Novel PHEX Variants and Splicing Mutations in Patients with X-Linked Hypophosphatemia

Xuesha Xing

China Medical University

Jinlan Gao

China Medical University

Hongwei Ma

China Medical University Second Hospital: Shengjing Hospital of China Medical University

Lina Zhang

Peking Jabrehoo Med Tech Co.

Fang Li

China Medical University Second Hospital: Shengjing Hospital of China Medical University

Leilei Yang

Peking Jabrehoo Med Tech Co.

Yang Luo (✉ yluo@cmu.edu.cn)

China Medical University <https://orcid.org/0000-0003-1349-0443>

Research

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Abstract

Background: X-linked hypophosphatemia rickets (XLH) is a genetic disorder of phosphate wasting that causes the majority of inherited hypophosphatemic rickets. The disease is caused by mutations in the phosphate-regulating endopeptidase gene (*PHEX*). All types of mutations have been detected in the *PHEX* gene. There is no clear preference for the position and the type of mutation.

Methods: In this study, we performed DNA sequencing and ex vivo splicing analysis to study the *PHEX* gene in ten sporadic patients and six core families with XLH.

Results: A total of 25 patients were studied. Fifteen different mutations were detected in these patients, including five missense mutations, five splicing mutations, two nonsense mutations, two small deletions, and one small insertion. Five mutations were not previously reported. We also characterized the splicing mutations identified in our study, which consisted of exon skipping and activation of new splice sites in an exon or intron. Most of the observed changes resulted in a frameshift of the *PHEX* open reading frame.

Conclusions: The genetic etiology in patients with XLH were successfully identified. This study expands the mutation database of the *PHEX* gene. We also explored the pathogenesis of XLH caused by splice site mutations. These results will contribute to the diagnosis of XLH and the pathogenicity analysis of mutations in patients.

Introduction

X-Linked Hypophosphatemia rickets (XLH, OMIM 307800) is the most prevalent inherited form of hypophosphatemic rickets, with a prevalence of 1:20,000 [1]. It is characterized by hypophosphatemia with low renal phosphate reabsorption, normal or low serum levels of vitamin D (1,25(OH)₂D₃, or calcitriol), normal serum calcium levels and PTH levels, hypocalciuria, and increased serum alkaline phosphatase (ALP) activity, which impairs bone mineralization, leading to rickets in childhood and persistent osteomalacia in adults [2].

The genetic basis for XLH is associated with mutations in the phosphate-regulating endopeptidase gene (*PHEX*, MIM 300550). *PHEX* is located on Xp22.11, which consists of 22 exons. To date, a wide variety of gene defects, over 400 *PHEX* mutations, have been reported in the Human Gene Mutation Database (HGMD, <http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html>, accessed November 2017), including nonsense mutations, missense mutations, splicing site mutations, insertions, and deletions at different positions in *PHEX*. Loss-of-function mutations lead to the overproduction of fibroblast growth factor 23 (FGF23, MIM 605380), a critical regulator of phosphate and vitamin D homeostasis, in osteocytes through a still unknown mechanism [3, 4]. Excess FGF23 can influence the renal synthesis of 1, 25-dihydroxyvitamin D and reduce the expression of renal tubular sodium phosphate co-transporters, leading to decreased proximal renal phosphate reabsorption and chronic hypophosphatemia [5].

The main purpose of this study was to investigate the etiology of 16 patients affected with XLH and identify pathogenic *PHEX* mutations.

Subjects And Methods

Patient information

Patients with XLH and their parents were recruited for this study. A total of 16 unrelated Chinese families were examined, including ten families containing sporadic XLH cases and six core families (Fig. 1A). The diagnosis of XLH was mainly based on clinical manifestations. All patients or their guardians provided signed informed consent, and the Institutional Review Board of China Medical University approved the study.

Genomic DNA isolation

Genomic DNA from peripheral blood leucocytes was isolated using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Dalian, China). Quality and consistency were determined using a spectrophotometer. The OD260/OD280 range for all samples was 1.8–2.0. Samples were stored at -30°C.

PHEX gene mutation analysis

PHEX gene mutations were analyzed by amplifying all 22 exons and flanking intronic regions. The primers and polymerase chain reaction (PCR) conditions were described previously [2]. The PCR products were separated on 1% agarose gels, and DNA sequencing was performed by Sangon Biotech (Shanghai, China). The *PHEX* gene sequences were compared with the reference sequence database 2013GRCh38/hg38 (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). Mutation detection was performed for 100 unrelated controls using restriction fragment length polymorphism analysis to ensure that the changes were not sequence polymorphisms.

Construction of plasmids

The pCAS-minigene splicing reporter vector (a gift from Dr. Mario Tosi) was used to evaluate whether mutations affected the recognition of normal splice sites [6]. Wild-type and mutant *PHEX* exons with approximately 500 bp of 5' and 3' intronic flanking sequences were PCR amplified from the genomic DNA samples of patients and unrelated controls. *Bam*H I and *Mlu* I restriction sites were included in the 5' tails of the forward and reverse primers, respectively, to allow the insertion of the PCR products into the pCAS vector (Fig. 2). The wild-type and mutant constructs were confirmed by DNA sequencing.

Cell culture and transfection

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum in an atmosphere of 5% CO₂ at 37°C. For transfection, 5×10⁶ HeLa cells were seeded into six-well plates in a volume of 2 mL and cultured for 24 h. Cells were transiently transfected for 24 h at 50–70% confluency with 2 µg wild-type or mutant *PHEX* constructs or empty vector using jetPRIME transfection reagent (Polyplus-transfection S.A., New York, USA).

Ex vivo splicing assay

Total RNA was extracted from cells 24 h post-transfection using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was performed using an AMV reverse transcriptase kit (TaKaRa, Dalian, China), PCR analysis was performed using primers that recognized vector sequences (Forward primer: 5'-CTGACCCTGCTGACCCTCCT-3', Reverse primer: 5'-TTGCTGAGAAGGCGTGGTAGAG-3'). The PCR products were separated with agarose gel and sequenced directly.

Construction of 3D models of mutant PHEX proteins.

Crystallographic modeling of wild-type PHEX protein was performed using SWISS-MODEL based on the crystal structure of neprilysin (PDB ID: 5JMY). Both neprilysin and PHEX are members of the M13 peptidase family and have the maximum homology. All nonsense and splicing mutations identified in this study were constructed into putative 3D mutant PHEX protein models.

Results

Clinical features of XLH patients

In this study, all the patients were born of a normal height and weight. There was no family history of consanguineous marriages or other specific genetic diseases. Ten of the patients were sporadic cases; one case was male. The age of the patients at diagnosis was 4 to 34 years old. All the patients exhibited varying degrees of short stature, growth retardation, hypophosphatemia, markedly elevated serum alkaline phosphatase levels and normal serum calcium. Seventy percent of the patients (12/16) had some bowing of the lower extremities, and almost all patients had "O" type bending. Only three boys showed "X" type bending of the lower extremities (Table 1). X-rays demonstrated that lucency existed in the zone of provisional calcification in the femurs and tibiae (Fig. 1B, C, D).

***PHEX* gene mutational analysis**

PHEX gene mutational analysis of 25 patient samples (10 sporadic, six pedigrees) revealed 15 different mutations, including 12 point mutations, two small deletions, and one small insertion (Table 2, Fig. 3). The 12 point mutations contained five missense mutations, five splicing mutations, and two nonsense mutations. All the *PHEX* mutations were distributed throughout the coding sequences and flanking intronic sequences of the *PHEX* gene. All these mutations appeared once, except the missense mutation c.2048T>C (p.Leu683Pro).

Five of the genetic changes had not been reported previously, including one small insertion, one nonsense mutation, one missense mutation, and two splicing mutations. These novel mutations were not detected in 100 unrelated, healthy Chinese people (200 control chromosomes), indicating that these mutations were not single nucleotide polymorphisms but causative mutations.

Ex vivo analysis of *PHEX* splice site mutations

In this study, six pairs of wild-type and mutant constructs were generated. Although our study found two deletions, one of the deletions included the last base of exon 14 and the first base of intron 14 and may affect the recognition of the splice site. Differences between the wild-type and mutant sequences were

detected by RT-PCR. Because the same primers were used to detect the wild-type and mutant fragments, it was easy to detect the abnormal fragments. The mutation IVS20+1G>A generated two new transcripts (Fig. 4F). The other five mutations resulted in only one transcript. Three mutations (IVS14+4A>T, IVS20-1G>T, and IVS20+1G>A) caused exon skipping (Fig. 4A, B, F). Four mutations activated new splice sites. Mutations c.1586_1586+1delAG and IVS5+1G>A activated new splice sites in exons 14 and 5, respectively, which resulted in shorter exons (Fig. 4C, D). Mutation IVS15+1G>A and IVS20+1G>A activated new splice sites in introns 15 and 20, respectively (Fig. 4E, F). Except for the exon skipping of IVS20+1G>A that caused an in-frame mutation, the mutations changed the open reading frame, resulting in premature stop codons.

3D homology modeling of PHEX mutant proteins

SWISS-MODEL was used to construct the presumptive 3D structure of mutant PHEX protein. All splice site mutations and nonsense mutations identified in this study were modeled based on the homology model of neprilysin. The sequence of the mutant PHEX protein carrying a splice mutation was inferred from the *ex vivo* splicing analysis. The modeling showed that the mutations caused changes in the PHEX protein structure even though the amino acid sequence before the mutation was the same (Fig. 5).

Discussion

Our mutational analysis identified 15 different *PHEX* gene mutations in ten sporadic XLH cases and six XLH pedigrees (Fig. 6). There were 20 female and five male patients in this study. The sex ratio of four females to one male conforms to the mode of XLH transmission [7]. Although the 20 female patients were heterozygous and had one normal X chromosome, there were no significant differences between the two genders in terms of clinical and biochemical measurements. The phenotypes among family members of the same pedigree did not offer necessary connections. In families 2 and 3, “X”-type bending of the lower extremities was found in the sons, while “O”-type bending on lower extremities was found in their mothers. These conditions were consistent with a previous report [8]. Recently, a report observed that hyperparathyroidism might be a frequent complication in adult XLH patients [9]. However, we did not find a similar symptom in our preliminary testing; however, we will follow patients for this condition by long-term observation.

As of last year, 623 different mutations have been identified and recorded in the HGMD (2020.4 version). Although several reports have presented the statistics of the different mutations, there were no explicit hotspots confirmed. Variant c.1601C > T(p.Pro534Leu) was mentioned as a potential hotspot, as it was found in five unrelated patients in southern China, two patients in other areas of China, and two patients in other counties [10–15]. Three other mutations (Glu579Arg, Arg747*, c.1645 + 1G > A) might also represent hotspot mutations [13]. None of these mutations were detected in our study. However, another variant, c.2078G > A(p.Cys693Tyr), appeared twice in the current study. This specific mutation has also been reported in three previous studies, including in one Chinese patient [13, 16, 17]. Thus, it might represent a hotspot.

Missense and nonsense mutations, splicing mutations, regulatory mutations, small deletions, insertions, or indels, gross deletions, insertions or duplications, and complex rearrangements have been detected in the *PHEX* gene. Splicing mutations are the third most frequent type of mutation found in the *PHEX* gene. Splicing mutations have been found in every intron (Fig. 7). However, mutations have not been reported in the

acceptor sites of introns 12 and 18. Splicing mutations in introns 3, 4, 10, 15, and 17 have been reported more frequently. Most splicing mutations occur within ten bases upstream or downstream of a splice site. Interestingly, a mutation located 73 bases upstream of the donor site of intron 5 and a mutation found 1268 bases downstream of the donor site of intron 7 could impact the recognition of the splice site [18, 19]. These mutations were detected in the RNA from patient blood samples; however, patient blood RNA samples are not always available. Therefore, we used a splicing reporter minigene assay in the current study. According to other studies, this approach is a relatively accurate method [20, 21]. In this study, we analyzed six splicing mutations using the minigene approach, including one small deletion in the donor site of intron 14 (c.1586_1586 + 1delAG). Coincidentally, the two bases before the deletion were also AG and recognized as an alternative donor site. Thus, we prefer to describe this mutation as c.1585_1586delGA (p.Glu529Valfs*52). In the other five splicing mutations, two variants (IVS14 + 4A > T and IVS20-1G > T) caused exon-skipping, resulting in a frameshift (p.Ile495Valfs*52 and p.Val691Glyfs*6). Two other variants (IVS15 + 1G > A and IVS5 + 1G > A) activated a preexisting pseudo splicing site within the exon, causing a frameshift (p.Arg549Hisfs*21 and p.Leu206Phefs*11). The variant IVS20 + 1G > A resulted in two outcomes. One resulted in the recognition of a new splice site in intron 20, and the other outcome was exon-skipping, which only accounted for a small portion comparing with the exon-skipping.

In previous studies, more than half of the identified mutations resulted in truncated proteins. In this study, all the splicing mutations caused frameshifts and truncated protein. However, no phenotypic differences have been observed between patients with truncating or non-truncating mutations except the condition that mutations in the first 649 amino acids led to earlier disease onset and higher iFGF23 levels [11, 13]. Our 3D homology modeling of the PHEX mutant proteins demonstrated a difference in the structures of wild-type and mutant PHEX proteins. Even the mutant proteins of IVS20 + 1G > A, IVS20-1G > T, c.1586_1586 + 1delAG, and c.1709_1710insTGAG(p.Tyr570Glyfs*12), which contained most of the wild-type protein (i.e., they were not truncated by the mutation), were structurally different from the wild-type PHEX, including changes in the position of the helix and the length of the loop. These alterations might affect the exposure of the functional domain and cause loss of function.

Patients in our study have been treated at the time of diagnosis. Oral phosphate supplements and active vitamin D analogs (calcitriol) were gradually given for medical management in the first one or two months. Oral phosphate was administered four times per day, with the dosage varying from 15 to 100 mg/kg/day depending on individual patient requirements and the occurrence of phosphate-induced diarrhea. ALP was measured one hour after taking the phosphate to ensure its levels had returned to the normal range. It was also important to monitor PTH levels in the patients because they might increase when phosphate is used alone, or the calcitriol dosage used in combination is insufficient, leading to secondary hyperparathyroidism [22, 23]. We did not detect high PTH levels in our study. Because increased FGF23 levels are responsible for phosphate wasting, the development of the anti-FGF23 monoclonal antibody (burosumab) has been met with great expectations [24, 25]. Clinical trials for burosumab in children have received market approval by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) and are underway [26–30]. A phase 3 trial evaluating the efficacy of burosumab in adult XLH patients is also being performed [31]. Thus far, the reported burosumab side effects are generally tolerable. The long-term effects require further investigation.

Conclusions

Our study identified 15 different *PHEX* gene mutations in ten XLHR sporadic cases and six XLH pedigrees. We investigated the effect of the splicing mutations. Ex vivo analysis showed that the splicing mutations could cause both exon skipping and initiation of covert splicing sites. The appearance of early termination codons was the most common outcome of the splicing mutations. Our results expand the mutation spectrum of the *PHEX* gene and will be helpful for genetic counseling.

Abbreviations

XLH: X-linked hypophosphatemia rickets; PHEX: phosphate-regulating endopeptidase; ALP: alkaline phosphatase; HGMD: Human Gene Mutation Database; FGF23: fibroblast growth factor 23; PCR: polymerase chain reaction; DMEM: Dulbecco's Modified Eagle's Medium; RT: Reverse transcription.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of China Medical University.

Consent for publication

Consent for publication was acquired from all participating subjects.

Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

All the authors declare no competing interests

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Authors' contributions

YL designed the study, HWM, FL, LNZ and LLY recruited patients and recorded clinical data, X SX performed experiments and wrote the manuscript, JLG performed the construction of plasmids, Cell culture and transfection, YL revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Clinical features of patients

Number	Gender	Age, y	Height, cm	Short degree	Bowed lower extremities	Bending type, X/O	Knee/ankle space, cm	Family history
Case 1	female	6	113.3	-0.72SD	severe	O	10	no
Case 2	female	19	140.5	-4.5SD	severe	O	10	no
Case 3	female	6	103	-3SD	severe	O	12	no
Case 4	female	9	116.4	-3.1SD	middle	O	4.2	no
Case 5	female	6	114.1	-1.5SD	mild	O	2	no
Case 6	female	7	105.9	-4SD		O	–	no
Case 7	male	8	125.4	+0.28SD	middle	X	3.7	no
Case 8	female	4	109	+0.3SD		O	–	no
Case 9	female	10	148.4	+0.8SD	middle	O	3.1	no
Case 10	female	34	–	–		O	–	no
Family 1	male	4.5	91.5	-2.33SD		O	–	yes
	female	36	140	-3.8SD		O	–	yes
	female	12	130.7	-3.5SD		O	–	yes
Family 2	male	4	97.3	-2.5SD	severe	X	8.3	yes
	female	30	138.8	-4.0SD		O	–	yes
Family 3	male	9	117	-3.1SD	middle	X	4.5	yes
	female	46	130	SD		O	–	yes
Family 4	female	1	82.3	-1SD		O	–	yes
	female	30	142	SD		O	–	yes
Family 5	female	4	95.3165	-3SD	mild	O	1.4	yes
	female	30	98.3	SD		O	–	yes
Family 6	female	3	–	–	mild	O	2.2	yes
	female	30	137	-4.4SD		O	–	yes

Table 2 Mutational analysis of *PHEX* gene

Number	Mutation type	Nucleotide change	Codon change	Exon/ intron	Zygosity	Mutation
Case 1	Small deletion	c.1586_1586+1delAG	p.Glu529Valfs*52	14	Het	Reported
Case 2	Small deletion	c.412delC	p.Leu138PhefsX6	4	Het	Reported
Case 3	Small insertion	c.1709_1710 insTGAG	p.Tyr570Glu fsX12	17	Het	Novel
Case 4	Missense mutation	c.2078G>A	p.Cys693Tyr	21	Het	Reported
Case 5	Nonsense mutation	c.1285G>T	p.Glu429*	11	Het	Novel
Case 6	Missense mutation	c.2155G>A	p.Gly719Ser	22	Het	Reported
Case 7	Nonsense mutation	c.1699C>T	p.Arg567*	16	Hemizygous	Reported
Case 8	Missense mutation	c.503C>T	p.Pro168Leu	5	Het	Novel
Case 9	Splicing mutation	IVS15+1G>A		15	Het	Reported
Case 10						Not found
Family 1 II-2 I-2 II-1	Missense mutation	c.2048T>C	p.Leu683Pro	20	Hemizygous Het Het	Novel
Family 2 II-1 I-2	Splicing mutation	IVS20+1G>A		20	Hemizygous Het	Novel
Family 3 II-1 I-2	Splicing mutation	IVS20-1G>T		20	Hemizygous Het	Reported
Family 4 II-1 I-2	Missense mutation	c.1735G>A	p.Gly579Arg	17	Het Het	Reported
Family 5 II-1 I-2	Splicing mutation	IVS5+1G>A		5	Het Het	Novel

Figure 2

Structures of pCAS-minigene splicing reporter vectors. The construct was composed of a pcDNA3.1-based vector and exons 2 and 3 of the SERPING1/C1NH gene (pink squares). Wild-type and mutant PHEX exons and their 5' and 3' intronic flanking sequences (orange squares) were inserted into the vectors between the BamHI and MluI restriction sites.

Figure 3

PHEX gene mutation analysis in XLH patients. A Eight missense mutations were detected in the XLH patients in this study. The top line displays the mutant DNA sequence, while the second line shows the conservation of the amino acid sequence corresponding to the mutation. The height of the letter represents the proportion of the amino acid in the 46 species. The sequence alignment of the mutant position was generated using the UCSC genome browser (<http://genome.ucsc.edu/index.html>.) and WebLogo 3 (<http://weblogo.threeplusone.com/>). The red squares represent the amino acid of the mutant position. B Three del/ins mutations and five splicing mutations were detected in the XLH patients of this study. The vertical arrows show the mutations.

Figure 4

Ex vivo splicing assays for the PHEX mutations. The results of six PHEX splicing mutations. The RT-PCR products were analyzed by agarose gel electrophoresis and sequencing. The purple squares indicate the exons of the SERPING1/CINH gene. The pink and green squares indicate the exons and introns of the PHEX gene.

Figure 5

Construction of 3D models of mutant PHEX protein. A The wild-type PHEX protein model. B–G Mutant PHEX protein models with splicing mutations: B IVS5+1G>A; C IVS14+4A>T; D IVS15+1G>A; E, F IVS20+1G>A; G IVS20-1G>T. H–I Mutant PHEX protein models with nonsense mutations: H c.1699C>T p.Arg567*; I c.1285G>T p.Glu429*. J–L Mutant PHEX protein models with del/ins mutations: J c.412delC; K c.1586_1586+1delAG; L c.1709_1710insTGAG. Blue, pink, and orange represent helix, sheet and loop structures. The initiation codon is shown in green.

Figure 6

Distribution of splicing mutations in the PHEX gene. Dark red squares represent the mutations found in this study.