A mosaic mutation of phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) in X-linked hypophosphatemic rickets with mild bone phenotypes

Shoko Asano1, Saori Sako1, Yuka Funasaki1, Yumie Takeshita1, Yo Niida2 and Toshinari Takamura1

1) Department of Endocrinology and Metabolism, Kanazawa University Graduate School of Medical Sciences, Ishikawa 920-8640, Japan
2) Division of Genomic Medicine, Department of Advanced Medicine, Medical Research Institute, Kanazawa Medical University, Ishikawa 920-0293, Japan

Abstract. X-linked hypophosphatemic rickets (XLH) is primarily characterized by renal phosphate wasting with hypophosphatemia, short stature, and bone deformity of the leg. Here we present a male case of XLH with relatively mild bone deformity caused by a mosaic mutation of the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX). Polymerase chain reaction (PCR) direct sequencing revealed a novel in-frame deletion, NM-000444.6:c.671-685del p.Gln224-Ser228del, at exon 6 in PHEX as a mosaic pattern. This mutation was not found in any database and may result in a significant change in higher-order protein structure and function. TA cloning of the PCR product and clone sequencing estimated the mutation allele frequency at 21%. Literature review of the previously reported three cases with novel mosaic mutations in PHEX, together with the present case, suggests that the rates of the mutation allele correlate with phenotype severity to some extent. We initially treated him with nutritional vitamin D supplements and phosphate salts. However, to avoid the development of secondary/tertiary hyperparathyroidism, we had switched nutritional to active vitamin D supplementation with reduced phosphorus salts. The present report contributes to understanding the relationship between the mosaic rate, in addition to the mutation locus, of the PHEX gene, and clinical features of XLH.

Key words: X-linked hypophosphatemic rickets, Phosphate-regulating gene with homologies to endopeptidases on the X chromosome, Rickets, Mosaicism

Submitted Dec. 22, 2020; Accepted Apr. 1, 2021 as EJ20-0809
Released online in J-STAGE as advance publication Apr. 28, 2021
Correspondence to: Toshinari Takamura, MD, PhD, Department of Endocrinology and Metabolism, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan.
E-mail: ttakamura@med.kanazawa-u.ac.jp

©The Japan Endocrine Society
Case Report

A 61-year-old Japanese man presented to our hospital for a detailed examination of his coxodynia. He was unmarried and had no children. He had short stature (150 cm), bow legs, and scoliosis. He had never experienced any low-trauma fracture and lacked a family history of short stature, pathological fractures, and any congenital diseases. He was born at full term by normal vaginal delivery. He had been short in stature since his childhood. He was diagnosed with rickets at 5 years of age and received treatment with active vitamin D by age 10. During his growth spurt, he grew 30 cm to reach 150 cm tall. He had suffered from coxodynia since he was in his 40s. During the same period, he developed multiple caries.

The patient’s laboratory test data on admission (Table 1) revealed hypophosphatemia, slightly elevated bone-specific alkaline phosphatase, reduced renal function, reduced 25-hydroxyvitamin D3 (25-[OH]D3), elevated parathyroid hormone (PTH) level, reduced renal tubular phosphorus reabsorption, and elevated FGF23 (assayed using the FGF-23 ELISA Kit, Kainos Laboratories, Tokyo). X-ray examinations revealed curvature of the spine (Fig. 1A), Looser’s zones in the femurs, and ectopic ossification in the pelvis (Fig. 1B). The space of both hip joints was narrow with irregular margins (Fig. 1B). Based on the findings of bone changes, hypophosphatemia, and elevated FGF23 levels, FGF23-related hypophosphatemic rickets was suspected.

Because the patient’s bone pain symptom was mild and because his 25-(OH)D3 level was as low as 10.6 ng/mL, we initially treated him with nutritional vitamin D supplements (25 μg/day) and phosphate salts (600 mg/day). Nutritional vitamin D supplementation normalized his 25-(OH)D3 and 1,25-(OH)2D3 levels from 10.6 to 34.2 ng/mL (>20 ng/mL) and from 42.3 to 69.4 pg/mL (20–60 pg/mL), respectively (Table 1). However, considering that FGF23 suppresses the activity of 1α-hydroxylase, the conversion of 25-(OH)D3 to 1,25-(OH)2D3 may be relatively insufficient. Indeed, PTH levels were elevated from 102 to 248 pg/mL during the course. To avoid the development of secondary/tertiary hyperparathyroidism, we thereafter had treated him with...
active vitamin D and phosphate salts, which reduced his PTH level to 168 pg/mL after 3 months (Table 1).

**Materials and Methods**

Genomic DNA was extracted from whole blood using a rapid extraction method [4]. The mutation was screened by CHIPS (CEL nuclease mediated heteroduplex incision with polyacrylamide gel electrophoresis and silver staining) assay, a highly sensitive enzyme mismatch cleavage assay described elsewhere [5, 6]. Subsequently, the mutation was confirmed by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3100XL Genetic Analyzer (Thermo Fisher Scientific). PCR primers used in the CHIPS and direct sequencing were shown in Table 2. To distinguish the shorter fragment of the mutation allele from the wild-type allele, we set PCR primers as forward primer PHEX-6F: 5'-ATATGGCTGGGATGACAGC-3' and reverse primer PHEX-6AS: 5'-GAGTAAACTTACAGACTTGGCTTC-3' as shown in Fig. 2A. TA cloning of this PCR product was performed with pT7 Blue T-vector (Novagen). The mutation allele frequency was estimated by colony direct PCR and 12% polyacrylamide gel electrophoresis for 100 colonies.

Written informed consent was obtained from the patient after he received courteous genetic counseling and an explanation of the genetic testing, as approved by the institutional review board (No. G111).

**Genetic Analyses**

We performed a genetic analysis for the candidate genes involved in FGF23-related hypophosphatemic rickets. We identified a shorter PCR fragment derived from the PHEX gene at exon 6 (Fig. 2A) in the patient. Sanger sequencing revealed a PHEX in-frame deletion NM_000444.6:c.671_685del p.Gln224_Ser228del as a mosaic pattern. Clone PCR of 100 colonies estimated the mutation allele frequency 21% (Fig. 2B), suggesting the mosaic mutation. This mutation was not found in any database, including the Genome Aggregation Database (http://gnomad.broadinstitute.org), the Exome Aggregation Consortium (http://exac.broadinstitute.org), and the Human Gene Mutation Database, suggesting that this mutation is a novel and not a normal variant.

The deleted region of the PHEX gene (Gln224_Ser228) in this patient is completely conserved among mammalian species, for example, human, mouse, and bat. Using the software tool Sequence to Function Annotation Server (http://citeseerx.ist.psu.edu), we could see that in the high-order structure of the DNA around the mutation, the deletion exists between the β-sheet structure and α-helix structure. The deletion causes the shortage of the β-sheet structure and makes the protein structure unstable. Using the software tool PROVEAN (http://provean.jcvi.org/index.php), we also predicted the impact of this novel mutation on protein structure by in silico analyses. These in silico prediction engines resulted in a score of −21.52, which suggests deleterious damage in PHEX function. These results suggest that this region is critical for the function of the protein and is causal for XLH. From these results, we diagnosed the patient’s illness as XLH caused by a mosaic novel PHEX mutation.

**Discussion**

We report a novel PHEX in-frame deletion mutation as a mosaic pattern in a sporadic male case of XLH with
Table 2  *PHEX* each exon PCR primer for CHIPS and direct sequencing

| Exon* | Primer Name | Primer Seq | Product size (bp) |
|-------|-------------|------------|------------------|
| 1     | PHEX-1F     | 5'-GAGCAAGAAAGCCTTGGATG-3' | 334              |
|       | PHEX-1R     | 5'-ACCTATGAAGCAGCGAACAC-3' |                 |
| 2     | PHEX-2F     | 5'-TGTTTCCGAGGGTGTTTAC-3' | 266              |
|       | PHEX-2R     | 5'-CAAAATGCTTCTGACTTGG-3' |                 |
| 3     | PHEX-3F     | 5'-GGGAAAAATATAATGCAAGG-3' | 345              |
|       | PHEX-3R     | 5'-TGTTTCTTGTCAAAATATGCTC-3' |               |
| 4     | PHEX-4F     | 5'-TTTCTGAGGTTGAATTGGT-3' | 324              |
|       | PHEX-4R     | 5'-CTTTCAAACTCATTCTTTAAACC-3' |            |
| 5     | PHEX-5F     | 5'-CTAGTGTCGATCCAGTTTG-3' | 371              |
|       | PHEX-5R     | 5'-GGCAGCATGAGTCTCTTTAC-3' |                 |
| 6     | PHEX-6F     | 5'-AATATGGCTGGGAGTAGCAG-3' | 293              |
|       | PHEX-6R     | 5'-CCTGCAATGGGAAATATGGTC-3' |             |
| 7     | PHEX-7F     | 5'-TTCTGCTTCTTCATGTCCTCA-3' | 298              |
|       | PHEX-7R     | 5'-ATTTCTATTGAGATATCTGCT-3' |             |
| 8     | PHEX-8F     | 5'-CAGATGTTTTGGCACAATTGAG-3' | 278              |
|       | PHEX-8R     | 5'-ACCAAGACTGAGACGAGTMTTAC-3' |          |
| 9     | PHEX-9F     | 5'-GATTTCTCATTCTGGTTTCTC-3' | 260              |
|       | PHEX-9R     | 5'-AAAGGATGTAGAAGGAAGGC-3' |                 |
| 10    | PHEX-10F    | 5'-GGAGCTTTGCAACAATCTGTT-3' | 294              |
|       | PHEX-10R    | 5'-CCCCCTGCTTAATCTCCAAAAGATG-3' |         |
| 11    | PHEX-11F    | 5'-TTCAGCCATGGGTTTTATCC-3' | 282              |
|       | PHEX-11R    | 5'-CTACACCTGGAAGGCTGAC-3' |                 |
| 12    | PHEX-12F    | 5'-GCTTCTTGGCTTGGAGGTT-3' | 263              |
|       | PHEX-12R    | 5'-GGGGTCATTCAGAGTCAACAG-3' |             |
| 13    | PHEX-13F    | 5'-GAAGGAGGCGATTCTACTAC-3' | 273              |
|       | PHEX-13R    | 5'-TTGGTTTCTTCTGACATAC-3' |                 |
| 14    | PHEX-14F    | 5'-TTGCTCTTCCTCTGATCTAG-3' | 300              |
|       | PHEX-14R    | 5'-AACTGGGCAAGCAGCTACTC-3' |             |
| 15    | PHEX-15F    | 5'-GTCCAACATCCCCATTTGC-3' | 297              |
|       | PHEX-15R    | 5'-AGCATACACCTGCAACTTTTG-3' |             |
| 16    | PHEX-16F    | 5'-GGAGGATGTTGCTTTCAAGATG-3' | 276              |
|       | PHEX-16R    | 5'-CCATGCGCTTTCTCTGCTG-3' |                 |
| 17    | PHEX-17F    | 5'-CAAGCATTATGTGGTCTGATG-3' | 264              |
|       | PHEX-17R    | 5'-AAGCTATGATGCTGCTATG-3' |                 |
| 18    | PHEX-18F    | 5'-CTTGTCAGGGGAGGAAAG-3' | 287              |
|       | PHEX-18R    | 5'-TGTTTAAGCAAAGCTAATGTCC-3' |          |
| 19    | PHEX-19F    | 5'-GCCTACCTGATTTTATTTATGAGTG-3' |        |
|       | PHEX-19R    | 5'-AGAAGAGCAATGGCTATGATGTAATTG-3' |       |
| 20    | PHEX-20F    | 5'-TTTGCTATTCTGTTGCTAGCTG-3' | 240              |
|       | PHEX-20R    | 5'-GGGAGCAAACTCAAGTCTCTG-3' |                 |
| 21    | PHEX-21F    | 5'-ATTGAGAGCTTAAACACGACG-3' | 239              |
|       | PHEX-21R    | 5'-TCTGCGAGCGGGCTTTGGATG-3' |                 |
| 22    | PHEX-22F    | 5'-AGAAATGCCAACCTTCTTCTAGC-3' | 299              |
|       | PHEX-22R    | 5'-TCTCCAGGGCTAAAGCAATG-3' |                 |

* Exon number is according to Genbank NM_000444.5, transcript variant 1 (https://www.ncbi.nlm.nih.gov/nuccore/NM_000444.5)
relatively mild bone deformity. We found it difficult to diagnose this patient as XLH because of his relatively mild symptoms and absence of family history. Of note, frequency of sporadic XLH are as high as 30% [7], 53% [8], and 54% [9].

Some studies have reported genotype-phenotype correlations in XLH; however, there has been no definite relationship found between disease severity and type of mutation [7, 10]. The mosaic mutation in PHEX appears to be rare. Indeed, there have been only three cases with novel mosaic mutations in PHEX reported in males [11-13]. Table 3 summarizes the clinical features of these cases. Among these cases, the Chinese boy [11] and the Japanese man [12] with a higher mutation rate seem to present relatively severer phenotypes. On the other hand, the Korean man [13] and the present patient with a lower mutation rate present a relatively milder phenotype. This trend in findings suggests that, in these cases, the rates of the mutation allele correlate with phenotype severity to some extent. Therefore, in patients with mild phenotypes, genetic analyses are strongly recommended to determine the mosaic somatic mutation of PHEX. However, we must keep in mind that mutation rates may vary among organs.

In the XLH subjects without impaired kidney function, serum FGF23 level negatively correlates with serum phosphate. However, FGF23 increases exponentially as eGFR declines [14]. Also, in the present patient, moderately impaired renal function is attributable to his elevated FGF23 levels. In this setting, elevated FGF23 levels are no longer consistent with his mild symptoms and the mosaic mutation of PHEX. Unfortunately, besides the present case, FGF23 levels were not reported in other cases with the PHEX mosaic mutation (Table 3). Accumulating similar cases will clarify the regulatory mechanisms of FGF23 production independently of the PHEX function as well as the role FGF23 plays in the disease severity of XLH.

Unfortunately, he had initially been treated with nutritional vitamin D supplements and phosphate salts. The majority of patients with XLH (83.3%) were reported to have evidence of secondary hyperparathyroidism [15]. Transient increase in serum phosphorus after phosphate doses is expected to stimulate PTH production due to unidentified mechanism in XLH patients [16]. Indeed, prolonged use of high-dose phosphate therapy has been identified as a risk factor for PTH elevation [17]. Active vitamin D supplementation reduces elevated PTH levels. Of note, FGF23 suppresses 25-hydroxyvitamin D-1α-hydroxylase and activates 25-hydroxyvitamin D-24-hydroxylase in the kidney [2]. In patients with XLH whose FGF23 levels are elevated, the nutritional vitamin D supplementation insufficiently elevates 1,25-(OH)2D3 levels. In addition, serum 1,25-(OH)2D3 levels represent 1,25-(OH)2D3 production in the renal tubule, which correlates with PTH levels, while actual action of 1,25-(OH)2D3 in the intestine is only associated with 1,25-(OH)2D3 production in the intestine by local 1α-hydroxylase and irrelevant to serum 1,25-(OH)2D3 levels. Therefore, his elevated serum 1,25-(OH)2D3 level after nutritional vitamin D supplementation may be attributable to the elevated PTH levels and does not represent his actual local 1,25-(OH)2D3 activity in the intestine. The treatment with phosphate under insufficient active vitamin D supplementation may aggravate...
his secondary hyperparathyroidism. Therefore, we had switched nutritional to active vitamin D supplementation with reduced phosphorus salts to prevent the development of secondary/tertiary hyperparathyroidism according to the clinician’s guide to XLH [16], resulting in a reduction in his PTH level.

Recently, burosumab, a human anti-FGF23 antibody, was developed as a potential treatment for XLH. In children with XLH, treatment with burosumab increased renal tubular phosphate reabsorption, serum phosphorus levels, linear growth, and physical function and reduced the pain and severity of rickets [18]. In adults with XLH, the treatment increased serum phosphorus levels and mobility and reduced pain and the severity of rickets [19]. Therefore, to improve patients’ quality of life, early definite diagnosis is of great importance in XLH, although the indication for burosumab treatment in adults with XLH should be carefully discussed.

In summary, we found a novel PHEX in-frame deletion NM_000444.6:c.671_685del p.Gln224_Ser228del mutation as a mosaic pattern in a sporadic male case of XLH with relatively mild bone deformity. The mutation may result in a significant change in higher-order protein structure and function and might, through an increase in the production of FGF23, cause XLH. Mild symptoms of rickets may be associated with the PHEX mutation presented as a mosaic pattern. We believe the present report contributes to understanding the relationship between the mosaic rate, in addition to the mutation locus, of the PHEX gene, and clinical features of XLH.

Table 3: Summary of the clinical features of previously reported cases of X-linked hypophosphatemic rickets with a mosaic mutation of PHEX

| Case reference number | 11 | 12 | 13 | Present case |
|-----------------------|----|----|----|--------------|
| Age (year)/Gender/ Nationality | 4.5/M/China | 35/M/Japan | 26/M/Korea | 61/M/Japan |
| Family history | — | Proband’s daughter | — | — |
| Bone phenotypes | Caput quadratum Bow legs | Bow legs | Mild osteoarthritis in both joint Multiple old fractures | Bow legs Curvature of his spine |
| Serum FGF23 level | N.D. | N.D. | N.D. | 104 pg/mL |
| Serum phosphate level | 2.0 mg/dL | 1.7 mg/dL | 2.0 mg/dL | 1.8 mg/dL |
| Serum alkaline phosphatase level | 616 IU/L | 215 IU/L | 99 IU/L | 373 IU/L |
| Mutation in PHEX* | c.1769-1G>A p.Gly590GlufsTer28 | c.1669C>T p.Arg567Ter | c.589C>T p.Gln197Ter | c.671_685del p.Gln224_Ser228del |
| Mosaic rate | 42% | 60% | 38% | 21% |

N.D., not determined; * mRNA reference number is NM_000444.5

References

1. Endo I, Fukimoto S, Ozono K, Namba N, Inoue D, et al. (2015) Nationwide survey of fibroblast growth factor 23 (FGF23)–related hypophosphatemic diseases in Japan: prevalence, biochemical data and treatment. Endocr J 62: 811–816.
2. Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, et al. (2004) FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res 19: 429–435.
3. Beck L, Soumounou Y, Martel J, Krishnamurthy G, Gauthier C, et al. (1997) Pex/PEX tissue distribution and evidence for a deletion in the 3′ region of the Pex gene in X-linked hypophosphatemic mice. J Clin Invest 99: 1200–1209.
4. Lahiri DK, Schnabel B (1993) DNA isolation by a rapid method from human blood samples: effects of MgCl$_2$, EDTA, storage time, and temperature on DNA yield and quality. *Biochem Genet* 31: 321–328.
5. Niida Y, Kuroda M, Mitani Y, Okamura A, Yokoi A (2012) Applying and testing the conveniently optimized enzyme mismatch cleavage method to clinical DNA diagnosis. *Mol Genet Metab* 107: 580–585.
6. Tsuji T, Niida Y (2008) Development of a simple and highly sensitive mutation screening system by enzyme mismatch cleavage with optimized conditions for standard laboratories. *Electrophoresis* 29: 1473–1483.
7. Holm IA, Nelson AE, Robinson BG, Mason RS, Marsh DJ, et al. (2001) Mutational analysis and genotype-phenotype correlation of the PHEX gene in X-linked hypophosphatemic rickets. *J Clin Endocrinol Metab* 86: 3889–3899.
8. Gaucher C, Walrant-Debray O, Nguyen TM, Esterle L, Garabédian M, et al. (2009) PHEX analysis in 118 pedigrees reveals new genetic clues in hypophosphatemic rickets. *Hum Genet* 125: 401–411.
9. Basu D, Pettifor JM, Kromberg J (2004) X-linked hypophosphatemia in South Africa. *S Afr Med J* 94: 460–464.
10. Song HR, Park JW, Cho DY, Yang JH, Yoon HR, et al. (2007) PHEX gene mutations and genotype-phenotype analysis of Korean patients with hypophosphatemic rickets. *J Korean Med Sci* 22: 981–986.
11. Weng C, Chen J, Sun L, Zhou ZW, Feng X, et al. (2016) A de novo mosaic mutation of PHEX in a boy with hypophosphatemic rickets. *J Human Genet* 61: 223–227.
12. Goji K, Ozaki K, Sadewa AH, Nishio H, Matsuo M (2006) Somatic and germline mosaicism for a mutation of the PHEX gene can lead to genetic transmission of X-linked hypophosphatemic rickets that mimics an autosomal dominant trait. *J Clin Endocrinol Metab* 91: 365–370.
13. Yang M, Kim J, Yong A, Jang J, Jeon TY, et al. (2018) A novel de novo mutation in PHEX in a Korean patient with hypophosphatemic rickets. *Ann Pediatr Endocrinol Metab* 23: 229–234.
14. Wolf M (2012) Update on fibroblast growth factor 23 in chronic kidney disease. *Kidney Int* 87: 737–747.
15. Delacey S, Liu Z, Broyles A, El-Azab SA, Guandique CF, et al. (2019) Hyperparathyroidism and parathyroidectomy in X-linked hypophosphatemia patients. *Bone* 127: 386–392.
16. Carpenter TO, Imel EA, Holm IA, Jan de Beur SM, Insogna KL (2011) A clinician’s guide to X-linked hypophosphatemia. *J Bone Miner Res* 26: 1381–1388.
17. Makitie O, Kooh SW, Sochett E (2003) Prolonged high-dose phosphate treatment: a risk factor for tertiary hyperparathyroidism in X-linked hypophosphatemic rickets. *Clin Endocrinol (Oxf)* 58: 163–168.
18. Carpenter TO, Whyte MP, Imel EA, Boot AM, Högler W, et al. (2018) Burosumab therapy in children with X-linked hypophosphatemia. *N Engl J Med* 378: 1987–1998.
19. An Insogna KL, Briot K, Imel EA, Kamenický P, Ruppe MD, et al. (2018) A randomized, double-blind, placebo-controlled, phase 3 trial evaluating the efficacy of burosumab, anti—FGF23 antibody, in adults with X-linked hypophosphatemia: Week 24 primary analysis. *J Bone Miner Res* 33: 1383–1393.