Seven novel and six *de novo PHEX* gene mutations in patients with hypophosphatemic rickets

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Abstract. Inactivating mutations in phosphate-regulating gene with homologies to endopeptidase on the X chromosome (*PHEX*) have been identified as a cause of X-linked hypophosphatemic rickets (XLH; OMIM 307800). In the present study, we enrolled 43 patients from 18 unrelated families clinically diagnosed with hypophosphatemic rickets and 250 healthy controls. For each available individual, all 22 exons with their exon-intron boundaries of the *PHEX* gene were directly sequenced. The levels of serum fibroblast growth factor 23 (FGF23) were measured as well. Sequencing analysis detected 17 different *PHEX* gene mutations, and 7 of these were identified as novel: 3 missense mutations, including c.304G>A (p.Gly102Arg) in exon 3, c.229T>C (p.Cys77Arg) in exon 3 and c.824T>C (p.Leu275Pro) in exon 7; 2 deletion mutations, including c.528delT (p.Glu177LysfsX44) in exon 5 and c.1234delA (p.Ser412ValfsX12) in exon 11; and 2 alternative splicing mutations, including c.436_436+1delAG in intron 4 at splicing donor sites and c.1483-1G>C in intron 13 at splicing acceptor sites. Moreover, 6 mutations were proven to be *de novo* in 6 sporadic cases and the probands were all females. No mutations were found in the 250 healthy controls. The serum levels of FGF23 varied widely among the patients with XLH, and no significant difference was found when compared with those of the healthy controls. On the whole, the findings of this study provide new insight into the spectrum of *PHEX* mutations and provide potential evidence of a critical domain in PHEX protein. In addition, the finding of an overlap of the serum FGF23 levels between the patients with XLH and the healthy controls indicates its limited diagnostic value in XLH.

Introduction

Inherited hypophosphatemic rickets, characterized by bone mineralization disorders due to hypophosphatemia, secondary to a leak of phosphate from the kidneys, was first introduced by Albright *et al* (1). The clinical characteristics of the disease include growth retardation, bending of the weight-bearing extremities, and the resistance to vitamin D therapy during early childhood. Subsequently, patients suffer from spontaneous dental abscesses, extensive bone pain and arthropathy with aging. X-linked hypophosphatemic rickets (XLH; OMIM 307800), with an occurrence of appproximately 1 in 20,000 live births, is known as the most prevalent form of inherited hypophosphatemic rickets (2,3). The mutation in the phosphate-regulating gene with homologies to endopeptidase on the X chromosome (*PHEX*; MIM 300550) was identified in 1995 as the causative gene for the disease (4). Thereafter, the genetic basis of other less prevalent types of inherited hypophosphatemic rickets, such as autosomal dominant hypophosphatemic rickets (ADHR; OMIM 193100) and autosomal recessive hypophosphatemic rickets (ARHR1; OMIM 241520), was successively clarified (5,6). Nevertheless, the underlying pathogenesis for some sporadic cases without known genetic mutations remains to be determined and the significant findings of key roles of microRNAs (miRNAs of miRs) involved in bone formation may provide potential research issues (7-9).

The *PHEX* gene, located on X chromosome Xp22.1, is composed of 22 exons spanning 243 kb and encodes for 749 amino acid proteins (10). It exhibits great homology to the M13 zinc metallopeptidases, a class of type 2 integral membrane glycoproteins that includes neprilysin (*NEP*), endothelin-converting enzymes 1 and 2 (*ECE-1* and *ECE-2*), as well as the Kell antigen (*KELL*) (11). These proteins show the common structural traits of a short N-terminal cytoplasmic domain, a single transmembrane hydrophobic region and a large extracellular domain. The latter contains 10 highly conserved cysteine residues and two zinc-binding motifs and is involved in the secondary structure conformation or catalytic activity of the protein (11). It has been demonstrated that the tissue-specific expression of the *PHEX* gene and its mRNA expression

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is predominantly detected in bone lineage cells; for instance, osteoblasts, osteocytes and odontoblasts (10,12). However, the exact mechanisms through which inactivated *PHEX* leads to abnormal skeletal and renal manifestations in XLH have not yet been fully clarified. Studies have suggested the existence of phosphatonin that is responsible for the regulation of phosphate homeostasis in the *Hyp* mouse (13), an animal model of XLH with a 3' deletion of the *PHEX* gene. Based on the finding of increased fibroblast growth factor 23 (FGF23) transcripts in the *Hyp* mouse, FGF23 was then regarded as a leading candidate for phosphatonin downstream of *PHEX* (14).

An extensive mutation analysis of the genetic defects of the *PHEX* gene only revealed a few cases in China (15-21). In the present study, we screened a total of 18 affected families for mutations in the *PHEX* gene in order to interpret the mutation traits in Chinese patients and potentially provide evidence of a critical domain in *PHEX* protein. Moreover, the serum FGF23 levels in affected individuals were also measured as this may also contribute to our understanding of the molecular basis of XLH.

Materials and methods

Ethics statement and study subjects. This study was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China) and all subjects or their guardians (for the under-aged participants) provided written informed consent prior to enrollment. The subjects enrolled in this study were from the Department of Osteoporosis and Bone Diseases in Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China over the past 3 years. A total of 18 unrelated Chinese families with hypophosphatemic rickets was examined and all subjects were of Han ethnicity. The pedigrees of the families with hypophosphatemic rickets are shown in Fig. 1. The diagnosis of hypophosphatemic rickets was mainly based on a history of childhood rickets, clinical manifestations and biochemical tests indicating hypophosphatemia along with elevated levels of serum alkaline phosphatases. Patients with secondary rickets due to malnutrition, medication or tumor-induced osteomalacia were excluded. Finally, 65 individuals, including 43 patients, and 250 unrelated healthy controls were recruited and subjected to blood sampling followed by DNA analyses. In addition, another 95 healthy controls with normal serum phosphate and calcium levels and normal renal function were randomly selected to carry out measurements of serum levels of FGF23 in order to derive a reference range.

Mutation screening. To detect mutations in the *PHEX* gene, all DNA samples from the probands were initially analyzed. When a *PHEX* mutation was confirmed, DNA from first-degree relatives, as well as the symptomatic individuals was then screened for the detected mutation. A proband was identified as a sporadic case if the *PHEX* mutation was not detected in the parents, namely if both were asymptomatic.

Mutation analyses of the affected individuals were performed by the direct sequencing of polymerase chain reaction (PCR) products amplified from genomic DNA. The DNA was extracted from peripheral white blood cells by proteinase K digestion followed by purification with phenol/chloroform

and isopropyl alcohol precipitation. The DNA sequence for the *PHEX* gene was obtained from the available online database (NCBI Reference Sequence Accession no. NG_007563.2). All the 22 exons with their adjacent intronic sequences of the *PHEX* gene were amplified by PCR with 21 pairs of sequencing primers designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences are presented in Table I. Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 and the PCR products were directly sequenced using an automated ABI PRISM 3130 sequencer (both from Applied Biosystems, Foster City, CA, USA). Simultaneously, single-nucleotide polymorphisms (SNPs) were identified using PolyPhred (http://droog.gs.washington.edu/polyphred/) and novel mutations were identified using HGMD (http://www.hgmd.cf.ac.uk/). Mutations were checked using Mutalyzer 2.0 (http://mutalyzer.nl/check). The DNA sequences obtained were aligned with homologous sequences that had been deposited into GenBank using the CluxtalX 1.83 algorithm. To predict the impact of missense mutations on protein structure and function, Polymorphism Phenotyping v2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2) (22) and Sorting Tolerant from Intolerant (SIFT; http://sift.jcvi.org) (23) were used based on sequence alignments.

Measurement of circulating intact FGF23 levels. Serum FGF23 leels were measured using an intact human FGF23 enzyme-linked immunosorbent assay (ELISA) kit obtained from Kainos Laboratories Inc. (Tokyo, Japan). The detectable concentration ranged from 3 to 800 pg/ml. This assay has been validated in previous studies (16,24).

Statistical analyses. Normally distributed variables are presented as the means ± SD, and non-normally distributed variables as the median (2.5th and 97.5th percentiles). Comparisons between groups were made using unpaired Student's t-tests for normal data, and the Wilcoxon rank sum test for non-normal data. Values of P<0.05 were considered to indicate statistically significant differences. Statistical analyses were performed using SPSS 13.0 software.

Results

The baseline clinical data and *PHEX* gene mutation analyses of 65 participants originating from 18 families are summarized in Table II. A total of 43 patients was confirmed to have XLH according to their clinical and genetic evidence. All the patients exhibited varying degrees of growth retardation, bending legs, dental anomalies, hypophosphatemia, markedly elevated serum alkaline phosphatase levels and normal serum calcium. The median (25th and 75th percentiles) age of the patients was 22.0 (8.3, 29.5) years and the median serum phosphate levels were 0.70 (0.57, 0.80) mmol/l.

We identified 17 different mutations in the *PHEX* gene from 18 unrelated families and the distribution of *PHEX* mutations were 6 alternative splicing mutations (35.3%), 5 missense mutations (29.4%), 3 nonsense mutations (17.6%), 2 deletion mutations (11.8%) and 1 insertion mutation (5.9%) (Fig. 2). Among these, 7 mutation sites were identified as novel and they were respectively detected from families 4, 6, 7, 8, 12, 13 and 16: c.1483-1G>C in intron 13 at splicing acceptor sites

Table I. List of primer sequences used for PCR amplification of the PHEX gene.

Exons	Forward primer (5'→3')	Reverse primer (5'→3')
1	AGGGACTTTGCTGAGGGAGAG	CCACTCGAAGCCACTTACACC
2	TGGGTTTTGGAATACCGTGTC	AAGAGAGGCCATTCAGCCTTC
3	CAAGGCTTGGAAACTGGTTGA	TTATGTTGAGATCTGGGAGTCCA
4	GGCACCATATGTGGGTGGATA	GTTTGCCCTGCTGACTTTGTC
5	CACATTGAAGCGTGGATCGTA	CGGGAGAAGGGAATATTCTGG
6	GCTCTGCCCAATCATGTTACC	GCAGCCTGGTAAGGCACATAG
7	GGGTGCCTGGTATTGCATAAT	CCAATGGGCAATGACACAAA
8	ACCACACCAAAGCCTTGAAAA	GAGCCAATGCCAACAATTACC
9	GGATGGCAATGATCAGGAGTT	GACAGTGCTTTTGGCCAGTTC
10	ATGTTCACTCTGAGGGCTGGA	GGCTACAAACTCCCCCTGTCT
11	CAGCCATGGGTTTTATCCAAA	CCCACTCCCCTGGAAAACTAC
12	AGTGTTGCCAGAGCATGGAGT	AGGAAAGGCCGAATTACAAGG
13	TCGATTCAGTCACCTTCTCCA	GAAAGGCACAAGGCCAGTAAA
14	TGACTGATGCAGCTTCTCTGC	ATGCTAGAAATGGGGGACCTG
15	GCAGGGACAGCCCTTTAGATT	GCCACTTTTGGGGGAAATAAG
16	GTGCAAAATGGTTTCCCTGAA	GTCCAGCCATACACCCTGGTA
17	AAGCAGTTTATCTTGGCTTTCCA	CAAGCCATCACAGCAAGACAC
18	CTGCTTTTTGAAGGCTTGTCG	ATGCCTGGTTAAGGGATGACC
19	TTGATGCCTCTTGCTGAATGA	AAATGAACCTAGCCCCAAGGA
20	TGGTAAGCAACAGGACATGGA	AGGGCTGCTAACCCATTTGAT
21	TTCCTGGGCACATATACGATTC	TTTTGGCTGCAAAATGGAAAT
22	CAGAACCTGTTGATGTGCAAGA	GCCAACACCCTAAAATGGACA

PCR, polymerase chain reaction; PHEX, phosphate-regulating gene with homology to endopeptidase on the X chromosome.

leading to truncated protein, c.1234delA (p.Ser412ValfsX12) in exon 11, c.436_436+1delAG in intron 4 at splicing donor sites leading to truncated protein, c.824T>C (p.Leu275Pro) in exon 7, c.304G>A (p.Gly102Arg) in exon 3, c.229T>C (p.Cys77Arg) in exon 3 and c.528delT (p.Glu177LysfsX44) in exon 5. PolyPhen-2 and SIFT were performed to assess the missense mutational consequence of *PHEX*, and all 3 missense mutations were predicted to be probably damaging with a score of 0.930 for L275R, 0.917 for G102R and 1.000 for C77R, and a SIFT score of <0.05. Notably, the amino acid residues at p.275, p.102 and p.77 were evolutionarily highly conserved across 12 different species as shown in Fig. 3. In addition, the probands from families 9, 10, 11, 16, 17 and 18 were identified as sporadic cases and the detected *PHEX* mutations were likely to be *de novo*, and the 6 probands were all female patients.

Serum FGF23 levels were not normally distributed in either the normal controls or the XLH patients. The median value for intact serum FGF23 levels from the 95 healthy individuals aged between 22 to 77 years was 40.6 pg/ml and the reference range (2.5th and 97.5th percentiles) was from 24.6 to 136.8 pg/ml (Table III). For the patients with XLH, as shown in Table IV, the serum FGF23 levels were below the reference range in 4 of 11 subjects, within the range in 4 subjects, and mildly elevated in 3 subjects. The serum FGF23 levels exhibited a wide variation in the patients with XLH, and no significant differences were found when compared with those of the normal controls (p>0.05).

Discussion

Based on the *PHEX* mutation database (http://www.*PHEX*db. mcgill.ca), the frequencies of different mutation types were 25% frameshifts, 23% alternative splicing, 22% missense, 18% nonsense, 8% deletion and 4% polymorphisms. The mutations were scattered throughout the gene and the majority would potentially influence the pattern of post-translational modification of the protein and alter its secondary structure, resulting in the loss of *PHEX* function (25,26). In this study, we identified 17 different *PHEX* mutations from 18 unrelated Chinese families, and to the best of our knowledge, 7 were novel.

Three novel missense mutations, namely, Cys77Arg, Gly102Arg and Leu275Pro, were detected. The residue p.77 was amongst the 10 highly conserved cysteine residues that were critical for disulfide-bond formation and protein folding (25,26). Thus, the single base change at this position would likely alter the secondary structure of the protein and render it out of action. Gly102 has been found to be conserved among *NEP*, *PHEX* and *ECE-1* according to the multiple sequence alignment analysis in molecular research (27). Additionally, the glycine to arginine exchange was likely to increase the local charge of the *PHEX* gene product as arginine was positively charged, while glycine had an uncharged polar group. Therefore, it is possible that this base alteration plays a role in a different spatial conformation. The novel missense mutation detected in exon 7 involved a
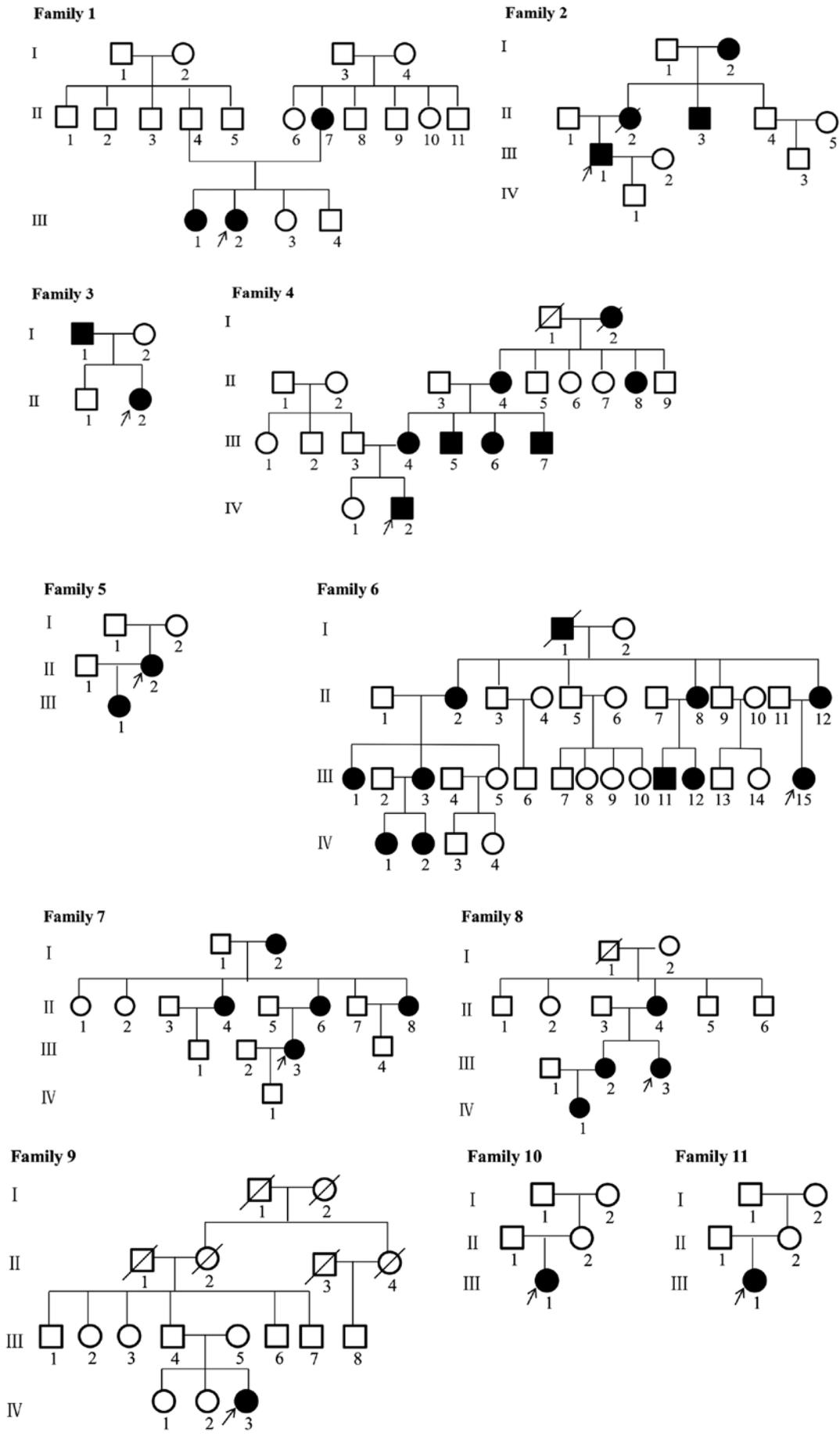
Figure 1. Pedigree of families 1-11 with hypophosphatemic rickets. The black symbols represent the affected individuals, and the open symbols represent the unaffected individuals. The circles and squares indicate females and males, respectively. The arrows identify the probands in the families.
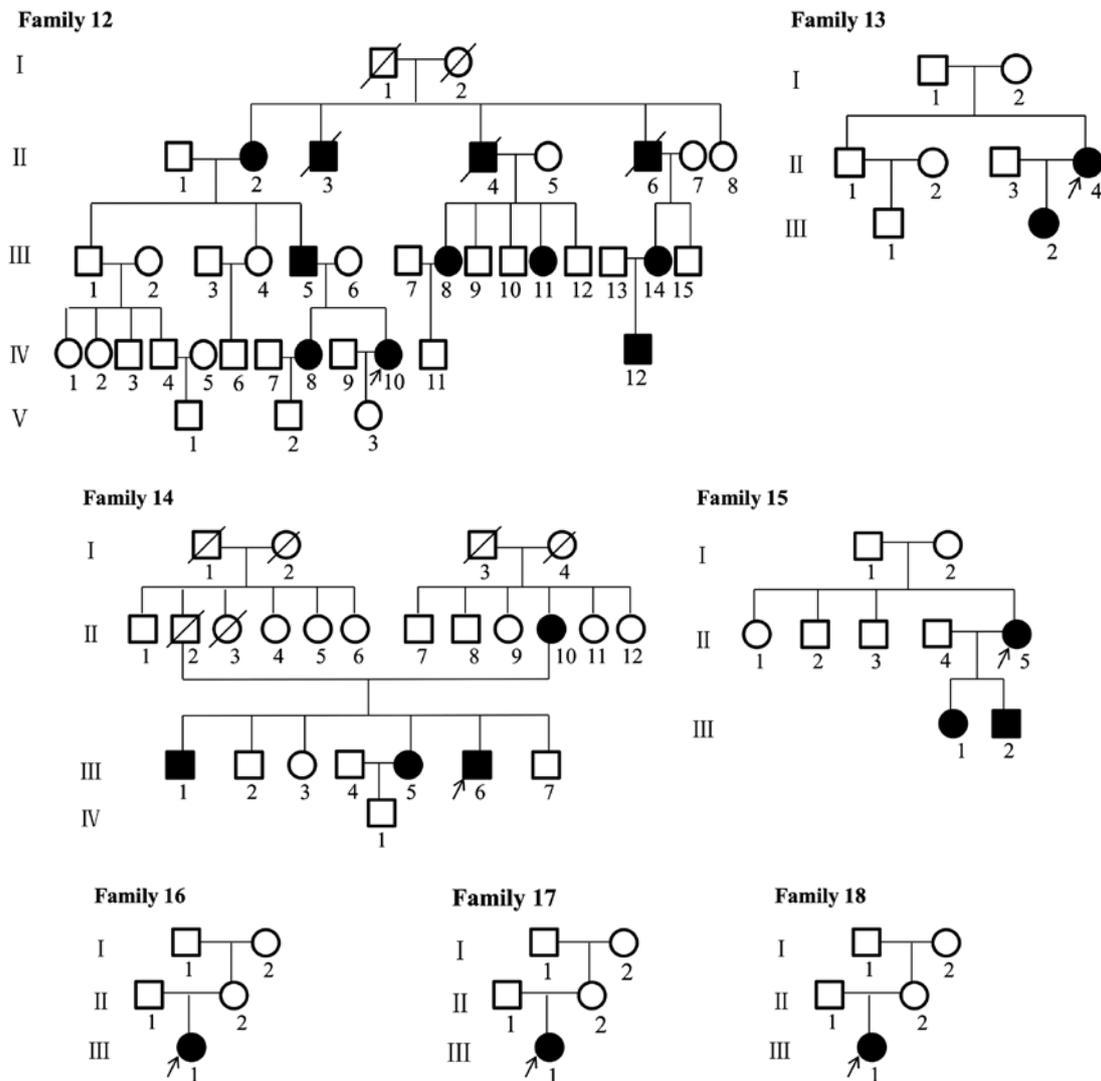
Figure 1. Continued. Pedigree of families 12-18 with hypophosphatemic rickets. The black symbols represent the affected individuals, and the open symbols represent the unaffected individuals. The circles and squares indicate females and males, respectively. The arrows identify the probands in the families.

substitution of proline for leucine at residue 275. To date, there are only 14 different mutations confirmed in exon 7 and it is among the rarest mutant exons in line with the *PHEX* mutation database. Moreover, this site was estimated to be conserved with the replaced leucine occurred in both *ECE-1* and *PHEX*. The 3 novel missense mutations identified in this study were shown to be highly conserved under the protein alignment of the *PHEX* gene from 12 different species and were verified to be pathogenic by bioinformatics tools (PolyPhen-2 and SIFT), which also rendered the evidence of their potential value for the phenotype in patients with XLH.

Three nonsense mutations: Lys432X in exon 11, Arg291X and Gln311X in exon 8 were detected in this study, all of which would cause the translation of truncated protein with accidental loss of C-terminal region. It was demonstrated that the C-terminal region in the large extracellular domain was abundant in conserved cysteine residues and contained the zinc-binding motifs in exons 17 and 19 (10,11). The cysteine residues are responsible for the secondary structure formation and contribute to conformation integrity. The highly conserved zinc-binding motifs among *NEP*, *PHEX*, *ECE-1* and *KELL*,

are essential for the catalytic activity of the protein. Therefore, these 2 nonsense mutations would inevitably lead to impaired PHEX protein function.

Six of the 17 *PHEX* mutations were identified as alternative splicing mutations, including 3 splice acceptor mutations and 3 splice donor mutations. Among these, 2 were novel mutations: 436_436+1delAG in the splice donor site of exon 4 and an alteration of C to G in intronic sequence 5' to initiate exon 11, -1 bp upstream. These 2 splicing mutations were predicted to result in the skipping of exons 4 and 11, respectively. Notably, both of the 2 affected exons comprised one conserved cyteine residue that was relevant for secondary structure transformation and thus protein function. That is, the newly identified 2≈splicing mutations were associated with the onset of XLH.

The remaining 3 mutations were 1234delA in exon 11, c.528delT in exon 5 and 1843dupA in exon 18, characterized as frameshift mutations. The mutation occurring in exon 18 was first reported by Gauche *et al* (28). It caused the replacement of aspartic acid for threonine at residue 615 and opened a new reading frame for 6 amino acids and deemed to disrupt the overall integrity of the PHEX protein. Moreover, the hetero-

Table II. Clinical findings and *PHEX* mutations identified in families affected by XLH.

Family no.	Patient[a]	Gender	Age (years)	Clinical characteristics	Serum P-value (mmol/l)[b]	Mutation site	Mutation type	DNA level	Protein level
1	III-2	F	21	Growth retardation; teeth falling out; genu valgum	0.68	Exon 15	Missense	c.1601C>T	p.P534L
	III-1	F	22	Growth retardation; genu valgum	0.73	Exon 15	Missense	c.1601C>T	p.P534L
	II-7	F	48	Short stature; teeth falling out; genu valgum	0.75	Exon 15	Missense	c.1601C>T	p.P534L
2	III-1	M	30	Growth retardation; teeth falling out; genu varum	NA	Exon 11	Nonsense	c.1294A>T	p.K432X
	I-2	F	78	Short stature	0.56	Exon 11	Nonsense	c.1294A>T	p.K432X
	II-3	M	51	Short stature; failure to walk; teeth falling out; genu varum	0.64	Exon 11	Nonsense	c.1294A>T	p.K432X
3	II-2	F	23	Growth retardation; genu varum	0.86	Exon 22	Missense	c.2192T>C	p.F731S
	I-1	M	53	Short stature; genu varum	0.72	Exon 22	Missense	c.2192T>C	p.F731S
4	IV-2	M	5	Growth retardation; genu varum	0.84	Intron13	Alternative splicing	c.1483-1G>C	p.?
	III-4	F	39	Short stature	0.54	Intron 13	Alternative splicing	c.1483-1G>C	p.?
5	II-2	F	28	Short stature; genu varum	0.60	Intron 10	Alternative splicing	c.1174-1G>A	p.?
	III-1	F	4	Growth retardation; genu varum	0.67	Intron 10	Alternative splicing	c.1174-1G>A	p.?
6	III-15	F	11	Growth retardation; teeth falling out; genu varum	0.75	Exon 11	Deletion	c.1234delA	p.S412VfsX12
	II-12	F	36	Short stature; teeth falling out; genu varum	0.60	Exon 11	Deletion	c.1234delA	p.S412VfsX12
	II-8	F	43	Short stature; teeth falling out; genu varum	0.77	Exon 11	Deletion	c.1234delA	p.S412VfsX12
	III-11	M	11	Growth retardation; genu varum	0.68	Exon 11	Deletion	c.1234delA	p.S412VfsX12
	III-12	F	12	Growth retardation; genu varum	0.77	Exon 11	Deletion	c.1234delA	p.S412VfsX12
7	III-3	F	28	Short stature; genu varum	0.69	Intron 4	Alternative splicing	c.436_436+1delAG	p.?
	II-4	F	59	Short stature; genu varum	NA	Intron 4	Alternative splicing	c.436_436+1delAG	p.?
	II-6	F	56	Short stature; genu varum	0.54	Intron 4	Alternative splicing	c.436_436+1delAG	p.?
	II-8	F	52	Short stature; genu varum	NA	Intron 4	Alternative splicing	c.436_436+1delAG	p.?
8	III-3	F	27	Short stature; genu varum	0.55	Exon 7	Missense	c.824T>C	p.L275P
	III-2	F	30	Short stature; genu varum	0.42	Exon 7	Missense	c.824T>C	p.L275P
	IV-1	F	8	Growth retardation; genu varum	0.90	Exon 7	Missense	c.824T>C	p.L275P
	II-4	F	54	Growth retardation; genu varum; teeth falling out	0.62	Exon 7	Missense	c.824T>C	p.L275P
9	IV-3	F	19	Growth retardation; genu valgum	0.50	Exon 8	Nonsense	c.931C>T	p.E311X
10	III-1	F	2	Genu varum; rib eversion; pectus carinatum	0.75	Intron 7	Alternative splicing	c.849+1G>C	p.?
11	III-1	F	10	Growth retardation; genu varum	0.70	Intron 4	Alternative splicing	c.436+1G>C	p.?

Table II. Continued.

Family no.	Patient[a]	Gender	Age (years)	Clinical characteristics	Serum P-value (mmol/l)[b]	Mutation site	Mutation type	DNA level	Protein level
12	IV-10	F	24	Short stature; genu varum; thoracic deformity	0.55	Exon 3	Missense	c.304G>A	p.G102R
	III-5	M	53	Short stature; genu varum; spinal deformity; teeth falling out	0.32	Exon 3	Missense	c.304G>A	p.G102R
	IV-8	F	27	Short stature; genu varum; loose teeth	0.64	Exon 3	Missense	c.304G>A	p.G102R
13	II-4	F	27	Short stature; genu varum; teeth falling out	0.56	Exon 3	Missense	c.229T>C	p.C77R
	III-2	F	1.5	Genu varum; pillow bald	0.88	Exon 3	Missense	c.229T>C	p.C77R
14	III-6	M	30	Short stature; teeth falling out; lower limb deformity; limited mobility	0.53	Exon 18	Insertion	c.1843dupA	p.T615nfsX6
	II-10	F	63	Short stature; lower limb deformity	0.40	Exon 18	Insertion	c.1843dupA	p.T615nfsX6
	III-1	M	36	Short stature; lower limb deformity	0.52	Exon 18	Insertion	c.1843dupA	p.T615nfsX6
	III-5	F	31	Short stature; lower limb deformity	0.75	Exon 18	Insertion	c.1843dupA	p.T615nfsX6
15	II-2	M	8	Growth retardation; genu varum; pectus carinatum; rib eversion	0.87	Intron 14	Alternative splicing	c.1586_1586+1delAG	p.?
	III-1	F	10	Growth retardation; genu varum	0.54	Intron 14	Alternative splicing	c.1586_1586+1delAG	p.?
	II-5	F	37	Short stature; genu varum	0.65	Intron 14	Alternative splicing	c.1586_1586+1delAG	p.?
16	III-1	F	5	Genu varum	0.78	Exon 5	Deletion	c.528delT	p.E177KfsX44
17	III-1	F	4	Genu varum	1.02	Intron 14	Alternative splicing	c.1586_1586+1delAG	p.?
18	III-1	F	15	Genu varum; teeth falling out	0.54	Exon 8	Nonsense	c.871C>T	p.R291X

[a]All the patients were pre-treated with calcitriol and phosphate apart from patient II2 in family 3 (F3-II2); [b]The normal range for serum phosphate is 0.8-1.6 mmol/l. M, male; F, female; NA, not available.
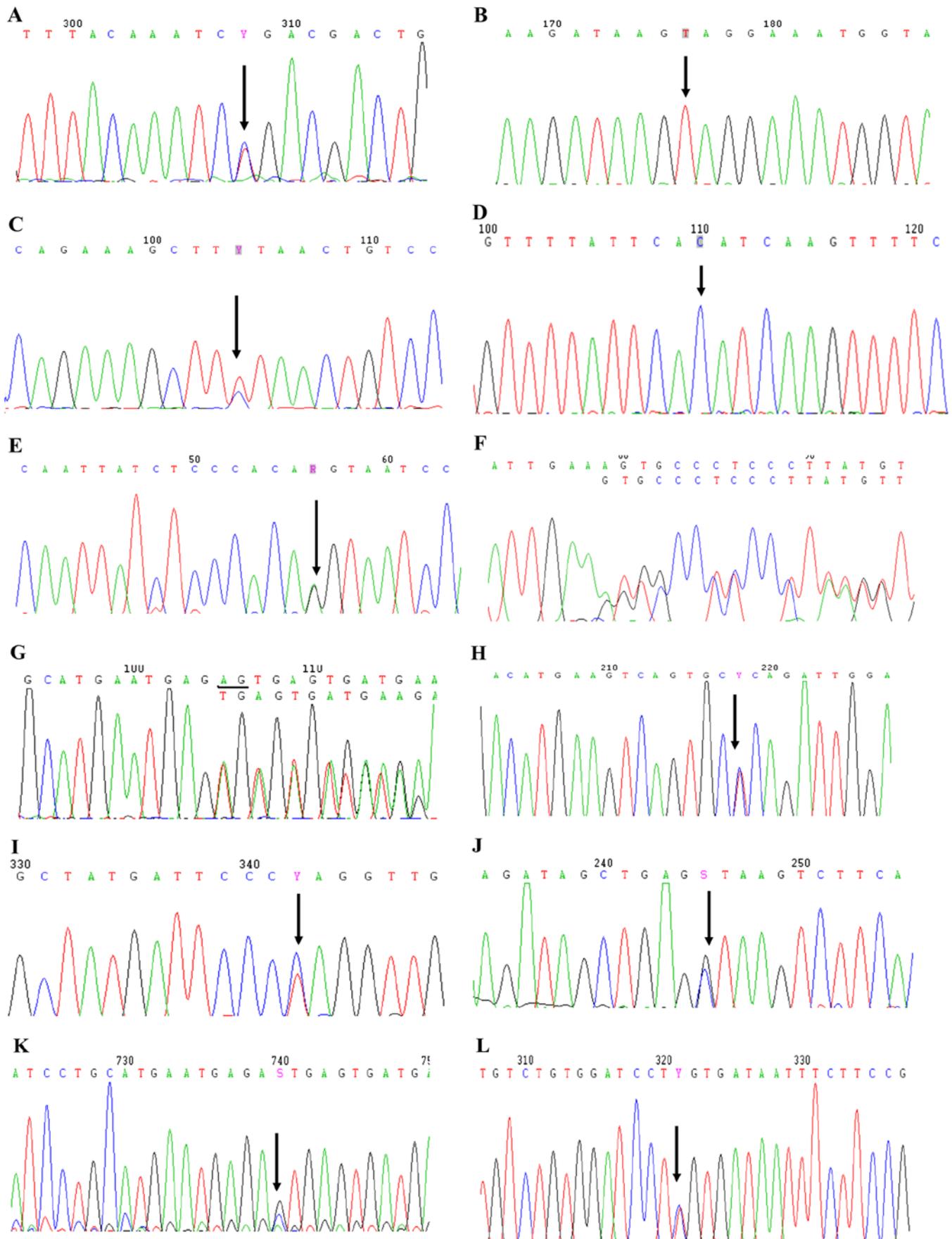
Figure 2. Mutational analyses of phosphate-regulating gene with homology to endopeptidase on the X chromosome (*PHEX*) gene in patients with hypophosphatemic rickets. (A) c.1601C>T in exon 15, (B) c.1294A>T in exon 11, (C) c.2192T>C in exon 22, (D) c.1483-1G>C in intron 13, (E) c.1174-1G>A in intron 11, (F) c.1234delA in exon 11, (G) c.436_436+1delAG in intron 4, (H) c.824T>C in exon 7, (I) c.931C>T in exon 8, (J) c.849+1G>C in intron 7, (K) c.436+1G>C in intron 4, (L) c.229T>C in exon 3.
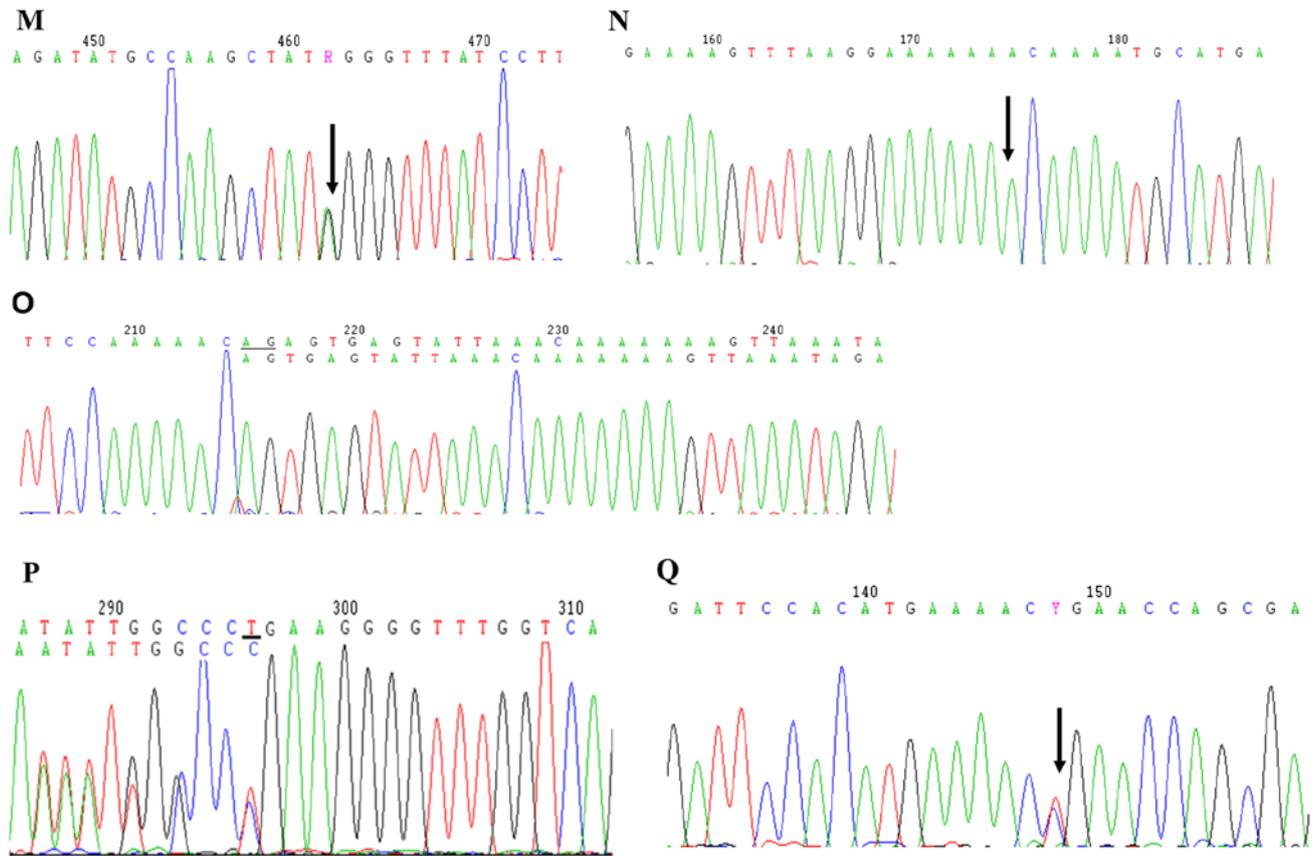
Figure 2. Continued. Mutational analyses of *PHEX* gene in patients with hypophosphatemic rickets. (M) c.304G>A in exon 3, (N) c.1843dupA in exon 18, (O) c.1586_1586+1delAG in intron 14, (P) c.528delT in exon 5 and (Q) c.871C>T in exon 8.

Figure 3. Evolutionary conservation of the residues p.77, p.102 and p.275. The C77R, G102R and L275P mutations occur at a highly conserved position in phosphate-regulating gene with homology to endopeptidase on the X chromosome (*PHEX*) gene, as shown by comparing the corresponding sequences of 12 different species.

zygous deletion of one adenine nucleotide and one thymine nucleotide in exons 11 and 5 were 2 novel mutations identified in our study. These mutations would result in premature termination of the PHEX protein.

Table III. Serum intact human FGF23 levels in healthy controls.

Subjects	FGF23 (pg/ml)	Subjects	FGF23 (pg/ml)	Subjects	FGF23 (pg/ml)	Subjects	FGF23 (pg/ml)	Subjects	FGF23 (pg/ml)
1	49.3	20	45.7	39	26.8	58	47.2	77	53.9
2	62.0	21	20.7	40	61.0	59	50.3	78	56.9
3	37.0	22	31.9	41	47.2	60	29.9	79	25.8
4	33.0	23	37.0	42	60.0	61	30.4	80	29.6
5	33.5	24	34.5	43	49.3	62	43.2	81	40.2
6	26.3	25	51.3	44	40.6	63	40.1	82	34.0
7	37.0	26	34.0	45	47.7	64	60.5	83	37.8
8	41.6	27	93.1	46	40.1	65	37.6	84	41.4
9	153.8	28	42.1	47	56.9	66	53.9	85	34.1
10	31.9	29	31.4	48	35.0	67	51.3	86	38.3
11	23.8	30	68.1	49	59.0	68	50.3	87	47.6
12	44.2	31	71.7	50	28.4	69	30.9	88	49.0
13	35.0	32	75.3	51	136.0	70	48.3	89	39.3
14	44.7	33	65.6	52	26.3	71	41.1	90	47.5
15	116.6	34	50.3	53	38.1	72	64.6	91	137.4
16	116.1	35	39.1	54	35.5	73	35.0	92	28.7
17	51.8	36	39.6	55	27.4	74	41.1	93	35.1
18	33.5	37	82.4	56	67.6	75	30.9	94	39.6
19	30.9	38	37.6	57	33.5	76	38.1	95	54.6

FGF23, fibroblast growth factor 23.

Table IV. Serum intact human FGF23 in patients with XLH.

Family no.-Patient[a]	Gender	Age (years)	Serum P-value (mmol/l)[b]	Serum FGF23 (pg/ml)[c]	Mutations in *PHEX* gene
F2-III1	M	30	NA	38.7	c.1294A>T (Exon 11)
F2-II3	M	51	0.64	40.4	c.1294A>T (Exon 11)
F3-II2	F	23	0.86	21.1	c.2192T>C (Exon 22)
F5-II2	F	28	0.60	7.8	c.1174-1G>A (Intron 10)
F6-III15	F	11	0.75	171.5	c.1234delA (Exon 11)
F9-IV3	F	19	0.50	29.7	c.931C>T (Exon 8)
F10-III1	F	2	0.75	111.4	c.849+1G>C (Intron 7)
F11-III1	F	10	0.70	15.7	c.436+1G>C (Intron 4)
F12-III5	M	53	0.32	24.0	c.304G>A (Exon 3)
F12-IV10	F	24	0.55	162.2	c.304G>A (Exon 3)
F12-IV8	F	27	0.64	143.2	c.304G>A (Exon 3)

[a]All the patients were pre-treated with calcitriol and phosphate apart from patient II-2 from family 3 (F3-II2). [b]The normal range for phosphate is 0.8-1.6 mmol/l. [c]Serum FGF23 in healthy controls (n=95): median 40.6 pg/ml, reference range (2.5th and 97.5th percentiles) 24.6-136.8 pg/ml. FGF23, fibroblast growth factor 23; M, male; F, female.

No hotspot mutations were found in XLH according to the present study in accordance with previous studies on *PHEX* mutations in Chinese patients (15-21). However, it was valued that exceeding 50% mutations occurred around exons 18 to 22 in the C-terminal region based on the mutation analyses in PHEXdb and it was speculated that this region may be the critical domain for PHEX function (29). We failed to determine the gene dosage effect on disease severity by comparing the

phenotypes of hemizygous males to those of heterozygous females from the same family. An evidence-based study also indicated that there was no difference in severity of the disease between genders in mutant *Hyp* mouse (30). Theoretically, heterozygous females should have a less severe phenotype due to random X-inactivation, the process of transcriptional silencing of one of the X chromosomes bringing about half of the normal alleles, while males have none. Sabbagh *et al* owed the absence of gene dose effect to a threshold of PHEX activity that was required for maintaining normal protein function (25). In addition to the gender impact, questions remain as to the possibility of correlations among either mutation location or type with phenotype severity. In the present study, the association between genotype and phenotype in patients with XLH was not observed. It has been proposed that there is a trend towards a more severe phenotype with mutations located in the C-terminal region or with truncating mutations (31); however, this has yet to be verified in a larger sample size.

The aberrant activity of FGF23 was revealed as a common fundamental mechanism for the development of defects in phosphate and vitamin D metabolism in several hypophosphatemic diseases, including ADHR, XLH and tumor-induced osteomalacia (TIO) (5,32,33). The *Hyp* mouse, an animal model of XLH, provided evidence of increased levels of FGF23 transcripts due to the inactivated mutations of *PHEX*. Furthermore, an injection of FGF23 antibodies or the deletion of *Fgf23* from the *Hyp* mouse has been shown to ameliorate or reverse the phosphate metabolic disorders (14,34). These findings strongly indicate the essential role of FGF23 in the regulation of systemic phosphate homeostasis in the *Hyp* mouse. However, we failed to observe a significant increase in serum FGF23 levels in all affected individuals compared with the healthy controls. Moreover, 4 of 11 patients with XLH even exhibited serum FGF23 levels below the reference range. Despite of the effect of confounding factors, the wide variation in serum FGF23 levels in XLH, as well as the overlapping FGF23 levels in XLH and healthy controls revealed its limited diagnostic value in patients with suspected XLH. Moreover, it was speculated that other factors, such as parathyroid hormone and 1,25-dihydroxyvitamin-D, were responsible for hypophosphatemia in patients with XLH with inappropriate low to normal serum FGF23 levels, and for the normal serum phosphorus in healthy subjects with high serum FGF23 levels (33,34). Additionally, it was suggested that hypophosphatemia alone was not entirely responsible for the skeletal phenotype in XLH and the potential direct local effects of FGF23 may be involved in bone mineralization independent of its systematic action in phosphate homeostasis (35,36).

In conclusion, we identified 17 different mutations in the *PHEX* gene in 18 unrelated Chinese families with hypophosphatemic rickets and 7 of these were novel. It should be noted that that 6 of the 17 *PHEX* mutations have been proven to be *de novo*, which suggests the frequent occurrence of sporadic cases of XLH in the Chinese population. The findings of the present study highlight the major role of *PHEX* gene mutations in hypophosphatemic rickets and emphasize the significance of genetic diagnosis in suspected cases to ascertain the clinical diagnosis of XLH, enabling timely intervention. Further studies are warranted in order to perform more extensive mutation analyses in affected individuals in order to elaborate the key domain in *PHEX* and the identification of unequivocal FGF23

function in the pathogenesis of XLH, subsequently, exploring for more effective treatments, not only for XLH, but also for associated diseases sharing similar molecular mechanism.

Acknowledgements

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