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Functional analysis of a *de novo* mutation c.1692 del A of the *PHEX* gene in a Chinese family with X-linked hypophosphataemic rickets

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Objectives

X-linked hypophosphataemic rickets (XLHR) is a disease of impaired bone mineralization characterized by hypophosphataemia caused by renal phosphate wasting. The main clinical manifestations of the disorder are O-shaped legs, X-shaped legs, delayed growth, and bone pain. XLHR is the most common inheritable form of rickets, with an incidence of 1/20000 in humans. It accounts for approximately 80% of familial cases of hypophosphataemia and serves as the prototype of defective tubular phosphate ($PO4^{3+}$) transport, due to extra renal defects resulting in unregulated *FGF23* activity. XLHR is caused by loss-of-function mutations in the *PHEX* gene. The aim of this research was to identify the genetic defect responsible for familial hypophosphataemic rickets in a four-generation Chinese Han pedigree and to analyze the function of this mutation.

Methods

The genome DNA samples of all members in the pedigree were extracted from whole blood. We sequenced all exons of the *PHEX* and *FGF23* genes, as well as the adjacent splice site sequence with Sanger sequencing. Next, we analyzed the *de novo* mutation c.1692 del A of the *PHEX* gene with an online digital service and investigated the mutant *PHEX* with SWISS-MODEL, immunofluorescence, and protein stability detection.

Results

Through Sanger sequencing, we found a *de novo* mutation, c.1692 del A, in exon 16 of the *PHEX* gene in this pedigree. This mutation can make the PHEX protein become unstable and decay rapidly, which results in familial XLHR.

Conclusion

We have found a *de novo* loss-of-function mutation, c.1692 del A, in exon 16 of the *PHEX* gene that can cause XLHR.

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Keywords: Hypophosphataemia, *PHEX*, Sanger sequencing

Article focus

- There are no previous studies that report on mutation c.1692 del A of the *PHEX* gene in relation to X-linked hypophosphataemic rickets (XLHR).
- We investigated the association between mutation c.1692 del A of the *PHEX* gene and XLHR.

Key message

- The *de novo* loss-of-function mutation c.1692 del A of the *PHEX* gene can cause XLHR.

Strengths and limitations

- This is the first report of mutation c.1692 del A of the *PHEX* gene.
- Our findings show that c.1692 del A of the *PHEX* gene is a loss-of-function mutation and is directly associated with XLHR.
- More studies should be conducted to investigate the relationship between *PHEX* and innate *FGF23*, in order to investigate how to treat, or even prevent, XLHR.

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Introduction

X-linked hypophosphataemic rickets (XLHR) is a rare disorder associated with low levels of phosphate in the serum, caused by renal tubular inorganic phosphate wasting. XLHR, however, is resistant to treatment with vitamin D. It is the most common heritable form of rickets, with an incidence of 1/20000.[1] The main clinical manifestations of the disorder are O-shaped and X-shaped legs, delayed growth, and bone pain. Although there is a large phenotypic variability, patients with XLHR exhibit a degree of disproportional dwarfism, leading to shortening of the lower limbs, low mineral density with rickets in children, and osteomalacia in adults.[2] XLHR is caused by loss of function in the *PHEX* gene, located in X chromosome p22.1-p22.2, which encodes a zinc-dependent metalloprotease.[3] The phosphate-regulating neutral endopeptidase (PHEX) protein has an important role in the regulation of renal tubular phosphate reabsorption and subsequent bone mineralization.[4]

In this paper, we have studied a four-generation Chinese Han pedigree with XLHR with a germline *de novo* mutation in the *PHEX* gene.

Materials and Methods

Family recruitment and clinical evaluations. A four-generation family consisting of 16 members, who all presented with segregating XLHR, was identified. Medical histories were obtained using a questionnaire covering the following issues: subjective degree of leg bowing; age at onset; short stature; joint pain and impaired mobility; cranial structure; dental abnormalities; and hearing loss. Medical examinations were performed on the proband and his younger brother.

***In silico* analysis.** Mutation Taster software (http://www.mutationtaster.org/) was used to determine possible changes in protein structure that might affect the phenotype.

Construction of *PHEX* lentiviral vectors. The *PHEX* cDNA was obtained from an associate's laboratory and subcloned into the pCDH-CMV-vector (Youbio Co. Ltd, Hunan, China), which contains a CMV promoter to drive cDNA expression, and a puromycin acetyltransferase marker, to construct a pCDH-CMV-*PHEX*-WT recombinant vector. The pCDH-CMV-*PHEX* (c.1692 del A) mutant was constructed using the Mut Express MultiS Fast Mutagenesis Kit V2 (Vazyme Biotech Co. Ltd, Nanjing, China) according to the manufacturer's instructions. FLAG-tagged wild-type PHEX (FLAG-WT-PHEX) and FLAG-tagged mutant PHEX (FLAG-MUT-PHEX) were subcloned by this vector. The mutation was introduced, and 5′-ATGGATTACAAGGATGACGACGATAAG-3′(FLAG) was inserted before the initiation codon, using the Mut Express MultiS Fast Mutagenesis Kit V2.

Both expression vectors were sequenced entirely by Sanger sequencing to verify their identity.

Cell culture and transfection. The human embryonic kidney (HEK) 293T cell was grown in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Beit HaEmek, Israel) with 10% foetal bovine serum (FBS) and 1% Pen-Strep antibiotics in a cell incubator with 5% CO_2 at 37°C. Before transfection, cells were seeded into six-well culture dishes at a density of 5×10^5 cells/well. The HEK-293T cells were transiently transfected with FLAG-WT-PHEX and FLAG-MUT-PHEX expression vectors separately (a total of 2.5 μg for each vector DNA) and were mediated by lipid transfection using lipofectamine 3000 (Invitrogen, Carlsbad, California) The medium was changed the next day and cells were harvested 24 hours later.

Expression detection of FLAG-WT-PHEX and FLAG-MUT-PHEX. Transfected cells were lysed in radio-immunoprecipitation assay (RIPA, Beyotime Biotechnology, Shanghai, China) buffer with 1% phenylmethylsulfonyl fluoride (PMSF) to acquire the total cellular proteins, and protein concentration was evaluated with a G-250 Bradford kit (Beyotime Biotechnology). To detect the expression of FLAG-WT-PHEX and FLAG-MUT-PHEX in the transfected HEK-293T cells, the soluble proteins were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane, where FLAG-PHEX ran at a size of approximately 180 kDa. The membrane was blocked in 5% milk, incubated with primary FLAG monoantibody (1:5000; Abmart, Shanghai, China), and diluted in 5% bovine serum albumin (BSA, CWBIO Technology Co. Ltd, Beijing, China) overnight at 4°C. The membrane was then washed with tris-buffered saline using Tween 20 (TBST; Beyotime Biotechnology) buffer three times, each for 15 minutes. Afterwards, the membrane was incubated with secondary horseradish peroxidase (HRP)-conjugated antibody (antimouse, 1:5000; CWBIO Technology Co. Ltd) diluted in 5% milk. Enhanced chemiluminescence (ECL) mix (Merck Millipore, Darmstadt, Germany) was used to visualize proteins on radiographs.

Cellular localization testing. Transfected cells for immunofluorescence (IF) experiments were grown on glass slips to 70% to 80% confluence, washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. The cells were permeabilized by adding PBS with 0.5% Triton (CWBIO Technology Co. Ltd) and blocked with 0.5% BSA with PBS-Triton-X100. Cells were incubated with anti-FLAG antibody (1:200; Abmart) and subsequently with Rho-conjugated goat antimouse immunoglobulin G (IgG). Cell nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Immunofluorescence images were captured using a confocal microscope (Leica Microsystems Inc. Heidelberg, Germany), with a 63× objective with fixed optical slice, laser power, and detector/amplifier settings, for all samples across each individual experiment to allow for comparison.

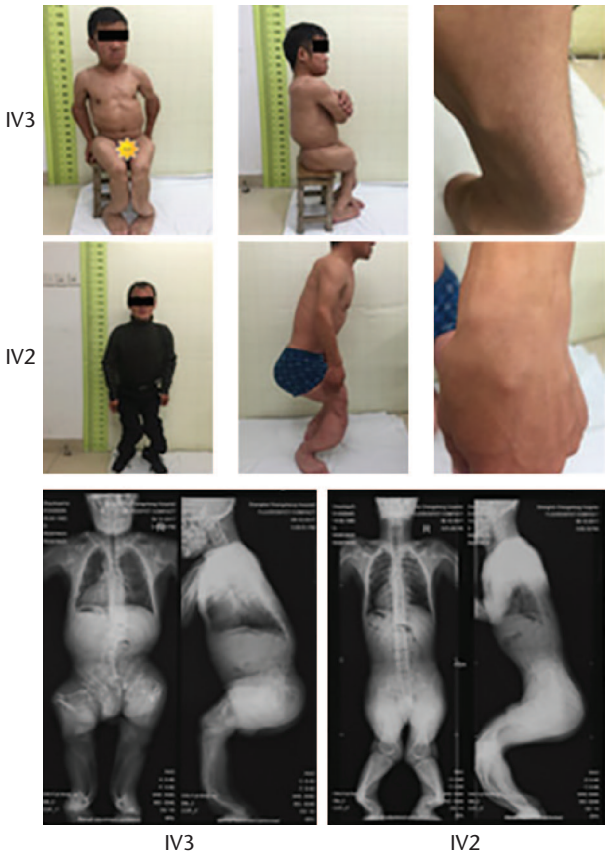

Fig. 1a

(Continued)

Protein stability analysis. The stability of PHEX protein was detected using cycloheximide (CHX, Yeasen Co. Ltd, Shanghai, China), inhibitor of protein *de novo* synthesis, and proteasome inhibitor MG132 (Yeasen Co. Ltd). The HEK-293T cells were seeded into six-well plates. When the confluence of cells grown on the glass reached between 70% and 80%, we began transfecting cells with FLAG-WT-PHEX or FLAG-MUT-PHEX vectors for 48 hours, and subsequently treated cells with 0.1 mM CHX and MG132 (Yasen Electronic Co. Ltd, Jiangsu, China) with 20 nM for the indicated timepoints. Transfected cells were lysed in RIPA with 1% PMSF to prepare the total cellular protein sample and protein concentration was evaluated using the G-250 Bradford kit. The soluble proteins were then separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane, where FLAG-PHEX ran at a size of approximately 180 kDa. The membrane was blocked with 5% milk, incubated with primary FLAG monoantibody (1:5000; Abmart), and diluted in 5% BSA overnight at 4°C. The membrane was then washed with TBST buffer three times, each for 15 minutes. Afterwards, the membrane was incubated with secondary HRP-conjugated antibody (antimouse, 1:5000; CWBIO Technology Co. Ltd) diluted in 5% milk. The ECL mix was used to visualize proteins on radiographic film.

Results

In this study, we found a pedigree with HR (Figs 1a and 1b). All affected members of this family presented with a short stature and bowed legs. Two of them had severe skeletal abnormalities involving the lower limbs (Fig. 1a) as well as bone pain. These features were the same in both the male and female members of the family, but the severity of the condition varied. We collected biochemical parameters including serum concentration of phosphate, calcium, alkaline phosphatase, intact parathyroid hormone, 25-hydroxyvitamin D (25(OH)D), urinary phosphate, and urinary calcium from two patients of this family. The clinical, biochemical, radiological, and genetic data of two male patients with the *PHEX* c.1692 del A mutation are shown in Table I. To further determine the way hypophosphataemia was inherited, we performed Sanger sequencing of *PHEX* and *FGF23* coding regions and adjacent splice sites. The primers are shown in Supplementary Table i. We identified a novel hemizygous deletion mutation in male patients and a heterozygous deletion mutation in female patients, in exon 16 of the *PHEX* gene (Fig. 1c). *PHEX* C.1692 del A is a frameshift mutation that substitutes 568 serine with a stop codon. Therefore, this mutation breeds a truncated protein that lost 181 amino acids from the C terminal (Figs 2a and 2c).

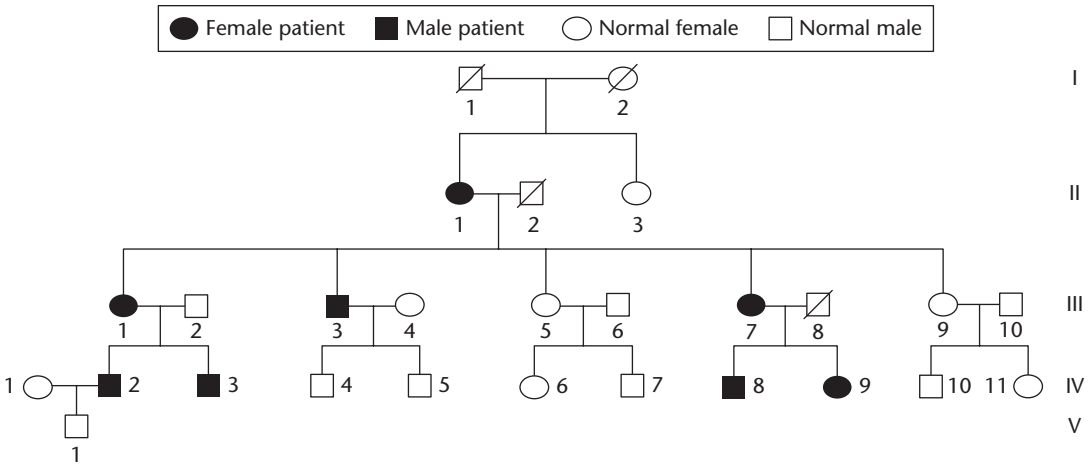

Fig. 1b

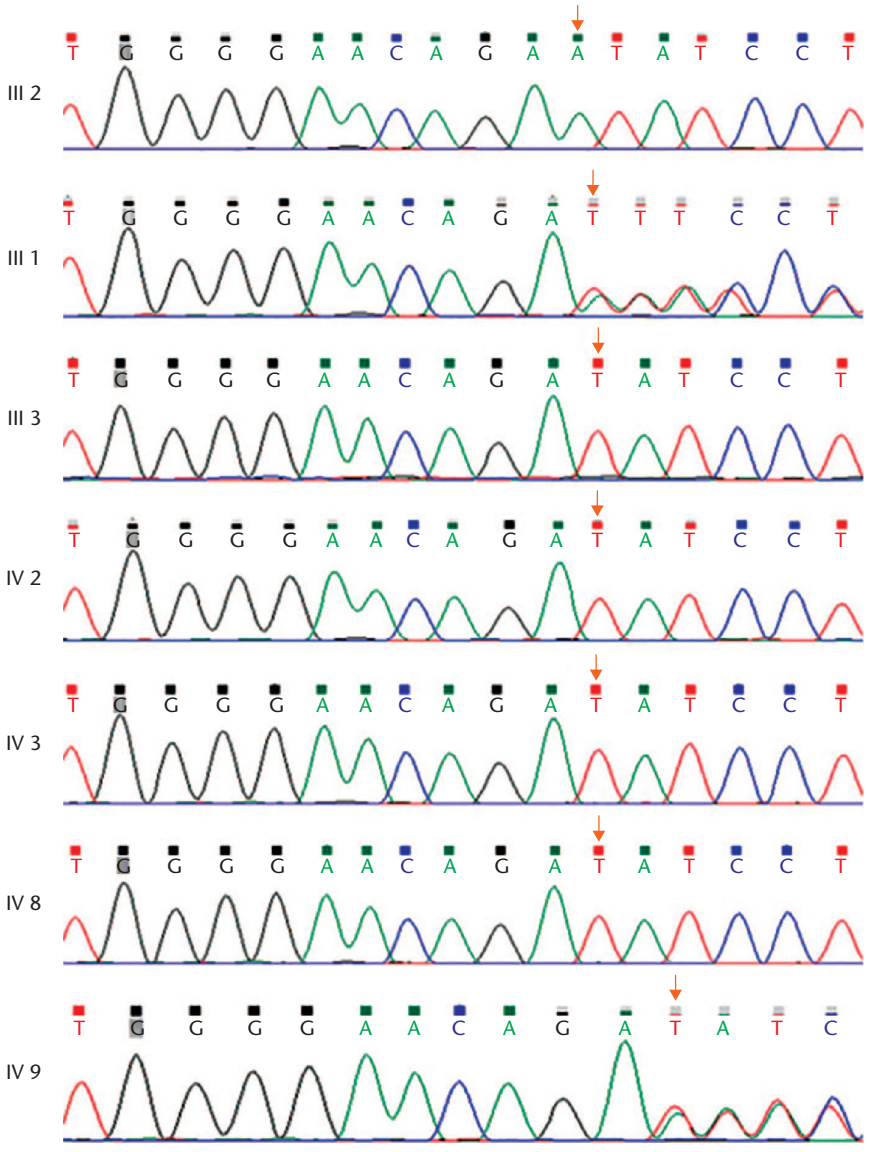

Fig. 1c

a) Photographs and radiographs of two brothers with the *PHEX* c. 1692 del A mutation: the proband (IV2) aged 39 years and his younger brother (IV3) aged 35 years. b) Pedigree of the family with X-linked hypophosphataemic rickets showing affected cases; c) Sanger sequencing chromatograms indicating male individuals affected with hemizygous *PHEX* c.1692 del A mutation and female individuals affected with heterozygous *PHEX* c.1692 del A mutation.

Table I. Clinical, biochemical, radiological, and genetic data of two male patients with the *PHEX* c.1692 del A mutation: the proband (IV_2) and his younger brother (IV_3)

Subject	IV2	IV3
Sex	Male	Male
Age, yrs	39	35
Genotype	Hemizygote	Hemizygote
Weight, kg	40	36
Height, cm	110	100
Delayed growth	Yes	Yes
Bowing of legs	Yes	Yes
Inability to walk	No	Yes
Bone pain	Yes	Yes
Skeletal deformities	Yes	Yes
Dental anomalies	No	No
Parathyroid hormone	79.9 pg/ml ↑	117.2 pg/ml ↑
Serum phosphate	0.71 mmol/l ↓	0.68 mmol/l ↓
Serum calcium	2.31 mmol/l	2.25 mmol/l
Serum AKP	205 U/l ↑	194 U/l ↑
Urine calcium	3.34 mmol/l ↑	0.93 mmol/l
Urine phosphate	10.29 mmol/l ↑	7.91 mmol/l ↑
Serum FGF23	637.1875 pg/ml ↑	618.4375 pg/ml ↑

↑, increased value; ↓, decreased value; AKP, alkaline phosphatase; FGF23, fibroblast growth factor 23

This mutation has never been reported before. According to our prediction, the *PHEX* c.1692 del A mutation was pathogenic. These predictions were processed with Mutation Taster (http://www.mutationtaster.org). We found that the PHEX truncated protein lost many important domains that contain putative active sites and zinc binding sites (Fig. 2b). The protein encoded by the *PHEX* gene is a transmembrane endopeptidase that belongs to the type II integral membrane zinc-dependent endopeptidase family. This truncated protein is therefore dysfunctional and the *PHEX* c.1692 del A mutation is disease-causing.

The PHEX protein localized in cell membrane. To further confirm the pathogenicity of this mutation, HEK-293T cells were transfected with vectors that express FLAG-WT-PHEX or FLAG-MUT-PHEX. In anti-FLAG western blot, we successfully detected the presence of FLAG-WT-PHEX and FLAG-MUT-PHEX in HEK-293T cell line. The PHEX protein was putative transmembrane endopeptidase, and we further confirmed the subcellular localization of the FLAG-WT-PHEX and FLAG-MUT-PHEX protein in HEK-293T cell line. After transient overexpression of wild and mutant type PHEX protein in cell, anti-FLAG IF was used to facilitate direct examination of the truncated product's localization. As the results show, WT PHEX was located in the cell membrane (Figs 3a to 3c). However, except for that located in the cell membrane, the mutant type PHEX was dispersed in the cytoplasm (Figs 3d to 3f). Thus, the cell localization of mutant PHEX protein was partially influenced.

***PHEX* c.1692 del A results in PHEX protein degradation.** The anti-FLAG Western blot suggests that this mutation may result in PHEX protein degradation. The stability of wild-type PHEX and mutant PHEX proteins in HEK-293T cells was analyzed using the CHX and MG132 (Yeasen Co. Ltd) chase protein degradation assay. For the CHX chase assay, we transfected HEK-293T cells with FLAG-WT-PHEX or FLAG-MUT-PHEX vectors. The PHEX protein stability was assessed by incubating cells for a maximum of 12 hours with 0.1 mM of CHX, a known inhibitor of eukaryotic translation. The PHEX protein expression was quantified at four different timepoints (baseline, four hours, eight hours, and 12 hours) after CHX treatment. Western blotting results suggested that wild-type PHEX remained stable even after 12 hours, whereas mutant PHEX protein showed significant protein degradation (Fig. 4a). MG132 was used to inhibit 20S proteasome and found that there was no difference among different groups of wild-type PHEX protein. However, there was a significant difference between the groups of mutant PHEX protein at baseline *versus* at four hours (Fig. 4b). Results from protein degradation analysis revealed that *PHEX* c.1692 del A mutation results in PHEX protein degradation. This therefore confirmed the mutation to be a loss-of-function mutation.

Discussion

Hypophosphataemic rickets is a condition in which there is impaired bone mineralization that is characterized by hypophosphataemia and caused by renal phosphate wasting. Heritable forms of HR include X-linked dominant HR caused by inactivating mutations in the *PHEX* gene, autosomal recessive HR (ARHR) due to inactivating mutations in the gene that encodes dentin matrix acidic phosphoprotein 1 (*DMP1*)[5-8] (MIM#241520) or the *ENPP1*[9,10] (MIM#613312) gene, autosomal dominant HR (ADHR, MIM#193100) induced by inactivating mutations in the *FGF23* gene,[11] and X-linked recessive HR (MIM#300554) caused by inactivating mutations in the *CICN5* gene.[12]

Autosomal recessive HR is a rare form of HR that is due to mutations in the *DMP1* gene. The *DMP1* gene is highly expressed in mineralized tissues, especially in osteoblasts and osteocytes, and is a key regulatory protein that is required for the normal growth and development of bone, cartilage, and dentin. Dentin matrix, acidic phosphoprotein 1 (DMP1) undergoes phosphorylation during the early phase of osteoblast maturation and is then exported into the extracellular matrix (ECM), where it acts as a regulator for the nucleation of hydroxyapatite. Levy-Litan et al[9] described an inactivating mutation in the *ENPP1* gene that resulted in ARHR. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is a cell surface enzyme that generates inorganic pyrophosphate and has an important role in the deposition of this compound.

ADHR is a rare genetic disorder. The gene causing ADHR was identified to encode fibroblast growth factor 23

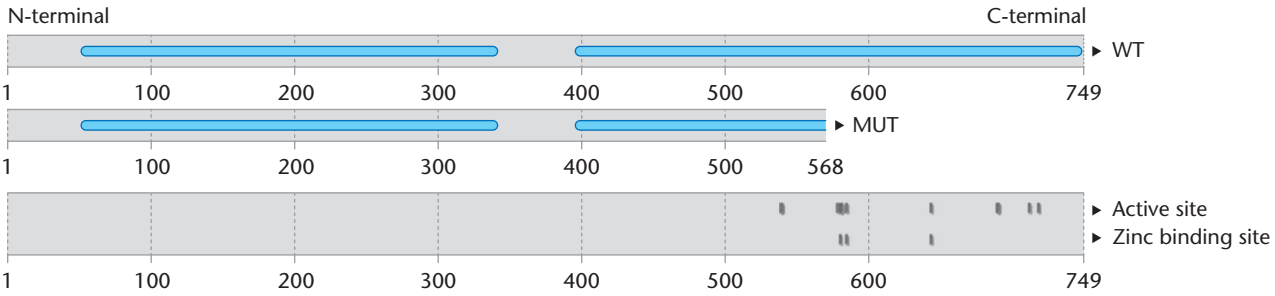

Fig. 2a

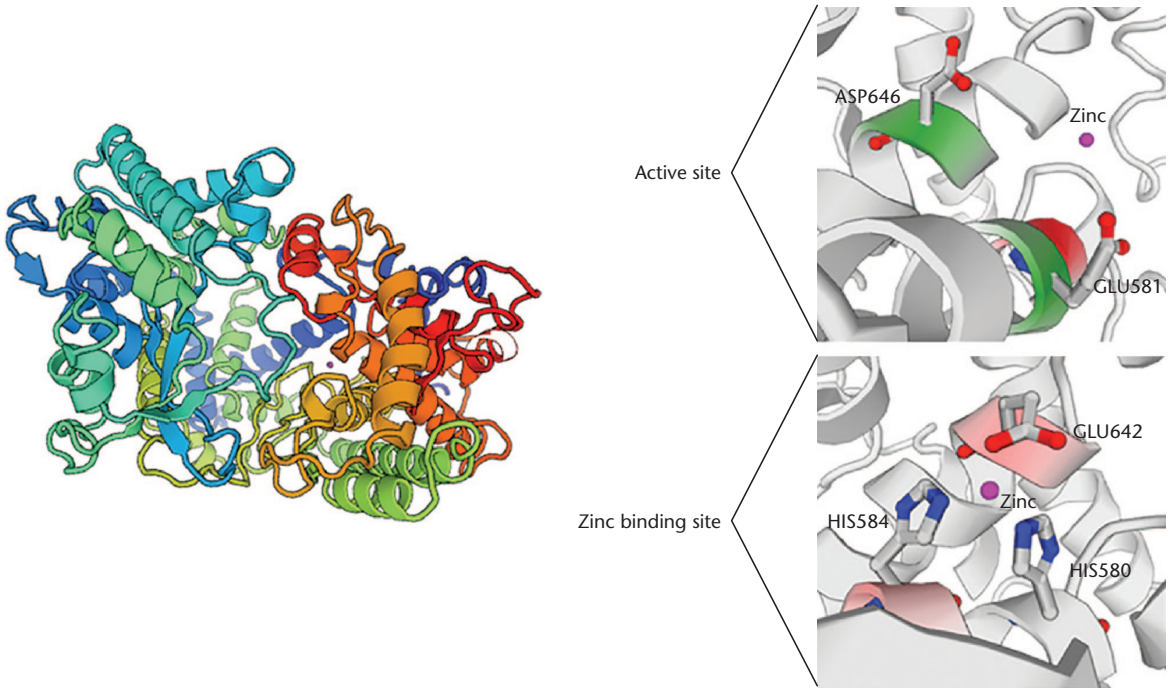

Fig. 2b

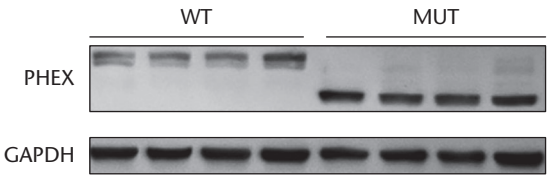

Fig. 2c

a) The wild-type (WT) and mutant (MUT) phosphate-regulating neutral endopeptidase, X-linked (PHEX) protein. *PHEX* c.1692 del A mutation resulted in a truncated protein, as well as the loss of zinc binding sites and most active sites. b) 3D structure of the WT PHEX protein model by SWISS-MODEL (https://swissmodel.expasy.org). c) Western blot detection of the WT and MUT PHEX protein. GADPH, glyceraldehyde 3-phosphate dehydrogenase.

(FGF23). FGF23 is a 251-amino acid protein and a circulatory hormone produced by osteocytes, but is also found in the heart and liver. FGF23 helps to maintain phosphorus homeostasis by inducing renal phosphate excretion and is a potent regulator of vitamin D metabolism, which acts by suppressing renal 1-α-hydroxylase activity.

According to the pedigree we have studied, HR is inherited as an autosomal dominant form in this family. Therefore, gene-specific DNA sequencing of *PHEX* and *FGF23* coding regions and adjacent splice sites was performed. A novel mutation in the *PHEX* gene was found, indicating that this familial HR is XLHR. The mutation of the *PHEX* gene, which comprises 22 exons coding for a 749-amino acid protein, is the cause of the most common XLHR. In this study, we have identified a novel germline mutation in the *PHEX* gene c.1692 del A in exon 16. Predictions of the impact of this mutation through different online software programs (SIFT (https://sift.bii.a-star.edu.sg/), PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2), and Mutation Taster) come to the same conclusion that this mutation causes disease. The truncated PHEX protein lost its zinc binding sites and its

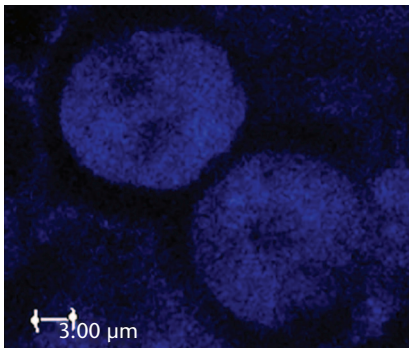

Fig. 3a

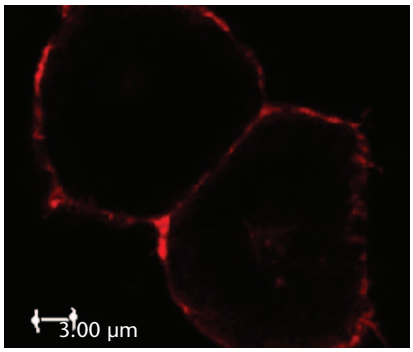

Fig. 3b

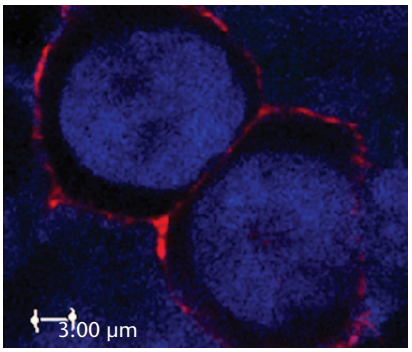

Fig. 3c

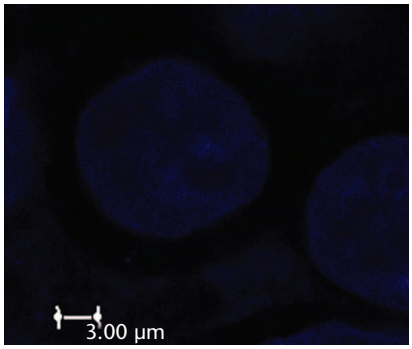

Fig. 3d

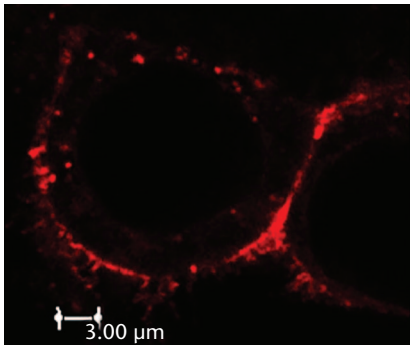

Fig. 3e

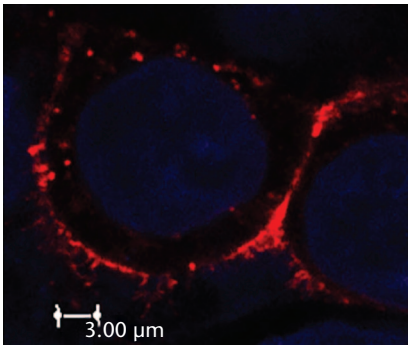

Fig. 3f

The immunofluorescent distribution of phosphate-regulating neutral endopeptidase, X-linked (PHEX) protein in a normal HEK-293T cell. The HEK-293T cell was transfected with a) to c) the PHEX wild-type overexpression vector and d) to f) the PHEX mutant overexpression vector, using nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; blue) and PHEX-FLAG antibody (red).

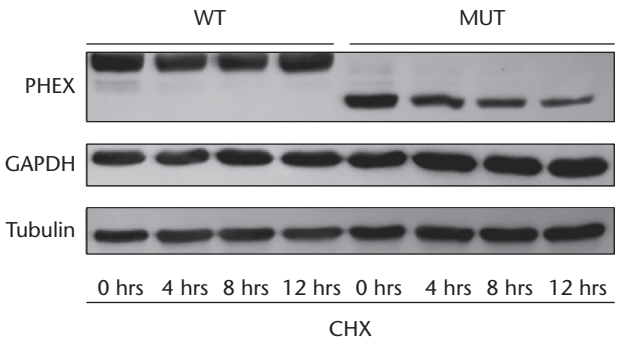

Fig. 4a

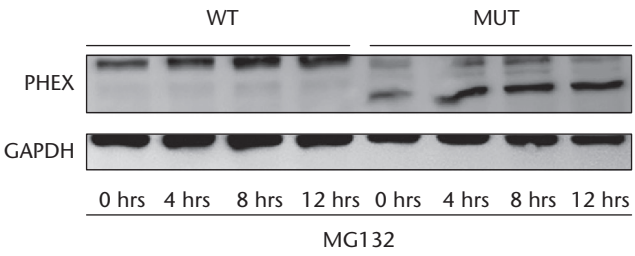

Fig. 4b

Mutant (MUT) phosphate-regulating neutral endopeptidase, X-linked (PHEX) protein degrades more rapidly than wild-type (WT) protein. a) PHEX bands at baseline, four hours, eight hours, and 12 hours with cycloheximide (CHX) treatment. b) PHEX bands at baseline, four hours, eight hours, and 12 hours with MG132 treatment. GADPH, glyceraldehyde 3-phosphate dehydrogenase.

most putative active sites, which may cause PHEX loss of function and protein instability (Figs 4a and 4b). PHEX is vital for normal bone and dentin mineralization and renal phosphate reabsorption, so the degradation of PHEX can result in low serum phosphate concentration and abnormal bone development.[13-15]

To date, more than 400 mutations in the *PHEX* gene have been identified and the connection between XLHR and *PHEX* is well defined.[16] However, the exact mechanism of the disorder is unclear. Initially, it was hypothesized that FGF23 is a substrate of the PHEX protein and that mutated PHEX protein cannot deactivate FGF23.[17] However, data from later studies of whether FGF23 is a substrate of PHEX have been conflicting and few studies have been able to demonstrate the PHEX-dependent cleavage of intact FGF23.[18,19] An intermediate pathway between *PHEX* and *FGF23* has therefore been proposed, in which inactivation of FGF23 is prevented, which leads to increased FGF23 levels and to decreased proximal tubule NaPi-II cotransporters' expression and activity, and therefore leads to phosphaturia.[20-25]

Delayed growth and rickets in XLHR start to occur in the first two years of life, when growth is at its physiological maximum.[26] Therefore early initiation of therapy is of vital importance, before growth deceleration and the overt manifestation of rickets develop. Up to now, pharmacological treatment in XLHR patients has consisted of oral phosphate supplementation, 1-alpha hydroxy-vitamin D derivative administration, and growth hormone treatment,[27] but the treatment efficiency is suboptimal.[27,28] On 17 April 2018, the United States Food and Drug Administration approved Crysvita (burosumab-twza; Kyowa Hakko Kirin Co., Ltd. and Ultragenyx Pharmaceutical Inc., Novator, California), an antagonist of intact FGF23, the first drug approved to treat XLHR in adults and children aged one year and older. The safety and efficacy of Crysvita were studied and found to have a positive therapeutical effect. However, it also had some adverse reactions.[29,30] In adults, these complications included back pain, headaches, restless leg syndrome, decreased vitamin D, dizziness, and constipation. In children, complications included headaches, injection site reaction, vomiting, decreased vitamin D, and pyrexia. With the rapid development of gene-editing technologies and gene therapy, methods that could cure XLHR patients permanently need to be investigated further.[30]

In this paper, we present a novel frameshift mutation in the *PHEX* gene in a family with XLHR. The protein translated by this mutated gene is a truncated protein that our experiments have shown to be a loss-of-function mutation. The discovery of this *PHEX* mutation enriches the *PHEX* mutation database and will help develop a molecular diagnosis with XLHR. Through a combination of genetic counselling and gene therapy, we are hopeful that XLHR will be eradicated soon.

Supplementary Material

Table showing primers revealed by exon sequencing of *PHEX* and *FGF23* genes.

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