A 22-year-old Woman with Hypocalcemia and Clinical Features of Albright Hereditary Osteodystrophy Diagnosed with Sporadic Pseudohypoparathyroidism Type Ib Using a Methylation-specific Multiplex Ligation-dependent Probe Amplification Assay

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Abstract

A 22-year-old woman presented to us with seizures of a few minutes duration. She had clinical features of Albright hereditary osteodystrophy (AHO), including hypocalcemia, hyperphosphatemia and resistance to parathyroid hormone. Genetic testing revealed a sporadic form of pseudohypoparathyroidism type Ib (PHP-Ib). This is the first Japanese case involving overlap between pseudohypoparathyroidism type Ia (PHP Ia) associated with AHO and PHP Ib. It is important to perform both DNA sequencing and methylation status analyses in cases of suspected PHP in patients with signs of AHO.

Key words: seizure, pseudohypoparathyroidism, hypocalcemia, Albright hereditary osteodystrophy, MS-MLPA

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Introduction

Pseudohypoparathyroidism (PHP) is a disease of hormone resistance resulting from the reduced responsiveness of parathyroid hormone (PTH)-target cells. PHP is a heterogeneous and rare disease with a prevalence of 3.4 per million people in Japan (1). Two subtypes of PHP type I are well known: PHP type Ia (PHP-Ia) presents with features of Albright hereditary osteodystrophy (AHO) and multiple hormone resistance, whereas AHO is classically absent and hormone resistance is limited to PTH and TSH in patients with PHP type Ib (PHP-Ib) (2, 3). A 22-year-old woman presented to us with seizures due to hypocalcemia. She had brachydactyly of both fifth fingers and the fourth and fifth toes bilaterally. Genetic testing revealed abnormal patterns of methylation known to be present in patients with sporadic PHP-Ib (4). She also exhibited overlap at the molecular level between PHP-Ia, as defined by the classic clinical diagnosis, and PHP-Ib, as defined by the genetic diagnosis. This is the first such case in Japan, and no other overlapping cases have previously been reported. We herein describe this case with a discussion of the relevant literature. Prior to genetic testing, the procedures were fully explained, and written informed consent was obtained from the patient. The study was approved by our institution’s review board.

Case Report

A 22-year-old woman visited the National Hospital Organization Disaster Medical Center with a chief complaint of seizures of a few minutes duration. She had undergone surgery for a right slipped femoral capital epiphysis at 7 years of age and bilateral femoral bone cysts at 10 years of age...
The knuckle sign was positive. Chvostek’s and Trousseau’s both fifth fingers and the fourth and fifth toes bilaterally.

The patient was a high school graduate, and her school performance of the use of supplements, such as vitamin D. The patient had taken any regular medications. There was no history of supplements, such as brachydactyly or subcutaneous calcification. The initial tonic-clonic seizure episode occurred at 7 years of age, at which time an electroencephalogram (EEG) showed spike waves. The second seizure episode occurred at 9 years of age. The patient was not treated with antiepileptic agents and experienced no seizure recurrence thereafter. She noticed at 14 years of age that both of her fifth fingers and fourth and fifth bilateral toes were short. Stiffness in the right arm and nausea had developed after childbirth at 22 years of age, a tonic-clonic seizure suddenly occurred, followed by impaired consciousness lasting for several tens of minutes, and she was emergently admitted to the National Hospital Organization Disaster Medical Center.

On admission, the patient’s height was 151 cm (−1.38 SD) and her weight was 49 kg, with a body mass index (BMI) of 22.3 kg/m² and a round face. She had brachydactyly of both fifth fingers and the fourth and fifth toes bilaterally. The knuckle sign was positive. Chvostek’s and Trousseau’s signs were negative. The shortened fingers and toes are presented in Fig. 1. Upon arrival, her consciousness was clear, and she had neither dysphagia nor a gait disturbance. No neck stiffness, Kernig’s sign or hyperesthesia were detected. The deep tendon reflexes were bilaterally equal, and she had no pathologic reflexes. The Mini-Mental State Examination (MMSE) score was 30/30. The Wechsler Adult Intelligence Scale (WAIS) III showed a verbal IQ of 77, performance IQ of 74, full-scale IQ of 73, verbal comprehension index of 82, perceptual organization index of 63, working memory index of 74 and processing speed index of 89, indicating borderline intelligence.

The laboratory test results showed a serum blood urea nitrogen (BUN) level of 9.5 mg/dL and a creatinine level of 0.42 mg/dL, without renal impairment. The sodium, potassium, chloride, calcium, inorganic phosphate and magnesium levels were 139 mEq/L, 3.2 mEq/L, 99 mEq/L, 4.7 mg/dL, 5.8 mg/dL and 2.0 mg/dL, respectively, indicating hypocalcemia, hyperphosphatemia and hypokalemia. The fractional potassium excretion was 6.54% and the urinary potassium-creatinine ratio was 49.8 mEq/g-cre, findings not suggestive of the renal loss of potassium. The intact serum PTH level was highly elevated at 719 pg/mL. The calcitonin level was 21 pg/mL, while that of 1,25-dihydroxyvitamin D (1,25(OH)₂VD) was 89 pg/mL. The level of 1,25(OH)₂VD was slightly higher. The free triiodothyronine (T3), free thyroxine and thyroid-stimulating hormone levels were 1.78 pg/
mL, 0.97 ng/dL and 1.13 μIU/mL, respectively, indicating no evidence of thyroid dysfunction. In addition, there was no growth hormone (GH) deficiency (GH, 5.09 ng/mL). The Ellsworth-Howard test showed negative phosphaturic ([U4+U5] - [U2+U3] = 28 mg/2 h) and cyclic AMP ([U4-U3]= 0.00625 μmol/h and U4/U3=1.8 times) responses. The serum glucose level was 91 mg/dL and the glycosylated hemoglobin (HbA1c) level was 5.6% (the Japanese Diabetes Society: JDS), indicating no glucose intolerance. The HbA1c level was calculated according to the method recommended by JDS. This level can be compared to the National Glycohemoglobin Standardization Program (NGSP) value of HbA1c calculated using the formula NGSP value=JDS value (%) ×1.02+0.25%. JDS values are generally lower than NGSP values by 0.3-0.4%. Tests for autoimmune antibodies, including antinuclear antibodies, anti-SS-A antibodies, anti-SS-B antibodies, MPO-ANCA and PR3-ANCA, were negative. A cerebrospinal fluid examination showed an opening pressure at 16 cm of water, with a cell count of 1/mm³ (monocytes, 100%) and a protein level of 16.7 mg/dL. An electrocardiogram (ECG) showed QT prolongation with a QTc interval of 0.527. On EEG, the background activity was 10 to 12 Hz, with α wave dominance over the parietal and occipital lobes. High-amplitude sharp waves appeared sporadically over an extensive area and were more frequent during hyperventilation. As shown in Fig. 1, plain radiographs of the extremities revealed shortened fifth metacarpals and fourth and fifth metatarsals. As shown in Fig. 2, computed tomography (CT) of the brain demonstrated hyperdense areas in the bilateral basal ganglia and cerebellar dentate nuclei as well as the gray-white matter interface extending from the frontal to the lateral and parietal lobes. Magnetic resonance imaging (MRI) of the brain disclosed a low signal intensity in the bilateral basal ganglia and cerebellar dentate nuclei on T2-weighted images. Neither brain atrophy nor cortical abnormalities were detected.

A diagnosis of PHP was made based on the findings of hypocalcemia, hyperphosphatemia and an elevated iPTH level with a normal renal function. Furthermore, the patient’s seizures were considered to be associated with PHP (5). The Ellsworth-Howard test was negative for phosphaturic and cAMP responses, suggesting PTH resistance in the renal tubules. The physical examination and imaging studies showed features of AHO. Her mother underwent blood testing, brain CT and plain radiographs of the extremities; however, no signs of PHP or AHO were observed.

Figure 2. Computed tomography (CT) of the brain. These scans show hyperdensity in the bilateral basal ganglia and cerebellar dentate nuclei as well as the gray-white matter interface extending from the frontal to the lateral and parietal lobes.
The administration of oral calcitriol at a dose of 0.5 μg daily was initiated, and the patient was discharged home on the third hospital day. There has been no recurrence of seizures since discharge. The patient’s stiffness in the arms, queasiness and hypocalcemia continued to gradually improve. A follow-up EEG showed fewer high-amplitude sharp waves, and a follow-up ECG demonstrated a normalized QTc interval.
A sufficient explanation of genetic testing was given to the patient and her family members, all of whom provided their informed consent. Genetic testing of the patient and her mother was performed at the National Hospital Organization Kyoto Medical Center. All GTP-binding protein alpha subunit (Gsa) gene (GNAS) exons were analyzed using PCR direct sequencing. Mutations and deletions were analyzed using multiplex ligation-dependent probe amplification (MLPA). The methylation status was evaluated using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). The NESP55, AS, XL and exon A/B DMRs (differential methylated regions) were assessed using MS-MLPA. MLPA identified no deletions in the mother’s GNAS complex, and PCR direct sequencing revealed no mutations in GNAS exons 1 to 13. MS-MLPA showed no methylation abnormalities. In the patient, MLPA identified no deletions in the GNAS complex, and PCR direct sequencing disclosed no mutations in GNAS exons 1 to 13 (Fig. 3). MS-MLPA demonstrated biallelic methylation of the NESP DMR and biallelic demethylation of the AS, XL and A/B DMRs (differential methylated regions) were assessed using MS-MLPA. MLPA identified no deletions in the mother’s GNAS complex, and PCR direct sequencing revealed no mutations in GNAS exons 1 to 13. MS-MLPA showed no methylation abnormalities. In the patient, MLPA identified no deletions in the GNAS complex, and PCR direct sequencing disclosed no mutations in GNAS exons 1 to 13 (Fig. 3). MS-MLPA demonstrated biallelic methylation of the NESP DMR and biallelic demethylation of the AS, XL and A/B DMRs (Fig. 4). These results suggested the hypermethylation of the NESP DMR on the maternal allele and demethylation of the AS, XL and A/B DMRs on the maternal allele. Given previously reported findings (4), the patient was considered to have sporadic PHP-Ib based on the genetic testing.

Discussion

PHP, hypoparathyroidism due to resistance to the actions of PTH, is a heterogeneous and rare disease with a prevalence of 3.4 per million people in Japan (1). Following the injection of exogenous PTH in the Ellsworth-Howard test, classic PHP type I characteristically presents with a lack of urinary cAMP elevation, whereas PHP type II is associated with increased urinary cAMP excretion. PHP is further divided into subtypes according to abnormalities in the signaling sites of PTH receptors, coupled to the G protein. PTH receptors activate adenylate cyclase to produce cAMP through the stimulatory Gsα in renal tubules. In PHP-Ia patients, the Gsα protein itself is inactivated. In contrast, in PHP-Ib patients, the Gsα protein is lacking. PHP-Ic patients exhibit significant decreases in the manganese-stimulated adenylate cyclase activity in fibroblast membranes, raising the possibility of a second defect of the cAMP pathway leading to the phenotype of PHP-Ic. In PHP-II patients, PTH infusion normally increases the urinary cAMP level; however, it does not elicit a phosphaturic response (6). PHP-II may be diagnosed in some patients with renal tubular damage or vitamin D deficiency or those receiving treatment with anticonvulsants. PHP-Ic has been identified in an extremely small number of cases beyond those initially reported. Both PHP-Ia and PHP-Ib are often diagnosed after the development of seizures around 10 years of age (7). Treatment with active vitamin D supplementation results in favorable outcomes in patients with both subtypes. The GNAS is involved in the pathogenesis both of PHP-Ia and PHP-Ib (2). The GNAS is located on chromosome 20q13 with a coding region consisting of 13 exons that encode the Gsα protein. Multiple DMRs are identified at the 5’-upstream region of exon 1, and allele-specific methylation is observed. GNAS is known to be paternally imprinted and exhibits tissue-specific repression of the gene expression. In renal tubules, the GNAS is primarily expressed by the maternal allele (8). PHP-Ia is considered to be caused by loss-of-function mutations, such as point mutations and deletions, in GNAS that result in reductions in the Gsα-protein expression or activity. Because GNAS is transcribed from both maternal and paternal alleles in most tissues, cells with a defective mutation in one allele continue to produce half the normal level of Gsα proteins. Indeed, patients with PHP-Ia display approximately 50% of the normal Gsα-protein activity in the erythrocyte membrane. The expression of GNAS in the renal tubules is influenced by paternal imprinting and tissue-specific inhibition. If the abnormal gene is inherited from the mother, PTH resistance is observed in the renal tubules. In contrast, if the abnormal gene is inherited from the father, no PTH resistance is observed in the renal tubules. In cases involving paternal transmission, physical features of AHO without evidence of PHP may be noted; this condition is called pseudopseudohypoparathyroidism (PPHP). AHO is characterized by a round face, obesity, soft tissue calcification, short stature, abnormal teeth, mental retardation and brachydactyly. The features of AHO are thought to be caused by resistance to PTH, the resistance of PTH-related proteins (PTHrPs) in cartilage and bone and abnormal lipid metabolism. However, AHO features are found not only in patients with PHP-Ia and PPHP, but also in those with Ehlers-Danlos syndrome, sarcoidosis and dermatomyositis (9). The presence of calcification in the cerebral basal ganglia is a well-known form of ectopic calcification; however, this finding is also observed in patients with idiopathic hypoparathyroidism. Soft tissue calcification is reportedly a typical feature of AHO. Brachydactyly is the most typical, and most specific, feature of AHO, being present in approximately 70% of patients with PHP-Ia (10). The Gsα deficiency observed in patients with PHP-Ia is associated with resistance to not only PTH, but also other hormones, such as thyroid-stimulating hormone, gonadotropins and growth hormone-releasing hormone (GHRH) (11). Therefore, PHP-Ia patients may also manifest thyroid and gonadal dysfunction (6). PHP-Ia is reported to cause bone diseases, such as osteitis fibrosa, due to the maintenance of the PTH response in bones (12).

On the other hand, PHP-Ib is considered to be caused by methylation abnormalities at DMRs that decrease the Gsα expression in the renal tubules, and PHP-Ib patients classically display no features of AHO (2). Classic PHP-Ib patients have a normal level of Gsα-protein activity in the erythrocyte membrane. Four DMRs, NESP55, AS, XL and A/B (also referred as 1A), are well known to be involved in the pathogenesis of PHP-Ib. PHP-Ib is also known to have cerebral basal ganglia is a well-known form of ectopic calcification; however, this finding is also observed in patients with idiopathic hypoparathyroidism. Soft tissue calcification is reportedly a typical feature of AHO. Brachydactyly is the most typical, and most specific, feature of AHO, being present in approximately 70% of patients with PHP-Ia (10). The Gsα deficiency observed in patients with PHP-Ia is associated with resistance to not only PTH, but also other hormones, such as thyroid-stimulating hormone, gonadotropins and growth hormone-releasing hormone (GHRH) (11). Therefore, PHP-Ia patients may also manifest thyroid and gonadal dysfunction (6). PHP-Ia is reported to cause bone diseases, such as osteitis fibrosa, due to the maintenance of the PTH response in bones (12).
both familial and sporadic presentations. Most patients with familial PHP-Ib have a partial deletion in the STX16 gene located approximately 220 kb upstream from the GNAS. In most cases of familial PHP-Ib, a 3-kb deletion in STX16 is identified (13). In one family, a 4.4-kb deletion partially overlapping the 3-kb deletion in STX16 was identified (14). In four other families, deletions in NESP55 were identified (15-17). The maternal transmission of these deletions results in the loss of methylation on the maternal allele at exon A/B DMR of the GNAS, while biallelic demethylation at this exon induces a decreased Gs\(\alpha\)-protein expression in the renal tubules. A study of sporadic cases demonstrated biallelic demethylation at exon A/B DMR and methylation at the NESP 55, XL and AS DMRs, i.e., all except the exon A/B DMR. Deletions in STX16 or the NESP DMR have not been found in sporadic cases (3). With respect to sporadic PHP-Ib, neither the cause nor inheritance of the methylation abnormalities have yet been elucidated.

An earlier study reported the absence of mutations in GNAS exons in 20-30% of PHP-Ia patients, in particular those who exhibit AHO features and a reduced erythrocyte membrane Gs\(\alpha\)-protein activity with PTH resistance (4). It is possible that these patients have some GNAS mutations that cannot be detected with PCR direct sequencing. In one reported case of PHP-Ia, the patient carried an 850-kb submicroscopic deletion identified with MLPA and array comparative genomic hybridization (CGH), but not PCR direct sequencing (18). These findings highlight the potential shortcomings associated with genetic testing. In addition to technical challenges, there is an increasing number of reported cases that do not fit the stereotype, i.e., a clinical diagnosis of PHP-Ia in patients with GNAS mutations identified using genetic testing or a clinical diagnosis of PHP-Ib in patients with an abnormal methylation pattern on genetic testing. Recent reports have included patients clinically diagnosed with PHP-Ia who were shown on genetic testing to have an abnormal methylation pattern indicative of familial or sporadic PHP-Ib (19-22). These reports support the existence of molecular overlap between PHP-Ia and PHP-Ib in cases in which the clinical and genetic diagnoses differ. A study in Italy reexamined 40 patients clinically diagnosed with PHP-Ia based on the presence of AHO features and multiple hormone resistance who did not have GNAS mutations on genetic testing. The investigation revealed an abnormal methylation pattern corresponding to PHP-Ib in 24 of the patients (60%) (21). Another recent report described overlap between PHP-Ia and PHP-Ib, as demonstrated by a reduced Gs\(\alpha\)-protein activity in the erythrocyte membrane in patients with a clinical diagnosis of PHP-Ib and features of AHO (23). Based on recent molecular and biological findings, researchers have suggested that a new classification of PHP is required (24). Little is known about the mechanisms of the GNAS function or methylation of DMRs, and the association with AHO has yet to be fully elucidated. Further studies of PHP cases are therefore needed.

Our patient experienced her first seizure at 7 years of age and had features of AHO, such that PHP-Ia was suspected in the clinical diagnosis. The seizures occurred during the growth and postpartum periods, when the demand for calcium is increased. When she first visited our hospital, she had mild hypokalemia with a slightly elevated serum level of 1.25(OH)\(2\)D. She described a history of suffering from a ‘cold,’ nausea and vomiting for a few days prior to the visit. The fractional potassium excretion was 6.54% and the urinary potassium-creatinine ratio was 49.8 mEq/g·cre, findings not suggestive of the renal loss of potassium. The hypokalemia was considered to be caused by the loss of body fluid and an inadequate dietary intake, particularly since the patient’s condition rapidly improved with fluid replacement and no abnormalities of the serum potassium level were noted thereafter. The serum 1.25(OH)\(2\)D level may increase in association with vitamin D insufficiency (25). Involvement of vitamin D insufficiency cannot be excluded in this case because we did not determine the patient’s serum 25(OH)D level. On the other hand, the results of the Ellsworth-Howard test and molecular analysis cannot be explained by vitamin D insufficiency alone. Slight increases in the serum 1.25(OH)\(2\)D level have also been reported in a Japanese family with PHP-Ib, although the underlying mechanism remains unknown (26).

Genetic testing yielded a diagnosis of sporadic PHP-Ib in this case. The present patient represents the first Japanese patient with overlap between PHP-Ia, as defined by the clinical diagnosis, and PHP-Ib, as defined by the results of genetic testing. The aberrant methylation observed in this patient, that is, hypermethylation on the maternal allele at the NESP DMR and demethylation at the AS, XL and A/B DMRs, matches the subcluster 3.3 pattern identified in sporadic PHP-Ib patients in a study conducted in France (4).

The MLPA technique was first developed by Schouten et al. in the Netherlands in 2002 (27). Using this method, copy number changes in up to -50 regions can be quantitatively determined using multiple probe pairs that are hybridized to target regions, ligated, PCR amplified and electrophoresed. Conventional PCR direct sequencing can be used to detect small deletions and duplications; however, it is ineffective if these features are larger than a few bps. In contrast, MLPA can be used to detect large deletions and duplications up to several Mbps. MLPA is well known for its application in the genetic diagnosis of Duchenne and Becker muscular dystrophy (28) and has been clinically applied in other genetic tests as well (29). One important application of MLPA is the analysis of DNA methylation. This method, MS-MLPA, involves the use of methylation-specific restriction enzymes (30). Conventional analyses of DNA methylation utilize bisulfite treatment to induce base substitutions and the concentration of methylated DNA. MS-MLPA is used to genetically diagnose well-known genomic imprinting-related diseases, such as Prader-Willi syndrome and Angelman syndrome (29). MS-MLPA is also useful in the genetic diagnosis of PHP-Ib (7, 31-33), and is considered to be superior to conventional methods, as it allows for easier manipulation,
requires a smaller sample volume and enables the analysis of multiple gene deletions simultaneously (7, 33).

In conclusion, the case described herein represents the first report of a Japanese patient who exhibited molecular overlap between PHP-Ia and PHP-Ib, i.e., clinical symptoms of AHO and PTH resistance in the renal tubules that were strongly suggestive of classic PHP-Ia in conjunction with an aberrant methylation pattern on genetic testing consistent with a diagnosis of sporadic PHP-Ib. It is very important to consider a diagnosis of PHP in patients with seizures associated with hypocalcemia. In addition, even if no definitive features of AHO are present, both DNA sequencing and methylation analyses should be performed for genetic testing.

The authors state that they have no Conflict of Interest (COI).

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