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Vitamin D 1α-Hydroxylase Gene Mutations in Patients with 1α-Hydroxylase Deficiency

Chan Jong Kim,* Larry E. Kaplan,* Farzana Perwad, Ningwu Huang, Amita Sharma, Yong Choi, Walter L. Miller, and Anthony A. Portale

Department of Pediatrics (C.J.K., L.E.K., F.P., N.H., W.L.M., A.A.P.), University of California, San Francisco, San Francisco, California 94143; Pediatric Nephrology (A.S.), MassGeneral Hospital for Children, Massachusetts General Hospital and Harvard School of Medicine, Boston, Massachusetts 02114; and Department of Pediatrics (Y.C.), Seoul National University Children's Hospital, Seoul 110-744, Korea

Context: Vitamin D 1α-hydroxylase deficiency, also known as vitamin D-dependent rickets type 1, is an autosomal recessive disorder characterized by the early onset of rickets with hypocalcemia and is caused by mutations of the 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase, *CYP27B1*) gene. The human gene encoding the 1α-hydroxylase is 5 kb in length, located on chromosome 12, and comprises nine exons and eight introns. We previously isolated the human 1α-hydroxylase cDNA and gene and identified 19 different mutations in 25 patients with 1α-hydroxylase deficiency.

Objectives, Patients, and Methods: We analyzed the 1α-hydroxylase gene of 10 patients, five from Korea, two from the United States, and one each from Argentina, Denmark, and Morocco, all from nonconsanguineous families. Each had clinical and radiographic features of rickets, hypocalcemia, and low serum concentrations of 1,25-dihydroxyvitamin D_3.

Results: Direct sequencing identified the responsible 1α-hydroxylase gene mutations in 19 of 20 alleles. Four novel and four known mutations were identified. The new mutations included a nonsense mutation in exon 6, substitution of adenine for guanine (2561G→A) creating a stop signal at codon 328; deletion of adenine in exon 9 (3922delA) causing a frameshift; substitution of thymine for cytosine in exon 2 (1031C→T) causing the amino acid change P112L; and a splice site mutation, substitution of adenine for guanine in the first nucleotide of intron 7 (IVS7+1 G→A) causing a frameshift.

Conclusions: Mutations in the 1α-hydroxylase gene previously were identified in 44 patients, to which we add 10 more. The studies show a strong correlation between 1α-hydroxylase mutations and the clinical findings of 1α-hydroxylase deficiency. **(*J Clin Endocrinol Metab* 92: 3177–3182, 2007)**

VITAMIN D IS PRESENT in two forms, ergocalciferol (vitamin D_2) produced by plants and cholecalciferol (vitamin D_3) produced by animal tissues or by the action of UV light on 7-dehydrocholesterol in human skin. Both forms of vitamin D are biologically inactive prohormones that must undergo sequential hydroxylations in the liver and the kidney before they can bind to and activate the vitamin D receptor (1, 2). The hormonally active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D], plays a critical role in calcium and phosphate metabolism, bone growth, and cellular differentiation. Renal synthesis of 1,25$(OH)_2$D from its endogenous precursor, 25-hydroxyvitamin D (25OHD), is the rate-limiting, hormonally regulated step in the bioactivation of vitamin D and is catalyzed by the mitochondrial cytochrome P450 enzyme, 25OHD-1α-hydroxylase (1α-hydroxylase, P450c1α) (3, 4). 1α-Hydroxylation occurs primarily in the kidney, although other tissues such as epidermal keratinocytes, macrophages, and osteoblasts express enzyme activity. Renal 1α-hydroxylase activity is tightly regulated by PTH, calcium, phosphorus, and 1,25$(OH)_2$D itself.

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* C.J.K. and L.E.K. contributed equally to this work.

Abbreviations: 1,25$(OH)_2$D, 1,25-Dihydroxyvitamin D_3; 25OHD, 25-hydroxyvitamin D; 1α-OHD, 1α(OH) vitamin D_3; VDDR-I, vitamin D-dependent rickets type I.

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Vitamin D 1α-hydroxylase deficiency, also referred to as vitamin D-dependent rickets type I (VDDR-I) or pseudovitamin D deficiency rickets, is an autosomal recessive disorder characterized clinically by hypotonia, muscle weakness, growth failure, hypocalcemic seizures in early infancy, and radiographic findings of rickets (5–7). Characteristic laboratory features are hypocalcemia, increased serum concentrations of PTH, and low or undetectable serum concentrations of 1,25$(OH)_2$D despite normal or increased concentrations of 25OHD. Physiological doses of 1α(OH) vitamin D_3 (1α-OHD) or 1,25$(OH)_2D_3$ induce remission of the clinical and laboratory abnormalities (5). We (8), as have others (9–12), cloned the cDNA encoding 1α-hydroxylase, cloned the full-length human gene (*CYP27B1*) (13), and demonstrated that VDDR-I is caused by mutations of this gene (8), identifying 19 different mutations in 25 patients from multiple ethnic backgrounds (8, 14, 15). Others have identified 13 other mutations (16–20); the 32 different mutations described to date include missense mutations, deletions, duplications, and splice site mutations. We now report 1α-hydroxylase mutations in 10 patients with clinical, laboratory, and radiographic features of 1α-hydroxylase deficiency.

Patients and Methods

Patients

We analyzed DNA from 10 unrelated patients from nonconsanguineous families with the clinical diagnosis of 1α-hydroxylase deficiency; patients numbered 1–5 were from Korea, and patients 6–9 were Cau-

casians from Morocco, United States, Argentina, and Denmark, respectively, and patient 10 was African-American. Specimens from the parents and siblings of some of the patients also were analyzed. This study was approved by the Committee on Human Research, University of California, San Francisco. The clinical data for these patients are summarized in Table 1.

Patient 1 presented with bowing of the legs at 12 months of age. He had a prominent forehead with craniotabes, rachitic rosary, genu varus, and enlargement of the wrists and ankles. Laboratory data revealed hypocalcemia, high serum alkaline phosphatase and PTH concentrations, and radiographic features of rickets. With initial treatment with high doses of ergocalciferol, the serum 25OHD concentration was increased, whereas that of $1,25(OH)_2D$ was low. Subsequent treatment with 1α-OHD resulted in improvement of clinical, radiographic, and laboratory abnormalities.

Patient 2 developed recurrent seizures at 5 months of age. At 15 months of age, she was found to have kyphosis, prominent rachitic rosary, a widely open anterior fontanel, varus deformity of the elbow, and x-ray findings of severe rickets with fractures of both fibulae. Laboratory data revealed hypocalcemia, high serum alkaline phosphatase and PTH concentrations, and low $1,25(OH)_2D$ concentration. She was successfully treated with 1α-OHD.

Patient 3 presented with a hypocalcemic seizure at 5 months of age. At 25 months of age, she was found to have a prominent forehead, rachitic rosary, and bowing of the legs. Laboratory evaluation showed hypocalcemia, high serum alkaline phosphatase activity, and low $1,25(OH)_2D$ concentration. Radiographs revealed features of severe rickets. She responded well to 1α-OHD.

Patient 4 presented with a femur fracture after minor trauma at the age of 14 months. He had hypocalcemia, high alkaline phosphatase activity, low $1,25(OH)_2D$ concentration, and rachitic changes on x-ray. Administration of 1α-OHD resulted in normalization of clinical, radiographic, and laboratory abnormalities.

Patient 5 presented with a hypocalcemic seizure at 5 months of age and was treated with calcium and vitamin D. At 17 months of age, he was noted to have rachitic rosary, prominent forehead, and enlargement of wrists and ankles. Laboratory data revealed hypocalcemia, high serum alkaline phosphatase concentration, and rachitic changes on x-ray. Serum concentration of 25OHD was above the normal range, whereas that of $1,25-(OH)_2D$ was not detectable. He responded well to administration of 1α-OHD.

Patient 6 presented with severe malnutrition, hypotonia, and respiratory distress at 22 months of age. Physical and x-ray examinations revealed findings consistent with severe rickets and pneumonia with pleural effusion. Laboratory examination showed hypocalcemia, hypophosphatemia, and high concentrations of alkaline phosphatase and PTH. Serum 25OHD concentration was within the normal range, whereas that of $1,25(OH)_2D$ was low. He developed bilateral pneumothoraces and multiple organ failure and died.

Patient 7 presented with rachitic rosary, genu varus, and fractures of the midregion of both ulnae, left tibia, and femur (four fracture sites) at 12 months of age. Laboratory examination showed normal serum calcium concentration, hypophosphatemia, high alkaline phosphatase and PTH concentrations, high normal 25OHD concentration, undetectable $1,25(OH)_2D$ concentration, and generalized aminoaciduria. He initially was treated with sodium citrate and citric acid solution, phosphate supplements, and calcitriol with good response. Renal ultrasound performed after 8 months of therapy showed nephrocalcinosis. He remains on treatment with calcitriol.

Patient 8 presented with seizures and hypocalcemia at 2 months of age. He was treated with calcium, diphenylhydantoin, and phenobarbitol. At 3 months of age, x-rays of the wrists showed severe rickets. He had hypocalcemia, high serum alkaline phosphatase and PTH concentrations, normal 25OHD and low $1,25(OH)_2D$ concentrations, and x-ray findings of severe rickets. With administration of calcitriol, the clinical, radiographic, and laboratory abnormalities became normal.

Patient 9 presented with delayed motor development and was found to have Harrison's grooves and delayed bone development, prompting the clinical diagnosis of rickets at the age of 10 months. Laboratory data revealed hypocalcemia, normophosphatemia, high alkaline phosphatase and PTH concentrations, normal 25(OH)D concentration, and low $1,25(OH)_2D$ concentration. She was successfully treated with calcium and 1α-OHD.

Patient 10, an African-American female, was evaluated for clinical signs of rickets at 12 months of age. Serum calcium was low, and $1,25(OH)_2D$ was not detectable. She responded well to treatment with calcitriol.

PCR amplification and sequence analysis of 1α-hydroxylase gene

Genomic DNA was extracted from leukocytes, and the human 1α-hydroxylase gene was amplified by PCR as previously described (14). PCR products were purified through QIAquick PCR purification columns (QIAGEN, Valencia, CA) and sequenced directly using an automated ABI PRISM 3700 sequencer (PerkinElmer Corp., Foster City, CA). The primers used for DNA sequencing of patients 1–5 were as previously described (14) except for exons 7 and 8, for which the following primers were used: exon 7, sense, 5′-ACTAGTGGATGGAAGCAGGGA-3′, and antisense, 5′-GGTCAAGGGGAGTGTTT-GAAG-3′; and exon 8, sense, 5′-TTCAAACACTCCCCTTGACC-3′, and antisense, 5′-CAGGGGAAAGAGCTCACAAC-3′. The primers used for DNA sequencing of patients 6–10 are shown in Table 2. Putative mutations were

TABLE 1. Clinical and laboratory findings in 10 patients with 1α-hydroxylase deficiency

Patient no.	Location	Age at diagnosis (months)	Clinical presentation	Serum concentration						Mutation no.
				Ca (mmol/liter)	P (mmol/liter)	ALP (IU/liter)	PTH (IU/liter)	25OHD (ng/ml)	$1,25(OH)_2D$ (pg/ml)	
1	Korea	12	Bowed legs	2.1	0.8	1050	560[b]	336[a]	<7	1/1
2	Korea	5	Seizures	1.6	1.0	2043	1320[b]	27	9	2/2
3	Korea	5	Seizure	1.4	2.0	6665	750[b]	38	<5	1/2
4	Korea	14	Femur fracture	1.8	1.6	1437	340[c]	16	<5	1/2
5	Korea	5	Seizure	1.5	1.2	1326	750[b]	77	<4	1/3
6	Morocco	22	Severe rickets	1.3	0.8	957	198[c]	42	6	4/4
7	United States	12	Rachitic rosary, fractures, bowed legs	2.5	0.6	3952	874[c]	49	<5	5/5
8	Argentina	2	Seizures, hypocalcemia, rickets	1.9	1.4	2477	164[c]	19	11	6/7
9	Denmark	10	Rachitic rosary	1.7	1.2	6507	1.58[d]	37	<5	7/?
10	United States	12	Rickets	Low	NA	NA	NA	NA	<10	8/8
Normal pediatric ranges				2.2–2.7	1.4–2.0	80–260		15–60	20–80	

To convert the values for 25OHD to nmol/liter, multiply by 2.5; to convert the values for $1,25(OH)_2D$ to pmol/liter, multiply by 2.4. ALP, Alkaline phosphatase; NA, not available.

[a] During treatment with cholecalciferol.

[b–d] PTH normal ranges: [b] 150–450 pg/ml; [c] 10–65 pg/ml; [d] 0.22–0.5 μg/liter.

TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing of the *CYP27B1* gene in patients 6–10

Exon	Sense primer	Antisense primer	Size (bp)
1	ACCACTCAGGAGGAGGGATTG	CTTATTTCATGGGCATCCGTTC	450
2	AGAAGCTCCCTATTCCCAAGC	TAGAGTGGGACAGCCGACCTC	409
3	CAGGTTTCCGTACCCCAAGG	GGATGAAGGTCTCCGTGTCG	500
4	TACAAGTTCGGACTGGAAGGTG	AGACTCCAGGTCCTTCTCGG	466
5	AGACTGGGACCAGATGTTTGC	CTAAGCCAAGCTGGTCTACGTG	398
6	GACCAGCTTGGCTTAGCACC	TTTTGGCCAGGAGTAGAGGG	393
7	AAAATGGCCCTCTACTCCTGG	GTCAAGGGGAGTGTTTGAAGG	298
8	GGAGATCCTTCCAAAAGTAGCC	GCCTATAATGGGCTTATCATATTTCAG	482
9	CCACCCAATCATTGACCATTC	TTTGGTCAGATAGGCATTAGGG	343

confirmed twice by PCR amplification and sequencing of the affected exon directly from genomic DNA of the patients and their parents. Nucleotides are numbered from the experimentally determined transcriptional start site as described (13); the reference sequence is GenBank accession no. AF 027152.

Results

Clinical characteristics

The diagnosis of 1α-hydroxylase deficiency was made in each of the 10 patients based on their clinical features and laboratory findings; the parents and siblings were asymptomatic. The 10 patients showed symptoms within the first 22 months of life, and their physical findings included rachitic rosary, genu varus or valgus, metaphyseal widening, and frontal bossing (Table 1). All patients had radiographic signs of active rickets. Laboratory testing revealed hypocalcemia, increased serum concentrations of alkaline phosphatase and PTH, normal or increased serum concentrations of 25OHD, and greatly reduced concentrations of $1,25(OH)_2D$. Patients responded well to treatment with 1α-OHD or calcitriol.

Mutations in the 1α-hydroxylase gene

To determine whether patients with the clinical syndrome of 1α-hydroxylase deficiency had mutations in the 1α-hydroxylase gene, we analyzed genomic DNA extracted from leukocytes. We found mutations in the 1α-hydroxylase gene on both alleles in nine of the 10 patients. Patient 1 was homozygous for a splice site mutation, substitution of an adenine for a guanine in the first nucleotide of intron 3 (IVS3 + 1 G→A). This mutation, reported previously in a Japanese patient (18), disrupts the splice donor site, resulting in retention of intron 3. The retained intron would create a frameshift after codon 196 and a translational termination signal 63 bp downstream from the end of exon 3, resulting in a severely truncated protein that cannot have enzymatic activity. Patient 2 was homozygous for a seven-nucleotide insertion in exon 8; this previously reported mutation alters the reading frame downstream of codon 442 and creates a premature TGA stop signal at codon 446 (14). Patients 3 and 4 were compound heterozygous for the two mutations detected in patients 1 and 2. Patient 5 was compound heterozygous for the mutation detected in patient 1, IVS3 + 1 G→A, and for a novel nonsense mutation, 2561G→A, in exon 6 that creates a premature TAG stop signal at codon 328. Patient 6 was homozygous for a novel deletion in exon 9, 3922delA. This frameshift mutation bypasses the termination signal normally found at codon 509 (TAG), permitting translation into the 3′ untranslated region of the gene to reach a TAA stop signal 92 bp downstream. Patient 7 was homozygous for a deletion in exon 2, 958delG, a known frameshift mutation that results in a severely truncated protein. We previously showed that 958delG is the founder mutation observed in French Canadian patients originating from the Charlevoix-Saguenay-Lac Saint Jean region of Quebec, in which the prevalence of 1α-hydroxylase deficiency is unusually high (14, 17). Patient 8 was heterozygous for a novel missense mutation in exon 2, 1031C→T, causing the amino acid change proline (P)112 to leucine (L), and a known missense mutation in exon 7, 2947G→A, causing the amino acid change arginine (R) 389 to histidine (H) (14, 15). Patient 9 was heterozygous for the same novel missense mutation identified in patient 8, 2947G→A (R389H); however, we were unable to detect a second mutation in this patient. Patient 10 was homozygous for the novel missense mutation 2998G→A, resulting in the splice donor site mutation IVS7 + 1 G→A.

Analysis of the family members showed that the asymptomatic parents and the brother of patient 1 were heterozygous for the IVS3 + 1 G→A mutation. The mother and the

TABLE 3. Mutation numbers

No.	Nucleotide change[a]	Amino acid change	Location
1	IVS3+1 G→A (1796G→A)	Frameshift after 196E	Intron 3
2	3398–3406insCCCACCC	Frameshift after 441H	Exon 8
3	2561G→A	W328X	Exon 6
4	3922delA	Frameshift after 498E	Exon 9
5	958delG	Frameshift after 87Y	Exon 2
6	1031C→T	P112 L	Exon 2
7	2947G→A	R389H	Exon 7
8	IVS7+1 G→A (2997G→A)	Frameshift after 405N	Intron 7

[a] Nucleotide numbers refer to genomic DNA and are numbered from the transcription start site (13). The reference sequence is available on the NCBI, Entrez, Nucleotide database (http://www.ncbi.nlm.nih.gov/Entrez; accession no. AF 027152).

TABLE 4. Known mutations of *CYP27B1* in patients with vitamin D 1α-hydroxylase deficiency

Nucleotide change[a]	Amino acid change	Exon	Ref.
Missense mutations			
246G→T	Q65H	1	14
1016G→A	R107H	2	16, 18
1031C→T	P112 L	2	This study
1070G→A	G125E	2	16
1634C→T	P143 L	3	18
1696G→A	D164N	3	18
1772A→G	E189G	3	15
1771G→A	E189K	3	14
2337C→G	T321R	5	18
2546C→A	S323Y	6	19
2582G→C	R335P	6	16
2605C→T	L343F	6	15
2925C→T	P382S	7	16
2946C→T	R389C	7	18
2946C→G	R389G	7	15
2947G→A	R389H	7	14, 15, and this study
3299C→T	T409I	8	14, 15
3359G→C	R429P	8	14
3430C→T	R453C	8	14
3680T→G	V478G	9	19
3917C→G	P497R	9	14
Nonsense mutations			
2014G→A	W241X	4	14
2561G→A	W328X	6	This study
3372G→A	W433X	8	18
Deletions			
212delG	Frameshift from 55K	1	14
958delG	Frameshift from 87Y	2	14, 17, and this study
1921delG	Frameshift after 209C	4	8, 14
1984delC	Frameshift after 230V	4	8, 14
3922delA	Frameshift after 498E	9	This study
Insertions			
3398–3406insCCCACCC	Frameshift after 441H	8	14, 17, 19, and this study
3398–3408insCCCACACCC	Frameshift after 441H	8	14
Deletion-insertion			
897–901delGGGCG; 897–902insCTTCGG	Frameshift after 66V	2	15
Splice-site mutations			
IVS2+1 G to A (1083G→A)	Frameshift after 129A	Intron 2	15
IVS3+1 G to A (1796G→A)	Frameshift after 196E	Intron 3	18 and this study
IVS6+1 G to A (2715G→T)	Frameshift after 379R	Intron 6	20
IVS7+1 G to A (G2997G→A)	Frameshift after 405N	Intron 7	This study

[a] Nucleotide numbers refer to genomic DNA and are numbered from the transcription start site (13). The reference sequence is available on the NCBI, Entrez, Nucleotide database (http://www.ncbi.nlm.nih.gov/Entrez; accession no. AF 027152).

brother of patient 2 were heterozygous for the seven-nucleotide insertion in exon 8; DNA from the father was not available for study. The mother of patient 3 was heterozygous for IVS3 + 1 G→A, and the father and sister were heterozygous for the seven-nucleotide insertion in exon 8. The mother of patient 4 was heterozygous for the seven-nucleotide insertion in exon 8; the father was not studied. The mother and brother of patient 5 were heterozygous for the IVS3 + 1 G→A, and the father was heterozygous for the 2651G→A mutation in exon 6. The father and mother of patient 6 were both heterozygous for the deletion 3922delA in exon 9. Both parents of patient 9 were heterozygous for 2947G→A in exon 7. DNA from the parents of patients 7, 8, and 10 was not available for analysis. A distant cousin of patient 7 was a compound heterozygote for the missense mutation R389H in exon 7, which was devoid of enzyme activity when expressed *in vitro*, and for the deletion 958delG in exon 2, which leads to a premature stop (Table 3, patient 2 of Ref. 14).

Discussion

$1,25(OH)_2D$ is the most potent metabolite of vitamin D and mediates its hormonal actions through the vitamin D receptor. $1,25(OH)_2D$ is synthesized from 25OHD by 25OHD-1α-hydroxylase, a mitochondrial (type I) cytochrome P450 enzyme that functions as an oxidase, using electrons from reduced nicotinamide adenine dinucleotide phosphate and molecular oxygen. The P450 moiety binds the substrate, receives electrons and molecular oxygen, and catalyzes the

reaction using the iron of the heme group to coordinate oxygen (21). The cloning of the 1α-hydroxylase gene after a long effort (8–13) has permitted determination of the molecular genetic basis of vitamin D 1α-hydroxylase deficiency. To date, a total of 36 mutations in the 1α-hydroxylase gene have been identified in 54 patients (108 alleles) with 1α-hydroxylase deficiency, including those in the present report (Table 4). All but one of the frameshift and premature translation-arrest mutations described eliminate the heme-binding site of P450c1α; hence, the resultant protein cannot have 1α-hydroxylase activity.

Two mutations described in *CYP27B1* are relatively more common than the others. Deletion of guanine 958 (958delG), as numbered from the gene's transcriptional start site (13), was found on 20 French Canadian alleles (14, 17); microsatellite haplotype analysis showed that these arose from a single founder (14, 22). A seven-nucleotide duplication/insertion in exon 8 was found in 14 affected alleles (14, 17, 19); these arose from various ethnic groups with different microsatellite haplotypes (14).

We have now shown that the splice site mutation, IVS3 + 1 G→A also is common, occurring in five of 10 Korean alleles. Guanine to adenine substitution in the first nucleotide of an intron is a common mutation in many genes and usually results in skipping of the 5′-exon or activation of potential splice sites (23). However, in short introns, retention of the intron may also occur (23), as has been demonstrated for IVS3 + 1 G→A in the 1α-hydroxylase (18). The retention of intron 3 causes altered translation and a premature stop codon 63 bp downstream from the end of exon 3. The resulting truncated 1α-hydroxylase would have only 197 of 508 amino acids, would lack the heme-binding domain, and hence would have no enzyme activity. Two other splice site mutations have been reported in the 1α-hydroxylase gene: IVS2 + 1 G→A and IVS6 + 1 G→A (15, 20). We now have identified a fourth splice site mutation, IVS7 + 1 G→A.

The 1α-hydroxylase gene contains the normally duplicated sequence 5′-CCCACCC CCCACCC-3′ in exon 8, which encodes residues 438–442 (Pro-Thr-Pro-His-Pro). In the present report, we found triplication rather than duplication of this seven-nucleotide sequence on four alleles. This seven-nucleotide insertion alters the reading frame after codon 441 and creates a premature TGA stop signal 25 codons downstream; this disrupts the heme-binding domain; hence, the resulting truncated protein is devoid of activity (14). We previously reported that six families of widely divergent ethnic backgrounds (Filipino, Polish, Chinese, Caucasian-American, African-American, and Hispanic) carried this seven-nucleotide insertion in association with four different microsatellite haplotypes (14). Thus, the ethnic and haplotype diversity associated with the seven-nucleotide insertion suggests that it has risen by several independent *de novo* events, probably as the result of a slipped-strand mispairing mechanism during meiosis (24). Kitanaka *et al.* (18) found 10 different 1α-hydroxylase gene mutations in 20 Japanese alleles, suggesting that there is no founder effect in that ethnic group. By contrast, we found only three different mutations in ten Korean alleles, suggesting that in this ethnic group, the genetic defect in the 1α-hydroxylase gene is more homogeneous.

The novel missense mutation identified in patient 6, Pro 112 to Leu, immediately precedes the B′ α-helix, which comprises residues 113–118 (14). Changing this residue from Pro to Leu is predicted to reorient dramatically the direction of the Cα carbon peptide backbone, disrupting the sterol-binding pocket of the enzyme. The resultant protein is predicted to be devoid of enzymatic activity.

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Address all correspondence and requests for reprints to: Anthony A. Portale, M.D., University of California, San Francisco, 533 Parnassus Avenue, Box 0748, Room U-585, San Francisco, California 94143-0748. E-mail: aportale@peds.ucsf.edu.

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Present address for C.J.K.: Department of Pediatrics, Chonnam National University Medical School, Gwangju, Korea.

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