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Three Novel PHEX Gene Mutations in Japanese Patients with X-Linked Hypophosphatemic Rickets

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ABSTRACT

X-linked hypophosphatemic rickets (XLH) is an X-linked dominant disorder characterized by renal phosphate wasting, abnormal vitamin D metabolism, and defects of bone mineralization. The phosphate-regulating gene on the X-chromosome (PHEX) that is defective in XLH has been cloned, and its location identified at Xp22.1. It has been recognized to be homologous to certain endopeptidases. So far, a variety of PHEX mutations have been identified mainly in European and North American patients with XLH. To analyze the molecular basis of four unrelated Japanese families with XLH, we determined the nucleotide sequence of the PHEX gene of affected members. We detected a new nonsense mutation (R198X) in exon 5, a new 3 nucleotides insertion mutation in exon 12 and a new missense mutation (L160R) in exon 5 as well as a previously reported nonsense mutation in exon 8 (R291X). These results suggest that: *1*) PHEX gene mutations are responsible for XLH in Japanese patients, and *2*) PHEX gene mutations are heterogeneous in the Japanese population similarly to other ethnic populations. **(*Pediatr Res* 48: 536–540, 2000)**

Abbreviations

XLH, X-linked hypophosphatemic rickets
PHEX, phosphate-regulating gene with homologies to endopeptidases on X-chromosome

Hypophosphatemic rickets is the most common non-nutritional rickets. The usual mode of inheritance is an X-linked dominant, although some forms are transmitted as an autosomal dominant fashion (1–5). This disorder is characterized by a defect in renal phosphate transport, causing phosphate wasting, hypophosphatemia, aberrant vitamin D metabolism, and defective bone mineralization (1–5). Affected patients have rickets, short stature, and poor dental development. The abnormalities in bone mineralization and growth are only partially corrected by treatment with high doses of phosphate and 1,25-dihydroxy vitamin D3 (1–4).

The genetic region responsible for X-linked hypophosphatemic rickets (XLH) was identified on Xp22.1 by linkage analysis. The human gene, phosphate-regulating gene with homologies to endopeptidases on X-chromosome (PHEX), was cloned and consists of 22 exons encoding a 749 amino acid protein (6–10), which is homologous to members of the family of neutral endopeptidase (10–14). It has been shown that transient expression of PHEX *in vitro*, degraded exogenously added PTH derived peptides, indicating that indeed PHEX has a function as an endopeptidases (10), and suggest that PHEX regulates a protein that is involved in phosphate transport (10). Moreover, the mRNA of PHEX in human fetal tissue has been found to be preferentially expressed in early bone development (9). In the hypophosphatemic (Hyp) mouse model of XLH, the murine phosphate-regulating gene (Pex) mRNA expression is also seen mainly in bone (9), and bone formation is not normalized after transplantation of bone cells from Hyp mice into the normal mice (15–18). These findings indicate that PHEX protein not only has endopeptidase-like function, but is also important for bone formation (9, 15–18).

Recently, extensive mutation analysis of the PHEX gene has revealed a wide variety of gene defects in XLH. These include nonsense mutations, missense mutations, splice site mutations,

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insertions and deletions (Fig. 1) (8, 19–23). Characterization of the mutations of the PHEX gene in XLH has clarified the distribution of the mutations and revealed no association with specific clinical phenotypes (19–23).

To elucidate the characteristics PHEX gene mutations in Japanese patients with XLH, we analyzed the PHEX gene in four unrelated Japanese families with XLH. We describe three new mutations including a nonsense mutation (Y198X in exon 5), a 3 nucleotides insertional mutation, and a new missense mutation (L160R in exon 5) as well as one previously reported nonsense mutation (R291X) (19, 21, 22).

SUBJECTS AND METHODS

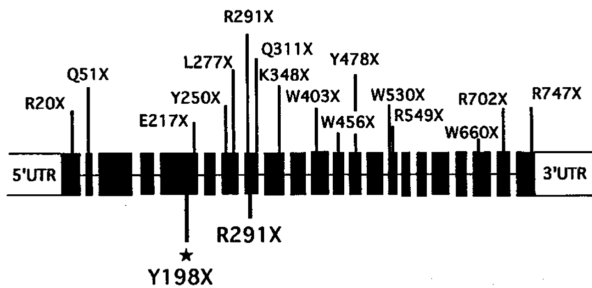
Patients

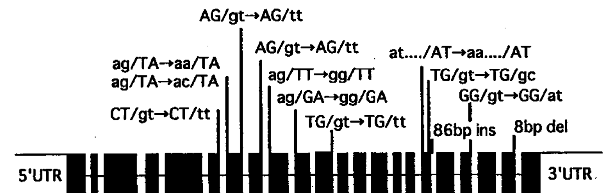
Ten affected members from four unrelated Japanese families in which hypophosphatemic rickets seemed to be inherited in an X-linked dominant mode were studied. Relevant laboratory findings are shown in Table 1. All laboratory data were obtained before the initiation of phosphorus and 1α-hydroxyvitamin D3 therapy. Affected individuals were frequently noticed by genu valgum. Hypophosphatemic rickets was diagnosed based on the medical history and physical examination, radiologic evidence of rachitic disease, unremarkable serum calcium, and electrolyte concentrations, and hypophosphatemia cause by selective renal phosphate wasting for which no etiology was found (1).

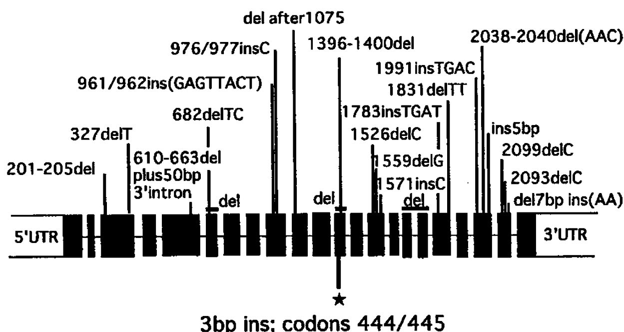
Polymerase-Chain-Reaction (PCR) and Direct Sequence of the PHEX Gene

The institutional review board approved this protocol, and informed consent for DNA analysis was obtained from their parents and/or patients. Among 10 affected members from 4 Japanese families, 8 patients were subjected to DNA analysis. Genomic DNA was extracted from peripheral white blood cells. All 22 exons and exon-intron boundaries of the PHEX gene were amplified by PCR (PCR) with specific primers according to a previous publication (20, 21). We made new primers for amplification of exon 16 as follows: 5′-CCAGGTACTCATCATTGAATC-3′ (sense) and 5′-CCATGGCTTCTT TCTGCTGA-3′ (antisense). All these primers were located approximately 20–100 bases away from the intron-exon boundaries (16, 17). Amplitaq-Gold (Perkin-Elmer) and its standard buffer were used in all reactions. All exons were amplified by PCR under following conditions: initial denaturation at 95°C 7 min, followed by 30 cycles at 94°C 1 min, 55°C 1 min, and 72°C for 2 min. The amplified products were directly purified by Wizard PCR preps columns (Promega Corp., Madison, WI), and the purified PCR products were sequenced by an automated sequencer according to the manufacturer's protocol (24).

When nonspecific bands in PCR reactions were found on 2% agarose gel-electrophoresis, the bands of expected size were







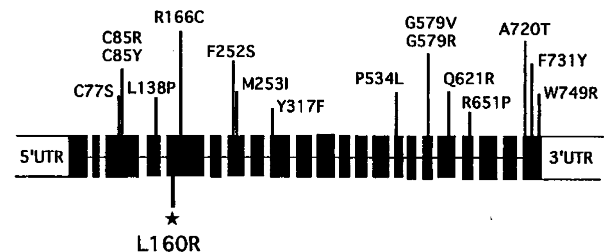


Figure 1. PHEX gene mutations causing hypophosphatemic rickets. Nonsense, splice, frameshift, and missense mutations are shown. Previously reported mutations are displayed above the PHEX schema, and the mutations described in this study are displayed below the line. Asterisks indicate that these are novel mutations. Note the broad distribution of mutations (data derived from this report and mutations reported in references (8, 19–23).

Table 1. *Laboratory findings without treatment and PHEX gene mutations in 10 affected members in 4 Japanese families with XLH*

Patient	Serum Ca (mg/dl)	Serum P (mg/dl)	Serum ALP (K-A U)	Serum PTH* (ng/ml)	Serum 1, 25-$(OH)_2D_3$ (pg/ml)	%TRP	Exon	Mutations
Family 1								
I-2	8.4	1.4	9.6	NT	NT	NT	Exon 5	TAC (Tyr) → TAG(Stop) at codon 198
II-1	9.5	1.7	55.2	0.26 (M)	50	46	Exon 5	TAC (Tyr) → TAG (Stop) at codon 198
Family 2								
I-2	8.8	1.8	6.3	<0.2 (C)	NT	66		not analyzed
II-1	9.5	2.2	42.3	<0.2 (C)	NT	68	Exon 12	Asn insertion between codon 444 and 445
II-2	9.8	2.6	65.9	0.26 (M)	NT	81	Exon 12	Asn insertion between codon 444 and 445
Family 3								
I-2	8.6	2.2	4.9	NT	48	NT		not analyzed
II-1	9.6	2.6	56.9	0.4 (C)	NT	70	Exon 5	CTA (Leu) → CGA(Arg) at codon 160
II-2	10.8	2.5	50.5	0.3 (C)	32	NT	Exon 5	CTA (Leu) → CGA (Arg) at codon 160
Family 4								
I-2	9.1	1.9	5.9	NT	NT	NT	Exon 8	CGA (Arg) → TGA (Stop) at codon 291
II-1	8.2	2.5	44.8	NT	NT	NT	Exon 8	CGA (Arg) → TGA (Stop) at codon 291
Normal range	(8.7–10.2)	Adult;(2.4–4.3) Child;(4.0–7.0)	Adult;(2.7–10) Child;(10–20)	M; (0.16–0.52) C; (<0.5)	(20–70)	(80–96)		

* Serum PTH levels were determined by midmolecule assay (M), and by C-terminal assay (C). NT; not tested; %TRP; tubular reabsorption of phosphate.

excised from the agarose gel, column purified, and then sequenced by an automated sequencer as mentioned.

RESULTS

Direct sequencing of PCR-amplified genomic DNA of the proband (II-1) in family 1 revealed a G to C transition, changing Tyr at codon 198 to a stop codon (Y198X) (Fig. 2*A*). Sequencing of his mother (I-2) with hypophosphatemic rickets showed both the wild and mutant alleles, confirming an X-linked dominant inheritance (Fig. 2*A*). In family 2, both affected brothers (II-1 and II-2) had 3 nucleotides insertion (A, A and C) in exon 12 (Fig. 2*B*), which introduces one amino acid (Asn) between codon 444 and 445, but the open reading frame after this insertion is not altered. While their mother (I-2) was also diagnosed as hypophosphatemic rickets, she was not subjected to DNA analysis. The proband (II-1) in family 3 showed a T to G change, causing amino acid substitution of Arg for Leu at codon 160 in exon 5 (L160R) [Fig. 2*C*]. His affected younger sister (II-2) also shared the same heterozygous mutation [Fig. 2*C*]. DNA form their affected mother was not available. To determine whether the 3-base insertion in family 2 and the L160R mutation in family 3 are not merely polymorphisms, DNA from 50 unrelated normal Japanese individuals were tested for these changes by PCR-direct sequencing. These mutations were not present in any of these control samples. In family 4, the proband (II-1) carried the *nonsense* mutation (R291X) in exon 8 previously reported (19, 21, 22). His affected mother (I-2) had both mutant and wild type alleles, indicating an X-linked dominant trait in this family [Fig. 2*D*].

DISCUSSION

We identified four PHEX gene mutations in four Japanese families with XLH. Each of these families had a different mutation. One mutation (R291X) had been previously reported (19, 21, 22). This report reveals three novel mutations in the PHEX gene. Previous molecular analysis suggests that 60% to 80% of patients with hypophosphatemic rickets in north American, European, African American, Saudi Arabian, Southeast Asian and subcontinent Indian had various mutations of the PHEX gene and these mutations were scattered throughout all exons except transmembrane region (Fig. 1) (8, 19–23). In our study, all four families had mutations of the PHEX gene. Since we analyzed only limited numbers of affected individuals, we cannot conclude the frequency and distribution of PHEX gene mutations among Japanese patients. For that purpose, it will be necessary to analyze more XLH patients.

In family 1 and 4, the Y198X and R291X mutations were identified, respectively. These nonsense mutations presumably result in a truncated protein, leading to the loss of function of PHEX. Several identical mutations including R291X were observed in different ethnic populations (19, 21, 22). Moreover, the frequency of *de novo* mutations has been estimated as approximately 20% (15, 16, 18). These findings suggest that the PHEX gene appears to be particularly prone to mutations for unknown reasons.

In family 2, the insertion of one amino acid (Asn) between codon 444 and 445 of exon 12 was detected. Since Arg at codon 443, Ala at codon 445, Phe at codon 446 and Ile at codon 447 are highly conserved residue in ECE-1, Kell, 24.11 and PHEX pro-

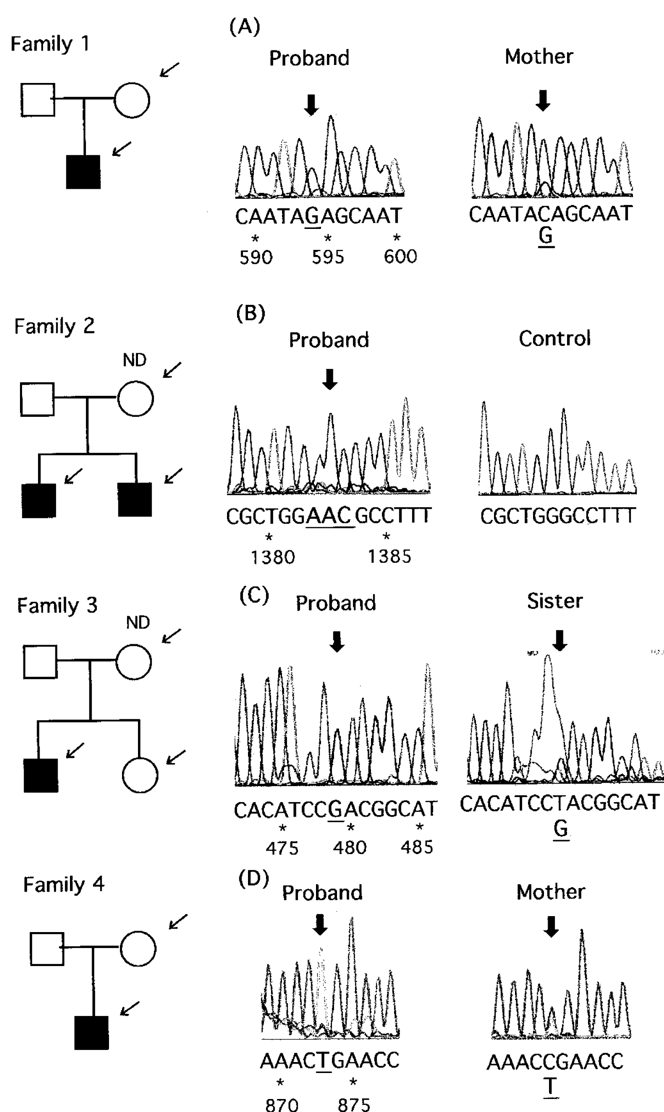


Figure 2. Mutation analysis of the PHEX gene in four unrelated Japanese families with hypophosphatemic rickets. The half-solid circles (females) indicate that they have heterozygous mutations. The solid squares (males) indicate that they have hemizygous mutations. Arrows denote individuals with the clinical features of XLH. ND indicates that DNA was not available for analysis. (*A*) A nonsense mutation of exon 5 (TAC to TAG, codon; 198 in the proband (II-1). His affected mother (I-2) had both the mutant and wild alleles. (*B*) A 3 base insertion in exon 12. This insertion mutation added one amino acid (Asn) between codon 444 (Trp) and codon 445 (Ala), but the open reading frame after the mutation site was not changed. This mutation was present in the proband (II-1) and his affected younger brother (II-2). DNA from their mother was not available. (*C*) A novel missense mutation at codon 160 in exon 5 (CTA to CGA, Leu to Arg) in the proband (II-1). In this family, his affected younger sister (II-2) also carried the wild and mutant alleles. (*D*) The mutation [CGA (Arg) to TGA (Stop)] was identified in the proband (II-1), and his affected mother (I-2) had both the mutant and wild type alleles.

tein, it is conceivable that this amino acid insertion might impair the function of the PHEX protein (20).

A third new mutation described here is a novel missense mutation (L160R) in exon 5. So far, 14 missense mutations were described in exon 3, 4, 5, 7, 9, 15, 17, 18, and 22 (19, 20, 22). Most of these mutations occurred at the conserved residues in the PHEX and other endopeptidase groups. One reported mutation in exon 5 is the transition of Arg to Cys at codon 163 (19). While both of R163C and L160R did not occur at endopeptidases groups conserved residues, these mutations caused XLH, indicating that two amino acids in exon 5 might be crucial for the normal function of PHEX protein.

In summary, we identified three novel mutations of the PHEX gene in Japanese patients with XLH. Because the various mutations causing XLH have so far only been described in North America and Europe, complete sequencing analysis of mutations will be required for each individual patient outside of these regions. Future analysis of larger numbers of Japanese patients with XLH will clarify the distribution, the frequency of PHEX gene mutations and the phenotype-genotype relation in Japanese XLH. Expanding the knowledge on the molecular basis of XLH may provide new insights and approaches to diagnosis and treatment.

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