ORIGINAL ARTICLE

A *de novo* mosaic mutation of *PHEX* in a boy with hypophosphatemic rickets

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X-linked dominant hypophosphatemic rickets (XLHR), is characterized mainly by renal phosphate wasting with hypophosphatemia, short stature and abnormal bone mineralization. *PHEX*, located at Xp22.1–p22.2, is the gene causing XLHR. We aim to characterize the pathogenesis of a Chinese boy who is apparently ‘heterozygous’ in *PHEX* gene. Direct sequencing showed two peaks: one was a wild-type ‘G’ and the other was one base substitution to ‘A’, though the patient was a male. TA clone assay clearly showed each sequences and the ratios. The mutation effect was predicted via bioinformatics and validated by exon-trapping assay. Real-time PCR was applied to determine the copy number of *PHEX*. TA clone assay showed the frequency of normal (G) to mutant allele (A) as 19:13. Normal karyotype and real-time PCR results indicate the normal copy number of *PHEX*. This splice site mutation leads to 4 bp of exon 18 skipping out causing frame shift p.Gly590Glufs*28 that ends up with a loss of active site and Zn²⁺-binding site of *PHEX*, which probably interfere with renal phosphate reabsorption and bone mineralization. In conclusion, mutation at conserved splice acceptor site resulted in aberrant splicing, ending up with a damaged protein product. This novel mutation is *de novo* in mosaic pattern that may be induced during early postzygotic period. Taking mosaic somatic mutation of *PHEX* into consideration is strongly suggested in genetic counseling and etiology research for XLHR. *Journal of Human Genetics* (2016) 61, 223–227; doi:10.1038/jhg.2015.133; published online 12 November 2015

INTRODUCTION

X-linked hypophosphatemic rickets or X-linked vitamin D-resistant rickets (XLHR, MIM#307800) is a disorder associated with low levels of phosphate in the blood (hypophosphatemia) caused by a decreased renal tubular reabsorption of inorganic phosphates. Additionally, the normal physiological response of an elevation of 1,25(OH)₂ vitamin D toward hypophosphatemia is absent. The incidence of XLHR is approximately 3.9–5/100 000 live birth.¹ Clinical manifestations include short stature, bone pain, dental anomalies, lower extremity deformities and radiographic evidence of rickets in children.^{2,3} XLHR is caused by mutations in *PHEX* gene that is located at Xp22.1–p22.2. *PHEX* encodes proteins that has an important role in regulation of phosphate transport and bone mineralization.⁴ *PHEX* protein shows high homology to membrane-bound zinc metalloproteases with the metal-binding site and active site close to C termination.⁵

In this study, we report an unusual case in that a Chinese boy with XLHR was identified with an apparently ‘heterozygous’ *de novo* splice site mutation in *PHEX* gene. We examined the mutant frequency and gene copy number and analyzed the mutation effects, which implies that the patient is mosaic with normal and mutated *PHEX* gene.

MATERIALS AND METHODS

Patient

The proband is a 4.5-year-old boy, presenting gait abnormalities and bone pain for 2 years. He does not have any siblings. No relevant family history was recorded. His parents showed normal appearance with no positive symptoms. They claimed that karyotyping was performed for the proband with a normal 46, XY reported.

We performed physical examination, X-ray and ultrasonic examinations. We measured biochemical parameters and bone mineral density. Informed consent was obtained and the study was approved by the ethics committee of the School of Medicine, Zhejiang University.

Molecular diagnosis

The proband’s DNA was extracted from peripheral white blood cells by RelaxGene Blood DNA system (TianGen, Beijing, China). We designed 22 primers (Table 1) that cover all exons and their exon–intron boundaries of *PHEX* based on the reference sequence (NC_000023.11) using standard PCR followed by Sanger sequencing.

TA cloning and real-time PCR (quantitative PCR (qPCR))

Using the pMD19-T Vector Cloning Kit (Takara, Shiga, Japan) we cloned PCR products of *PHEX*-exon18. After transformation and overnight culture, 32 colonies were selected randomly and directly sequenced. Quantization of the gene copies of *PHEX* of the proband was completed by qPCR assay with ABI

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Received 10 July 2015; revised 13 September 2015; accepted 9 October 2015; published online 12 November 2015

Table 1 Primer for PCR amplification of *PHEX* gene

Primer name	Primer sequence (F), 5' → 3'	Primer sequence (R), 5' → 3'	Product size (bp)
PHEX-exon1	gggggaaagccaaggcaacca	aacgcaggcaaacagccct	452
PHEX-exon2	tgcgtatgtttccgagggtgt	ggcaggcaagtcagaccaca	529
PHEX-exon3	aggcttggaactggttgatagga	agtgagaagtcagcttcaatccc	358
PHEX-exon4	acttgccaccatgtgggtgga	gccagacttgccctggcttct	462
PHEX-exon5	ccctgtcagctctcctggca	cccaggatggcaccacca	537
PHEX-exon6	aagcaggggcaggtctctga	gggagtgctgtctccaagc	520
PHEX-exon7	tgcctgtattgcataatccctgc	ttggccacagctgagcaacg	548
PHEX-exon8	ttggggaccaccaaaagcct	atgataaccaagactgagagcaggt	327
PHEX-exon9	ggcttgatggcaatgatcaggagt	caccagacagtgcttttgccagt	487
PHEX-exon10	actctgagggtcggagctgga	agctgggctacaaactcccct	467
PHEX-exon11	tgggttaggtgtgctagttttgt	tggaggctgacattagcctgttg	330
PHEX-exon12	tgccagagcatggagtcaagctg	ggcagcaagccatgtgcctc	528
PHEX-exon13	tgccttcacagtggcttgc	accactcagctacgcatcgtt	440
PHEX-exon14	gctgagagaggtgtgactgatgc	actggcaagccagctactctga	337
PHEX-exon15	tccatttcaggagacagccctt	accctgccacttttggggga	501
PHEX-exon16	ccagtgcaggaggagtgcc	gagtacaggcgaccacgccc	311
PHEX-exon17	ttcagagagtagggcactaaggttc	cccgatcaccaatgggaacaa	525
PHEX-exon18	aggctgtcaggtgaggga	tcaggaaaaagtcatgcctggt	432
PHEX-exon19	tccagagcaccttgcaggat	agggtcaatggggagacacact	413
PHEX-exon20	tgcattttgcacgtggcaggagt	accacagggtcctaacca	453
PHEX-exon21	acactgtctgattcctgggcac	tggatggctgggtggggag	438
PHEX-exon22	gggcatgtgcttggtcattcagga	agatccaagcaggtcacctacca	458
PHEX-qPCR	tgcaggagctgatctcagga	tgagtacaactgtggctaaggg	290
PHEX-et_18 ^a	gtcagaattccactggggctattcctatgg	gtcactcgagatgagcagtggtctcacttg	943
SD6 and SA2 ^b	ttgtctaccttgaccaccaga	cccctgaacctgaacataaaat	^c

Abbreviation: qPCR, quantitative PCR.

^aPrimer for exon trapping that covers restriction enzyme cutting site.^bPrimers of pSPL3.^cProduct size varies.

7900HT (ABI, Los Angeles, CA, USA) Real-time PCR System using the primer PHEX-qPCR (Table 1). Two normal males and two females were taken into the assay as controls. β -Globin gene was tested as internal reference gene. SYBR Green was applied as nucleic acid stain (Bestar qPCR mastermix, DBI, Shanghai, China).

Computational analysis

After genotyping, the impact of this splicing mutation was evaluated by bioinformatic tools online: Human-Splicing-Finder (<http://www.umd.be/HSF/>), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html), and NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html).

Exon-trapping assay

To examine the predicted results, a mini-gene assay was carried out. We designed a pair of primers (Table 1, PHEX-et_18) covering exon 18 and exon-intron boundaries (943 bp). PCR products and pSPL3 vector (Invitrogen Corporation, Carlsbad, CA, USA) were double digested by EcoRI and XhoI followed by a ligation reaction. After transformation into competent cell DH5 α , plasmid DNA was prepared by the NucleoBond Xtra Midi Kit (MN, Neumann-Neander-Straße, Germany) for transfection. African green monkey cell line COS7 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO₂ at 37 °C. We transfected COS7 with empty pSPL3 vector as well as normal/mutant recombinant plasmids using PolyJet reagent (SignaGen Laboratories, Gaithersburg, MD, USA), respectively. After 24 h, cells were harvested and total RNA was extracted. Then reverse transcriptase-PCR was performed using primers SD6 and SA2 (Table 1) followed by agarose gel electrophoresis and sequencing.

Table 2 Biochemical parameters of the proband at 4.5 years

Blood profile	Value	Reference range
Serum alkaline phosphatase	616 U l ⁻¹	<383 U l ⁻¹
Serum calcium	2.40 mol l ⁻¹	2.25–3.75 mol l ⁻¹
Serum phosphate	0.65 mol l ⁻¹	1.0–1.95 mol l ⁻¹
24-h urine calcium	0.7 mmol per 24 h	2.5–7.5 mmol per 24 h
24-h urine phosphate	11.7 mmol per 24 h	0–32.3 mmol per 24 h
Bone mineral density	Z = -3.96	Z = -1 to +1

RESULTS

The patient was 93 cm (below 0.4th percentile), weighing 14 kg (2nd percentile), with a stature shorter than peers. The shape of his head became box-like (caput quadratum) owing to thickening of the parietal and frontal bones. The patient had a typical hypophosphatemia and elevated serum alkaline phosphatase for his age (Table 2). In addition, his chest and extremities X-ray indicated an enlargement of head of rib, decreased bone density and rough metaphysis of both bilateral scapula and upper segment of humerus. Bilateral left radius, distal end of femur, ulna, tibia and fibula had cup-shaped change, rough margin of metaphysis, uneven bone density and sparse bony trabeculae. Bilateral distal femoral ossification centers had uneven density and rough margin; bilateral femora slightly curved outward and medial tibial cortex thickened. Anteroposterior pelvis X-ray showed coarse bone trabecula, increased bone density and slightly wider space between bone trabecula that arrayed in a grid-like arrangement. The upper outer margins of both femoral heads were

vague, while the central part had focal reduced bone density and patchy increased bone density. These physical examination and radiographic findings implied an active rickets. Biochemical testing showed a normal serum calcium level and typical hypophosphatemia. Therefore, this case was highly suspected to be a category of hereditary hypophosphatemic rickets.

We first focused on *PHEX* gene as XLHR is the most common form of hereditary hypophosphatemic rickets. Through direct sequencing, a novel splice site mutation was found at c.1769-1 (IVS 17) on the *PHEX* gene of the proband, changing from G to A (Figure 1b), which has not been documented by 1000 Genomes Projects yet. This result indicated that the patient is likely affected with XLHR. Interestingly, rather than a single peak at the mutation site, the sequencing result showed two peaks (Figure 1b). This is not common considering that *PHEX* is located on X chromosome of the proband who is a male. In order to confirm the result and distinguish the different alleles, we carried out a TA clone assay and picked out 32 colonies. It is shown that both c.1769-1G and c.1769-1A indeed exist (Figures 1c and d) and the frequency of G to A is 19:13. Karyotype is claimed normal 46, XY (data not shown). qPCR was applied to further confirm this karyotype result and investigate the copy number of *PHEX* gene. The average $2^{-\Delta\Delta Ct}$ values of proband/control male and proband/control female are 1.002 and 0.541, respectively, which indicates that the patient has the same amount of gene copies of *PHEX* as normal males do (Figure 1e).

In order to evaluate the impact of this mutation, Human Splicing Finder was applied to analyze the adjacent DNA sequence.⁶ It suggested that the native splicing acceptor (c.1769-2_c.1769-1) is destroyed, and there are two loci (c.1769-1_c.1769 and c.1771_c.1772), conforming to the criteria of a typical alternative splicing site, which would probably be adopted instead (Table 3).

The results of exon trapping, a cell-based assay, demonstrated that c.1771_c.1772 is actually the cryptic site. The first 4 bp (GTAG) of exon 18 were skipped out abnormally in the mutational group (Figure 2). A deletion of 4 bp in the transcripts will lead to a frame shift during translation (p.Gly590Glufs*28). A premature stop codon occurred at p.617 that truncated 133 amino acids.

DISCUSSION

Hereditary hypophosphatemic rickets are a group of genetically heterogeneous disorders. Mutations in *PHEX* gene cause the most common X-linked dominant form. Mutations in *FGF23* gene causes autosomal-dominant form (ADHR), *DMP1* and *ENPP1* gene for autosomal-recessive form (ARHR) and *CLCN5* gene for X-linked recessive form. Given an isolated patient, all forms are possible and thus molecular diagnosis is critical. ADHR and ARHR are much rarer than XLHR.^{7,8} X-linked recessive form is associated with increased 1,25(OH)₂ vitamin D and hypercalciuria that was not observed in this case.⁹ Accordingly, we focus on *PHEX* gene in the first place. This gene, which comprises 22 exons coding for a 749 amino-acid protein

Figure 1 Sanger and TA clone sequencing and real-time PCR of proband. (a) Pedigree of *PHEX* family. Black symbol shows an affected individual for hypophosphatemic rickets. The letter 'P' and an arrow show the proband. (b) Sanger sequencing of the proband. The arrow indicates two peaks: one is a wild-type 'G' and the other is one base substitution to 'A'. (c, d) show TA clone sequencing; the arrow shows the different alleles that are isolated (A/G). (e) Real-time PCR of *PHEX* gene including c.1769-1G>A mutation demonstrates that the proband has a normal copy number of *PHEX* as male controls do. Ctrl (M) and Ctrl2 (M): male control; Ctrl3 (F) and Ctrl4 (F): female control. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 3 Potential splice site prediction by HSF matrices

Position	Splice site type	Ref motif	Mut motif	WT	MU	Variation%
c.1769-1 (WT)	Acceptor	tccttttgctaggt	tccttttgctaaGT	84.4	55.51	-34.27 (broken)
c.1769	Acceptor	ccttttgctaggta	ccttttgctaaGTA	47.43	76.37	+61.03
c.1772	Acceptor	tttgctaggtagaa	tttgctaagtagAA	77.23	77.3	+0.09

Abbreviations: HSF, Human Splicing Finder; MU, mutant; WT, wild type.

Figure 2 pSPL3 exon-trapping analysis and sequencing. The illustrations of partial structure of the recombinant plasmids are shown at the bottom, with the wild type on the left and the mutant on the right. Agarose gel electrophoresis and sequencing of RT-PCR products are shown above. Lane M: DNA marker; Lane 1: normal splicing RT-PCR product (394 bp); Lane 2: aberrant splicing RT-PCR products losing 4 bp due to the splice site mutation (390 bp); Lane 3: empty pSPL3 vector (263 bp). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Figure 3 Prediction of the functional influence of *PHEX* mutation c.1769-1G>A. (a) A predicted amino-acid sequence of normal and mutant transcript. The aberrant splicing leads to p.G590E fs*28. (b) The position of potential premature stop codon and associated functional domain. The numbers indicate residues' number. Two important functional domains are damaged owing to premature stop codon.

(NP_000435.3), is the cause of the most common XLHR. In this study, we identified a novel splice site acceptor mutation in *PHEX* gene c.1769-1G>A in intron 17. Splicing acceptor site is highly conserved and crucial for accurate splicing process. Predictions of the impact of this mutation through different algorithms as well as the mini-gene assay come into the same conclusion that the splicing acceptor would be damaged and a cryptic acceptor is probably generated. The truncated *PHEX* proteins lost its active site, Zn^{2+} -binding site and four conserved Cytosines, which probably causes loss of function and structural instability (Figure 3). *PHEX* protein is essential in bone and dentin mineralization and renal phosphate reabsorption, and thus the loss of function of *PHEX* is probably the cause of the low phosphate concentration and the abnormal bone mineral density. Moreover, it is known that splice site mutations of *PHEX* always lead to typical phenotype, such as lower tubular resorption of phosphate and lower calcitriol levels.^{10,11} Therefore, we consider that this novel splice site mutation is probably pathogenic.

Although DNA sample of patient's mother was unavailable, we ruled out the possibility of his phenotypic normal mother as a carrier because the penetrance of XLHR is often said to be 100% by age 1 year.¹² The father was validated negative as expected by sequencing analysis. Thereby, this novel mutation is deduced to be *de novo* rather than by inheritance.^{13,14}

The proband is a boy who carried two alleles on X chromosome, which is absolutely uncommon. The chromosome analysis displayed a normal 46, XY, which ruled out the possibility of heterozygous XXY. Also, there is no duplication of *PHEX* that occurred on X chromosome, because the copy number of *PHEX* is identical to normal males based on qPCR. As those possibilities ruled out, the proband is probably a somatic mosaicism, which was rarely reported before.

Generally, *de novo* mutations are present in the sperm or egg of one parent or in a fertilized egg. Once inherited to daughter cells, they would be present in all tissues of an individual.^{13–15} On the other hand, it is reported that *de novo* mutations in *PHEX* gene are mostly found in females; obviously,^{13,14} it is, however, not the case here. Given the observation of the mosaic mutations in the peripheral blood of this patient, we think that this mutation could be generated at an early postzygotic period so that mutation presented in most tissues, including leukocytes, and in a mosaic pattern according to similar studies in neurological disease.^{16,17} In terms of the phenotype of this patient, he displayed typical hypophosphatemia and skeleton deformity but without severe lower extremity bowing. Considering that the frequency of normal (G) to mutant allele (A) is 19:13, we suppose this, to some extent, supports the point of view that the dosage effect (mutant copy number) have influence on skeleton phenotype while mineral ion homeostasis remain unaffected.¹⁸

To date, >200 mutations in *PHEX* have been identified, yet the somatic mutation is rarely reported with the only case documented in Japan in 2006.¹⁷ Goji *et al.*¹⁷ reported a girl with XLHR in Japan whose father turned out to have mosaic mutation, indicating that germline cells are involved in mutant type as well. Additionally, Saito *et al.*¹⁹ reported three kinds of *PHEX* alleles in a female through single-nucleotide polymorphism analysis, suggesting a somatic mosaicism. Both studies are similar to our observation of the *de novo* mosaic mutation, which may provide an exciting opportunity for further study through cell lineage tracing in order to understand the etiology of XLHR at the cellular level. On the other hand, we realized that it is not easy to detect mosaic mutations for *PHEX*, especially in females, because they could probably be regarded as common heterozygous. As a result, we suspect that some of the mosaic mutations might have been missed out in previous study.^{13,14} Moreover, it is noteworthy that somatic mosaic mutation could change the mode of inheritance, which may confuse the genetic diagnosis and genetic counseling.

In summary, we identified a novel splice site mutation that caused an aberrant splicing, ending up with a loss of active site and Zn²⁺-binding site of PHEX protein. This apparently 'heterozygous' mutation in an affected male had a normal karyotype and *PHEX* copy number and his parents show completely normal phenotype. Therefore, we suppose that it is a *de novo* mutation generated at early postzygotic period as a mosaic pattern was even observed in peripheral blood. This study emphasizes the significance to consider the possibility of *de novo* mutation in mosaic pattern during molecular diagnosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the families of the patient for their cooperation in the study. The study was partially supported by the grant National Natural Science Foundation of China (31371271), grant Natural Science Foundation of Zhejiang province, China (LZ14C06000) and Zhejiang province medical and health science and technology project (201493975). We sincerely thank Professor Jörg Gromoll for providing pSPL3 vector.

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