SUPPLEMENTAL DATA

Autoimmune Hyperphosphatemic Tumoral Calcinosis in a Patient with FGF23 Autoantibodies

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Patient

This study was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research, NIH. The parents of the minor subject provided written consent to the study protocol; the subject provided written assent. The subject underwent a set of evaluations that included detailed medical and family history, physical examination, standard biochemical evaluation, detailed laboratory evaluation of calcium and phosphorus metabolism including 1,25D, intact parathyroid hormone (iPTH), TRP, iFGF23, and C-terminal FGF23, genetic testing, and standard radiographs. Clinical evaluations performed by subject's home physicians occurred prior to consenting to the NIH study, and the subject permitted use of this data for research purposes/publication upon signing informed consent.

Biochemical

Testing included routine blood and urine chemistries, complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), iPTH (electrochemiluminescence immunoassay on Roche Cobas e601 analyzer; NIH, Bethesda, MD, USA), 1,25D (radioimmunoassay or liquid chromatography-tandem mass spectrometry, Mayo Medical Laboratories, Rochester, MN, USA), insulin (electrochemiluminescence immunoassay on Roche Cobas e601 analyzer; NIH, Bethesda, MD, USA), and C-peptide (electrochemiluminescence immunoassay on Roche Cobas e601 analyzer; NIH, Bethesda, MD, USA). Hemoglobin A1c was determined via high performance liquid chromatography (NIH Clinical Center Department of Laboratory Medicine, Bethesda, MD, USA). iFGF23 and C-terminal FGF23 were measured

by second generation enzyme-linked immunosorbent assay (ELISA) (Immutopics International, San Clemente, CA, USA). The iFGF23 assay measures only the intact protein while the C-terminal FGF23 assay reflects both the intact and C-terminal fragments. Serial dilutions (1:1, 1:10, 1:20, 1:50, 1:100, 1:500 and 1:1000) of plasma samples was performed using sample diluent as recommended by the kit manufacturer to assess for interfering antibodies and then evaluated via iFGF23 and C-terminal FGF23 ELISA's.

Genetic analysis

Genomic DNA was isolated from leukocytes using standard procedures. All coding exons and their adjacent intronic sequences in *FGF23*, *KLOTHO,* and *FGFR1* were amplified by PCR, using Multiplex PCR Kit (QIAGEN Inc., Valencia, CA, USA). Approximately 100 ng of purified PCR products were directly sequenced from PCR primers, using Big-Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) [1].

Whole exome sequencing

The sample was prepared for whole exome sequencing using the Nextera Rapid Capture Exome Kit (Illumina, San Diego, CA) according to manufacturer's protocols. Sequencing was completed on an Illlumina HiSeq 2500 instrument with TruSeq v4 reagents, yielding paired end 125 nucleotide reads, with 6.9 GB of data resulting in a mean 50X coverage. Gapped alignment to reference sequences (GRCh37.p5) was performed with GSNAP and the GATK and analysis completed using custom-developed software, RUNES and VIKING as previously reported [2, 3].

Imaging

Tumoral calcinosis was assessed by conventional radiography and magnetic resonance imaging.

Generation of mammalian expression vectors for light-emitting fusion proteins

Light-emitting luciferase fusion proteins for FGF23, FGFR1 and Klotho were generated for use with the LIPS antibody profiling technology [4]. To generate the fusion proteins, commercially available plasmids were employed to clone the specific cDNA fragment for each gene and the strategies utilized are available upon request. For FGF23, the entire molecule (amino acid resides 1-251) was generated, while for FGFR1, only the extracellular region excluding the transmembrane region was used (amino acid residues 1-285). For Klotho [5], a DNA fragment generated by restriction enzyme digestion containing amino acid residues 1-967 and containing its signal peptide was subcloned. The mammalian expression constructs for N-terminal FGFR1 and FGF23 fusion proteins contained their endogenous start methionine along with signal peptide sequences and were built in the pGAUS3 [6] vector expressing *Gaussia* luciferase (Gluc). A new vector pNANO3, replacing the Gluc fragment with NanoLuc [7] was used to make the N-terminal fusion protein for Klotho. Plasmids for these different mammalian expression vectors containing the correct inserts were prepared using a Qiagen Midi kit. DNA sequencing confirmed integrity of the three constructs.

LIPS autoantibody analysis

The LIPS technology was performed as previously described [8]. The constructs for the potential autoantigens were transfected into Cos1 cells (ATCC) with Fugene-6 transfection reagent (Promega) and cell lysates harvested 48 hours later to obtain crude cell extracts. For autoantibody testing, serum samples were diluted 1:10 in assay buffer A (20 mM Tris, pH 7.5,

150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) and diluted aliquots (10 μl) were then evaluated

using a 96-well plate format as described previously [8]. For these tests, serum (equivalent to

1 μl of serum), 40 μl of buffer A and 50 μl of Cos1 cell extract containing 10^7 light units (LU) of

a particular luciferase-antigen extract were used. After incubation at room temperature for one

hour, a microtiter filter plate (Millipore Sigma) containing protein A/G beads (Protein A/G

UltraLink Resin, Invitrogen) captured the IgG antibody-antigen complexes for one hour

incubation. The antibody-antigen-bead complexes were then washed eight times with buffer A

and twice with PBS on a microtiter filter plate to remove unbound antigens. After the final wash,

light units (LU) were measured in a Berthold LB 960 Centro microplate luminometer (Berthold

Technologies, Bad Wildbad). While injection of coelenterazine substrate (Promega) was used

for detection of Ruc and Gluc reporter activity, the Nano-Glo® substrate (Promega) was used for

NanoLuc LU measurements.

Cell culture

Human embryonic kidney cells (HEK293) stably transfected with full-length transmembrane form

of mouse α-Klotho (KL) was a kind gift from Makoto Kuro-o, University of Texas Southwestern,

Dallas, TX [9]. HEK293-KL cells were cultured in DMEM-H21 containing 10% fetal bovine

serum (FBS) (HyClone™) and 1% Geneticin (Gibco). All culture plates were coated with 50μg/ml

gelatin (Sigma-Aldrich) in phosphate buffered saline (PBS) for 30min at 37°C. On day 1,

HEK293-KL cells were seeded in 6 or 24 well plates at a density of 650,000 or 130,000 cells per

well, respectively. On day 2, media was changed to reduced serum media (1% FBS). On day 3,

cells were treated with either vehicle (PBS) or recombinant human FGF23 R176Q (25, 50, or 100

ng/ml) (Amgen) with 2% human patient plasma or 2% human control plasma. As a negative

control, parallel experiments were set up with vehicle (PBS containing 0.1% bovine serum albumin) or recombinant human bFGF 10 ng/ml (R&D Systems). Treatment time was 15 min for detection of phosphorylated ERK1/2 and 2 hours for determination of *EGR1* mRNA abundance.

RNA extraction and quantitative PCR (qPCR)

All qPCR reagents are from Life Technologies unless stated otherwise. Total mRNA was extracted with TRIzol™ followed by purification with NucleoSpin® RNA Miniprep (Clonetech) including DNAse digestion. Total mRNA extractions were analyzed for quality, purity, and concentration using the NanoDrop ND-1000 spectrophotometer (NanoDrop products). RNA samples were diluted to a final concentration of 100 ng/µl and cDNA was prepared as described below. In a reaction volume of 40 µl, 300 ng of RNA was used as template and mixed with the following final concentrations of RT buffer (1x), $MgCl_2$ (5.5 mmol/l), random hexamers (2.5 µmol/l), dNTP mix (500 µmol/l each), RNase inhibitor (0.4 U/µl), multiscribe reverse transcriptase (1.25 U/µl), and RNAse-free water. Reverse transcription was performed with thermocycling conditions set at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min on a thermocycler (Eppendorf North America). qPCR was performed on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). *EGR1* primers (forward 5'-AGCAGCACCTTCAACCCTC-3', reverse 5'-GTCTCCACCAGCACCTTCTC-3') were designed using Primer 3 software [10, 11] and *18SrRNA* (control) primers were purchased from Life Technologies. Primers were chosen to span intron - exon boundaries to exclude genomic DNA detection. The specificity of all primers was tested by agarose gel electrophoresis to confirm amplification of a single product of the expected size (data not shown). qPCR reactions were performed using either PowerUp™ SYBR™ Green Master Mix or TaqMan® Fast Advanced Master Mix.

Protein extraction and western blot

Proteins were extracted with RIPA buffer containing 20mM Tris-HCl, 150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1mM EDTA, 0.1% SDS, 1mM PMSF (Sigma-Aldrich), and 1x Complete® protease inhibitor (Roche). Protein concentration was measured with Pierce ® BCA Protein Assay (Thermo Scientific). 30μg protein were mixed with loading buffer containing DTT and separated on a 10% polyacrylamide gel. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Millipore). After blocking with Odyssey Blocking Buffer (PBS) (LI-COR Biosciences) for 1 hour, blots were incubated with primary antibodies p44/42 MAP Kinase (L34F12) mouse monoclonal antibody (1:2000) and Phospho-p44/42 MAP Kinase (Thr202/Tyr204) (D13.14.4E) XP® rabbit monoclonal antibody (1:2000) (Cell Signaling Technology) for 2 hours at room temperature or overnight at 4°C. Membranes were incubated with secondary donkey anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680 antibodies (LI-COR Biosciences) (1:25000) in Odyssey Blocking Buffer (PBS). The protein signal was detected with LI-COR Odyssey infrared imager and quantified with Image Studio Lite software version 5.2.

Lymphocyte immunophenotyping of human blood using flow cytometry

Whole blood was harvested into EDTA tubes, red blood cells were lysed using BD FACS Lysing Solution (BD Biosciences), and staining was performed using fluorochrome-conjugated (FITC, PE, PE-Cy7, APC, APC-eFluor 780, Alexa Fluor 700, eFluor 450, PerCP-Cy5.5) antibodies against human CD45 (HI30), CD19 (SJ25C1), CD27 (TNFRSF7), and CD10 (CB-CALLA) (eBioscience); CD3 (SK7), CD16 (SK7), CD56 (SK7), CD20 (L27), CD62L (SK11), and CD38

(HB7) (BD Biosciences); IgM (MHM-88) and CD21 (Bu32) (BioLegend); CD4 (S3.5) and CD8 (3B5) (ThermoFisher Scientific); and CD45RA (ALB11) (Beckman Coulter) for 30 minutes on ice. After incubation, the cells were washed with FACS buffer and the samples were acquired using a FACSCanto (BD Biosciences). FlowJo (TreeStar) was used for the final analysis. Cell numbers were quantified from the FACS plot percentages using the patient's same-day absolute lymphocyte count obtained from the complete blood cell with white count differential.

Assessment of T- and B-Cell clonality

DNA was extracted from peripheral blood cells using a PSS USA Magnatron System 8LX automated DNA extraction robot and PCR amplified for detection of immunoglobulin (IGH and IGk loci) and T-cell receptor gene (TRG locus) rearrangements.   Sizing control reactions indicated that the quality of the extracted DNA was adequate for all PCR assays.

For the IGH locus, two separate reactions were performed, the first using consensus primers to framework region III and the joining region of the immunoglobulin heavy chain gene (FRIII-IGH PCR), and the second using consensus primers to framework region II and the joining region of the immunoglobulin heavy chain gene (FRII-IGH PCR) [12]. For both reactions, the joining region primer was covalently linked to a fluorescent dye FAM to allow for fluorescence detection. The products were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer, and electropherograms were analyzed using GeneMapper software version 4.0 (ABI). Polyclonal rearrangement patterns were detected in both reactions.

Two additional reactions were performed for the IGk locus using the Biomed II primer set [13], and supplied by InVivoScribe Technologies (IGK Gene Clonality Assay - ABI Fluorescence Detection). These reactions interrogate rearrangements involving the Vk loci and Jk (Tube A), the Vk locus and the kDE locus (Tube B), and the k intron RSS locus and the kDE locus (Tube B). The products were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer, and electropherograms were analyzed using GeneMapper software version 4.0 (ABI).

IG PCR is capable of detecting a clonal population comprising 2%-10% (FRIII-IGH=3%, FRII-IGH=10%, IGk Tube A=2% and Tube B=10%) of the total B-cell population. The combined analysis of IGH and IGk can identify approximately 85-90% of all clonal B-cell proliferations. Failure of amplification of any of the above four independent reactions results in a lower overall clinical sensitivity.

For the TRG locus, a single multiplexed PCR reaction was performed [14] using primers that interrogate TRG rearrangements involving all of the known Vg family members, and the Jg1/2, JP1/2 and JP joining segments. To allow for fluorescence detection, each joining region primer was covalently linked to a unique fluorescent dye. The products were analyzed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer, and electropherograms were analyzed using GeneMapper software version 4.0 (ABI). TRG PCR is capable of detecting a clonal population comprising a minimum of 2-5% of the total T-cell population, and can identify >90-95% of all TRG gene rearrangements occurring in clonal T-cell proliferations.

These tests were developed and their performance characteristics determined by the Molecular Diagnostics Unit of the Laboratory of Pathology, NCI, in Bethesda, MD, USA.

Evaluation of additional autoantibodies

Chemiluminescence was used to measure autoantibodies targeted against thyroid peroxidase and anti-thyroglobulin, and immunoglobulin levels were measured on Roche Cobas 6000 Analyzer (NIH Clinical Center Department of Laboratory Medicine, Bethesda, MD, USA). Tissue transglutaminase IgA was measured via enzyme-linked immunosorbent assay. Radioimmunoassay was used to measure autoantibodies targeted against GAD65, islet antigen-2, and insulin, and immunoabsorption assay was used to measure autoantibodies targeted against 21-hydroxylase. Intrinsic factor blocking antibody and parietal cell IgG antibody measurements in the serum of the patient using a competitive binding immunoenzymatic assay and enzyme-linked immunosorbent assay, respectively. These tests were performed at Mayo Medical Laboratories, Rochester, MN, USA.
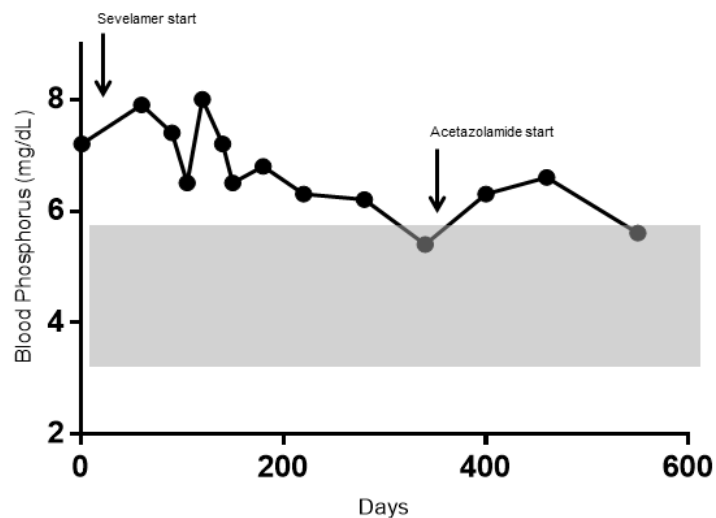
Supplemental table

Supplemental Table 1 Intact FGF23 and C-terminal FGF23 serial dilutions assessed via ELISA

Dilution	Intact FGF23[1] (pg/mL)		C-terminal FGF23[2] (RU/mL)	
	ELISA Readout	Calculated Intact FGF23	ELISA Readout	Calculated C-terminal FGF23
1:1	944		913	
1:10	447	4470	908	9078
1:20	257	5145	856	17112
1:50	142	7094	532	26614
1:100	79	7925	275	27512
1:500	15	7599	55	27682

[1]Upper limit of detection for intact FGF23 ELISA in our lab is 658 pg/mL, and the lower limit of detection is 1.5 pg/mL
[2]Upper limit of detection for C-terminal FGF23 ELISA in our lab is 448 RU/mL, and the lower limit of detection is 1.5 RU/mL

Supplemental Figure 1

Supplemental Figure 1. Blood phosphorus in tumoral calcinosis patient on phosphate-lowering medical therapy. The patient was initially prescribed a low phosphorus diet and sevelamer, a phosphate binder,1600 mg with meals, 800 mg with snacks. Approximately 14 months after starting this regimen, acetazolamide 125 mg twice daily was added in an effort to promote renal phosphate wasting. The blood phosphorus level fluctuated on phosphate-lowering medical therapy but overall declined with a nadir of 5.4 mg/dL, which is in the normal range for age.

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