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Three novel *PHEX* gene mutations in four Chinese families with X-linked dominant hypophosphatemic rickets

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ABSTRACT

Background: X-linked hypophosphatemia (XLH), the most common form of inherited rickets, is a dominant disorder that is characterized by renal phosphate wasting with hypophosphatemia, abnormal bone mineralization, short stature, and rachitic manifestations. The related gene with inactivating mutations associated with XLH has been identified as *PHEX*, which is a phosphate-regulating gene with homologies to endopeptidases on the X chromosome. In this study, a variety of *PHEX* mutations were identified in four Chinese families with XLH.

Methods: We investigated four unrelated Chinese families who exhibited typical features of XLH by using PCR to analyze mutations that were then sequenced. The laboratory and radiological investigations were conducted simultaneously.

Results: Three novel mutations were found in these four families: one frameshift mutation, c.2033dupT in exon 20, resulting in p.T679H; one nonsense mutation, c.1294A > T in exon 11, resulting in p.K432X; and one missense mutation, c.2192T > C in exon 22, resulting in p.F731S.

Conclusions: We found that the *PHEX* gene mutations were responsible for XLH in these Chinese families. Our findings are useful for understanding the genetic basis of Chinese patients with XLH.

1. Introduction

Hypophosphatemic rickets (HR) is a group of disorders accompanied by a defect in bone mineralization due to renal phosphate wasting that leads to hypophosphatemia [1–3]. Four main subtypes have been identified: X-linked dominant hypophosphatemic rickets (XLH), autosomal dominant hypophosphatemic rickets (ADHR), hereditary hypophosphatemic rickets with hypercalciuria (HHRH), and tumor-induced osteomalacia (TIO) [1–3]. XLH (MIM 307800) is the most prevalent genetic form of HR with an occurrence of approximately 1 per 20,000 [4,5]. Laboratory indices of the disease include low serum phosphorus levels, normal serum calcium levels, increased activity of serum alkaline phosphatases, and inappropriately normal or decreased levels of 1,25-dihydroxyvitamin D3. The clinical manifestations of HR include short stature, bone pain, dental anomalies, lower extremity deformities, and radiographic evidence of rickets in children [6,7].

XLH is mainly caused by inactivating mutations in the *PHEX* (MIM 300550) gene, a phosphate-regulating gene with homology to endopeptidases on the X chromosome, which was identified in the Xp22.1 region by positional cloning [8,9]. The gene is a member of the M13 membrane-bound zinc metalloendopeptidase family involving 22 exons and encoding a 749-amino-acid protein, which consists of an intracellular region, a transmembrane domain, and an extracellular domain and is expressed in bone lineage cells, including osteoblasts, osteocytes, and odontoblasts [10–14].

Recently, a wide variety of *PHEX* gene defects in XLH, including missense mutations, nonsense mutations, splice site mutations, insertions and deletions, have been revealed [4]. In this study, we identified three novel mutations of the *PHEX* gene: one frameshift mutation, c.2033dupT in exon 20, resulting in p.T679H; one

Abbreviations: ADHR, Autosomal dominant hypophosphatemic rickets; FGF23, Fibroblast growth factor 23; HHRH, Hereditary hypophosphatemic rickets with hypercalciuria; HR, Hypophosphatemic rickets; PHEX, Phosphate-regulating gene with homology to endopeptidases on X chromosome; TIO, Tumor-induced osteomalacia; XLH, X-linked dominant hypophosphatemic rickets.

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nonsense mutation, c.1294A > T in exon 11, resulting in p.K432X; and one missense mutation, c.2192T > C in exon 22, resulting in p.F731S. These mutations were found in four unrelated Chinese families with XLH. Additionally, we found another missense mutation in two members of the same family, namely, c.1601C > T in exon 15, resulting in p.P534L, which was present in the *PHEX* mutation database (http://www.phexdb.mcgill.ca). The laboratory and radiographic findings in these patients were also analyzed.

2. Materials and methods

2.1. Patients

This study was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The subjects provided informed consent before participating in the study, and clinical data and DNA samples were obtained. We investigated four families with hypophosphatemic rickets, all of whom were members of the Han ethnic group in China. Eighteen members of these families were diagnosed with XLH based on their medical histories, clinical manifestations, physical examination, laboratory indices, and radiologic evidence. The general features and laboratory findings are shown in Table 1, and all of the laboratory data were collected prior to surgical or medical treatment. The pedigrees of the families with X-linked hypophosphatemic rickets are shown in Fig. 1.

In family 1 (F1), the proband (III-2) referred to our clinic was a 21-year-old woman who was born of a non-consanguineous marriage. The woman was born to a G2P2 mother in a normal spontaneous vaginal delivery, had no delay in initial crying, and had a birth weight of 2.3 kg. At the time of presentation to us, she presented with short stature (133 cm), serious lower extremity genu valgum deformities, and poor dental development with three teeth absent. The proband's older sister (III-1), a 22-year-old woman, also presented to us. Her height (135 cm) was similar to that of the proband, her lower extremity deformities were mild and her dental development was normal. Related X-rays of the proband and her older sister are shown in Fig. 2A–C. It was clear that the proband's disease manifestations were much more severe. Their 48-year-old mother (II-2) also exhibited similar manifestations. Other members (I-2, II-1, III-3, and III-4) of the proband's family were healthy.

In family 2 (F2), the proband (IV-6), a 10-year-old boy, presented to our clinic. He was born of a non-consanguineous marriage at full term. The boy had exhibited lower extremity genu varus deformities and poor dental development since 2 years of age. His vision and hearing were normal. His 44-year-old mother (III-6) had exhibited "bowing of legs" since her childhood. She also reported a history of tooth loss in early adulthood. She had not been formally accessed until her son's diagnosis was determined. Both the outward appearance and radiological evidence of the proband (IV-6) and his mother (III-6) are exhibited in Fig. 2D–F. In this family, there were three additional members (II-2, II-4, and III-2) with similar medical histories and clinical features. Unfortunately, no detailed information was available.

In family 3 (F3), the proband (II-3), a 53-year-old man of notably short stature (140 cm) who reported pain in the lumbar spine was subjected to a physical examination that revealed genu varus deformities of the lower limbs, dental defects, and walking difficulties. His X-rays showed high bone mineral density and apparent deformities of the lower extremity, which can be observed in Fig. 2G–I. Accompaning him to our clinic was his 30-year-old nephew (III-1), who had been noted to have "bowing of legs" and a shorter right lower limb when he started walking at 4 years of age. The nephew had presented with poor dental development at age 24. In their family, the proband's sister (II-2) and mother (I-2) exhibited similar clinical manifestations. Unfortunately, his sister had passed away, and his mother was too old to come to our clinic; therefore, there was no evidence of any bone disease in this family.

In family 4 (F4), the proband (III-4), a 23-year-old woman, was referred to our clinic. She was born of a non-consanguineous marriage in a normal spontaneous vaginal delivery, had no delay in initial crying and was late to walk at 3 years of age. She presented to us with short stature (150 cm), poor dental development with four absent teeth, lower extremity genu varus deformities, and occasional tinnitus aurium. Her lower extremity deformities are shown in Fig. 2J–L. Her 45-year-old father (II-6) had also been noted to have quite short stature (148 cm) in childhood, had dental anomalies since he was young, and had pain in both knees at age 30. At the time of presentation to us, he had no teeth left. In this family, there were four additional patients (I-2, II-4, II-5, and III-1) with similar clinical features, but no detailed information was available.

Laboratory analysis of the patients' sera revealed low phosphorus levels, normal calcium levels and elevated alkaline phosphatase levels, which are listed in Table 1.

2.2. Methods

Genomic DNA was extracted from peripheral white blood cells that were collected in ethylenediaminetetraacetic acid (EDTA) tubes using conventional methods. We screened for *PHEX* gene mutations among seven patients, other normal members of the four families, and the control group, which included 250 volunteers (male: 125, female: 125) without XLH. The DNA sequence of the *PHEX* gene was obtained from the available online database (GenBank accession NO. NC_000012). The primers (21 pairs [4,15]) were designed using Primer-3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All 22 exons and their exon–intron boundaries in the *PHEX* gene were amplified by polymerase chain reaction (PCR). Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Foster, CA, USA), and the sequences were analyzed with an ABI Prism 3130 automated sequencer. Single nucleotide polymorphisms (SNPs) were identified using Polyphred (http://droog.mbt.washington.edu/poly_get.html), and novel mutations

Table 1
General features of and laboratory findings in the XLH patients studied.

Family no.	Patient no.	Gender	Age (yr)	Ht (cm)	Wt (kg)	Ca (mmol/L)	P (mmol/L)	ALP (U/L)
1	III-2	F	21	133	48	2.17	0.82	198
1	III-1	F	23	135	50	2.19	0.73	137
2	IV-6	M	10	123.5	26	2.29	0.88	612
2	III-6	F	44	146	39.5	2.05	0.54	80
3	II-3	M	53	140	50	2.35	0.73	165
3	III-1	M	30	148	56	2.32	0.43	109
4	III-4	M	23	150	48	2.33	0.62	89

The normal range for phosphate is 0.8–1.6 mmol/L; for calcium, 2.08–2.60 mmol/L; and for ALP, 15–112 U/L. In the table, F indicates female, and M indicates male.

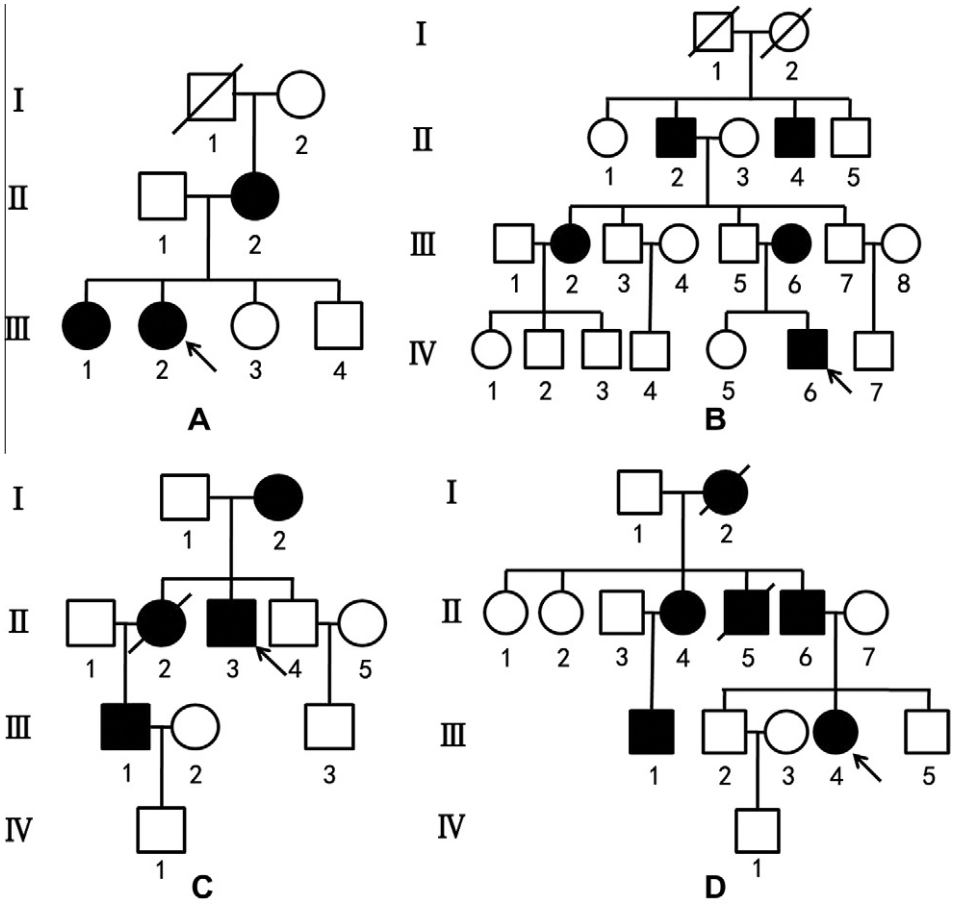

Fig. 1. Pedigrees of families with X-linked hypophosphatemic rickets. The black symbols represent the affected individuals, and the open symbols represent the unaffected individuals. The circles and squares indicate females and males, respectively. The arrows identify the probands in the families. (A) F1: the proband (III-2), her sister (III-1), and her mother (II-2) were affected by XLH. (B) F2: the proband (IV-6), his mother (III-6), his mother's sister (III-2), his grandfather (II-2), and his grandfather's brother (II-4) suffered from XLH. (C) F3: the proband (II-3), his nephew (III-1), his sister (II-2), and his mother (I-2) suffered from XLH. (D) F4: the proband (III-4), her father (II-6), her uncle (II-5), her aunt (II-4), her cousin (III-1), and her grandmother (I-2) were affected by XLH.

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 in GenBank using the ClustalX 1.83 algorithm [16].

3. Results

In F1, we found a recurrent missense mutation of exon 15 in III-1 and III-2 (proband). This mutation was a heterozygous C-to-T transition at c.1601, which resulted in a proline (CCG)-to-leucine (CTG) substitution at p.534 (Fig. 3A).

In F2, a frameshift mutation of exon 20 was found in III-6 and IV-6 (proband), which was a heterozygous c.2033dupT mutation. This mutation inserted a thymidine that changed the codon ACA to CAT and resulted in the subsequent change of a threonine codon to a histidine codon at p.679 and truncation at p.717 (Fig. 3B).

In F3, a nonsense mutation of exon 11 was present in II-3 (proband). This mutation was an A-to-T transition at c.1294, which changed codon AAG to TAG and transformed a lysine codon to a stop codon, resulting in truncation at p.432 (Fig. 3C).

In F4, the proband (III-4) and her father (II-6) harbored a missense mutation at c.2192 of exon 22, which was a heterozygous T-to-C transition that caused a subsequent change in the phenylalanine codon (TTT) to a serine codon (TCT) at p.731 (Fig. 3D).

In addition, by comparing the DNA sequence of the *PHEX* gene among 13 different biological species, we found that two gene sites, p.534 and p.731, were conserved (Fig. 4A, B).

We did not detect the above *PHEX* gene mutations in the other normal family members or in the 250 healthy volunteers.

4. Discussion

To date, there have been 248 identified XLH cases characterized by different types of *PHEX* gene mutations, the observed frequencies of which are 25% frameshifts, 23% abnormal splicing, 22% missense, 18% nonsense, 8% deletions, and 4% polymorphisms according to the *PHEX* mutation database (http://www.phexdb.mcgill.ca). If these mutations were translated, the *PHEX* protein would be truncated and lose function [17]. Of these XLH cases, only five Chinese cases were reported; one consisted of a deletion mutation, one consisted of a nonsense mutation, one consisted of a frameshift mutation, and two consisted of missense mutations [18,19]. In our study, all of the patients were of Han Chinese ethnicity, and three mutations identified had not been previously reported.

Two missense mutations of *PHEX*, c.1601C > T in exon 15 in F1 and c.2192T > C in exon 22 in F4, were found. These mutations resulted in p.P534L and p.F731S, respectively. According to the *PHEX* mutation database (http://www.phexdb.mcgill.ca), seven publications [4,7,15,17,20–22] had reported the missense mutation p.P534L. Only

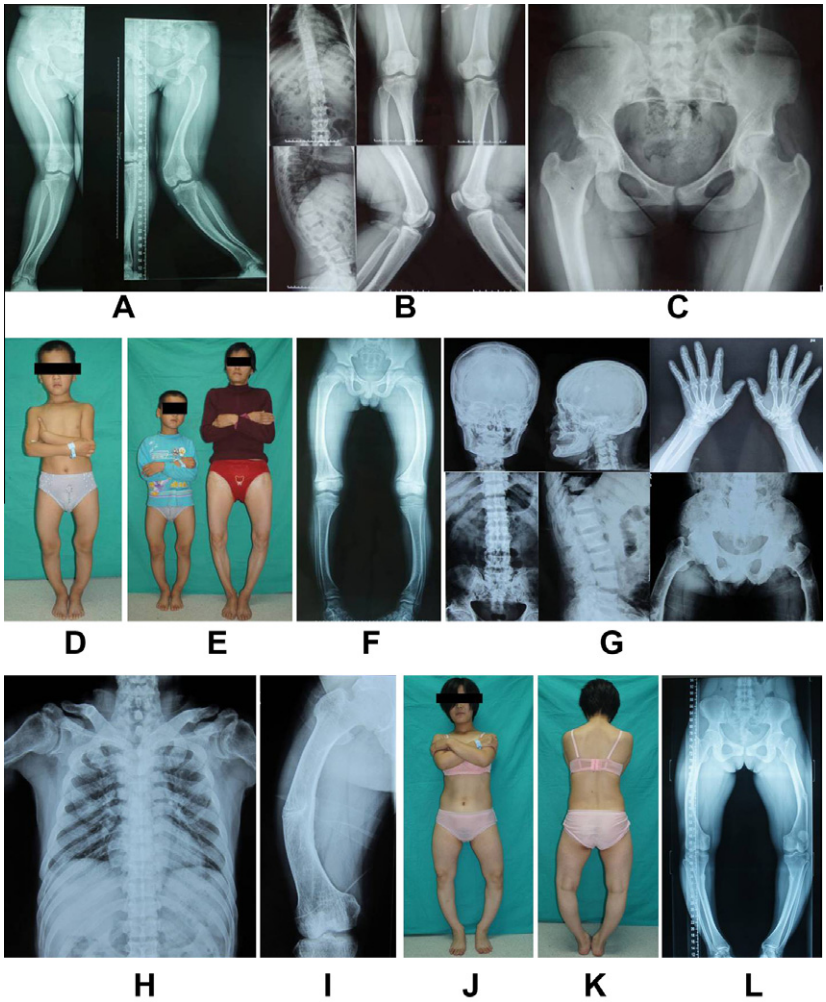

Fig. 2. The outward appearance and radiological evidence of XLH in our patients. (A) III-2 of F1: the radiographs demonstrate her serious lower extremity genu valgum deformities. (B) and (C) III-1 of F1: the radiographs of the spine, knees, and hip are normal. (D) IV-6 of F2: the image demonstrates his lower extremity genu varus deformities. (E) IV-6 and III-6 of F2: the picture shows lower extremity genu varus deformities of the proband (IV-6) and his mother (III-6). (F) IV-6 of F2: the radiograph shows his lower extremity genu varus deformities. (G–I) II-3 of F3: the radiographs of the head, hands, spine, hip, femur, and chest show high bone mineral density. In addition, the femur and hip radiographs show genu varus deformities of the lower limbs. (J–L) III-4 of F4: the front view, the dorsal view, and the radiograph demonstrate her lower extremity genu varus deformities. All of the images are published with permission from the affected individuals.

three cases [20–22] were described as manifesting the clinical phenotype of XLH. In this study, the proband (III-2) of F1 presented with typical clinical manifestations, whereas her older sister (III-1) exhibited much milder symptoms. The other missense mutation, p.F731S, was novel. Sabbagh et al. [23,24] studied the structure and function of the *PHEX* gene and indicated that certain *PHEX* missense mutations (such as p.Y317F and p.F731Y) altered the conformation of the protein by subjecting the proteins to limited protease digestion with trypsin or endoproteinase Glu-c. Moreover, p.F731Y was significantly more sensitive to proteolytic digestion compared with the wide-type protein [24]. In our study, the missense mutation p.F731S might be similar to p.F731Y, which might also prevent the interaction of the *PHEX* ectodomain with putative partners at the cell surface and might cause a similar loss of function. Additionally, the conservation of p.534 and p.731 in the DNA sequence of the *PHEX* gene among the 13 different biological species we examined suggested that the two missense mutations might lead to loss of function of the *PHEX* product. Furthermore, we failed to detect the mutations in 250 normal members of the population, which strongly suggested that p.P534L and p.F731S were truly responsible for XLH.

One frameshift mutation of *PHEX*, namely c.2033dupT in exon 20 of F2, was identified. Searching the *PHEX* mutation database (http://www.phexdb.mcgill.ca), we found that there were 15 mutations in exon 20: ten deletion mutations, three nonsense mutations, and two missense mutations. In this study, the novel mutation in III-6 and IV-6 (proband) of F2 was an insertion that changed a threonine to a histidine and resulted in a movement of bases that subsequently led to a premature truncation at p.717 and a loss of function of the *PHEX* product.

One nonsense mutation of *PHEX* was found, namely, c.1294A > T in exon 11 in II-3 (proband) of F3, which transformed a lysine to a stop codon and led to an untimely truncation at p.432. Notably, exon 11 contains the conserved residues that encode the extracellular domain, and the mutated region of *PHEX* in II-3 was close to that domain. Thus, the mutated region may have been more likely to affect the function of the *PHEX* product.

In the present study, we found that the relationship between the phenotype and genotype of the *PHEX* gene was not invariant. Patients III-1 and III-2 of F1 had the same mutation type, but their clinical manifestations were different. Davidai et al. [25] had drawn the conclusion that the phenotypic heterogeneity common to XLH might be due, in part, to genetic variability, and they suspected that such heterogeneity was due to altered phosphorus transport at variable segments of the proximal convoluted tubule.

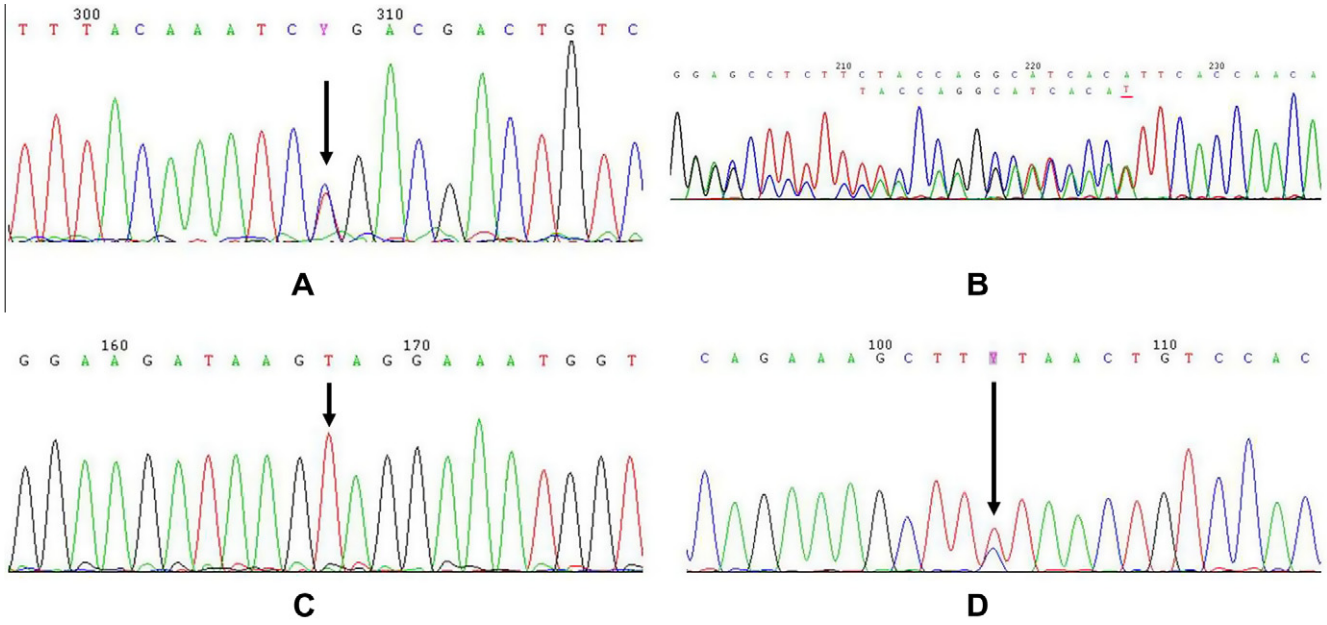

Fig. 3. Mutational analysis. (A) A missense mutation, c.1601C>T in exon 15 of the *PHEX* gene, was found in proband III-2 and III-1 of F1. (B) A frameshift mutation, c.2033dupT in exon 20 of the *PHEX* gene, was found in proband IV-6 and III-6 of F2. (C) A nonsense mutation, c.1294A>T in exon 11 of the *PHEX* gene, was found in proband II-3 of F3. (D) A missense mutation, c.2192T>C in exon 22 of the *PHEX* gene, was found in proband III-4 and her father II-6 of F4.

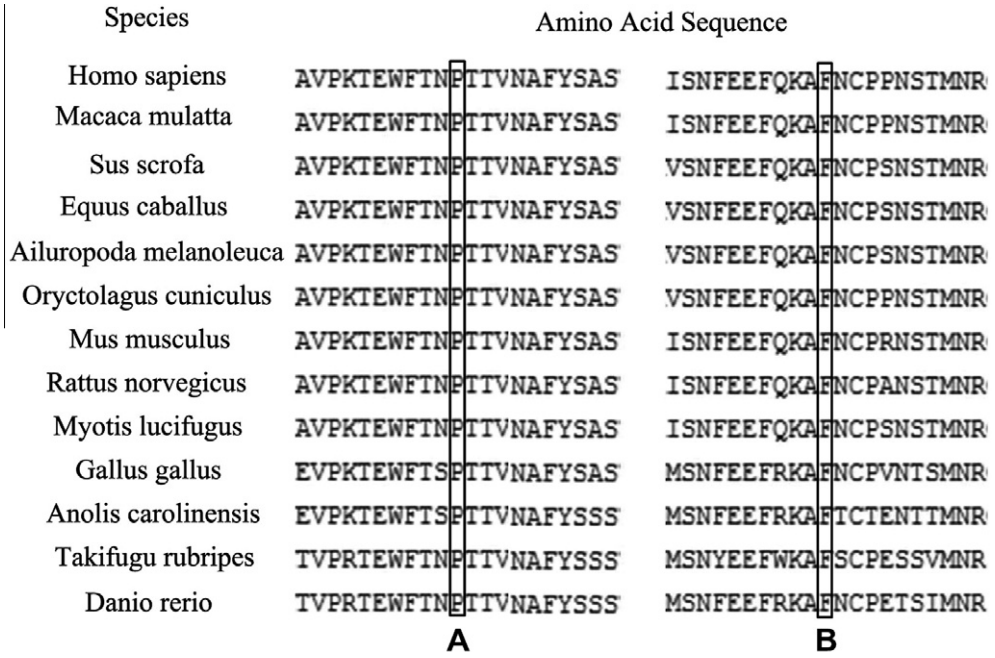

Fig. 4. Evolutionary conservation of the residues p.534 and p.731. Partial amino acid sequences encoded by exon 15 (A) and exon 22 (B) are shown. The residues that were mutated in F1 and F4 are marked.

Studies by Yuan et al. [26] had provided evidence that aberrant *PHEX* function in osteoblasts and/or osteocytes alone was sufficient to result in the HYP-mouse phenotype. The HYP Consortium [8] had also proposed that the *PHEX* gene was involved in XLH. Therefore, we believe that the relationship between the phenotype and genotype of the *PHEX* gene is complex. In addition, Popowska et al. [20] mentioned that phosphate and vitamin D3 supplementation could slow progressive growth retardation and leg bowing. As our patients had not received formal treatment before coming to our clinic, II-2 of F1, IV-6 of F2, and III-4 of F4 underwent osteotomies, and they were satisfied with their results.

In addition, another factor, fibroblastic growth factor-23 (FGF23), the product of *FGF23*, might be responsible for XLH. FGF23 contains an RXXR structure, which is the typical site of action of proteolytic enzymes. It has been confirmed that FGF23 is a major candidate for the phosphatonin function, which regulates the metabolism of circulating phosphorus [27,28]. Due to the mutation of the *PHEX* in XLH, the function of protein hydrolysis is lost, leading to the accumulation of its substrate; thus, serum FGF23 levels are elevated [29,30]. Previous studies suggested that hypophosphatemic rickets, including XLH, might be caused by the overexpression of full-length *FGF23* [29–32], thereby warranting the investigation conducted in the present study.

In conclusion, we have described three novel mutations in the *PHEX* gene in four unrelated Chinese families with XLH whose clinical manifestations and laboratory indices were typical of XLH patients. In the future, as knowledge of the molecular basis of genetics increases and a much greater number of Chinese XLH patients is available, there will be opportunities to elucidate the complex phenotype-genotype relationship and function of the *PHEX* gene and the precise interactions between *PHEX* and *FGF23*, which may contribute to the early molecular diagnosis and treatment of XLH.

Acknowledgments

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