Three Novel Mutations in the \textit{PHEX} Gene in Chinese Subjects with Hypophosphatemic Rickets Extends Genotypic Variability

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Abstract Mutations in the phosphate-regulating endopeptidase homolog, X-linked, gene (\textit{PHEX}), which encodes a zinc-dependent endopeptidase that is involved in bone mineralization and renal phosphate reabsorption, cause the most common form of hypophosphatemic rickets, X-linked hypophosphatemic rickets (XLH). The distribution of \textit{PHEX} mutations is extensive, but few mutations have been identified in Chinese with XLH. We extracted genomic DNA and total RNA from leukocytes obtained from nine unrelated Chinese subjects (three males and six females, age range 11–36 years) who were living in Taiwan. The \textit{PHEX} gene was amplified from DNA by PCR, and the amplicons were directly sequenced. Expression studies were performed by reverse-transcription PCR of leukocyte RNA. Serum levels of FGF23 were significantly greater in the patients than in normal subjects (mean 69.4 ± 18.8 vs. 27.2 ± 8.4 pg/mL, \( P < 0.005 \)), and eight of the nine patients had elevated levels of FGF23. Germ-line mutations in the \textit{PHEX} gene were identified in five of 9 patients, including novel c.1843 delA, donor splice site mutations c.663-2delT and c.1899+2T>A, and two previously reported missense mutations, p.C733Y and p.G579R. These data extend the spectrum of mutations in the \textit{PHEX} gene in Han Chinese and confirm variability for XLH in Taiwan.

Keywords X-linked hypophosphatemic rickets · \textit{PHEX} · FGF23 · Mutation analysis

Although acquired vitamin D deficiency is the principal cause of rickets worldwide, rickets (and osteomalacia) can also arise in patients with defects that perturb vitamin D metabolism or responsiveness or with conditions that cause hypophosphatemia. Phosphate homeostasis is principally controlled by changes in the tubular reabsorption of phosphate, which is regulated, directly or indirectly, by PTH, phosphatonin such as FGF23, and 1,25(OH)\(_2\)D. Thus, hypophosphatemia and hypophosphatemic rickets most commonly occur in patients who have conditions that depress renal tubular phosphate transport. A variety of acquired and genetic disorders have been described in which decreased renal tubular phosphate reabsorption leads to the development of hypophosphatemic rickets. An acquired form of hypophosphatemia occurs in patients with fibrous dysplasia or mesenchymal tumors that overexpress FGF23, a phosphatonin that reduces expression of the NaPi2a and NaPi2c sodium–phosphate cotransporters in renal proximal tubular cells and impairs synthesis of 1,25(OH)\(_2\)D [1, 2]. In addition, at least five inherited forms of hypophosphatemic rickets have now been characterized:
X-linked hypophosphatemic rickets (XLH; MIM 307800, 307810) is caused by mutations in the PHEX gene located at Xp22.1 [3], autosomal dominant hypophosphatemic rickets (MIM 193100) is caused by mutations in the FGF23 gene [4], autosomal recessive hypophosphatemic rickets (MIM 600980) is caused by mutations in the DMP1 [5, 6] or ENPP1 [7, 8] gene, and autosomal recessive hypophosphatemic rickets with hypercalciuria (HHRH) is caused by mutations in SLC34A3 encoding the NaPi-IIc renal sodium–phosphate cotransporter [9–11]. XLH is the most prevalent form of inherited rickets in humans, with an incidence of 1 in 20,000 individuals [12, 13]. It is a dominant disorder characterized by hypophosphatemia secondary to renal phosphate wasting, abnormal regulation of renal metabolism of vitamin D, rickets and osteomalacia, growth failure, and resistance to vitamin D therapy. Despite marked hypophosphatemia, these patients seldom present with muscle weakness [13–15].

The PHEX gene encompasses 22 exons and encodes a 749-amino acid protein with significant structural homology to members of the type II integral membrane zinc-dependent endopeptidase family, with short N-terminal cytoplasmic domains, a single transmembrane domain, and a long extracellular domain [3]. Defective PHEX function is associated with increased serum concentrations of the phosphatonin FGF23, which likely accounts for the metabolic defects in hypophosphatemic rickets. More than 250 mutations in the PHEX gene have been identified to date in patients with XLH and are catalogued in a locus-specific database [16](www.phexdb.mcgill.ca). The mutations, which are scattered throughout the gene, include deletions, splice junction and frameshift mutations, duplications, insertions, and missense and nonsense mutations and are consistent with loss of function of the PHEX protein. XLH due to unique mutations in PHEX has been reported in subjects of East Asian ethnicity including Japanese, Korean, and Chinese living in China and Taiwan [17–25]. Mutational founder effects for a variety of genetic disorders, as well as haplotype sharing in only a small chromosomal interval, have been found in East Asians that are not replicated in Caucasians, suggesting that specific mutations are ancient and occurred after the divergence of East Asians and Caucasians. In this study, we sought to identify PHEX gene mutations in Chinese patients with hypophosphatemic rickets who are living in Taiwan.

Patients and Methods

Patients

We studied nine unrelated Chinese subjects (three males and six females, age range 11–36 years) with clinical and biochemical manifestations of early-onset or congenital hypophosphatemic rickets, including hypophosphatemia, short stature, and lower extremity deformities (Table 1). There were three familial (cases 1, 2, and 5) that showed dominant transmission of rickets and six sporadic cases. At the time of these studies, all affected subjects except proband 1 and his sister and proband 2 and her brother were receiving treatment with an active form of vitamin D plus phosphate. This study was approved by the institutional review board of the Taipei Veterans General Hospital, and informed consent was obtained from each individual.

Serum Measurements

Serum concentrations of intact FGF23 were measured using a two-site enzyme-linked immunosorbent assay (FGF23 ELISA Kit; Kainos, Tokyo, Japan) according to the manufacturer’s recommendations. All samples were assayed at the same time in the same assay. Circulating levels of intact FGF-23 were measured in serum from nine patients, two siblings, and 11 age- and sex-matched normal individuals. Serum 1,25(OH)2D levels were measured with RIA (DiaSorin, Stillwater, MN). The TRP was calculated using the standard, formula and TmP/GFR was determined using the nomogram of Bijvoet [12, 26, 27].

PHEX Mutational Analysis

Genomic DNA was isolated from EDTA-preserved whole blood using the GFX Genomic Blood DNA purification kit (Amersham Biosciences, Piscataway, NJ). Twenty-two pairs of PHEX-specific primers [22] were used for PCR amplification of the coding exons and adjoining splice sites in a 25-μL reaction containing 200 ng of genomic DNA, 2.0 mM MgCl2, 0.2 mM of each dNTP, 0.15 M of each primer, 1x reaction buffer, and 1 unit of FastStart Taq DNA polymerase (Roche, Indianapolis, IN). The DNA sequences of both strands were determined by Taq polymerase cycle sequencing using fluorescence-labeled dideoxyterminators (BigDye Terminator V3.1 Cycle Sequencing Kits; Applied Biosystems, Foster City, CA) and resolved on an automated detection system (ABI 377-36 sequencer, Applied Biosystems). Mutations are reported according to the guidelines of the Human Genome Variation Society; based on the cDNA sequence, +1 is the A of the ATG start codon [28]. Novel mutations were defined by their absence from the Human Gene Mutation Database (http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html) and from mutations previously reported in the PHEX-specific database [16](www.phexdb.mcgill.ca). The functional consequences of novel splice variants were predicted with the NNSPLICE 0.9 automated splice site analysis program on the web (http://www.fruitfly.org/seq_tools/splice.html) [29].
Total RNA was extracted from peripheral whole blood using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was generated with SUPERSCRIPT II reverse transcriptase (Invitrogen, Carlsbad, CA) using an oligo(dT) 12–18 primer and subsequently used as a template for PCR. For proband 2, the forward primer (ggtctcttaagtctccaagc) was located in exon 2 and the reverse primer (tttgtgtacatggcctcgc) was located in exon 8. PCR products of female subjects were cloned into pGEM-T vector (Promega, Madison, WI), and 10 clones were selected for sequence analysis (Applied Biosystems). For proband 3, the forward primer (atccgacgactgtcaatgcc) was located in exon 15 and the reverse primer (ttggacttgttctcgggcag) in exon 21. The products of RT-PCRs for cases 2 and 3 were electrophoresed on 3% agarose gel and stained with ethidium bromide. The identity of the amplicons was confirmed by nucleotide sequence analysis.

Statistical Analysis

All data are expressed as the mean ± SD, and comparison between groups was calculated using unpaired Student’s t-tests. Linear regression was used to compare phenotypic features and genotypes. P < 0.05 was considered significant. GraphPad InStat, version 3.06 (GraphPad, San Diego, CA), was used to perform statistical analyses.

Results

Clinical Features

The clinical, molecular, and biochemical data for all nine patients are shown in Table 1. Patients 4 and 5 had experienced multiple fractures of the lower extremities. The mean serum FGF23 level was significantly higher in patients than in normal subjects (69.4 ± 18.8 vs. 27.2 ± 8.4 pg/mL, P < 0.005) and frankly elevated in eight of nine patients (Table 1). At the time of sampling, probands 1 and 2 were not taking either calcitriol or phosphate; other subjects either were taking low doses of calcitriol and phosphate or were poorly compliant.

Mutations of the PHEx Gene

DNA analysis of the entire coding region together with the associated splice sites and 5’ and 3’ untranslated regions of the PHEx gene disclosed mutations in five of

Table 1  Clinical, biochemical, and molecular data for all patients

| Probanda | PHEx mutation | Sex | Age (years) | Height (cm) | Z scores for height | Weight (kg) | S.Ca (mmol/L) |
|----------|---------------|-----|-------------|-------------|--------------------|-------------|---------------|
| 1        | nt 1843 del A, exon 18 | M   | 29          | 150         | -3.69              | 50          | 2.40          |
| 2        | IVS5 + 2 del t, intron 5 | F   | 20          | 133         | -5.00              | 47          | 2.40          |
| 3        | IVS18+2 t→a, intron 18 | M   | 30          | 148         | -4.03              | 53          | 2.40          |
| 4        | p.C733Y, exon 22 | F   | 33          | 144         | -2.92              | 62          | 2.27          |
| 5        | p.G579R, exon 17 | F   | 36          | 142         | -3.30              | 42          | 2.35          |
| 6        | N              | F   | 15          | 143         | -2.76              | 49          | 2.47          |
| 7        | N              | F   | 30          | 136         | -4.43              | 46          | 2.35          |
| 8        | N              | F   | 16          | 142         | -3.15              | 43          | 2.32          |
| 9        | N              | M   | 11          | 128         | -2.23              | 34          | 2.50          |
| Normal   |                |     |             |             |                    |             | 2.15–2.60     |

| Probanda | U.Ca/Cr (mmol/L) | S.Phos (mmol/L) | TRP (mmol/L) | TmP/GFR (mmol/L) | 1,25 (OH)2D (pmol/L) | FGF23 (pg/mL) |
|----------|-----------------|----------------|-------------|-----------------|---------------------|----------------|
| 1        | 0.23            | 0.52           | 0.90        | 0.46            | 85                  | 55.8           |
| 2        | 0.17            | 0.61           | 0.82        | 0.50            | 95                  | 55.8           |
| 3        | 0.12            | 0.39           | 0.54        | 0.20            | 48                  | 88.8           |
| 4        | 0.03            | 0.68           | 0.80        | 0.53            | 34                  | 63.3           |
| 5        | 0.14            | 0.77           | 0.78        | 0.47            | 235                 | 50.1           |
| 6        | 0.22            | 0.87           | 0.90        | 0.77            | 151                 | 55.8           |
| 7        | 0.04            | 0.61           | 0.65        | 0.39            | 83                  | 85.0           |
| 8        | 0.01            | 0.81           | 0.68        | 0.54            | 123                 | 104.3          |
| 9        | ND              | 0.81           | 0.81        | 0.65            | 86                  | 89.5           |
| Normal   | <0.62           | 0.8–1.45       | >0.90       | 0.80–1.35       | 39–102              | 18.6–50.8      |

a Cases 1, 2, and 5 were familial
N no detectable mutation, ND not done
the 9 patients we studied (Table 1, Fig. 1). Patient 1 was hemizygous for a single nucleotide deletion (c.1843 delA) in exon 18 of the PHEX gene, resulting in a frameshift and early translational termination that is predicted to produce a protein that is truncated at amino acid residue 617. Patient 2 was heterozygous for a donor site mutation (c.663+2delT) in intron 5. RT-PCR of RNA from patient 2 produced two amplicons, one that corresponded to the expected size of 771 bp and a shorter amplicon of 694 bp (Fig. 1b). Analysis of this mutation by NNS-PLICE 0.9 [29] predicted aberrant splicing of the PHEX mRNA, which was confirmed by RT-PCR of leukocyte RNA and nucleotide sequence analysis, which confirmed the usage of an alternative 5'-splice site in exon 5 that was located 77 nucleotides upstream of the normal donor splice site (Fig. 1). Patient 3 was hemizygous for a donor site transversion (c.1899+2T>A) in intron 18 that also was predicted to result in aberrant splicing of the PHEX mRNA. RT-PCR of leukocyte RNA from this patient revealed a PHEX cDNA that utilized an alternative 5'-splice site that is 131 nucleotides upstream of the normal donor site and results in skipping of exon 18 (Fig. 1c). The amplicon corresponding to wild-type cDNA is 521 bp, and the corresponding mutant amplicon is 390 bp. Patient 4 had a C733Y missense mutation (TGT → TAT) in codon 733 of exon 22. Patient 5 had a G579R missense mutation (GGA → AGA) in codon 579 of exon 17.

Fig. 1 Molecular analysis of PHEX. a Probando had a c.1843 delA mutation in his copy of the PHEX gene. b Probando 2 was heterozygous for c.663+2delT, resulting in alternative splicing of mRNA from this allele. The amplicons generated by RT-PCR of RNA from a normal subject and the patient are shown and indicate that the patient had a smaller band (694 bp) than expected (771 bp) for the wild-type allele. The RT-PCR product of this patient was sequenced, and the 5'-splice site of intron 5 was shifted 77 nucleotides upstream in exon 5. c Patient 3 was hemizygous for the c.1899+2T>A mutation that resulted in an abnormally spliced mRNA in which the 5'-donor splice site of intron 18 was shifted 131 nucleotides upstream of the native donor splice site, resulting in skipping of exon 18. The amplicon corresponding to wild-type cDNA is 521 bp, and the corresponding mutant amplicon is 390 bp. d Patient 4 had a C733Y missense mutation (TGT → TAT) in codon 733 of exon 22. e Patient 5 had a G579R missense mutation (GGA → AGA) in codon 579 of exon 17.
these sequence changes was present in genomic DNA from 50 unrelated subjects.

Patients 4 (C733Y) and 5 (G579R) were heterozygous for missense mutations that have been previously described [30, 31].

Patients 1, 2, and 5 have familial hypophosphatemic rickets. The affected brother and mother of proband 2 had the same genotype, and all of them had severe genu varum and short stature. Proband 2 had severe genu varum and had undergone osteotomy of the lower extremities four times. All of the clinically affected relatives of proband 5 carried the same mutation and presented with genu varum and short stature. Regrettably, neither of the two affected family members of proband 1 agreed to participate in this study.

There was no relationship between height SD and presence or absence of a \textit{PHEX} mutation, nor were there any relationships between type of mutation and height SD, serum concentration of FGF23, and serum phosphate levels. Moreover, we found no evidence for a relationship between serum concentrations of FGF23 and serum levels of phosphate or calcitriol.

**Discussion**

We analyzed the \textit{PHEX} gene in nine unrelated Chinese subjects with early-onset hypophosphatemic rickets, including three patients with familial rickets and six patients with sporadic rickets. Our mutation detection rates in the \textit{PHEX} gene were 100% in familial cases and 33% in sporadic cases, which are similar to the results of most previous studies in which the mutation detection rates ranged 51–86% in familial cases and 22–57% in sporadic cases [18, 30–34]. One notable exception is a single study from Finland, in which the mutation detection rate was 100% for familial cases and 93% for sporadic cases [35].

The lower mutation detection rate in apparently sporadic cases might reflect nongenetic causes of renal phosphaturia, autosomal recessive forms of hypophosphatemic rickets due to mutations in the \textit{DPMP1} [5, 6] or \textit{ENPP1} [7, 8] gene, or the presence of de novo mutations in the \textit{FGF23} gene [4].

Our work raises several points that are worth noting. First, all of the \textit{PHEX} mutations that we identified in this study were different from the mutations that have been previously described in Chinese patients with XLH [19] (Fig. 2), indicating that there is significant variability in \textit{PHEX} mutations in this population. Second, the mutations in the coding exons of \textit{PHEX} were found in the 3’ end of the gene, which is consistent with the results of previous studies [36, 37] and mutations entered in \textit{PHEXdb} [16], which suggests that this region might encode for a critical region of the \textit{PHEX} protein. Third, the two donor splice site mutations both resulted in an aberrant 5’-splice site. As is typical of mutations at donor or acceptor sites, the two mutations that we identified utilized upstream cryptic splice sites that in one case resulted in skipping of the upstream exon. Mutations affecting splice sites are common in human genetic disease and account for 10–15% of gene mutations. The majority of these are single-point mutations affecting the conserved bases at the donor or acceptor splice sites. A study of more than 100 splice-site mutations [38, 39] showed that point mutations affecting the 5’ donor splice site were more common than those at the 3’ acceptor site (62% vs. 26%). At the 5’ donor splice site, mutations affecting the G residue at position +1 are the most common, followed by mutations at position +5. Mutations at these two positions are thought to significantly reduce the pairing of the donor splice site with the complementary site in the small nuclear ribonucleoprotein particle U1snRNP, which is one of the first steps in the complex process of mRNA splicing [40, 41].

Lastly, we found elevated serum levels of intact FGF23 in eight of nine patients with hypophosphatemic rickets.

![Fig. 2](image-url)  
**Fig. 2** The distribution of \textit{PHEX} gene mutations among Chinese with XLH is shown. Mutations in exon 18, in which the zinc-binding domain is located, are the most common and account for 30% (4/13) of \textit{PHEX} mutations in Chinese subjects.
Our results confirm the association of elevated circulating FGF23 and hypophosphatemia in subjects with XLH and other disorders characterized by hypophosphatemia [2, 42–44]. Recent studies show that most subjects with XLH demonstrate increases in serum FGF23 in response to treatment with calcitriol and phosphate, with significant and nonsignificant positive associations with daily phosphate and calcitriol doses, respectively [45]. However, the relatively low doses of calcitriol and phosphorus used by the mostly adult subjects in our study, as well as generally poor compliance in our population, did not permit us to identify similar associations. Moreover, the cross-sectional design of our study makes it very difficult to determine if an association or a lack of such is caused by differences in treatment or by primary differences in the phenotype.

The sodium–phosphate cotransporters NaPi-IIc and NaPi-IId are coexpressed in the renal brush border membrane of the proximal tubule, where most filtered phosphate is reabsorbed. NaPi-IId and NaPi-IIc are both regulated by PTH, FGF23, and dietary phosphate; PTH and FGF23, in response to elevated levels of dietary serum phosphate, decrease the abundance of cotransporters at the cell membrane and thereby reduce the tubular reabsorption of phosphorus from the glomerular filtrate [9, 46, 47]. Serum levels of FGF23 are inappropriately normal or elevated in XLH and other forms of hypophosphatemic rickets [1, 2] except ADHR [11, 48].

A limitation of our study is the small sample size, which may have restricted our ability to identify potential genotype–phenotype relationships between XLH and any of the mutations that we identified. Although Holm et al. [31] identified a trend between truncating mutations and more severe skeletal disease in a familial group (P = 0.072), at present no consensus has been reached regarding the correlation between the disease phenotypes and PHEX mutation locations, including those in this study [16, 18, 20, 22, 33, 37, 49, 50].

In conclusion, we have identified five mutations in the PHEX gene in Han Chinese patients with XLH in Taiwan, including three novel mutations. Our results, taken in the context of prior studies of PHEX gene analyses in East Asians, confirm the notion that variable context of prior studies of PHEX mutation locations, including those in this study [16, 18, 20, 22, 33, 37, 49, 50].

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