A study on the function of novel *PHEX* mutations p.Glu145* and p.Trp749Arg in families with X-linked hyphosphatemic rickets

Abstract

X-linked hypophosphatemic rickets (XLH) is characterized by increased circulating fibroblast growth factor 23 (FGF23) levels caused by *PHEX* (NM_000444.5) mutations. Renal tubular resorption of phosphate is impaired, resulting in rickets and impaired bone mineralization. By phenotypic-genetic linkage analysis, two *PHEX* pathogenic mutations were found in two XLH families: c.433G>T, p.Glu145* in exon 4 and c.2245T>C, p.Trp749Arg in exon 22. Immunofluorescence showed that the localization of p.Glu145* and p.Trp749Arg mutant and secretory PHEX (secPHEX) changed, with decreased expression. In a HEK293T cell model co-transfected with *PHEX*, sec*PHEX*, and *FGF23*, wild-type PHEX, secPHEX, and FGF23 proteins were distributed in the cell membrane or endoplasmic reticulum, while the mutant was located in the nuclear membrane and cytoplasm. qPCR of p.Glu145* revealed decreased *PHEX* and sec*PHEX* mRNA expression in cells, with no difference in mRNA expression of p.Trp749Arg. Both mutations decreased intracellular PHEX endopeptidase activity. Western blot analysis showed decrease in mutant and secPHEX protein expression and no FGF23 protein expression in single-transfected PHEX and secPHEX cells. In cells co-transfected with FGF23, *PHEX* and sec*PHEX* mutation promoted FGF23 expression. Thus, p.Glu145* mutation may lead to XLH through mRNA decay, while p.Trp749Arg missense may cause disease by affecting the spatial structure of the protein.

Keyword: X-linked hypophosphatemic rickets (XLH), phosphate regulating endopeptidase homolog X-linked (*PHEX*), gene mutations, fibroblast growth factor 23 (*FGF23*)

Abbreviations : PHEX: Phosphate regulating endopeptidase homolog X-linked; FGF23: Fibroblast growth factor 23; XLH : X-linked hypophosphatemic rickets; WT: Wild type; Mut: Mutant; sec: Secretory; GFP: Green fluorescent protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NEP: Neprilysin; ECE-1: Endothelin-converting enzyme-1; PC2: Proprotein convertase 2; DMPl: Dentin matrix acidic phosphoprotein 1; NMD: Degrading mRNA via nonsense - mediated mRNA decay; HEK : Human embryonic kidney; SPC: Subtilisin-like proprotein convertase; ADHR: Autosomal dominant hypophosphatemic rickets; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; FAM20C Family with sequence similarity 20, member C; SLC34A3 Solute carrier family 34 member 3; TIO: Tumor-associated

hypophosphatemia; NADPH: Nicotinamide adenine dinucleotide phosphate; MEK-ERK: Mitogen-activated protein kinase kinase -- extracellular signal-regulated kinase; ASARM: ; HIF-1a: Hypoxia inducible factor 1 subunit alpha; SPC: Subtilisin-like proprotein convertase; GALNT3: Polypeptide N-acetylgalactosaminyltransferase 3; SGK1: Serum/glucocorticoid regulated kinase 1; NHERF-1: Na(+)/H(+) exchange regulatory cofactor 1; NaPi-2a: Sodium-phosphate cotransporter; DCT: Distal convoluted tubule; MAPK: Mitogen-activated protein kinase

INTRODUCTION

X-linked dominant hypophosphatemic rickets (XLH, MIM307800) is the most common type of human hereditary rickets, with an incidence of approximately 1:20,000 [1,2]. XLH is characterized by hypophosphatemia secondary to kidney phosphate consumption and abnormal vitamin D metabolism, leading to bone mineralization disorders. Patients often report growth retardation, bone deformity, abnormal teeth, and other symptoms in the early stage; hearing impairment, joint pain, spontaneous fracture, and attachment point disease may occur in the later stage [3-5]. Sagittal suture fusion and Chiari I malformation are common complications of XLH [6] with clinical characteristics of young age, serious illness, and poor curative effect, which seriously endangers human health.

In 1995, the Hyperparathyroidism Consortium identified a phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (*PHEX*, NM_000444.5) as the pathogenic gene of XLH[7]. The gene is located on chromosome X (Xp22.11), contains 22 exons, and has a full-length size of 2861 bp. The protein encodes 749 residues (NP_000435.3), and is a member of the M13 family of type II transmembrane metalloproteases. Its structure is composed of a short chain intracellular domain, a transmembrane domain, and a large extracellular domain containing a zinc-binding reion and conserved cysteine residues. It has significant homology with enkephalin (NEP), endothelin converting enzyme 1 and 2 (ECE-1 and ECE-2), and Kell antigen (Kell) [8-10]. A unique hydrophobic peptide (SA domain) at the N-terminal not only guides protein transport through the rough endoplasmic reticulum as a signal peptide, but also anchors the protein to the plasma membrane of the cell as a transmembrane domain [11]. Although PHEX expresses a protease, it is considered to mainly affect the expression of fibroblast factor 23 (FGF23), rather than promoting the degradation of FGF23 [12]. Pathogenic mutations in *PHEX* lead to a decrease in endopeptidase activity, protein transport, and protein conformation abnormality, resulting in the loss of protein function [1]. The types of mutations include missense and nonsense mutations, deletions, small internal exon insertions and deletions, repetition, and splicing site variants [13,14]. At present, the molecular mechanism by which *PHEX* regulates renal phosphate metabolism is unclear. Most studies have found that PHEX is mainly expressed in osteoblasts, bone cells, and odontoblast

cells, but not in renal epithelial cells [15]; the specific regulatory mechanism remains to be studied.

FGF23, which is mainly produced by bone cells and osteoblasts, is a secretory protein containing 249 residues; the N-terminal has a signal peptide comprising 24 residues, which is one of the core hormones of calcium and phosphorus regulation. FGF23, FGF19, and FGF21 belong to the "endocrine FGFs" subgroup [16]. The excessive and deficient effects of FGF23 lead to hypophosphatemia and hyperphosphatemia [17]. FGF23 mainly has a role as a phosphorus-regulating factor in the kidney, parathyroid gland, and other tissues. On the one hand, it can act independently on proximal and distal renal tubule epithelial cells, downregulate the expression of Na/Pi in renal proximal convoluted tubules, reduce the reabsorption of phosphate, and thus reduce the concentration of blood phosphorus [18]. On the other hand, FGF23 also inhibits the expression of 1 α-hydroxylase and promotes the effect of 24 hydroxylase, inhibits the production of 1,25-dihydroxyvitamin D [1,25-(OH)2D], promotes its degradation, and affects intestinal phosphate transport [19]. FGF23 is a key factor in the development of XLH. Pathogenic mutations in *PHEX* lead to an increase in FGF23 levels [20]. Almost the same incremental FGF23 levels have been detected in bone cells of *PHEX* or *DMPl* gene knockout mice, while the clinical features also overlapped, including hypophosphatemia, abnormal vitamin D metabolism, rickets, and osteomalacia [21,22]. The same characteristics appear in the XLH rabbit model [23]. In addition, Burosumab, a human cloned antibody against FGF23, has also been approved for use in XLH patients in Europe, the United States, and other regions, and has been demonstrated to be safe and effective [24,25]. In the initial study, FGF23 was thought to be directly regulated by endopeptidase encoded by *PHEX*, but it has been confirmed that FGF23 is not the direct substrate of the PHEX protein [26]; however, it is undeniable that FGF23 is closely related to *PHEX*.

Secretory *PHEX* (sec*PHEX*) refers to the use of gene-directed mutagenesis to convert the SA domain of PHEX into a signal peptide with cleavage activity. It is mainly used to explore the substrate of the PHEX enzyme and its main advantage is that it is easy to purify from the used medium without the need for detergent. It has been reported that there are differences in the properties of enzymes encoded by sec*PHEX* and wild type *PHEX* [27]. In this study, the difference in function between the sec*PHEX* gene produced by SA domain mutation and the enzyme expressed by the wild-type sec*PHEX* gene was determined based on the pathogenic mutation of *PHEX*. To identify the function of the mutant gene at the molecular and cell levels, wild type *PHEX*, mutant *PHEX*, wild sec*PHEX*, and mutant sec*PHEX* were constructed in a lentivirus expression vector and the lentivirus was packaged and transferred into HEK293T cells. The expression of the *PHEX* gene and protein was detected in different transfer groups by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot, and the localization of the PHEX protein was detected in cells using immunofluorescence. The activity of the PHEX endopeptidase was determined using a peptide Abz-GFSDYK(Dnp)-OH

hydrolysis function experiment. At the same time, an *FGF23* expression vector was constructed and co-transfected into HEK293T cells withe four *PHEX* gene expression vectors. The expression of PHEX and FGF23 in different transfer groups was detected by western blot, and the differences in cell localization and expression of different constructs were detected by immunofluorescence.

Materials and Methods

Case presentation

In the author's previous clinical practice, one novel mutation was identified in the genetic analysis of two Han X-linked hypophosphatemic rickets (XLH) families, where the probands clinically manifested gait instability, square skull, costal margin valgus, tooth loss, bracelet, and late tooth emergence. Serum phosphorus was significantly decreased (0.7, 0.69 mmol/L), and 24 h urine phosphorus was significantly increased (68.4, 58.0 mmol/day, respectively). X-rays of the limbs and chest showed signs of partial rickets: the epiphysis was found to be sunken and cupped (Mouth-like), there were brush-like changes, thin bone cortex, osteoporosis, and bending deformity of the tibia. In the family survey, it was learned that there were two or three patients in each family, who were short, or had been diagnosed with rickets (Figure 1a) in infancy or childhood. The *PHEX* gene (NM_000444.5) was identified in Exon 4 (c.433G>T, p.Glu145*), as well as two heterozygous missense mutations in Exon 22 (c.2245T>C, p.Trp749Arg). In line investigation and genetic screening was found to be consistent with family genetic characteristics. Through clinical phenotypic-genetic linkage analysis, the data suggest these sites are two responsible mutations [22].

Construction and identification of plasmids

The plasmid synthesis scheme was as follows (Figure 1b, 1d) using the expression vector pBoBi-N-3*Flag. To synthesize the *PHEX* gene, the insert size was 2262 bp and the restriction site used the restriction enzymes XbaI-BsrGI (see Supplement 1 for the sequence). The following mutant plasmids were then constructed. Construct mutant plasmid 1: pBoBi-N-3*Flag-secPHEX, where secPHEX was derived from amino acids 109-120. For the deletion mutation of PHEX, the insert size was 2250 bp [27] (see Supplement 2) and the restriction site was XbaI-AgeI. For construction of mutant plasmid 2, the pBoBi-N-3*Flag-PHEX-mut145, the 433G>T(p.Glu145*) mutation of *PHEX* was introduced. For construction of mutant plasmid 3, the pBoBi-N-3*Flag-PHEX-mut749, the 2245T>C(p.Trp749Arg) mutation of *PHEX* was introduced. For construction of mutant plasmid 4, the pBoBi-N-3*Flag-secPHEX-mut145, using the pBoBi-N-3*Flag-secPHEX plasmid, the 421G>T mutation of sec*PHEX* was introduced. For construction of mutant plasmid 5, pBoBi-N-3*Flag-secPHEX-mut749, using the pBoBi-N-3*Flag-secPHEX plasmid, the 2233T>C mutation of sec*PHEX* was introduced. The target gene was amplified, digested with DpnI, and the digested product was transformed into *Escherichia coli* DH5a. Colonies were screened with antibiotics, monoclonal colonies were selected, and sequencing identification was conducted. The *PHEX*-WT, *PHEX*-mut, sec*PHEX*-WT, sec*PHEX*-mut gene cloning, and related PCR primer synthesis were performed

by General Biosystems (Anhui, China). *FGF23* gene synthesis and plasmid construction were performed by Shanghai Generay Biotech Co., Ltd (Shanghai, China). *FGF23* was 765 bp in length and was cloned into pBOBI-cs2-c-3HA using the cloning site BamHI/XhoI.

Cell culture, transfection, expression level, and functional identification

HEK293T cells were cultured in Dulbecco's modified Eagle medium containing high glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin. HEK293T cells were digested with trypsin and seeded into 24-well plates. When the cells reached 50–60% confluency, the single *PHEX* gene and the co-expression plasmid of *PHEX* and *FGF23* were used for transformation. The grouping was as follows: (1) *PHEX*-WT + pLKO-*GFP*, (2) *PHEX*-mut145 + pLKO-*GFP*, (3) sec*PHEX*-WT + pLKO-*GFP*, (4) sec*PHEX*-mut145 + pLKO-*GFP*, (5) *PHEX*-mut749 + pLKO-*GFP*, and (6) sec*PHEX*-mut749 + pLKO-*GFP*. The HEK293T cell line was provided by the Life Technology Co., Ltd. (Xiamen, China).

For the *PHEX* single gene cell transformation, 10 μg plasmid was mixed with 1000 μL basic medium, then 20 μL Turbofect was added to the culture medium and allowed to stand for 20 min. The mixture was dripped onto a 10 cm disk containing 70–80% cultured cells, the disk was shaken gently for mixing, and then cultured at 37 °C for 36 h.

For the *PHEX* and *FGF23* cell co-transfection, two separate solutions were prepared. Mixture 1 contained 25 μL OPTI-MEM + *PHEX* (WT/sec) and the *FGF23* plasmid (1 or 0.5 μg, 1:1), plus 1 μL P3000. Mixture 2 contained 25 μL OPTI-MEM + 1.5 μL Lipo3000. After mixing the prepared Mixtures 1 and 2, they were incubated at room temperature 22 °C for 5 min, added dropwise to the cell culture medium, which was gently shaken, and placed in a 37 °C cell incubator for 48 h. A sterilized cover glass was placed into a 6-well plate, ~ 500 cells/well were placed on the cover glass, and the plate was gently shaken and placed in a cell culture box at 37 °C for culture.

Detection of PHEX protein and FGF23 protein expression by western blot

HEK293T cell total protein was extracted by culture lysis and the concentration of the PHEX protein was detected. Protein samples and a protein marker were added to the 10% electrophoresis gel in the desired order. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, soaked in 5% skim milk for 1 h before the primary antibody solution was added (rabbit anti Flag, 1:1000 or mouse anti FGF23, 1:50) and incubated at 4 °C overnight. After washing the membrane three times, the secondary antibody (goat anti rabbit/mouse 1:5000) was added for 1 h. After washing an additional three times, the membrane was exposed.

Detection of PHEX mRNA expression by RT-PCR

Cellular RNA was extracted according to the instructions of the animal total RNA rapid extraction kit, and the first chain of cDNA was synthesized according to the instructions of 5× All-In-One RT Master Mix. The reaction included: 5× All-In-One RT Master Mix 4 μL, total RNA 2 μg, and nuclease-free H_2O to 20 μL. The reaction conditions were as follows: 25 ℃ for

10 min, 42 ℃ for 15 min, and 85 ℃ for 5 min. The Primer 3 software (version 0.4.0, San Francisco, USA) was used to design *PHEX*-, *eGFP*-, and *GAPDH*-specific primers, as follows: h*PHEX* (F: CAGGCATCACATTCACCAAC, R: GCCTCTGTTCATCGTGGAAT); *GFP* (F: ACGTAAACGGCCACAAGTTC, R: AAGTCGTGCTGCTTCATGTG); *GAPDH* (F: CAAGGTCATCCATGACAACTTTG, R: GTCCA CCACCCTGTTGCTGTAG). The qRT reaction system was 20 μL and included: template, 8 μL; 2 μmol ·L^{-1} upstream and downstream primers, 1 μL; and 2× SYBR Green PCR Master Mix, 10 μL. The qRT reaction conditions were: pre-denaturation (95 °C for 10 min), denaturation (95 °C for 15 s), annealing (60 °C for 20 s), and extension (72 °C for 25 s), 39 cycles. Each cell sample was duplicated using three techniques.

Cellular immunofluorescence

After the transfected HEK293T cells were fixed, permeabilized, and blocked, the primary antibody (Flag, 1:100; FGF23 1:200) was added and incubated overnight at 4 °C. The next day, cells were washed with phosphate-buffered saline (PBS) three times, and the fluorescent secondary antibody (1: 500, Flag488, FGF23-594) was added and incubated for 1 h at room temperature. The nucleus was stained with Hoechst (1:1000) and incubated at room temperature for 20 min in the dark. The cells were washed with PBS three times, incubated with 4′,6-diamidino-2-phenylindole at room temperature for 5 min, mounted with 50% glycerol, and photographed with a laser confocal microscope (LSM780, Carl Zeiss AG, Jena, Germany).

Determination of PHEX protein activity

The cultured HEK293T cells were collected and lysed in radioimmunoprecipitation assay buffer and the supernatant of the lysate was used for enzyme activity determination. For the enzyme activity assay (Varioskan LUX, Thermo Scientific, Waltham, US), the polypeptide Abz-GFSDYK (Dnp)-OH (10 μM) and 100 μg total protein were mixed and incubated at 37 °C for 30 min. The absorbance was measured in a microplate reader using an excitation wavelength of 420 nm and an emission wavelength of 320 nm.

RESULTS

Double enzyme digestion and sequencing verification of plasmid

After cloning the *PHEX*-WT, *PHEX*-mut, sec*PHEX*-WT, sec*PHEX*-mut and *FGF23* genes, the genes were packaged into a lentiviral vector, transformed into *Escherichia coli* cells, subjected to double enzyme digestion (Figure 1c, 1e, 1f), and sequencing identification (Figure 1g-1j). The *PHEX*-WT, *PHEX*-mut, sec*PHEX*-WT, and sec*PHEX*-mut genes were successfully cloned and by comparison with the known sequences in the National Center for Biotechnology Information (NCBI), the results were consistent. Thus, these plasmids can be used in subsequent experiments.

Immunofluorescence localization of PHEX, secPHEX, and FGF23 proteins in cells

The packaged *PHEX*-WT, *PHEX*-mut145, *PHEX*-mut749, sec*PHEX*-WT, sec*PHEX*-mut145, and sec*PHEX*-mut749 plasmids were separately transfected into HEK293T cells for 24 h. Cells were then incubated with Flag-PHEX, co-stained with the PHEX protein, secPHEX, and the cell structure was observed by fluorescence microscopy. Wild type PHEX and secPHEX proteins were mainly located in the cell membrane and cytoplsm, while the location of mutant PHEX and secPHEX proteins changed after p.Glu145* and p.Trp749Arg mutations, and were widely distributed. The expression of mutant PHEX and secPHEX proteins decreased significantly, especially in the p.145* mutant (Figure 2).

The packaged *PHEX*-WT, *PHEX*-mut145, *PHEX*-mut749, sec*PHEX*-WT, sec*PHEX*-mut145, sec*PHEX*-mut749 plasmids, and the constructed *FGF23* plasmid were co-transfected into HEK293T cells for 24 h. After being fixed and incubated with Flag-PHEX and FGF23 antibodies, the distribution of the PHEX and FGF23 proteins in HEK293T cells was observed by fluorescence microscopy. Wild type PHEX, secPHEX, and FGF23 proteins in the *PHEX*-WT and sec*PHEX*-WT groups were distributed in the cell membrane or endoplasmic reticulum, while the mutant PHEX and secPHEX proteins in the *PHEX*-mut145 and sec*PHEX*-mut145 groups were mostly co-localized with FGF23 in the nucleus, nuclear membrane (possibly endoplasmic reticulum) and cytoplasm (Figure 3).

Expression levels of PHEX and secPHEX mRNA

The expression of *PHEX*, sec*PHEX*, green fluorescence protein (*GFP*), and the internal reference glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, Figure 4a, 4b) was detected by qPCR. Under the condition that the *GAPDH* level of the internal reference was relatively consistent, the expression levels of *GFP* in the external reference were the same, indicating that the level of cell transfer among each group was the same. Using *GFP* as the external parameter, the expression of *PHEX* mRNA in cells was determined. The *PHEX* p.Glu145* nonsense mutation led to a decrease in intracellular *PHEX* mRNA expression ($p < 0.01$) and the sec*PHEX* p.Glu145* nonsense mutation caused a decrease in sec*PHEX* mRNA expression ($p < 0.01$). Regarding *PHEX* or sec*PHEX* p.Trp749Arg missense mutations, there was no significant difference in *PHEX* or sec*PHEX* mRNA expression in wild-type and mutant cells ($p > 0.05$).

PHEX endopeptidase activity

The results of the enzyme activity test showed that both the *PHEX* p.Glu145* nonsense mutation and p.Trp749Arg missense mutation led to a decrease in PHEX gene endopeptidase activity during hydrolysis (Figure 4c).

Differences in the expression of PHEX, secPHEX, and FGF23 proteins in HEK293T cells

The expression of PHEX, GFP, and GAPDH was detected by western blot after the *PHEX* and sec*PHEX* plasmids were transfected into HEK293T cells (Figure 4d). Under the condition that the GAPDH of the internal reference was relatively consistent among each group, the expression level of GFP in the external reference was the same, indicating that the level of cell transfer among each group was the same. Using GFP as the external parameter,

the expression of PHEX in cells was determined. The *PHEX* p.Glu145* nonsense mutation led to a decrease in intracellular PHEX protein expression. The sec*PHEX* p.Glu145* nonsense mutation led to a decrease in secPHEX protein expression, while the *PHEX* or sec*PHEX* p.Trp749Arg missense mutation resulted in a decrease in PHEX or secPHEX protein expression. In addition, no expression of FGF23 was detected in the four groups.

After *PHEX* and sec*PHEX* plasmids were co-transfected with *FGF23* plasmids into HEK293T cells, western blot analysis detected the expression of PHEX, FGF23, and the internal reference β-actin (Figure 4e, 4f). The expression of PHEX and secPHEX decreased and the band size was smaller after the p.Glu145* and p.Trp749Arg mutations in *PHEX* and sec*PHEX*, which promoted the expression of the FGF23 protein, especially the p.Glu145* mutation. It might be that the function of the PHEX protein was significantly decreased due to the truncation of the protein caused by the mutation of p.Glu145*.

DISCUSSION

Hereditary hypophosphatemic rickets is a group of metabolic osteopathies characterized by increased excretion of renal phosphate and decreased blood phosphorus[3]. According to the mode of inheritance, in X-linked hypophosphatemic rickets (XLH), the pathogenic gene is *PHEX*; the pathogenic gene of autosomal dominant inherited hypophosphatemic rickets (ADHR) is *FGF23*; autosomal recessive inherited hypophosphatemic rickets, is divided into three types of diseases: ARHR1, ARHR2, and ARHR3. The pathogenic genes are dentin matrix acidic phosphoprotein 1 (*DMP1*), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*), and family with sequence similarity 20,member C (*FAM20C*); in hereditary hypophosphatemic rickets with hypercalciuria (HHRH), the pathogenic gene is the solute carrier family 34 member 3 (*SLC34A3*); and in tumor-associated hypophosphatemic rickets (TIO) [28-32], approximately 80% are X-linked hypophosphatemic rickets. Until 2020-4-01, the ClinVar gene database (https://www.ncbi.nlm.nih.gov/clinvar) showed 529 types of pathogenic and 16 types of *PHEX* mutations with a likely pathogenic classification. Molecular sequences included Frameshift (106), Missense (42), Nonsense (91), Splice site (70), ncRNA (14), Near gene (0), untranslated region (UTR, 13), and other mutations (https://www.ncbi.nlm.nih.gov/clinvar). Frameshift mutations accounted for the majority of pathogenic or likely pathogenic mutations.

Nonsense mutations in most genes lead to the occurrence of disease by degrading mRNA via a nonsense-mediated mRNA decay (NMD) mechanism [33]. This mechanism degrades the newly synthesized mRNA and synthesizes the premature termination codon (PTC) mRNA, resulting in a pathogenic truncated protein with loss of protein function [4]. The newly discovered p.Glu145* nonsense mutation may cause the occurrence of XLH through an NMD mechanism, as nonsense mutations cause gene translation stops ahead of time, which may lead to defects in protein transport. If the mutation site is located in the zinc-binding region, it may affect the catalytic activity of the PHEX protein [4]. In addition, truncated proteins produced by some nonsense mutations may affect the splice mode of RNA

precursors (such as c.436+1 G>C), thereby affecting the conformation and function of proteins [34]. Some missense mutations (such as pC85r, 579 rpGpP 558 a) influence the PHEX protein spatial structure and cause the mutated PHEX protein to be isolated within the endoplasmic reticulum; thus, it cannot reach the plasma membrane and influence function and disease [1,35,36]. In the C-terminal area, extracellular missense mutations affect the formation of disulfide bonds, leading to secondary protein structural defects, and destroy function by inhibiting the enzyme activity of the PHEX protein [37]. In this study, there was no significant difference in mRNA expression after the p.Trp749Arg mutation, but the prin content decreased after the mutation. Immunofluorescence localization indicated that the localization of the mutated PHEX protein was different from that of the wild-type protein; p.Trp749Arg may cause disease by affecting the spatial structure of the PHEX protein, resulting in a decrease in the expression of PHEX, or by changing the location of the protein. p.Trp749Arg is the substitution of a large positively charged residue for a small residue, which increases the local charge of the *PHEX* gene product and may play a role in different spatial conformations [36]. Sako S et al. found a new mutation in Exon 22 of PHEX (Nm_000444.6): c.2202 del(p.Asn736Ilefs*4), which is located near the 3' UTR region encoding the C-terminal extracellular domain, which does not contain assumed zinc binding sites or active sites, but is generally considered a pathogenic mutation of XLH [2]. Li et al. found that a new nonsense mutation p.Trp403* variant does not cause an NMD mechanism, but significantly increases the expression levels of FGF23 mRNA in patients. p.Trp403* significantly reduces the phosphorylation of P38 mitogen activated protein kinase (MAPK) instead of extracellular signal regulated kinase (ERK)1/2, and the overexpression of FGF23 significantly reduces the phosphorylation of p38MAPK, while siRNA-induced *FGF23* mRNA knockdown significantly increases the phosphorylation of p38MAPK, strongly suggesting that the new signaling mechanisms of PHEX, FGF23, and p38MAPK may lead to XLH [38]. Combined with these studies and previous family genetic linkage analysis [22], these data strongly suggest that the p.Glu145* nonsense variant and p.Trp749Arg missense variant in *PHEX* cause XLH.

The kidney is one of the important target organs of phosphate homeostasis *in vivo*. In the process of phosphate processing, there are two important hormones with complex relationships: parathyroid hormone (PTH) and FGF23; FGF23 inhibits 1 α-hydroxylase, but promotes the expression of 24-hydroxylase, while PTH has the opposite role [39]. However, PTH and FGF23 also have some overlapping functions, such as inhibiting phosphate reabsorption of proximal renal tubules by targeting the stent protein Na+/H+ exchange to regulate the phosphorylation of cofactor -1 (NHERF-1) [40], and promoting calcium reabsorption of distal tubules through an increase of potential transient receptor potential of calcium channels in epithelial cells (vanilloid-5, TRPV5) expression and/or open frequency [41]. Although the signal mechanism is different, the proximal and distal target molecules of PTH and FGF23 are the same or there is a cross-feedback mechanism. At present, it has been confirmed that PTHrP107-139 is a direct substrate of PHEX [26,27]. If so, PHEX can regulate PTH and regulate phosphate metabolism, which may be directly related to FGF23 regulation

The active form of FGF23 is the full-length protein from the 25th to 251st amino acids (227 amino acids in total) after the transcription of *FGF23* and fragmentation from position 24.

Under physiological conditions, the hydrolysis of Arg176-R-R-Arg179 leads to the non-active form of FGF23. During secretion in HEK293 and COS-7 cells, FGF23 is processed by a subtilisin-like proprotein convertase (SPC) cleavage site (RXXR motif) at the C-terminal of amino acids 179-180, and the expression of FGF23 cannot be detected in cells [42]. The expression of FGF23 protein was not detected in HEK293T cells after the *PHEX* and sec*PHEX* overexpression vectors were separately transfected. Therefore, the *FGF23* overexpression vector, and the *PHEX* and sec*PHEX* overexpression vectors were selected for co-transfer into HEK293T cells. There was a negative regulation between the PHEX protein and *FGF23*. In addition, after the PHEX protein lost its transmembrane binding domain (secPHEX protein), the ability to regulate *FGF23* was further decreased.

FGF23 plays an important role in kidney and bone metabolism. Hypophosphatemia caused by FGF23 elevation is the main pathophysiological mechanism of FGF23 and XLH. The expression of FGF23 is mainly regulated by serum phosphate and ossified triol. The increase in serum FGF23 induced by phosphate mainly occurs in bone, which may be related to nicotinamide adenine dinucleotide phosphate (NADPH)-induced reactive oxygen species (ROS) and the mitogen-activated protein kinase kinase (MEK)-ERK pathway [43]. The molecular mechanisms related to FGF23 expression are *FAM20C*, *ENPP1*, *DMP1*, and acidic serine- and aspartate-rich matrix extracellular phosphoglycoprotein (MEPE)-associated motif (ASARM) peptides [44,45]. FGF23 signals are mainly coordinated by competitive substitution of *DMP1* and ASARM motifs and the interaction between free ASARM peptides and PHEX. The binding of PHEX to *DMP1* through the *DMP1* ASARM motif leads to a decrease in active FGF23 [46]. The mutant PHEX may not be able to bind to the above molecules because of the change in spatial structure, thus affecting the degradation of FGF23 [46]. In a TIO study, HIF-1α and FGF23 are co-localized in fusiform cells adjacent to blood vessels [47]. HIF-1α may be a direct transcriptional activating factor of *FGF23*, affecting the expression of *FGF23* [47]. The active product of FGF23 can be hydrolyzed by SPC, while SPC can be hydrolyzed by o-glycosylation at the hydrolysis site of the N-acetylgalactosaminyltransferase 3 (GalNAcT3) protein, encoded by *GALNT3*. Therefore, a *GALNT3* mutation leads to hyperphosphatemia and extraosseous calcification [48]. At the same time, iron deficiency and inflammation also affects the expression and fragmentation of FGF23 [49].

The mechanism underlying the increase in FGF23 levels caused by *PHEX* mutation in XLH patients is not clear. One of the mechanisms by which full-length PHEX regulates serum FGF23 may be through the direct lysis of proprotein convertase subtilisin/kexin type 9 (PC2), and PC2 can be upregulated by PHEX [46]. PC2 promotes the formation of the PHEX-DMP1-integrin complex, which inhibits FGF23 [50] when PC2 and its chaperone, neuroendocrine polypeptide 7B2 (7B2 PC2), is activated. However, there is no possibility of direct interaction between 7B2 PC2 and FGF23, so it is not clear whether PHEX directly regulates FGF23 [51]. In recent years, the FGF23-Klotho signal axis has become the focus of XLH research. *KLOTHO* was originally found to be a gene regulatingsenescence, encoding a type I single-pass transmembrane protein, which exists in the form of a full-length membrane (mKlotho) protein and soluble cyclic (sKlotho) protein. At present, the expression

of *KLOTHO* has been detected in proximal convoluted tubules, distal convoluted tubules, bone cells, parathyroid glands, and epithelial cells of the cerebral choroid plexuses [52]. Klotho can be used as a synergistic receptor to enhance the affinity and specificity of FGF to FGFRs, thus activating FGF23-mediated receptors [52]. The close relationship between Klotho and FGF23 has been verified in *KLOTHO* deficient (*KLOTHO* -/-) mice. Even when *FGF23* was overexpressed in *KLOTHO* (-/-) mice, its expression was very similar to that of *FGF23* deficient (*FGF23* -/-) mice [53]. The receptors of FGF23, including FGF receptors (FGFR) 1, FGFR2, FGFR3, and FGFR4, all of which are receptor tyrosine kinases, initiate an intracellular phosphate cascade after ligand-induced dimerization, while FGFR activates a variety of signaling pathways, including the phosphorylation of mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase phospholipase CC [54]. The MAPK inhibitor PD0325901 has been shown to increase the levels of phosphate and 1, 25 (OH) D in Hyp mice and long-term application enhances bone mineralization and bone mineral density [55]. FGF23 mainly plays a role through MAPK as the downstream signaling pathway of FGF receptor/klotho complex binding. In renal proximal convoluted tubules, FGF23 induces phosphorylation of NHERF-1 through the α-Klotho/FGFR1c receptor signal cascade, ERK1/2, and serum/glucocorticoid regulated kinase 1 (SGK1), which leads to the internalization and degradation of sodium phosphate cotransporter (NaPi-2a) and (NaPi-2c) [40], thus inhibiting the increase of phosphate reabsorption and excretion in proximal tubular cells. In the distal tubule (DCT), FGF23 increases the apical membrane abundance of vanillod-5 and sodium-sodium cotransporter (NCC) through the ERK1/2, SGK1, and no - lysine kinase-4 (WNK4) signaling cascade, and increases the reabsorption of calcium and sodium [41]. Therefore, FGF23 has physiological significance in the preservation of calcium and sodium in distal renal tubules.

In addition to these effects on phosphate and calcium metabolism, abnormal FGF23 also has a "miss" effect, affecting cardiovascular, immune, and central nervous system functions. The levels of FGF23 increased with aggravation of renal function damage [56]. FGF23 levels are significantly correlated with left ventricular hypertrophy and mortality in patients with chronic renal failure. In patients with renal failure, a high level of FGF23 is an independent risk factor for cardiovascular disease, but high circulation levels of FGF23 in the hypophosphatemic environment does not induce cardiac hypertrophy [56,57]. Hepatocytes of two infants with congenital biliary atresia have ectopic overexpression of FGF23 protein [4], which is also worthy of attention.

PHEX-WT and secPHEX-WT and FGF23 are co-localized on the cell membrane or endoplasmic reticulum, while most are co-localized with FGF23 on the nuclear membrane and cytoplasm after mutation. One possible mechanism is that the mutation makes the PHEX and FGF23 proteins obtain the function of nuclear entry, or the mutation leads to the loss of the localization ability of PHEX and FGF23 proteins or the loss of selectivity of the nuclear membrane to the two proteins. In summary, through clinical characterization and a series of functional studies of two XLH families, it is preliminarily confirmed that p.Glu145* and p.Trp749Arg are pathogenic mutations. It is speculated that these mutations may be related to the localization ability of the PHEX and FGF23 proteins. These data provide some reference for the molecular genetic pathogenesis of XLH.

DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 81874379), Fujian Province Medical Innovation Foundation (No. 2019-CXB-3, 2018-CXR-2), and the Special Research Foundation of Fujian Provincial Department of Finance (No. 2018-643#), China.

ACKNOWLEDGMENTS

We thank General Biosystems (Anhui, China) and Shanghai Generay Biotech Co., Ltd. (Shanghai, China) for the gene synthesis technology and plasmid construction. We are very grateful to the Life Technology Co., Ltd. (Xiamen, China) for assisting in the completion of transfection and immunofluorescence experiments.

ORCID

Jiewei Luo http://orcid.org/0000–0003–4271–4848

Faqiang Tang http://orcid.org/0000–0002–0625–8470

REFERENCES

1. Sabbagh Y, Boileau G, Campos M, Carmona AK, Tenenhouse HS. Structure and function of disease-causing missense mutations in the PHEX gene. J Clin Endocrinol Metab. 2003;88(5):2213-2222.
2. Sako S, Niida Y, Shima KR, Takeshita Y, Ishii KA, Takamura T. A novel PHEX mutation associated with vitamin D-resistant rickets. *Hum Genome Var*. 2019;6:9. Web of Science
3. Fuente R, Gil-Pena H, Claramunt-Taberner D, et al. X-linked hypophosphatemia and growth. *Rev Endocr Metab Disord*. 2017;18(1):107-115.
4. Gao Y, Wang ZM, Li XL. Analysis of 2 novel mutations of PHEX gene inducing X-linked dominant hypophosphatemia rickets in 2 families: Two case reports. *Medicine (Baltimore)*. 2018;97(31):e11453. Web of Science
5. Beck-Nielsen SS, Mughal Z, Haffner D, et al. FGF23 and its role in X-linked hypophosphatemia-related morbidity. *Orphanet J Rare Dis*. 2019;14(1):58.
6. Vega RA, Opalak C, Harshbarger RJ, et al. Hypophosphatemic rickets and craniosynostosis: a multicenter case series. *J Neurosurg Pediatr*. 2016;17(6):694-700.
7. A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium. *Nat Genet*. 1995;11(2):130-136. Web of Science
8. Turner AJ, Isaac RE, Coates D. The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays*. 2001;23(3):261-269.
9. Li SS, Gu JM, Yu WJ, He JW, Fu WZ, Zhang ZL. Seven novel and six de novo PHEX gene mutations in patients with hypophosphatemic rickets. *Int J Mol Med*. 2016;38(6):1703-1714.

10. Ruppe MD, Brosnan PG, Au KS, Tran PX, Dominguez BW, Northrup H. Mutational analysis of PHEX, FGF23 and DMP1 in a cohort of patients with hypophosphatemic rickets. *Clin Endocrinol (Oxf).* 2011;74(3):312-318.
11. Wickner WT, Lodish HF. Multiple mechanisms of protein insertion into and across membranes. *Science.* 1985;230(4724):400-407. Web of Science
12. Murali SK, Andrukhova O, Clinkenbeard EL, White KE, Erben RG. Excessive Osteocytic Fgf23 Secretion Contributes to Pyrophosphate Accumulation and Mineralization Defect in Hyp Mice. *PLoS Biol.* 2016;14(4):e1002427.
13. Goljanek-Whysall K, Tridimas A, McCormick R, et al. Identification of a novel loss-of-function PHEX mutation, Ala720Ser, in a sporadic case of adult-onset hypophosphatemic osteomalacia. *Bone.* 2018;106:30-34.
14. Forero-Delgadillo JM, Cleves D, Ochoa V, et al. PHEX Gene Mutation in a Patient with X-Linked Hypophosphatemic Rickets in a Developing Country. *Appl Clin Genet.* 2020;13:57-62.
15. Yuan B, Takaiwa M, Clemens TL, et al. Aberrant Phex function in osteoblasts and osteocytes alone underlies murine X-linked hypophosphatemia. *J Clin Invest.* 2008;118(2):722-734.
16. Fukumoto S, Yamashita T. FGF23 is a hormone-regulating phosphate metabolism--unique biological characteristics of FGF23. *Bone.* 2007;40(5):1190-1195. Web of Science
17. Fukumoto S. FGF23-FGF Receptor/Klotho Pathway as a New Drug Target for Disorders of Bone and Mineral Metabolism. *Calcif Tissue Int.* 2016;98(4):334-340. Web of Science
18. Larsson T, Marsell R, Schipani E, et al. Transgenic mice expressing fibroblast growth factor 23 under the control of the alpha1(I) collagen promoter exhibit growth retardation, osteomalacia, and disturbed phosphate homeostasis. *Endocrinology.* 2004;145(7):3087-3094.
19. Shimada T, Kakitani M, Yamazaki Y, et al. Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest.* 2004;113(4):561-568. Web of Science
20. Jonsson KB, Zahradnik R, Larsson T, et al. Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. *N Engl J Med.* 2003;348(17):1656-1663.
21. Liu S, Zhou J, Tang W, Menard R, Feng JQ, Quarles LD. Pathogenic role of Fgf23 in Dmp1-null mice. *Am J Physiol Endocrinol Metab.* 2008;295(2):E254-261.
22. Lin X, Zhu Y, Luo J, Huang J. Genetic analysis of three families with X-linked dominant hypophosphatemic rickets. *J Pediatr Endocrinol Metab.* 2018;31(7):789-797.
23. Sui T, Yuan L, Liu H, et al. CRISPR/Cas9-mediated mutation of PHEX in rabbit recapitulates human X-linked hypophosphatemia (XLH). *Hum Mol Genet.* 2016;25(13):2661-2671.
24. Whyte MP, Carpenter TO, Gottesman GS, et al. Efficacy and safety of burosumab in children aged 1-4 years with X-linked hypophosphataemia: a multicentre, open-label, phase 2 trial. *Lancet Diabetes Endocrinol.* 2019;7(3):189-199.
25. Lambert AS, Zhukouskaya V, Rothenbuhler A, Linglart A. X-linked hypophosphatemia: Management and treatment prospects. *Joint Bone Spine.* 2019;86(6):731-738.
26. Benet-Pages A, Lorenz-Depiereux B, Zischka H, White KE, Econs MJ, Strom TM. FGF23 is processed by proprotein convertases but not by PHEX. *Bone.* 2004;35(2):455-462. Web of Science
27. Boileau G, Tenenhouse HS, Desgroseillers L, Crine P. Characterization of PHEX endopeptidase catalytic activity: identification of parathyroid-hormone-related peptide107-139 as a substrate and osteocalcin, PPi and phosphate as inhibitors. *Biochem J.* 2001;355(Pt 3):707-713.

28. Goldsweig BK, Carpenter TO. Hypophosphatemic rickets: lessons from disrupted FGF23 control of phosphorus homeostasis. *Current osteoporosis reports.* 2015;13(2):88-97.
29. Ichikawa S, Gerard-O'Riley RL, Acton D, et al. A Mutation in the Dmp1 Gene Alters Phosphate Responsiveness in Mice. *Endocrinology.* 2017;158(3):470-476.
30. Steichen-Gersdorf E, Lorenz-Depiereux B, Strom TM, Shaw NJ. Early onset hearing loss in autosomal recessive hypophosphatemic rickets caused by loss of function mutation in ENPP1. *J Pediatr Endocrinol Metab.* 2015;28(7-8):967-970.
31. Hasani-Ranjbar S, Amoli MM, Ebrahim-Habibi A, et al. SLC34A3 intronic deletion in a new kindred with hereditary hypophosphatemic rickets with hypercalciuria. *J Clin Res Pediatr Endocrinol.* 2012;4(2):89-93.
32. Wang X, Wang S, Li C, et al. Inactivation of a novel FGF23 regulator, FAM20C, leads to hypophosphatemic rickets in mice. *PLoS Genet.* 2012;8(5):e1002708.
33. Kurosaki T, Maquat LE. Nonsense-mediated mRNA decay in humans at a glance. *J Cell Sci.* 2016;129(3):461-467.
34. Ichikawa S, Traxler EA, Estwick SA, et al. Mutational survey of the PHEX gene in patients with X-linked hypophosphatemic rickets. *Bone.* 2008;43(4):663-666. Web of Science
35. Xia W, Meng X, Jiang Y, et al. Three novel mutations of the PHEX gene in three Chinese families with X-linked dominant hypophosphatemic rickets. *Calcif Tissue Int.* 2007;81(6):415-420.
36. Sabbagh Y, Boileau G, DesGroseillers L, Tenenhouse HS. Disease-causing missense mutations in the PHEX gene interfere with membrane targeting of the recombinant protein. *Hum Mol Genet.* 2001;10(15):1539-1546.
37. Huang Y, Mei L, Pan Q, et al. Novel de novo nonsense mutation of the PHEX gene (p.Lys50Ter) in a Chinese patient with hypophosphatemic rickets. *Gene.* 2015;565(1):150-154.
38. Li W, Tan L, Li X, et al. Identification of a p.Trp403* nonsense variant in PHEX causing X-linked hypophosphatemia by inhibiting p38 MAPK signaling. *Hum Mutat.* 2019;40(7):879-885.
39. Yu X, White KE. FGF23 and disorders of phosphate homeostasis. *Cytokine Growth Factor Rev.* 2005;16(2):221-232. Web of Science
40. Andrukhova O, Zeitz U, Goetz R, Mohammadi M, Lanske B, Erben RG. FGF23 acts directly on renal proximal tubules to induce phosphaturia through activation of the ERK1/2-SGK1 signaling pathway. *Bone.* 2012;51(3):621-628.
41. Andrukhova O, Smorodchenko A, Egerbacher M, et al. FGF23 promotes renal calcium reabsorption through the TRPV5 channel. *EMBO J.* 2014;33(3):229-246.
42. White KE, Carn G, Lorenz-Depiereux B, Benet-Pages A, Strom TM, Econs MJ. Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. *Kidney Int.* 2001;60(6):2079-2086. Web of Science
43. Scanni R, vonRotz M, Jehle S, Hulter HN, Krapf R. The human response to acute enteral and parenteral phosphate loads. *J Am Soc Nephrol.* 2014;25(12):2730-2739.
44. Liu S, Rowe PS, Vierthaler L, Zhou J, Quarles LD. Phosphorylated acidic serine-aspartate-rich MEPE-associated motif peptide from matrix extracellular phosphoglycoprotein inhibits phosphate regulating gene with homologies to endopeptidases on the X-chromosome enzyme activity. *J Endocrinol.* 2007;192(1):261-267.
45. Beck-Nielsen SS, Brixen K, Gram J, Brusgaard K. Mutational analysis of PHEX, FGF23, DMP1, SLC34A3 and CLCN5 in patients with hypophosphatemic rickets. *J Hum Genet.* 2012;57(7):453-458.

46. Rowe PS. Regulation of bone-renal mineral and energy metabolism: the PHEX, FGF23, DMP1, MEPE ASARM pathway. *Crit Rev Eukaryot Gene Expr.* 2012;22(1):61-86.
47. Zhang Q, Doucet M, Tomlinson RE, et al. The hypoxia-inducible factor-1alpha activates ectopic production of fibroblast growth factor 23 in tumor-induced osteomalacia. *Bone Res.* 2016;4:16011. Web of Science
48. Kato K, Jeanneau C, Tarp MA, et al. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. *J Biol Chem.* 2006;281(27):18370-18377.
49. Francis C, David V. Inflammation regulates fibroblast growth factor 23 production. *Curr Opin Nephrol Hypertens.* 2016;25(4):325-332.
50. Yuan B, Feng JQ, Bowman S, et al. Hexa-D-arginine treatment increases 7B2*PC2 activity in hyp-mouse osteoblasts and rescues the HYP phenotype. *J Bone Miner Res.* 2013;28(1):56-72.
51. Yamamoto H, Ramos-Molina B, Lick AN, et al. Posttranslational processing of FGF23 in osteocytes during the osteoblast to osteocyte transition. *Bone.* 2016;84:120-130.
52. Kurosu H, Kuro OM. The Klotho gene family as a regulator of endocrine fibroblast growth factors. *Mol Cell Endocrinol.* 2009;299(1):72-78. Web of Science
53. Razzaque MS, Sitara D, Taguchi T, St-Arnaud R, Lanske B. Premature aging-like phenotype in fibroblast growth factor 23 null mice is a vitamin D-mediated process. *FASEB J.* 2006;20(6):720-722.
54. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* 2005;16(2):139-149. Web of Science
55. Ranch D, Zhang MY, Portale AA, Perwad F. Fibroblast growth factor 23 regulates renal 1,25-dihydroxyvitamin D and phosphate metabolism via the MAP kinase signaling pathway in Hyp mice. *J Bone Miner Res.* 2011;26(8):1883-1890.
56. Minamizaki T, Konishi Y, Sakurai K, et al. Soluble Klotho causes hypomineralization in Klotho-deficient mice. *J Endocrinol.* 2018;237(3):285-300.
57. Liu ES, Thoonen R, Petit E, et al. Increased Circulating FGF23 Does Not Lead to Cardiac Hypertrophy in the Male Hyp Mouse Model of XLH. *Endocrinology.* 2018;159(5):2165-2172.

Figure Legends

Figure 1 a, X-linked dominant hypophosphatemic rickets (XLH) pedigree diagram. b, pBOBI-cs2-c-3HA-*FGF23* plasmid vector schematic diagram. c, *FGF23* cloned plasmid identified by double enzyme digestion. Lane 1 is plasmid digested with BamHI/XhoI; Lane 2 is DNA marker. d, pBoBi-N-3*Flag-*PHEX* plasmid vector schematic diagram. e, *PHEX* cloned plasmid identified by double enzyme digestion. Lane 1 is plasmid; Lane 2 is plasmid digested with XbaI-BsrGI; and Lane M is DNA marker. f, sec*PHEX* cloned plasmid identified by double enzyme digestion. Lane 1 is plasmid, Lane 2 is plasmid digested with XbaI-AgeI, Lane M is DNA marker. g-j, *PHEX* cloned gene sequencing map; g, c.433T (p.145*), 145 mut type; h, c.433G (p.145Glu), 145 WT type; i, c.2245C(p.749Arg), 749 mut type; and j, c.2245T (p.749Trp), 749WT type.

Figure 2 The localization of wild type and mutant PHEX and secPHEX, as detected by immunofluorescence in HEK293T cells single-transfected with *PHEX and secPHEX. Wild type PHEX and secPHEX proteins are mainly located in the cell membrane and cytoplasm, while the localization of p.Glu145* and p.Trp749Arg mutated PHEX and secPHEX proteins in the cells changes such that they are widely distributed. At*

the same time, the expresion of mutant PHEX and secPHEX proteins decreases significantly, especially the p.145 mutants.*

Figure 3 The localization of wild type and mutant PHEX, secPHEX, and FGF23, as detected by immunofluorescence in HEK293T cells co-transfected with *PHEX*, sec*PHEX*, and *FGF23*. Wild type PHEX, secPHEX, and FGF23 proteins are distributed in the cell membrane or endoplasmic reticulum, while the mutant is located in the nuclear membrane (possibly endoplasmic reticulum) and cytoplasm. WT, wild type; mut, mutation type; sec, secretory type.

Figure 4 a-b, The expression of *PHEX*, sec*PHEX* and green fluorescence protein (*GFP*) in transfected HEK293T cells, as detected by quantitative polymerase chain reaction (qPCR). Under the condition that the internal reference glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) is relatively consistent among each group, the expression levels of *GFP* in the external reference are the same. The expression of mRNA in cells decreases by *PHEX* and sec*PHEX* p.Glu145* nonsense mutations (*, $p < 0.01$); there is no difference in the expression of mRNA in p.Trp749Arg. c, p.Glu145* and p.Trp749Arg mutants all lead to the decrease in intracellular PHEX endopeptidase activity (***, $p < 0.001$; ****, $p < 0.0001$). d, Western blot (WB) detected that the PHEX and secPHEX proteins mutated by p.Glu145* and p.Trp749Arg are obviously decreased in single transfected *PHEX* and sec*PHEX* cells. e-f, the expression of PHEX and secPHEX mutant proteins is decreased by WB, while the expression of FGF23 protein increases in *PHEX*, sec*PHEX* and *FGF23* co-transfected cells. β-actin is the internaleference. WT, wild type; mut, mutation type; sec, secretory type; GFP, external reference; GAPDH and β-actin, internal reference.