Mutational analysis of patients with FGF23-related hypophosphatemic rickets

Yuka Kinoshita¹, Tasuku Saito², Yuichiro Shimizu¹, Michiko Hori¹, Manabu Taguchi¹, Takashi Igarashi², Seiji Fukumoto¹ and Toshiro Fujita¹

¹Division of Nephrology and Endocrinology, Department of Medicine and ²Department of Pediatrics, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

(Correspondence should be addressed to S Fukumoto; Email: fukumoto-tky@umin.ac.jp)

Abstract

Objective: X-linked hypophosphatemic rickets (XLHR) caused by mutations in the PHEX gene is considered to be the most frequent cause of fibroblast growth factor 23 (FGF23)-related congenital hypophosphatemic rickets. In previous studies, mutations in the PHEX gene were detected in 60–70% of patients with clinical diagnoses of XLHR. This leads to the question whether current screening methods for mutations in the PHEX gene are inadequate or whether there is a substantial number of patients with other genetic causes of hypophosphatemic rickets. We conducted a genetic analysis of patients with FGF23-related hypophosphatemic rickets to clarify their etiology and evaluate the prevalence of XLHR among this group.

Design and methods: We studied 27 patients with familial and sporadic congenital hypophosphatemic rickets in whom serum FGF23 was above 30 pg/ml using an assay for the full-length protein. Exons and exon–intron junctions of genomic DNA of causative genes for FGF23-related hypophosphatemic rickets were sequenced. PHEX mRNA from peripheral blood was analyzed in some patients.

Results: Direct sequencing of genomic DNA identified 11 novel and four known mutations in the PHEX gene. Additionally, there was a large PHEX gene deletion in one case and abnormal PHEX mRNA splicing in another. In summary, 26 patients (96%) had XLHR and one patient had autosomal recessive hypophosphatemic rickets 2.

Conclusions: XLHR is by far the most prevalent cause of FGF23-related hypophosphatemic rickets. We propose that analysis of PHEX mRNA from peripheral blood would be appropriate for the first screening step in determining the etiology of FGF23-related hypophosphatemic rickets.

Introduction

Hypophosphatemic rickets and osteomalacia are diseases that are characterized by impaired bone mineralization due to defects in phosphate metabolism. The serum phosphate level is regulated by hormones such as fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D (1,25(OH)₂D). Of these, FGF23 reduces serum phosphate by inhibiting the expression of type 2a and 2c sodium phosphate cotransporters in the brush border membrane of the proximal renal tubules (1). FGF23 also decreases the circulating 1,25(OH)₂D level both by suppressing the expression of 25-hydroxyvitamin D-1α-hydroxylase and by enhancing the expression of 25-hydroxyvitamin D-24-hydroxylase (1). FGF23 was cloned in the year 2000 and tagged as the gene responsible for autosomal dominant hypophosphatemic rickets (ADHR; OMIM No. 193100) (2). FGF23 was also identified as a responsible humoral factor for tumour-induced osteomalacia, a rare paraneoplastic syndrome caused by tumors that overproduce FGF23 (3). These results indicated that dysfunctional FGF23 can cause hypophosphatemic rickets/osteomalacia. Since then, it has become clear that several types of hypophosphatemic diseases are caused by overactive FGF23 (4).

There are several types of congenital hypophosphatemic rickets such as X-linked hypophosphatemic rickets (XLHR: OMIM No. 307800), ADHR, and autosomal recessive hypophosphatemic rickets 1 and 2 (ARHR1: OMIM No. 241520 and ARHR2: OMIM No. 613312) that are characterized by renal phosphate wasting and inappropriately suppressed levels of 1,25(OH)₂D for the degree of hypophosphatemia. XLHR is believed to be the most prevalent form of genetic hypophosphatemic rickets and is caused by inactivating mutations in the phosphate-regulating endopeptidase homolog, X-linked (PHEX) gene (5).

Approximately 300 mutations of the PHEX gene have been reported, mostly within 22 coding exons or exon–intron junctions (6). It was also shown that FGF23 expression was enhanced in the bones of
Hyp mice, a murine homolog of XLHR, in which the 3′ region of the Phec gene is deleted (7). Alternatively, ADHR is caused by missense mutations in the FGF23 gene (2), which prevent the proteolytic cleavage of FGF23 protein into inactive fragments (8, 9). The responsible genes for ARHR1 and ARHR2 are dentin matrix protein 1 (DMP1) (10, 11) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) (12, 13) respectively. It was reported that the circulating FGF23 is elevated in patients with ARHR1 and ARHR2 most likely because of enhanced FGF23 expression in bone. However, it remains unclear how mutations in the PHEX, DMP1, and ENPP1 genes result in overexpression of FGF23.

We previously showed that FGF23 measurements are useful for the differential diagnosis of FGF23-related hypophosphatemic diseases (14). An ELISA used to detect full-length FGF23 demonstrated that the FGF23 cutoff value to distinguish FGF23-related hypophosphatemic rickets from hypophosphatemia of other causes, such as vitamin D deficiency and Fanconi syndrome, is 30 pg/ml. Despite the apparently different modes of inheritance and a high frequency of XLHR, it is difficult to identify the exact cause of congenital hypophosphatemic rickets by family history alone. It is true that genetic analysis can help to identify the responsible genes, but the reported frequency of mutations in the PHEX gene among patients with clinically diagnosed XLHR is ~60–70% (15, 16, 17). From these results, we questioned whether the current genetic analysis techniques used to search for mutations in the PHEX gene are insufficient or whether there is a substantial number of patients with congenital FGF23-related hypophosphatemic rickets that is not XLHR. Therefore, we conducted a detailed analysis to identify the etiology of FGF23-related hypophosphatemic rickets in 27 consecutive Japanese patients.

Materials and methods

Patients

Patients were included in this study based on the clinical and biochemical manifestations of congenital hypophosphatemic rickets with FGF23 above 30 pg/ml (14). This study was approved by the institutional review board of the University of Tokyo, and written informed consent was obtained from either the participants or their parents.

Biochemical measurements

Serum biochemical parameters were measured at the time of diagnosis or at the time of genetic consultation. Normal values for adults are 8.4–9.7 mg/dl for calcium, 2.5–4.5 mg/dl for phosphate, 20–60 pg/ml for 1.25(OH)2D, and 10–50 pg/ml for FGF23. Samples for FGF23 measurements were stored at −20 °C until analysis and measured using a FGF23 ELISA Kit (Kainos, Tokyo, Japan) that detects only full-length FGF23 (14).

Genomic DNA analysis of the PHEX, FGF23, DMP1, and ENPP1 genes

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen). First, all 22 coding exons and exon–intron junctions of the PHEX gene were amplified by PCR. Then, the Wizard SV Gel and PCR Clean-Up System (Promega) was used. The genes were directly sequenced with dye-deoxy termination cycle sequencing using either the same primers used in the PCR or the redesigned sequencing primers. In patients with no PHEX mutations, all coding exons and exon–intron junctions of the DMP1 and ENPP1 genes were similarly analyzed. For the FGF23 gene, R176Q, R176W, R179Q, and R179W were the only known mutations that caused ADHR (2, 18). Therefore, the genomic region containing codons 176 and 179 was amplified by PCR and sequenced. Primer sequences can be obtained on request. Mutations were considered to be novel when they were not found on the PHEX mutational database (www.phexdb.mcgill.ca). For all missense mutations, we screened the corresponding sequences in 20 control subjects and confirmed that these mutations were not found in healthy controls.

Multiplex ligation-dependent probe amplification analysis

For the analysis of large deletions or duplications in the PHEX and FGF23 genes, a multiplex ligation-dependent probe amplification (MLPA) analysis was performed with the SALSA MLPA Kit (P223-B1 PHEX; MRC-Holland, Amsterdam, The Netherlands).

Analysis of PHEX mRNA

RNA was extracted from peripheral blood using the Blood Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) or the NucleoSpin RNA Blood (Macherey-Nagel, Duren, Germany), and it was transcribed to cDNA using the PrimeScript RT Master Mix (TAKARA BIO, Shiga, Japan). The coding region of PHEX mRNA was amplified as six overlapping PCR products using the SapphireAmp Fast PCR Master Mix (TAKARA BIO). PCR conditions were as follows: 1 min at 94 °C, followed by 40 cycles for 5 s at 98 °C, 5 s at 60 °C, and 5 s at 72 °C, with a final extension for 3 min at 72 °C. The primer sets were 5′-GCCACCAAACACCGAAAGT-3′ and 5′-GGCTTGGCATCTGTTTCTC-3′ for segment 1, 5′-CATCCTGCTGTAATGAGAAGC-3′ and 5′-GGCATCCCGATAAGACTT-G-3′ for segment 2, 5′-TCTTGAGCTGGACCAAGCA-3′.
Table 1  Biochemical data of patients and mutations in the PHEX gene.

<table>
<thead>
<tr>
<th>Case</th>
<th>Family</th>
<th>Age/sex</th>
<th>Ca (mg/dl)</th>
<th>P (mg/dl)</th>
<th>1,25(OH)2D (pg/ml)</th>
<th>FGF23 (pg/ml)</th>
<th>Mutation in PHEX (NM_000444)</th>
<th>Amino acid</th>
<th>Location in PHEX</th>
<th>PHEXdb ID** reference</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>39/F</td>
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<td>95.7</td>
<td>Exons 1–3 deletion</td>
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<td></td>
<td></td>
</tr>
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<td>37/M</td>
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<td>28.0</td>
<td>541.6</td>
<td>Exons 1–3 deletion</td>
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<td></td>
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</tr>
<tr>
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<td>35/M</td>
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<td>1.9</td>
<td>38.4</td>
<td>38.4</td>
<td>c.1946G&gt;A</td>
<td>G649D</td>
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<td>286</td>
</tr>
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<td>2</td>
<td>4/F</td>
<td>8.5</td>
<td>2.1</td>
<td>NE</td>
<td>233.6</td>
<td>c.1946G&gt;A</td>
<td>G649D</td>
<td>Exon 19</td>
<td>286</td>
</tr>
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<td>2/F</td>
<td>9.4</td>
<td>2.9</td>
<td>NE</td>
<td>281.7</td>
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<td>G649D</td>
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<td>33/M</td>
<td>9.0</td>
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<td>NE</td>
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<td>P534T</td>
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<td>0/F</td>
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<td>4.6</td>
<td>30.3</td>
<td>45.4</td>
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<td>P534T</td>
<td>Exon 15</td>
<td>294</td>
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<td>45/M</td>
<td>10.0</td>
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<td>125 (20)</td>
</tr>
<tr>
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</tr>
<tr>
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<td>2.0</td>
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<td>75.3</td>
<td>IVS20-2(A&gt;G)</td>
<td></td>
<td>Intron 20</td>
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</tr>
<tr>
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<td>F684C</td>
<td>Exon 20</td>
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<tr>
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<td>9.3</td>
<td>2.3</td>
<td>49.0</td>
<td>80.0</td>
<td>(ARHR2 with a homozygous mutation in the ENPP1 gene)</td>
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<td></td>
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<tr>
<td>13</td>
<td>7</td>
<td>51/F</td>
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<td>Exon 9</td>
<td>299</td>
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<td>14</td>
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<td>2/F</td>
<td>9.6</td>
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<td>IVS3-1(G&gt;A)</td>
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<td>45/F</td>
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<td>2.2</td>
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<td>80.0</td>
<td>c.1735G&gt;A</td>
<td>G579R</td>
<td>Exon 17</td>
<td>90 (16, 21, 22, 23, 24, 25, 26)</td>
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<tr>
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<tr>
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<td>3/F</td>
<td>9.1</td>
<td>2.3</td>
<td>81.0</td>
<td>58.9</td>
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<td>18</td>
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<td>41/F</td>
<td>8.8</td>
<td>2.3</td>
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<tr>
<td>19</td>
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<td>47/M</td>
<td>9.5</td>
<td>2.3</td>
<td>NE</td>
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<td>c.564_573del</td>
<td></td>
<td>Exon 5</td>
<td>293</td>
</tr>
<tr>
<td>20</td>
<td>13</td>
<td>23/F</td>
<td>9.2</td>
<td>2.8</td>
<td>NE</td>
<td>62.1</td>
<td>c.564_573del</td>
<td></td>
<td>Exon 5</td>
<td>293</td>
</tr>
<tr>
<td>21</td>
<td>13</td>
<td>20/F</td>
<td>9.6</td>
<td>2.6</td>
<td>NE</td>
<td>83.3</td>
<td>c.564_573del</td>
<td></td>
<td>Exon 5</td>
<td>293</td>
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<tr>
<td>22</td>
<td>13</td>
<td>18/F</td>
<td>9.4</td>
<td>1.9</td>
<td>NE</td>
<td>65.5</td>
<td>c.564_573del</td>
<td></td>
<td>Exon 5</td>
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<tr>
<td>23</td>
<td>14</td>
<td>9/F</td>
<td>10.3</td>
<td>2.8</td>
<td>NE</td>
<td>140.7</td>
<td>c.1368G&gt;A</td>
<td>W456X</td>
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<td>10.0</td>
<td>2.2</td>
<td>61.5</td>
<td>153.3</td>
<td>IVS13-1(G&gt;A)</td>
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<td>296</td>
</tr>
<tr>
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<td>16</td>
<td>29/F</td>
<td>9.1</td>
<td>2.0</td>
<td>70.8</td>
<td>155.1</td>
<td>t(X;4)(p11.4;q21.1), aberrant PHEX mRNA</td>
<td></td>
<td>Exons 12–22</td>
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<tr>
<td>26</td>
<td>17</td>
<td>3/F</td>
<td>9.4</td>
<td>2.9</td>
<td>52.8</td>
<td>95.1</td>
<td>c.1034del1A</td>
<td></td>
<td>Exon 9</td>
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<tr>
<td>27</td>
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<td>3/F</td>
<td>9.2</td>
<td>2.4</td>
<td>97.0</td>
<td>290.7</td>
<td>c.1646G&gt;C</td>
<td>R549P</td>
<td>Exon 16</td>
<td>290</td>
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</table>

Fourteen patients from five families and 13 sporadic patients were analyzed. FGF23 level is above 30 pg/ml in all patients. There are 11 novel mutations (bold) and five known mutations in the PHEX gene. Additionally, there is aberrant PHEX mRNA secondary to chromosomal abnormalities in one patient and a homozygous mutation in the ENPP1 gene in another. NE, not examined. Reference numbers are shown in parentheses.

and 5’-TTGTTGTCCTGGATTAC-3’ for segment 3, 5’-CCTAGGCCGTTCTCAGT-3’ and 5’-AATCGGATCTGTTGTTGGA-3’ for segment 4, 5’-AATCCGATCGACTGTCAATGC-3’ and 5’-AATCGGATCGACTGTCAATGC-3’ for segment 5, and 5’-TGGAAGAAAGCTGGCTT-3’ and 5’-TCTCCAGGCCTAAAGCAATG-3’ for segment 6. PCR products were then gel purified as mentioned earlier or purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and directly sequenced by dye-deoxy termination cycle sequencing using the same primers. The 3′ RACE was conducted using the 3′ RACE system (Invitrogen) and the SMARTer RACE cDNA Amplification Kit (Clontech and TAKARA BIO).

Results

Clinical data

Twenty-seven patients from 18 families with FGF23-related hypophosphatemic rickets were enrolled in the study (Table 1). There were 14 familial cases and 13 sporadic cases. All the patients presented with hypophosphatemic rickets and had FGF23 levels above 30 pg/ml. Serum phosphate levels at the time of evaluation were above the lower limit in some patients because of treatment with neutral phosphate and/or active vitamin D$_3$.

Mutational analysis of genomic DNA

XLHR, which is caused by inactivating mutations in the PHEX gene, has been considered to be the most prevalent cause of congenital FGF23-related hypophosphatemic rickets. Therefore, we first screened mutations in the coding exons and exon–intron junctions of the PHEX gene by directly sequencing genomic DNA. Fifteen distinct mutations, such as missense mutations, nonsense mutations, or several base deletions were found in 23 patients (Table 1). The results of direct genomic DNA sequencing are shown in Fig. 1. While only the sequences of the sense strands are shown, all mutations were confirmed by sequencing the antisense strands as well (data not shown).

A large deletion in the PHEX gene of two patients in family 1 had already been reported (19). Direct sequencing of genomic DNA in a female patient who was heterozygous for this mutation (case 1) did not show any apparent abnormality in the PHEX gene. However, no PCR products corresponding to exons 1–3 were obtained from the genomic DNA of her brother (case 2). Subsequent analysis of his genomic DNA revealed a 50 kbp deletion that included exons 1–3 of the PHEX gene (19). Both were considered to have XLHR. Thus far, we have detected 11 novel and five known mutations in 16 families (16, 20, 21, 22, 23, 24, 25, 26) . In the remaining two cases, cases 12 and 25, we examined the FGF23, DMP1, and ENPP1 genes. There was one case of ARHR2, which had been previously reported (case 12) (27). Her parents were first cousins and one of her sisters died at age 45 due to congenital rickets. A homozygous splice donor site mutation was found at the exon–intron junction of exon 21 in the ENPP1 gene (IVS21 C1–3 GTA CACC). As for case 25, direct sequencing of these genes did not reveal any mutations.

MLPA analysis of the PHEX gene and analysis of PHEX mRNA

For case 25, the possibility remained that there were either large heterozygous deletions or duplications in
the PHEX gene, or PHEX mRNA abnormalities, neither of which had been identified by direct sequencing of genomic DNA. Therefore, we next studied the PHEX gene by MLPA analysis. While MLPA analysis showed a large deletion in the PHEX gene in case 1, which was used as a positive control, no gross abnormality was detected in case 25 (Fig. 2). Finally, we conducted PHEX mRNA analysis. While the PHEX gene is mainly expressed in cartilage, osteoblasts, and odontoblasts (28, 29), the most easily accessible tissue is blood. Therefore, we extracted RNA from peripheral blood and examined the validity of analyzing peripheral blood RNA in determining the etiology of XLHR. First, we amplified PHEX mRNA using peripheral blood RNA from a healthy control and patients with known PHEX mutations. The coding region of PHEX mRNA was amplified as six overlapping RT-PCR products in a healthy control (Fig. 3). We then used the RNA from case 24, in whom a splice acceptor site mutation in intron 13 had been found by direct sequencing of genomic DNA. In this case, RT-PCR showed a shorter segment 4 that resulted from exon 14 skipping (Fig. 3). Subsequently, we analyzed peripheral blood RNA from patients with missense, nonsense, and microdeletions (1–3 bp) in the PHEX gene, which had already been found by direct sequencing of genomic DNA. All mutations were correctly demonstrated by mRNA analysis (data not shown). Therefore, we confirmed that mutations in the PHEX gene were accurately reflected in PHEX mRNA isolated from peripheral blood.

As for case 25, segments 4–6, which corresponded to the 3′ end of PHEX mRNA, were not amplified (Fig. 3). Further, RT-PCR analysis with additional primers showed the presence of PHEX mRNA corresponding to exons 1–11 of the PHEX gene; however, exons 12–22 were not represented. The 3′ RACE system was used to determine the 3′ end of the patient’s PHEX mRNA. With a forward primer designed against exon 11 of the PHEX gene, we have obtained a 1.7 kb mutant product. The sequence 5′-ggaagtcctccttgtggaagattgtgtagattgtgtactcaggaagaataagaagaa-3′ in exon 11 of the PHEX gene was connected to the sequence 5′-agaactcagttgctctggaaagagttgtaatgag-3′ in chromosome 4q21.23 (Fig. 4a and b). This suggested the presence of a chromosomal translocation in this patient. Actually, high-resolution chromosome banding revealed a balanced translocation between chromosome X and chromosome 4, t(X;4) (p11;q21.1). Furthermore, we obtained a 2 kb PCR product from genomic DNA with a forward primer designed against intron 11 of the PHEX gene (5′-GAGCCCTTTCTCCTAGGCGCTAGG-3′) and with a reverse primer designed against chromosome 4q21.23 (5′-GACGCCCTTCTCCTAGGCCCTTCAA-3′), which was located 3 kb upstream of the above-mentioned transcribed region (Fig. 4a). Direct sequencing of this PCR product revealed a break point.

![Image](https://example.com/figure4.png)
sequence of t(X;4) (Fig. 4c). Therefore, the diagnosis of this patient resulted in XLHR due to aberrant PHEX mRNA secondary to chromosomal abnormalities.

Discussion

We identified 16 different mutations in the PHEX gene and one PHEX mRNA abnormality in 26 out of 27 patients (96%). This is consistent with the previous studies which concluded that XLHR was the major cause of congenital FGF23-related hypophosphatemic rickets. The frequency of mutations in the PHEX gene among patients with clinical diagnoses of XLHR reported previously in the literature is \( \sim 60-70\% \) (15, 16, 17). Alternatively, Morey et al. (30), who used MLPA analysis and RT-PCR along with direct sequencing of genomic DNA of the PHEX gene, diagnosed all the 43 patients from 36 families of hypophosphatemic rickets as XLHR. These results indicate that XLHR is by far the most prevalent cause of both familial and sporadic FGF23-related hypophosphatemic rickets. Along with common mutations, such as missense mutations, microdeletions, and splice site mutations, our study included a case with a 5 kb deletion in the PHEX gene in family 1 and a patient (case 25) with abnormal splicing of PHEX mRNA caused by a translocation between chromosome X and chromosome 4.

In case 25, who was a female patient with two X chromosomes, neither the RT-PCR product corresponding to exons 12–22 of the PHEX gene nor the wild-type 3’ RACE product was obtained. This suggested that transcription of the PHEX gene from a normal allele is suppressed in this patient. It is known that in a balanced X chromosome, autosome translocation, the normal X chromosome is usually inactivated to prevent deleterious monosomy of the translocated autosomal segment (31, 32, 33). This phenomenon, referred to as skewed X chromosome inactivation, may explain the absence of both the RT-PCR products corresponding to exons 12–22 of the PHEX gene and the wild-type 3’ RACE product in this patient. Contrarily, because genomic DNA encoding the PHEX gene was intact for at least one allele, MLPA analysis failed to detect any abnormalities due to a chromosomal translocation in this patient.

Large deletions have been reported to account for \( \sim 10\% \) of all PHEX mutations (21). With direct sequencing of PCR products from genomic DNA, it is difficult to detect large deletions in the PHEX gene in female patients who have two X chromosomes. Clausmeyer et al. (15) used MLPA analysis to detect large deletions in the PHEX gene. They reported four types of large deletions: loss of exon 22, loss of exons 21–22, loss of exons 1–9, and loss of exons 4–20. We also confirmed the deletion of exons 1–3 in a female patient (case 1) by MLPA analysis (Fig. 2).

Analysis of PHEX mRNA has been reported by several authors. Christie et al. (34) analyzed RNA extracted from Epstein–Barr virus-transformed lymphoblastoid cell lines obtained from peripheral blood cells, and the RT-PCR products confirmed the presence of the PHEX pseudogenes in a family with XLHR. Additionally, two recent studies that used peripheral blood RNA for RT-PCR were published during the course of this study (30, 35). Although the PHEX gene is mainly expressed in cartilage, osteoblasts, and odontoblasts (28, 29), these results together with ours indicate the utility of analyzing PHEX mRNA in peripheral blood. Analyzing PHEX mRNA has several advantages over direct sequencing of PCR products from genomic DNA. To search for mutations in the PHEX gene with genomic DNA, 22 exons should be amplified and sequenced. In contrast, we have created six pairs of primers that span the coding region of PHEX mRNA that can be relatively easily sequenced. As shown in case 25, analysis of PHEX mRNA could reveal mRNA abnormalities that would not be detected by direct sequencing of genomic DNA. Furthermore, we could detect missense and nonsense mutations, microdeletions, and splice site mutations using mRNA (case 24. Fig. 3: other data not shown), which confirmed the validity of the analysis of PHEX mRNA in peripheral blood. Therefore, we propose that analysis of PHEX mRNA from peripheral blood would be appropriate for the first screening step in determining the etiology of FGF23-related hypophosphatemic rickets. Moreover, analysis of mRNA may contribute to finding more mutations in the PHEX gene in patients with hypophosphatemic rickets, which could potentially result in a changed frequency of XLHR.

In conclusion, we analyzed 27 patients with congenital FGF23-related hypophosphatemic rickets and confirmed that XLHR is the most prevalent form of FGF23-related hypophosphatemic rickets in both familial and sporadic cases. Investigating PHEX mRNA in peripheral blood is an effective screening method for identifying the cause of FGF23-related hypophosphatemic rickets.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Mutational analysis of rickets


