Green I kit (Roche Applied Science, Basel, Switzerland). The primer sequences used in this study were: HK2 sense, 5′-CAAAGTGACAGTGGGTGTGG-3′; HK2, antisense, 5′-GCCAGGTCCTTCACTGTCTC-3′; PDK1 sense, 5′-CCAAGACCTCGTGTTGAGACC-3′; PDK1 antisense, 5′-AATACAGCTTCAGGTCTCCTTGG-3′; PDP2 sense, 5′-ACCACCTCCGTGTCTATTGG-3′; PDP2 antisense, 5′- CCAGCGAGATGTCAGAATCC-3′. Data were normalized to expression of a control gene (b-actin) for each experiment. Data represent the mean ± SD of three independent experiments.

Statistical analysis. JMP pro 10.0.2 software (SAS Institute, Cary, NC, USA) was used to perform statistical analysis. The Kaplan–Meier method was used to estimate tumor recurrence in CRC, and the log-rank test was used to determine the statistical significance. Associations between discrete variables were assessed using the χ^2 test or Fisher's exact test as appropriate. Mean values were compared using the Mann–Whitney *U*-test. *P*-values <0.05 were considered to indicate statistical significance.

Results

HK2 in CRC. We performed immunohistochemical staining of HK2 in clinical samples of CRCs. The intensities of HK2 staining rated in three stages were assigned to two groups: the HK2-negative group included scores of 0 or 1, while the HK2-positive group included score of 2 (representative cases are shown in Fig. 1b). As summarized in Table 1, HK2 expression was significantly associated with extensive tumor diameter ($P = 0.0460$), advanced tumor depth ($P = 0.0395$), and positive lymph node metastasis ($P = 0.0409$), suggesting that the HK2-positive group had more patients with advanced stages than the HK2-negative group. HK2 expression was not associated with the backgrounds of the patients, including serum tumor markers, tumor locations, and histological types of tumors.

p-PDH in CRCs. Immunohistochemical staining of p-PDH was performed in a manner similar to that of immunohistochemical analysis of HK2 (Fig. 1c). Correlations between p-PDH expression and clinicopathological factors are summarized in Table 2. In contrast to HK2 expression, p-PDH expression did not show any correlations with tumor depth, lymph node metastasis, or other patient backgrounds. Assessment of tumor locations indicated that p-PDH expression tended to be higher in right-sided CRCs than in left-sided CRCs, but did not reach statistical significance. We also performed immunohistochemical analysis to detect the total amount of PDH-E1α regardless of phosphorylation status in 19 colorectal cancer tissues. The result showed that total PDH-E1α-positive cases showed p-PDH positive or negative expression (Fig. 2; Figs S2 and S3), whereas the total PDH-E1α-negative cases (intensity score 0) was absent for p-PDH expression, although the low expression cases (score 1) could be p-PDH positive (Table 3; data not shown), suggesting that the phosphorylation event is independent of the protein amount, and that phosphorylation control may be critical in clinical status of tumors.

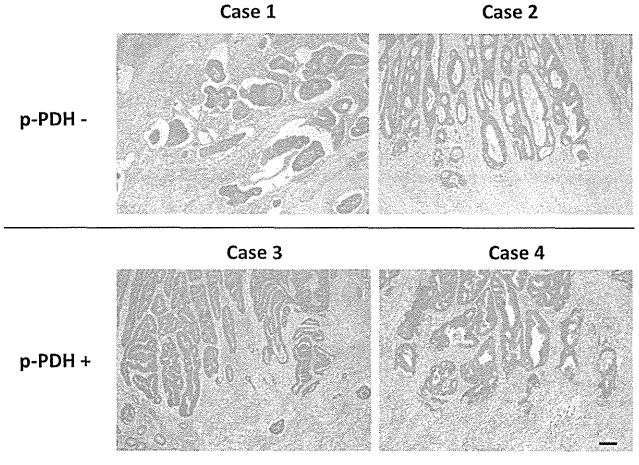

Fig. 2. Immunohistochemical analysis of PDH-E1α. Representative images of PDH-E1α staining in p-PDH negative cases (case 1 and 2) and p-PDH positive cases (cases 3 and 4). Scale bar, 100 μm.

Table 3. Correlation between the immunohistochemical staining of phosphorylation status of PDH-E1α

	p-PDH		*P*
	Positive	Negative	
Total PDH-E1α			
Positive	6	8	1.0000
Negative	2	3	

Heterogeneity of HK2 or p-PDH staining in CRC and in normal mucosa. We examined whether the heterogeneity of HK2 or p-PDH staining in CRC might be observed, and moreover, the staining intensities in normal mucosa. As shown in Table 4, positive correlation could be observed between the staining intensities in the deep part and in the superficial part of tumors regarding HK2 and p-PDH expression. In relation to the expression in normal mucosa, both expressions could scarcely be observed and any correlations with the staining in the deep part of tumor could not be observed.

Recurrence-free survival. We studied the correlations of HK2 or p-PDH expression with recurrence-free survival (RFS) in all the patients except for nine Stage IV patients who underwent non-curable resection. HK2 could separate the patients by prognosis, with positive HK2 expression being associated with

Table 4. Assessment of the staining heterogeneity of HK2 and p-PDH in tumor superficial and deep part and in normal mucosa

	HK2 staining in deep part		*P*
	Positive	Negative	
HK2 staining in superficial part			
Positive	30	10	0.0074*
Negative	31	33	
HK2 staining in normal mucosa			
Positive	1	1	1.0000
Negative	54	38	
	p-PDH staining in deep part		***P***
	Positive	**Negative**	
p-PDH staining in superficial part			
Positive	28	36	0.0024*
Negative	6	34	
p-PDH staining in normal mucosa			
Positive	0	0	NA
Negative	31	66	

*Statistically significant. In the assessment of staining in normal mucosa, the cases that did not contain normal mucosa in paraffin section were not included.

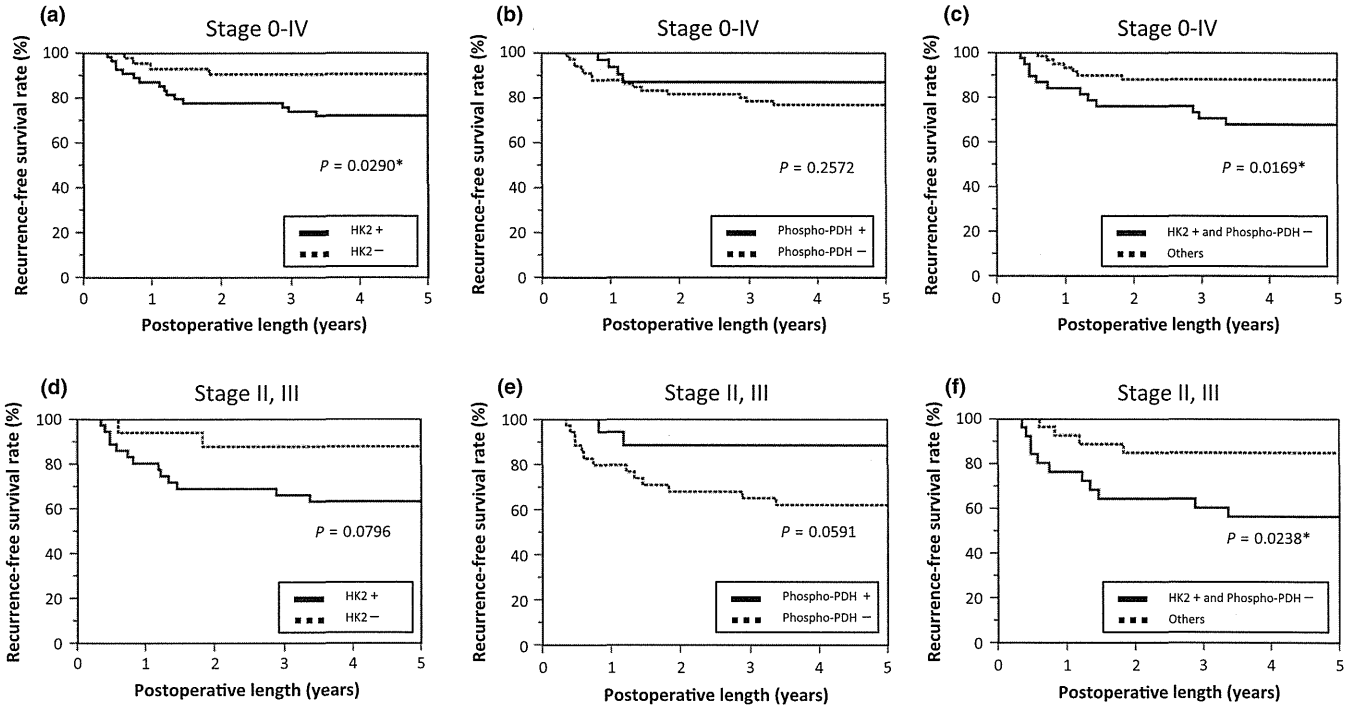

Fig. 3. Recurrence-free survival curves of the analyzed patients. (a) Patients in all stages were analyzed based on the level of HK2 expression. (b) Patients in all stages were analyzed based on the level of p-PDH expression. (c) Patients in all stages were analyzed based on combined expression of HK2 and p-PDH. (d) Patients in stage II and III were analyzed based on the level of HK2 expression. (e) Patients in stage II and III were analyzed based on the level of p-PDH expression. (f) Patients in stage II and III were analyzed based on combined expression of HK2 and p-PDH. *Statistically significant.

a poor survival rate (P = 0.0290) (Fig. 3a). In contrast, negative p-PDH expression tended to correlate with poor RFS, but this difference was not statistically significant (P = 0.2572) (Fig. 3b). We then assessed the ability of the combination of two metabolic markers to predict aggressive phenotypes of tumors and survival of patients. We classified the patients into

Table 5. Results of univariate and multivariate Cox regression analysis for Stage 0–IV patients

Variables	Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P
Age	1.017 (0.977–1.063)	0.4066		
Gender				
Female	Reference			0.0120*
Male	3.015 (1.004–12.963)	0.0491*	4.186 (1.365–18.220)	
Tumor diameter	1.014 (0.994–1.032)	0.1515		
Location				
Right-sided	Reference	0.2371		
Left-sided	1.806 (0.691–5.588)			
Tumor type				
Type 0	Reference	0.0062*		
Type 1/2/3/4/5	7.787 (1.608–140.039)			
Histological type				
tub1/tub2/pap	Reference	0.5763		
por/muc	1.557 (0.247–5.434)			
Depth				
T0/T1/T2	Reference	0.0028*		0.0162*
T3/T4	4.497 (1.631–15.781)		3.695 (1.257–13.500)	
Lymph node metastasis				
N0	Reference	0.0009*		0.0190*
N1/N2/N3	4.716 (1.892–12.704)		3.156 (1.207–8.912)	
Immunohistochemistry				
Others	Reference	0.0198*		0.0656
HK2+ and p-PDH-	2.952 (1.187–7.933)		2.383 (0.946–6.475)	

*Statistically significant. CI, confidence interval; HR, hazard ratio.

Table 6. Results of immunohistochemistry for Stage II and III cases based on HK2 and p-PDH expression

	HK2 expression	
	Positive	Negative
p-PDH expression		
Positive	10	6
Negative	25	11

two groups: the combined HK2-positive and p-PDH-negative group and the group consisting of the other cases. This immunohistochemical evaluation of combined enzyme expression showed that positive HK2 expression combined with negative p-PDH expression was associated with poor RFS rates (P = 0.0169), which could be considered as more sensitive prognostic factor than HK2 alone (Fig. 3a–c). In the multivariate analysis, tumor depth and lymph node metastasis were found to be independent prognostic factors, while the combined evaluation showed the statistical significance in univariate, but not in multivariate analysis (Table 5).

Furthermore, we carried out a similar analysis of RFS for the 52 patients, including 24 Stage II patients and 28 Stage III patients, who had a certain level of recurrence risk and might benefit from adequate estimation of recurrence risk in that the necessity of adjuvant therapy could be evaluated (Table 6).[23] HK2 and p-PDH expression tended to separate the patients by prognosis; however, significant differences were not observed (P = 0.0796 and 0.0591, respectively; Fig. 3d,e). Interestingly, the immunohistochemical evaluation of combined enzyme expression showed that positive HK2 expression combined with negative p-PDH expression significantly correlated with poor RFS rates (P = 0.0238) (Fig. 3f). Multivariate analysis showed the combination of positive HK2 and negative p-PDH expression was independently associated with poor prognosis (P = 0.0389) (Table 7).

Budding. To confirm the reason why the combined evaluation of both HK2 and p-PDH expression strongly correlated with RFS in colorectal cancer patients especially in Stage II and III, we analyzed the association between the combined evaluation and "budding". As a result, positive HK2 and negative p-PDH associated with the increased number of budding (P = 0.0199) (Fig. 4).

HK2 and PDH activity analysis. In the process of invading into stroma, cancer cells acquire the ability to detach from the epithelial lining and migrate, which is regulated by the mechanism of EMT, in a manner similar to the developmental program of an embryo. A set of pleiotropically acting genes orchestrates the EMT process by evoking loss of adherent junctions, conversion to spindly shapes, increased motility, and resistance to apoptosis in invasive front lesions of tumors.[24] To study the underlined mechanism in the present observation, we induced EMT to colon cancer cell line SW480, as a model of invading colorectal cancer cells, according to the previously described procedure followed by the analyses of HK and PDH activity.[25–28] In response to EMT stimulation, cell morphology changed from epithelial to fibroblastic-like spindle shape (Fig. 5a) and expression of E-cadherin was decreased and Vimentin was increased (Fig. 5b). By acquiring mesenchymal phenotype, HK2 expression and phosphorylation level of PDH were up-regulated (Fig. 5b). Corresponding to these shifts in the expression of the two enzymes, HK activity and PDH activity were augmented in biochemical analyses (Fig. 5c,d). Activity of PDH is regulated by two key PDH-modifying enzymes; PDK1 phosphorylates PDH to suppress the function, whereas PDP2 dephosphorylates PDH to stimulate it.[17] We found that PDP2 expression was increased in EMT condition, but PDK1 expression was stable, which might explain why p-PDH was down-regulated (Fig. 5e).

Table 7. Results of univariate and multivariate Cox regression analysis for Stage II and III patients

Variables	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	1.026 (0.976–1.077)	0.3052		
Gender				
Female	Reference	0.0166*		0.0270*
Male	4.657 (1.284–29.806)		4.740 (1.175–32.086)	
Tumor diameter	1.011 (0.989–1.030)	0.3140		
Location				
Right-sided	Reference	0.0974		0.9038
Left-sided	2.496 (0.8526–9.011)		0.923 (0.270–3.821)	
Histological type				
tub1/tub2/pap	Reference	0.8206		
por/muc	1.193 (0.186–4.321)			
Depth				
T0/T1/T2	Reference	0.3387		
T3/T4	2.390 (0.4803–43.287)			
Lymph node metastasis				
N0	Reference	0.0813		0.1745
N1/N2/N3	2.616 (0.892–9.458)		2.284 (0.705–8.957)	
Immunohistochemistry				
Others	Reference	0.0230*		0.0389*
HK2+ and p-PDH-	3.451 (1.179–12.463)		3.143 (1.058–11.461)	

*Statistically significant. CI, confidence interval; HR, hazard ratio.

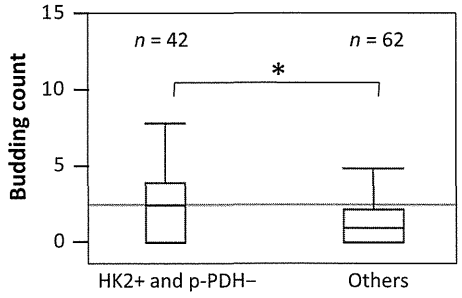

Fig. 4. The budding count in colorectal cancer tissues. The budding count of HK2+ and p-PDH- cancers was significantly greater than that of the others (P = 0.0199). *Statistically significant.

Discussion

An increasing amount of evidence has shown that cancer-specific alterations in metabolism, that is, enhanced glucose uptake and successive preferential conversion to lactate, are important factors in cancer metabolism (the Warburg effect)[6,29] and constitute tumor growth. This system is beneficial for the biosynthetic and bioenergetic demands of proliferation by diverting glycolytic intermediates to an alternative biosynthetic, the pentose phosphate pathway. Moreover, these metabolic systems are attractive targets for possible therapeutic interventions and currently research is ongoing to demonstrate the definite mechanism of cancer metabolism.[30] Although aerobic glycolysis is intimately linked to tumor growth and cancer cell proliferation, how glycolysis is involved in cellular invasion remains unclear. Invasion and metastasis are hallmarks of cancer and are closely associated with the development of pathological stages of cancer arising from precancerous lesions in epithelial tissues.[24] Distant metastasis becomes clinically evident as a consequence of multistep cascades that initially occur in local invasions.[31,32] Thus, the study of the effect of cancer metabolism in invasion may be beneficial for elucidating the novel mechanism of invasion and metastasis. To the best of our knowledge, this is the first study to demonstrate a significant association between the expression of the biomarkers HK2 and p-PDH and patient survival.

Although tumor heterogeneity is largely a common feature for the generation of biological plasticity, genetic diversification,

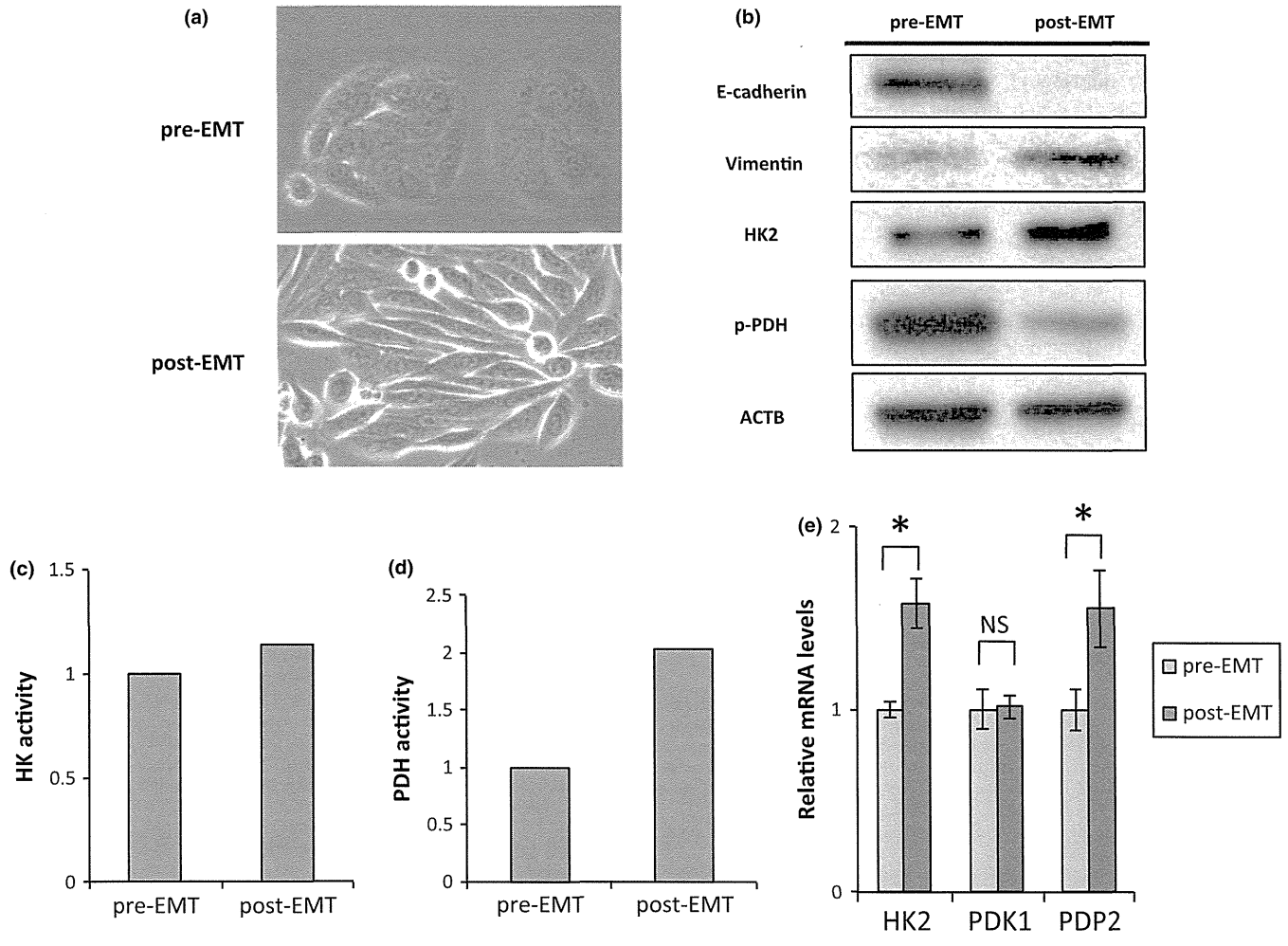

Fig. 5. Epithelial–mesenchymal transition (EMT) accompanies HK2 upregulation and p-PDH downregulation. (a) Photomicrographs of the morphological change of SW480 cells. (b) Western blot assays of E-cadherin, Vimentin, HK2, p-PDH, and ACTB expression in pre-EMT and post-EMT cells. Samples of post-EMT cells were harvested at 72 h. (c) Hexokinase activity in pre-EMT and post-EMT cells. Samples of post-EMT cells were harvested at 72 h. (d) Pyruvate dehydrogenase activity in pre-EMT and post-EMT cells. Samples of post-EMT cells were harvested at 72 h. (e) Relative transcript (mRNA) levels of HK2, PDK1, and PDP2 after inducing EMT for 0 and 48 h. The values at 0 h have been normalized to 1, and the data are expressed as fold. *Statistically significant.

and intractableness of tumors in advanced stages,[33] the presence of subpopulations with a high invasive potential (termed "budding") characterizes tumor heterogeneity in CRCs.[19,20] Budding is defined as detachment from tumor tissues into single or up to five cancer cell clusters at invasive front lesions of CRCs.[19,20] Previous reports, including our own, indicate that tumor budding undergoes EMT.[34–36] The clinical guidelines of the European Society for Medical Oncology[37] and the Japanese Society for Cancer of the Colon and Rectum[38] include tumor budding. Based on these backgrounds, we aimed to examine the glycolytic characteristics of the deepest part of tumor and those of the cancer cells undergoing EMT in this study.

According to the previous studies, the association between HK2 and RFS has not been clear and consistent results could not be acquired yet.[39–43] A possible explanation for this controversy is that the samples analyzed in these studies might be obtained from the superficial tissues of tumor. In considering the role of HK2 in aerobic glycolysis in the invasive front lesions of CRCs, where the cancer cells are usually located in the deep parts of tumors and are stimulated to invade and metastasize, the present study focused on samples from the invasive front lesions of CRCs. The present study showed that enhanced glucose uptake and glycolysis in the deeper parts of the tumor was associated with tumor growth and invasion, as shown by the data of lymph node metastasis samples. Thus, in invasive fronts, our results suggest that cancer metabolism may be reprogrammed and dominantly shifted toward active glycolysis.

We also studied the expression of p-PDH. Considering that p-PDH is involved in the OxPhos inhibition in the mitochondria and contributes to the establishment of aerobic glycolysis, it is possible that high p-PDH is associated with poor prognosis. Contrary to this original expectation, the present analysis of the invasive fronts of CRCs showed that low p-PDH was associated with poor prognosis, and evaluation of combined expression of p-PDH and HK2 demonstrated a clear association with patient prognosis. The results suggest that p-PDH plays a unique role in the malignant behavior of CRCs. Interestingly, recent studies have identified two subpopulations of cancer cells that are distinct in their energy-generating pathways.[44–46] One subpopulation depends on anaerobic glycolysis and secretes massive lactate. The other subpopulation can use lactate from upstream glycolysis in individual cells as well as from surrounding cells, and therefore the metabolites produced by the abovementioned subpopulation. The latter subpopulation can use lactate as the energy source by employing OxPhos in the mitochondria.[46] Also as suggested by our *in vitro* experiments, we speculate that CRC cells perform OxPhos in invasive front lesions. This hypothesis may be further supported by the observations that the citric cycle generates reactive oxygen species by OxPhos, which promote EMT and further cancer invasion.[47] Assessment of combined expression of HK2 and p-PDH may be useful for detecting highly malignant CRC cells. Further investigation should be performed via more detailed mechanistic studies of cancer metabolism associated with invasion and budding to identify more accurate predictors of patient prognosis and to regulate cancer invasion and metastasis.

In conclusion, combined expression of HK2 and p-PDH, as a novel cancer metabolomics-associated biomarker measure, may be clinically useful for predicting tumor aggressiveness and survival in CRC.

Acknowledgments

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Disclosure Statement

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Western blot analysis of extracts from HEK293, HeLa cells and three clinical samples of colorectal cancer using Hexokinase 2 antibody.

Fig. S2. Western blot analysis of extracts from HEK293 cell and three clinical samples of colorectal cancer using p-PDH antibody.

Fig. S3. Western blot analysis of extracts from HEK293 cell and three clinical samples of colorectal cancer using PDH-E1α antibody.

Fig. S4. Absorption test of HK2 antibody on pancreatic cancer (A) and colorectal cancer tissues (B–D). Scale bar, 100 μm.

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Case Report

Hypophosphatemic osteomalacia and bone sclerosis caused by a novel homozygous mutation of the FAM20C gene in an elderly man with a mild variant of Raine syndrome

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Cortical hyperostosis

ABSTRACT

Background: Hypophosphatemia and increased serum fibroblast growth factor 23 (FGF23) levels have been reported in young brothers with compound heterozygous mutations for the FAM20C gene; however, rickets was not observed in these cases. We report an adult case of Raine syndrome accompanying hypophosphatemic osteomalacia with a homozygous *FAM20C* mutation (R408W) associated with increased periosteal bone formation in the long bones and an increase in bone mineral density in the femoral neck.
Case: The patient, a 61-year-old man, was born from a cousin-to-cousin marriage. A short stature and severe dental demineralization were reported at an elementary school age. Hypophosphatemia was noted inadvertently at 27 years old, at which time he started to take an active vitamin D metabolite (alphacalcidol) and phosphate. He also manifested ossification of the posterior longitudinal ligament. On bone biopsy performed at the age of 41 years, we found severe osteomalacia surrounding osteocytes, which appeared to be an advanced form of periosteocytic hypomineralized lesions compared to those reported in patients with X-linked hypophosphatemic rickets. Laboratory data at 61 years of age revealed markedly increased serum intact-FGF23 levels, which were likely to be the cause of hypophosphatemia and the decreased level of $1,25(OH)_2D$. We recently identified a homozygous *FAM20C* mutation, which was R408W, in this patient. When expressed in HEK293 cells, the R408W mutant protein exhibited impaired kinase activity and secretion.
Discussion: Our findings suggest that certain homozygous *FAM20C* mutations can cause FGF23-related hypophosphatemic osteomalacia and indicate the multiple roles of FAM20C in bone.

Introduction

In 1989, Raine syndrome was first reported as a lethal disease with generalized osteosclerotic bone dysplasia inherited in an autosomal recessive fashion [1]. It is characterized by increased periosteal bone formation, which differs from osteopetrosis [2]. In 2007, *FAM20C* was identified as the gene responsible for a lethal type of Raine syndrome [3], and about 24 patients were previously reported [4,5]. However, *FAM20C* is also associated with a non-lethal type of Raine syndrome with generalized osteosclerosis. In 2009, two patients with this type of Raine syndrome were reported and one patient showed hypophosphatemia [6]. In 2013, Rafaelsen et al. reported that patients with compound heterozygous mutations in this gene had hypophosphatemia due to increased serum fibroblast growth factor 23 (FGF23) levels [7]. In addition, homozygous inactivation of *FAM20C* leads to hypophosphatemic rickets in mice [8]. These results indicate that the FAM20C gene is responsible for the distinct type of autosomal recessive hypophosphatemia with abnormal bone metabolism.

Concerning the function of *FAM20C* products, *FAM20C* encodes a Golgi kinase that phosphorylates proteins [9] including small integrin-binding ligand N-linked glycoproteins (SIBLINGs) [10], some of which

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are known to be inhibitors of mineralization through binding to hydroxyapatite [11]. Thus, a physiological function of FAM20C may be to inhibit mineralization. From a clinical point of view, mutations of the FAM20C gene in patients with Raine syndrome are considered to be inactivating ones because the disease is transmitted in an autosomal recessive fashion. Therefore, inactivation of the FAM20C gene may be associated with osteosclerosis in patients with Raine syndrome due to the promotion of mineralization. However, this hypothesis cannot explain the cause of hypophosphatemia due to increased serum FGF23 levels [7]. The increased production of FGF23 by bone tissues as well as hypophosphatemic rickets has also been reported in the *FAM20C* knock-out (KO) mouse model [8]. These observations suggest that FAM20C gene abnormalities may cause both hypophosphatemic disease and osteosclerosis at the same time.

In addition, Raine syndrome is associated with features that are different from those found in FGF23-related hypophosphatemic rickets such as X-linked hypophosphatemic rickets (XLH), autosomal dominant hypophosphatemic rickets (ADHR), and autosomal recessive hypophosphatemic rickets (ARHR) 1 and 2 [12]. These features are intracerebral calcification and a higher incidence of dental anomalies. Since severe cases of Raine syndrome are lethal, it is unlikely that mutant proteins have no enzymatic activity in adult patients. However, the residual activity of mutated FAM20C found in patients with FGF23-related hypophosphatemia has not been shown in function assays [7].

Here, we report the case of an elderly man with a non-lethal variant of Raine syndrome caused by a homozygous R408W mutation in the FAM20C gene associated with hypophosphatemic osteomalacia, increased periosteal bone formation and femoral neck bone mineral density, early loss of teeth, and intracerebral calcification. The R408W mutant exhibited residual kinase activity that phosphorylated osteopontin.

Materials and methods

Laboratory data

General laboratory data were obtained at Minoh City Hospital, Osaka, Japan. Serum 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) and 25-hydroxyvitamin D (25OHD) levels were measured with a radioreceptor assay and competitive protein binding assay, respectively. Serum intact and high sensitive parathyroid hormone levels were measured with an electrochemiluminescent immunoassay and a radioimmunoassay, respectively. Serum intact FGF23 (iFGF23) levels were measured with the FGF-23 ELISA Kit (Kainos Inc., Tokyo, Japan).

The maximal tubular reabsorption of phosphate per glomerular filtration rate (TmP/GFR) was calculated with Bijvoet's nomogram [13]. The bone mineral density was measured with a dual-energy X-ray absorptiometry (DXA) scanner (Hologic Inc., Bedford, MA, USA).

Mutational analysis

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). All coding exons and exon–intron junctions of the PHEX, DMP1, ENPP1, FGF23, and FAM20C genes were amplified by PCR. PCR products were then gel-purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Duren, Germany) and directly sequenced by dye-deoxy termination cycle sequencing using the same primers. Primer sequences can be obtained upon request.

Construction of expression vectors

Human FAM20C cDNA was amplified by RT-PCR from peripheral blood leukocytes and cloned into pcDNA 3.1/*myc*-His A (Invitrogen) with the 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 pcDNA 3.1/*myc*-His A.

Site-specific mutagenesis was performed using the 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.

Functional analysis of FAM20C

HEK293 cells were grown in 6-well dishes to 70–80% confluency and transfected with 2 μg of pcDNA 3.1/*myc*-His A-FAM20C (or mutants) with 5 μL of FuGENE-HD (Roche) as recommended by the manufacturer. Cells were transfected with 0.4 μg of pcDNA 3.1/*myc*-His A-FAM20C (or mutants) and 1.6 μg of pcDNA 3.1/*myc*-His A-OPN for co-transfection analysis. The medium was replaced with 1 mL of Opti-MEM Reduced Serum Medium 24 h after transfection. Conditioned medium was 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 of Opti-MEM Reduced Serum Medium, 55 μL of FastBreak Cell Lysis Reagent (Promega), 5 μL of Protease Inhibitor, and 0.5 μL of 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 osteopontin, as recommended by the manufacturer. A 10-μL aliquot 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 an anti-c-Myc antibody (Santa Cruz Biotechnology) overnight at 4 °C. The membrane was then incubated with an anti-mouse 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.

Bone histology

The patient underwent lumbar fenestration surgery at the age of 41 years old due to intermittent claudication and numbness of both legs, during which time iliac bone biopsy was performed. Bone specimens were fixed in neutral formaldehyde, dehydrated in ethanol, and embedded in methylmethacrylate, followed by the preparation of undecalcified sections. Sections were stained with Villanueva bone stain in Japan followed by Goldner staining at Shriners Hospitals for Children in Montreal, Canada.

Ethics

The study protocol was approved by the Review Board of the Minoh City Hospital Clinical Ethics Committee. We obtained informed consent from the patient using written documents.

Case report

The patient is a 61-year-old Japanese man born from a cousin-to-cousin marriage. His weight and height at 58 years old were 61 kg (−0.67 SD) and 153 cm (−2.8 SD), respectively. His sister and brother did not have a short stature or symptoms of metabolic bone diseases. Although he had mild bowlegs in childhood, his short stature was initially reported at an elementary school age. He had lost all his teeth by the age of 17 years as they had been gradually worn down (Fig. 1). He had no history of abscessed teeth, and the lost teeth were replaced with dentures. He was incidentally diagnosed with hypophosphatemia associated with elevated urinary loss of phosphate at 27 years old; however, no tumors were found on computerized tomography (CT) scan and magnetic resonance imaging, which suggested that he did not have tumor-induced osteomalacia. He was treated with an active vitamin D metabolite (alphacalcidol) and phosphate. Ossification of the posterior

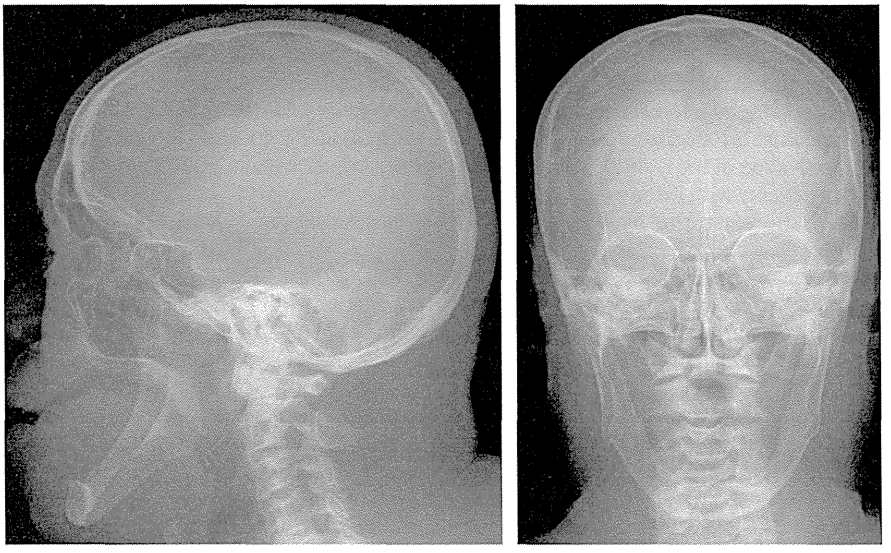

Fig. 1. Skull radiographs at the age of 61 years. Note the absence of teeth.

longitudinal ligament was revealed at the age of 35 years (Fig. 2). When the patient was 37 years old, we performed the Ellsworth–Howard test after 2 weeks of treatment discontinuation, and found normal responses of urinary phosphate and cyclic AMP excretions to exogenous parathyroid hormone (PTH).

His serum high sensitive parathyroid hormone level was slightly increased to 340 pg/mL (normal control: 90–270) at the age of 37 years. We stopped treatment with phosphate but continued active vitamin D administration at the age of 41 years because of a slightly deteriorated renal function, which was possibly derived from nephrocalcinosis because of the phosphate therapy. Bone biopsy specimens revealed large areas of osteomalacia surrounding osteocytes (Fig. 3), which appeared to be advanced forms of periosteocytic hypomineralized lesions compared to those reported in patients with X-linked hypophosphatemic rickets [14].

The most recent laboratory test following the cessation of therapy for 10 days when the patient was 61 years old revealed that his serum $1,25(OH)_2D$ level was low (12 pg/mL; normal range: 20–60) in spite of a decrease in his serum phosphate concentration (1.9 mg/dL; normal

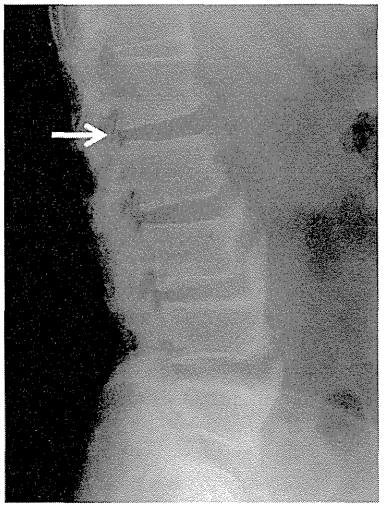

Fig. 2. A lumbar spine radiograph at the age of 50 years. Note anterior osteophytes with a coarse trabecular pattern from L3 to L5 regions. The arrow denotes ossification of the posterior longitudinal ligament.

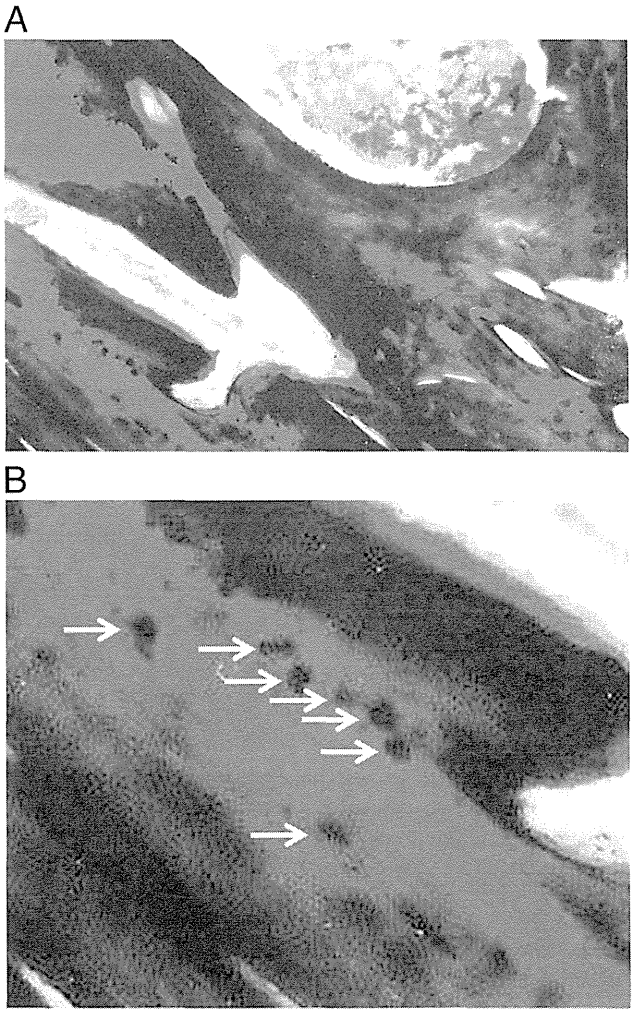

Fig. 3. (A) Iliac crest biopsy specimen was stained by Villanueva bone and Goldner's methods. Large areas of osteomalacia surrounding osteocytes were observed, being much more severe compared to hypomineralized periosteocytic lesions, halos of unmineralized bone surrounding osteocyte lacunae, reported in XLH patients. (B) A higher magnification of Fig. 3A. The arrows indicate osteocytes.

Table 1
Laboratory data at 61 years old (before and after stopping medication).

	Before	After	Reference range
Serum Ca (mg/dL)	10.3	9.2	8.7–11.0
Serum P (mg/dL)	2.1	1.9	2.7–4.4
Serum Cr (mg/dL)	1.40	1.31	0.6–1.1
ALP (IU/L)	347	288	104–338
Intact-PTH (pg/mL)	32	60	10–65
$1,25(OH)_2D$ (pg/mL)	52	12	20–60
25OHD (ng/mL)	27.1	ND	
Intact FGF23 (pg/mL)	386	309	10–50
Urine P (mg/dL)	70.3	32.0	0–80
Urine Cr (mg/dL)	145	52	
%TRP (%)	67.7	57.6	>80
TmP/GFR (mg/dL)	1.5	1.1	2.8–4.2

ND, not done; Ca, calcium; P, phosphorus; Cr, creatinine; ALP, alkaline phosphatase; PTH, parathyroid hormone; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; 25OHD, 25-hydroxyvitamin D; %TRP, percentage of tubular reabsorption of phosphate; and TmP/GFR, the maximal tubular reabsorption of phosphate per glomerular filtration rate.

range: 2.7–4.4) (Table 1). The serum intact-PTH level was 60 pg/mL (normal range: 10–65). %TRP and TmP/GFR values were 57.6% (normal range >80) and 1.1 mg/dL (normal range: 2.8–4.2), respectively. His serum iFGF23 level was elevated to 309 pg/mL (normal range: 10–50). We identified intracerebral calcification in the basal ganglia by CT (Fig. 4). Bone radiographs revealed increased periosteal bone formation in the humerus (Fig. 5). Bone mineral densities determined by DXA analysis in the bilateral femoral neck were elevated at 1.034 g/cm^2 (Z-score +2.5) and 1.156 g/cm^2 (Z-score +3.6) on the right and left sides, respectively. The lumber bone mineral density (L2–L4) was also elevated at 2.007 g/cm^2 (Z-score +6.3).

In order to identify the cause of FGF23-related hypophosphatemic disease in this patient, we sequenced all coding exons and exon–intron junctions in PHEX, DMP1, ENPP1, and FGF23 genes. However, no mutation was observed in these genes. In contrast, a novel homozygous missense mutation, c.1222C>T, R408W, was found in exon 6 of the FAM20C gene (Fig. 6). This base change has not been reported in the database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=56975). We could not investigate his sister and brother because they did not agree to genetic testing. *FAM20C* mutations associated with hypophosphatemia in the literature are shown in Table 2, confirming that R408W mutation has not previously been reported. We then analyzed the secretion and kinase activity of the mutant FAM20C protein. When wild-type FAM20C was expressed in vitro, Western blotting revealed more FAM20C protein in the conditioned medium than in the cell lysate (Fig. 7A).

In contrast, most R408W mutant protein was observed in the cell lysate, indicating that secretion of the mutant protein was impaired (Fig. 7A). A kinase-dead mutant (D478A) also exhibited partially impaired secretion (Fig. 7A), consistent with a previous report [9]. Since wild-type FAM20C was shown to increase the molecular weight of osteopontin by phosphorylation [9], the kinase activity of wild-type and mutant FAM20C proteins was examined by analyzing changes in the molecular weight of osteopontin. When wild-type FAM20C and osteopontin were co-expressed, phosphorylated osteopontin had a higher molecular weight than that of unphosphorylated osteopontin without FAM20C expression (Fig. 7B). The kinase-dead D478A mutant FAM20C did not alter the molecular weight of osteopontin, whereas the R408W mutant FAM20C slightly increased it, which indicated the impaired but residual kinase activity of this mutant protein (Fig. 7B).

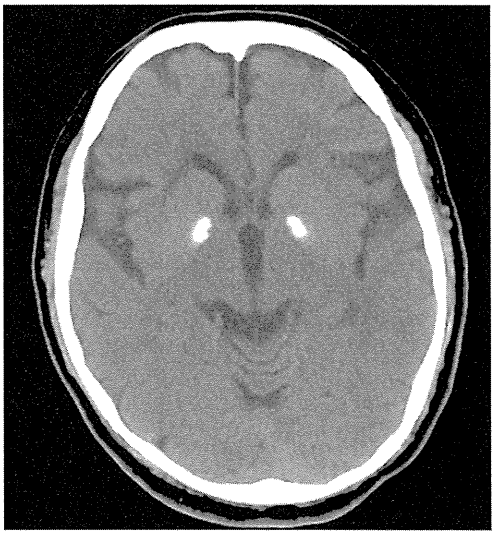

Fig. 4. Cranial computerized tomography at the age of 61 years. Note intra-cerebral calcification in the region of the basal ganglia.

Discussion

We reported a patient with a homozygous mutation in the FAM20C gene that was R408W. He had FGF23-related hypophosphatemic osteomalacia complicated by cortical hyperostosis and increased bone mineral density in the femoral neck and lumbar vertebrae, suggesting that the FAM20C gene has the dual effect of increasing bone formation and causing osteomalacia in humans.

FGF23-related hypophosphatemic rickets with a genetic background consists of XLH, ADHR, and two forms of ARHR (ARHR1 and ARHR2). The genes responsible for this type of rickets were identified as *PHEX*, *FGF23*, *DMP1*, and *ENPP1*, respectively [12]. The FAM20C gene was recently shown to be the gene responsible for autosomal recessive hypophosphatemia, although it was originally identified as the causal gene of Raine syndrome [3,6,7]. Since the loss-of-function mutations in *FAM20C* seem to impair the phosphorylation of SIBLING proteins, some of which are inhibitors of mineralization, and are expected to destroy the inhibitory function, osteomalacia was an unexpected observation. Thus, our patient suggested the possibility that hypophosphatemia due to the elevated serum levels of FGF23 partly overcomes the role of FAM20C in bone leading to osteomalacia. Thus, the present case provides additional evidence to show that the FAM20C gene may be responsible for ARHR in addition to DMP1 and ENPP1 gene, although we had no evidence that the patient suffered from rickets in childhood. Dental abnormalities have been reported in patients with ARHR1 [15]; however, the premature loss of teeth is rare [16]. In contrast, FAM20C was reported to be essential for the formation of murine teeth [17], and the premature loss of teeth was reported in patients with compound heterozygous mutations in *FAM20C* [7]. Our patient did not have a remarkable history of abscessed teeth. Moreover, his teeth were worn down, showing a gradually decreased volume. These findings are compatible with a recent report on the presence of amelogenesis imperfecta in *FAM20C* null mice [18]. Thus, the premature loss of teeth may be an index suggesting the presence of a *FAM20C* mutation in patients with sporadic non-lethal Raine syndrome with hypophosphatemia. In addition, hyperostosis of the cortical bones appears to be characteristic of these patients because no hyperostosis was reported in cortical bone in patients with ARHR1 or XLH. A recent study using peripheral quantitative computed tomography revealed a reduced bone mineral density in the forearms of XLH patients [19]. Thus, the clinical key to diagnosing hypophosphatemic rickets caused by mutations in *FAM20C* is the premature loss of teeth and coexistence of osteomalacia and cortical hyperostosis. The presence of cerebral calcification in the basal ganglia suggests the possibility of *FAM20C* mutations; however, the same findings have also been reported in healthy individuals [20] and patients with several diseases such as hypoparathyroidism and Fahr disease [21].

The mechanism by which osteomalacia coexists with cortical hyperostosis combined with an increased bone mineral density in the femoral

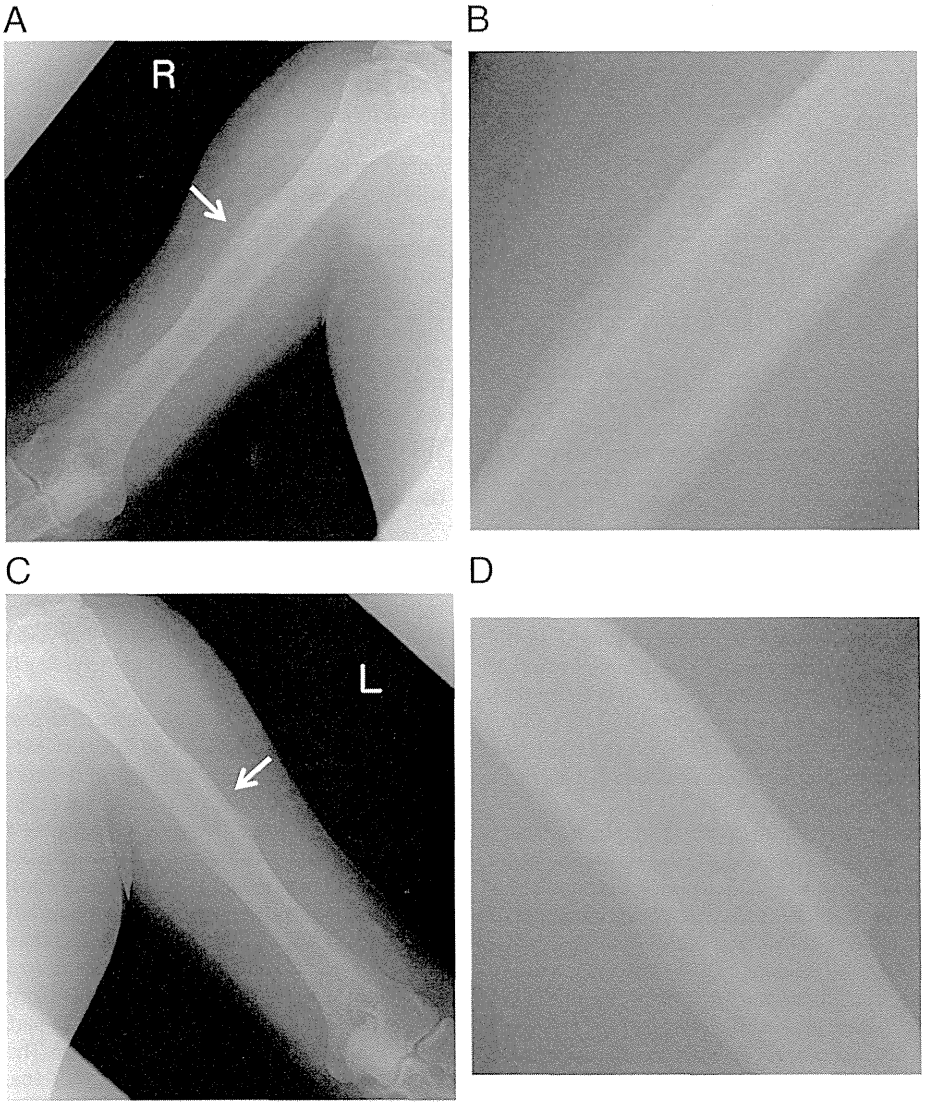

Fig. 5. Radiographs of the (A and B) right and (C and D) left humerus at the age of 61 years. The arrows indicate periosteal hyperostosis. (B and D) A close-up view of periosteal hyperostosis.

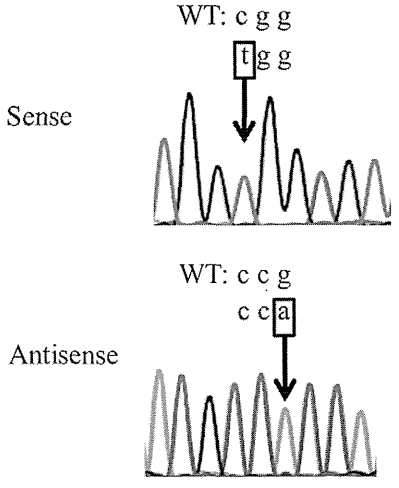

Fig. 6. Mutational analysis of *FAM20C*. The novel homozygous missense mutation c.1222C>T, R408W in exon 6 of the FAM20C gene is shown.

neck and lumbar vertebrae remains unknown. However, osteomalacia of our patient can be explained by the increased serum FGF23 levels. Increased serum FGF23 levels have recently been reported in FAM20C gene KO mice [8]. Markedly increased serum FGF23 levels (309 pg/mL; normal range: 10–50) were observed in our patient after stopping the medication of alfacalcidol (1 μg) for 10 days, being similar to reports on XLH patients [22]. The serum FGF23 level under therapy was 386 pg/mL (normal range: 10–50), being thought to be increased by the therapy [23].

Therefore, it is likely that serum FGF23 levels play an important role in hypophosphatemic rickets in humans with *FAM20C* mutations. Another possibility is the abnormal differentiation of osteoblasts due to impaired FAM20C functions. This mechanism was supported by

Table 2
FAM20C mutations in non-lethal Raine syndrome.

Protein	Phenotype	Hypophosphatemia	Reference
D451N	Raine	+	[6]
P328S	Raine	+	[27]
T268M + Y305X	Hypophosphatemia	+	[7]
R408W	Raine	+	

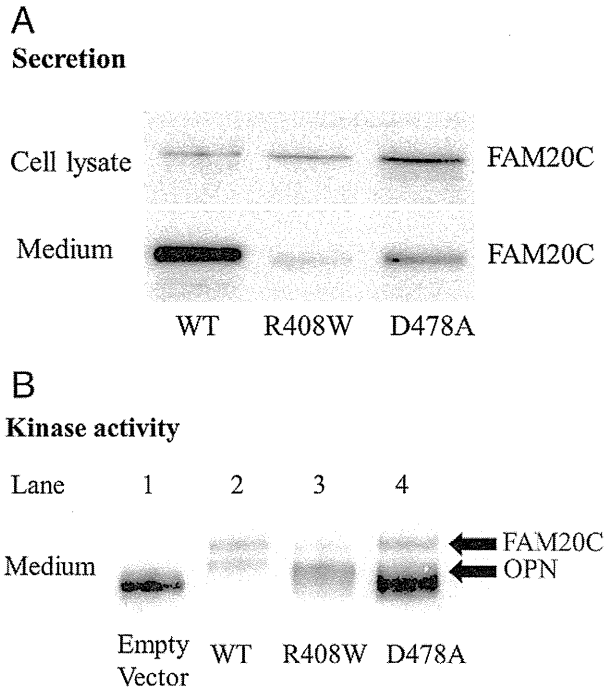

Fig. 7. Secretion and kinase activity of wild-type and mutant FAM20C proteins. (A) While the medium contained more wild-type FAM20C protein than the cell lysate, most R408W mutant FAM20C was present in the cell lysate. (B) When wild-type FAM20C was co-expressed with OPN (Lane 2), the molecular weight of OPN was higher than that without FAM20C expression (Lane 1). Kinase-dead D478A did not alter the molecular weight of OPN (Lane 4), whereas R408W slightly increased it (Lane 3). WT, wild-type; R408W, our patient; D478A, previously reported kinase-dead mutant; OPN, osteopontin.

histological findings in the long bones of FAM20C gene KO mice [8]. The impaired maturation of osteocytes with a wider periosteocytic region and loss of osteocyte processes was also reported in these mice. These findings are consistent with a large area of osteomalacia surrounding osteocytes in the iliac crest bone biopsy specimen of the present patient (Fig. 3). Moreover, they showed the down-regulation of the DMP1 gene in the calvaria of *FAM20C*-deficient mice. These lines of evidence support the hypothesis that the impaired maturation of osteoblasts and osteocytes due to *FAM20C* mutations causes osteomalacia. However, whether or not the mechanism of osteomalacia associated with *FAM20C* mutations is shared in humans and mice has not yet been confirmed.

The patient was complicated by ossification of the posterior longitudinal ligament, an advanced form of enthesopathy [24], characterized by mineral deposition near the tendon at the spine and lower extremities. Enthesopathies have been reported in patients with XLH, ARHR2 [25], and possibly ADHR [26], in which serum FGF23 levels are elevated.

Our patient has the novel homozygous missense mutation c.1222C>T, R408W in the FAM20C gene. Other non-lethal mutations in the FAM20C gene that cause hypophosphatemia have been reported: D451N [6], P328S [27], and compound heterozygous mutations of T268M and Y305X [7] (Table 2). The number of cases is too small to explain what mutation causes hypophosphatemia. We speculate that all non-lethal mutations in the FAM20C gene cause hypophosphatemia.

In our patient, we showed a homozygous missense mutation of R408W in the FAM20C gene and detected the impaired kinase activity and secretion of the mutated FAM20C protein using an assay for the phosphorylation of osteopontin. However, we have no clear explanation for the reduced amount of osteopontin in lanes 2 and 3 (Fig. 7B), but it is possible that the phosphorylation of osteopontin affects the stability of this protein, although we did not confirm the result using antibodies against FAM20C and osteopontin. Further studies are required to clarify the genotype–phenotype relationship in patients with mutations of the FAM20C gene.

Osteopontin is one of the SIBLING proteins, such as MEPE, DMP1, and DSPP [10]. The acidic serine- and aspirate-rich motif (ASARM) peptides are produced from SIBLING proteins and have been shown to inhibit bone formation [27]. In addition, osteopontin KO mice exhibited cortical hyperostosis in response to PTH [28]. Based on these two observations, it is possible that the impaired function of the osteopontin-derived ASARM peptide due to inactivated mutations in *FAM20C* increased cortical bone formation in response to PTH. The reduced function of the MEPE-derived ASARM peptide, for example, may also explain the phenomena of increased bone mineral density in the femoral neck and lumbar vertebrae of the patient because of the increased mass of the cancellous bone in *MEPE* KO mice [29]. Another hypothesis to explain the patient's high bone mineral density is the long-term treatment by active vitamin D metabolite and phosphate, which were shown in XLH patients [19,30].

In conclusion, we herein describe the first reported case of hypophosphatemic osteomalacia in a human caused by a novel homozygous mutation of the FAM20C gene, which was R408W. The transfection experiments suggested the impaired secretion and kinase activity of the R408W mutant FAM20C. It is interesting that osteomalacia and increased periosteal bone formation in the upper extremities with increased bone mineral densities of the femoral neck and lumbar vertebrae coexisted in our patient, suggesting the dual functions of FAM20C in bone.

Acknowledgments

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Serum Fibroblast Growth Factor 23 Is a Useful Marker to Distinguish Vitamin D-Deficient Rickets from Hypophosphatemic Rickets

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Key Words
Vitamin D · Fibroblast growth factor 23 · Rickets · Hypophosphatemia · Children

Abstract
Background/Aims: Vitamin D-deficient rickets (DR) has recently re-emerged among developed countries. Vitamin D deficiency can influence biochemical results of patients with fibroblast growth factor 23 (FGF23)-related hereditary hypophosphatemic rickets (HR), making differential diagnosis difficult. In the present study we evaluated the utility of serum FGF23 levels in the diagnosis of DR and during its treatment. ***Methods:*** The study group comprised 24 children with DR and 8 children with HR. Serum FGF23 levels and bone metabolism-related measurements were assessed. ***Results:*** Serum FGF23 levels in patients with DR were less than 19 pg/ml, while those in patients with HR were more than 57 pg/ml. There were significant differences in serum levels of calcium, phosphate, parathyroid hormone, and 1,25-dihydroxyvitamin D, as well as tubular maximum phosphate reabsorption per glomerular filtration rate between patients with DR and HR, but these values were not fully mutually exclusive. In addition, serum FGF23 and phosphate levels were increased following treatment. ***Conclusion:*** Serum FGF23 level is the most critical biochemical marker for distinguishing DR from HR and might be a good indicator of biochemical response to the intervention. Serum FGF23 levels show utility for the diagnosis of DR and in the assessment of its response to treatment.

Introduction

Rickets is caused by defective mineralization in the growth plate of cartilage and in the matrix of bone in a growing child [1, 2]. Bowed legs, enlargement of the wrists and knees, rachitic rosary, craniotabes, growth retardation, delayed initiation of walking, and waddling gait are often associated with rickets. Diagnosis of rickets requires radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone.

The most common cause of rickets is vitamin D deficiency, although genetic or acquired disorders of the gut, liver, kidney, and metabolism of vitamin D can cause rickets [2]. Increased numbers of patients with vitamin D deficiency have been reported among children in recent years throughout the world [3–5], including Japan [6–9]. Circulating 25-hydroxyvitamin D [25(OH)D] concentration is the best clinical indicator of vitamin D repletion in the body. Vitamin D deficiency is diagnosed by the mea-

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surement of serum 25(OH)D concentration below 20 ng/ml in adults [10, 11]. In addition, many experts have commonly proposed a cutoff value of 20 ng/ml for serum 25(OH)D concentration to designate vitamin D deficiency in children [12, 13]. Treatment of vitamin D deficiency with native vitamin D or active vitamin D is effective for the correction of rickets [8, 14].

X-linked hypophosphatemic rickets (HR) is the most common form of heritable rickets and is manifested by fibroblast growth factor 23 (FGF23) excess and renal phosphate wasting [15, 16]. Clinical and radiographic features are mostly similar to vitamin D-deficient rickets (DR). Biochemical findings include hypophosphatemia and low-to-normal circulating 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. Serum concentrations of parathyroid hormone (PTH) are usually normal or modestly elevated in some cases. Other forms of FGF23-related hereditary HR have been described, including an autosomal dominant form caused by mutations in FGF23 and autosomal recessive forms caused by mutations in dentin matrix protein 1 and in ectonucleotide pyrophosphatase/phosphodiesterase 1. The prevalence of these forms of HR appears much less than that of X-linked HR. Serum FGF23 concentrations are increased in patients with HR [17, 18]. FGF23 decreases serum phosphate concentrations by the inhibition of renal proximal tubular phosphate reabsorption and the suppression of 25(OH)D-1α-hydroxylase [19]. Vitamin D and phosphate are necessary for the treatment of HR [15, 20].

In collaboration with other institutes, we previously reported on the diagnostic utility of serum FGF23 measurement in patients with hypophosphatemia [21]. However, it remains unclear whether serum FGF23 measurement is useful for differentiating DR and HR, especially in the case of comorbidity of HR plus vitamin D deficiency. Thus, in the current study, we report the diagnostic utility of serum FGF23 measurements to distinguish patients with DR from those with HR.

Subjects and Methods

Subjects

This study included 32 patients who attended Osaka University Hospital or Minoh City Hospital (Osaka, Japan) from January 2003 through June 2012 and who were diagnosed with DR or HR based on clinical, laboratory, and radiographic findings, as well as clinical course. In detail, the diagnostic criteria of DR included radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone, high serum levels of alkaline phosphatase (AP) and PTH, and low 25(OH)D levels. Vitamin D deficiency was defined as serum 25(OH)D levels less than 20 ng/ml [13]. The diagnosis of DR was confirmed by no recurrence of rickets after discontinuation of treatment. The diagnostic criteria of HR included radiographic signs such as cupping, splaying, or fraying in the metaphysis of a long bone, low serum phosphate concentrations, and tubular maximum phosphate reabsorption per glomerular filtration rate (TmP/GFR), high AP levels, and normal levels of PTH, 1,25$(OH)_2$D, and 25(OH)D. Although 2 patients did not meet the criteria of HR due to low 25(OH)D levels, they were diagnosed with HR because of high FGF23 levels and resistance to α-calcidol treatment. Other disorders which could develop rickets were excluded, including malabsorption, liver and renal tubular diseases, parathyroid disorders, type I and II vitamin D-dependent rickets, hypophosphatasia, primary disorders of bone matrix, drug-induced mineralization defects, and tumors. Twenty-four patients (11 boys, 13 girls) were diagnosed with DR and 8 (2 boys, 6 girls) with HR. Seven of the 8 patients with HR were sporadic, while 1 patient inherited HR from her mother. Physical examinations were made, and blood and urine samples were taken. Radiography demonstrated rachitic signs in the metaphysis of a long bone in all the patients. Complaints, feeding type before solid food, restricted and/or unbalanced diet, and sunlight exposure were evaluated for DR patients. Dietary content and sun exposure were based on information obtained from parents or guardians. When patients were not given some foods because of concern about allergy, it was considered as a restricted diet. When patients did not take certain foods, it was considered as an unbalanced diet. Playing outside twice a week or less was regarded as insufficient sun exposure. Laboratory data without serum FGF23 levels of 3 DR patients and those with serum FGF23 levels of 2 HR patients were included in previous publications by our group [9, 21]. Measurement of serum FGF23 levels was approved by the institutional review board of Osaka University Hospital and written informed consent was obtained from the parents or guardians of the patients. Patients with DR were treated with α-calcidol suspension because neither cholecalciferol nor ergocalciferol suspension is available on prescription or on the market in Japan.

Measurements

Laboratory measurements included serum levels of calcium (reference range: 8.4–10.0 mg/dl), phosphate (4.2–6.2 mg/dl for the age of 1 year), AP (353–1,009 U/l for the age of 1 year), PTH (10–60 pg/ml), 1,25$(OH)_2$D (20–60 pg/ml), 25(OH)D (the lower limit, 20 ng/ml [13]), and FGF23 (10–50 pg/ml for adults [21]), as well as TmP/GFR (2.7–6.3 mg/dl for the ages 1–24 months [22]) and urine calcium/creatinine ratio (U-Ca/Cr). TmP/GFR was calculated from the formula: TmP/GFR = serum phosphate – urine phosphate × serum creatinine/urine creatinine [23]. Serum 25(OH)D levels were measured in 3 out of 8 with patients with HR. Serum FGF23 levels were measured by an ELISA method that recognizes only full-length biologically active FGF23 (Kainos Laboratories, Japan). The lowest reportable value of FGF23 was 10 pg/ml. Serum 25(OH)D levels were measured by a competitive immunoluminometric direct assay (LIAISON 25OH Vitamin D TOTAL Assay; DiaSorin, USA, 20 samples) and by competitive protein-binding assays (Mitsubishi Chemical Medience, Japan, 6 samples; BML, Japan, 1 sample) because of differences of assay costs.

Statistics

Data were analyzed by a Mann-Whitney U test, ROC analysis, or paired t test using JMP (SAS Institute, USA) and SPSS (IBM SPSS, USA) statistical software.

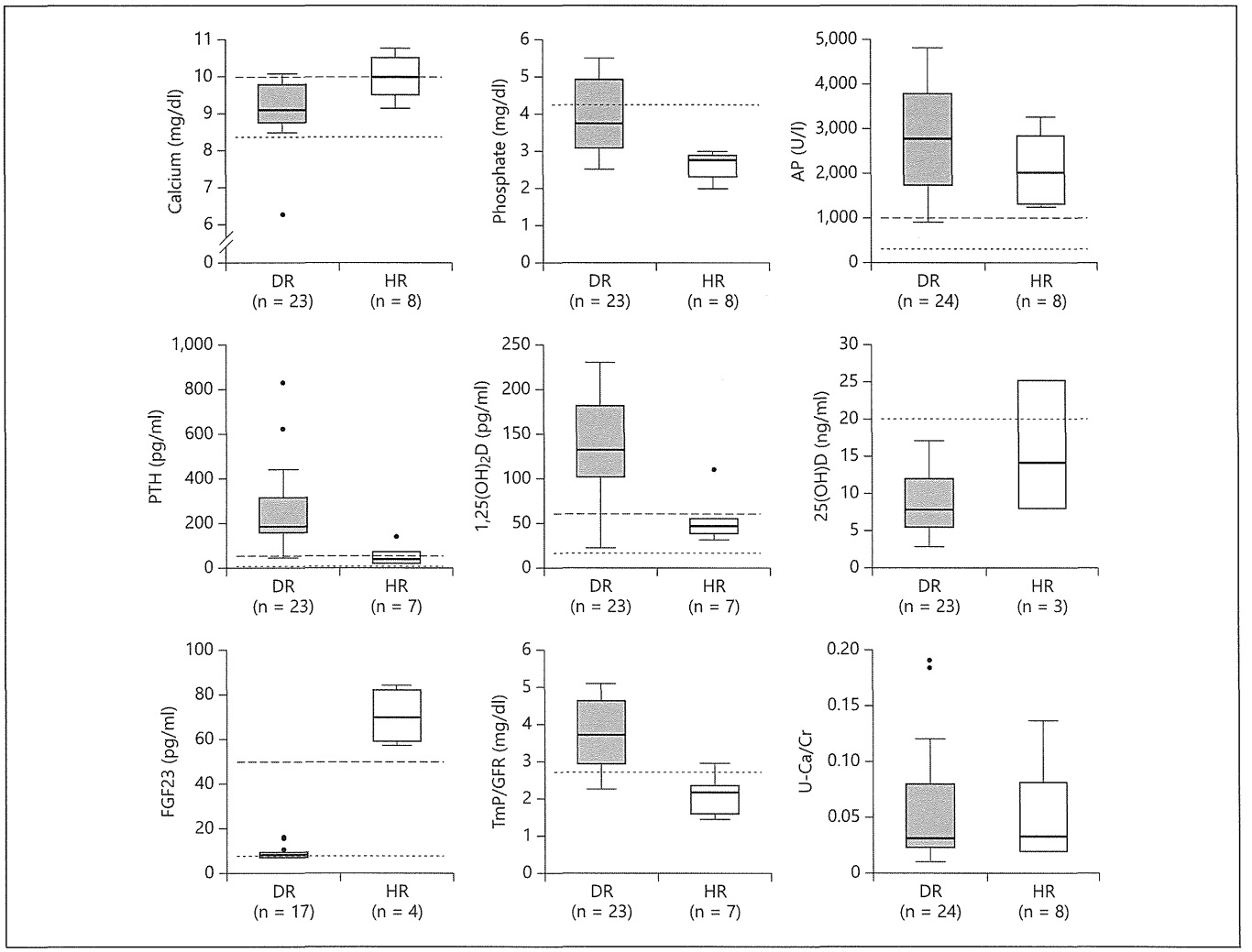

Fig. 1. Biochemical measurements of patients with DR and HR before treatment. Note that only the serum FGF23 level is exclusive between DR and HR. The data are presented as box plots. $p < 0.01$ for calcium, phosphate, PTH, $1,25(OH)_2D$, FGF23, and TmP/GFR. Dashed and dotted lines are the upper and lower limit of reference ranges, respectively.

Results

Clinical Features of DR Patients

Clinical features, including complaints, feeding type before solid foods, restricted and/or unbalanced diet restriction, and sunlight exposure were evaluated in the 24 DR patients. Complaints consisted of bowed legs (n = 18, 75%), elevated serum AP level (n = 5, 21%), and convulsions (n = 1, 4%). Feeding type before solid food was exclusively breast milk (n = 21, 89%) and breast plus formula milk (n = 1, 4%). Twelve patients (50%) had a restricted and/or unbalanced diet. There were 6 patients (25%) with insufficient sun exposure.

Characteristics of DR and HR Patients

There were no differences in age, height, and weight between DR and HR patients. Age was 17 ± 7 (mean ± SD) months, height was −1.3 ± 1.5 SD score (SDS), and weight was −0.5 ± 1.3 SDS in DR patients compared to age 21 ± 8 months, height −1.9 ± 1.0 SDS, and weight −0.1 ± 0.8 SDS in HR patients.

Utility of Serum FGF23 Levels to Distinguish HR and DR Patients

Laboratory findings of DR and HR patients were determined before treatment (fig. 1). Serum calcium concentration was lower in patients with DR than those

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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

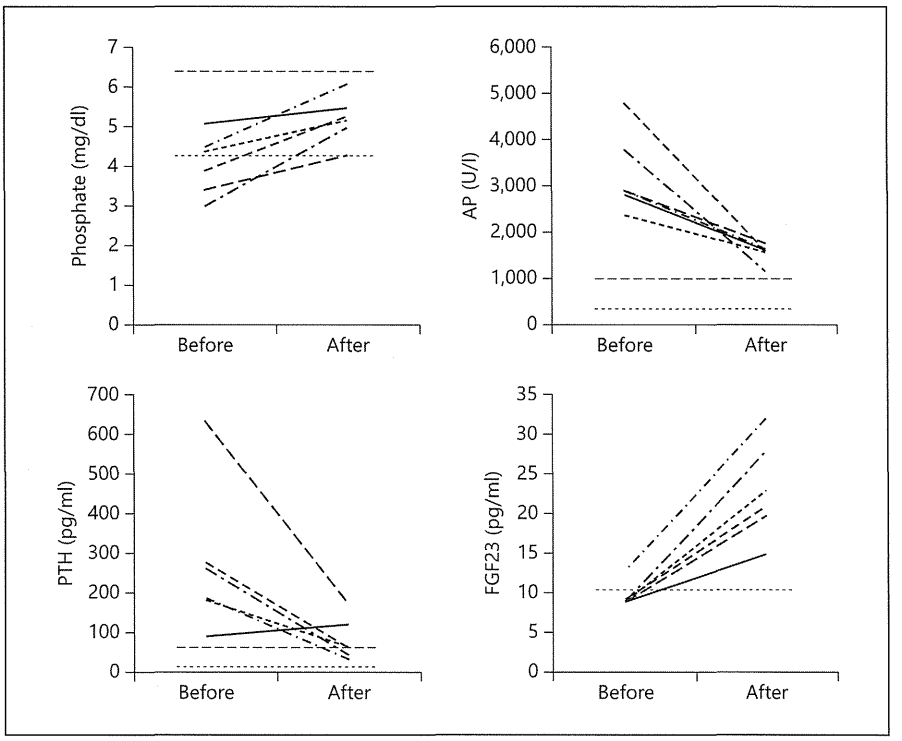

Fig. 2. Response of biochemical measurements to intervention in patients with DR. Serum levels of phosphate, PTH, AP, and FGF23 were significantly changed after the intervention compared to those before. $p < 0.01$ for phosphate, AP, and FGF23; $p < 0.05$ for PTH. Dashed and dotted lines are the upper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