

ORIGINAL ARTICLE

Fibroblast Growth Factor 23 in Oncogenic Osteomalacia and X-Linked Hypophosphatemia

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ABSTRACT

BACKGROUND

Mutations in fibroblast growth factor 23 (FGF-23) cause autosomal dominant hypophosphatemic rickets. Clinical and laboratory findings in this disorder are similar to those in oncogenic osteomalacia, in which tumors abundantly express FGF-23 messenger RNA, and to those in X-linked hypophosphatemia, which is caused by inactivating mutations in a phosphate-regulating endopeptidase called PHEX. Recombinant FGF-23 induces phosphaturia and hypophosphatemia in vivo, suggesting that it has a role in phosphate regulation. To determine whether FGF-23 circulates in healthy persons and whether it is elevated in those with oncogenic osteomalacia or X-linked hypophosphatemia, an immunometric assay was developed to measure it.

METHODS

Using affinity-purified, polyclonal antibodies against [Tyr223]FGF-23(206–222)amide and [Tyr224]FGF-23(225–244)amide, we developed a two-site enzyme-linked immunosorbent assay that detects equivalently recombinant human FGF-23, the mutant form in which glutamine is substituted for arginine at position 179 (R179Q), and synthetic human FGF-23(207–244)amide. Plasma or serum samples from 147 healthy adults (mean [±SD] age, 48.4±19.6 years) and 26 healthy children (mean age, 10.9±5.5 years) and from 17 patients with oncogenic osteomalacia (mean age, 43.0±13.3 years) and 21 patients with X-linked hypophosphatemia (mean age, 34.9±17.2 years) were studied.

RESULTS

Mean FGF-23 concentrations in the healthy adults and children were 55±50 and 69±36 reference units (RU) per milliliter, respectively. Four patients with oncogenic osteomalacia had concentrations ranging from 426 to 7970 RU per milliliter, which normalized after tumor resection. FGF-23 concentrations were 481±528 RU per milliliter in those with suspected oncogenic osteomalacia and 353±510 RU per milliliter (range, 31 to 2335) in those with X-linked hypophosphatemia.

CONCLUSIONS

FGF-23 is readily detectable in the plasma or serum of healthy persons and can be markedly elevated in those with oncogenic osteomalacia or X-linked hypophosphatemia, suggesting that this growth factor has a role in phosphate homeostasis. FGF-23 measurements might improve the management of phosphate-wasting disorders.

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MUTATIONS IN THE GENE ENCODING fibroblast growth factor 23 (FGF-23) (Online Mendelian Inheritance in Man [OMIM] number 605380) cause autosomal dominant hypophosphatemic rickets (OMIM number 193100).¹ As is consistent with a presumed role in phosphate homeostasis, FGF-23 messenger RNA (mRNA) and protein are markedly overexpressed in tumors that are responsible for oncogenic osteomalacia,²⁻⁴ and recombinant FGF-23 induces hypophosphatemia in vivo as a result of urinary phosphate wasting.^{3,5,6}

FGF-23 is initially produced as a 251-amino-acid precursor, with residues 1 through 24 serving as signal peptide that allows efficient secretion of the mature protein.^{1,3,7} Tumors responsible for oncogenic osteomalacia and cells expressing wild-type FGF-23 produce at least two molecular forms of FGF-23 (the full-length, 32-kD protein and a 12-kD fragment). Since only the larger fragment is present in cells carrying one of the FGF-23 missense mutations identified in autosomal dominant hypophosphatemic rickets,^{2,3,6,8} it has been proposed that these mutations affect sites of cleavage by a furin-type proprotein convertase (an endoprotease that cleaves protein precursors after pairs of basic residues). Thus, the mutations would impair FGF-23 degradation and thereby enhance or prolong its biological activity.^{3,6,8-10}

In addition to being a substrate for furin-type enzymes, FGF-23 may also be a substrate for PHEX (the phosphate-regulating protein with homologies to endopeptidases encoded by a gene on the X chromosome), which is mutated in patients with X-linked hypophosphatemic rickets (OMIM number 307800).^{9,11,12} Different molecular mechanisms — overproduction of FGF-23 by the tumors responsible for oncogenic osteomalacia,^{2,3} generation of a mutant FGF-23 that is resistant to cleavage by furin-type enzymes,^{2,3,6,8,10} and impaired FGF-23 degradation due to reduction or loss of PHEX activity⁹ — may thus lead to elevated FGF-23 concentrations and consequently to urinary phosphate wasting.

To explore the role of FGF-23 in the regulation of phosphate homeostasis, we developed a two-site enzyme-linked immunosorbent assay (ELISA) that detects the carboxy-terminal portion of FGF-23, and we measured the circulating concentrations of this growth factor in healthy persons and in patients with oncogenic osteomalacia or X-linked hypophosphatemia.

METHODS

PEPTIDE SYNTHESIS AND ANTIBODY PRODUCTION

Peptides were synthesized by the Biopolymer Core Facility, Massachusetts General Hospital, Boston. Peptides representing portions of the FGF-23 precursor — [Cys70]FGF-23(51–69)amide, [Tyr185]FGF-23(186–206)amide, [Tyr223]FGF-23(206–222)amide, and [Tyr224]FGF-23(225–244)amide — were coupled to keyhole limpet hemocyanin, emulsified with complete Freund's adjuvant, and used for subcutaneous immunization of eight goats (with approximately 100 µg per animal); each peptide was injected into two animals. Subsequent booster injections (approximately 50 µg per animal) were performed every four weeks with peptides emulsified with incomplete Freund's adjuvant. Each peptide was covalently coupled to agarose (AminoLink Kit, Pierce Chemical), and 30-to-200-ml quantities of each crude polyclonal antiserum were affinity-purified with the use of the appropriate immobilized peptide, as previously described.¹³

GENERATION OF RECOMBINANT FGF-23

The complementary DNA (cDNA) encoding full-length human FGF-23 was amplified by the polymerase chain reaction from a cDNA library derived from a previously described oncogenic osteomalacia tumor.² To express recombinant FGF-23, the polymerase-chain-reaction product was ligated into the vector pIZ/V5-His (InsectSelect cloning kit, Invitrogen) to yield a plasmid called [V5-His]rhFGF-23(1–251); the two epitope tags were located at the carboxy-terminal end of the encoded FGF-23; the nucleotide sequence of the construct was then confirmed by sequence analysis. Sf-9 insect cells, which allow high-level protein expression, were then transfected (Insectine-Plus transfection reagent, Invitrogen) with the [V5-His]rhFGF-23(1–251) plasmid. The transfected cells were maintained at 25°C and selected for resistance to Zeocin, an antibiotic of the bleomycin family (300 µg per milliliter, Invitrogen). Polyclonal cell lines with high levels of [V5-His]rhFGF-23(1–251) expression were expanded and maintained in 250-ml spinner flasks with serum-free insect medium (Trichoplusia ni Medium Formulation Hink [TNF-H], GIBCO) supplemented with Zeocin (50 µg per milliliter) and a combination of amphotericin B, penicillin, and streptomycin. Conditioned medium from stably transfected cells expressing mature, [V5-His]-tagged FGF-23 and the [V5-His]-tagged

Table 1. Laboratory Findings in Patients with Documented or Suspected Oncogenic Osteomalacia and in Patients with X-Linked Hypophosphatemia.*

Diagnosis and Patient No.	Age yr	Sex	Surgery	Phosphate mg/dl	Calcium mg/dl	Intact Parathyroid Hormone pg/ml	1,25-Dihydroxy-vitamin D pg/ml	Alkaline Phosphatase U/liter	Tubular Reabsorption of Phosphate %	Fibroblast Growth Factor 23 RU/ml	Family History
Confirmed oncogenic osteomalacia											
1	50	F	Before	1.8	10.2	32	15	330	ND	426	
			After	4.2	10.1	33	33	204	ND	154	
2	53	F	Before	1.2	9.0	42	1	470	57	7970	
			After	4.1	9.0	47	87	373	94	31	
3	31	F	Before	0.9	8.7	ND	8	528†	70	6170	
			After	3.4	9.0	ND	53	700†	98	128	
4	46	M	Before	1.6	8.8	25	39	699	39	82	
			After	3.7	9.2	56	86	1343	94	51	
5	43	F	Before	1.4	9.5	ND	10	215	75	658	
			After	3.3	9.5	ND	59	173	ND	40	
6	30	M	Before	2.7	9.4	23	5	666	69	240	
			After	4.2	9.6	33	54	510	84	ND	
Suspected oncogenic osteomalacia											
7	32	M		1.5	8.6	36	7	845	71	166	
8	59	F		1.5	9.3	ND	ND	ND	79	496	
9	14	M		2.0	7.7	19	<10	428†	ND	376	
10	46	M		1.1	9.5	ND	7	359	43	1731	
11	37	F		1.6	9.2	27	12	797	82	116	
12	53	F		1.3	8.3	22	3	517	74	239	
13	31	F		1.2	9.0	ND	12	946	82	168	
14	45	M		2.4	9.2	43	30	231	64	32	
15	62	M		1.4	10.5	43	22	172†	ND	666	
16	35	F		1.2	9.4	ND	16	270†	ND	1168	
17	64	F		1.9	9.4	ND	ND	100†	ND	139	

carboxyl-terminal fragment (collectively referred to as [V5-His] rhFGF-23) was collected every other day and kept at -20°C until use. Bacterially produced [V5-His]rhFGF-23(25-251) protein, the mutant protein in which glutamine is substituted for arginine at position 179 ([V5-His;R179Q]rhFGF-23(25-251)), and fibroblast growth factor 19 were kindly provided by Drs. Susan Schiavi and Marlon Pragnell (Genzyme).

DEVELOPMENT OF AN ELISA FOR THE DETECTION OF FGF-23

Eight affinity-purified antibodies were immobilized

on microtiter-plate wells and biotinylated for subsequent detection with horseradish peroxidase conjugated to avidin (Pierce Chemical) to test different combinations of capture and detection antibodies in a two-site ELISA. The best recognition and sensitivity for the detection of [V5-His]rhFGF-23 were observed when anti-[Tyr-223]FGF-23(206-222)amide was used as a capture antibody and anti-[Tyr-224]FGF-23(225-244)amide was used as a detection antibody. In the assay, 150 µl of recombinant or synthetic FGF-23 or unknown sample and 50 µl of biotinylated detection antibody were used in each microtiter-plate well. After mixing and in-

Table 1. (Continued.)

Diagnosis and Patient No.	Age	Sex	Surgery	Phosphate	Calcium	Intact Parathyroid Hormone	1,25-Dihydroxyvitamin D	Alkaline Phosphatase	Tubular Reabsorption of Phosphate	Fibroblast Growth Factor 23	Family History
	yr			mg/dl			pg/ml	U/liter	%	RU/ml	
X-linked hypophosphatemia‡											
18	38	M		2.3	10.0	27	ND	432	65	307	+
19	55	F		2.9	10.0	23	ND	212†	ND	2335	-
20	16	F		1.9	9.4	57	ND	444†	ND	122	+
21	21	F		1.9	9.2	59	ND	149†	ND	46	+
22	55	F		2.4	9.9	34	ND	68†	ND	221	+
23	36	M		1.6	9.7	160	28	316	60	347	-
24	21	F		1.9	8.9	100	48	252	68	31	-
25	63	F		2.0	10.2	ND	ND	220	67	373	+
26	31	M		1.4	9.6	ND	ND	394	75	375	+
27	32	M		1.6	9.4	43	30	262	64	60	-
28	58	F		1.6	8.7	36	29	299	74	99	+
29	30	F		2.2	9.2	70	ND	165	82	242	-
30	28	F		1.9	9.8	82	26	173	76	169	-
31	59	F		1.9	9.6	ND	ND	294	49	1079	-
32	49	M		1.5	9.4	64	ND	131†	ND	143	+
33	16	F		1.9	9.4	36	ND	130†	ND	140	+
34	2	F		2.9	9.8	30	75	851†	66	234	-
35	22	M		2.0	10.2	46	ND	43 (bone-specific)§	62	575	+
36	55	F		1.9	8.8	54	28	102	ND	71	+
37	29	M		2.1	9.0	122	ND	232	ND	236	+
38	19	F		1.5	9.9	10	40	362	66	203	-

* The normal range for phosphate is 2.5 to 4.3 mg per deciliter; for calcium, 8.5 to 10.4 mg per deciliter; for intact parathyroid hormone, 10 to 65 pg per milliliter; for 1,25-dihydroxyvitamin D, 20 to 60 pg per milliliter; for alkaline phosphatase, 50 to 350 U per liter (in adults); and for tubular reabsorption of phosphate, 75 to 95 percent. To convert values for phosphate to millimoles per liter, multiply by 0.3229. To convert values for calcium to millimoles per liter, multiply by 0.250. F denotes female, M male, ND not done, plus signs a family history, and minus signs no family history. Data on Patients 1 and 6 were previously reported by White et al.² Data on Patient 3 were previously reported by White et al.² and Yang et al.¹⁴

† The normal range at the laboratory where the sample was tested is 30 to 120 U per liter.

‡ With the exception of Patient 37, all the patients with X-linked hypophosphatemia received treatment with phosphate and 1,25-dihydroxyvitamin D.

§ The normal range of bone-specific alkaline phosphatase is 24.2 to 89.5 μ g per liter.

cubation for 18 to 24 hours at room temperature, the wells were rinsed five times with normal saline containing 0.05 percent Tween 20 (350 μ l per well), and the immobilized "sandwich" complex was then incubated with horseradish peroxidase-conjugated avidin (200 μ l) in the dark for 1 hour to allow the enzyme-avidin complex to bind to the biotinylated

antibody. After rinsing (with 350 μ l per well, five times), the enzyme bound to the wells was incubated in the dark with tetramethylbenzidine substrate solution (200 μ l) for 30 minutes and immediately measured in a spectrophotometric plate reader at 620 nm. The reaction was then stopped by the addition of 1 M sulfuric acid (50 μ l per well); after

brief mixing, the plate was again read at 450 nm. This approach, involving dual reading at 620 and 450 nm, ensured that the assay had a wide dynamic range (3 to 2300 RU per milliliter).

STUDY SUBJECTS

To derive a reference range, blood samples (plasma in EDTA or serum) were collected from 85 healthy women and 62 healthy men (mean [\pm SD] age, 48.4 \pm 19.6 years) and 26 children (mean age, 10.9 \pm 5.5 years) with normal serum calcium and phosphate concentrations and normal renal function. The samples were obtained after the subjects had fasted overnight, and all the measurements were made from the same blood-sample collection. The study was approved by the human-studies committees of the Massachusetts General Hospital, Indiana University School of Medicine, St. Thomas' Hospital, National Hyogo-Chuo Hospital, and the University of Uppsala. Written or oral informed consent was obtained before the samples were collected.

Seventeen patients with confirmed or suspected oncogenic osteomalacia were studied (Table 1). Patients 1, 3, and 6 have been previously described,^{2,14} and the measurements were performed on stored samples. In five of the patients with confirmed oncogenic osteomalacia, samples were obtained for measurement of FGF-23 before and after surgical removal of the tumor^{2,14}; in one patient, whose disease was cured after tumor resection, only a preoperative sample was available. Oncogenic osteomalacia was suspected in 11 patients on the basis of clinical, radiologic, and laboratory findings, but a tumor had not been identified. The diagnosis of X-linked hypophosphatemia was based on a consistent medical history, a positive family history (in some cases), findings on physical examination, radiologic evidence of childhood rickets, and hypophosphatemia due to increased renal losses, as indicated by diminished tubular reabsorption of phosphate (Table 1), with initially normal serum concentrations of calcium and parathyroid hormone and normal concentrations of other serum electrolytes. All the patients with X-linked hypophosphatemia except one (Patient 37) were treated with oral phosphate and 1,25-dihydroxyvitamin D.

RESULTS

CHARACTERISTICS AND PERFORMANCE OF THE FGF-23 IMMUNOASSAY

An immunometric assay for detecting the carboxy-

terminal portion of FGF-23 was established with an affinity-purified antibody raised against [Tyr223]-FGF-23(206–222)amide as a capture antibody and an affinity-purified biotinylated antibody raised against [Tyr224]FGF-23(225–244)amide as a detection antibody (Fig. 1A). Since no synthetic or natural (purified) full-length FGF-23 is available, dilutions of supernatant from Sf-9 cells expressing [V5-His]-rhFGF-23 were used as a standard. The concentrations of standards and controls are expressed in reference units (RU) per milliliter relative to a specific lot of conditioned cell-culture medium (Fig. 1B) (available on request from Dr. Jüppner).

The sensitivity of the assay, 3.0 RU per milliliter, was determined by the 95 percent confidence limit (\pm 2 SD) in 20 duplicate determinations of the standard (0 RU per milliliter). The intraassay variation was less than 5.0 percent, and the interassay variation less than 7.3 percent. Blood samples, collected either as plasma (in EDTA or heparin) or as serum, showed no appreciable differences in the detectable concentration of immunoreactive FGF-23 (data not shown). Serial dilutions of samples from two patients with oncogenic osteomalacia showed parallelism to the standard curve (Fig. 1C). Studies in which [V5-His]rhFGF-23 was added to serum or plasma samples from healthy persons yielded recoveries of 89 to 115 percent. [V5-His]rhFGF-23 from Sf-9 cells and [V5-His]rhFGF-23(25–251) from bacterial cultures (data not shown), [V5-His;R179Q]rhFGF-23(25–251) from bacterial cultures, or synthetic FGF-23(207–244)amide showed parallel dose dilutions (Fig. 1B). Recombinant human fibroblast growth factor 19 at concentrations of up to 1.8 μ g per milliliter showed no cross-reactivity (data not shown).

CIRCULATING FGF-23 CONCENTRATIONS IN HEALTHY PERSONS AND IN PATIENTS WITH PHOSPHATE-WASTING DISORDERS

Samples from the 147 healthy adults with normal concentrations of calcium, phosphate, and creatinine revealed mean FGF-23 concentrations of 55 \pm 50 RU per milliliter (52.9 \pm 20.8 RU per milliliter in women and 42.0 \pm 15.8 RU per milliliter in men) (Fig. 2). The mean concentrations in samples from the 26 healthy children were 69 \pm 36 RU per milliliter, and samples taken every four hours during the day (8 a.m., noon, 4 p.m., and 8 p.m.) from six healthy adults eating a regular diet revealed no major differences in mean FGF-23 concentrations (35 \pm 4.1, 44 \pm 6.6, 35 \pm 14.6, 77 \pm 17.0, 58 \pm 5.3, and

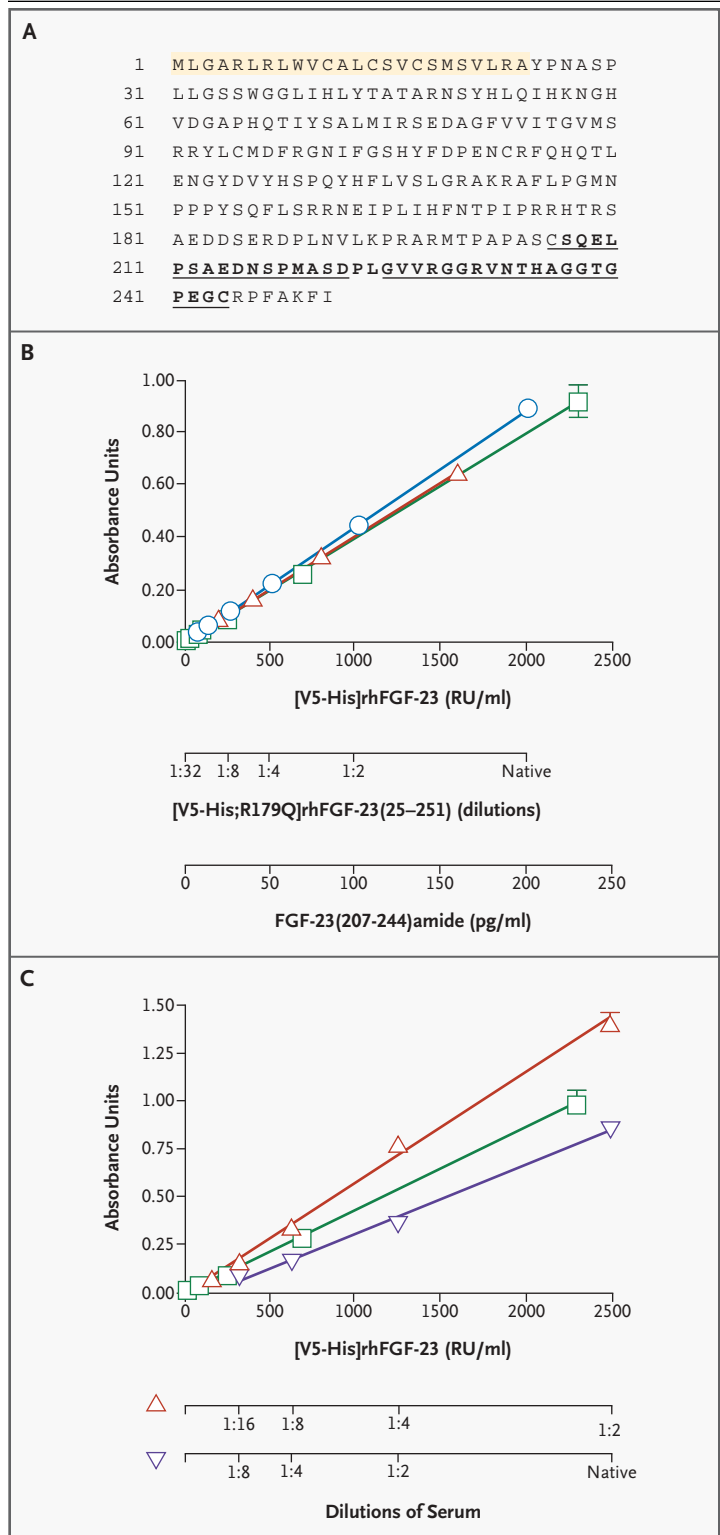
42±7.6 RU per milliliter for each adult, respectively). The 10 patients with end-stage renal disease who were undergoing dialysis had elevated levels of FGF-23 (range, 162 to 5820 RU per milliliter); dilutions of these samples ran parallel to the standard curve.

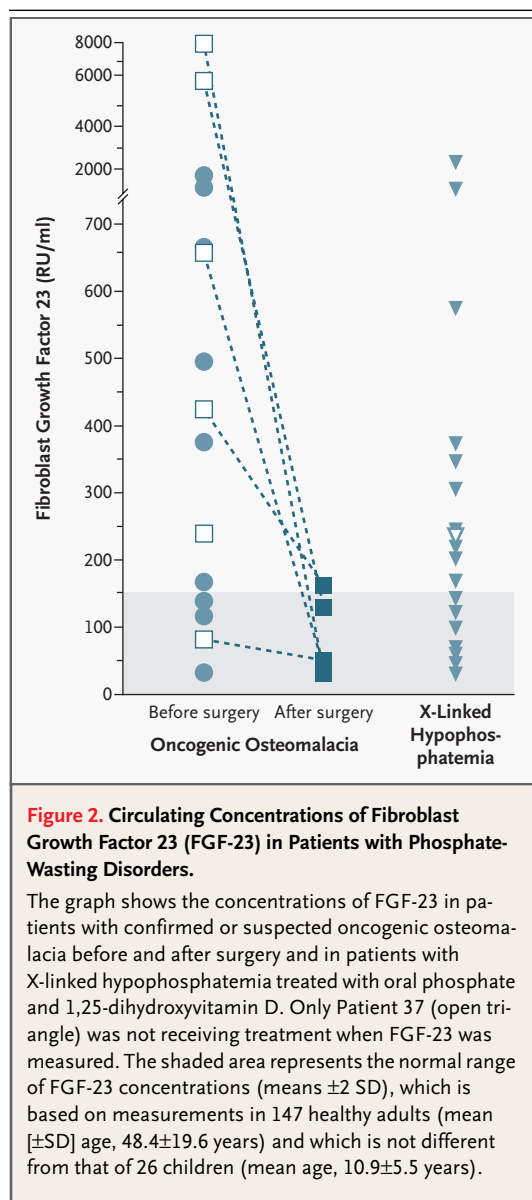
As indicated in Table 1, six patients with oncogenic osteomalacia had complete resolution of renal phosphate wasting and consequent normalization of phosphate concentrations in the blood after tumor resection. Before tumor resection, five of these patients had markedly elevated FGF-23 concentrations (Fig. 2); after tumor removal, their FGF-23 concentrations were within or slightly above the normal range. Patient 4, who had oncogenic osteomalacia, had FGF-23 concentrations well within the range for healthy controls before and after surgery (Table 1), although removal of the tumor led to normalization of urinary phosphate excretion and serum phosphate concentrations. Of samples from 11 other patients with suspected oncogenic osteomalacia, the FGF-23 concentrations were elevated in 8 but normal in 3.

Thirteen of the 21 patients with X-linked hypophosphatemia (mean age, 34.9±17.2 years) had el-

Figure 1. Characteristics of the Two-Site Enzyme-Linked Immunosorbent Assay for Fibroblast Growth Factor 23.

Panel A shows the amino acid sequence of human fibroblast growth factor (FGF-23). The putative signal peptide (residues 1 to 24) is shown on a shaded background; the amino acid sequence of the predicted mature protein is shown without shading; the amino acid sequence of synthetic FGF-23(207–244) amide is shown in bold type; and the sequences that were used for immunization are underlined. Either Tyr223 or Tyr224 was added to the peptides used for immunization. As shown in Panel B, microtiter-plate wells were coated with anti-[Tyr223]FGF-23(206–222) amide as a capture antibody. The plates were then incubated with increasing concentrations of [V5-His]rhFGF-23 (green squares), serial dilutions of [V5-His;R179Q]rhFGF-23(25–251) (blue circles), or FGF-23(207–244) amide (red triangles). After extensive rinsing, the biotinylated detection antibody directed against [Tyr-224]FGF-23(225–244) amide was added for quantification with horseradish peroxidase-conjugated avidin. Recombinant fibroblast growth factor 19, at concentrations as high as 1.8 µg per milliliter, was not detected (data not shown). Panel C shows increasing concentrations of [V5-His]rhFGF-23 (green squares) and serial dilutions of serum from Patients 2 (red triangles) and 10 (purple triangles). In Panels B and C, the I bars represent standard deviations.





elevated FGF-23 concentrations (>155 RU per milliliter [2 SD above the mean for healthy adults]) (Fig. 2). The eight patients with X-linked hypophosphatemia and normal FGF-23 values had serum phosphate concentrations well below the reference range (Table 1).

DISCUSSION

The ELISA for FGF-23 that was developed and clinically evaluated detects full-length FGF-23 as well as a synthetic carboxyl-terminal fragment, FGF-23 (207–244)amide. Because two affinity-purified an-

tibodies were raised against synthetic FGF-23 fragments, this assay does not detect fibroblast growth factor 19, a member of the fibroblast growth factor family of proteins that is more closely related to FGF-23 than other fibroblast growth factors. Furthermore, the substitution of glutamine for arginine at position 179 of [V5-His]rhFGF-23(25–251) did not alter cross-reactivity, making it likely that this assay can detect wild-type FGF-23 and the mutant forms identified in autosomal dominant hypophosphatemic rickets^{1,2,10} with equal efficacy. However, in patients with various phosphate-wasting disorders, fragments of FGF-23 in addition to full-length wild-type and mutant forms of protein^{2,3,8,10} may exist in the circulation, and the fragments may have altered affinity for one or both of the two antibodies. In our study, circulating FGF-23 concentrations were readily detectable in healthy adults and children, increasing the likelihood that this growth factor may be involved in the physiologic regulation of phosphate homeostasis. However, FGF-23 expression could be detected only at low levels and only in a few tissues,^{1-3,7} and its normal source in healthy persons therefore remains uncertain.

In oncogenic osteomalacia, tumor tissue is the major source of FGF-23, as previously shown by the abundant amounts of FGF-23 mRNA and protein produced by these often remarkably small and hard-to-find tumors.^{2-4,15} In some cases, elevated FGF-23 concentrations normalized after tumor removal—an observation that suggests that the ELISA for FGF-23 might be a useful tool for locating occult FGF-23-producing tumors through selective venous sampling and for monitoring the surgical or medical treatment of patients with this disorder.¹⁵

Patient 4, who had oncogenic osteomalacia, had concentrations of FGF-23 well within the normal range both before and after the removal of his tumor, although his urinary phosphate excretion and, consequently, his serum phosphate concentration normalized after resection. The tumor from this patient expressed FGF-23 mRNA, as determined by the reverse-transcriptase polymerase chain reaction, but it is currently unknown whether the mRNA was as abundantly expressed as in previously reported cases.^{2-4,15} Thus, it remains uncertain whether FGF-23 or other proteins with phosphaturic activity could be responsible for the disease in this and possibly other patients.

No attempts were made to measure FGF-23 in patients with X-linked hypophosphatemia after the discontinuation of regular medication, since the

half-life of FGF-23 is currently unknown and since prolonged withdrawal of therapy might affect patient outcomes. However, the finding that FGF-23 is elevated in some, but not all, patients with X-linked hypophosphatemia may have implications for our current understanding of this genetic disorder.

The FGF-23 assay may facilitate studies that address the role of FGF-23 in normal phosphate homeostasis and that explore whether additional molecular species of this growth factor are present in the circulation of healthy persons and of patients with phosphate-wasting disorders or various stages of renal failure. FGF-23 measurements may thus offer improved insight into the normal regulation of phosphate homeostasis and have implications for the diagnosis and treatment of phosphate-wasting disorders.

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Dr. Jüppner reports having received honorariums from Genzyme and having received patents on parathyroid hormone and parathyroid hormone-related peptide (PTH/PTHrP) receptors and on PTH/PTHrP analogues activating these receptors. Drs. White and Econs report having a patent pending on FGF-23.

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