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Hereditary Hypophosphatemic Rickets With Hypercalciuria and Nephrolithiasis—Identification of a Novel SLC34A3/NaPi-IIc Mutation

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Abstract

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is characterized by rickets, hyperphosphaturia, hypophosphatemia, elevated 1,25-dihydroxyvitamin-D, increased gastrointestinal calcium absorption and hypercalciuria. Serum calcium, 25-hydroxyvitamin-D and PTH levels are normal. Here we describe a boy with HHRH, nephrolithiasis, and compound heterozygosity for one previously described mutation (g.4225_50del) and a novel splice mutation (g.1226G>A) in SLC34A3, the gene encoding the renal sodium-phosphate co-transporter NaPi-IIc. The patient's mother and grandmother are carriers of g.4225_50del, and both have a history of nephrolithiasis associated with hypercalciuria and elevated 1,25-dihydroxyvitamin-D. His three siblings (2–6 years old), who are also carriers of g.4225_50del, have hypercalciuria but so far their renal ultrasounds are normal. Thus, SLC34A3/NaPi-IIc mutations appear to be associated with variable phenotypic changes at presentation, which can include recurrent nephrolithiasis.

Keywords

rickets; hypophosphatemia; hypercalciuria; NaPi

INTRODUCTION

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH, OMIM 241530) was first described by Tieder et al. [1985]. The excessive renal losses of phosphorus result in hypophosphatemia, which appropriately stimulates renal production of 1,25-dihydroxyvitamin-D. This results in hypercalciuria via increased gastrointestinal absorption

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of calcium. The diagnosis of HHRH is generally established in childhood on the basis of rickets leading to bone pain, as well as muscle weakness and growth retardation. Serum calcium, 25-hydroxyvitamin-D and intact PTH levels are generally normal. Elevated 1,25-dihydroxyvitamin-D levels and hypercalciuria distinguish HHRH from other hereditary forms of hypophosphatemic rickets such as X-linked hypophosphatemia [Lyles et al., 1982] (XLH, OMIM 307800, *PHEX* gene mutations), autosomal dominant hypophosphatemic rickets [Econs et al., 1997] (ADHR, OMIM 193100, *FGF23* gene mutations), autosomal recessive hypophosphatemia [Feng et al., 2006; Lorenz-Depiereux et al., 2006a] (ARHP, OMIM 241520, *DMP1* gene mutations), and autosomal recessive hypophosphatemic rickets with generalized arterial calcifications (*ENPP1* gene mutations) [Levy-Litan et al., 2010; Lorenz-Depiereux et al., 2010]. Patients affected by Dent disease (OMIM 300009, *CLCN5* gene mutations) also present with hypophosphatemic rickets, appropriately elevated 1,25-dihydroxyvitamin-D levels and hypercalciuria; however, glucosuria, aminoaciduria, proteinuria, and/or hematuria, which can be observed in Dent disease, are absent in HHRH [Lloyd et al., 1996]. Genetic mapping studies have recently led to the identification of loss-of-function mutations in *SLC34A3*, the gene encoding the renal sodium-phosphate co-transporter NaPi-IIc, as the cause of HHRH [Bergwitz et al., 2006; Lorenz-Depiereux et al., 2006b]. Although these patients have hypercalciuria, there are only a few reports of renal calcifications, which have generally been attributed to treatment with vitamin D analogs [Chen et al., 1989; Tieder et al., 1993]. However, nephrolithiasis has been reported in some individuals with HHRH and their heterozygous relatives prior to therapy [Bergwitz et al., 2006; Ichikawa et al., 2006; Jaureguiberry et al., 2008; Kremke et al., 2009; Tencza et al., 2009].

Here, we describe a family with HHRH and nephrolithiasis in the index case. Nucleotide sequence analysis of the index case identified one known deletion mutation (g.4225_50del) [Jaureguiberry et al., 2008], and one novel splice-site mutation (g.1226G>A) of the *SLC34A3*/NaPi-IIc gene. Other family members, who were heterozygous carriers of the g.4225_50del mutation, had kidney stones or hypercalciuria. Although the present kindred is too small to support formal linkage of renal stones to g.4225_50del, our findings lend further support to the newly evolving view that renal calcifications in HHRH may be more common than previously thought.

CLINICAL REPORT

A 3-year-8-month-old Caucasian male (Fig. 1; III-1) was brought to the Pediatric Endocrinology clinic at the Connecticut Children's Medical Center for evaluation of nephrolithiasis. He was the product of a full-term pregnancy, and he was delivered vaginally without any complications. He had been exclusively breast fed until 1 year of age, and solids had been introduced but dairy had been limited due to a possible milk allergy. He met all his developmental milestones until age 21 months, when he developed pain with ambulation. Radiographs of both knees and wrists showed rickets. Serum tests showed a low serum phosphorous level of 2.8 mg/dl, a high alkaline phosphatase level of 956 U/L, normal total calcium level of 9.2 mg/dl, normal iPTH level of 15 ng/L, low 25-hydroxyvitamin-D level of 10 ng/ml, and a high 1,25-dihydroxyvitamin-D level of 114 pg/ml. He was prescribed vitamin D supplements but the family did not start this medication. The diagnosis of HHRH

was considered, but the patient was lost to follow-up. At age 3 years and 5 months, he was brought to the emergency room with flank pain and hematuria, and was diagnosed with two kidney stones by non-contrast computerized tomography (CT) scan. A 24-hr urine collection showed an elevated calcium of 15.1 mg/kg/day (normal is 8–16). The urine calcium to creatinine ratio was 1.05 mg/mg (normal for age is <0.28) [So et al., 2001]. The urine collection also showed a low uric acid 180 mg (250–750), that is, 0.48 g/1.73 m²/day (0.52 ± 0.15), low citric acid 99 mg/day (457 ± 164), and low oxalic acid 0.04 g/1.73 m²/day (0.52 ± 0.15). On physical examination he was irritable with mild genu valgus deformity and mild forehead frontal bossing. There was no evidence of epiphyseal flaring, rachitic rosary, craniostylosis, digital abnormalities, midfacial hypoplasia, clefts or dental abnormalities. He had symmetric upper and lower extremities. His fasting morning laboratory tests showed hypophosphatemia, hyperphosphaturia, and normal serum calcium. Random blood tests showed an elevated 1,25-dihydroxyvitamin-D level, slightly elevated serum calcium, a low 25-hydroxyvitamin-D level, and low PTH. The random urine sample showed hypercalciuria, which resolved with fasting (Table II).

The patient's 2-year-old sister (III-4) had normal blood tests but had hypercalciuria, and was on the 10th centile for height and 25th centile for weight. The twin 6-year-old siblings (III-2 and III-3) were found to have hypophosphatemia, hyperphosphaturia, and hypercalciuria (Table II). Both were on the 50th centile for height and 25th centile for weight. The 6-year-old brother was asymptomatic, while the 6-year-old sister complained of occasional leg pain. All three sibs had normal renal ultrasounds. The patient's father (II-2) was asymptomatic and had normal test results with the exception of a low normal serum phosphorus level. No further paternal family history was available. The mother (II-1) had a history of bone pain since late adolescence, and developed recurrent kidney stones at age 20 years requiring multiple sessions of lithotripsy. The maternal grandmother (I-1) also had bone pain since late adolescence and nephrolithiasis starting in her 30s, but never required surgical intervention. Both were diagnosed with sarcoidosis based on their elevated serum 1,25-dihydroxyvitamin-D levels, although neither had lung or skin involvement. Repeat evaluation of the mother showed hypophosphatemia and hyperphosphaturia. Her serum 1,25-dihydroxyvitamin-D level was normal. One maternal great-aunt was healthy, while a second maternal great-aunt had a history of nephrolithiasis. Both were unavailable for further studies. Treatment of our patient (III-1) with K-Phos Neutral (70 mg/kg/day of elemental phosphorus), resulted in marked clinical and biochemical improvement. His bone aches improved and repeat labs included a serum-phosphate of 4.2 mg/dl, 1,25-dihydroxyvitamin-D level of 77 pg/ml, and a normal random spot urine for calcium.

METHODS

Laboratory Assays

All laboratory studies, with the exception of genetic analysis, were performed at a local commercial laboratory (Clinical Laboratory Partners). The 25-hydroxyvitamin-D levels were tested by liquid chromatography with tandem mass spectroscopy. The 1,25-dihydroxyvitamin-D levels were determined by radioimmunoassay. Serum intact PTH levels were done by electrochemiluminescence immunoassay. The renal tubular reabsorption of

phosphorus was calculated using the following formula: $\% \text{ TRP} = 100 \times (1 - (\text{urine phosphorus} \times \text{serum creatinine}) / (\text{serum phosphorus} \times \text{urine creatinine}))$. A normal value is above 90% when the serum phosphorus is below the reference range for age. TmP/GFR (maximal reabsorption of phosphorus per unit of GFR) was estimated using the Walton and Bijvoet nomogram [Alon and Hellerstein, 1994]. TP/GFR was calculated using the following formula: $\text{serum phosphorus} - (\text{urine phosphorus} \times \text{serum creatinine} / \text{urine creatinine})$ [Alon and Hellerstein, 1994], using simultaneous urine and blood creatinine and phosphorus concentrations. TP indicates tubular phosphate reabsorption under basal conditions, without a phosphate load, and is preferred in pediatric patients since their serum phosphate levels often exceed the range provided by the Walton and Bijvoet nomogram, and results in an overestimation of TmP/GFR compared with TP/GFR [Alon and Hellerstein, 1994]. Average TP/GFR is 4.1 mg/dl for a 3- to 6-year-old child with an average serum phosphate of 4.5 mg/dl [Brodehl et al., 1982].

Mutation and Haplotype Analysis

Mutation and haplotype analysis of *SLC34A3* was performed after informed consent (approved by the institutional review board of Massachusetts General Hospital). The entire *SLC34A3* gene of the index case (III-1) was amplified by PCR at the Massachusetts General Hospital DNA Sequencing Core Facility. PCR assays for the confirmation in III-1, and analysis of g.4225_50del and g.1226G>A in all subsequent individuals, were designed as described [Bergwitz et al., 2006; Jaureguiberry et al., 2008] using Qiagen reagents (Valencia, CA) at standard PCR cycling conditions and the primers and restriction enzymes listed in Table I. Haplotype analysis was performed by using microsatellite markers from deCODE Genetics [Kong et al., 2002], *D9S1826* and *D9S1838*, and the markers *CB-9* and *CB-11* as described [Bergwitz et al., 2006] (using CHGR Genotyping Resource, Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA 02114). GenBank accession numbers for *SLC34A3* are as follows: genomic contig NT_024000.15; cDNA, NM_080877.1; protein, NP_543153.1.

RT-PCR Assay for Ectopic Expression of Mutant NaPi-IIc RNA in Lymphoblastoid Cells

To permit allele assignment, segregation analysis was performed for the SNPs, c.200G>A(p.R67H), c.1140C>T(p.L385L), and c.1538T>A(p.V513E) using genomic DNA of the index case III-1 and his parents. For this purpose, exons 5, 12, and 13 of the *SLC34A3* gene were amplified by PCR using Qiagen PCR reagents at standard PCR cycling conditions and the primers, followed by restriction enzymatic digestion to obtain allele-specific fragments as listed in Table I. Citrated whole blood of the index case III-1 was used for transformation to generate lymphoblastoid cells at the Biosamples Facility, Harvard Medical School and Partners Center for Genetics and Genomics, Cambridge, MA 02139. RNA then was extracted from lymphoblastoid cells using the Qiagen RNeasy Kit and subjected to RNase-free DNase treatment to remove residual genomic DNA followed by cDNA synthesis using the Qiagen Omniscript kit. Using forward primer 5'-CAGTCATCAATGCGGACTTC-3' and reverse primer 5'-TCCAGAGAATGGAGCCAGAC-3', followed by nested PCR with forward primer 5'-GCTGGCTCGGCGGCTACC-3' and reverse primer 5'-CGCCGCTGCAGGACAGTAAC-3', the region encompassing the marker SNPs c.

1140C>T(p.L385L), and c.1538T>A(p.V513E) and the mutation g.4225_50del was amplified with initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 65 or 72°C for 1 min, and 72°C 1 min, and a final extension at 72°C for 10 min. The nested-PCR product was purified using spin columns (Qiagen, Qiaquick PCR Purification Kit) and subjected to nucleotide sequence analysis with forward primer 5'-CTGGCTCGGCGGCTACCT-3', and reverse primer 5'-GGTACCACAGCAGGATGC-3' at the Massachusetts General Hospital DNA Sequencing Core Facility. Similarly, the region encompassing the marker SNP c.200G>A(p.R67H), and the mutation g.1226G>A was amplified using forward primer 5'-CCTCCAGTTCTGCTCCAGTC-3' and reverse primer 5'-CGCACCAGTGCTTAATGAGA-3' followed by nested PCR with forward primer 5'-TTGGAGGAAGGGGACACA-3' and reverse primer 5'-TGCTGTTAGTGGCGTTGC-3' with initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min. The obtained PCR product was 22 bp shorter than the expected 720 bp. It was subjected to nucleotide sequence analysis with the nested primers as well as forward primer 5'-GGGTGTCAGGCTGGCGGC-3', and reverse primer 5'-AGCATGGTGGCTGCTAAGC-3' at the Massachusetts General Hospital DNA Sequencing Core Facility. The variant-specific reverse primers 5'-GTCAGCACACGATGGAGGA-3', and nested primer 5'-CGGACAGTCAGCACACGAT-3', which anneal across the novel splice junction, were used to confirm presence of the alternatively spliced transcript for g.1226A in lymphoblastoid cells of III-1 and to exclude an in vitro artifact caused by skipping of the DNA polymerases used for RT-PCR and nucleotide sequencing analysis.

RESULTS

To search for mutations in NaPi-IIc, the entire *SLC34A3* gene was PCR-amplified in four overlapping fragments using DNA of the index case (III-1, see Pedigree and Table II) and these fragments were DNA sequenced. The index case was compound heterozygous for g.1226G>A (IVS5 + 1G>A, affecting the consensus splice donor site of intron 5) and g.4225_50del (IVS12-25_1del causing a deletion of the last 25 nucleotides of intron 12 and of the first base of exon 13). In addition, he was heterozygous or homozygous for several previously described SNPs in non-coding and coding regions of the gene: hom. g.(-)804AA, het. c.200G>A(p.R67H), het. g.2704TA, het. c.1140C>T(p.L385L), het. g.3296CG, hom. g.3701CC, hom. g.3736TT, hom. g.4107CC, het. c.1538T>A(V513E) [Bergwitz et al., 2006] and unpublished observations by C.B.

Haplotype analysis of the kindred indicated that g.1226G>A was inherited from the father (II-2), while g.4225_50del was inherited from the mother (II-1). The g.4225_50del mutation was also found on one *SLC34A3* allele of the maternal grandmother (I-1), who previously had recurrent kidney stones, and on one of alleles of the sibs III-2, III-3, and III-4. All three sibs had normal renal ultrasounds; however, all had hypercalciuria and two of them (III-2 and III-3) had mild fasting hypophosphatemia. The g.4225_50del mutation occurred based on haplotype analysis at positions c.1140 and c.1538 independently in another, compound heterozygous case with HHRH and renal stones on presentation [Jaureguierry et al., 2008], while it was absent in 124 control alleles. g.1226G>A is a novel mutation. It has not been previously reported and was not detected in 114 control alleles.

NaPi-IIc is predicted to have eight complete and four partially membrane-spanning domains, connected by intra- and extracellular loops and N- and C-terminal intracellular tails [Virkki et al., 2007]. The identified mutations are predicted to affect splicing of the messenger RNA leading to premature termination of the NaPi-IIc transcript after the 2nd (g.1226G>A) and 5th (g.4225_50del) membrane spanning domains. To test for alternative splicing experimentally, we performed RT-PCR of ectopic transcripts from lymphoblastoid cells of the index case III-1 as described in the Methods Section. Haplotype analysis indicated that III-1 is heterozygous for SNPs c.200G>A(p.R67H), c.1140C>T(p.L385L), and c.1538T>A(V513E), and inherited ATT from his mother and GCA from his father. RT-PCR using primers flanking these marker SNPs and the mutations g.1226G>A and g.4225_50del indicated that the mother's allele (ATT), carrying g.4225_50del was missing (Fig. 2A). This indicates that either the transcript is unstable (non-sense-mediated decay), or that a transcript is generated, which is missing the annealing sites of the primers. However, the latter possibility is very unlikely given that two non-overlapping amplicons failed to amplify the mutant allele. Furthermore, RT-PCR using primers flanking the marker SNP c.200G>A(p.R67H), and the mutation g.1226G>A yielded a single transcript with c.200G, which was lacking the last 22 bp of exon 5. This finding confirms that the father's allele is transcribed and suggests that g.1226G>A generates an alternative splice donor site in exon 5 that is located 22 bp upstream of the natural splice donor site. Deletion of 22 bp in the resulting transcript is predicted to cause a frame shift, which leads to a truncated NaPi-IIc transcript after the 2nd membrane-spanning domain (Fig. 2B).

DISCUSSION

The index case of this kindred is compound heterozygous for nucleotide changes in *SLC34A3*, which are predicted to lead to aberrant splicing and loss-of-function of the co-transporter NaPi-IIc. These nucleotide changes were not found in control subjects. In addition they co-segregate with HHRH in the present pedigree. Hence these mutations likely cause HHRH in this patient and hypophosphatemia and/or hypercalciuria in the respective heterozygous carriers.

HHRH was initially reported as an autosomal recessive form of rickets in a large consanguineous Bedouin kindred [Tieder et al., 1985]. The affected individuals had renal phosphate wasting with ensuing hypophosphatemia and an appropriate up-regulation of renal 1,25-dihydroxyvitamin-D synthesis, increased intestinal absorption of calcium and a rise in urinary calcium excretion [Tieder et al., 1992]. HHRH was recently mapped to a region of 9q34 containing the *SLC34A3* gene. [Bergwitz et al., 2006; Lorenz-Depiereux et al., 2006b]. A homozygous deletion of nucleotide C in cDNA position 228 was found to be the cause of HHRH in the original Bedouin kindred [Tieder et al., 1985]. Seventy percent of homozygous carriers had bone changes consistent with rickets and typical biochemical changes consistent with HHRH. Furthermore, seventy percent of the heterozygous relatives of the same tribe were found to be hypercalciuric and their serum biochemical parameters including phosphorus and 1,25-dihydroxyvitamin-D levels were intermediate between normal and affected individuals. Bone changes are generally missing in heterozygous carriers, although they can rarely be seen [father I-2 of kindred D Bergwitz et al., 2006]. None of the hypercalciuric members of the Bedouin kindred suffered from renal stone

disease. However, subsequently stone formation has been reported in individuals with HHRH, either upon presentation [Bergwitz et al., 2006; Jaureguiberry et al., 2008; Kremke et al., 2009; Tencza et al., 2009], or in the context of treatment with vitamin D analogs [Chen et al., 1989; Tieder et al., 1993]. One report furthermore described nephrolithiasis in several heterozygous carriers of a 101-bp deletion in intron 9 of *SLC34A3* [Ichikawa et al., 2006].

In our kindred, the index case presented with all the features of HHRH, while his heterozygous parents only displayed one or more biochemical abnormalities. This is consistent with an autosomal recessive disorder with haplo-insufficiency in the heterozygous carriers. Interestingly, several maternal relatives of our index case suffered from recurrent renal stones. In addition, all available relatives with kidney stones carry one of the splice mutations in NaPi-IIc, g.4225_50del, suggesting a dominant effect of this mutation with regard to renal calcifications. However, the siblings III-2, III-3, and III-4, who also carry g.4225_50del, have not developed renal stones, although presence of hypercalciuria may suggest an increased risk. An adult carrier of g.4225_50del in an unrelated kindred [Jaureguiberry et al., 2008] had recurrent renal stones. However, co-segregation could not be proven, since his father, who is carrier of g.4225_50del did not have stones, while the paternal grandmother, who had stone disease, was unavailable for genotyping. Since these findings raise the interesting possibility that some NaPi-IIc mutations such as g.4225_50del cause a dominant form of nephrolithiasis, we investigated whether mutant transcripts are present in the index case.

Using peripheral lymphoblastoid cells, we found that ectopic transcripts of the maternal allele carrying g.4225_50del are missing in III-1 and in the second, unrelated, compound heterozygous individual with HHRH [Jaureguiberry et al., 2008]. This finding would argue for complete loss-of-function and thus against a dominant effect of this mutation leading to renal calcifications. However, transcripts of g.4225_50del, although absent in peripheral lymphocytes, may be present in proximal tubular cells. In this case, the predicted NaPi-IIc protein would be lacking exon 13, which comprises the membrane-spanning domains 6–8, the 3rd intracellular loop and the C-terminal intracellular tail. We have previously studied one predicted transcript of g.4225_50del, V446Stop, after expression in *Xenopus* oocytes, which resulted in loss of sodium-phosphate co-transport [Jaureguiberry et al. 2008], further arguing for complete loss-of-function of g.4225_50del, and thus against a dominant effect. However, at the moment it cannot be excluded that g.4225_50del, if expressed and translated, has dominant effects on other proximal tubular functions leading to stone formation despite loss of sodium-phosphate co-transport properties.

Taken together with previously published mutations, the present pedigree further illustrates that renal stone disease can be a significant and sometimes presenting symptom of HHRH. Mutations in *SLC34A3*/NaPi-IIc thus need to be considered as a rare cause of nephrolithiasis, particularly, if the renal stone disease is recurrent and family history is present. It continues to be unclear, whether nephrolithiasis is part of the “loss-of-function phenotype” of NaPi-IIc or whether some mutations such as g.4225_50del have dominant effects, since our studies were unable to show ectopic transcripts and sodium-phosphate co-transport function of one possible transcript, V447Stop. Although the segregation in our

kindred suggests a dominant trait for nephrolithiasis, the theoretical lod-score is <3.0 (even under the assumption that follow-up clinical evaluation were to show nephrolithiasis in all mutation carriers). Additional individuals and collection of independent kindreds with g.4225_50del may thus be required before linkage of this particular NaPi-IIc mutation to kidney stone disease can be concluded.

Treatment and Screening Options

Phosphate supplementation may be a rational treatment for the heterozygous carriers of g.4225_50del in the present kindred and would be expected to reduce their hypercalciuria to potentially prevent recurrent nephrolithiasis. We are, however, carefully monitoring their kidneys by ultrasound while on phosphate therapy, since hyperphosphaturia alone may lead to stone formation, at least in the context of g.4225_50del. First-degree relatives of patients with HHRH could benefit by biochemical screening. Renal ultrasounds should be performed in these relatives if hypercalciuria is present. If the urine calcium is normal, Vitamin D levels should be screened because vitamin D deficiency may reduce urinary losses in calcium, while worsening the hypophosphatemia and bone disease. As indicated in Table II, the low vitamin D levels in individuals III-1, II-1, and I-1 may explain their normocalciuria. Since nephrolithiasis can be asymptomatic, renal ultrasounds should be considered even if hypercalciuria is absent, particularly in the setting of vitamin D deficiency. Hypercalciuria may return when the patient is on Vitamin D treatment or on phosphate supplementation, and follow up renal ultrasounds may be indicated. Furthermore genetic testing should be considered to establish a possible risk for the development of renal stone disease.

CONCLUSION

Here we describe a young male with HHRH and recurrent nephrolithiasis, who is compound heterozygous for a novel splice-site mutation of the *SLC34A3*/NaPi-IIc gene, g.1226G>A, and a previously described mutation, g.4225_50del. These mutations suggest alternative splicing leading to a frame-shift, which is predicted to give rise to premature truncation of peptide and non-sense-mediated decay resulting in loss-of-function of the co-transporter. Family members who were heterozygous carriers of the second mutation, g.4225_50del, suffered from recurrent kidney stones or hypercalciuria. Phosphate supplementation may be a rational treatment for these individuals to reduce hypercalciuria and to prevent recurrent kidney stones. Biochemical, renal ultrasounds, and genetic screening should be considered in first-degree relatives of a patient with HHRH.

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FIG. 1.
 A: Pedigree. Ages at presentation were: III-1 3 years 8 months old, III-2 and III-3 6 years old, and III-4 2 years old. I-1, II-1, and II-2 are adults. Solid square indicates the index case, who developed rickets during childhood along with renal phosphate-wasting, hypophosphatemia, and hypercalciuria. Open symbols indicate individuals who are healthy. Samples for individuals with dashed lines were unavailable for genotyping. B: Haplotypes for chromosome region 9q34 between markers D9S1826 and D9S1838. Alleles for microsatellite markers are designated as bp or coded. The haplotype associated with g.4225_50del is shaded in light gray, the haplotype associated with g.1226G>A is shaded in dark gray. The mutations and SNPs were identified by nucleotide sequencing analysis in III-1 and by PCR-based assays using restriction enzymatic digests in the other family members.

FIG. 2.

RT-PCR of maternal and paternal transcripts from lymphoblastoid cells of III-1. A: One microgram of total RNA from lymphoblastoid cells of III-1 were subjected to nested RT-PCR using primers flanking the mutations g.1226G>A (RT-PCR 1), g.4225_50del (RT-PCR 2) and three SNPs, c.200G>A(p.R67H), c.1140C>T(p.L385L), and c.1538T>A(V513E), as described in the Methods Section. The obtained PCR products were subjected to nucleotide sequence analysis, which indicates that the maternal transcript carrying the SNP haplotype ATT and the mutation g.4225_50del is missing entirely. B: The paternal transcript contained the SNP haplotype GCA and was found to lack the last 22 bp in exon 5, which precede the splice donor site altered by g.1226G>A. The novel transcript is predicted to generate a frame shift, a premature termination codon, and a truncated NaP-IIc protein. RT-PCR 3 uses a reverse primer, which anneal across the 22 bp deletion and was used to confirm presence of the altered paternal transcript in lymphoblastoid cells of III-1 (coding sequence is show in upper case, intronic sequence is shown in lower case).

TABLE I

Amplification and Restriction Endonuclease Digestion to Confirm Mutations/SNPs

Mutation/SNP	Exon	Primers (5' > 3')	Enzyme	Amplicon size (bp)	Genotype 11 (bp)	Genotype 12 (bp)	Genotype 22 (bp)
g.1226G>A	5	AGTCAGCCTGCGGCCACAGAGGGCCAGCCAGGGACA	<i>NlaIII</i>	377	GG: 377, 71	GA: 377, 71, 55, 16	AA: 377, 55, 16
g.4225_50del	13	CTGGTGACCCCACTCTGTTCCAGAGAAATGGAGCCAGAC	—	397	397	397, 371	371
c.200G>A(p.R67H)	5	GAGGGCCAGCCAGGGACAAATCAGCCTGCGGCCAC	<i>DraIII</i>	448	GG: 382, 66	GA: 448, 382, 66	AA: 448
c.1140C>T(p.L385L)	12	CAGGGCTGACCCAGCATCGCGCTGAGCATCCTGTCT	<i>BstUI</i>	419	CC: 261, 158	CT: 419, 283, 53	TT: 419
c.1538T>A(p.V513E)	13	CATCCACTTCTTCTTCAACCTGTCCAGAGAAATGGAGCCAGAC	<i>AluI</i>	336	TT: 196, 140	TA: 336, 196, 140	AA: 336

The region of *SLC34A3* flanking the mutation/SNP was amplified by PCR in a final volume of 20 µl using the indicated primers (Qiagen PCR kit, Qiagen, Valencia, CA) using standard thermal cycler conditions. PCR products were then subjected to restriction endonuclease digestion using the enzymes indicated to obtain the indicated genotype-specific fragments upon 2% agarose/TAE gel electrophoresis.

TABLE II

Labs and Imaging

	Index case		Mother		Father	Grandmother	6-year sister		6-year brother		2-year sister		2-year sister	
Pedigree	III-1	III-1	II-1	II-1	II-2	I-1	III-2	III-2	III-3	III-3	III-4	III-4	III-4	III-4
Time	Random	Fasting	Fasting	Random	Fasting	Fasting	Fasting	Fasting	Fasting	Fasting	Fasting	Fasting	Random	Random
S Phos (mg/dl)	3.1	3.1	2.6	2.6	2.7	3.9	3.7	3.9	3.8	3.8	4.9	4.5–6.7	5.5	5.5
Range	4.5–5.5	4.5–5.5	2.7–4.5	2.7–4.5	2.7–4.5	2.7–4.5	4.5–5.5	4.5–5.5	4.5–5.5	4.5–5.5	4.5–6.7	4.5–6.7	4.5–6.7	4.5–6.7
S Ca (mmol/L)	1.37	1.27	1.25	1.25	1.25	1.25	1.24	1.23	1.23	1.23	1.32	1.32	1.32	1.32
Range	1.20–1.35	1.20–1.35	1.17–1.33	1.17–1.33	1.17–1.33	1.17–1.33	1.20–1.35	1.20–1.35	1.20–1.35	1.20–1.35	1.15–1.40	1.15–1.40	1.15–1.40	1.15–1.40
S Creat (mg/dl)	0.5	0.5	0.7	0.7	1.2	0.6	0.5	0.5	0.5	0.5	0.3	0.3	0.6	0.6
Range	0.2–0.7	0.2–0.7	0.4–1.1	0.4–1.1	0.5–1.3	0.4–1.1	0.2–0.7	0.2–0.7	0.2–0.7	0.2–0.7	0.2–0.7	0.2–0.7	0.2–0.7	0.2–0.7
Spot/24 hr	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot	Spot
U Phos (mg/dl or mg)	11	74	55	7	76	54	56	590	58	546	80	80	80	80
U Ca (mg/dl or mg)	50	18	12	33	10	17	31	100	22	96	38	38	38	38
U Creat (mg/dl or mg)	47	56	66	84	221	146	83	440	67	450	88	88	88	88
%TRP	96.2	78.7	77.6	98	84.7	94	90.9	83	88.6	84	90.1	90.1	90.1	90.1
TP/GFR (mg/dl)	2.98	2.44	2.02	2.54	2.29	3.68	3.36	3.23	3.37	3.19	4.95 ^a	4.95 ^a	4.95 ^a	4.95 ^a
TmP/GFR (mg/dl)	3.6	2.45	2.05	3.55	2.40	4.40	3.80	3.30	3.65	3.25	0.43	0.43	0.43	0.43
UCa/Cr (mg/mg)	1.06	0.32	0.18	0.39	0.05	0.12	0.37	0.23	0.33	0.21	0.43	0.43	0.43	0.43
Range	<0.28	<0.28	<0.20	<0.20	<0.20	<0.20	<0.28	<0.28	<0.28	<0.28	<0.28	<0.28	<0.28	<0.28
25 vit D (ng/ml)	15		16	16		27	42	42	59	34	34	34	34	34
Range	>30		>30	>30		>30	>30	>30	>30	>30	>30	>30	>30	>30
1,25 vit D (pg/ml)	100		43	43		55	58	58	47	60	60	60	60	60
Range	15–90		21–65	21–65		21–65	15–90	15–90	15–90	15–90	15–90	15–90	15–90	15–90
iPTH (ng/L)	7		20	20		124	23	23	35	23	23	23	23	23
Range	15–65		15–65	15–65		124	15–65	15–65	15–65	15–65	15–65	15–65	15–65	15–65
Alk Phos (U/L)	384	338	47	47		32–122	361	361	305	278	278	278	278	278
Range	104–345	104–345	32–104	32–104		32–122	96–297	96–297	93–309	108–317	108–317	108–317	108–317	108–317
Renal US	Stones	Stones					Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Renal CT			Stones	Stones										

	Index case	Index case	Mother	Mother	Father	Grandmother	6-year sister	6-year sister	6-year brother	6-year brother	2-year sister	2-year sister
Renal stones by history		Yes	Yes			Yes	No		No		No	
X-rays wrists knees	Rickets						No rickets		No rickets		No rickets	

S, serum; U, urine; Ca, serum ionized calcium; Phos, phosphorus; Creat, creatinine; Alk Phos, alkaline phosphatase.

Abnormal results are in bold.

% TRP = $1 - (\text{urine phosphorus} \times \text{serum creatinine}) / (\text{serum phosphorus} \times \text{urine creatinine})$.

TP/GFR = $\text{serum phosphorus} - (\text{urine phosphorus} \times \text{serum creatinine} / \text{urine creatinine})$.

TmP/GFR mg/dl from Walton's nomogram.

SI unit conversions: To convert the values for phosphate and TP/GFR to mmol/L, multiply by 0.323; to convert the values for calcium to mmol/L, multiply by 0.250; to convert the values for 1,25-(OH)₂ vitamin D to pmol/L, multiply by 2.599; to convert the values for 25-OH vitamin D to nmol/L, multiply by 2.496; to convert the values for creatinine to μmol/L, multiply by 76.26. The random and fasting tests performed on each patient were within a few days of each other, and before any treatment.

^aWalton's nomogram only applies for a serum phosphorus <5 mg/dl.