which Gα is preferentially expressed from the maternal allele. In addition to synthesis of the signaling proteins Gα and XLαs, the GNAS locus also generates a distinct amino-terminally extended XLαs variant, termed XXLαs (11, 12), as well as alternative translational products of the XL and XXLαs transcripts, termed ALEX (13) and ALEXX (11), respectively.

PHP1a (MIM 103580; also iPPSD2) is caused by heterozygous mutations within the exons that encode Gα on the maternal GNAS allele, and deficiency of Gα in those tissues in which there is preferential transcription of Gα from the maternal allele leads not only to PTH resistance but reduced responsiveness to GHRH, TSH, gonadotropins, and calcitonin as well as obesity and neurocognitive defects (14). Patients with similar (or identical) mutations on paternal GNAS alleles express normal amounts of Gα derived from the maternal allele in these tissues and thereby retain normal hormone responsiveness, a condition that has been termed pseudopseudohypoparathyroidism (MIM 612463) (15). Patients with PHP1a and pseudopseudohypoparathyroidism manifest a similar constellation of developmental somatic defects, collectively termed Albright hereditary osteodystrophy (AHO), that includes short stature, brachydactyly, and heterotopic ossifications (2, 15, 16).

By contrast, reduced expression of Gα in patients with PHP1b (MIM 603233; also iPPSD3) is associated with imprinting defects that affect DMRs of the maternal GNAS allele (2, 14, 17-19). All cases of PHP1b described to date show a complete loss of methylation (LOM) of the maternal exon A/B DMR, which leads to biallelic expression of exon A/B transcripts and suppression of Gα transcription in those tissues in which Gα is normally monoallelically expressed (17, 20-22). In addition, there is evidence of reduced methylation of the minor GNAS-AS2:TSS-DMR as well (9, 10). In tissues such as the proximal renal tubule, hormone resistance arises as consequence of impaired Gs-dependent signaling from deficiency of Gα protein. In addition, hormone responsiveness may also be inhibited by inhibitory forms of Gα that result from translation of A/B transcripts from an initiator methionine located within exon 2 (23, 24).

It is likely that this epigenetic mechanism for hormone resistance is less severe than that caused by genetic defects in patients with PHP1a because PHP1b patients have a more modest phenotype: hormone resistance is usually limited to...
Figure 1. GNAS locus and differentially methylated regions (DMRs). The figure shows the organization of the GNAS locus and relevant genes (not drawn to scale) with solid black circles denoting methylation of DMRs on the maternal (M) or paternal (P) alleles. Solid arrows show the direction of transcription from promoters in all tissues; the dotted arrow denotes transcription of $\alpha$ from the paternal allele in only some tissues (see text). Nucleotides sequences (GRCh Build 37; hg19) in GNAS (20q13.32) that were analyzed by pyrosequencing were in the DMRs for NESP55 (ADS471; 20:g.57,415,807-57,415,853), XL (ADS470; 20:g.57,429,235-57,429,362), and exon A/B (ADS464; 20:g.57,464,773-57,464,927). The STX16 gene is located approximately 220kb centromeric of the GNAS locus.

Materials and Methods

Study population

We studied 53 subjects who met the criteria for PHP1b: biochemical evidence for PTH resistance, presence of mild or no features of AHO, and 2 wild-type copies of GNAS exons 1 through 13. DNA was extracted from peripheral blood leukocytes, and exons 1 through 13 of the GNAS gene were analyzed by Sanger sequencing as previously described (54, 55). This group consisted of 27 affected subjects from 12 families (Table 1) as well as 26 sporadic cases. Six of the PHP1b kindreds have been previously reported (54, 56, 57). We also studied 17 subjects who were either unaffected or obligate carriers of GNAS alleles with imprinting defects and a cohort of control subjects, which included 11 healthy individuals and 3 patients with PHP1a. The protocol was approved by the appropriate institutional review boards and informed consent/assent was obtained from all subjects. Epstein-Barr virus transformation of peripheral blood B-lymphocytes and growth of transformed lymphoblastoid cell lines (LCLs) were performed using standard techniques (58).

Quantitative pyrosequencing

Bisulfite treatment of genomic DNA and pyrosequencing analysis was performed using the PSQ 96HS system, as previously described (40), with unique sets of primers designed by EpigenDx (Hopkinton, MA). Nucleotides sequences (GRCh Build 37, hg19) in GNAS (20q13.32) that were analyzed were located within the DMRs for NESP55 (ADS471; 20:g.57,415,807-57,415,853), XL (ADS470; 20:g.57,429,235-57,429,362), and exon A/B (ADS464; 20:g.57,464,773-57,464,927) (Fig. 1). The NESP methylation assay covers 12 CG dinucleotides in NESP ranging from -50 to +96 from the transcriptional start site based on Ensembl Gene ID ENSG00000204531 and Transcript ID ENST00000259915. The human XL methylation assay covers 19 CG dinucleotides in the promoter region ranging to -50 and +75 on Ensembl Gene ID ENSG00000204531 and Transcript ID ENST00000259915.

Approximately 15% to 20% of cases of PHP1b demonstrate autosomal dominant transmission through a maternal GNAS allele (30). Subjects with familial PHP1b usually manifest complete LOM that is limited to the exon A/B DMR, and carry microdeletions within the maternal STX16 allele about 220 kb upstream of GNAS that correspond to 3 kb (missing exons 4-6) or 4.4 kb (missing exons 4-6) (18, 31, 32), although larger deletions have been identified in rare cases (33-35). Other mutations that lead to LOM that is limited to exon A/B include a few duplications/triplications involving the region centromeric of exon A/B (36-38). Of note, LOM of exon A/B (39, 40), in which cases the deletion of the methylated maternal DMR leads to an apparent LOM for the biallelic epigenotype. Other cases of familial PHP1b show additional methylation defects that affect other DMRs, and that are associated with microdeletions that include sequences associated with NESP and/or the AS transcript (41-45). In these cases, deletion of the corresponding unmethylated maternal DMR can give the appearance of a gain of methylation (GOM) at this DMR. By contrast, the genetic basis for most cases of sporadic PHP1b remains unknown, and does not appear to be associated with the GNAS locus (46). These patients often have broad epigenetic defects in methylation that affect all 4 GNAS DMRs. In some cases, partial (47-49) or complete (14, 47, 50-52) paternal uniparental disomy (patUPD) for chromosome 20 has been identified.

Here, we report the use of quantitative pyrosequencing (40, 53), TaqMan assays, and comparative genomic hybridization using a highly customized array (aCGH) to characterize the genetic and epigenetic defects in patients with familial or sporadic PHP1b. We identified distinctive patterns of methylation that corresponded to known and novel genetic and epigenetic mutations, and that further define the spectrum of molecular defects in PHP1b.
from -523 to -384 from the transcriptional start site based on Ensembl Gene ID ENSG00000102081 and Transcript ID ENST00000370475. The human exon A/B methylation assay covers 24 CpG dinucleotides in the promoter region ranging from -523 to -384 from the transcriptional start site based on Ensembl Gene ID ENSG00000102081 and Transcript ID ENST00000370475. We also used assay ADS1410 to analyze methylation of the DMR of the maternally imprinted gene NNAT encoding neuronatin on chromosome at 20q11.23 (20:g.36149607-36152092) as a control. Experimental conditions are available upon request. The amount of C relative to the sum of C and T at each CpG was determined, and we calculated the average of the percentage numbers for all sites in a particular DMR to determine the DMR methylation level (scale, 0-100).

Copy number variant analyses

We sought to identify microdeletions or duplications within the STX16-GNAS region using 2 complementary techniques, aCGH and TaqMan assays. We hybridized aliquots of genomic DNA (1.5 μg) to custom-designed NimbleGen microarrays using standard methods (59). The microarrays contained 135,000 oligonucleotide probes providing 10-bp tiling coverage of 20q13.3 from STX16 through GNAS. The array has a calculated sensitivity to detect duplications and deletions down to a size of 100 bases. The raw data from the normalized text files were analyzed using Nexus 5.1 software. The following preprocessing steps were applied to the data: (1) we performed sequential lowess regression to %GC content of the probe as well as a 4-kb window around the target to remove any possible systematic effect from these factors; (2) we recentered the data so the median probe value was on the 0 line; and (3) we used RankSegmentation to make the final determination. We used a rather stringent setting to identify deletion breakpoints, with the following parameters: a significance threshold = 1.0E-9, a minimum of 3 probes per segment, a 1 copy gain threshold of 0.18 and a 1 copy loss threshold of -0.2, and a high copy gain threshold of 0.7. We also used a homozygous loss threshold of -1.1.

We performed TaqMan assays to determine copy number of exons 3, 4, and 5 within STX16 and exon NESP because these sequences are often deleted in patients with familial PHP1b. Primers and probes were synthesized and labeled with FAM by Applied Biosystems (Applied Biosystems, Foster City, CA). A labeled RNaseP probe was coamplified as a reference gene and appropriate copy-number controls were included in each assay. Quantitative real-time PCR reactions were performed using an Applied Biosystems StepOne-Plus (Applied Biosystems, Carlsbad, CA) real-time PCR system. Primer sequences and reaction conditions are available upon request. Results were analyzed using Copy Caller Software version 1.0 (Applied Biosystems). Each copy number assessment is the difference in cycle threshold (Ct) between the genomic target sequence of unknown copy number and, RNaseP, an endogenous reference sequence. A statistical model was used to calculate ΔCt, the well level for each target/reference combination in each well which was then used to calculate a copy number assignment (60).

Deletion breakpoint analysis

Genetic deletions that were identified by aCGH were further characterized by PCR amplification of genomic DNA using primers that flank the presumed breakpoints followed by Sanger sequencing of both strands of the amplicons. Primer sequences and reaction conditions are available upon request.

Uniparental disomy analyses

We used microsatellite analysis to assess DNA samples from patients with sporadic PHP1b and broad GNAS methylation defects for patUPD of 20q. Microsatellite analyses were performed for 9 loci (D20s120; D20s100; D20s102; D20s149; D20s171; D20s481; D20s64; D20s173; D20s93) that extended over a 5.9-mbp region of chr20q13 (hg19 chr20:53005482-58919785) that included the GNAS locus as previously described (56).

Statistical analyses

Pyrosequencing results are presented as the mean ± SD, and comparisons between groups were analyzed by 1-way ANOVA with Dunnett’s posttest with group 1 as control or between groups with Tukey-Kramer posttest, using
Results
Clinical phenotypes
A summary of the clinical and biological characteristics of the affected members of the 12 PHP1b kindreds who were included in our study is presented in Table 1. An early age of onset was observed in all cases, with presentation as hypocalcemia resulting from PTH resistance. Vertical transmission of PHP1b was from an affected or unaffected mother in all kindreds. Mild features of AHO were present in some subjects and were manifest as brachydactyly type D and/or E. Patients with sporadic PHP1b manifested only PTH resistance. Many of the families were previously described in earlier publications, as noted in the table. Brief descriptions of the other families are presented here.

Family F6 is of Mexican origin with no evidence of consanguinity. The proband (F6-4), aged 9 years when evaluated, had hypocalcemia, hyperphosphatemia, and markedly elevated PTH. She has triplet younger siblings, 3 years of age, twin brothers who have evidence of PTH resistance (F6-5 and F6-6), and a sister (F6-1) who does not have evidence of PTH resistance. A maternal cousin (not available for study) also has PHP1b. None of the affected subjects has features of AHO or additional endocrine defects (eg, hypothyroidism). Methylation analysis of exon A/B had been previously performed at Johns Hopkins Clinical Genetics Laboratory on the kindred and showed loss of methylation that was limited to the affected children; STX16 deletion testing had also been performed and was negative.

Family F7 consisted of an affected mother and daughter (F7-1) with hypocalcemia, hyperphosphatemia, and elevated PTH. There were no signs of AHO or other endocrine defects. A younger brother was unaffected.

Family F8 consisted of a mother (F8-1) and son (F8-2) with hypocalcemia, hyperphosphatemia, and elevated PTH. There were no signs of AHO or other endocrine defects. Two younger siblings, a brother and sister, were unaffected.

Family F11 is shown in Fig. 2. The proband, F11-4 (II-1) and her sister, F11-2 (II-2) both presented in childhood

Figure 2. Array CGH and pedigree of patient F11-4 with a novel deletion of NESP. (A) The aCGH map for patient F11-1, corresponding to the region of chromosome 20q containing NESP, XL, and AS exons. The novel deletion of NESP is shown with breakpoints as indicated. (B) A 4-generation pedigree for family 11. The kindred designation and methylation pattern are indicated beneath each patient who was evaluated. Males are denoted by squares and females are denoted by circles.
with hypocalcemia, hyperphosphatemia, and elevated PTH. There was no evidence of other endocrine disorders and neither sister had any features of AHO. F11-2 has 2 children, F11-3 (III-2) and III-3, and F11-3 was diagnosed with PHP1b at age 5 years when he was evaluated for parasthesias and muscle cramping and was discovered to have hypocalcemia. The son of F11-3, F11-1, has normal serum levels of calcium, phosphorus, and PTH.

Family 12 is a 3-generation pedigree in which the proband, F12-3, presented at age 3 with developmental delays that led to a diagnosis of autism. At that time, her serum calcium was low and PTH was elevated with slightly elevated serum phosphorus level. She also had an elevated serum TSH (10 mIU/mL) with normal serum levels of free T4. She was begun on calcitriol, calcium carbonate, and levothyroxine. She was a naturally conceived single gestation at age 5 years when he was evaluated for parasthesias and muscle cramping and was discovered to have hypocalcemia. The son of F11-3, F11-1, has normal serum levels of calcium and phosphorus but an elevated serum PTH. She has normal thyroid function tests.

Quantitative pyrosequencing analysis

We used quantitative pyrosequencing (40) to assess the methylation status of CpG dinucleotides within 3 GNAS DMRs that are associated with methylation defects in subjects with PHP1b (17, 50, 54). We did not assess the ASI or AS2 DMRs. The AS1 DMR is closely located within 200 bp of that for XL (61), and previous studies have shown only rare lack of concordance between methylation results for these 2 DMRs (9, 18, 62). We also assessed the methylation status of the DMR for the centromeric imprinted gene NNAT (chr20:36,149,607-36,152,092, GRCh37/hg19), which was normal in all subjects except in subject S10, who had patUPD 20q (see the following section), indicating that genome- or chromosome-wide defects in methylation were not present in the subjects we analyzed.

Our pyrosequencing analyses resolved control and study subjects into 5 groups (Table 2), with significantly different degrees of methylation at various DMRs. Subjects in group 1, consisting of healthy subjects, PHP1a patients, and unaffected family members and obligate carriers had approximately 50% methylation at each DMR (Table 2 and Fig. 3), a pattern we term NNN. The heatmap indicates some variability in the amount of methylation at each CpG (Fig. 3); for example, there are 2 sites in NESP (CpG #43 and CpG #44) and 1 site in XL (CpG #35) where all of the control subjects have approximately 25% to 30% methylation. However, the consistency regarding which sites have less methylation than the expected 50% suggests that these deviations are systematic and not random errors, although we cannot determine whether they are of biological relevance. By contrast, several CpG nucleotides sites within the exon A/B DMR showed increased cytosine methylation in early pyrosequencing runs, but not in later runs, suggesting technical artifact.

Nearly all PHP1b patients (groups 2-4) had markedly reduced methylation of the exon A/B DMR (Table 2 and Fig. 3). Group 2 consisted of individuals in whom the defect in methylation was limited to exon A/B, with normal methylation at NESP and XL; we have termed this pattern NNL (to indicate normal, normal, and low methylation at the 3 sites, respectively) (Table 2). Most subjects in group

<table>
<thead>
<tr>
<th>Table 2. Methylation patterns</th>
</tr>
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<tbody>
<tr>
<td>Cohort</td>
</tr>
<tr>
<td>Group 1 (N = 31)</td>
</tr>
<tr>
<td>Healthy (N = 11)</td>
</tr>
<tr>
<td>PHP1a (N = 3)</td>
</tr>
<tr>
<td>Unaffected family members/obligate carriers (N = 17)</td>
</tr>
<tr>
<td>Group 2 (N = 21)</td>
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<tr>
<td>Group 3 (N = 6)</td>
</tr>
<tr>
<td>3A (familial, N = 3)</td>
</tr>
<tr>
<td>3B (sporadic, N = 3)</td>
</tr>
<tr>
<td>Group 4 (N = 21)</td>
</tr>
<tr>
<td>Group 5 (N = 5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly (P < 0.01) different from corresponding group 1 value, by Dunnett.

<sup>b</sup>Significantly (P < 0.001) different from corresponding group 4 value, by Tukey-Kramer.
Figure 3. Heatmap of the results of the pyrosequencing analysis at the 3 DMRs. Each row represents a subject, with PHP1b patients denoted as sporadic (S) or familial (F, with kindred number); the diagnosis and mutation when known are also shown (ND, not detected). Each column represents a CpG site that was assessed for percent methylation; the color indicates the degree of methylation (see legend on figure) and the actual percentage methylation is indicated in each box. The sections are divided into the DMRs for NESP, for XL and for A/B. The results are clustered in groups, as denoted in the rightmost column, that are described more fully in Table 2 and the text. The key for the heatmap colors is shown at the bottom of the figure.
2 had familial PHP1b, but 3 subjects with apparently sporadic PHP1b, S1, S23, and S24 (Fig. 3), also showed this very common pattern. By contrast, most subjects with sporadic PHP1b, as well as some patients with familial PHP1b, had more extensive methylation defects that corresponded to several different patterns (Fig. 3 and Table 2). The most common methylation pattern was represented by group 4, in which there was a gain of methylation at the DMR for \(NESP\) and loss of methylation at \(XL\) and exon A/B DMRs. This pattern is consistent with 2 paternal epigenotypes, and termed here HLL (to indicate high, low, and low methylation at the 3 sites) (Table 2). This pattern is well-described and is the most common epigenotype in subjects with sporadic PHP1b. There were additional PHP1b patients who showed a gain of methylation in the \(NESP\) DMR but whose epigenotypes differed somewhat from those of subjects in group 4. Patients in group 3 were similar to those in group 4 (HLL) but had normal methylation at the \(XL\) DMR (HNL, to indicate high, normal, and low). To our knowledge, this unusual pattern has been reported previously in only 1 subject (43). Finally, we identified a pyrogram pattern consistent with a partial methylation defect in 5 unrelated subjects with apparently sporadic PHP1b (S19, S20, S21, S22, and S25; Fig. 3 and Table 2). These subjects showed lesser gains of methylation and only moderate losses of methylation at the DMRs for \(NESP\), \(XL\), and A/B than subjects in group 4, and we term this pattern HMM (Table 2).

### Molecular genetic analyses

We used custom-designed aCGH to identify potential copy number variations (CNVs) that might account for abnormal epigenotypes. Our arrays disclosed microdeletions that included exons of \(STX16\) in 20 of 21 PHP1b patients with NNL epigenotypes (Table 3 and Fig. 3); in all cases, these deletions were confirmed by TaqMan assays. These mutations were also present in other affected relatives and obligate gene carriers but were not present in unaffected family members (Fig. 3). An apparently identical deletion of approximately 3 kb that represented the loss of exons 4 through 6 of \(STX16\) (Fig. 4) was present in 18 probands and 2 subjects with sporadic PHP1b (Table 3); these deletions correspond to the previously reported recurrent 3-kb deletion that has been found in most cases of familial PHP1b (31-34). Sequencing demonstrated that the left breakpoint is within

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**Table 3. Mutation analysis of deletions**

<table>
<thead>
<tr>
<th>Kindred or subject</th>
<th>Methylation pattern</th>
<th>TaqMan CNV</th>
<th>Deletion by aCGH</th>
<th>Breakpoints by Sanger sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left</td>
</tr>
<tr>
<td>F1</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 3, 4, and 5</td>
<td>STX16 and NPEPL1</td>
<td>chr20:57,151,892-57,289,110</td>
</tr>
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<td>F2</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
<tr>
<td>F3</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
<tr>
<td>F4</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
<tr>
<td>F5</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
<tr>
<td>F6</td>
<td>NNL</td>
<td>Normal</td>
<td>ND</td>
<td>chr20:57,243,741-57,243,927</td>
</tr>
<tr>
<td>F7</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
<tr>
<td>F8</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
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<tr>
<td>F11</td>
<td>HNL</td>
<td>1 copy of (NESP)</td>
<td>NESP</td>
<td>chr20:57,243,567-57,243,739</td>
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<tr>
<td>F12</td>
<td>NNL</td>
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<td>S23</td>
<td>NNL</td>
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<tr>
<td>S50</td>
<td>NNL</td>
<td>1 copy of (STX16) exons 4 and 5</td>
<td>STX16 exons 4-6</td>
<td>chr20:57,243,567-57,243,739</td>
</tr>
</tbody>
</table>

Abbreviations: aCGH, comparative genomic hybridization using a highly customized array; CNV, copy number variation; HNL, high, normal, and low; NNL, normal, normal, and low.
chr20:57,243,577-57,243,739 and the right breakpoint (BP) is within chr20:57,246,545-57,246,717 (Fig. 5) but could not be more precise because of the repetitive sequence in these areas. A slightly different microdeletion was identified in subject F7-1 (Fig. 4), in which the left breakpoint is within 20:57,243,741-57,243,927 and the right breakpoint is within 20:57,246,719-57,246,905 (Fig. 5). The deletions are located approximately 220 kb centromeric of GNAS exon A/B and are flanked by 2 391-bp direct repeats, which prevents more accurate delineation of the precise breakpoints. A mechanism involving homologous recombination between the flanking repeats likely accounts for the recurrent occurrence of these deletions in patients with PHP1b (31).

Proband F1-1 and other affected relatives had a large, approximately 205-kb deletion that contained the entire STX16 gene as well as the centromeric NPEPL1 gene (Fig. 6A). This was maternally inherited, and was not present in any unaffected family members. The aCGH predicted a single microdeletion with breakpoints at chr20:57,152,382-57,357,883, but Sanger sequencing (Fig. 6B) revealed 2 adjacent deletions, 20:57,151,892-57,289,110 and 20:57,289,120-57,358,140, which are separated by 9 bp (20:57,289,111-57,289,119). It is conceivable that this represents a single large deletion and a small random insertion of 9 bp during DNA repair (ie, an indel). Thus, this represents a novel nearly 206-kb deletion (20:57,151,892-57,289,110 and 20:57,289,120-57,358,140). Figure 7 compares this deletion with previous CNVs in this region. Of greatest relevance is the large 87.5-kb deletion (20:57,215,896 to 57,301,635) reported by Yang et al (35) in a single patient with PHP1b, which also includes the entire STX16 gene and the downstream NPEPL1 gene. Loss of 1 copy of NPEPL1, which encodes the widely distributed enzyme aminopeptidase-like 1 (EC 3.4.11.1), does not appear to have any phenotypic consequences on either the 4 affected subjects in F1 or the patient described by Yang et al (35), and no human diseases have been ascribed to this gene.

The aCGH analysis also identified a novel 9.5-kb NESP mutation in proband F11-4 (Fig. 2A), who, with affected relatives F11-2 and F11-3 (Fig. 2B), shared a very unusual HNL epigenotype of group 3 (Fig. 3). This deletion was confirmed by TaqMan assays (Table 3) and whole genome sequencing, and the breakpoints were mapped to ch20:57,406,457-57,415,987 by Sanger sequencing (Table 3). An unaffected relative, F11-1, did not carry the deletion and had a normal NNN epigenotype. A similar HNL
methylation defect was previously reported by Richard et al. (43) in 3 affected members of a PHP1b kindred who carried a 19-kb deletion (chr20:57,397,711 to 57,416,700) that removed NESP and a large part of AS intron 4 (Fig. 8). Both the deletion described by Richard et al. (43) and the 9.5-kb deletion that we have identified lead to loss of the unmethylated NESP DMR on the maternal allele (Fig. 8), and thereby lead to an apparent GOM rather than a true GOM. Nevertheless, the 19-kb deletion as well as the smaller deletion that we describe here cause loss of A/B methylation without affecting methylation of the XL/AS DMR, which is unusual for deletions in this region (Fig. 8). Previous work by Liu et al. (18) had shown that in virtually all cases, the imprinting status of the NESP and AS/XL promoters is concordant, suggesting that their imprinting is coregulated, whereas the imprinting of the AS/XL promoter region and XLαs first exon is not always concordant even though these regions are closely linked within the same DMR. In these cases, normal imprinting of the XLαs first exon is maintained even in the presence of an imprinting defect involving NESP, the AS/XL promoter, and the exon A/B DMR. These 3 patients had slightly different methylation patterns (3B, Table 2) than the 3 patients with the NESP deletion (3A, Table 3), but given the small number of subjects these differences failed to reach statistical significance. And aCGH also failed to identify CNVs for 19 sporadic cases in group 4 (HLL), 1 of whom (S10) was found to have partial paternal UPD that was confirmed by whole genome sequencing as isodisomy of the telomeric end of chromosome 20 (roughly chr20:44,097,455-qter).

Finally, we were unable to identify CNVs in the 5 subjects with sporadic PHP1b in group 5. These subjects carried an unusual HMM pattern of methylation that is statistically distinct from the HLL pattern of other groups (Table 2) and is consistent with an increase in methylation...
at the NESP DMR and partial loss of methylation at the XL and exon A/B DMRs. Close inspection of the methylation heatmap (Fig. 3) shows consistent levels of methylation across all the CpGs positions that were analyzed in each DMR, suggesting that the HMM pattern might be the result of postzygotic mosaicism in which there are 2 populations
of cells. To examine this hypothesis, we generated Epstein-Barr virus-transformed LCLs from subject S25 and performed pyrosequencing on DNA from these cell lines. These analyses included DNA from a second sample of whole blood and DNA isolated from a nonclonal population of LCLs. The second blood DNA sample (data not shown) had methylation results for the 3 DMRs that were nearly identical to the first DNA sample (S25 BLD, Fig. 3), indicating that this unusual HMM methylation pattern was not an artifact of DNA preparation or pyrosequencing. By contrast, DNA that was extracted from the nonclonal LCLs showed the HLL pattern that is characteristic of the global defect in methylation typical of patients with sporadic PHP1b (Fig. 3, S25 LCL). Similar results were obtained from pyrosequencing DNA from 30 clonal LCLs from subject S25 (data not shown). These results provide strong evidence of mosaicism as the basis for the HMM epigenotype.

Discussion
Genomic imprinting determines parental-specific monoallelic expression of a small number (approximately 200) of genes in mammals (63), and abnormal expression of imprinted genes has been implicated in a variety of human disorders of growth and development. These genes tend to be distributed in distinct clusters that are epigenetically regulated by one or more imprinting control regions (ICRs) (64). Analysis of DNA methylation at DMRs located at either ICRs or the promoters associated with specific genes has provided important insights into the genetic pathophysiology of imprinting disorders and at the same time, has also been used for diagnosis of conditions such as Beckwith-Wiedemann syndrome, Silver-Russell syndrome-1, Prader-Willi and Angelman syndromes, and Temple syndrome (65). PHP1 is now recognized as yet another disorder in which defective expression of an imprinted gene is responsible for the phenotype. Genetic mutations that affect the Gα coding exons of GNAS account for PHP1a, whereas genetic and epigenetic imprinting defects and patUPD that affect transcription of GNAS account for PHP1b. We report here a comprehensive analysis of subjects with familial and sporadic PHP1b using high-throughput methods to identify both epigenetic and genetic molecular defects.

Figure 8. Summary of deletions affecting NESP in patients with PHP1b. Breakpoint structures of deletions disrupting NESP in PHP1b patients. The region of chromosome 20q that contains STX16 through GNAS is depicted above an inset that shows the NESP-AS2 region (not drawn to scale) with genomic coordinates for each exon. The 7 previously described deletions are shown above the novel deletion that was identified in family 11 that removes the entire NESP exon. The methylation patterns for the 4 or 3 DMRs analyzed in previous studies and this study are shown to the right.
We first performed quantitative pyrosequencing to classify subjects based on specific methylation defects, and then sought CNVs by comparative genome hybridization using a customized high-density array. Our heatmap of patient pyrograms confirmed that LOM within the exon A/B DMR is a consistent finding in both sporadic and familial PHP1b (20, 21, 25, 54, 66). We also found that nearly all subjects with familial PHP1b, and some cases of sporadic PHP1b, showed the typical NNL methylation pattern in which the imprinting defect is limited to a LOM of maternal exon A/B, which is both necessary and sufficient to prevent transcription of Gsα protein from the maternal allele in imprinted tissues. Nearly all of our patients with the NNL epigenotype carried the common recurrent 3-kb deletion that removes exons 4 through 6 of the maternal STX16 allele (31, 32), indicating two of our subjects with sporadic PHP1b who presumably have de novo mutations. Our previous haplotype analysis of these kindreds excluded identity by descent for these STX16 mutations (data not shown). The 3-kb STX16 deletion has been reported previously in subjects with apparently sporadic PHP1b (31) and accounts for a form of “sporadic” PHP1b that is presumably hereditable unlike other forms of sporadic PHP1b (vide infra). Larger microdeletions within STX16 have also been reported (32-34), as well as an 85-kb deletion that, similar to the novel 206-kb deletion that we report here, removes the entire STX16 and NPEPL1 genes (35). This region has been proposed to contain a critical long-range cis-acting element, tentatively localized to exon 4 of STX16 (32), which has been proposed to function as 1 of at least 2 primary ICR within the GNAS locus (2, 14). Nevertheless, this region lacks typical features of an ICR (ie, it lacks CpG islands and differential methylation), and although this putative ICR controls methylation of the GNAS A/B:TSS-DMR in humans, deletion of this region in transgenic mice appears to have no effect on imprinting of Gnas (67). Although the mechanism by which these deletions lead to an imprinting defect in humans remains uncertain, STX16 may coordinate long-range chromatic interactions between enhancers and promoters via intrachromosomal loops.

Pyrosequencing also disclosed the HLL methylation pattern in many subjects, including patients with sporadic PHP1b and affected members of one PHP1b family (F9). Although we were unable to identify a genetic mutation in any of these subjects, we found patUPD for a portion of chromosome 20q in 1 of the 19 cases of sporadic PHP1b, which accounts for the global pattern of methylation defects (HLL) (32). Other studies that have used a wider variety of techniques have found patUPD to occur far more frequently (46-49, 68-71), with an overall prevalence of 13% in patients with sporadic PHP1b (32). In this study, we used microsatellite genotyping and whole genome sequencing to detect blocks of homozygosity, which represents a less sensitive procedure to identify isodisomy than high-density single nucleotide polymorphism (SNP) array, and we may have missed limited regions of genetic identity. We would also point out that we (and others) may have failed to ascertain some cases of patUPD because heterodisomy would be missed in the absence of parental samples.

The basis for the HLL imprinting defect in family F9 is likely to be a heritable genetic mutation that is too small to be detected by the techniques that we used in this study. On the other hand, the basis for the global imprinting defect in the patients with sporadic PHP1b remains unknown. In this regard, comprehensive genetic and epigenetic studies of subjects with other imprinting disorders have revealed potentially informative molecular mechanisms that account for epimutations. For example, determination of the grandparental origin of the incorrectly imprinted chromosome in patients with Prader-Willi syndrome and Angelman syndrome (72) has indicated that a failure in imprint erasure or a failure in imprint establishment and/or maintenance is the most likely explanation for the imprinting defect in these patients, respectively. Similar studies have suggested that errors in imprint establishment in the maternal germline or postzygotic maintenance of methylation are the likely causes of the imprinting defects in some patients with Beckwith-Wiedemann syndrome (73) and Temple syndrome (74). It is conceivable that aberrant imprinting in oocytes may be the result of exposure to environmental toxins such as the synthetic estrogen bisphenol A (75) or a consequence of suboptimal maternal nutritional status (65).

Our pyrogram heatmap also revealed unusual methylation patterns, HNL in members of 1 family (F11) and HMM in 5 subjects with sporadic PHP1b. We discovered that the HNL epigenotype in affected members of family F11 was due to a novel 9.5-kb deletion that included the entire NESP exon. A similar HNL methylation defect was previously reported by Richard et al (43) in 3 affected members of a PHP1b kindred who carried a larger 19-kb deletion that removed NESP and a large part of AS intron 4. The unusual pattern of methylation in these 2 families is relevant to understanding the mechanism that controls imprinting for the entire GNAS locus. The GNAS cluster contains 2 CpG islands with the characteristics of a germline ICR; these regions are differentially methylated in the germline and the differential methylation is maintained in the somatic tissues of the offspring. The principal ICR for the GNAS cluster contains the AS promoter, and lies within the AS and XL DMR (76, 77); a second germline ICR is within the exon A/B DMR (78), and specifically controls maternal expression of A/B transcripts and the imprinted expression...
of transcripts encoding Gα. By contrast, the third DMR in the GNAS locus encompasses the promoter and exon for NESP; this is a somatic DMR that becomes methylated on the paternal allele postfertilization as a consequence of transcription of AS (76, 78). NESP is maternally expressed for about 80 kb across the entire GNAS locus, and the long primary transcript is processed by splicing the open reading frame encoding NESP55 onto exons 2 through 13 or exons 2, 3, and N1 of Gα (79). Truncation (80, 81) or deletion (22) of Nesp in mice leads to failure to acquire normal methylation at the downstream Nespa/Gnasxl and exon 1a DMRs in the oocyte. These results recapitulate the epigenetic changes observed in PHP1b patients with deletions of NESP that include upstream AS exons 4 or 4 and 3 (41) and isolated deletions of AS exons 3-4 (42) and are consistent with a model in which DNA methylation is established in the oocyte when Dnmt3a:Dnmt3L complexes are recruited to a DMR in response to active transcription rather than by specific DNA sequence motifs or properties (82, 83). Thus, transcription initiating at the NESP promoter and proceeding through the GNAS locus is required for acquisition of maternally methylated germline DMRs at XL/AS and exon A/B. Therefore, the presence of normal methylation marks at the XL/AS DMR in the patients reported by Richard et al (43) and the patients in kindred F11 that we report here is unexpected. On the other hand, these findings are not unprecedented because they are similar to the outcomes in transgenic mice with maternal inheritance of a truncated Nesp allele (80, 81), which leads to consistent loss of exon 1A methylation but variable germline LOM of the Nespa-Gnasxl DMR. The basis for the inconsistent LOM at the Nespa-Gnasxl DMR in these 2 knockout mouse strains, and the 2 PHP1b families, is uncertain, but Chotalia et al (80) have proposed that the variable methylation could be explained by a stochastic failure to erase a preexisting paternal methylation pattern in the oocyte, because the methylated Nespa/Gnasxl DMR was apparently not heritable. By contrast, the HNL epigenotype is transmitted in family F11, suggesting instead that these deletions lead to initiation of transcription from alternative or cryptic promoters that are located upstream of the NESPAS-GNASXL DMR, with termination of transcription before traversing exon A/B DMR (84). Unfortunately, in the absence of oocytes from affected subjects, this hypothesis cannot be tested.

The second unusual methylation pattern, HMM, was present in 5 unrelated subjects with sporadic PHP1b (group 5). Previous work had demonstrated similar partial methylation defects in DNA from whole blood of some patients with sporadic PHP1b, and had proposed that this pattern represented the presence of somatic mosaicism for an epigenetic defect with contributions from normal and abnormal cell lines (45, 46, 85, 86). Elli et al (86) and (46) had been unable to demonstrate differences in the pattern of methylation between DNA from whole blood and other tissues or transformed B-lymphoblastoid cell lines, respectively, which had suggested that potential epimutations may have occurred very early during embryological development in these patients. By contrast, Takatani et al (45), were able to show that 2 subjects with partial methylation defects in DNA from whole blood had near-complete methylation defects (ie, HLL) in DNA from nonclonal transformed B-lymphoblastoid cell lines. Here, we were able to generate transformed B LCLs from 1 (S25) of our 5 sporadic PHP1b subjects with the partial methylation defect (HMM) to further evaluate the hypothesis of somatic cell mosaicism. In agreement with Takatani et al (45), we found that DNA isolated from a nonclonal LCL population (Fig. 3, S25 LCL) as well as DNA isolated from multiple clonal LCL populations (data not shown) had only the methylation pattern corresponding to the global epigenetic defect (HLL) in GNAS. Our results provide strong additional evidence for postzygotic somatic cell mosaicism and are consistent with the notion that the whole blood samples that were used for isolation of DNA contained significant numbers of nonhematopoietic cells such as bone marrow-derived progenitor and stem cells (87). Given that paternal UPD is a recognized mechanism for the global defect in methylation seen in some patients with sporadic PHP1b, we evaluated the possibility that this mosaic defect may have occurred as a result of segmental paternal UPD during postzygotic development. Using genome wide sequencing, we evaluated SNPs that corresponded to the GNAS locus in DNA from whole blood and LCLs in subject S25. SNP genotypes were identical in both DNA samples, without loss of heterozygosity that might suggest even partial UPD (data not shown). On the other hand, our confirmation that mosaicism is responsible for a subset of sporadic PHP1b provides important insight into the likely basis for imprinting defects in sporadic PHP1b in general. Epigenetic defects can arise because of an error in imprint erasure, an error in imprint establishment, or an error in imprint maintenance. To distinguish between these mechanisms for sporadic PHP1b, it is necessary to analyze DNA from a subject’s parents and maternal grandparents because an error in imprint erasure in maternal primordial germ cells will always occur on a GNAS allele of grandmaternal origin. By contrast, an error in imprint establishment or imprint maintenance can affect a GNAS allele of either grandmaternal or grandpaternal origin. Although we were unable to assess methylation status using DNA from PHP1b trios, the presence of somatic mosaicism in at least some subjects strongly suggests
that the underlying molecular mechanism of primary epimutations in PHP1b is an error in imprint maintenance because only this mechanism can also explain mosaicism, in which the failure takes place after fertilization in the early embryo so that only a subset of cells harbor an imprinting defect, whereas the other cells have a normal epigenotype.

Our study has significant strengths. First, we included many families and a large number of patients with sporadic PHP1b. Second, our quantitative pyrosequencing provided comprehensive information on the methylation status of discrete CpGs and enabled us to construct detailed heatmaps that disclosed novel patterns of methylation. Third, we identified novel mutations in STX16 and NESP that expand the catalog of genetic defects that cause PHP1b. And last, we have confirmed and extended prior published work that support postzygotic somatic cell epimutations as a basis for sporadic PHP1b in at least some patients. We also note that our study has several weaknesses. First, although our aCGH had greater sensitivity than previous arrays (88), and was predicted to be able to detect copy number variations down to 100 bp, we were still unable to identify mutations in all probands with familial PHP1b. In this case, we presume that the mutations were below the resolution of our array or were not located within the genomic region that we assessed. These mutations may be resolved by next-generation sequencing through the area from STX16 to GNAS. A second weakness is our incomplete ascertainment of relatives of patients with sporadic PHP1b, which hampered our ability to assess patUPD isodisomy or grandparental origin of the improperly imprinted GNAS allele. A third weakness is that we did not directly assess the methylation status of the AS1 and AS2 DMRs, which may have provided evidence for more discrete subclassification of our subjects (10).

In conclusion, our data suggest 4 plausible mechanisms for imprinting defects in PHP1b patients: (1) genetic mutations that disrupt imprint establishment; (2) paternal UPD; (3) failure to maintain the maternal methylation imprint during the global wave of demethylation in the zygote; or (4) during early embryonal development in the case of methylation mosaics. Future work to resolve these mechanisms will require comprehensive genetic and epigenetic analyses using 3-generation pedigrees.

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