Establishment of diagnosis by bisulfite-treated methylation-specific PCR method and analysis of clinical characteristics of pseudohypoparathyroidism type 1b

Kaori Kinoshita, Masanori Minagawa, Tomozumi Takatani, Rieko Takatani, Mika Ohashi and Yoichi Kohno

Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

Abstract. Pathogenesis of pseudohypoparathyroidism type 1b (PHP-1b) is related to the loss of methylation at the GNAS exon A/B region, which is combined with epigenetic defects at other differentially methylated GNAS regions in most sporadic cases. In this study, we established a method for evaluating the methylation status of a CpG island in exon A/B using a methylation-specific polymerase chain reaction (MSPCR). We designed two primer pairs specific for the methylated and unmethylated alleles and evaluated the methylation status of GNAS exon A/B in samples from PHP-1b patients and normal controls. We examined 20 Japanese patients and 20 normal controls, and one primer set was found to be appropriate for diagnosis. Furthermore, we evaluated the clinical data of patients. Weight and height of patients were not significantly different from the normal population. Short stature (adult height ≤ 2SD) was observed in one patient and short metacarpals in two. Obesity was observed in six patients, and no patient showed ectopic subcutaneous calcification. Seven patients showed subclinical hypothyroidism because of resistance to thyroid stimulating hormone, but no patient had an abnormally low free thyroxine level, and none received oral thyroid hormone replacement. For diagnosis of PHP-1b, only clinical data is not sufficient because a few PHP-1b patients show clinical features similar to PHP-1a, and hence, molecular biology techniques are required for correct diagnosis. We concluded that MSPCR is applicable for rapid molecular diagnosis of PHP-1b.

Key words: Pseudohypoparathyroidism type 1b, GNAS, exon A/B, Differentially methylated region, Methylation-specific polymerase chain reaction

PSEUODOHYPOPARATHYROIDISM (PHP; MIM 103580) refers to hypocalcemia caused by resistance to the action of parathyroid hormone (PTH) and is classified into two types, PHP-1a and PHP-1b. PHP-1a patients show, besides PTH resistance, resistance to other hormones as well as Albright’s hereditary osteodystrophy (AHO), a collection of features including short stature, obesity, brachydactyly, ectopic ossifications, and/or mental retardation [1]. PHP-1b patients do not typically show AHO and mental retardation. Nevertheless, genomic DNA from PHP-1b patients shows a loss of methylation at the GNAS exon A/B (also referred to as 1A) region [2], which is combined with epigenetic defects at other differentially methylated GNAS regions in most of sporadic cases [3]. Maternally inherited deletions within STX16 or NESP55 have been identified in familial PHP-1b patients [4-7]. Standard methods for genomic methylation analysis involve digestion of genomic DNA with methylation-sensitive restriction enzymes followed by Southern blotting [3].

In this study, the methylation status in the CpG island of exon A/B region was evaluated by a methylation-specific polymerase chain reaction (MSPCR). Bisulfite treatment of DNA converts all unmethylated cytosines to uracils; however, methylated cytosines in the CpG dinucleotides are resistant to this chemical modification. On the basis of this differential effect, the bisulfite-modified DNA sequence of a methylated allele (MET) was successfully distinguished from that of an unmethylated allele (UNMET) using two sets of allele-specific primer pairs, MET-specific primer (M) and UNMET-specific primer (U) [8].

Some PHP-1b patients with methylation defect in
exon A/B region had mild AHO like phenotype [9, 10]. We previously reported that the adult height of male PHP-1b patient was slightly lower than average. An early growth spurt was observed only in male patients with PHP-1b and it may reduce the adult height of male patients with PHP-1b [12]. We evaluated the symptoms and clinical data of patients and examined whether the AHO phenotype, especially short stature, were present in Japanese PHP-1b patients.

**Methods**

**Subjects and Materials**

Subjects were 20 Japanese PHP patients (10 females and 10 males) and 20 normal controls. Clinical diagnosis was based upon the presence of PTH resistance (hypocalcemia, hyperphosphatemia, and increased serum PTH levels in the absence of vitamin D deficiency) without or with mild AHO phenotype (three or less of 6 symptoms; short stature, obesity, round face, short metacarpals, subcutaneous calcification and mental retardation). Genomic DNA was extracted from whole blood cell using the Blood & Cell Culture DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany). Genetic analyses were performed after written informed consent had been obtained and upon approval by the Ethics Committee of Chiba University.

**Southern Blotting**

Molecular diagnosis of PHP-1b was achieved by Southern blotting. Genomic DNA was digested with either *Pst*I or *Pst*I plus either of the methylation-sensitive enzymes *Asc*I or *NgoMIV*. The product was electrophoresed on a 1.5% agarose gel and transferred to Nylon Membranes, positively charged (Roche Diagnostics, Indianapolis, USA). Membranes were hybridized with an exon A/B-specific probe (28852-29847, according to GenBank accession number AL121917) labeled with DIG PCR DIG probe synthesis Kit (Roche Diagnostics, Indianapolis, USA). Hybridization signals were detected by a chemiluminescent reaction using a DIG luminescent detection kit for nucleic acids and CDP-Star (Roche Diagnostics, Indianapolis, USA).

**Polymerase Chain Reaction**

From the genomic sequence of *GNAS* (GenBank accession number AL121917) exon A/B region, DNA sequences of MET and UNMET after bisulfite modification were deduced. Several sets of primers were designed using MethPrimer [12]. Among these primer sets, only two sets showed successful PCR amplification. We designed primers for two regions near the digestion sites of methylation-sensitive restriction enzymes in exon A/B (A/B-1, A/B-2) and two sets of primers for one region, one set specific for MET and a second for modified UNMET (Figs. 1 and 2). The expected product sizes from M-A/B-1, M-A/B-2, U-A/B-1, U-A/B-2 primer pairs were 151, 191, 153, and 194 base pairs, respectively. Primer designs are as follows (Fig. 2).

**M-A/B-1F, 5’-CATAAAAAAAGCAAGCTTCTATGCT-3’ **
**M-A/B-1R, 5’-GACGCTTACCCCTACTATACGC -3’**
**U-A/B-1F, 5’-CATAAAAAAAACAAACTTCTATACT -3’**
**U-A/B-1R 5’-ACAACACTTACCCCTACTATACACC -3’**
**M-A/B-2F, 5’-GCAAAAAAACAAAAAACCAAAG -3’ **
**M-A/B-2R, 5’-CCCGACGACTCTTACCTACG -3’ **
**U-A/B-2F, 5’-CACAAAAAAACAAAAAACCAAAAC-3’ **
**U-A/B-2R, 5’-AACCCAACAACTCTTACCTACACC-3’**

Bisulfite treatment with the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) was performed and the DNA was subjected to MSPCR using M or U primer pairs.

For A/B-1 primers, PCR amplification of the bisulfate-modified DNA was performed in 20 μL reaction mixtures containing 0.4 μL bisulfite-treated genomic DNA, 0.2mM dNTPs, and 1.5mM MgCl₂. One unit of Expand High Fidelity Enzyme mix (Roche Diagnostics, Indianapolis, USA) and 0.3 μM of forward and reverse primers were added. After initial denaturation for 2 min at 94°C, one cycle at 94°C for 15 s, 54°C for 30 s, and 72°C for 30 s was repeated 10 times. The cycle at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s (5 s longer after one additional cycle) was then repeated 25 times with a final elongation step at 72°C for 7 min.

For A/B-2 primers, PCR amplification of the bisulfate-modified DNA was performed in 20 μL reaction mixtures containing 0.4 μL bisulfite-treated genomic DNA, 0.2mM dNTPs, and 2.5 mM MgCl₂ for M-A/B-2 and 1.5 mM MgCl₂ for U-A/B-2. One unit of Expand High Fidelity Enzyme mix and 0.3 μM of forward and reverse primers were then added. After initial denaturation for 2 min at 94°C, one cycle at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s was repeated 10 times. The cycle of 94°C for 15 s, 54°C for 30 s, and 72°C for 30 s (5 s longer after one additional cycle) was then repeated 30 times with a final elongation step at 72°C for 7 min.

The product was electrophoresed on a 3% agarose
Diagnosis and characteristics of PHP-1b

Fig. 1 Southern blotting of exon A/B (patient and normal control)

Upper panels show the schematic representation of DNA fragment created by PstI digestion (horizontal bar) and restriction map for methylation-sensitive enzyme (Ascl or NgoMIV). Gray box on the horizontal bar indicates exon A/B. Primer pairs for MSPCR are also indicated with arrows (M-A/B-1 and U-A/B-1, or M-A/B-2 and U-A/B-2). Lower panels show the representative results of Southern analysis. Genomic DNA from PHP-1b patient (Pt) and normal control (C) were digested with PstI, alone (A) or in combination with Ascl (A) or NgoMIV (B).

A Single digestion of genomic DNA with PstI produced approximately 2.8 kb fragments both in PHP-1b patient and normal control. In PstI+Ascl digestion of normal control, a 2.8 kb band and a 1.6 kb band were obtained due to the Ascl restriction site. In PHP-1b patient, the 2.8 kb band was absent due to loss of methylation at Ascl site.

B In PstI+NgoMIV digestion of normal control, in addition to a 2.8 kb band, a 1.5 kb band and a 1.3 kb band were observed due to the NgoMIV restriction site. In PHP-1b patient, the 2.8 kb band was absent due to loss of methylation at NgoMIV site.
Fig. 2  Target sequences of the primer pairs for MSPCR at the promoter region of the *GNAS* gene

MET and UNMET sequences show nucleotides after bisulfite treatment in methylated and unmethylated allele, respectively. CpG dinucleotides are indicated in NATIVE sequences with shade. Arrows indicate primer pairs for MSPCR with orientation.

**A** The native DNA sequences located position 28771 through 29100 of GenBank Accession number AL121913 are shown as NATIVE. Bold underline indicates *AscI* site used for Southern blotting analysis.

**B** The native DNA sequences located position 29321 through 29570 of GenBank Accession number AL121913 are shown as NATIVE. Bold underline indicates *NgoMIV* site used for Southern blotting analysis. The sequences of exon A/B are shown in bold-italic.
Evaluation of Clinical Characteristics
Clinical data was retrospectively collected from medical record. We compared the height SD scores of male PHP-1b patients with female patients, and these of adult PHP-1b patients with patients before reaching adult height. Statistical significance of observed differences between groups was determined by the Mann-Whitney U test for unpaired samples using GraphPadPrism3 software.

Results

Southern Blotting
Southern blotting confirmed the lack of DNA methylation at *Ascl* and *NgoMIV* sites in all patients. The representative results are shown in Fig. 1. In normal controls, double digestions produced both a 2.8 kb band representing intact *PstI* fragment and smaller bands resulting from digestion by methylation-sensitive enzymes, consistent with the presence of MET and UNMET. In contrast, the 2.8 kb fragment was completely digested by both enzymes in all 20 patients, consistent with a total loss of the maternal-specific methylation of exon A/B region.

Polymerase Chain Reaction
The results of MSPCR analysis in PHP-1b patients and normal controls are shown in Fig. 3. Bisulfite-modified DNA from normal controls was amplified with both M and U primer pairs in A/B-1 and A/B-2 regions (Fig. 3, C1-7). The remaining 13 data from normal controls are not shown. Modified DNA from 12 PHP-1b patients was amplified only with the U-A/B-1 primer pair, whereas that from eight patients was amplified only with the M-A/B-2 primer pair.
amplified with both M-A/B-1 and U-A/B-1 primer pairs. When we used M-A/B-2 and U-A/B-2 primer pairs, modified DNA from all 20 PHP-1b patients was amplified only with the U-A/B-2 primer pair.

**Evaluation of Clinical Characteristics**

Clinical data of patients is summarized in Table. The ages at diagnosis ranged from 3 to 38 years. Typical symptoms caused by hypocalcemia were observed in 14 patients, but five had no symptoms and were diagnosed by chance or due to a family history. Seven patients (P7, 23, 24, 25, 34, 42, 42S) had subclinical hypothyroidism because of resistance to thyroid stimulating hormone, but no patients had a low free thyroxine level (data not presented) and none received oral thyroid hormone supplements. Short stature was observed in one patient (adult height ≤ 2 SD). Obesity was observed in six patients (Body mass index >25kg/m² in adults or degree of obesity >20% in children). Round face was present in 6 patients. Furthermore, short metacarpals were observed in two patients. No patient had ectopic subcutaneous calcification, but one patient (P40) had calcification in carotid artery. Mental retardation was reported in one patient complicated with sensorineural hearing loss. Intracranial calcifications were found in 10 patients out of 17 patients investigated. One sporadic case (P34) and three familial cases (the proband is P42, P42M and P42S are mother and younger sister of P42) had a 3.3 kb STX16 deletion detected by a previously described PCR method [3] (data not shown).

The comparison of height SD scores between males (n=10) and females (n=10) didn’t show statistical difference (Fig. 4A). The height SD scores of patients before reaching adult height (n=9) were slightly larger than adult patients (n=11), however, the difference was not statistically significant (Fig. 4B).

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age at diagnosis (years)</th>
<th>Symptoms (*1)</th>
<th>Serums biochemical values</th>
<th>AHO features</th>
<th>Extra features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Calcium (mg/dL)</td>
<td>Phosphate (mg/dL)</td>
<td>Intact PTH(*2) (pg/mL)</td>
<td>ALP (IU/L)</td>
</tr>
<tr>
<td>P3</td>
<td>M</td>
<td>14</td>
<td>none</td>
<td>8.6</td>
<td>6.0</td>
<td>239</td>
</tr>
<tr>
<td>P7</td>
<td>M</td>
<td>6</td>
<td>loc, c</td>
<td>4.6</td>
<td>10.3</td>
<td>347</td>
</tr>
<tr>
<td>P8</td>
<td>M</td>
<td>3</td>
<td>c</td>
<td>5.2</td>
<td>6.8</td>
<td>720</td>
</tr>
<tr>
<td>P9</td>
<td>F</td>
<td>9</td>
<td>loc</td>
<td>6.3</td>
<td>7.0</td>
<td>NA</td>
</tr>
<tr>
<td>P13</td>
<td>F</td>
<td>6</td>
<td>c</td>
<td>6.0</td>
<td>6.6</td>
<td>NA</td>
</tr>
<tr>
<td>P18</td>
<td>M</td>
<td>12</td>
<td>muscle cramp</td>
<td>4.8</td>
<td>9.1</td>
<td>139</td>
</tr>
<tr>
<td>P20</td>
<td>M</td>
<td>11</td>
<td>c</td>
<td>7.8</td>
<td>9.6</td>
<td>137</td>
</tr>
<tr>
<td>P21</td>
<td>F</td>
<td>12</td>
<td>c</td>
<td>5.7</td>
<td>9.1</td>
<td>NA</td>
</tr>
<tr>
<td>P23</td>
<td>F</td>
<td>8</td>
<td>c</td>
<td>6.7</td>
<td>8.2</td>
<td>134</td>
</tr>
<tr>
<td>P24</td>
<td>F</td>
<td>35</td>
<td>tetany</td>
<td>5.3</td>
<td>5.2</td>
<td>96</td>
</tr>
<tr>
<td>P25</td>
<td>M</td>
<td>8</td>
<td>c</td>
<td>7.1</td>
<td>8.6</td>
<td>340</td>
</tr>
<tr>
<td>P27</td>
<td>F</td>
<td>12</td>
<td>c</td>
<td>5.4</td>
<td>9.5</td>
<td>360</td>
</tr>
<tr>
<td>P29</td>
<td>M</td>
<td>15</td>
<td>c</td>
<td>4.8</td>
<td>7.2</td>
<td>190</td>
</tr>
<tr>
<td>P32</td>
<td>M</td>
<td>9</td>
<td>none</td>
<td>6.7</td>
<td>7.4</td>
<td>360</td>
</tr>
<tr>
<td>P34</td>
<td>M</td>
<td>4</td>
<td>none</td>
<td>6.6</td>
<td>8.2</td>
<td>1088</td>
</tr>
<tr>
<td>P40</td>
<td>F</td>
<td>9</td>
<td>headache</td>
<td>8.3</td>
<td>5.9</td>
<td>480</td>
</tr>
<tr>
<td>P42</td>
<td>F</td>
<td>10</td>
<td>c</td>
<td>5.4</td>
<td>8.2</td>
<td>510</td>
</tr>
<tr>
<td>P42M</td>
<td>F</td>
<td>38</td>
<td>none</td>
<td>5.5</td>
<td>4.6</td>
<td>270</td>
</tr>
<tr>
<td>P42S</td>
<td>F</td>
<td>8</td>
<td>none</td>
<td>8.4</td>
<td>4.6</td>
<td>240</td>
</tr>
<tr>
<td>P54</td>
<td>M</td>
<td>13</td>
<td>c</td>
<td>5.7</td>
<td>9.4</td>
<td>146</td>
</tr>
</tbody>
</table>

NA, not available; SS, short stature; Ob, obesity; RF, round face; SM, short metacarpals; SC, subcutaneous calcification; MR, mental retardation; IC, intracranial calcification. *1 c, convulsion; loc, loss of consciousness  *2 Elevated PTH was confirmed by mid-PTH assay, where intact PTH was not available.  *3 Height SD score below -2SD   *4 BMI > 25 for adults or obesity index > 20% for children
Diagnosis and characteristics of PHP-1b

Fernandez-Rebollo recently reported a deletion of 30,431 bp extending from the intronic region between exons XL and A/B to intron 5 in a PHP-1a patient who had typical AHO features including subcutaneous ossifications and brachymetacarpia [11, 16]. This case showed loss of methylation only in exon A/B region and was presented first as pseudohypoparathyroidism with mild features of AHO [11]. Molecular testing for methylation status in exon A/B region is not appropriate for discriminating such case with deletion including exon A/B and coding exons of Gsα protein from ‘true’ PHP-1b. Fluorescence in situ hybridization, comparative genomic hybridization array or quantitative PCR may be employed for correct diagnosis of the cases with large GNAS deletion in maternal allele.

Clinical diagnosis of PHP-1b may be concordant with molecular pathogenesis only when PTH resistance without multiple hormone resistance is present and AHO is absent. Mantovani reported that 24 of 40 patients with AHO and without mutations in Gsα coding regions showed a methylation defect in the A/B region [9]. In those patients, 6 patients showed all the 6 AHO symptoms and no correlation was observed in their study between the degree of methylation defects and the severity of AHO. On the other hand, de Sanctis reported 43 patients with PTH resistance and/or AHO, and sequencing of the whole coding region of GNAS identified 11 mutations in 18 PHP-1a patients [10]. They all had at least 3 symptoms of 6 AHO symptoms.

Discussion

MSPCR was invented by Herman for rapid detection of DNA methylation pattern in CpG islands [8], and was clinically applied for rapid diagnosis of Prader-Willi and Angelman syndromes by Kosaki [14]. Weinhaeusel also reported about diagnostic testing of PHP-1b with MSPCR, but his method needs DNA digestion with restriction enzyme prior to PCR reaction [15]. Our method is more rapid and simple. In the present study, we have shown that MSPCR can be used to evaluate the methylation status of the GNAS A/B region. When we used M-A/B-2 and U-A/B-2 primer pairs, specificity and sensitivity of the assay was 100%; however, when we used M-A/B-1 and U-A/B-1 primer pairs, specificity was 100% (12/12) and sensitivity was 60% (12/20). Furthermore, we did not observe any false positive results for PHP-1b in normal controls. When we used the A/B-2 primer pairs, MSPCR could detect loss of CpG methylation specific to PHP-1b patient in a rapid and cost-effective manner. Thus, this method should be considered during initial evaluation of all patients in whom PHP-1b is suspected. The reason for the false negative result when A/B-1 primer pairs are used is not known, but we suspect that the CpG dinucleotides in particular A/B-1 region (5’ upstream of exon A/B) have a mosaic methylation pattern or that the methylation of CpG dinucleotides matched to the primer sequences are not critical for the maintenance of tissue-specific imprinting of Gsα expression (Fig. 2). Further investigation is needed to determine the mechanism of CpG methylation and Gsα expression.

Fernandez-Rebollo recently reported a deletion of 30,431 bp extending from the intronic region between exons XL and A/B to intron 5 in a PHP-1a patient who had typical AHO features including subcutaneous ossifications and brachymetacarpia [11, 16]. This case showed loss of methylation only in exon A/B region and was presented first as pseudohypoparathyroidism with mild features of AHO [11]. Molecular testing for methylation status in exon A/B region is not appropriate for discriminating such case with deletion including exon A/B and coding exons of Gsα protein from ‘true’ PHP-1b. Fluorescence in situ hybridization, comparative genomic hybridization array or quantitative PCR may be employed for correct diagnosis of the cases with large GNAS deletion in maternal allele.

Clinical diagnosis of PHP-1b may be concordant with molecular pathogenesis only when PTH resistance without multiple hormone resistance is present and AHO is absent. Mantovani reported that 24 of 40 patients with AHO and without mutations in Gsα coding regions showed a methylation defect in the A/B region [9]. In those patients, 6 patients showed all the 6 AHO symptoms and no correlation was observed in their study between the degree of methylation defects and the severity of AHO. On the other hand, de Sanctis reported 43 patients with PTH resistance and/or AHO, and sequencing of the whole coding region of GNAS identified 11 mutations in 18 PHP-1a patients [10]. They all had at least 3 symptoms of 6 AHO symptoms.

Fig. 4 Height of PHP-1b patients. NS, not significant
We compared height SD score of male patients (square) and female patients (triangle) (A) as well as adults (square) and children (triangle) (B). We assumed height SD score of children are slightly higher than that of adults, but the difference was not statistically significant.
including mental retardation. In our study, patients with exonA/B methylation defects had at most 3 symptoms of 6 AHO symptoms, and without mental retardation, except for one patient. Originally PHP-1a and 1b were classified only by clinical symptoms. Molecular diagnosis helped us to distinguish two types of PHP, but genetic and epigenetic defect doesn’t always correspond to specific symptoms. The distinction between PHP-1a and 1b remains unclear. We believe the most important information for PHP patients is about inheritance, and our method must contribute to patients to give rapid information about it.

It is known so far that Gsα protein is paternally imprinted in a tissue-specific manner only in the renal proximal tubules, pituitary, thyroid gland, ovaries, and megakaryocytes. The defects associated with abnormal loss of methylation in exon A/B region of GNAS must be limited to these ‘imprinted tissues or cells’. Mild or subclinical hypothyroidism observed in PHP-1b may be caused by partial imprinting of GNAS gene in thyroid. In the view of AHO-like features, one patient had short stature and two had short metacarpals in our study subjects. We assume that the tissue-specific imprinting partially exists in the subset of bone cells. We have previously evaluated the growth curve of PHP patients. We found that male PHP-1b patients had low normal adult height while female PHP-1b patients had almost mean height, and male PHP-1b patients had an early growth spurt resulting in slightly reduced adult height [12]. Therefore, we expected to have some difference in comparison between males and females or between adults and children in this study. No significant difference between groups was observed in the results, however, longitudinal assessment is required to conclude whether the skeletal growth is disturbed in PHP-1b or not.

**Conclusion**

MSPCR is applicable for rapid PHP-1b diagnosis. For correct PHP-1b diagnosis, we need not only clinical data but also molecular biology techniques.

**Acknowledgments**

The authors sincerely thank Dr. Hiroshi Mochizuki, Dr. Akira Otake, Dr. Seiki Wada, Dr. Hisayuki Oda, Dr. Kazuko Yonamine, Dr. Fumio Morohashi, Dr. Naooaki Hori, and Dr. Ayuko Narita, who provided samples from PHP-1b patients. This study was supported in part by grants from the Ministry of Health, Labor and Welfare, Japan.

This study was also partially supported by a grant for the Global COE Program, “Global Center for Education and Research in Immune System Regulation and Treatment,” from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


