High iFGF23 level despite hypophosphatemia is one of the clinical indicators to make diagnosis of XLH

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Abstract. X-linked hypophosphatemic rickets (XLH) is caused by inactivating mutations in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) gene. Deletion of Phex leads to increased serum fibroblast growth factor 23 (FGF23) levels in mouse. The aim is to assure the clinical usefulness of FGF23 determination in the diagnosis of XLH. Participants were 21 patients with XLH having abnormalities in PHEX (PtPHEX: 1 to 42 years old; 10 males, 11 females) and 55 healthy controls (1 month to 18 years old; 27 males, 28 females). Temporal changes in FGF23 were determined by a single oral phosphate administration in PtPHEX and an ad lib diet in controls. Reference ranges of intact FGF23 (iFGF23) for children were determined. iFGF23 level which distinguish between controls and PtPHEX were validated. Correlations between iFGF23 and the severity of XLH (gender, age of onset, bone deformity, the ratio of maximum rate of renal tubular reabsorption of phosphate to glomerular filtration rate (TmPO4/GFR), inorganic phosphate (IP), Alkaline Phosphatase (ALP), therapeutic dose) were investigated. Increasing tendency after phosphate administration and no general tendency after breakfast in iFGF23 were observed. Reference range (5th and 95th percentiles) of iFGF23 for children (12.9 and 51.2 pg/mL) was similar to that for adults. iFGF23 did not correlate with any index of severity of XLH. Relatively high iFGF23 despite hypophosphatemia is one of the clinical indicators to diagnose XLH.

Key words: X-linked hypophosphatemic rickets (XLH), FGF23, PHEX mutation, Reference range, Phosphate loading

FOUR genes responsible for hereditary hypophosphatemic rickets have been reported: X-linked hypophosphatemic rickets (XLH) caused by abnormalities in phosphate-regulating gene to endopeptidases on the X chromosome (PHEX) gene, autosomal dominant hypophosphatemic rickets (ADHR) caused by mutations in fibroblast growth factor 23 (FGF23) gene [1], hypophosphatemic rickets with hypercalciuria caused by mutations in solute carrier family 34 member 3 (SLC34A3) gene, and autosomal recessive hypophosphatemic rickets caused by mutations in dentin matrix protein 1 (DMP1) [2, 3] or ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) genes [4, 5]. The most common cause of hereditary hypophosphatemic rickets is XLH.

XLH is a disorder of mineralization of bone matrix caused by combined defects of phosphate reabsorption in the proximal tubule and calcitriol synthesis. Patients with XLH are treated with phosphate and vitamin D. More than 160 inactivating PHEX mutations are responsible for the clinical presentation of patients with XLH [6]. However, the mechanism whereby loss of PHEX function leads to the disease remains unknown. The current view is that, loss of PHEX function probably results in accumulation of phosphatonin which are circulating factors that regulate phosphate homeostasis and mineralization of bone. FGF23 [7], matrix extracellular phosphoglycoprotein (MEPE) [8], frizzled related protein 4 (FRP-4) [9] and FGF7 are the putative phosphatonin [10, 11].

FGF23 is a 32-kDa (251 amino acids) protein with an N-terminal region containing the FGF-homology
domain and a unique 71 amino acid C-terminus. It has been reported that full length FGF23 reduces serum phosphate and 1,25(OH)2D levels by reducing the renal sodium/inorganic phosphate co-transporter, type IIc (NaPi IIc) and type IIa (NaPi IIa) and by controlling renal expression of key enzymes of the vitamin D metabolism, respectively [12, 13].

FGF23 is central to the pathogenesis of XLH, for the following reasons: 1) the phenotype of patients with abnormalities in \textit{PHEX} is similar to that of patients with abnormalities in \textit{FGF23}[10], 2) serum FGF23 levels are high in the Hyp mouse [10], a model of XLH which harbors large deletions in the 3' region of the \textit{Phex}, and 3) ablation of FGF23 in the Hyp mouse ameliorates the hypophosphatemic phenotype [7].

Serum FGF23 levels in patients with XLH are expected to be high since serum concentrations of FGF23 in Hyp mice are reported to be 10-fold higher when compared with normal mice [10]. Indeed, patients with XLH are reported to have high serum FGF23 levels [14-16]. However, diagnosis was not confirmed genetically in some patients. Serum FGF23 levels are also increased in patients with mutations in \textit{FGF23, DMP1} and \textit{ENPP1} called FGF23-related hypophosphatemic diseases [17]. It had been reported that low and high phosphate diet for more than 4 days reduces and increases FGF23 levels, respectively in both human [18-20]and mouse [21]. However, the short term effect of an oral phosphate load on FGF23 levels remains unknown. The clinical usefulness of FGF23 levels for the diagnosis of XLH with abnormalities in \textit{PHEX} remains to be elucidated. The reference range of FGF23 levels for children has not been established, although that for adults is reported [14]. Whether FGF23 levels can predict disease severity also remains to be elucidated.

**Objectives**

The aims of the present study were the following: 1) to establish the temporal change in FGF23 levels in response to the short term administration of an oral phosphate load and diet, 2) to investigate the clinical usefulness of FGF23 determination in the diagnosis of XLH with abnormalities in \textit{PHEX}. In order to investigate the second aim, we performed the following three sub studies: a) establish the reference range of FGF23 in the first two decades of life, b) validate the serum FGF23 levels which distinguish between healthy subjects and patients with abnormalities in \textit{PHEX}, c) explore the correlation between the severity of XLH and FGF23 levels.

**Participants**

\textit{Patients with XLH bearing abnormalities in PHEX}

Forty five patients (15 male, 30 female) with hypophosphatemic rickets from 35 kindred have been followed in our hospital from 1966 to 2007. Twenty-one of 45 patients (ages 1 to 42 years; 10 male, 11 female: 14 patients under 20 years old and 7 patients aged 20 years or over)) from 13 separate kindred who provided informed consent and were diagnosed as having abnormalities in \textit{PHEX} were included in this study. Twenty-four patients were excluded from this study for the following reasons: 1) 22 patients had no gene analysis, 2) two patients had no abnormalities in \textit{PHEX}. Among the 21 patients with abnormalities in \textit{PHEX}, 20 patients were treated with 1-\alpha hydroxyl vitamin D3 (alfarol®) and phosphate at the time of the study, and one patient was not on medications. Treatment dosages of phosphate were adjusted individually to achieve an increase in serum inorganic phosphate (IP) levels of 2.5 and 1.0 mg/dL after oral administration, during childhood and adulthood, respectively. Treatment included: 1-\alpha hydroxyl vitamin D3 of 0.05 to 0.1 micro-grams/kg/day and 1.5 to 2.0 micro-grams/day in children and adults, respectively, and phosphate as PO4 of 120 to 360 mg/kg/day and 36 to 160 mg/kg/day in children and adults, respectively. Renal function in all patients was normal determined by estimated glomerular filtration rate (GFR) calculated using the original Schwartz equation (72 to 191 mL/min/1.73m²) and no proteinuria. Renal calcification was observed in 11 out of 19 patients who underwent ultrasonography.

Diagnosis of hypophosphatemic rickets was made based on the findings of hypophosphatemia, renal phosphate wasting, elevated alkaline phosphatase levels, absence of aminoaciduria and hypercalciuria, and radiological evidence of rickets or osteomalacia. Mutations in \textit{PHEX} were confirmed by direct sequence for all participants.

**Healthy individuals**

Healthy control individuals were recruited to participate in this study to establish a reference range for serum FGF23 levels. For a reference range for adults, 111 healthy individuals (from 21 to 63 years old; 34
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Correlation between iFGF23 levels and IP was evaluated. Data were available from 23 loading tests performed in 14 patients with abnormalities in \( \text{PHEX} \) (ages 1 to 36 years).

As for the influence of diet in FGF23, serum samples were obtained prior to and subsequently 60 minutes after breakfast in seven healthy control adults (ages 30 to 49 years). General amount of phosphorus in the typical Japanese breakfast is 200 mg to 500 mg.

Analyses of correlation between severity of XLH and iFGF23 levels

The correlations between iFGF23 levels and severity of XLH were investigated. The severity of XLH was determined by gender, age of onset, existence or nonexistence of bone deformity, the ratio of maximum rate of renal tubular reabsorption of phosphate to glomerular filtration rate (TmPO\(_{4}/\text{GFR}\)), serum IP levels, serum ALP levels, therapeutic dose of phosphate, or phosphate dosage which increased serum IP levels by 1.5 mg/dL after oral administration.

Because phenotypic difference may be influenced by genotype of abnormalities in \( \text{PHEX} \) and by gender, the correlations between iFGF23 levels and severity of XLH were also investigated among the members in the same kindred or the same gender siblings.

Statistical analysis

The reference range for serum iFGF23 level was defined as the level between the 5th and the 95th percentiles of healthy individuals. Comparison of iFGF23 levels for each gender and the existence or nonexistence of bone deformity was analyzed by Mann-Whitney’s U test. Correlations between iFGF23 levels and age of onset, TmPO\(_{4}/\text{GFR}\), serum IP levels, serum ALP levels, therapeutic dose of phosphate, or phosphate dosage which increased serum IP levels by 1.5 mg/dL after the

Methods

**Biochemical analysis of FGF23**

All the samples were obtained in the morning and stored at -20°C before analysis. Full-length FGF23 (iFGF23) levels in samples were determined by the sandwich ELISA as previously reported with FGF-23 ELISA kit (Kainos, Japan) [14]. Detection limits of the assay ranged from 3 to 800 pg/mL. Inter-assay error was less than 10%. If iFGF23 levels exceeded 800 pg/mL, samples were diluted with standard reagent in the kit and reanalyzed.

When two or more determinations per patient were available, the lowest level was selected as data for use in this study to elucidate the lowest level of serum FGF23 in patients with XLH with abnormalities in \( \text{PHEX} \) that could distinguish from healthy subjects. Serum samples were obtained before the meal and oral phosphate administration. Samples were obtained before initiation of therapy with phosphate in four patients, and during periods of noncompliance over more than six years in two patients. Serum samples were obtained from 15 other patients who were current being treated with phosphate. However, compliance was poor in two of the 15 patients upon reviewing the frequency of prescription refills. Serum iFGF23 levels with and without phosphate therapy were available in three of the six patients who did not take phosphate.

**Single oral phosphate load and diet**

A single oral phosphate load and diet were evaluated as factors which may alter serum iFGF23 levels. As for a single oral phosphate load, serum samples were obtained prior to then subsequently 30, 60, 90, and 120 minutes after oral phosphate administration in patients with abnormalities in \( \text{PHEX} \). The phosphate load was administered more than two hours after the patient’s last meal. Loading dosages of phosphate were the single therapeutic dose in each patient (30 to 115 and 10 to 40 mg/kg as PO\(_{4}\) in children and adults, respectively). Serum iFGF23 and IP levels were then determined. Correlation between iFGF23 levels and IP was evaluated. Data were available from 23 loading tests performed in 14 patients with abnormalities in \( \text{PHEX} \) (ages 1 to 36 years).
oral administration were analyzed by Pearson’s correlation coefficient. $P<0.05$ was defined as being statistically significant.

**Results**

**Temporal change in iFGF23 levels in response to single oral phosphate load and diet**

No general tendency was observed in iFGF23 levels after breakfast in seven healthy individuals (Fig. 1). iFGF23 levels were increased ($>5$ pg/mL) in three and decreased ($>5$ pg/mL) in one of seven individuals and not significantly changed ($<5$ pg/mL) in the other three individuals (Fig. 1). An increasing trend was shown in iFGF23 levels after oral phosphate administration in patients with abnormalities in PHEX (Fig. 2). A representative course of iFGF23 and IP levels is shown in Fig. 2a. The increase in iFGF23 levels was subsequent to an increase in IP levels. All time courses of iFGF23 levels from 23 loading tests derived from 14 patients are shown in Fig. 2b. iFGF23 levels increased by over 18 pg/mL from base line in 14 tests from 10 patients, and did not increase (increment $<10$ pg/mL) in 9 tests from

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**Fig. 1** iFGF23 levels after breakfast

Solid line (---): iFGF23 levels obtained before and after breakfast in seven healthy individuals. Double line (-----): Two individuals who showed increase in iFGF23 levels after breakfast ($>10$ pg/mL). Dashed line (----): Mean and SD of the iFGF23 levels of all healthy individuals. No general tendency was observed in iFGF23 levels after breakfast in healthy individuals.

**Fig. 2** iFGF23 levels after oral phosphate administration

An increasing trend was shown in iFGF23 levels after oral phosphate administration in patients with abnormalities in PHEX. Fig. 2a: A representative course of iFGF23 and IP levels. The increase in iFGF23 levels was subsequent to an increase in IP levels. Fig. 2b: All time courses of iFGF23 levels from 23 tests derived from 14 patients. iFGF23 levels increased over 18 pg/mL ($18 \sim 111$ pg/mL) from baseline in 14 determinations from 10 patients, and did not increase (increment $<10$ pg/mL) in 9 determinations from 7 patients. Among six patients who had two or more trials of iFGF23 levels changed with phosphate loading, elevation only, reduction only and both elevation and reduction were observed in two, one, and three patients, respectively. There was no relationship between the amount of change in iFGF23 levels and IP levels.
High iFGF23 is indicator of XLH

Table 1 Reference range for iFGF23 levels in normal individuals

<table>
<thead>
<tr>
<th>Age (years old)</th>
<th>n (male : female)</th>
<th>iFGF23 levels (pg/mL)</th>
</tr>
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<tr>
<td>0 – 5</td>
<td>24 (8 : 16)</td>
<td>12.7 - 45.0</td>
</tr>
<tr>
<td>6 – 10</td>
<td>15 (7 : 8)</td>
<td>11.3 - 44.0</td>
</tr>
<tr>
<td>11 – 18</td>
<td>16 (12 : 4)</td>
<td>18.3 - 55.6</td>
</tr>
<tr>
<td>0 – 18</td>
<td>55 (27 : 28)</td>
<td>12.9 - 51.2</td>
</tr>
<tr>
<td>21 – 63</td>
<td>111 (34 : 77)</td>
<td>11.5 - 48.9</td>
</tr>
</tbody>
</table>

Reference range for serum iFGF23 level was defined as the level between the 5th and the 95th percentiles of healthy individuals.

There was no significant difference in iFGF23 levels among those three age groups. The reference range across all age groups (from 0 to 18 years old) was from 12.9 to 51.2 pg/mL. The reference range for adults (from 21 to 63 years old) was from 11.5 to 48.9 pg/mL (previously reported) [14]. iFGF23 levels in the first two decades of life were not significantly different from those in adults.

Serum iFGF23 levels in XLH with abnormalities in PHEX ranged from 40 to 4710 pg/mL before a meal and oral phosphate administration (Fig. 3). Among 14 children (under 20 years old), iFGF23 levels were over 51.2 pg/mL in all patients except one (40.0 pg/mL in one female who was 1 year and 3 months old, and >57.8 pg/mL in the other 13 patients). Among 7 adults (20 years and older), iFGF23 levels were over 48.9 pg/mL.

Fig. 3 iFGF23 levels in XLH with abnormalities in PHEX


7 patients. Among six patients who had two or more trials of iFGF23 levels changed with phosphate loading, elevation only, reduction only and both elevation and reduction were observed in two, one, and three patients, respectively. There was no relationship between the amount of change in iFGF23 levels and IP levels.

Reference range of iFGF23 in the first two decades of life

The reference ranges (5th and 95th percentiles) for each age group (from 1 month to 5 years old, from 6 to 10 years old, from 11 to 18 years old) are shown in Table 1. There was no significant difference in iFGF23 levels among those three age groups. The reference range across all age groups (from 0 to 18 years old) was from 12.9 to 51.2 pg/mL. The reference range for adults (from 21 to 63 years old) was from 11.5 to 48.9 pg/mL (previously reported) [14]. iFGF23 levels in the first two decades of life were not significantly different from those in adults.
iFGF23 levels should be determined in serum obtained before oral phosphate administration and a meal. FGF23 may be not only a chronic but also an acute regulator of phosphorus homeostasis. An upward trend was shown in iFGF23 levels after oral phosphate administration in patients with abnormalities in \textit{PHEX}. A change in iFGF23 levels was observed after a meal in two of seven healthy individuals, however no general tendency was observed. It was reported that low and high phosphate diet for more than 4 days reduced and increased FGF23 levels, respectively in both human [18-20] and mouse [21]. This is the first report to determine that a single oral phosphate load also did increase iFGF23 levels within two hours. A reason why no increase was observed in iFGF23 levels after a meal in five healthy individuals may be because blood glucose lowers serum IP levels, or non-standardized diets contained variable amount of phosphate, although changes in serum IP levels were not available.

An iFGF23 level over 40 pg/mL with hypophosphatemia is one of the clinical indicators to help establish a diagnosis of XLH as well as family history, bone deformity, increased ALP, and low TmPO\textsubscript{4}/GFR. In our study where participants included only patients with XLH who were diagnosed genetically, iFGF23 levels were above the upper limit of the reference range in 19 of 21 patients with abnormalities in \textit{PHEX}. Our human data is congruent to the increased FGF23 levels found in the Hyp mouse. However, in the other two

<table>
<thead>
<tr>
<th>sex</th>
<th>age (years)</th>
<th>iFGF23 (pg/mL)</th>
<th>bone deformity</th>
<th>pretreatment IP (mg/dL)</th>
<th>ALP (IU/L)</th>
<th>TmPO\textsubscript{4}/GFR</th>
<th>therapeutic dosage of PO\textsubscript{4} before 15 y.o. (mg/day)</th>
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<tbody>
<tr>
<td>F1-1 M</td>
<td>12</td>
<td>359.5</td>
<td>NP</td>
<td>1.9</td>
<td>1957</td>
<td>1.88</td>
<td>1.5</td>
</tr>
<tr>
<td>-2 M</td>
<td>10</td>
<td>286.4</td>
<td>P</td>
<td>2.1</td>
<td>2040</td>
<td>3.18</td>
<td>1.66</td>
</tr>
<tr>
<td>F2-1 F</td>
<td>3</td>
<td>70.1</td>
<td>P</td>
<td>2.4</td>
<td>1762</td>
<td>1.45</td>
<td>1.66</td>
</tr>
<tr>
<td>-2 F</td>
<td>2</td>
<td>181.6</td>
<td>NP</td>
<td>1.9</td>
<td>2081</td>
<td>2.95</td>
<td>360</td>
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<tr>
<td>F3-1 F</td>
<td>24</td>
<td>84.5</td>
<td>P</td>
<td>1.7</td>
<td>695</td>
<td>1.64</td>
<td>160</td>
</tr>
<tr>
<td>-2 F</td>
<td>21</td>
<td>47.5</td>
<td>NP</td>
<td>1.9</td>
<td>813</td>
<td>1.71</td>
<td>140</td>
</tr>
</tbody>
</table>

Two patients from each family (F1~3) are siblings. M: male, F: female  P : presented, NP : not presented, y.o.: years old

**Discussion**

The reference range (5\textsuperscript{th} and 95\textsuperscript{th} percentiles) for iFGF23 level for ages 18 years and younger was from 12.9 to 51.2 pg/mL. It was not significantly different from that for adults (11.5 to 48.9 pg/mL). iFGF23 level in healthy children was reported to be higher than that in adults [22]. The difference from our data may be due to small samples and wide variation of iFGF23 levels, considering wide SD range in the previous report as our data.
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patients, iFGF23 levels were below the upper limit of the reference range (40.0 and 47.5 pg/mL). The lowest iFGF23 level in patient with XLH was reported to be 38.0 pg/mL [15]. Patients with XLH may be unusual in that their iFGF23 levels were measurable despite hypophosphatemia, considering that highest iFGF23 levels in hypophosphatemic patients without XLH was 35.8 pg/mL in our clinic (data not shown) and reported to be 23.9 pg/mL [15]. Although treating XLH with phosphate and calcitriol was reported to associated with increases in FGF23 concentrations [23], ifGF23 levels in patients who do not undergo therapy were even higher than in healthy individuals in this study. Although FGF23-related hypophosphatemic disease other than XLH could not be excluded, majority of FGF23-related hypophosphatemic disease are XLH and the treatment for patients with FGF23-related hypophosphatemic disease are identical regardless of the cause.

The reason why iFGF23 levels in human patients with XLH were not consistently high, unlike in the Hyp mouse, remains unknown. Overlaps in FGF23 levels between patients with XLH and healthy control were reported previously, using the c-terminal ELISA assay [24, 25] and the intact FGF23 assay [15, 25, 26]. There are three possible reasons for the overlaps: 1) phosphatonin other than FGF23 such as MEPE, secreted frizzled-related protein-4 (sFRP-4) and FGF7 may be a modifier in the pathogenesis of XLH in patients with abnormalities in PHEX, 2) iFGF23 may decrease due to degradation when samples are stored long-term, 3) patients with low iFGF23 levels may have milder symptoms. One patient whose iFGF23 level was 40.0 pg/mL never had treatment, because her symptoms were mild. However, some patients whose iFGF23 levels were below 100 pg/mL were severely affected, and there was no correlation between iFGF23 levels and any of the indicators for the severity of XLH.

Considering that FGF23 is regarded as a major phosphatonin, FGF23 levels are predicted to correlate with the severity of XLH, which was not true in this study. It is reported that serum phosphate levels are negatively correlated with circulating FGF23 levels (C-terminal ELISA assay) in 11 patients with XLH, of which four of the 11 patients were diagnosed solely by clinical means [24]. However, serum phosphate levels did not correlate with iFGF23 levels in this study. Because XLH is an X-linked disorder, iFGF23 levels are expected to be higher in male patients than in female patients. However, even within the same kindred, iFGF23 levels in female patient were higher than in male patients, as was reported previously[25].

There are two possible reasons why iFGF23 levels do not correlate with disease severity in XLH. One is that phosphatonins other than FGF23 may be involved in determining the severity in XLH, as is discussed above. The other is that iFGF23 levels may be affected by treatment with oral phosphate administration. iFGF23 levels were reported to be elevated after long term phosphate loading [15, 18 -21]. However, even when patients undergoing treatment with phosphate are excluded, there was still no correlation between iFGF23 levels and disease severity (data not shown). Furthermore, iFGF23 levels were not significantly different in three patients that were either treated or untreated (83.8 and 71.1, 84.5 and 117.7, 47.5 and 83.6 pg/mL treated and untreated for each patient, respectively) (Fig. 3).

**Conclusion**

Increasing tendency after single phosphate administration and no general tendency after ad lib diet in iFGF23 were observed. The reference range for children (12.9~51.2 pg/mL) was similar to that for adults. iFGF23 levels were above the reference range in 19 of 21 patients with abnormalities in PHEX (>40.0 pg/mL). Relatively high iFGF23 level despite hypophosphatemia is one of the clinical indicators to help establish a diagnosis of XLH when there is also a positive family history of XLH, bone deformity, increased ALP, and a low TmPO4/GFR. iFGF23 did not correlate with any index of severity of XLH.

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