Table 1. ROC analysis of biochemical data for the diagnosis of DR and HR

Measurement	AUC	SE	95% CI	p
FGF23	1.00	0.00	1.00, 1.00	<0.01
25(OH)D	0.82	0.11	0.61, 1.04	0.09
Calcium	0.57	0.20	0.17, 0.96	0.72
Phosphate	0.14	0.09	−0.03, 0.32	0.06
$1,25(OH)_2D$	0.12	0.11	−0.10, 0.34	<0.05
PTH	0.11	0.08	−0.05, 0.27	<0.05
TmP/GFR	0.02	0.03	−0.04, 0.09	<0.05

Serum FGF23 level is the most significant measurement for distinguishing patients with DR from those with HR on ROC analysis (1 = DR, 0 = HR).

with HR, although they stayed within the reference range (8.4–10.0 mg/dl) in most patients with DR (fig. 1). Serum phosphate concentration was higher in patients with DR than those with HR. Some patients with DR had reference serum phosphate concentrations (4.2–6.2 mg/dl for the age of 1 year). Serum levels of PTH and $1,25(OH)_2D$ and TmP/GFR were increased in patients with DR compared to those with HR. While there were significant differences in serum levels of calcium, phosphate, PTH, and $1,25(OH)_2D$, as well as TmP/GFR, between patients with DR and with HR, a clear overlap of the data existed between the two groups. Of note, a patient with HR had an obvious increase in serum PTH (145 pg/ml) and $1,25(OH)_2D$ (110 pg/ml). All serum 25(OH)D levels in patients with DR were less than 18 ng/ml. Patients with HR had serum 25(OH)D levels of 8.2, 14.1, and 25.2 ng/ml before the treatment, indicating the presence of vitamin D deficiency in at least 2 patients with HR. Serum FGF23 levels were different and, notably, exclusive between patients with DR and HR. Serum FGF23 levels in all patients with DR were 18 pg/ml or less, while 72% had levels less than 10 pg/ml (reference range for adults: 10–50 pg/ml [21]). In contrast, serum FGF23 levels in patients with HR were 58 pg/ml and more. These results indicate that the measurement of serum FGF23 levels is useful to distinguish patients with DR from those with HR. There were no differences in serum AP levels and U-Ca/Cr between patients with DR and HR. Furthermore, ROC analysis showed the AUC of serum FGF23 had a more significant p value among the measurements, including TmP/GFR, PTH, and $1,25(OH)_2D$ (table 1). These results suggest that serum FGF23 measurements may be more useful compared to measurements including TmP/GFR, PTH, and $1,25(OH)_2D$ to discriminate patients with DR from those with HR.

Response of Serum FGF23 Levels to Intervention among DR Patients

Laboratory data of patients with DR were determined in the period before intervention and 1–3 months following treatment using α-calcidol combined with lifestyle advice for adequate sun exposure and diet (fig. 2). Both data before and 1–3 months after intervention were obtained in 6 patients with DR. Serum levels of FGF23, as well as phosphate, PTH, and AP, were significantly changed after intervention compared to those before. In addition, percent increases in FGF23 levels after intervention were positively correlated with those in serum phosphate concentrations ($p < 0.05$, $r = 0.87$; data not shown), and tended to be inversely correlated with percent decreases in PTH levels ($p = 0.06$, $r = -0.79$). Serum calcium and $1,25(OH)_2D$ levels, TmP/GFR, and U-Ca/Cr were not obviously changed by intervention. These results suggest that serum FGF23 level might be a good indicator of biochemical response to treatment and for lifestyle advice to patients with DR.

Discussion

Our study demonstrated that serum FGF23 is clearly suppressed in infants with DR. Thus, it is suggested that FGF23 is a useful marker to distinguish DR from HR, although not all patients with DR require FGF23 measurement. Indeed, serum levels of calcium, phosphate, PTH, and $1,25(OH)_2D$, as well as TmP/GFR, are also useful to distinguish DR from HR; however, only serum FGF23 levels were mutually exclusive between DR and HR. In addition, serum FGF23 levels were increased following treatment with α-calcidol and lifestyle advice on sun exposure and diet, suggesting that serum FGF23 level might be a good indicator of biochemical response to intervention. At least 2 patients with HR had low serum 25(OH)D levels and one of them had high PTH and $1,25(OH)_2D$ levels. These results suggest hypovitaminosis D may influence the biochemical data of patients with HR and make diagnosis of HR difficult. On the other hand, FGF23 remained high in patients with HR even when hypovitaminosis D is a complicating factor.

Serum PTH levels in patients with HR are usually normal or modestly elevated in some case [16]. However, given that patients with HR may have low serum 25(OH)D

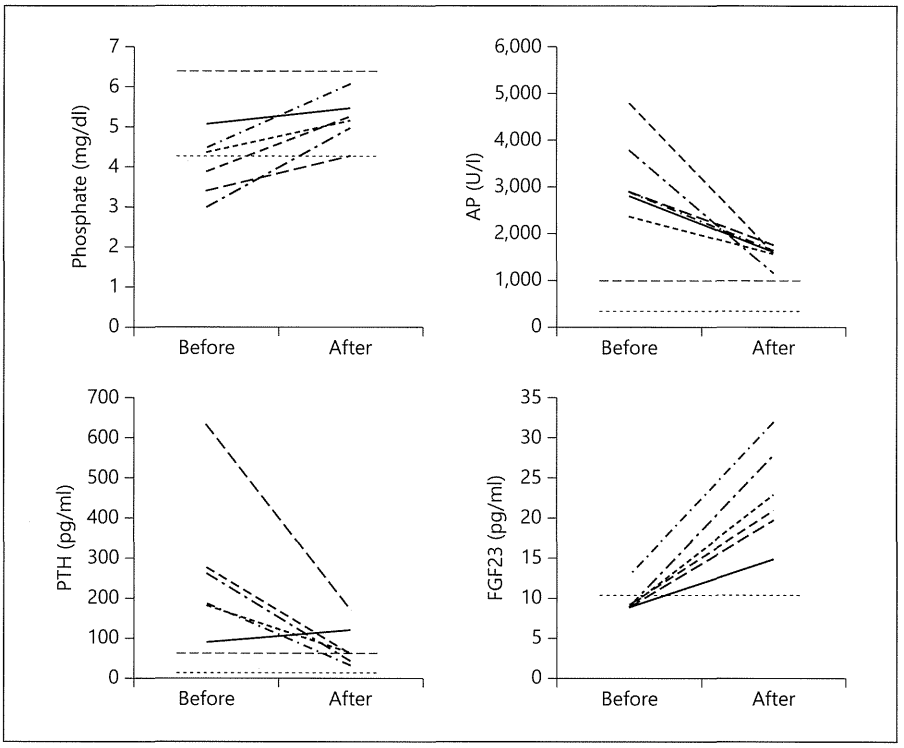

Fig. 2. Response of biochemical measurements to intervention in patients with DR. Serum levels of phosphate, PTH, AP, and FGF23 were significantly changed after the intervention compared to those before. $p < 0.01$ for phosphate, AP, and FGF23; $p < 0.05$ for PTH. Dashed and dotted lines are the upper and lower limit of reference ranges, respectively.

levels, clinicians should bear in mind that high serum PTH levels do not preclude a possibility of HR. In this study, there was a clear and exclusive difference in serum FGF23 levels between infants with DR and HR. These results indicate that a finding of the decrease in serum FGF23 levels is useful for differentiating DR from HR.

It is unclear to what extent serum FGF23 levels are affected in patients with DR. This study showed that serum FGF23 levels were 18 pg/ml or less in 18 infants with DR and was below 10 pg/ml in 72% of these. We previously described a maximum serum FGF23 level of 23.9 pg/ml in hypophosphatemic patients with vitamin D deficiency, Fanconi syndrome, and Cushing's syndrome whose ages ranged from 1 to 75 years [21]. Other researchers have reported that serum intact FGF23 levels were 23.4 pg/ml (mean) in adult females with vitamin D deficiency, a 36% reduction compared to those [36.7 pg/ml (mean)] in healthy control subjects [24]. Serum phosphate concentrations were not reduced in patients with vitamin D deficiency, while bone mineralization was not assessed. In our study, the mean serum FGF23 levels in 18 infants with DR was 8.4 pg/ml, a 71% reduction compared to those [28.9 pg/ml (mean)] in healthy control adults [25], although there could be a difference in FGF23 levels between healthy infants and adults. Thus, serum FGF23 levels in patients with vitamin D deficiency might vary depending on age, serum phosphate concentration, or defective bone mineralization. FGF23 levels less than 19 pg/ml might be useful to distinguish patients with rickets due to vitamin D deficiency from those with rickets due to FGF23 excess who might also be vitamin D deficient.

Serum FGF23 levels in patients with HR were 70.8 ± 11.5 pg/ml (mean ± SD) with a minimum value of 58 pg/ml. This is consistent with previous reports [25, 26] and also our previous study which suggested that FGF23 levels more than 30 pg/ml with hypophosphatemia indicate the presence of excessive FGF23-related diseases such as HR [21].

The nutritional and lifestyle survey of our patients showed that a majority with DR had been exclusively fed with breast milk before solid foods and that some patients had a restricted and/or unbalanced diet and limited sun exposure. Limited vitamin D intake and sun exposure are causes of DR without intrinsic diseases [13]. Supplementation with vitamin D for infants is recommended in many countries [27]. However, no recommendation for vitamin D supplementation is given in Japan. Considering patients with DR were mostly fed exclusively with breast milk, a recommendation concerning vitamin D

supplementation for infants is necessary in Japan to decrease the prevalence of DR.

Patients with DR exhibited an increase in serum PTH and $1,25(OH)_2D$ levels. Although $1,25(OH)_2D$ and PTH are thought to induce the expression of FGF23 in bone [17, 28], this is not the case in our study. The finding of the striking reduction of FGF23 levels in patients with DR indicates hypovitaminosis D and/or a chronic decrease in serum phosphate levels might have more influence on the decreased FGF23 expression, thereby overcoming any increase caused by 1,25(OH)2D and PTH. On the other hand, the patient with HR complicated by vitamin D deficiency displayed an increased FGF23 level, suggesting that the intrinsic genetic abnormality may have more of an impact on FGF23 expression than that caused by vitamin D deficiency. Further study is necessary to elucidate the mechanism(s) regulating FGF23 synthesis in osteocytes.

Serum FGF23 levels were increased after intervention using α-calcidol combined with lifestyle advice. The increases in FGF23 levels were accompanied by increases in serum phosphate concentrations, raising the possibility that increased serum phosphate could increase FGF23. Although FGF23 regulates serum phosphate by inhibiting renal tubular reabsorption, the effects of phosphate on FGF23 remain unclear [29]. However, it is known that $1,25(OH)_2D$ is an important systemic regulator of FGF23 that induces FGF23 expression. Thus, in our study, $1,25(OH)_2D$ derived from α-calcidol might directly increase FGF23. Another possible mechanism for the increased FGF23 is that lifestyle advice for adequate sun exposure and diet might improve hypovitaminosis D although 25(OH)D levels were not measured after intervention.

Our study has several limitations. First, the sample size is small. However, our results indicate that serum FGF23 levels are distinctly decreased in patients with DR compared to those with HR. Second, we did not have a control group composed of healthy infants, although it does not seem ethical to obtain blood samples from healthy infants. Third, genetic analysis was not performed in patients with HR. However, X-linked HR is the most common type of HR and other forms of HR are much less prevalent.

In summary, our study revealed that the measurement of serum FGF23 levels was highly useful for distinguishing infants with HR from those with DR, as serum FGF23 levels were exclusive between DR and HR. It is also suggested that serum FGF23 levels might be a good indication of biochemical response to the intervention in patients with DR.

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Original Article

Therapeutic Use of Oral Sodium Phosphate (Phosribbon® Combination Granules) in Hereditary Hypophosphatemic Rickets

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Abstract. Oral sodium phosphate formulations indicated for hypophosphatemia are commercially available worldwide. In Japan, however, many medical institutes have used hospital dispensary or foreign over-the-counter formulations because no such medication with an indication covered by the health insurance system is domestically available. To address this problem, we initiated the development of Phosribbon®. The present study evaluated the efficacy and safety of Phosribbon® in 16 patients with hereditary hypophosphatemic rickets. The optimal dosage and an administration pattern were also investigated. Administration of the agent resulted in an increase in the level of serum phosphorus in all patients, which implied that the employed dosage was appropriate. The dosage and administration pattern were adjusted based on comprehensive considerations, including changes in clinical laboratory values such as serum phosphorus, alkaline phosphatase and intact PTH, the dosage of a concomitantly administered activated vitamin D formulation and characteristics of individual patients. Adverse drug reactions were observed in 2 patients, neither of which were serious or necessitated therapy dose reduction or discontinuation. We conclude that Phosribbon® is a safe and effective treatment for patients with hypophosphatemic rickets and that dose adjustment in this therapy can be guided by the results of regular clinical examination and renal ultrasonography. (ClinicalTrials.gov Identifier: NCT01237288)

Key words: oral sodium phosphate, hypophosphatemic rickets/osteomalacia, hypophosphatemia, FGF23

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Introduction

Hereditary hypophosphatemic rickets is a group of diseases that develop due to congenital impairment of phosphorous reabsorption in proximal renal tubules. The rickets may presumably cause overexpression of fibroblast growth factor 23 (FGF23), a factor that regulates phosphate metabolism, which results in hypophosphatemia, hyperphosphaturia and inhibition of vitamin D activation in the kidney (1–6). Therefore, formulations of activated vitamin D and oral sodium phosphate have been employed for the treatment of the rickets (2–6). Although oral sodium phosphate formulations are commercially available in such countries as the United States, the United Kingdom, Germany and France, they have not been covered for hypophosphatemia by the health insurance system in Japan. As a result, many medical institutes in Japan, following review by ethical boards, have employed (a) phosphate reagents for research use; (b) formulations obtained by the pulverization of Visiclear® combination tablets, an oral bowel cleaner containing phosphoric acid; or (c) unapproved drugs after individual import of foreign over-the-counter (OTC) medications (7–9).

In order to address this problem, we initiated a development of Phosribbon® combination granules, an oral sodium phosphate formulation. We obtained approval for production and sales of this agent for use in patients with hypophosphatemia in December 2012 from the Japanese Ministry of Health, Labour and Welfare. This report describes the efficacy and safety results associated with the use of Phosribbon® in patients with hereditary hypophosphatemic rickets.

Subjects and Methods

Four medical institutes in Japan performed an open-label, uncontrolled study from September 2010 to December 2011 in an attempt to examine the efficacy and safety of Phosribbon® and to optimize the dosage and administration pattern for it when given orally to patients with hereditary hypophosphatemic rickets. Patients aged 1 to 14 yr were enrolled if they were diagnosed as having hereditary hypophosphatemic rickets based on such things as family history, genetic test results and clinical examination values and a finding of rickets by means of bone X-ray examination. Patients with hyperparathyroidism were excluded.

The study drug contained 330 mg of sodium dihydrogen phosphate monohydrate and 119 mg of anhydrous disodium hydrogen phosphate per envelop (equal to 100 mg of phosphorus). The defined total daily dosage of phosphorus ranged from 300 to 3,000 mg and was to be orally administered 3 or 4 times a day. Patients who had previously received an oral sodium phosphate formulation received the equivalent dosage of phosphorus at the initial administration, while other patients received an initial dose of 30 mg/kg/day of phosphorus 4 times a day (3 times a day if the patient was less than 10.0 kg in weight). The amount of the present formulation was increased in cases where the serum phosphorus value at 1 to 2 h after administration was less than 130% of the value at baseline or in cases where the serum alkaline phosphatase (ALP) level after administration was 150% or more of the level at baseline. Conversely, the drug dose was reduced in cases in which the level of intact parathyroid hormone (PTH) at 1 to 2 h after administration exceeded 130 pg/mL. Investigators were instructed to report reasons for any deviations from the protocol.

Table 1 shows the items of examination, observation, and survey in each period.

Efficacy variables

With regard to serum phosphorus, the intervention was considered to be successful when the value for each treatment period (1 to 2 h after administration) was 130% or more of the baseline. For serum ALP, within 150% of the

Table 1 Items of examination/observation/survey in each period

Schedule	Observation period	Treatment period								
	Before dosing	Dose initiation W0	W4	W8	W12	W16	W20	W24	W36	W48
Predetermined days of visit (Day 1 defined as initial dosing day)	Day −27 to initiation of dosing	1	29	57	85	113	141	169	253	337
Informed consent/patient characteristics	○									
Height/body weight	○	○	○	○	○	○	○	○	○	○
Blood/urine sampling (pre-administration)	○									
Blood/urine sampling (1 to 2 h post-administration)		○	○	○	○	○	○	○	○	○
Bone X-ray/renal ultrasonography	○							○		○
Decision of initial dosage	○									
Review of dosage and administration*		○	○	○	○	○	○	○	○	○
Survey of study drug compliance			○	○	○	○	○	○	○	○
Survey of adverse events		◀	—	—	—	—	—	—	—	▶

* Dosage and administration patterns were reviewed within 7 days of blood sampling. W: Week.

baseline was set as the successful range.

In addition, the frequencies of alterations in dosage and administration patterns were calculated.

A bone X-ray examination was also utilized to compare the findings of rickets between the observation period and each treatment period (wk 24 and 48). The findings were evaluated as either improved, no change or aggravated based on the judgment of physicians.

Safety variables

Safety profiles were investigated using blood and urine tests. In addition, renal ultrasonography was employed to examine renal conditions, such as renal calcification and renal cysts. Furthermore, adverse events and the causal relationship between the interventions were investigated, with adverse events defined as any undesirable or unintended signs, symptoms or diseases that developed when the study drug was administered.

Ethical considerations

The present clinical trial was performed in accordance with ethical principles based on the Declaration of Helsinki and good clinical practice. The parents were well informed of the purpose, and potential risks and benefits of the study using a patient information sheet approved by the institutional review board. Written informed consent was obtained from these parents prior to enrollment. (ClinicalTrials.gov Identifier: NCT01237288)

Results

All 16 patients whose parents provided written informed consent were included in the efficacy and safety analysis sets, and all the patients completed the study. Drug compliance among the 16 patients generally exceeded 80%; but one patient achieved only 50 to 80% compliance with the prescribed treatment in a certain period of time during the treatment period; the patient had trouble remembering to take the drug. Table 2 shows the patient backgrounds. Fifteen out

Table 2 Patient background

Items		N=16
Sex	Male Female	3 13
Age (in years)	Mean ± SD Minimum – maximum	8.1 ± 3.0 3–14
Height (cm)	Mean ± SD Minimum – maximum	120.07 ± 17.78 86.8–156.2
Body weight (kg)	Mean ± SD Minimum – maximum	26.78 ± 11.48 14.2–60.2
Renal ultrasonography	Normal Abnormal (kidney calcification)	9 7
Use of oral sodium phosphate (pretreatment drugs)	Not used Used	1 15
Use of activated vitamin D formulation (pretreatment drugs)	Not used Used	0 16
Use of GH formulation*	Not used Used	9 7
Use of activated vitamin D formulation*	Not used Used	0 16
25-OH vitamin D value (ng/mL)	Mean ± SD Minimum – maximum	25.0 ± 5.5 16–38
1.25-$(OH)_2$ vitamin D value (pg/mL)	Mean ± SD Minimum – maximum	71.01 ± 32.05 18.0–132.0
FGF23 value (pg/mL)	Mean ± SD Minimum – maximum	319.5 ± 614.6 69–2600

* Formulation used during treatment period.

of 16 patients received oral sodium phosphate formulations prior to initiation of the study. All the patients had been treated with activated vitamin D formulations at a dose of 0.037 to 0.129 μg/kg/day. Also, 7 out of 16 patients received GH formulations for the treatment of short stature. FGF23 was determined during the observation period by SRL using an FGF23 ELISA Kit (Kainos Laboratories, Inc., Tokyo, Japan). Its values were over 30 pg/mL in all the patients, which is consistent with the characteristics of hereditary hypophosphatemic rickets defined in an accepted reference source (10).

Throughout this study, the dosage of Phosribbon® in all the patients ranged from 400 to 2,000 mg/day of phosphorus, which was equivalent to 19.7 to 59.9 mg/kg/day of phosphorus.

Efficacy evaluation

The proportion of patients who experienced successful intervention in terms of the levels of serum phosphorus for each period ranged from 37.5 to 81.3%, and the corresponding serum phosphorus values (mean ± standard deviation) ranged from 3.58 ± 0.70 to 4.07 ± 0.74 mg/dL, which exceeded the baseline of 2.86 ± 0.65 mg/dL. The mean relative values [95% confidence

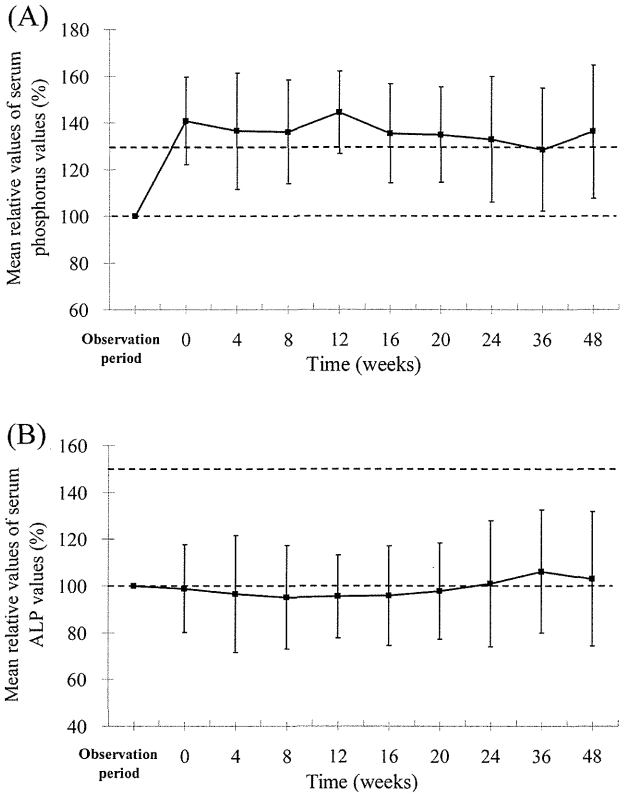

Fig. 1. Mean relative values of (A) serum phosphorus and (B) serum ALP. The value for the observation period was the pre-administration value, and the values for each period were those at 1–2 h post administration.

interval] (calculated on the assumption that the value at the observation period was 100%) ranged from 128.52% [114.46, 142.58] to 144.31% [134.89, 153.73] (Fig. 1-A).

In regard to serum ALP, the proportion ranged from 93.8 to 100%, and the corresponding serum ALP values ranged from 1,479.4 ± 472.8 to 1,620.1 ± 549.9 U/L, which were almost the same as those at baseline, i.e., 1,554.6 ± 494.3 U/L. The mean relative values ranged from 95.19% [89.68, 100.69] to 106.21% [91.86, 120.55] (Fig. 1-B).

Fifteen patients (59 times in total) met the dose increase and/or dose reduction criteria. Sorted by these criteria, 1 patient (twice in total) met the dose increase criteria for serum ALP, and 14 patients (52 times in total) met the dose increase criteria for serum phosphorus. By contrast, 5 patients (9 times in total) met the dose reduction criteria for intact PTH. Eight patients (13 times in total) experienced changes in dosages and administration patterns; 5 patients (7 times in total) required a dose increase, and 5 patients (6 times in total) required a dose reduction.

Comparison of bone X-ray examination findings of rickets between the observation period and each treatment period (wk 24 and 48) showed that 6 patients experienced improvement and that the remaining 10 patients underwent no change. No aggravation of the disease was observed.

Table 3 Adverse events developed in two or more patients

Name of events	N	(%)
Nasopharyngitis	9	56.3
Influenza	4	25.0
Pain in extremity	4	25.0
Upper respiratory tract inflammation	4	25.0
Diarrhoea	3	18.8
Abdominal pain	2	12.5
Seasonal allergy	2	12.5
Otitis media	2	12.5
Parotitis	2	12.5
Dermatitis atopic	2	12.5
Pharyngitis	2	12.5
Myalgia	2	12.5

MedDRA/J Ver.14.0.

Safety evaluation

Sixteen patients reported adverse events, but none of the patients died or experienced serious adverse events or adverse events requiring discontinuation of the study drug. Table 3 shows the adverse events that developed in 2 or more patients.

Two out of 16 patients experienced the following adverse drug reactions: 1 patient experienced abdominal pain and allergic dermatitis, and the other experienced diarrhea.

The two gastrointestinal adverse drug reactions (i.e., abdominal pain and diarrhea) were mild in severity and did not necessitate dose reduction or discontinuation of the study drug.

Allergic dermatitis was moderate in severity, but the intervention was continued because neither dose reduction nor discontinuation was required; the resultant dermatitis was not aggravated. We failed to deny a causal relation between the intervention and this reaction because the allergic dermatitis developed after initiation of the intervention.

The results of renal ultrasonography showed that 1 patient developed newly reported kidney calcification at wk 48. However, the event was not classified as an adverse event because the kidney calcification was not rapidly aggravated in comparison with the observation period. Some patients reported intact PTH levels of more than 130 pg/mL, but these values were within the range of physiological change, and no patient was diagnosed with hyperparathyroidism.

Discussion

Oral administration of sodium phosphate may result in the improvement of hypophosphatemia, which may lead to remission or improvement in clinical symptoms, as described in several previous reports (2, 3, 11). In Japan, however, no such treatment with an indication for management of hypophosphatemic rickets is covered by the health insurance system, and no integrated therapeutic method has been established.

Therefore, we conducted a study in which Phosribbon® was administered at a predetermined dosage and administration pattern to patients with hereditary hypophosphatemic rickets. Although some decisions made by the investigators deviated from predetermined criteria, we confirmed that administration of Phosribbon® elevated serum phosphorus values. The phosphorus-equivalent dosage of Phosribbon® employed in this study was the same as that described for other formulations in textbooks and in the package inserts of the oral sodium phosphate formulations used in other countries (2, 4–6, 12–15). The dosage and administration patterns were modified based on comprehensive considerations, including changes in serum phosphorus, serum ALP and intact PTH, and the doses of concomitantly administered activated vitamin D formulations, in accordance with the therapeutic guidelines for X-linked hypophosphatemia reported by Carpenter *et al.* (3). In particular, intact PTH was carefully monitored because its value may affect the safety profile. Also taken into account were the characteristics of individual patients by means of bone X-ray and renal ultrasonography.

As Phosribbon® is an oral sodium phosphate, care must be taken with regard to gastrointestinal disorders, hyperparathyroidism and kidney calcification, although the present study failed to find clinically significant issues related to these conditions.

Marketing approval for Phosribbon® may enable patients suffering from adverse drug reactions to derive benefits from the relief system for sufferers. In addition, the use of this approved formulation will obviate not only the need for the use of non-approved formulations and off-label use but also the associated needs for ethical review, informed consent acquisition, exclusive preparation and individual import. The present data suggest that Phosribbon® is suitable as a common treatment for hypophosphatemia and may be useful as a treatment for hereditary hypophosphatemic rickets.

Conclusions

The present study revealed that Phosribbon® combination granules are a safe and effective treatment for patients with hypophosphatemic rickets and that dose adjustment in this therapy can be guided by results of regular clinical examination and renal ultrasonography.

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We appreciate the contributions of all the patients and their parents to the study.

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Original Full Length Article

Functional analysis of mutant FAM20C in Raine syndrome with FGF23-related hypophosphatemia

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ABSTRACT

Raine syndrome is an autosomal recessive disorder characterized by generalized osteosclerosis with periosteal bone formation and a distinctive facial phenotype. Either homozygous or compound heterozygous mutations in *family with sequence similarity 20, member C* (*FAM20C*) have been reported to cause this syndrome. Recently, it was reported that fibroblast growth factor 23 (FGF23)-related hypophosphatemia was found in patients with non-lethal Raine syndrome, and *Fam20c* conditional knockout mice presented Fgf23-related hypophosphatemic rickets. To clarify the mechanism of how FAM20C regulates FGF23, we performed functional analysis of mutant FAM20C proteins reported in Raine syndrome. We analyzed 6 mutant FAM20C proteins (T268M, P328S, R408W, D451N, D478A, and R549W) for their distributions, kinase activities, and effects on *dentin matrix protein* (*DMP1*) promoter activity. We also analyzed the effect of *Fam20c* knockdown on *Dmp1* and *Fgf23* mRNA levels in UMR-106 cells. As a result, all the mutant FAM20C proteins showed decreased kinase activities compared to wild-type (WT) FAM20C, and most of them also showed impaired secretion. Overexpression of WT FAM20C increased *DMP1* promoter activity in Saos-2 cells while mutant FAM20C did not. *Fam20c* knockdown decreased *Dmp1* mRNA and increased *Fgf23* mRNA in UMR-106 cells. In conclusion, our results suggest that FAM20C suppresses FGF23 production by enhancing DMP1 expression, and inactivating mutations in *FAM20C* cause FGF23-related hypophosphatemia by decreasing transcription of *DMP1*.

Introduction

Raine syndrome is an autosomal recessive disorder characterized by generalized osteosclerosis with periosteal bone formation and a distinctive facial phenotype. It was first reported in 1989 by Raine et al. as a lethal osteosclerotic bone dysplasia [1]. Later, Simpson et al. found that mutations in *family with sequence similarity 20, member C* (*FAM20C*) is responsible for this syndrome [2], and non-lethal cases were reported afterwards [3–5].

FAM20C is a member of a four-jointed family of protein kinases. Four-jointed is a kinase that localizes to the Golgi in *Drosophila* and phosphorylates the secretory proteins [6]. Among four-jointed family members, only FAM20C and FAM20A have been shown to be associated with human diseases. FAM20A is one of the close relatives of FAM20C in a phylogenic tree, and is also a protein kinase. FAM20A is expressed mainly in the parathyroid glands and the cells comprising the tooth and gingivae, such as ameloblasts, odontoblasts, dental pulp cells, and suprabasal cells of the gingivae, and mutations in *FAM20A* cause amelogenesis imperfecta (AI) and gingival hyperplasia syndrome in humans [7].

FAM20C was shown to be expressed in a wide variety of tissues [8]. In the skeletal and dental tissues, FAM20C was detected in the osteoblasts, odontoblasts, and ameloblasts, and was proposed to act as an inhibitor for mineralization during osteogenesis [9]. It is also shown that secretory calcium-binding phosphoprotein (SCPP) members whose genes cluster on chromosome 4 in humans, have multiple S-x-E/pS motifs and are phosphorylated by FAM20C [10]. The small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) are a subfamily of the SCPPs and comprise dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), osteopontin (OPN), matrix extracellular phosphoglycoprotein (MEPE), and bone sialoprotein (BSP). The contribution of these proteins to mineralization is complicated. Some proteins such as OPN are considered to inhibit the mineralization process [11], while others such as DMP1 are reported to promote tissue mineralization [12]. Therefore, these phosphorylated secretory proteins may regulate proper growth/formation of hydroxyapatite crystals. Actually, abnormal phosphorylation of these proteins has been shown to be responsible for the deranged mineralization in Raine syndrome [13,14].

Besides the abnormal mineralization, the association with fibroblast growth factor 23 (FGF23)-related hypophosphatemia has been shown in patients with Raine syndrome. In 2013, whole-exome sequencing revealed compound heterozygous mutations in *FAM20C* in Norwegian brothers with FGF23-related hypophosphatemia, dental anomalies, and ectopic calcifications [5].

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FGF23 is a hormone that regulates phosphate homeostasis [15]. Several types of FGF23-related congenital hypophosphatemic rickets have been reported. Among them, X-linked hypophosphatemic rickets (XLHR: OMIM#307800) 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 [16]. Autosomal dominant hypophosphatemic rickets (ADHR: OMIM#193100) is caused by missense mutations in the *FGF23* gene [17], which prevent the proteolytic cleavage of FGF23 protein into inactive fragments [18,19], and autosomal recessive hypophosphatemic rickets 1 and 2 (ARHR1: OMIM#241520 and ARHR2: OMIM#613312) are caused by inactivating mutations in *DMP1* [20,21] and *ectonucleotide pyrophosphatase/phosphodiesterase 1* (*ENPP1*) [22,23], respectively.

Along with the FGF23-related hypophosphatemia in the Norwegian brothers with compound heterozygous mutations in *FAM20C* [5], coexisting hypophosphatemia have been reported in other non-lethal patients with Raine syndrome [3,4]. These results suggest the possibility that *FAM20C* is another candidate gene that regulates FGF23 production. In addition, *in vivo* analysis of *Fam20c* knockout mice confirmed the association between Fam20c and Fgf23 [24]. *Fam20c* conditional knockout mice, in which exons 6–9 were ablated, developed Fgf23-related hypophosphatemic rickets but not osteosclerosis [24]. It is noteworthy that down regulation of *Dmp1* was observed in the calvaria of *Fam20c* conditional knockout mice [24]. Since DMP1 is a target for phosphorylation by FAM20C [13] and *Dmp1* knockout mice present Fgf23-related hypophosphatemic rickets [20], Dmp1 might be a key regulator of Fgf23 in *Fam20c* knockout mice. However, it is not clear how mutations in *FAM20C* result in elevation of FGF23 in patients with Raine syndrome.

Therefore, we performed functional analysis of mutant FAM20C proteins to clarify the mechanism of FGF23-related hypophosphatemia in patients with Raine syndrome.

Methods

Construction of vectors

Human *FAM20C* cDNA was amplified by RT-PCR from peripheral blood leukocytes and cloned into pcDNA 3.1/*myc*-His A (Invitrogen) with In-Fusion HD Cloning Kit (Clontech). Human *OPN* cDNA was amplified by PCR with pDest490-OPN-a (Addgene plasmid 17590) as a template and similarly cloned into pSecTag/FRT/V5-His-TOPO vector (Invitrogen). Human *DMP1* cDNA was amplified by RT-PCR from peripheral blood leukocytes and cloned into either pcDNA 3.1/*myc*-His A or pcDNA3 mCherry LIC cloning vector (6B) (Addgene plasmid 30125) with In-Fusion HD Cloning Kit (Clontech). Site-specific mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (TAKARA BIO), PrimeSTAR HS DNA polymerase with GC Buffer (TAKARA BIO), or Tks Gflex DNA polymerase (TAKARA BIO). Primer sequences are available upon request.

For construction of human *DMP1* promoter, the promoter region of *DMP1* gene, −3 base to −501 base from the ATG start codon, and about a 2 kb region in the intron 3 which includes multiple transcription factor binding sites [UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly, Transcription Factor ChIP-seq from ENCODE] were amplified by PCR and cloned into pGL3-Basic vector (Invitrogen) with In-Fusion HD Cloning Kit (Clontech) (Fig. 1).

Functional analysis of FAM20C

HEK293 cells were plated in 12-well dishes to 70–80% confluency and were transfected with 0.2 μg pcDNA 3.1/*myc*-His A-*FAM20C* (or mutants) and either 0.8 μg pcDNA 3.1/*myc*-His A-*OPN* or 0.8 μg pcDNA3 mCherry *DMP1* with 2.5 μl FuGENE-HD (Promega) as recommended by the manufacturer. The media were replaced with 1 ml Opti-MEM Reduced Serum Medium (Gibco) 24 h after transfection. Conditioned media were removed 48 to 72 h after transfection and centrifuged at 1000 ×*g* for 5 min to remove cell debris. Cells were washed twice with PBS and lysed with 500 μl Opti-MEM reduced serum medium (Gibco), 55 μl FastBreak cell lysis reagent (Promega), 5 μl protease inhibitor, and 0.5 μl DNaseI for 20 min. The cell lysate was centrifuged at 13,000 ×*g* for 10 min and the supernatant was collected. The MagneHis Protein Purification System (Promega) was used to purify polyhistidine-tagged FAM20C and OPN as recommended by the manufacturer. 10 μl from 100 μl of the elutant was separated by SDS-PAGE and transferred to a PVDF membrane, which was blocked in ImmunoBlock (DS Pharma Biomedical) and probed with either anti-c-Myc antibody (9E10, Santa Cruz Biotechnology) for FAM20C or V5 Tag antibody (GTX117997, GeneTex) for OPN overnight at 4 °C. The membrane was then incubated with either anti-mouse IgG antibody or anti-rabbit IgG antibody with horseradish peroxidase (GE Healthcare) for 1 h at room temperature. The ECL Select Western Blotting Detection Reagent (GE Healthcare) was used for chemiluminescent detection.

It has been shown that phosphorylated and unphosphorylated peptides can be separated in agarose gel electrophoresis by fluorescent labeling [25]. To analyze the phosphorylation of fluorescence-tagged DMP1, 1 ml of the conditioned medium was concentrated with Amicon Ultra-4 (Millipore) at 4000 ×*g* for 8 min. 20 μl of the concentrate was agarose gel electrophoresed and fluorescence-tagged DMP1 was detected with UV light and photographed on a trans-illuminator.

Endoplasmic reticulum (ER) stress associated with FAM20C overexpression

It has been shown that the mutant FAM20C proteins with impaired secretion mislocalized from the Golgi to the ER [14]. Thus, we assessed

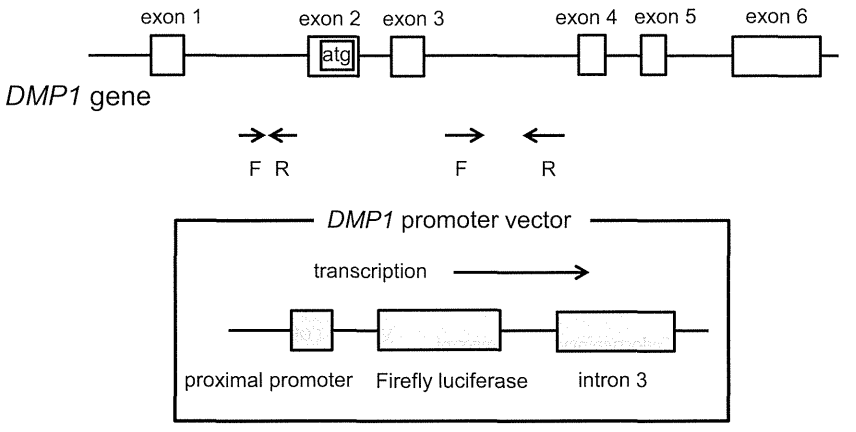

Fig. 1. Construction of homo *DMP1* promoter vector. Proximal promoter region of human *DMP1* gene, −3 base to −501 base from the ATG start codon, and about 2 kb region in intron 3 were amplified by PCR and cloned into pGL3-Basic vector. Arrows indicate primers used for PCR amplification of both regions. F, forward primer: R, reverse primer.

ER stress in the cells overexpressing WT and mutant FAM20C proteins using a luciferase reporter vector, p5xATF6-GL3 (Addgene plasmid 11976) [26]. HEK293 cells were plated at 10×10^4 cells/well in 24-well dishes in DMEM (Gibco) plus 10% FBS, and incubated overnight. Cells were then transiently co-transfected with 0.1 μg vectors expressing either wild-type (WT) or mutant *FAM20C*, 0.4 μg p5xATF6-GL3, and 2.5 ng Renilla luciferase control reporter vector (pRL-TK) (Promega), using 1.25 μl FuGENE HD (Promega). After 48 h, cells were washed twice with PBS and lysed in 100 μl/well Passive Lysis Buffer (Promega). Relative luciferase activity, the ratio of firefly luciferase to Renilla luciferase activity, was measured in a Centro LB960 (Berthold Technologies) using the Dual-Luciferase Reporter Assay System (Promega).

The effect of FAM20C overexpression on DMP1 promoter activity

To examine the effect of WT and mutant FAM20C proteins on *DMP1* transcription in the osteoblastic lineage, we used human osteosarcoma Saos-2 cells. Saos-2 cells were plated at 10×10^4 cells/well in 24-well dishes in alpha-MEM (Gibco) plus 10% FBS, and incubated overnight. Cells were then transiently co-transfected with 0.1 μg vectors expressing either WT or mutant *FAM20C*, 0.4 μg *DMP1* promoter vector and 2.5 ng pRL-TK (Promega) using 2 μl FuGENE HD (Promega). After 48 h, relative luciferase activity was measured as previously described. To examine the effect of inhibitors, conditioned media were changed 4 h after transfection for serum free alpha-MEM containing either a MEK inhibitor U0126, a focal adhesion kinase (FAK) inhibitor PF573228, or DMSO, and incubated for an additional 48 h.

The effect of Fam20c knockdown on Dmp1 mRNA and Fgf23 mRNA

1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] has been shown to increase *Fgf23* mRNA in UMR-106 cells [27] and also increase *Fgf23* promoter activity in ROS cells [28]. It has been shown that *Dmp1* is expressed in UMR-106 cells [29] and we found that *Fam20c* is also expressed in this cell line. Thus, we analyzed the effect of *Fam20c* knockdown on *Dmp1* and *Fgf23* mRNA in UMR-106 cells with or without $1,25(OH)_2D_3$ treatment.

UMR-106 cells were plated at 5×10^4 cells/well in 24-well dishes in DMEM plus 10% FBS, and incubated overnight. Cells were then transiently co-transfected with 20 pmol of either *Fam20C* siRNA (rat), SR511577 (ORIGENE) or control siRNA, SR30004 (ORIGENE), using 10 μl siGENE (Promega). After 24 h, cells were treated with 1 nM $1,25(OH)_2D_3$ or ethanol. After 48 h of incubation with $1,25(OH)_2D_3$, total RNA was extracted using NucleoSpin RNA (Macherey-Nagel) and reverse-transcribed to cDNA using PrimeScript RT Master Mix (TAKARA BIO). Real time PCR was performed with cDNA equivalent to 50 ng total RNA in a StepOnePlus (Applied Biosystems) using SYBR Premix Ex Taq (TAKARA BIO). *Gapdh* was used as an internal control.

The effect of DMP1 overexpression on Fgf23 mRNA

UMR-106 cells were plated at 5×10^4 cells/well in 24-well dishes in DMEM plus 10% FBS, and incubated overnight. Cells were transiently transfected with 0.5 μg vectors expressing *DMP1* or pcDNA 3.1/*myc*-His A for control using 1.25 μl FuGENE HD (Promega). After 24 h, cells were treated with either 1 nM or 10 nM $1,25(OH)_2D_3$. After 48 h of incubation with $1,25(OH)_2D_3$, total RNA was extracted and real time PCR was performed as previously described.

Data analysis

The differences between the groups were analyzed by ANOVA followed by Bonferroni–Dunn test. $P < 0.05$ was considered statistically significant.

Results

Functional analysis of FAM20C

The kinase domain of FAM20C protein is from 354 to 565 amino residues, and D478A [13] is an artificially created kinase-dead mutant (Fig. 2A). In addition to this mutant, we analyzed 4 types of mutant proteins (T268M, P328S, R408W, and D451N) which were found in patients with non-lethal Raine syndrome who also showed hypophosphatemia [3–5]. We also included one mutant protein (R549W) which was found in a patient with lethal Raine syndrome [2]. When expressed in HEK293 cells, WT FAM20C protein was secreted and found in the media (Fig. 2B). T268M and D478A mutants were similarly secreted into the media as WT FAM20C. However, secretion was impaired in R408W, and was almost completely blocked in D451N, P328S, and R549W mutant proteins. This impaired secretion was confirmed by evaluating ER stress in cells expressing these mutant FAM20C proteins. ER stress was significantly elevated in cells transfected with the mutant FAM20C proteins that were not secreted in the media (Fig. 3).

When OPN was co-expressed with WT FAM20C in HEK293 cells, the molecular weight of the secreted OPN in the media was higher than that without FAM20C, indicating that WT FAM20C phosphorylated OPN (Fig. 2B). In contrast, the kinase-dead D478A mutant did not change the molecular weight of OPN. When co-expressed with other mutant FAM20C proteins, changes of molecular weight of OPN were negligible or less than that with WT FAM20C. We also analyzed the kinase activities of the mutant FAM20C proteins with a different method using DMP1 to confirm these findings. The band of fluorescence-tagged DMP1 moved more efficiently toward the anode when co-expressed with WT FAM20C compared to that with control empty vector or mutant FAM20C proteins (Fig. 2C). Relative migration distances of OPN and DMP1, which were the ratio of the migration distances of OPN or DMP1 co-expressed with mutant FAM20C to that with WT FAM20C, showed that only WT FAM20C could fully phosphorylate OPN and DMP1. All the mutant FAM20C proteins had less kinase activities and the relative migration distance was less than 0.5 (Fig. 2D).

The effect of FAM20C overexpression on DMP1 promoter activity

We then utilized human osteoblastic cell line, Saos-2, to investigate the effect of FAM20C on DMP1 production. In Saos-2 cells, co-transfection with the WT *FAM20C* expression vector increased *DMP1* promoter activity compared to that with control empty vector (Fig. 4A). However, co-transfection with either R408W, T268M, or D478A expression vector did not increase *DMP1* promoter activity. A MEK inhibitor U0126 diminished the positive effect of WT FAM20C on *DMP1* promoter activity, while a FAK inhibitor PF573228 did not (Fig. 4B). These results suggest that FAM20C enhances *DMP1* transcription in osteoblastic cells *via* extracellular signal-regulated kinase (ERK) signaling pathway but not *via* integrin signaling pathway, and that FAM20C kinase activity is necessary for its stimulatory effect on *DMP1* transcription.

The effect of Fam20c knockdown on Dmp1 and Fgf23 mRNA

Finally, we analyzed the relationship between FAM20C and FGF23. For this purpose, it was necessary to utilize cells that can produce FGF23. Therefore, we adopted UMR-106 cells that were already known to express *Fgf23* mRNA.

Fam20c knockdown with siRNA decreased *Fam20c* mRNA by approximately 50% (Fig. 5A). Treatment with 1 nM $1,25(OH)_2D_3$ significantly increased both *Dmp1* and *Fgf23* mRNA in UMR-106 cells (Figs. 5B, C). *Fam20c* knockdown with siRNA significantly decreased *Dmp1* mRNA levels compared to control siRNA in both 0 nM and 1 nM $1,25(OH)_2D_3$ treatment groups (Fig. 5B), and significantly increased *Fgf23* mRNA in the 1 nM $1,25(OH)_2D_3$ treatment group (Fig. 5C).

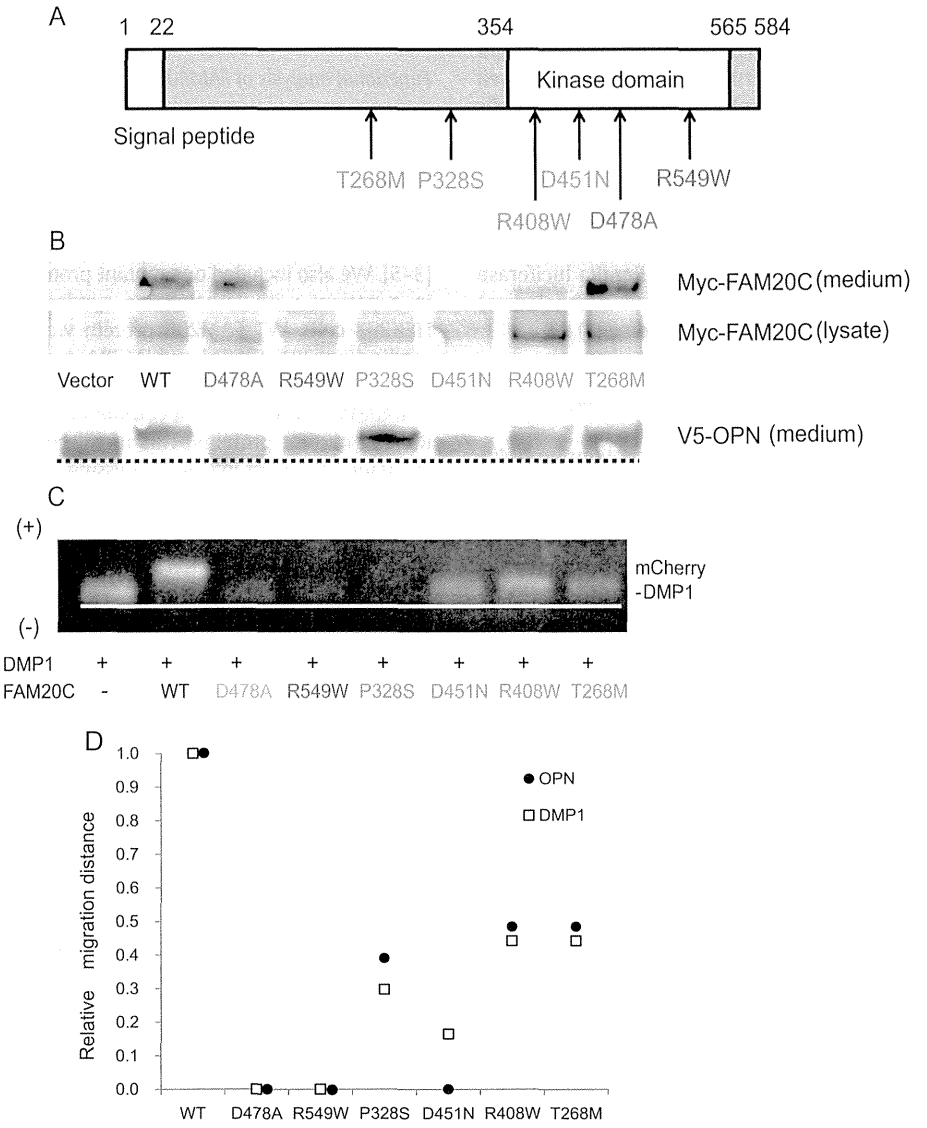

Fig. 2. A, Schematic structure of FAM20C. We analyzed 6 mutant FAM20C proteins. Non-lethal mutations with hypophosphatemia, T268M, P328S, R408W, and D451N, are shown in green, a lethal mutation R549W in red, and an artificially created kinase-dead mutation D478A in blue. B, *In vitro* analysis of WT and mutant FAM20C proteins. *myc*-His-tagged FAM20C and V5-His-tagged OPN were co-expressed in HEK293 cells. Conditioned media and lysate were purified by His-tag, subjected to SDS-PAGE, and probed with either anti-c-Myc or V5-tag antibody, and visualized by horseradish peroxidase conjugated secondary antibody. T268M and D478A mutants were similarly secreted into the media as WT FAM20C, but secretion was impaired in R408W, and was almost completely blocked in D451N, P328S, and R549W mutant proteins. The molecular weight of OPN co-expressed with WT FAM20C was higher than that with empty vector, indicating that WT FAM20C phosphorylated OPN. Kinase-dead D478A mutant did not change the molecular weight of OPN. Other mutant FAM20C proteins change the molecular weight of OPN less efficiently compared to WT FAM20C. C, Concentrated conditioned media were analyzed by agarose gel electrophoresis and fluorescence-tagged DMP1 was detected with UV light. The band of DMP1 co-expressed with WT FAM20C moved more efficiently toward the anode compared to that with empty vector or mutant FAM20C, indicating that DMP1 had more negative charge when phosphorylated by WT FAM20C. D, Relative migration distances of OPN and DMP1, which were the ratio of the migration distances of OPN or DMP1 co-expressed with mutant FAM20C to that with WT FAM20C, showed that only WT FAM20C could fully phosphorylate OPN and DMP1. The average scores of two independent studies were shown.

The effect of DMP1 overexpression on Fgf23 mRNA

In order to further investigate the involvement of DMP1 in FGF23 production, we examined the effect of overexpression of *DMP1* in UMR-106 cells. *DMP1* overexpression significantly decreased *Fgf23* mRNA in the 10 nM $1,25(OH)_2D_3$ treatment group (Fig. 5C).

Discussion

Previous studies have reported that mutant FAM20C proteins found in patients with Raine syndrome showed either decreased kinase activity, impaired secretion, or both [13,14]. To clarify the characteristics of mutant FAM20C proteins which are important in deciding the phenotypes of patients, such as lethal or non-lethal, and normophosphatemic or hypophosphatemic, we performed *in vitro* analysis of six types of mutant FAM20C proteins on their distributions and kinase activities. Among them, T268M, P328S, R408W, and D451 are found in patients with nonlethal Raine syndrome accompanied by hypophosphatemia [3–5]. R549W is found in a patient with lethal Raine syndrome [2], and D478A is an artificially created kinase-dead mutant [13]. Functional analysis showed that all the mutant FAM20C proteins have less kinase activities compared to WT FAM20C, which suggested that mutations that result in impaired kinase activities are pathogenic. Some of the mutant proteins also showed impaired secretion from cells. However, there seems to be no correlation between the *in vitro* secretion of FAM20C proteins and their kinase activities. For example, the kinase dead D478A mutant was secreted in the media, but for mutant proteins with partially preserved

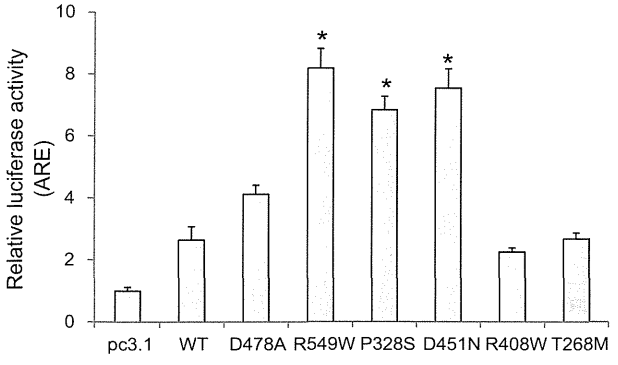

Fig. 3. ER stress associated with *FAM20C* overexpression. Relative luciferase activity represented ER stress caused by *FAM20C* overexpression. ER stress was significantly elevated in cells with R549W, P328S, and D451N mutant FAM20C proteins that were not secreted in the media (*P < 0.01 greater than WT). Values shown are the mean + SEM (n = 4).

kinase activities, such as P328S and D451N, secretion was completely lost. Moreover, *in vivo* analysis using pig ameloblasts and odontoblasts has revealed that FAM20C localizes intracellularly in the Golgi and is not present in the extracellular matrix during enamel and dentin biomineralization [30], which suggested that *in vitro* secretion of FAM20C into the media may be just an artifact of overexpression. Therefore, it is possible that the loss of *in vitro* secretion into the media represents the misfolding of mutant FAM20C proteins and their mislocalization from the Golgi to the ER. Actually, ER stress was elevated in cells expressing mutant FAM20C proteins with impaired secretion, such as P328S, D451N, and R549W. In addition, there seems to be no genotype–phenotype correlation because FAM20C with a lethal R549W mutation showed similar defects to FAM20C with other non-lethal mutations.

The findings that *Fam20c* knockout mice showed Fgf23-related hypophosphatemic rickets and down regulation of *Dmp1* was observed in their calvaria suggested that loss of Dmp1 activity leads to Fgf23 elevation in *Fam20c* knockout mice [14]. To clarify the relationship between FAM20C and DMP1, we constructed a *DMP1* promoter vector and examined the effect of WT or mutant *FAM20C* expression on *DMP1* transcription. As a result, *DMP1* promoter activity was significantly elevated with WT *FAM20C* expression in Saos-2 cells, but neither with R408W, D478A, nor T268M mutant *FAM20C* expression. The ChIP-seq data from ENCODE project and TFBIND, a software for searching transcription factor binding sites (http://tfbind.hgc.jp/), showed that there are multiple consensus sites for AP-1 components, such as c-Fos, c-Jun, and JunD, or p53 in both *DMP1* proximal promoter and intron 3. When co-transfected with WT *FAM20C* expression vector, the reporter vector containing both the proximal promoter and a 2 kb region of the intron 3 of the *DMP1* gene showed a larger increase in luciferase activity compared to the vector containing the proximal promoter alone (data not shown). We assumed that the intron 3 of the *DMP1* gene serves as a transcriptional enhancer, so we used the vector containing both the proximal promoter and the intron 3 for the analysis. Our findings that a MEK inhibitor U0126 diminished the stimulatory effect of WT FAM20C on *DMP1* promoter activity, while a FAK inhibitor PF573228 did not, suggest that the activation of ERK signaling but not integrin signaling pathway is important in the up-regulation of *DMP1* transcription by FAM20C. Further study is needed to clarify how FAM20C activates the ERK signaling pathway.

In our study, $1,25(OH)_2D_3$ increased both *Dmp1* and *Fgf23* mRNA as previously reported [29]. It was suggested that the increased *Dmp1 mRNA* by $1,25(OH)_2D_3$ is a part of a negative feedback loop to repress elevated Fgf23 [29]. In addition, *Fam20c* knockdown by siRNA resulted in decreased *Dmp1* mRNA and increased *Fgf23* mRNA in UMR-106 cells treated with $1,25(OH)_2D_3$, which suggested that FAM20C promotes *DMP1* expression and in turn decreases *FGF23* expression. Finally, we showed that overexpression of *DMP1* resulted in decreased *Fgf23* mRNA in UMR-106 cells, which confirmed that DMP1 has a negative effect on *Fgf23* mRNA.

Extracellular DMP1 has been shown to bind to integrin family members with its RGD motif [31]. Integrin family members are membrane receptors involved in cell adhesion, and DMP1 has been shown to activate intracellular FAK and MAPK signaling pathways, which involves phosphorylation of ERK, JNK, and c-Jun [31]. Recombinant DMP1 in the conditioned media has been shown to reduce *Fgf23* expression induced by $1,25(OH)_2D_3$ in UMR-106 cells [32], which indicated that DMP1 extracellularly regulates *Fgf23* expression. However, when we added a FAK inhibitor, PF573228, to UMR-106 cells with *DMP1* overexpression, we could not recover decreased *Fgf23* expression (data not shown). Some reports have shown that DMP1 could serve as a transcriptional factor in the nucleus: DMP1 activates the expression of the *DSPP* gene through binding to the *DSPP* promoter in an odontoblast-like cell line T4-4 [33] and the C-terminal region of DMP1 is considered to be important in its binding to DNA. Thus, it is possible that nuclear localized DMP1 directly regulates FGF23 transcription and that integrin signaling pathway is not mainly involved in the regulation of FGF23 by DMP1. Further studies are required to clarify the mechanism of how DMP1 regulates *FGF23* expression.

A recent study demonstrated that *Dmp1* transgene failed to rescue the bone and dentin phenotypes, and did not decrease serum FGF23 levels of *Fam20c* conditional knockout mice [34]. These results seem to suggest that reduced expression of DMP1 does not explain phenotypes

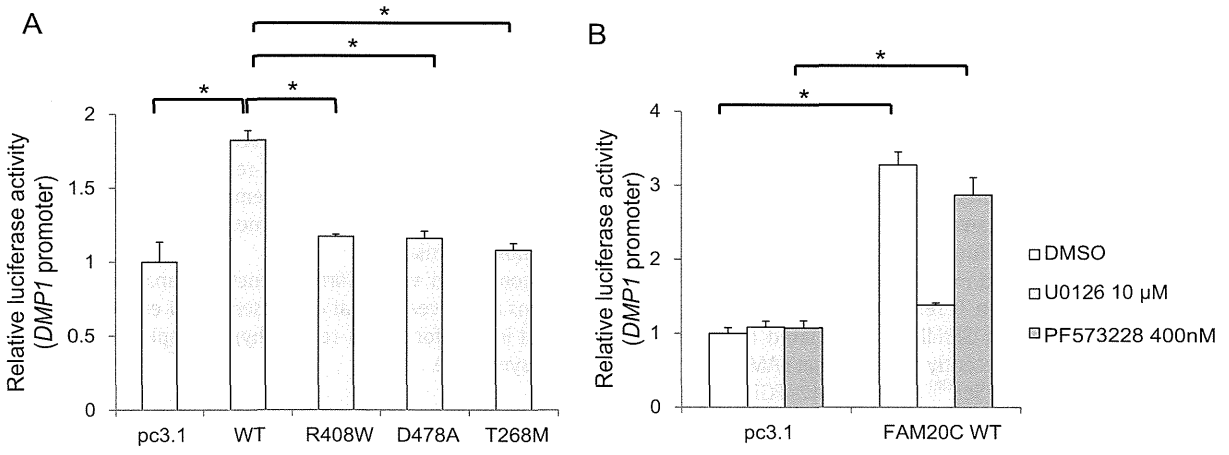

Fig. 4. A, The effect of *FAM20C* overexpression on *DMP1* promoter activity. Co-transfection with WT *FAM20C* expression vector increased *DMP1* promoter activity compared to that with control empty vector in Saos-2 cells. However, co-transfection with either R408W, T268M, or D478A expression vector did not increase *DMP1* promoter activity (*P < 0.001). Values shown are the mean + SEM (n = 4). B, A MEK inhibitor U0126 diminished the stimulatory effect of WT FAM20C on *DMP1* promoter activity, while a FAK inhibitor PF573228 did not.

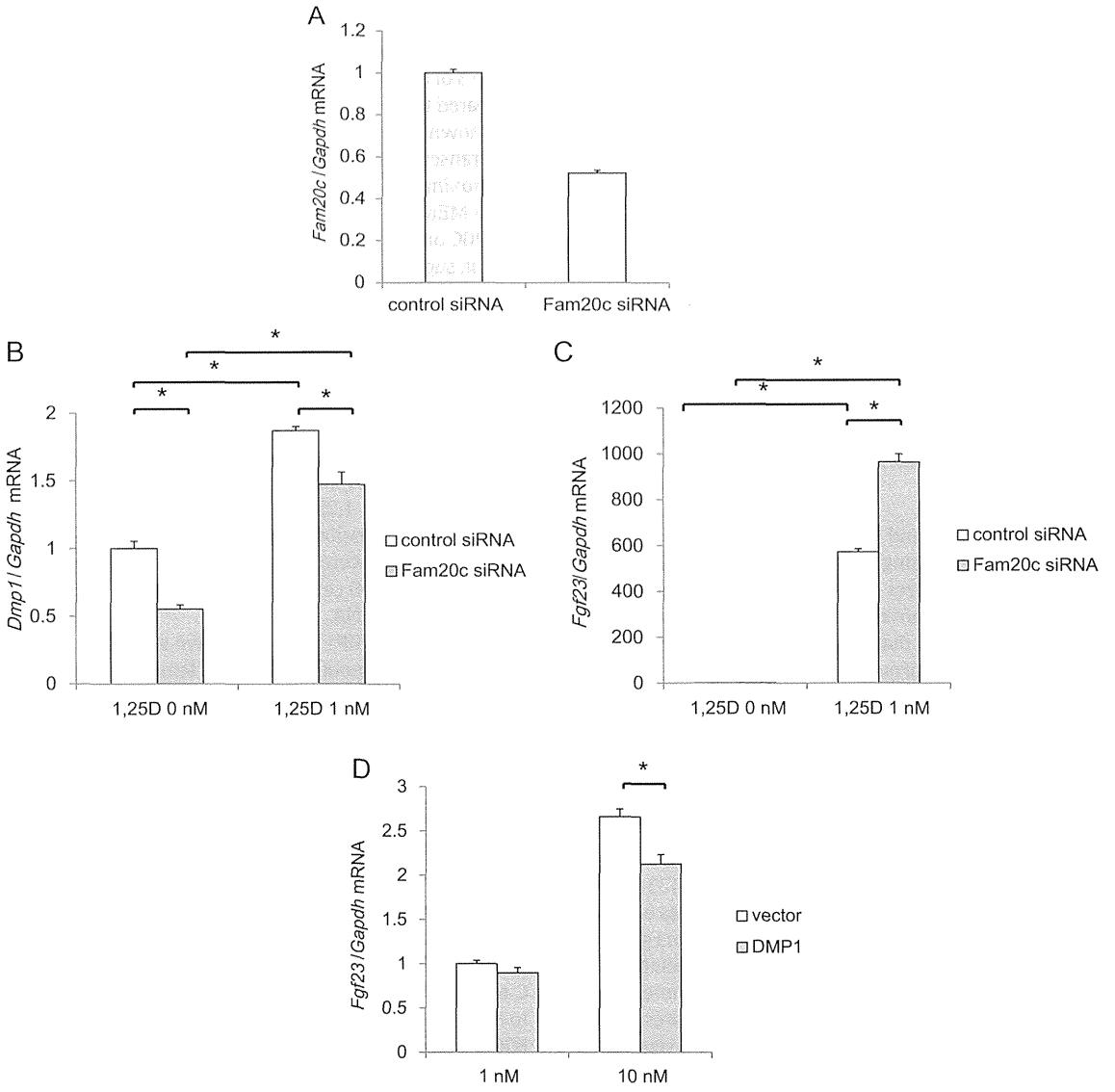

Fig. 5. A, *Fam20c* siRNA decreased *Fam20c* mRNA by approximately 50%. Values shown are the mean + SEM (n = 4). B, The effect of *Fam20c* knockdown on *Dmp1* mRNA levels in UMR-106 cells treated with $1,25(OH)_2D_3$ (1,25D). $1,25(OH)_2D_3$ significantly increased *Dmp1* mRNA. *Fam20c* knockdown with siRNA significantly decreased *Dmp1* mRNA in both 0 nM and 1 nM $1,25(OH)_2D_3$ treatment groups (*P < 0.001). Values shown are the mean + SEM (n = 4). C, $1,25(OH)_2D_3$ significantly increased *Fgf23* mRNA in UMR-106 cells. *Fam20c* knockdown with siRNA increased *Fgf23* mRNA in the 1 nM $1,25(OH)_2D_3$ treatment group (*P < 0.001). Values shown are the mean + SEM (n = 4). D, The effect of *DMP1* overexpression on *Fgf23* mRNA. *DMP1* overexpression significantly decreased *Fgf23* mRNA in UMR-106 cells treated with 10 nM $1,25(OH)_2D_3$ (*P < 0.001). Values shown are the mean + SEM (n = 4).

in patients with mutations in *FAM20C*. However, it is also likely that the expressed Dmp1 in this study is not correctly phosphorylated because they overexpressed *Dmp1* in *Fam20c* conditional knockout mice. As mutant FAM20C proteins showed impaired kinase activity, we assume that both decreased *DMP1* expression and abnormal phosphorylation of DMP1 at least partly explain the elevation of FGF23 by mutant FAM20C. In Raine syndrome, decreased FAM20C kinase activity may lead to osteosclerosis and dental anomalies by impaired phosphorylation of SIBLINGs that regulate biomineralization, and FGF23-related hypophosphatemia by decreased expression of *DMP1* and impaired phosphorylation of DMP1 protein. It remains to be clarified whether phosphorylation defect of other SIBLINGs are related to increased expression of FGF23. It was also recently reported that FAM20C directly phosphorylates FGF23 protein on Ser^{180} and promotes FGF23 cleavage, and that mutant FAM20C with decreased kinase activity leads to elevation of intact FGF23 [35]. This mechanism may also contribute to the development of hypophosphatemia in patients with mutant *FAM20C*. However, microarray analyses and real time PCR using the calvaria of *Fam20c* conditional knockout mice showed that *FGF23* mRNA was elevated by more than 100 folds compared to that of WT mice [24]. Therefore, it is unlikely that the abnormal posttranslational modification of FGF23 protein is the only mechanism for high FGF23 levels in patients with mutations in *FAM20C*.

Since Raine syndrome has been recognized only from its osteosclerotic aspect, its association with FGF23-related hypophosphatemia has not been fully examined. Therefore, it remains to be clarified whether or not all the patients with non-lethal Raine syndrome develop FGF23-related hypophosphatemia.

In conclusion, we performed functional analysis of mutant FAM20C proteins and revealed that decreased *DMP1* expression is responsible at least in part for FGF23-related hypophosphatemia in patients with Raine syndrome.

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Diagnostic Modalities for FGF23-Producing Tumors in Patients with Tumor-Induced Osteomalacia

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Fibroblast growth factor 23 (FGF23) is a hormone that is produced by osteocytes and regulates phosphate and vitamin D metabolism through binding to the Klotho-FGF receptor complex. Excessive actions of FGF23 cause several kinds of hypophosphatemic rickets/osteomalacia. Tumor-induced rickets/osteomalacia (TIO) is a paraneoplastic syndrome caused by overproduction of FGF23 from the responsible tumors. Because TIO is cured by complete resection of the causative tumors, it is of great clinical importance to locate these tumors. Several imaging methods including skeletal survey by magnetic resonance imaging and octreotide scintigraphy have been used to identify the tumors that cause TIO. However, none of these imaging studies indicate that the detected tumors are actually producing FGF23. Recently, systemic venous sampling was conducted for locating FGF23-producing tumor in suspected patients with TIO and demonstrated that this test might be beneficial to a subset of patient. Further studies with more patients are necessary to establish the clinical utility of venous sampling in patients with TIO.

Keywords: Fibroblast growth factor 23; Hypophosphatemia; 1,25-Dihydroxyvitamin D; Sampling

INTRODUCTION

Rickets and osteomalacia are diseases characterized by impaired mineralization of bone matrix. While rickets and osteomalacia are caused by the same etiologies, rickets develops in children before the closure of growth plates. There are diverse causes of rickets and osteomalacia. However, chronic hypophosphatemia underlines most cases of rickets and osteomalacia. Fibroblast growth factor 23 (*FGF23*) was identified as a responsible gene for autosomal dominant hypophosphatemic rickets (ADHR) by positional cloning [1]. Almost simultaneously, FGF23 was cloned as a causative humoral factor for tumor-induced rickets/osteomalacia (TIO), a paraneoplastic syndrome with hypophosphatemia [2]. Since then, it has been shown that FGF23 is produced mainly by osteocytes, binds to the Klotho-FGF receptor complex and works as a phosphotropic hormone as a physiological regulator of serum phosphate level [3]. In this review, diseases caused by excessive actions of FGF23 are summarized with an emphasis on TIO.

ACTIONS OF FGF23

FGF23 is one of the FGF family members which are defined as humoral factors with a FGF homology region [4]. There are 22 FGF family members in humans and these FGF family members are divided into several subfamilies. FGF23 belongs to the FGF19 subfamily together with FGF19 and FGF21 [4]. After the cloning of *FGF23*, actions of FGF23 were examined using the recombinant FGF23 protein. Single injection of recombinant FGF23 in mice caused reduction in serum phos-

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phate and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] levels [5]. Serum phosphate level is mainly determined by renal handling of phosphate. Most phosphate filtered from glomeruli is reabsorbed in proximal tubules by the actions of type 2a and 2c sodium-phosphate cotransporters (NaPi-2a, 2c). FGF23 reduces the expression of these cotransporters and inhibits phosphate reabsorption [5]. At the same time, FGF23 modifies the expression of vitamin D-metabolizing enzymes. The 1,25$(OH)_2$D is produced from 25-hydroxyvitamin D [25(OH)D] by the action of 25(OH)D-1α-hydroxylase. On the other hand, 25(OH)D-24-hydroxylase converts 25(OH)D to 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] and also 1,25$(OH)_2$D to 1,24,25-trihydroxyvitami D [1,24,25$(OH)_3$D]. Therefore, 25(OH)D-1α-hydroxylase is an enzyme that increases serum 1,25$(OH)_2$D, while 25(OH)D-24-hydroxylase works to reduce serum 1,25$(OH)_2$D. FGF23 reduces 1,25$(OH)_2$D levels by suppressing the expression of 25(OH)D-1α-hydroxylase and also enhancing 25(OH)D-24-hydroxylase expression [5]. Because 1,25$(OH)_2$D increases intestinal phosphate absorption, FGF23 decreases serum phosphate by suppressing both proximal tubular reabsorption and intestinal phosphate absorption. FGF23 is produced by bone [6] and works in the kidney, indicating that there should be a specific receptor for FGF23 in the kidney. While the affinity of FGF23 to canonical FGF receptors is low, it was shown that FGF23 can transduce signals through the Klotho-FGF receptor complex [7,8].

FGF23-RELATED HYPOPHOSPHATEMIC DISEASES

Table 1 summarized the disease of FGF23-related hypophosphatemia. The actions of FGF23 mentioned above have been confirmed in humans by the demonstration that high FGF23 levels cause several hypophosphatemic rickets/osteomalacia. Hypophosphatemia is one of the stimulators of 25(OH)D-1α-hydroxylase and usually enhances serum 1,25$(OH)_2$D [9]. However, in these hypophosphatemic diseases caused by excessive actions of FGF23, serum 1,25$(OH)_2$D remains low to low normal in the presence of frank hypophosphatemia with impaired proximal tubular phosphate reabsorption. Several kinds of hypophosphatemic rickets with different modes of inheritance were believed to be caused by enhanced expression of FGF23 in bone and high circulatory FGF23. In particular, X-linked hypophosphatemic rickets (XLHR) is caused by mutations in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (*PHEX*) [10]. As mentioned above, *FGF23* was identified as a responsible gene for ADHR [1]. Furthermore, autosomal recessive hypophosphatemic rickets 1 and 2 (ARHR1, 2) are caused by mutations in dentin matrix protein 1 (*DMP1*) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*), respectively [11-14]. Mutations in *PHEX*, *DMP1*, and *ENPP1* in these diseases are considered to be inactivating mutations. However, it is not clear at the moment how inactivating mutations in these genes cause enhanced expression of FGF23.

Table 1. FGF23-Related Hypophosphatemic Diseases

X-linked dominant hypophosphatemic rickets (XLHR) Mutations in *PHEX* gene
Autosomal dominant hypophosphatemic rickets (ADHR) Mutations in *FGF23* gene
Autosomal recessive hypophosphatemic rickets (ARHR1) Mutations in *DMP1* gene
Autosomal recessive hypophosphatemic rickets (ARHR2) Mutations in *ENPP1* gene
McCune-Albright syndrome/fibrous dysplasia
Linear sebaceous nevus syndrome
Hypophosphatemic disease with dental anomalies and ectopic calcification Mutations in *FAM20C* gene
Tumor-induced rickets/osteomalacia (TIO)
Hypophosphatemic rickets/osteomalacia by saccharated ferric oxide or iron polymaltose

PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; *FGF23*, fibroblast growth factor 23; *DMP1*, dentin matrix protein 1; *ENPP1*, ectonucleotide pyrophosphatase/phosphodiesterase 1; *FAM20C*, family with sequence similarity 20, member C.

A part of the FGF23 protein is proteolytically cleaved between ^{179}Arg and ^{180}Ser by enzymes that recognize the ^{176}Arg-X-X-^{179}Arg sequence before or during the process of secretion [15]. Mutations in patients with ADHR replace either ^{176}Arg or ^{179}Arg with other amino acids and destroy the consensus ^{176}Arg-X-X-^{179}Arg sequence recognized by enzymes that process FGF23 [1]. Therefore, the mutant FGF23 protein was shown to be resistant to the processing, suggesting that this resistance to the processing caused high FGF23 levels [16,17]. However, it is not entirely clear how mutations in *FGF23* cause hypophosphatemic rickets. Because FGF23 works as a phosphotropic hormone, the production of FGF23 should be tightly regulated. This implies that even when the mutant FGF23 proteins produced by mutations in this gene are more stable than wild-type FGF23, this does not result in excessive actions of FGF23 if the regulatory mechanisms of FGF23 production remain intact. Actually, it has been shown that FGF23