Molecular and biochemical analyses of FGF23 mutations in familial tumoral calcinosis

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FGF23 is a hormone required for normal renal phosphate reabsorption. FGF23 gain-of-function mutations result in autosomal dominant hyperphosphatemic rickets (ADHR), and FGF23 loss-of-function mutations cause familial hyperphosphatemic tumoral calcinosis (TC). In this study, we identified a novel recessive FGF23 TC mutation, a lysine (K) substitution for glutamine (Q) (160 C > A) at residue 54 (Q54K). To understand the molecular consequences of all known FGF23-TC mutants (H41Q, S71G, M96T, S129F, and Q54K), these proteins were stably expressed in vitro. Western analyses revealed minimal amounts of secreted intact protein for all mutants, and ELISA analyses demonstrated high levels of secreted COOH-terminal FGF23 fragments but low amounts of intact protein, consistent with TC patients’ FGF23 serum profiles. Mutant protein function was tested and showed residual, yet decreased, bioactivity compared with wild-type protein. In examining the role of the FGF23 COOH-terminal tail (residues 180–251) in protein processing and activity, truncated mutants revealed that the residues downstream from the known FGF23 SPC protease site (176RXXR179/S180) were not required for protein secretion. However, residues adjacent to the RXXR site (between residues 188 and 202) were required for full bioactivity. In summary, we report a novel TC mutation and demonstrate a common defect of reduced FGF23 stability for all known FGF23-TC mutants. Finally, the majority of the COOH-terminal tail of FGF23 is not required for protein secretion but is required for full bioactivity.

fibroblast growth factor 23; phosphate; hyperphosphatemia; Klotho

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mutations and determined the significance of the FGF23 COOH-terminal region. These studies expand our current understanding of the molecular genetic and metabolic etiologies of TC and reveal a common pathogenic mechanism for TC cases due to mutations in the FGF23 gene.

MATERIALS AND METHODS

TC patients. All subjects provided written, informed consent, in accordance with the Institutional Review Board of Indiana University, which approved the study. The kindreds were of Iranian descent. Routine serum biochemistries were assessed using standard protocols.

FGF23 serum assays. Intact FGF23 serum concentrations were determined using an ELISA according to the manufacturer’s protocol (Kainos Laboratories International, Tokyo, Japan). This assay uses monoclonal antibodies and has been shown to recognize full-length human and rodent FGF23/Fgf23 (34). Serum FGF23 concentrations were also assessed using a COOH-terminal FGF23 serum assay kit (Immutopics, San Clemente, CA) (17) according to the manufacturer’s protocol. This kit is a two-site sandwich ELISA that recognizes the COOH-terminal portion of human FGF23, thus reacting with both full-length and COOH-terminal fragments of FGF23.

FGF23 and GALNT3 mutational analyses. Genomic DNA was extracted from blood samples using the Qiamp DNA Blood Extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Gene exons, including the intron-exon splice junctions, were PCR-amplified with intronic primers (available upon request), using 20 ng of genomic DNA as template. PCR conditions for all experiments were 1 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 57°C, 1 min at 72°C, and a final extension of 7 min at 72°C. Amplified exons were analyzed by DNA sequencing with the appropriate forward primers for each exon using Big Dye Terminator Chemistry.

FGF23 exon 1 controls. A primer pair (forward: 5’AATCTCAG- CACAGCCACTC 3’; reverse: 5’GATGGACACAAAGGGTGCTC 3’) was used to amplify a 270-bp region containing exon 1 of the FGF23 gene in the patients and in 200 control alleles. PCR conditions were the same as those described above for mutational analyses. The resulting PCR products were digested with Pstl for 18 h at 37°C and analyzed on 2% ethidium bromide-stained agarose gels.

Site-directed mutagenesis. Point mutations within the FGF23 cDNA were generated with the QuickChange II XL site-directed mutagenesis kit (Stratagene) using pCMV-FLAG containing wild-type (WT), full-length FGF23 (33) to create the FGF23-TC cDNAs: H41Q, Q54K, S71G, M96T, and S129F. Complimentary primer pairs (15–25 bases in length, available upon request) were designed to create the TC mutations using the PrimerX primer design program (http://bioinformatics.org/primerx/). PCR conditions for site-directed mutagenesis were 1 min at 95°C followed by 35 cycles of 50 s at 95°C, 50 s at 60°C, 7 min at 68°C, and a final extension of 7 min at 68°C. The mutagenesis reactions were digested with DpnI for 1 h at 37°C to remove template WT plasmid DNA. The plasmids containing mutant FGF23 cDNAs were sequenced to confirm replacement of the targeted residues.

TC mutant FGF23 in vitro expression and Western analyses. The expression vectors containing the Q54K, H41Q, S71G, M96T, and S129F mutant FGF23 cDNAs were transiently expressed in human embryonic kidney (HEK)-293 cells as previously performed using the Fugene6 transfection reagent (Roche) (21, 32). The cellular lysates and media were collected in 1X lysis buffer (Cell Signaling) and assessed for the presence of FGF23 protein by Western analysis. The samples were electrophoresed on 15% SDS-PAGE minigels (Bio-Rad) and electrotransferred to PVDF membranes (Bio-Rad). The membranes were then probed with an anti-FLAG antibody (1:1,000;

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**Fig. 1.** Family pedigree. Tumoral calcinosis (TC) patients 2 and 3 are members of a large consanguineous family (inset). Patient 2 had calcific lesion surrounding the large right toe and right elbow.
Table 1. TC patient serum biochemistries

<table>
<thead>
<tr>
<th>Patient 1 (20 yr old)</th>
<th>Patient 2 (11 yr old)</th>
<th>Patient 3 (5 yr old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate, mg/dl</td>
<td>7 (2.7–4.5)</td>
<td>8.8 (4–6.5)</td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>9 (9–10.5)</td>
<td>9.3 (8.5–10.4)</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>12 (9–55)</td>
<td>17 (9–55)</td>
</tr>
<tr>
<td>1.25(OH)2D, pg/ml</td>
<td>32 (15–60)</td>
<td>42 (20–55)</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.9 (0.6–1.2)</td>
<td>1 (0.4–1.5)</td>
</tr>
</tbody>
</table>

TC, tumoral calcinosis; PTH, parathyroid hormone. Normal ranges are shown in parentheses.

RESULTS

Clinical assessment of TC patients. Three patients presented with a phenotype consistent with tumoral calcinosis. Patient 1 was an isolated case, whereas patients 2 and 3 were members of a large consanguineous family (Fig. 1). Patient 1, a 20-yr-old male, was referred for calcific lesions in the elbow, hip, and knee. Analyses of serum biochemistries revealed persistent hyperphosphatemia and normocalcemia as well as normal parathyroid hormone (PTH) and 1,25(OH)2 vitamin D concentrations (Table 1). The lesion in the patients’ elbows had been surgically removed due to restriction of movement, but it recurred.
Patient 2, an 11-yr-old male, was originally referred for the development of a painful lesion in the right big toe following physical trauma. The lesion in the toe was surgically removed, but it recurred (Fig. 1, inset). An additional lesion causing movement limitation developed in the patient’s right elbow (Fig. 1, inset). Serum biochemistries revealed persistent hyperphosphatemia and normocalcemia as well as normal PTH and 1,25(OH)2 vitamin D (Table 1).

Patient 3, a 5-yr-old male, was referred following the development of a lesion in the left big toe. No physical trauma to the affected area was noted; however, the mass of the lesion increased over an 8-mo period. Serum biochemistries were significant for mild hyperphosphatemia with normocalcemia as well as normal PTH and 1,25(OH)2 vitamin D levels (Table 1).

FGF23 serum assays. Serum FGF23 levels were assessed in these patients using two assays, the first being a “COOH-terminal” ELISA that detects both full-length and COOH-terminal fragments of FGF23 and the second being an “intact” ELISA that utilizes epitopes encompassing the 176RXXR179/S180 SPC protease site and therefore recognizes intact FGF23 protein. The COOH-terminal ELISA results showed that patients 1, 2, and 3 had FGF23 concentrations roughly nine, 17, and six times the normal mean, respectively (Fig. 2). In contrast, the intact FGF23 concentrations for the three patients were found to be inappropriately normal considering the marked degree of persistent hyperphosphatemia (Fig. 2). These values of elevated COOH-terminal FGF23 concentrations in parallel with normal levels of intact FGF23 are consistent with the aberrant circulating FGF23 profile detected in other TC cases (3, 22).

Mutational analyses. Genomic DNA from patients 1–3 was assessed for GALNT3 mutations as the etiology for the TC phenotype; however, no mutations were detected within the exons or intronic splice sites. In contrast, direct DNA sequence analyses of the patients’ FGF23 exon 1 revealed a homozygous guanine-to-adenine (159 G > A) silent transition in concert with a cytosine-to-adenine transversion (160 C > A). The C > A change at position 160 resulted in a novel glutamine (Q)-to-lysine (K) amino acid substitution at position 54 (Q54K) (Fig. 3A). All other FGF23 exons and splice junction sites were negative for nucleotide substitutions.

FGF23 exon 1 controls. The 159 G > A substitution and the 160 C > A substitution that results in the Q54K change were not found in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). Normal controls were then assessed by restriction fragment length polymorphism (RFLP) for the nucleotide changes, because either sequence alteration would interrupt a PstI site containing nucleotides 159 and 160. PCR amplification of FGF23 exon 1 results in a 270-bp DNA product containing two PstI restriction sites. Digests of the 270-bp exon 1 product from normal individuals with PstI create 3 fragments that are 46 (not visible), 109, and 115 bp in size (lane 2). The presence of the guanine (G) to adenine (A) transition at position 159 (159 G > A) and cytosine (C) to adenine (A) transversion at position 160 (160 C > A) mutations interrupt the PstI restriction site between the larger 109- and 115-bp fragments and results in the appearance of a 224-bp DNA fragment (lanes 4, 5, and 6). Position of molecular size markers are shown for reference at left. H, histidine; L, leucine; K, lysine; I, isoleucine; Q, glutamine.

FGF23 mutant expression in vitro. Previous studies indicated that FGF23 mutations resulting in TC caused destabilization of the intact FGF23 protein (21). The molecular consequences of the Q54K mutation on FGF23 protein stability, as well as other novel FGF23-TC mutants, including H41Q (23) and M96T (6), have not been evaluated; thus whether a common cellular mechanism exists for TC is currently unknown. Therefore, site-directed mutagenesis was used to produce FGF23 cDNAs harboring each of these three untested mutations (Q54K, H41Q, and M96T) as well as the S71G and S129F (Ser71Gly and Ser129Phe) FGF23-TC mutations that we have previously assessed for expression in vitro (22). These mutant FGF23 constructs were individually transfected into HEK-293 cells. As determined by Western analyses of the cellular lysates using an anti-FLAG antibody that recognizes an NH2-terminal FLAG epitope, all of the FGF23 TC mutants were retained within the cells, and intact FGF23 protein was not detectable in the unconcentrated medium.
from the same cells (Fig. 4A). In contrast, WT FGF23 displayed a known distribution pattern of low intracellular expression and high extracellular expression due to efficient processing of the native molecule (Fig. 4A). These results demonstrating similar aberrant protein production by the FGF23-TC mutants were then confirmed using the COOH-terminal and intact FGF23 ELISA on the cell medium. This assay showed markedly elevated levels of COOH-terminal fragments (Fig. 4B). Concomitant with these findings, low levels of intact FGF23 were detected by ELISA analysis in the same media samples (Fig. 4B), consistent with similar COOH-terminal and intact FGF23 ELISA patterns from the patients’ serum samples (Fig. 2). Taken together, these results demonstrate that the known FGF23-TC mutants, including the novel Q54K mutation, have a common molecular property that results in the destabilization of intact FGF23 protein.

**FGF23 mutant activity analyses.** FGF23 stimulates the transcription of EGR1 in HEK-293 and other cell lines in the presence of the coreceptor KL (31). An assay based upon these findings has been developed for determining FGF23 bioactivity (11, 14). To produce mutant FGF23 protein at levels greater than that which could be derived from transiently transfected cells, we developed HEK-293 cell lines stably producing each of the FGF23 mutants Q54K, H41Q, S71G, M96T, and S129F (Fig. 5A). Due to the increased expression levels in the stable cell lines, Western blot analyses revealed that these lines expressed truncated FGF23 analogs as a 34-kDa protein species, which represented a partially glycosylated full-length FGF23 protein (2). The FGF23 protein concentrations in the media which represented a partially glycosylated full-length FGF23 were treated with FGF23-conditioned medium from each mu-

**MOLECULAR ANALYSIS OF MUTANT FGF23 IN TC**

**Fig. 4. A:** Western blot analyses of TC-mutant FGF23 proteins. Western blot analyses of conditioned media from human embryonic kidney (HEK)-293 cells transiently expressing wild-type (WT) FGF23 revealed that the protein is produced and secreted into the media (top) as 36- (full-length) and 26-kDa (NH2-terminal domain) proteins. These same cells were negative for the presence of the protein in the lysate (bottom). Media collected from cells expressing FGF23 cDNAs containing the TC mutations were negative for the presence of FGF23 (top). Lysates from the same cells were positive for a 34-kDa protein (bottom). B: intact and COOH-terminal FGF23 concentrations in conditioned media. ELISA analyses were performed to quantify intact and COOH-terminal FGF23 protein present in media samples from the mutant FGF23 species. The COOH-terminal ELISA results indicated mutant FGF23 protein levels similar to WT (Log10 concentration shown; top); however, the intact ELISA results showed that intact protein levels were >200-fold lower for the mutant proteins compared with WT FGF23 (bottom).
showed that in contrast to changes within the FGF23 NH2-terminal domain (Fig. 4), modification of the COOH terminus did not alter secretion of FGF23 protein compared with WT protein.

Activity of truncated FGF23. As determined above, our truncation analyses showed that the COOH-terminal region of FGF23 does not play a predominant role in regulating protein secretion; however, the functional consequences of this domain on FGF23 bioactivity remain unexplored. Therefore, we next examined the truncated FGF23 proteins for activity in the HEK-293-KL cell line. Due to the fact that the serial truncations resulted in removal of the epitopes required for the intact and COOH-terminal FGF23 ELISAs, confirmation of protein expression was assessed through examination of the intact FGF23 bands upon Western analyses of the truncated mutants (comparison of the 33-, 25-, 23-, and 20-kDa species). These analyses demonstrated that the FGF23Δ19 and FGF23Δ48 truncations possessed signaling capabilities and increased EGR1 mRNA five- to sixfold, which was not different from WT FGF23 (P < 0.167 and 0.427, respectively; Fig. 6B). However, FGF23 protein lacking the 62 and 71 COOH-terminal residues had no activity compared with WT FGF23 or the FGF23Δ19 and FGF23Δ48 truncations (P < 0.0001; Fig. 6B). Thus the residues between −62 and −48 (i.e., residues between +189 and +203 in mature 251-residue FGF23) are required for FGF23 activity, whereas the FGF23 amino acids 3’ to residue 203 are not necessary to initiate FGF23-dependent intracellular signaling.

DISCUSSION

In the present study, we identified a novel Q54K mutation in the FGF23 gene in three patients with TC and, through in vitro studies, tested the etiology of TC using biochemical analyses of FGF23 processing and activity. FGF23 is required for the control of serum phosphate and vitamin D metabolism, as highlighted by its role in ADHR (1, 33) and familial TC (2, 3, 9, 22). Stabilizing mutations in FGF23 cause ADHR through the interruption of the SPC176RXXR179/S cleavage site (32), whereas destabilizing mutations in FGF23 lead to TC (3, 22). The phenotype of patients with TC varies considerably across and within kindreds. However, despite the clinical inconsistency in ectopic and vascular calcification severity, these patients share a common biochemical finding of persistent hyperphosphatemia. Furthermore, to date, all patients with TC due to GALNT3 or FGF23 mutations display markedly increased levels of COOH-terminal FGF23 fragments in tandem with low or low/normal levels of bioactive intact FGF23 protein (Fig. 2) (2, 9, 13, 15, 22). Importantly, all known FGF23-TC mutations occur in the portion of FGF23 conserved among the FGF family members NH2-terminal to the S176RXXR179/S SPC site.

The mutations in FGF23 that lead to TC have dramatic effects on the secretion of intact, active FGF23. Indeed, the ability of cells to secrete intact FGF23 protein containing the novel Q54K mutation and the untested H41Q and M96T alterations, as well as those mutations reported previously (2, 3, 6, 22, 23), was greatly reduced compared with wild-type FGF23, as shown by Western analyses of cellular lysates and conditioned media collected from cells transiently expressing FGF23 (Fig. 4). Consistent with our current findings, previous in vitro analyses of the FGF23 TC mutation S71G indicated that this amino acid alteration leads to the elevated secretion of FGF23 proteolytic fragments (3, 21). Disruption of the FGF23 S176RXXR179/S180 SPC cleavage site using the ADHR R176Q and R179Q mutations in tandem with the S71G TC mutation resulted in a protein species less susceptible to cleavage (21), consistent with increased sensitivity of the FGF23-TC mutants to intracellular proteases.

In parallel with the FGF23 mutations, the known GALNT3 inactivating mutations that result in TC lead to decreased
FGF23 protein is COOH-terminal and intact FGF23 ELISA profiles (10). The second, the NH2-terminal region produced by cleavage at the 176RXXR179/S180 SPC recognition site lacks signaling activity through EGR1, as shown by the FGF23 mutations or by KL-dependent signaling (Fig. 5); however, this activity was reduced over the dose and time tested compared with that of the wild-type FGF23 protein (Fig. 5). Determining that the FGF23-TC mutants possessed residual activity adds to our current understanding of the complexity of the disease etiology in TC patients. In this regard, our studies indicated that the decrease in circulating biologically active FGF23 in patients with TC is likely the primary cause for the persistent hyperphosphatemia in these patients. Taking into consideration the findings of the present study, as well as the normal or nearly normal intact serum levels of FGF23 in the current patients, as well as in other reported cases (2, 22), the decreased activity of the mutant protein may also have a role in TC. Decreased serum levels of biologically active FGF23 may be the initial step in disease pathology in patients with TC. As TC patients produce more FGF23 to attempt to compensate for their hyperphosphatemia, nearly normal serum levels of FGF23 are approached; however, these levels are insufficient to suppress the renal reabsorption of phosphate. Of note, Fgf23-null and KL-null mice display a severe hyperphosphatemic phenotype and survive an average of only 10–12 wk (26, 31). Therefore, the residual activity of the TC-mutant FGF23 isoforms is likely sufficient to sustain life in humans; however, these mutant isoforms are not produced in quantities high enough to adequately inhibit the renal reabsorption of phosphate.

Interestingly, the FGF23 mutations that are known to result in TC are located within the NH2-terminal region that shares a considerable degree of homology with all members of the FGF protein family (residues 25–180 in FGF23), and no disease-causing mutations have been found in the COOH-terminal variable region of FGF23. The consistent lack of COOH-terminal mutations in patients with disorders of phosphate homeostasis led to our investigation of this uncharacterized region. The deletion of 19 and 48 COOH-terminal residues had no effect on FGF23 processing or secretion (Fig. 6) or on FGF23 activity (Fig. 6). In contrast, the deletion of 62 COOH-terminal FGF23 residues, as well as the entire COOH-terminal variable region with the FGF23Δ71 truncation, did not affect protein production and secretion (Fig. 6) but abolished KL-dependent signaling (Fig. 6). These findings have two implications. First, FGF23 residues between 189 [the last residue in FGF23Δ62 (inactive)] and 203 [the last residue in FGF23Δ48 (active)] are critical for FGF23 bioactivity, and second, the NH2-terminal region produced by cleavage at the 176RXXR179/S180 SPC recognition site lacks signaling activity through EGR1, as shown by the FGF23Δ71 truncated species (Fig. 6). Since FGF23 is properly secreted and retains activity with large portions of the COOH-terminal tail truncated, this could also explain the lack of known disease-causing mutations within the nucleotides 3′ to the R176XXR179/S180 site in FGF23. In this regard, amino acid substitutions in this variable region are consistent with being less deleterious to FGF23 expression and function.
In summary, we report a novel recessive mutation in the FGF23 gene (Q54K) that results in familial TC. We have determined that all of the known FGF23 mutations that result in TC lead to destabilization of the intact molecule. We have also shown that the COOH-terminal variable region is not required for FGF23 processing and secretion; however, residues adjacent to the conserved NH2 terminus are necessary for full bioactivity. Collectively, these findings expand our molecular understanding of TC as well as define protein domains critical for FGF23 activity.

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REFERENCES


