BRIEF REPORT

Novel GALNT3 Mutations Causing Hyperostosis-Hyperphosphatemia Syndrome Result in Low Intact Fibroblast Growth Factor 23 Concentrations

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Context: Hyperostosis-hyperphosphatemia syndrome (HHS) is a rare metabolic disorder characterized by hyperphosphatemia and localized hyperostosis. HHS is caused by mutations in GALNT3, which encodes UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3. Familial tumoral calcinosis (TC), characterized by ectopic calcifications and hyperphosphatemia, is caused by mutations in the GALNT3 or fibroblast growth factor 23 (FGF23) genes.

Objective: Our objective was to identify mutations in FGF23 or GALNT3 and determine serum FGF23 levels in an HHS patient.

Design: Mutation detection in FGF23 and GALNT3 was performed by DNA sequencing, and serum FGF23 concentrations were measured by ELISA.

Patients or Other Participants: A 5-year-old French boy with HHS and his family members participated.

Results: The patient presented with painful cortical lesions in his leg. Radiographs of the affected bone showed diaphyseal hyperostosis.

HHS has biochemical characteristics similar to familial tumoral calcinosis (TC; OMIM no. 211900), which is defined by ectopic calcifications occurring mainly around the large joints as well as hyperphosphatemia and inappropriately normal or elevated levels of 1,25-dihydroxyvitamin D. In addition, the disease is often associated with dental abnormalities (5–7) and angioid streaks of the retina (8). On the molecular level, TC is caused by biallelic inactivating mutations in genes encoding fibroblast growth factor 23 (FGF23) (5, 9, 10) or UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc transferase 3; GALNT3) (7, 11–14). Interestingly, GALNT3 mutations were also found in patients with HHS (2). FGF23 is a hormone that regulates renal tubular phosphate reabsorption and 1,25-dihydroxyvitamin D metabolism. GalNAc transferase 3 is a Golgi-associated biosynthetic enzyme, which initiates mucin-type O-glycosylation of proteins. O-glycosylation of FGF23 by GalNAc transferase 3 is essential for the secretion of intact FGF23 because O-glycosylation at

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Abbreviations: FGF23, Fibroblast growth factor 23; GALNT3, GalNAc transferase 3 or UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3; HHS, hyperostosis-hyperphosphatemia syndrome; MRI, magnetic resonance imaging; RFLP, restriction fragment length polymorphism; TC, tumoral calcinosis.

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a subtilisin-like proprotein convertase recognition sequence motif prevents cleavage of FGF23 (15).

Herein we present a case of HHS caused by two novel mutations in the GALNT3 gene. These inactivating mutations and serum FGF23 levels found in the HHS case resemble those in TC, providing evidence that HHS and TC are two different phenotypic manifestations of the same disorder.

**Subjects and Methods**

**Study subjects**

Blood samples were collected from a family with HHS and healthy race-matched individuals. The study was approved by the Institutional Review Board of Indiana University-Purdue University Indianapolis. Written informed consent was obtained from all study subjects or their parents before participation in the study. Serum biochemistries of the family members were measured at Limoges University Teaching Hospital. The tubular maximum rate for phosphate reabsorption per deciliter of glomerular filtrate was determined using a nomogram (16).

**Mutation detection**

Genomic DNA was extracted from whole blood obtained from study subjects, using Nucleon BACC3 (Amersham, Little Chalfont, UK). All exons and their adjacent intronic sequences in the FGF23 and GALNT3 genes were amplified by PCR, using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR products were gel purified and directly sequenced from forward PCR primers, using Big-Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

**PCR-restriction fragment length polymorphism (RFLP) analysis**

The C insertion in exon 3 was PCR amplified with forward primer (5'-ACGTTGATGTCAGGCAACACTGCGC-3') and reverse primer (5'-ACGTTGATGCAAGGATAACTGGCTGAA-3') and digested with HaeIII (New England Biolabs, Beverly, MA) at 37°C. A mismatch nucleotide (underlined) was introduced in the forward primer to create a restriction nuclease HaeIII site in the insertion allele. The 10-bp tails (italicized) were added to each primer to improve separation between normal and insertion alleles during an agarose gel electrophoresis. The G-to-A transition in intron 8 was PCR amplified with forward primer (5'-GGCTGTTGAATTGCCTCTTG-3') and reverse primer (5'-GCAAGGATAACTGGCTGGAA-3') and digested with BstNI (New England Biolabs), which cuts only G allele because the transition disrupts the BstNI site. The digested PCR products were electrophoresed in an agarose gel and visualized under the UV light.

**RNA analysis**

Total RNA was isolated from whole blood, using RNeasy Mini Kit (QiAGEN, Courtabeuf, France). First-strand cDNA was synthesized from 1 μg each of total RNA, using Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). PCR amplification was performed with forward primer in exon 6 (5'-AGCTTTCATGACGGCACTCTCA-3') and reverse primer in exon 9 (5'-AACGAGACCTTGACAGCCAT-3'). The RT-PCR products were purified and sequenced as described above.

**FGF23 serum assays**

Serum FGF23 concentrations were determined using two different assays according to the manufacturer’s instructions. Intact FGF23 was measured using FGF23 ELISA Kit (Kainos Laboratories Inc., Tokyo, Japan). FGF23 was also measured using Human FGF-23 (C-Term) ELISA Kit (Immutopics International, San Clemente, CA), which detects both intact FGF23 and C-terminal FGF23 fragments.

**Results**

**Clinical findings**

A 5-yr-old French boy (II-2, Fig. 1A) presented with a 3-wk history of left leg pain. On physical examination, there was an inflammatory swelling on the upper third of his left tibial crest. Similar skin abnormalities were found on the right tibia and the left forearm. The rest of the physical examination was normal. Pain and erythema resolved after several days of treatment with nonsteroidal antiinflammatory drugs.

The patient was hyperphosphatemic with serum phosphate ranging from 2.40–2.87 mmol/liter (normal, 1.16–1.80 mmol/liter) and tubular maximum rate for phosphate reabsorption per deciliter of glomerular filtrate of 3.3 mmol/liter (normal, 1.30–2.58 mmol/liter). Serum 25-hydroxyvitamin D, calcium, creatinine, and alkaline phosphatase levels were normal. However, serum 1,25-dihydroxyvitamin D was inappropriately elevated (72.2 pg/ml, normal, 20–60 pg/ml), and PTH concentration was low normal (13.8 pg/ml, normal, 10–55 pg/ml). (To convert the values for 1,25-dihydroxyvitamin D to pmol/liter, multiply by 2.599.) His parents (I-1 and I-2) and brother (II-1) were asymptomatic with normal clinical examinations and normal biochemical values.

A skeletal survey revealed no ectopic calcifications. Diaphyseal hyperostosis was evident in the painful bones, as indicated by patchy condensation of the medullary canal, localized endosteal bone proliferation, and periosteal appositions (Fig. 2A). Magnetic resonance imaging (MRI) with short inversion time recovery sequence (echo time = 61.8 msec; repetition time = 2620 msec) revealed an abnormal signal in the medullary canal of the diaphyses (Fig. 2B). T1 sequence with gadolinium injection and fat saturation (echo time = 9 msec; repetition time = 400 msec) showed increased contrast enhancement at periosteal appositions. Technetium scintigraphy revealed colocalizing increased uptake at the left cubital and both tibial diaphyses (Fig. 2C).

The bone biopsy of the left tibia showed irregular, variably thickened, inter-anastomosing trabeculae of immature, woven bone surrounded by fibrous tissue (Fig. 2D). Plump osteoblasts rimmed the trabeculae throughout most of the specimen. Much less conspicuous were occasional, scattered osteoclasts. In areas, particularly toward the leading or active edge of bone deposition, the histology was reminiscent of that encountered in osteoblastoma or active fracture callous.

**Molecular analysis**

Due to a phenotype similar to TC, FGF23 and GALNT3 genes were screened for the presence of mutation. The affected child (II-2) has no mutations in the FGF23 gene. However, he has two novel mutations in GALNT3: a nucleotide C insertion in exon 3 (c.803–804insC or g.8761–8762insC) from the mother (I-2) and a G-to-A transition at the 5' splice site in intron 8 (IVS8+1G>A, c.1626+1G>A, or g.16075G>A) from the father (I-1) (Fig. 1, B and C). These mutations were not found in over 170 chromosomes in healthy Caucasian individuals by PCR-RFLP analysis (data not shown).

The C insertion in codon 268 results in premature termination at codon 271, truncating the encoded GalNAc trans-
ferase 3 in the middle of its glycosyl transferase domain (residues 188–374). The G-to-A transition in intron 8 disrupts a highly conserved 5′/H11032 splice site, resulting in inclusion of partial intron 8 (99 bp) due to cryptic splice site activation (P1, 537 bp) and skipping of exon 8 (P3, 336 bp) (Fig. 1D). The intensity of the RT-PCR products suggests that mRNA lacking exon 8 is the major product generated from the mutant allele.

Serum FGF23 levels

When measured using the C-terminal assay, FGF23 concentration in the affected child (II-2) was quite elevated (2113.9 RU/ml) compared with his family members, who had C-terminal FGF23 concentrations in the normal range (normal, 72.9 ± 38.2 RU/ml) (17). Intact FGF23 concentrations were inappropriately low normal in the patient (13.9 ± 3.1 pg/ml) and normal in other family members (25.7–34.3 pg/ml) (normal, 29.7 ± 20.7 pg/ml) (17), indicating that almost all of the immunoreactive FGF23 in the patient consisted of inactive C-terminal fragments.

Discussion

To date, GALNT3 mutations in HHS have been described only in Middle Eastern patients (2). The present study describes biallelic GALNT3 mutations in a European patient manifesting hyperphosphatemia associated with diaphyseal hyperostosis. These mutations cause premature termination of translation or aberrant splicing of mRNA, likely disrupting GalNAc transferase 3 activity.

In TC patients with inactivating FGF23 mutations, intact
Hematoxylin-eosin-saffron staining. showed the area between the bone spicules. L soluble fibrous tissue occupied the trabeculae, vascularized fibrous tissue lined the trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules. Histologically, areas of hyperostosis were composed of thickened, inter-anastomosing trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules. (Hematoxylin-eosin-saffron staining.)

FIG. 2. Clinical findings in the patient (II-2). A, Lateral and anteroposterior radiographs. Left tibia of the patient shows hyperostosis with patchy condensation of the medullary canal, thickening of the cortical bone, and periosteal apposition. B, MRI. The short inversion time recovery sequence on MRI shows increased signal in the medullary canal of both tibias. C, Technetium scintigraphy. Scintigraphy demonstrated abnormal uptake in both tibiae and in the left forearm, corresponding to areas of hyperostosis seen on plain radiographs (ulna and right tibia films not shown). D, The hyperostotic area of the left tibia. Histologically, areas of hyperostosis were composed of thickened, inter-anastomosing trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules. Histologically, areas of hyperostosis were composed of thickened, inter-anastomosing trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules. Histologically, areas of hyperostosis were composed of thickened, inter-anastomosing trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules. Histologically, areas of hyperostosis were composed of thickened, inter-anastomosing trabeculae of woven bone. Although osteoblasts were conspicuous and lined the trabeculae, vascularized fibrous tissue occupied the area between the bone spicules.

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serum FGF23 levels are rather low, whereas C-terminal fragments are highly elevated (9, 10). TC patients with GALNT3 mutations also have similar serum FGF23 levels (low or undetectable intact; high C-terminal) (11, 12). In this study, we demonstrated that GALNT3 mutations in an HHS patient also result in the same pattern of FGF23 levels. These findings indicate that hyperphosphatemia seen in both TC and HHS is caused by decreased circulating FGF23 regardless of whether FGF23 or GALNT3 is mutated. In this regard, a recent study demonstrated that GalNAc transferase 3 specifically O-glycosylates FGF23, thereby preventing cleavage of FGF23 by convertases and allowing secretion of intact (active) FGF23 in vitro (15).

Even before gene identification, HHS and TC were thought to share a common pathogenic mechanism based on the observation that cortical hyperostosis and ectopic calcifications coexisted in some patients (4, 18, 19). Furthermore, the identical GALNT3 mutation was found in both TC and HHS patients (2, 14). Recently, FGF23 mutation was also found in a TC patient with periosteal reaction and patchy medullary sclerosis in the leg (5). These genetic and phenotypic findings, along with the observed FGF23 levels, indicate that HHS and TC are different manifestations of the same disease. This recent study demonstrated that GalNAc transferase 3 specifically O-glycosylates FGF23, thereby preventing cleavage of FGF23 by convertases and allowing secretion of intact (active) FGF23 in vitro (15).

In conclusion, the presence of GALNT3 mutations and elevated C-terminal, but low intact serum FGF23 levels in this new case of HHS implies that HHS and TC are two different clinical presentations of the same disease. This study also demonstrates that low intact FGF23 concentrations, due to the absence of functional GalNAc transferase 3, are responsible for increased tubular phosphate reabsorption and hyperphosphatemia in HHS.

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