Published in final edited form as:

Bone. 2008 October ; 43(4): 663–666. doi:10.1016/j.bone.2008.06.002.

Mutational Survey of the *PHEX* Gene in Patients with X-linked Hypophosphatemic Rickets

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Abstract

X-linked hypophosphatemic rickets (XLH) is a dominantly inherited disorder characterized by renal phosphate wasting, aberrant vitamin D metabolism, and abnormal bone mineralization. XLH is caused by inactivating mutations in *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the X chromosome). In this study, we sequenced the *PHEX* gene in subjects from 26 kindreds who were clinically diagnosed with XLH. Sequencing revealed 18 different mutations, of which thirteen have not been reported previously. In addition to deletions, splice site mutations, and missense and nonsense mutations, a rare point mutation in the 3'-untranslated region (3'-UTR) was identified as a novel cause of XLH. In summary, we identified a wide spectrum of mutations in the *PHEX* gene. Our data, in accord with those of others, indicate that there is no single predominant *PHEX* mutation responsible for XLH.

Keywords

Mutation; *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the X chromosome); 3'-untranslated region; X-linked hypophosphatemic rickets

Introduction

X-linked hypophosphatemic rickets (XLH, OMIM #307800) is the most common genetic disorder of renal phosphate wasting with an approximate prevalence of 1 in 20,000. XLH is an X-linked dominant disease with high penetrance and little evidence of a gene dosage effect [1]. Clinical features of the disease include short stature, bone pain, enthesopathy (calcification of tendons and ligaments), and lower extremity deformities from rickets and osteomalacia. Biochemically, patients with XLH have hypophosphatemia secondary to renal phosphate wasting, as well as inappropriately normal 1,25-dihydroxyvitamin D concentrations.

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XLH results from mutations in *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) [2]. Despite the fact that *PHEX* mutations cause renal phosphate wasting, *PHEX* is not expressed in the kidney. Instead, *PHEX* is predominantly expressed in bone (osteoblasts and osteocytes) and teeth (odontoblasts). Recent data indicates that plasma concentrations of a phosphaturic hormone, fibroblast growth factor 23 (FGF23), are elevated in most affected individuals [3,4]. Furthermore, Fgf23 is overexpressed in the bone of the Hyp mouse, an animal model of XLH [5], suggesting that increased Fgf23 expression is the likely cause of the clinical phenotype of XLH.

Since the identification of the *PHEX* gene, over 180 mutations have been found in XLH patients (www.phexdb.mcgill.ca) [6]. Previously described *PHEX* mutations include deletions, insertions, and base-pair substitutions, all of which are predicted to be loss-of-function mutations. Even though the genetic cause of XLH is well established, the mechanism of the disorder has yet to be elucidated. However, identifying additional mutations, particularly missense mutations, may potentially reveal functionally important regions of the gene product. In this study, we report thirteen novel mutations.

Subjects and Methods

Study subjects

We evaluated subjects from 26 kindreds with a clinical history of hypophosphatemia and renal phosphate wasting, consistent with XLH. Multiple subjects from four kindreds (Kindreds G, S, U, and W) were included, resulting in 34 total subjects. We ascertained the presence of disease as previously reported [7]. Diagnosis was established based on physical examination, serum phosphorus concentration, and urinary phosphate excretion with or without skeletal radiographs. Some subjects had been previously treated for hypophosphatemia before recruitment. The study protocol was approved by the Indiana University-Purdue University Indianapolis (IUPUI)/Clarian and Duke University institutional review boards, and written informed consent was obtained from all subjects prior to participation.

Mutational analysis

Genomic DNA was extracted from whole blood samples using standard methods. All 22 exons, along with adjacent intronic sequences, were amplified using Multiplex PCR Kit (QIAGEN Inc., Valencia, CA). PCR primers are from Francis et al. [8] or redesigned to include larger non-coding sequences. Primer sequences are available upon request. PCR products were electrophoresed in 2% agarose gels and then purified for direct DNA sequencing. All exons were sequenced from forward or reverse PCR primers, using Big-Dye Terminator Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). In some instances, DNA sequencing was performed with Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit according to the manufacturer's specification (USB Corp., Cleveland, OH).

Because the patient A1 had two nucleotide changes in exon 22, this exon was amplified with PfuUltra HF DNA polymerase (Stratagene, La Jolla, CA) and cloned into pCR4Blunt-TOPO vector (Zero Blunt® TOPO® PCR Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA). Five clones were sequenced from T7 to verify whether the two changes occurred on the same chromosome or on separate chromosomes.

Sequencing results of the PCR products were aligned with the *PHEX* genomic sequence using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>). The effect of nucleotide changes occurring in adjacent intronic regions were analyzed using a splice site prediction tool (http://www.fruitfly.org/seq_tools/splice.html). The mutations are reported

according to mutation nomenclature guidelines given by the Human Genome Variation Society: Based on the cDNA sequence, +1 is the A of the ATG start codon [9].

Allele-specific PCR

To determine if the base pair change that was observed in the 3'-untranslated region (3'-UTR) of the patients was a mutation or a polymorphism, allele-specific PCR was performed in the patients and normal controls as previously described [10]. DNA from normal individuals was obtained from 341 whites (11 males, 330 females) and 99 blacks (2 males, 97 females). PCR primers were designed using Primer3 (Whitehead Institute; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Forward primers were designed so that each of the two alleles could be differentiated by both the size and the color of the PCR product when used with a common reverse primer: Each 5' end was labeled with either FAM or HEX, the 5' end of the FAM-labeled primer had two additional bases, and the final 3' nucleotide specifically matched the targeted allele (underlined in the sequences below). Using this technique, amplification occurs preferentially when the 3' end of the forward primer exactly matches the DNA sequence. PCR was performed with the two forward primers and one reverse primer in a single reaction. Primer sequences were: 5' CCTTCCCAGGTATTCATGCAC (reverse); 5' FAM-GTTTCTTTAGAAAATCAAACCAACA (forward, wild-type); and 5' HEX-TTCTTTAGAAAATCAAACCAACG (forward, mutant). PCR products were separated using a 3100 Genetic Analyzer (Applied Biosystems). The data were then analyzed using Genescan Analysis 3.7 software (Applied Biosystems).

Results

We identified 13 novel *PHEX* mutations and five mutations that have been previously described in the literature or entered in PHEXdb as personal submission [6] (Table 1). Of the 18 mutations, five were deletions, four were missense, seven disrupted normal splice sites, one was nonsense, and one was in the 3'-UTR. There was also a rare sequence variation in exon 3 (c.288A>G, Glu96Glu). We were unable to determine whether this is a rare synonymous polymorphism or a mutation affecting an exonic splicing enhancer. Sequence analysis revealed that several observed deletions occurred in short sequence repeats (Figure 1), which likely gave rise to misalignment during meiosis.

Due to its ambiguous mutation status, we further investigated the point mutation (c.*231A>G) that occurred in the 3'-UTR of the *PHEX* gene. This particular change was found in seven XLH patients (B1 to G2) from six different kindreds although these kindreds are from the same geographical region, consistent with a founder effect. The c.*231A>G transition is located three base pairs upstream of the putative polyadenylation signal (AAUAAA) (Figure 2A); however, it does not appear to affect any regulatory element in the region. Therefore, to determine if this change is a mutation or a polymorphism, we performed allele-specific PCR in 440 healthy individuals (427 females and 13 males; 867 chromosomes) as well as our seven patients. Following the allele-specific PCR, this particular change was found only in the DNA samples from the seven XLH patients, but not in the 440 healthy controls. The chromatogram of the PCR products shows the presence of both the mutated and normal alleles in a female XLH patient and the normal allele in a control female (Figure 2B). These results demonstrate that the c.*231A>G transition is a mutation and not a polymorphism. Subsequently, the same mutation was found in an unrelated XLH patient (A1) of a different race (i.e. African American compared to the seven American white patients) and geographic region than the other patients, increasing the number of patients with the 3'-UTR mutation to eight. Interestingly, this patient also had an additional 3'-UTR nucleotide change (c.*58C>T) on the other chromosome.

Discussion

In this study, we identified 18 different *PHEX* mutations and one synonymous change in 26 XLH kindreds. To the best of our knowledge, thirteen of these mutations are novel and have not been reported. Although a relationship between *PHEX* and XLH is well established, identification of new mutations, particularly missense mutations, has merit as these mutations may shed a light on domains important in *PHEX* function. In this regard, it is worth noting that the majority of the mutations were found in the 3' end of the gene (around exons 14 through 22). This trend appears to be consistent with a previous study [11] and mutations entered in PHEXdb [6], indicating that this region might encode for a critical region of the *PHEX* protein. Similarly, recurrent mutations found in unrelated kindreds have significant implications in genomic structure of *PHEX*. Mutations affecting the G nucleotide at 1735 have been found in multiple studies (Table 1), indicating that this may be a mutation hotspot. The presence of short sequence repeats around the deletions also suggests that those regions are susceptible to misalignment during meiosis.

Although various mutations in *PHEX* are known to cause XLH, a rare point mutation in the 3'-UTR (c.*231A>G) is noteworthy. This mutation upstream of the polyadenylation signal of *PHEX* was found in eight patients from seven different kindreds. To our knowledge, no mutations in the 3'-UTR have been associated with the occurrence of XLH. Since the identified change is not in the *PHEX* coding region, it is challenging to prove that this is indeed a disease-causing mutation. However, several observations support our conclusion: (1) This rare point mutation was found in all affected subjects from seven apparently unrelated kindreds (patients A1 to G2). However, it was not found in over 800 normal chromosomes. (2) Six of the seven kindreds came from the same geographical region, while the seventh with a different racial background was identified in a distant state. Although their relationship was not determined, it is possible that the mutation is a result of a founder effect. (3) Several of these patients were members of kindreds where linkage was established between XLH and Xp22.1 [12,13], making other genetic hypophosphatemic disorders (autosomal dominant hypophosphatemic rickets, autosomal recessive hypophosphatemic rickets and hereditary hypophosphatemic rickets with hypercalciuria) unlikely. (4) These XLH patients did not have any other nucleotide changes in *PHEX*. Since patient A1 had another rare variation in the 3'-UTR, we cannot rule out the possibility that the additional variation was pathogenic in this patient. However, these observations overwhelmingly support the notion that the 3'-UTR nucleotide variation (c.*231A>G) is in fact a mutation responsible for XLH.

The location of this mutation was three base pairs upstream of the potential polyadenylation signal (AAUAAA), and therefore, it might prevent generation of polyadenylated 3' end (poly (A) tail), resulting in less stable mRNA. Alternatively, since microRNA usually binds to the 3'-UTR of mRNA, this mutation may increase microRNA binding, leading to inhibition of protein translation or degradation of mRNA. In any event, if a protein were made from the mutant allele, we would predict that it would result in a normal protein. Thus, although a functional study is necessary, this mutation likely decreases mRNA levels or inhibits translation of the *PHEX* mRNA.

In conclusion, we identified a variety of *PHEX* mutations in patients with XLH, including thirteen novel mutations. Although there were a few recurrent mutations, no single mutation is predominant in XLH. This mutational survey also indicates that variation in the 3'-UTR could potentially cause XLH. Therefore, molecular diagnosis of XLH may require screening of untranslated regions of the *PHEX* gene.

Acknowledgements

This work was supported by NIH grants R01 AR42228 and M01 RR00750. SAE was supported by a grant to the IU Cancer Center, CA82706-04S1.

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Figure 1.

Sequence repeats near deletions. Horizontal bars indicate repeats in the genomic sequences, which may have promoted misalignment during meiosis. Two deletions (c.328_330delAAT and c.1809_1816delGTCTACTG) with no apparent repeats are not shown.

Figure 2.
A. Sequence of the 3'-UTR from XLH male and female patients alongside a normal control. Horizontal bars denote putative polyadenylation signal (AAUAAA). B. Allele-specific PCR chromatogram comparing a normal female and a XLH female. Chromatogram proves that the mutation is exclusive to individuals with XLH. Green, mutant allele; blue, normal allele.

Table 1

Mutational analysis of the XLH patients

Patient ^a	Region	Mutation	Type	Previous Report
A1	Exon 22	c.*58C>T; c.*231A>G	3'-UTR	
B1, C1, D1, E1, F1, G1–G2	Exon 22	c.*58C>T; c.*231A>G	3'-UTR	
H1	Intron 4	c.436+1G>C	Splice Site	
I1	Intron 4	c.436+5_8delGTGA	Splice Site	
J1	Intron 14	c.1586+3_6delGAGT	Splice Site	[11,14]
K1	Intron 14	c.1586+6T>C	Splice Site	
L1	Intron 15	c.1645+1G>A	Splice Site	[15,16]
M1	Intron 15	c.1645+6T>C	Splice Site	
N1	Intron 17	c.1768+1G>C	Splice Site	
O1	Exon 3	c.328_330delAAT (Asn110del)	Deletion	
P1	Exon 14	c.1525delA	Deletion	[14]
Q1	Exon 16	c.1685delG	Deletion	
R1	Exon 18	c.1809_1816delGTCTACTG	Deletion	Personal submission in PHEXdb [6]
S1–S6	Exon 18	c.1848delA	Deletion	
T1	Exon 10	c.1109T>G (Met370Arg)	Missense	
U1–U2	Exon 16	c.1646G>C (Arg549Pro)	Missense	
V1	Exon 17	c.1735G>A (Gly579Arg)	Missense	[8,11,16–18]
W1–W2	Exon 17	c.1757T>C (Phe586Ser)	Missense	
X1, Y1	Exon 16	c.1699C>T (Arg567Stop)	Nonsense	
Z1	Exon 3	c.288A>G (Glu96Glu)	Polymorphism?	

^a Alphabet, kindred; number, individual.