Genetic and epigenetic states of the GNAS complex in pseudohypoparathyroidism type Ib using methylation-specific multiplex ligation-dependent probe amplification assay

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Abstract

Context: Pseudohypoparathyroidism type Ib (PHP-Ib) is a rare disorder resulting from genetic and epigenetic aberrations in the GNAS complex. PHP-Ib, usually defined by renal resistance to parathyroid hormone, is due to a maternal loss of GNAS exon A/B methylation and leads to decreased expression of the stimulatory G protein α (Gsa) in specific tissues.

Objective: To clarify the usefulness of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), we evaluated genetic and epigenetic changes of the GNAS locus in Japanese PHP-Ib patients.

Design: Retrospective case series.

Patients: We studied 13 subjects with PHP-Ib (three families with eight affected members and one unaffected member and four sporadic cases).

Measurements: The methylation status of GNAS differentially methylated regions (DMRs) was evaluated using MS-MLPA. The main outcome measure was the presence of deletion mutations in the GNAS locus and STX16, which were assessed using MLPA.

Results: In all familial PHP-Ib cases, a ~3 kb deletion of STX16 and demethylation of the A/B domain were identified. In contrast, no deletion was detected throughout the entire GNAS locus region in the sporadic cases. Broad methylation abnormalities were observed in the GNAS DMRs.

Conclusions: MS-MLPA allows for precise and rapid analysis of the methylation status in GNAS DMRs as well as the detection of microdeletion mutations in PHP-Ib. Results confirm the previous findings in this disorder and demonstrate that this method is valuable for the genetic evaluation and visualizing the methylation status. The MS-MLPA assay is a useful tool that may facilitate making the molecular diagnosis of PHP-Ib.

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Introduction

Pseudohypoparathyroidism (PHP) is characterized by hypocalcemia and hyperphosphatemia due to the resistance to parathyroid hormone (PTH) (1). PHP type 1 (PHP-I) comprises a group of heterogeneous disorders caused by different genetic/epigenetic defects within the same locus (2). Analysis of PHP patients over the past several years has provided remarkable insight for understanding GNAS expression, function, and regulation (3). PHP-Ib patients present predominantly with renal PTH resistance and lack any features of Albright’s hereditary osteodystrophy in the majority of cases (1). Most PHP-Ib cases are sporadic, but some cases are familial and show an autosomal dominant mode of inheritance (AD-PHP-Ib) (4). In contrast to most cases of PHP-Ia, which are caused by a mutation in the coding sequence of the GNAS gene, the majority of PHP-Ib cases are reportedly caused by a methylation alteration of the GNAS locus (5, 6). The GNAS complex contains at least four distinct differentially methylated regions (DMRs) (7, 8, 9). By a parent-specific methylation pattern of most of its different promoters, the GNAS locus gives rise to several transcripts, including the α-subunit of the heterotrimeric stimulatory G protein α (Gsa), the Gsa extra-large variant (XLsa), a second alternative gene product encoded by the XL-exon 1 (ALEX), neuroendocrine protein 55 (NESP55), untranslated exon A/B (also known as exon 1A), and an additional antisense transcript (AS)
The XLαs, A/B, and AS transcripts derive exclusively from the paternal allele, whereas NESP55 is maternally derived (7, 9). Consistent with this imprinted expression pattern, the promoters of these genes are methylated on the silenced allele.

An epigenetic defect of exon A/B DMR that reduces the expression of Gαs from the maternal GNAS allele leads to PTH resistance in patients with PHP-Ib. AD-PHP-Ib, which is maternally transmitted in an autosomal dominant manner, is typically associated with loss of imprinting at exon A/B DMR due to microdeletions disrupting the upstream STX16 gene, which likely harbors a cis-acting control element crucial for establishing the methylation imprint at exon A/B (10). A heterozygous 3 or 4.4 kb deletion of STX16 has been identified in affected individuals and unaffected carriers of AD-PHP-Ib cases (11, 12, 13, 14) and a few apparently sporadic PHP-Ib cases (14). In addition, deletions of NESP and/or AS have been identified in AD-PHP-Ib patients (15, 16, 17). Interestingly, most sporadic PHP-Ib cases also exhibit GNAS imprinting abnormalities that involve multiple DMRs: NESP, AS, XL, and A/B (5, 6). Therefore, the loss of methylation of A/B DMR is common in all patients with PHP-Ib. Genetic deletions within STX16 and NESP55/AS are not present in most sporadic PHP-Ib cases (12, 13, 14, 18). In particular, the PTH concentration in the absence of treatment is positively correlated with the percent of methylation at the A/B DMR of GNAS (18).

Thus, molecular analyses including analysis of the methylation pattern of the GNAS locus provide useful information in PHP-Ib, but conventional techniques to detect large deletion or epigenetic alterations are both time-consuming and costly. Multiplex ligation-dependent probe amplification (MLPA) assays allow for relative quantification of ~50 different DNA sequences in a single reaction requiring only 20 ng human DNA. The MLPA technique is a powerful tool for evaluating genetic deletions that cannot be detected using PCR-based sequencing techniques. The MLPA assay has been adapted to detect aberrant methylation (19). Methylation-sensitive (MS)-MLPA is a modification of the MLPA assay with a methylation-sensitive restriction endonuclease that simultaneously detects the methylation state of CpG islands. The potential usefulness of MS-MLPA for analyzing PHP-Ib had been reported in some cases (20, 21, 22). In this study, we aimed to clarify the usefulness of this method by evaluating the MS-MLPA results for 13 Japanese PHP-Ib patients.

Materials and methods

Patients

We studied a total of 13 subjects with PHP-Ib (three families with eight affected members and one unaffected member and four sporadic cases). The clinical diagnosis was based on the physical and laboratory findings. The clinical background and the data are summarized in Table 1. Patient 4 was a 1-year-old girl with no features of PTH resistance; nevertheless, her parents requested that we perform presymptomatic genetic testing. We performed the analysis after providing careful genetic counseling to her parents. Informed consent was obtained from all patients or their parents.

PCR and direct sequencing of the GNAS gene

Genomic DNA was extracted from peripheral blood leukocytes or saliva. Genomic DNA was extracted using a QIAamp DNA Blood Kit (Qiagen) for blood samples and a PUREGENE DNA Purification kit for saliva. All 13 exons, including the GC-rich exon 1 and intron–exon boundaries of the GNAS gene, were amplified by PCR using specific primers. The PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MA, USA) and subsequently sequenced. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

MLPA and MS-MLPA assays

Aberrant methylation within the GNAS locus was identified by MS-MLPA using the SALSA MLPA kit ME031-A1 GNAS (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer’s protocol. After hybridization with the probe mix, each sample was divided into two aliquots. One tube was used to identify the presence of deletions in the GNAS locus, including DMRs and STX16 (MLPA). The other tube was treated with the HhaI restriction enzyme, which recognizes the unmethylated GCCG sequence, to assess methylation status (MS-MLPA). Following PCR amplification, the products were separated by electrophoresis. Each sample peak was assigned to a specific probe according to its length. The peak ratio was determined by dividing the sample peaks of patients by those of controls; undigested samples were used as controls for MS-MLPA. Automated fragment and data analysis for MLPA were performed using GeneMapper Software (Applied Biosystems).

Methylation-specific PCR

The DNA samples (control, case 9 for AD-PHP-Ib and case 11 for sporadic PHP-Ib) were treated with sodium bisulfite using the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures, North Ryde, NSW, Australia). DNA methylation of the NESP55 and A/B regions was analyzed using Epifect MSP Kit (Qiagen). Two sets of PCR primers were designed using Methyl Primer Express Software v1.0 (Applied Biosystems): one for unmethylated and one for
methylated DNA sequences in each region. The primer sequences are shown in supplementary Fig. 1C, see section on supplementary data given at the end of this article. The PCR involved an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 94 °C for 15 s, predetermined optimal annealing temperature of 45 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. Five microliters of PCR product were analyzed on a 3.0% agarose gel.

Microsatellite marker analysis and polymorphism genotyping

Eight previously described polymorphic microsatellite markers (D20S117, D20S889, D20S115, D20S112, D20S107, D20S196, D20S171, and D20S173) were labeled with fluorescent dye and used for PCR. The genomic DNA template (100 ng) was amplified by adding each marker. PCR products were subjected to capillary electrophoresis. Data were analyzed using GeneMapper Software (Applied Biosystems).

PCR-based detection of the 3 kb microdeletion

Exons 4–6 of the STX16 gene were amplified by PCR using the following primers: forward primer, 5'-CTCAAGAGGCCCTGAAGTCCTCCAGCTGTC-3'; reverse primer, 5'-GGTCTTGTGTAGGGATTTTCCCACCTGTGGC-3'. PCR was carried out in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 nM of each dNTP, 1 mM of each primer, 100 ng genomic DNA, and five units of LA Taq polymerase (Takara, Shiga, Japan). Amplifications were performed as follows: 95 °C for 30 s and 68 °C for 5 min. The PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore) and subsequently sequenced. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

As a GNAS mutation had been reported in some PHP-Ib patients (23), we first confirmed that none of the patients carried a mutation in the GNAS gene by PCR direct sequencing. Normal control subjects showed a 50% reduction of the signal intensity by HhaI digestion compared with nondigested samples in all the DMRs, suggesting a one-allele methylation in these domains (Fig. 1A, lower panel closed bars). These findings are consistent with the previously reported normal methylation pattern (Fig. 1A, upper panel). Although all nine cases of AD-PHP-Ib exhibited a methylation pattern identical to that of normal controls in the NESP55, AS, and XL regions, a complete loss of PCR products after HhaI digestion was observed in the two distinct A/B regions, suggesting a biallelic unmethylated A/B region.

### Table 1

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<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Calcium (mg/dl)</th>
<th>Phosphate (mg/dl)</th>
<th>iPTH (pg/ml)</th>
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<td>19.2</td>
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</table>

Age, age at diagnosis; AHO, Albright’s hereditary osteodystrophy (clinical features of either short stature < −2.5 SDS, shortened fourth and fifth metacarpals, or rounded facies); NA, data not available.

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or loss of methylation in the maternal A/B region. Figure 1B shows the MLPA analysis results for the nine AD-PHP-Ib patients. All the probes covering the GNAS locus regions (data not shown) and STX16 had two copy number amplifications in the normal control subjects (Fig. 1B, lower panel closed bars). All nine AD-PHP-Ib patients, however, had a copy number loss of exons 5 and 6 of STX16, suggesting a one-allele deletion in this region. PCR amplification and subsequent sequencing analysis of the corresponding region revealed that one of two direct repeats and the intervening nucleotides were deleted, thus removing a 3 kb region including STX16 exons 4, 5, and 6 as previously reported (Fig. 1C). Although the DNA of case 8 was extracted from saliva, we confirmed that the MS-MLPA results on saliva DNA were identical to the data obtained from DNA extracted from peripheral blood in control subjects (data not shown).

The results of the MS-MLPA in the four sporadic PHP-Ib patients (cases 10–13) are shown in Fig. 2. In these four cases, a gain of methylation of NESP55 in the maternal allele and a loss of methylation in the AS, XL, and A/B domains in the maternal allele were detected (Fig. 2A). We confirmed the methylation alterations in the NESP55 and A/B domains using a methylation-specific PCR method (supplementary Fig. 1). MLPA analysis showed no copy number alterations in any of the four patients (Fig. 2B). We performed microsatellite analysis to exclude paternal disomy. Analysis of the microsatellite markers indicated normal biparental inheritance, suggesting that paternal uniparental isodisomy did not occur (Table 2). As the parents’ DNA was not available, however, paternal heterodisomy could not be excluded from this study.

Discussion

In this study, we clearly identified the genetic and epigenetic alterations of GNAS in 13 of 13 Japanese

![Figure 1](https://www.eje-online.org/)

Figure 1 (A) MS-MLPA analysis of AD-PHP-Ib patients (cases 1–9). Upper panel shows a schematic representation of the GNAS promoter region. Gray bars represent the allele-specific methylation state. Lower panel shows the MS-MLPA results. Closed bar indicates the normal control. In the NESP55, AS, and XL regions, all the AD-PHP-Ib patients showed the same one-allele methylation pattern as the control. In contrast, in both A/B domains, all the patients showed a complete loss of PCR products after HhaI digestion, suggesting biallelic demethylation. (B) The upper panel shows the STX16 structure. The filled boxes indicate the exons, and the triangles indicate direct repeat sequences. Lower panel shows the MLPA analysis of AD-PHP-Ib patients. A copy number reduction was observed in exons 5 and 6 of STX16. (C) Upper right panel shows the PCR strategy for detecting the STX16 deletion. Triangles indicate direct repeat sequences and arrows indicate the primers used for PCR. Left upper panel shows the family tree of the AD-PHP-Ib and lower panel shows the PCR analysis corresponding to family members. Lower panel shows the electrophoresis of PCR products on agarose gel. In all the AD-PHP-Ib patients, the smaller product (763 bp), corresponding to a 3 kb deletion due to recombination between the direct repeat sequences, and wild-type product (3.7 kb) were observed.

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PHP-Ib patients using the MS-MLPA technique. We identified an identical 3 kb deletion of STX16 and a loss of methylation in the A/B domain in all nine Japanese AD-PHP-Ib patients from three families. Recent studies reported that most familial cases with PHP-Ib display a loss of methylation on maternally inherited exon A/B DMR without imprinting defects in other DMRs. In addition to those epigenetic alterations, genetic deletions are found within STX16, NESP55, or AS. A 3 kb deletion of STX16 was first identified and considered to be the most frequent mechanism of AD-PHP-Ib (11, 12, 13, 14). Several other deletions have been reported since then, including a 4.4 kb deletion within STX16 (24), a 19 kb deletion in NESP55 (15), 4 and 4.7 kb deletions of NESP55/AS (16), and a 4.2 kb deletion within AS (17). In our patient series, however, we detected none of these deletions other than the dominant 3 kb deletion of STX16.

None of the four Japanese sporadic PHP-Ib patients showed genetic alterations in the entire GNAS locus, including STX16 and NESP55, in MLPA analysis. Broad losses of methylation of DMRs were demonstrated by MS-MLPA. The maternal NESP55 was extra-methylated, and the AS, XL, and A/B domains of the maternal allele were demethylated. The resulting methylation pattern could be caused by paternal uniparental isodisomy. Uniparental isodisomy of the long arm is considered a plausible cause of the disease in a few sporadic PHP-Ib cases with broad GNAS methylation defects (25, 26). Although paternal heterodisomy could not be ruled out, microsatellite marker analysis did not identify paternal uniparental isodisomy of chromosome 20q in any of the present sporadic PHP-Ib cases (Table 2). The MLPA kit used in this study for the GNAS locus allows for analysis of most of the exons throughout the GNAS locus, including NESP55, but does not contain probes covering AS exons 2, 3, and 4. Therefore, the present MLPA analysis could not rule out the possibility of a recently identified 4.2 kb deletion in AS (17). Heterogeneity in the methylation abnormalities at the GNAS locus in patients affected with PHP-Ib was first reported by Liu et al. (6). Recent studies showed that methylation alterations in DMRs and/or deletion within STX16 play a critical role in PHP-Ib pathogenesis.

### Table 2: Genotyping of the polymorphic markers at the GNAS region.

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<th>Case 11</th>
<th>Case 12</th>
<th>Case 13</th>
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Moreover, PHP may be reclassified on the basis of these molecular analyses. The most commonly used assays for these purposes include restriction fragment length polymorphism analysis using methylation-sensitive enzymes followed by Southern blot analysis and methylation-specific PCR using bisulfite-treated DNA samples and allele-specific PCR followed by gel electrophoresis. These conventional techniques, although considered to be relatively efficient, are time-consuming and costly and do not provide accurate information on copy number variation (28). In contrast, MS-MLPA requires a smaller amount of DNA and the data generated can be used to determine both copy number changes and methylation status of multiple loci in a single-tube experiment. MS-MLPA was an easy to use and rapid molecular diagnostic tool for detecting both the genetic and/or the epigenetic defects underlying PHP-Ib. Recent data showed that GNAS-related disorders are more heterogeneous than previously understood. Molecular overlap between clinically diagnosed PHP-Ia and -Ib has been reported (29, 30, 31, 32). In addition to sequencing analysis of GNAS, MS-MLPA is a powerful tool for molecular diagnosis in individual patients and provides fruitful information that will lead to a better understanding of these disorders by revealing various genetic and/or epigenetic alterations in each clinical case. There are some limitations, however, to the use of MS-MLPA as follows. MS-MLPA probes select CpG islands only. Moreover, a single HhaI site methylation status may not be representative of the status of the entire CpG island. Furthermore, probe signals may also be affected by mutations and/or polymorphisms situated either in the vicinity of or at the probe ligation site. Interpretation of the test results should be conducted in the context of the patient’s ethnicity, clinical and family histories, and other laboratory test results and molecular analyses of family lineage. In conclusion, we identified genetic and/or epigenetic alterations in all 13 Japanese PHP-Ib patients using MS-MLPA and MLPA assays. This simple method provides important information regarding the genetic and epigenetic status of patients with PHP-Ib.

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Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-12-0548.

Declaration of interest
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