ORIGINAL ARTICLE

JBMR®

Vitamin D–Dependent Rickets Type 1B (25-Hydroxylase Deficiency): A Rare Condition or a Misdiagnosed Condition?

Arnaud Molin,[1,2,3,4*] Arnaud Wiedemann,[5,6*] Nick Demers,[7] Martin Kaufmann,[7] Jérémy Do Cao,[5,6] Laurent Mainard,[6,8] Brigitte Dousset,[9] Pierre Journeau,[6,10] Geneviève Abeguile,[1] Nadia Coudray,[1] Hervé Mittre,[1,2,4] Nicolas Richard,[1,3] Georges Weryha,[6,11] Arthur Sorlin,[12] Glenville Jones,[7] Marie-Laure Kottler,[1,2,3] and Francois Feillet[5,6,13]

[1]CHU de Caen, Department of Genetics, Molecular Genetics Laboratory and Reference Center for Rare Diseases of Calcium and Phosphorus Metabolism, Caen, France
[2]Université Caen Normandie, Medical School, Caen, France
[3]BioTARGEN, Université Caen Normandie, Caen, France
[4]OeReCa, Université Caen Normandie, Caen, France
[5]CHU de Nancy, Department of Pediatrics and Reference Center for Rare Hereditary Diseases of Metabolism, Vandoeuvre-lès-Nancy, France
[6]Université de Lorraine, Medical School, Vandoeuvre-lès-Nancy, France
[7]Queen's University, Department of Biomedical and Molecular Sciences, Kingston, Canada
[8]CHU de Nancy, Department of Radiology, Vandoeuvre-lès-Nancy, France
[9]CHU de Nancy, Department of Biochemistry, Vandoeuvre-lès-Nancy, France
[10]CHU de Nancy, Department of Orthopedic Pediatric Surgery, Vandoeuvre-lès-Nancy, France
[11]CHU de Nancy, Department of Endocrinology, Vandoeuvre-lès-Nancy, France
[12]CHU de Nancy, Department of Genetics, Vandoeuvre-lès-Nancy, France
[13]INSERM, U954 Nutrition - génétique et exposition aux risques environnementaux, Vandoeuvre-lès-Nancy, France

ABSTRACT

Vitamin D requires a two-step activation by hydroxylation: The first step is catalyzed by hepatic 25-hydroxylase (*CYP2R1*, 11p15.2) and the second one is catalyzed by renal 1α-hydroxylase (*CYP27B1*, 12q13.1), which produces the active hormonal form of 1,25-$(OH)_2$D. Mutations of *CYP2R1* have been associated with vitamin D–dependent rickets type 1B (VDDR1B), a very rare condition that has only been reported to affect 4 families to date. We describe 7 patients from 2 unrelated families who presented with homozygous loss-of-function mutations of *CYP2R1*. Heterozygous mutations were present in their normal parents. We identified a new c.124_138delinsCGG (p.Gly42_Leu46delinsArg) variation and the previously published c.296T>C (p.Leu99Pro) mutation. Functional in vitro studies confirmed loss-of-function enzymatic activity in both cases. We discuss the difficulties in establishing the correct diagnosis and the specific biochemical pattern, namely, very low 25-OH-D suggestive of classical vitamin D deficiency, in the face of normal/high concentrations of 1,25-$(OH)_2$D. Siblings exhibited the three stages of rickets based on biochemical and radiographic findings. Interestingly, adult patients were able to maintain normal mineral metabolism without vitamin D supplementation. One index case presented with a partial improvement with 1alfa-hydroxyvitamin D_3 or alfacalcidol (1α-OH-D_3) treatment, and we observed a dramatic increase in the 1,25-$(OH)_2$D serum concentration, which indicated the role of accessory 25-hydroxylase enzymes. Lastly, in patients who received calcifediol (25-OH-D_3), we documented normal 24-hydroxylase activity (*CYP24A1*). For the first time, and according to the concept of personalized medicine, we demonstrate dramatic improvements in patients who were given 25-OH-D therapy (clinical symptoms, biochemical data, and bone densitometry). In conclusion, the current study further expands the *CYP2R1* mutation spectrum. We note that VDDR1B could be easily mistaken for classical vitamin D deficiency. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: RICKETS; VITAMIN D; 25-HYDROXYLASE; GENETICS; PERSONALIZED MEDICINE

Received in original form February 1, 2017; revised form May 22, 2017; accepted May 25, 2017. Accepted manuscript online May 26, 2017.
Address correspondence to: Marie-Laure Kottler, MD, PhD, CHU de Caen – Site Clémenceau, Service de Génétique, Avenue de la Côte de Nacre, CS30001, 14033 CAEN cedex 9, France. E-mail: kottler-ml@chu-caen.fr
*AM and AW contributed equally to this work.
Additional Supporting Information may be found in the online version of this article.
Journal of Bone and Mineral Research, Vol. 32, No. 9, September 2017, pp 1893–1899
DOI: 10.1002/jbmr.3181
© 2017 American Society for Bone and Mineral Research

Introduction

Vitamin D requires a two-step activation by hydroxylation: The first step is catalyzed by 25-hydroxylase (*CYP2R1*, 11p15.2) and the second one is catalyzed by 1α-hydroxylase (*CYP27B1*, 12q13.1), which produces the active hormonal form of 1,25-$(OH)_2$D. The major function of vitamin D is to maintain serum calcium and phosphate levels within the normal physiological range to support most metabolic functions, neuromuscular transmission, and bone mineralization. An inadequate calcium-phosphate product leads to a bone mineralization defect that causes rickets in children and osteomalacia in adults. Classical vitamin D deficiency is the most common cause of rickets and has been used to justify public health recommendations for vitamin D supplementation in children.

Rickets with adequate dietary intake or sunlight exposure has been associated with rare hereditary disorders. Defects in 25- or 1α-hydroxylation lead to an inability to activate vitamin D, thus causing a vitamin D deficiency–like state that is resistant to physiologic doses of vitamin D. Vitamin D–dependent rickets type 1A (VDDR1A, MIM 264700) was first described and associated with loss-of-function mutations of *CYP27B1*. Mutations of *CYP2R1* have recently been associated with VDDR1B (MIM 600081), a very rare condition that has been reported to currently affect only 4 families.[1–5]

In this article, we described the molecular analysis of 7 patients from 2 unrelated families who presented with VDDR1B due to a loss-of-function mutation of *CYP2R1* and a new mutation. We noted the specific biochemical pattern of the disease and its dramatic improvement with 25-hydroxyvitamin D therapy.

Patients and Methods

In family 1 (F1) (Fig. 1*A*), the proband (II-3) is a 4-year-old boy, the third child of a non-consanguineous healthy French couple. He received daily vitamin D_3 supplementation according to French recommendations (1000 to 1200 IU/d or 25 to 30 μg/d of cholecalciferol) until the age of 18 months. He received medical attention for growth restriction (height, −4 SD; weight, −2 SD) with lower limb abnormalities (*genu varum*) and mild hypotonia. Biochemical analyses were suggestive of vitamin D deficiency and nutritional rickets, as revealed in the lower limb radiography images (Fig. 2). This patient was first diagnosed with 1α-hydroxylase deficiency and was treated with calcium supplementation and alfacalcidol (1α-OH-D_3). Once the exact diagnosis of VDDR1B was achieved, he was subsequently treated with calcifediol (25-OH-D_3).

In family 2 (F2) (Fig. 1*B*), the proband (II-1) is a 9-year-old boy, born at 40 gestational weeks (birth weight 3370 g, birth length 49 cm, birth head circumference 35 cm, Apgar 9/10), to consanguineous healthy parents from Moroccan extraction. Cognitive and motor skill development were normal except for a walking delay (23 months). Medical history began at the age of 5 years with the exploration of a waddling gait. He was supplemented with vitamin D during infancy (800 IU/d of cholecalciferol until age 18 months); later he received a yearly bolus of vitamin D (100,000 IU of cholecalciferol every autumn). At 7 years old, he complained of muscular pain and increased fatigability. He presented with proximal muscular deficiency and dramatic amyotrophy of the lower limbs suggestive of myopathy, but a muscular biopsy was normal. Finally, the diagnosis of VDDR1B rickets was suspected because of radiological (Fig. 2) and biological data (Table 1).

Written informed consent was obtained from the patients and/or their parents for the collection of clinical and laboratory data and for genetic investigation. The molecular analysis of relatives was suggested through genetic counseling after the identification of a mutation in the probands.

Biochemical investigations (Tables 1 and 2)

Routine laboratory data were collected at the time of the diagnosis, retrospectively and prospectively, using records from hospitals. Intact parathyroid hormone (PTH 1-84), 1,25-$(OH)_2$D and alkaline phosphatase (ALP) were quantified in the serum via a chemiluminescent immunoassay (LIAISON XL Analyzer, DiaSorin, Stillwater, MN, USA). 25-OH-D levels were assessed via the following two methods: a chemiluminescent immunoassay for the quantitative determination of total 25-OH-D and a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) assay for the simultaneous quantification of 25-OH-D_2, 25-OH-D_3, and 24,25-$(OH)_2D_3$. CYP24A1 (24-hydroxylase) enzyme activity was quantified by LC-MS/MS from the ratio of 25-OH-D_3: 24,25-$(OH)_2D_3$ as previously described.[6]

The analytical performance of the various methods is described in the supplemental material.

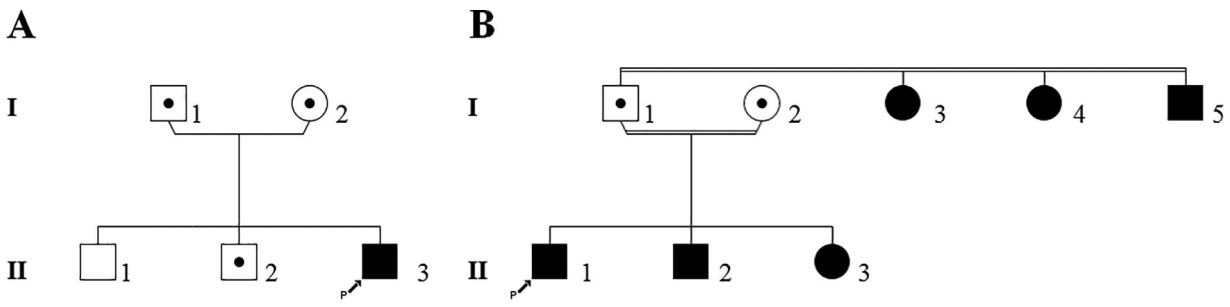

Fig. 1. (*A*) Pedigree tree of family 1 with the *CYP2R1* mutation c.124_138delinsCGG (p.Gly42_Leu46delinsArg). (*B*) Pedigree tree of family 2 with the *CYP2R1* mutation c.296T>C (p.Leu99Pro). ⊡ = heterozygous carrier; ■ = affected individuals; □ = unaffected individual without mutation; ═ = consanguinity; P = proband.

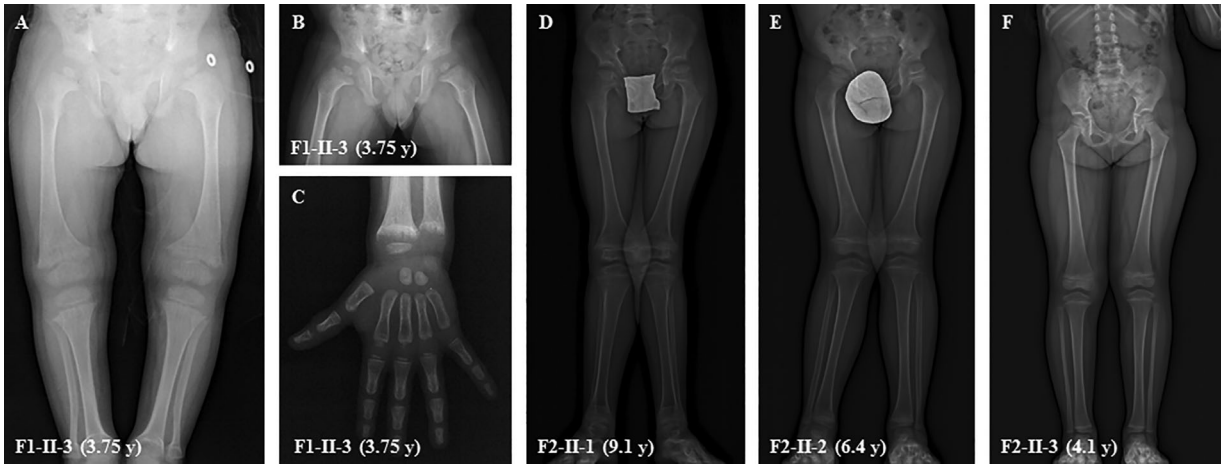

Fig. 2. Radiological features of rickets in VDDR1B patients with classical cupping and fraying of the metaphyseal region and bowing of the legs. Proband in family 1 (*A–C*) at 3.75 years. Proband in family 2 (*D*) and siblings (*E*, *F*) at the ages of 9.1, 6.4, and 4.1 years, respectively. Of note, patient F2-II-3 showed only very moderate metaphyseal dysplasia without bowing of the lower limbs.

Table 1. Biochemical Data and Clinical Evolution

		Routine serum assay					
		Ca	P	PTH (1-84)	ALP	25-OH-D	1,25-$(OH)_2$D
Patient age		mmol/L	mmol/L	pg/mL	UI/L	nmol/L	pmol/L
Normal range	Therapy	2.20–2.65	*	6.5–36.8	—	62.4–282	20–100
F1-II-3							
3.75 yr	D_3 25–30 μg/d	1.57	1.44	216	762	<10	194
3.8 yr	Ca 500 mg/d and 1α-OH-D_3 4 μg/d (after 1.5 m)	2.55	2.1	96.9	513	10	383
4.5 yr	25-OH-D_3 20 μg/d during 0.5 mo (after 2 mo)	2.6	1.6	14.7	302	268	221
5.5 yr	25-OH-D3 20 μg/d	2.48	1.84	21.7	218	182	187
F1-I-1 35 yr		2.3	0.8	29.2	71	59	179
F1-I-2 33.5 yr		2.4	1.3	24.9	62	38	150
F1-II-1 9.5 yr		2.45	1.71	22.5	256	60	157
F1-II-2 7 yr		2.43	1.74	16	232	39	165
F2-II-1							
8.75 yr		1.98	1.07	338	1921	<10	36
9.1 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 3 mo)	2.27	1.74	59.9	595	164	731
9.75 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 1 yr)	2.5	1.58	33.1	374	211	372
F2-II-2							
6.2 yr		1.38	1.29	345	581	<10	64
6.4 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 3 mo)	2.48	1.78	10.2	320	250	285
7.1 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 1 yr)	2.48	1.65	23.4	394	218	235
F2-II-3							
3.8 yr		2.45	1.42	130	266	<10	117
4.1 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 3 mo)	2.65	1.45	6.3	298	410	189
4.8 yr	Ca 500 mg/d and 25-OH-D_3 15 μg/d (after 1 yr)	2.45	1.36	24	294	480	214
F2-I-3							
17.75 yr	Ca 4.5 g/d and 25-OH-D_3 50 μg/d (after 1 yr)	2.28	0.78	37		153	68
20.9 yr	Ca 1.5 g/d and 25-OH-D_3 15 μg/d: bad compliance	2.3	0.9	39	128	<10	
F2-I-4							
11.6 yr	No treatment	2.3	0.7	375	3762	<10	83
11.7 yr	Ca 1 g/d and 25-OH-D_3 50 μg/d (after 1 mo)	2.5	1.4	59	1400	102.3	
16.5 yr	Ca 1 g/d and 25-OH-D_3 50 μg/d: bad compliance	2.2	1.3	109	255	<10	122.4
31.8 yr	No treatment	2.5			233		
F2-I-5							
11.8 yr	No treatment	2.1	0.7		1487	<10	83
12.25 yr	Ca 2 g/d and 25-OH-D_3 40 μg/d (after 3 mo)	2.6	1.6	14	551	185	145
30.7 y	No treatment	2.3		50		<10	208

Ca = calcium; yr = year(s); mo = month(s); d = day(s).

*Normal values for serum phosphate (P) according to age: 1–5 years: 1.3–2.4 mmol/L; 5–13 years: 1.3–1.9 mmol/L; >13 years: 0.9–1.8 mmol/L.

Table 2. Vitamin D Metabolites Identified Via LC-MS/MS

		LC-MS/MS assay			
		25-OH-D_3	25-OH-D_2	24,25-$(OH)_2$ D_3	Ratio
	Vitamin D therapy	nmol/L	nmol/L	nmol/L	$N < 25$
F1-II-3 4.7 yr	25-OH-D_3 20 μg/d	309.3	1.2	28.6	10.8
F2-II-1 9.1 yr	25-OH-D_3 15 μg/d	204.9	1.0	13.2	15.5
F2-II-2 6.4 yr	25-OH-D_3 15 μg/d	302.3	1.2	22.6	13.4
F2-II-3 4.1 yr	25-OH-D_3 15 μg/d	460.3	1.5	54.2	8.5
F2-I-5 30.7 yr	No treatment	6.7	0.2	0.2	33.5

Normal 25-OH-D_3: 24,25-$(OH)_2$ D_3 ratio in children receiving oral 25-OH-D_3 as a result of a normal 24-hydroxylase activity. Of note, patient F2-I-5 has a low but detectable 25-OH-D_3.

Bone densitometry

Bone densitometry was assessed in the lumbar spine and total hip with a QDR 4500 A Hologic device (Hologic, Stefanix, France). The results are given as *Z*-scores. The analytical performance of the method is described in the Supplemental Material.

CYP2R1 molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes via standard methods. We designed several sets of primers for the amplification and sequencing of *CYP2R1* exons 1–5 and their intron-exon junctions (available on request). PCR products were purified and sequenced with the CEQTMDTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) on a Beckman Coulter DNA Sequencer.

Genetic variations were studied in silico with prediction tools (Polyphen-2, Align GVGD, Mutation Taster, SIFT) and data frequencies from Exome Variant Server (EVS) and Exome Aggregation Consortium (EXAC) databases. Data on the functional activity of each variant were searched in published literature. The OPM server (Orientations of Proteins in Membranes; http://opm.phar.umich.edu/server.php) was used to determine the position of variants in vitamin D 25-hydroxylase and its consequences on membrane binding.

In vitro functional analysis of CYP2R1 variants

A *CYP2R1* mutation was introduced into the wild-type hCYP2R1 with QuikChange (Stratagene Corp., La Jolla, CA, USA), according to the manufacturer's protocol and oligonucleotide pair bases specific for each variation (Integrated DNA Technologies, Inc., Coralville, IA, USA). Mutations were confirmed by sequencing (Robarts Research Institute, London, Canada), and the plasmids used for transfection were purified with QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Canada).

Human wild-type and mutated *CYP2R1* constructs were transiently transfected into V79-4 Chinese hamster lung fibroblast cells. These cells have no *CYP2R1* basal activity and are a favorite host system for a variety of CYPs. Transfected cells were incubated with 500 nM 1α-OH-D_2, and the incubation media was extracted via the Bligh and Dyer method. Enzyme extracts were immunopurified over a column containing anti-1,25-$(OH)_2$D antibodies anchored to a resin. The aqueous phase was re-extracted with 0.1% glacial acetic acid and 2.5 mL of methylene chloride and dried. The samples were then derivatized by redissolving the dry residue in DMEQ-TAD as previously described.[6] The derivatized extract was dried and redissolved in 50 μL of 60:40 (vol/vol) methanol/water running solvent, and LC-MS/MS analysis was performed.[6] The 1,25-$(OH)_2D_2$ product was quantitated by LC-MS/MS using a deuterated internal standard for 1,25-$(OH)_2D_2$. The DMEQ-TAD adduct peaks exactly correspond to 1,25-$(OH)_2D_2$ at 12.5 and 13.4 minutes. The amount of CYP2R1 products that included 1,25-$(OH)_2D_2$ from 1α-OH-D_2 were used to compare the relative activities of variants to wild-type CYP2R1. The transient transfections were performed twice, each time with triplicate evaluations of each mutant and wild-type CYP2R1.

Results

Biochemical data are reported in Table 1.

At the time of diagnosis, the two probands presented with marked hypocalcemia, hypophosphatemia, high parathyroid hormone (PTH) and alkaline phosphatase (ALP) levels; undetectable 25-OH-D concentrations and high 1,25-$(OH)_2$D values led to the diagnosis of vitamin D deficiency, and they were treated accordingly.

Patient F1-II-3 was initially treated with calcium and oral alfacalcidol (1α-OH-D_3) that led to a relief of symptoms and normalized serum calcium and phosphate but not PTH and ALP. After 9 months with this therapy, hypercalcemia with hypercalciuria was observed and calcium supplementation was stopped. Once the final diagnosis was achieved, he was subsequently treated with calcifediol (25-OH-D_3). This treatment resulted in the prompt normalization of all biochemical parameters (including PTH and ALP) and the correction of long bone deformations. Biochemical parameters in his parents and brothers were within the normal ranges. We documented normal 24-hydroxylase activity when patients were given 25-OH-D_3.

In family 2, patient F2-II-2 (the proband's brother) exhibited *genu varum*, and presented the same biochemical pattern at the age of 6.2 years. Conversely, the sister (F2-II-3) was asymptomatic and had normal serum calcium and ALP but increased PTH and low/normal phosphate at the age of 3.8. She exhibited mild metaphyseal dysplasia at the age of 4.1 years.

An initial bone densitometry exploration showed low vertebral bone density in the 3 children. A dramatic amelioration of clinical and biochemical phenotypes and a major improvement in vertebral bone density were observed with 25-OH-D_3 treatment (Table 3). However, despite good compliance to the therapy, patient F2-II-2's *genu varum* was treated by epiphysiodesis at the age of 10 years.

Patients F2-I-3, F2-I-4, and F2-I-5 are the paternal aunts and uncle of the 3 children, also born to consanguineous parents.

Table 3. Bone Densitometry in Family 2, Proband F2-II-1, and Siblings F2-II-2 and F2-II-3, Before and After 6 Months of 25-OH-D_3 Treatment

	F2-II-1		F2-II-2		F2-II-3	
Age (years)	8.8	9.4	6.2	6.8	3.8	4.4
Z-score						
Femoral neck	−3.6	0.2	−1.8	1.5	—	—
Lumbar spine	−2.2	0.5	−1.2	1.2	−0.5	0.7
Whole body	−3.1	0.1	−0.5	1.8	1.5	2

They were followed during childhood for so-called "severe nutritional rickets" and were given 25-OH-D_3. Variable compliance over time of patients F2-I-3 and F2-I-4, confirmed by regularly undetectable levels of 25-OH-D, caused recurrent complaints of bone pain, back pain, paresthesias, and headaches. Patient F2-I-3 underwent epiphysiodesis. Conversely, patient F2-I-5 was given calcium 1500 mg/d and 25-OH-D_3 15 μg/d that corrected the lower limb deformity. He remained asymptomatic and reached a height of 170 cm. At the time of diagnosis, they were 20.9, 31.8, and 32.8 years old, respectively. Serum concentrations of calcium, PTH, and ALP were within the normal range. However, routine biochemical investigations showed an undetectable concentration of 25-OH-D with a normal/high 1,25-$(OH)_2D$.

The molecular analysis of *CYP2R1* identified a variation c.124_138delinsCGG (p.Gly42_Leu46delinsArg) and the mutation c.296T>C (p.Leu99Pro) in a homozygous state in both probands of family 1 and 2, respectively. Heterozygous mutations were present in their normal parents.

The p.Leu99Pro variant was previously described by Cheng and colleagues.[2] The p.Gly42_Leu46delinsArg variant was not reported in a study of 200 control patients. In silico studies showed that the protein lacked one of its fourth intramembranous hydrophobic domains, suggesting a modification of the three-dimensional conformation of the substrate accessing channel. Finally, in vitro studies found no activity over that in mock-transfected cells of both mutants compared with wild type (Fig. 3).

Discussion

This study underlines the misdiagnosis observed in patients with VDDR1B who exhibit normal/high 1,25-$(OH)_2D$ with low/undetectable 25-OH-D levels and the dramatic improvement when patients were given 25-OH-D_3. It provides evidence for the role of this enzyme in vitamin D activation in humans. The

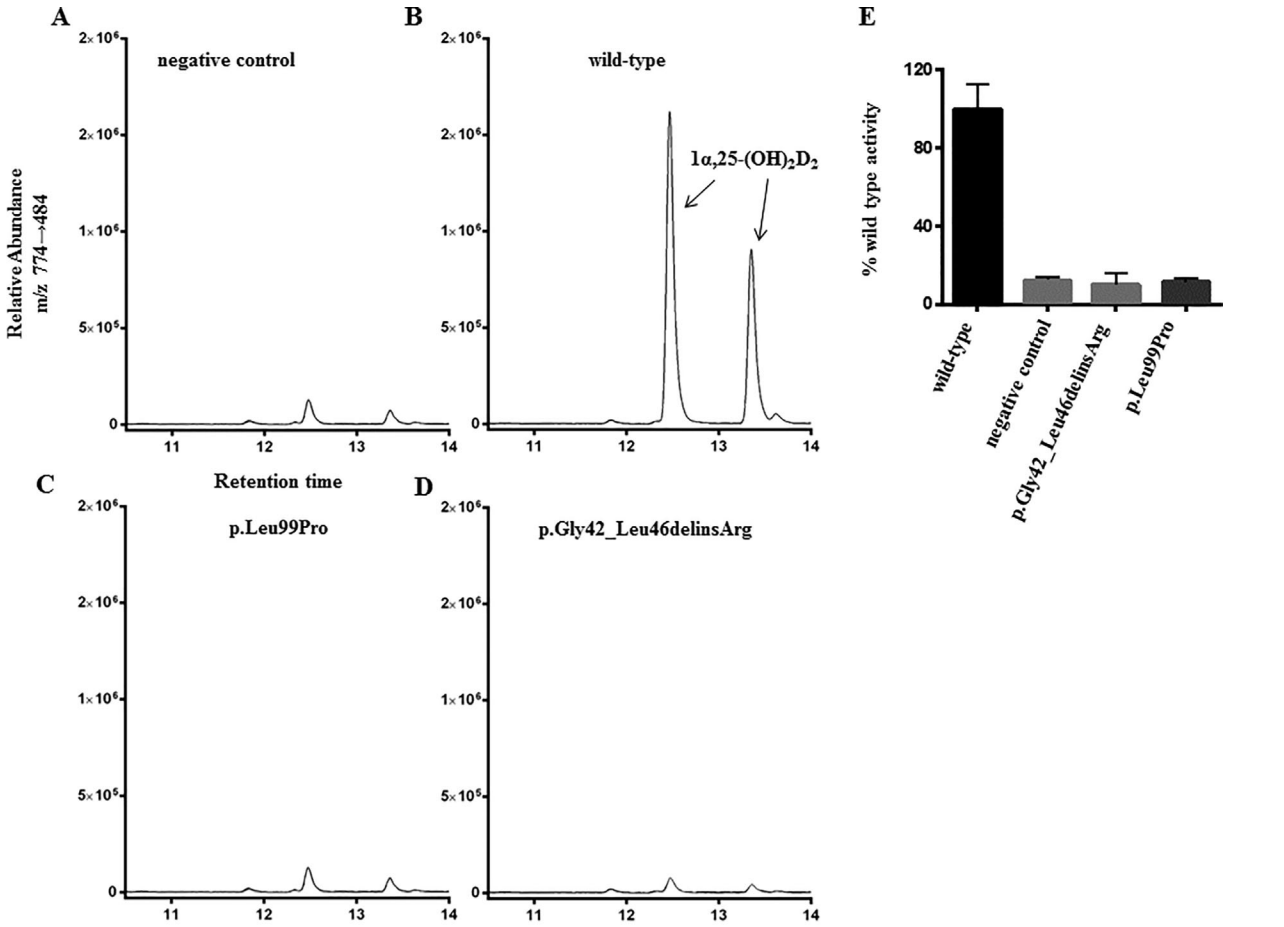

Fig. 3. Functional analysis of *CYP2R1* mutations. (*A–D*) Chromatographs showing deficient vitamin D 25-hydroxylase activity. (*E*) Histogram showing 25-hydroxylase enzymatic activities of the CYP2R1 variant.

scarcity of 25-hydroxylase deficiency cases may reflect a misdiagnosis, as many subjects with hereditary defects in vitamin D_3 metabolism receive dietary supplementation that results in normal levels of 1,25-$(OH)_2$D. Patients with VDDR1B carry mutations in a homozygous state. Relatives of these patients carry mutations in a heterozygous state, and in family 1, presented with normal clinical and laboratory parameters, according to a classic recessive autosomal inheritance.

To date, 4 *CYP2R1* mutations were characterized in vitro as 25-hydroxylase loss-of-function mutations: c.296T>C (p.Leu99Pro),[2] c.726A>C (p.Lys242Asn),[7] c.768dup (p.Leu257Serfs*6), and a splice mutation in the donor sequence of exon 2 c.367+1G>A.[5]

The c.296T>C (p.Leu99Pro) variation (rs61495246) was initially described in two unrelated Nigerian families[1] and was characterized as a loss-of-function mutation that was responsible for vitamin D_3 hydroxylase deficiency.[2] Its allelic frequency is approximately 0.46% in the African American population in EVS and 0.40% in the EXAC African population, suggesting a founder effect. However, linkage analysis that was performed by genotyping siblings of family 2 for single nucleotide polymorphisms ruled out any association with this population (data not shown). The c.124_138delinsCGG (p.Gly42_Leu46delinsArg) variation found in family 1 has never been described and was confirmed in vitro as a loss-of-function mutation.

Patients described herein exhibited a clinical history and classical features suggestive of rickets, and an initial laboratory analysis showed very low 25-OH-D, suggestive of classical vitamin D deficiency. These siblings reminded us of the evolving stages of rickets previously described in nutritional rickets, with considerable overlap[8]; they reflected the initiation of calcium homeostasis, followed by skeletal calcium depletion and increased vitamin D requirements for growth. The first stage, usually undiagnosed, is characterized by the establishment of chronic hypocalcemia, eventually marked by non-specific signs of hypocalcemia but without any bone lesions; the second stage is marked by secondary hyperparathyroidism to correct the serum calcium level, which leads to decreased serum phosphate and an elevation of ALP, osteomalacia, and the progressive apparition of skeletal signs of rickets (patient F2-II-3). The third stage is characterized by marked clinical features of rickets and symptomatic hypocalcemia, as the homeostatic mechanisms are no longer effective.

The finding that serum levels of 25-OH-D remain low despite the administration of supraphysiological doses of vitamin D without evidence of malabsorption suggested the diagnosis of VDDR1B. In this context, the normal concentration of 1,25-$(OH)_2$D, which was also described in other VDDR1B[1,3–5] patients, appeared particularly intriguing. Serum 1,25-$(OH)_2$D concentrations have been reported to be low, normal, or even elevated in patients with vitamin D deficiency.[9–12] However, these levels of 1,25-$(OH)_2$D could be considered inappropriately low for the degree of hyperparathyroidism, incapable of maintaining normal calcium homeostasis, and variable over time, depending on the different stages of the disease.[13,14] Moreover, patient F1-II-3 exhibited a dramatic increase in 1,25-$(OH)_2$D when they received 1α-OH-D_3, suggesting the presence of 25-hydroxylase activity. Similarly, previously described patients responded to treatment with large doses of vitamin D and did have measurable levels of 1,25-$(OH)_2D_3$.[1–3] Because 1α-OH-D_3 is known to be a good substrate for all 25-hydroxylases (eg, CYP2R1 and CYP27A1) and circulating 1,25-$(OH)_2$D is required only in the pmol/L range, the level of 1,25-$(OH)_2$D observed in our patients could be a product of a mutant CYP2R1 with residual activity or a product of alternative 25-hydroxylases as postulated previously. Similarly, 1,25-$(OH)_2$D detected in vitamin D_3–treated patients could be the product of small amounts of 25-OH-D_3 generated by defective CYP2R1 or from alternative 25-hydroxylases. Subsequent 1α-hydroxylation of the small amounts of 25-OH-D_3 that were formed would then be due to the massive stimulation of *CYP27B1* expression by high PTH levels, as observed in patient F1-II-3. Residual 25-hydroxylase activity for vitamin D_2 of p.Leu99Pro could result in 25-OH-D synthesis as previously suggested,[2] but the administration of supra physiological doses of vitamin D had a negligible effect on the serum level of 25-OH-D.[4,5] The presence of alternative vitamin D 25-hydroxylases is, therefore, likely to be the most plausible explanation for the 1,25-$(OH)_2D_3$ that was detected.[15]

Unlike CYP27B1, the unique vitamin D 1α-hydroxylase, several other cytochrome P450 enzymes have been suggested to have vitamin D 25-hydroxylase activity in a variety of biochemical and clinical studies.[16,17] Recent studies involving the murine knockout model Cyp2r1$^{-/-}$ and Cyp2r1$^{-/-}$/Cyp27a1$^{-/-}$ double knockout mice both showed similar circulating levels of 25-OH-D_3, which were 50% lower than in wild-type mice.[18] Similar mechanisms could be involved in humans.[19] CYP27A1, a mitochondrial liver sterol 27-hydroxylase, can contribute to the formation of 25-OH-D from cholecalciferol (vitamin D_3) but not from ergosterol (vitamin D_2). CYP2J2, the arachidonic acid epoxygenase, has preferential 25-hydroxylase activity toward vitamin D_2 over vitamin D_3. CYP3A4, another cytochrome P450 microsomal hepatic enzyme, activates 1α-OH-D_3 to form 1,25-$(OH)_2$D.[16,17,19,20] Although these alternate enzymes may contribute to the detectable levels of serum 25-OH-D_3, they are unable to generate the higher nanomolar serum concentrations that are usually observed in normal patients. On the other hand, these enzymes could be involved and even up-regulated, testifying to an adaptation in CYP2R1-deficient subjects to the need in active vitamin D. Consequently, the amounts of 25-OH-D_3 produced would be insufficient to satisfy the demands for growth and bone formation in order to prevent rickets during childhood. This accessory activity could also explain a later onset of VDDR1B compared with VDDR1A. Indeed, our clinical findings were similar to the Saudi and Nigerians cases, in which clinical symptoms appeared later (usually after 2 years) than in VDDR1A.[15] Accordingly, patient F2-II-3 was still asymptomatic at the age of 4 years.

Although a high-dose cholecalciferol[3,5] or 1α-OH-D_3 administration resulted in a dramatic increase in 1,25-$(OH)_2$D and normal or even high calcium levels and an improvement in deformities, the biochemical pattern was not fully normalized, as PTH and ALP levels remained above the normal values. These observations suggest that 1,25-$(OH)_2D_3$ is not the only biologically active metabolite of vitamin D, as was previously suggested.[21] Treatment of CYP2R1-deficient patients with 1,25-$(OH)_2D_3$ was not considered because of the risks of hypercalciuria and nephrocalcinosis.[22] Consistent with a concept of personalized medicine, we showed that a replacement therapy adjusted to VDDR1B physiopathology must be based on bypassing the enzymatic defect. Calcifediol (25-OH-D_3) takes advantage of the high PTH-induced *CYP27B1* expression and leads to a global improvement in clinical and laboratory data. Patient F2-II-3, who was treated before the clinical symptoms appeared, did not present any bone deformations.

Interestingly, adult subjects in family 2 are able to sustain near-normal serum levels of calcium, PTH, and ALP despite homozygosity for the non-functional p.Leu99Pro allele. This feature was also described in another patient with VDDR1B[4] and confirmed the need for 25-OH-D_3 or an active vitamin D during childhood.

Finally, in our study, we detected normal 24-hydroxylase activity (*CYP24A1*) in patients receiving 25-OH-D_3 with normal values for the 25-OH-D_3: 24,25-$(OH)_2D_3$ ratio (Table 2).[6] This 24-hydroxylase activity is very low when serum 25-OH-D_3 levels are below 25 nmol/L,[6] likely repressed by high PTH levels,[1,23] and very little or no 24,25-$(OH)_2D_3$ is likely to be produced, as observed in patient F2-I-5 in the untreated state.[23] Whether 24-hydroxylated vitamin D metabolites have health consequences, particularly fracture healing in patients with this disease, is still unknown.[24]

With the inclusion of 2 new families, this article extends the number of *CYP2R1* mutations responsible for VDDR1B to 5. We noted the difficulties in establishing the correct diagnosis: First, patients presented with low 25-OH-D levels and were treated as having classical nutritional vitamin D–deficiency rickets; second, the patients had normal/high 1,25-$(OH)_2D$ levels, leading to diagnostic uncertainty. The administration of calcifediol, which bypasses the enzymatic defect, is the adequate treatment to restore optimal vitamin D status.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We thank the patients and their families, who kindly consented to participate, and Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen. Grant support is from Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen APRIM - réf. CHU 16-048. The LC-MS/MS instrument used in these studies was provided by the Waters Corporation (Milford MA) as part of a Queen's University/Waters Corporation Research Agreement.

Authors' roles: AM performed the genetic analyses of *CYP2R1*, generated all figures and tables, and wrote the first draft of the manuscript. AW collected all laboratory data and wrote the first draft of the manuscript together with AM. GA, NC, NR, and HM helped with nucleotide sequence analyses and microsatellite analyses. JCD, AS, PJ, GW, and FF diagnosed and treated patients. BD performed all biochemical assays. LM performed radiological explorations. MK performed LC-MS/MS assays. ND performed functional in vitro analyses. M-LK and FF conceived of the project. FF was the principal investigator. M-LK wrote the final version of the manuscript. GJ reviewed and corrected the final version of the manuscript.

References

1. Casella SJ, Reiner BJ, Chen TC, Holick MF, Harrison HE. A possible genetic defect in 25-hydroxylation as a cause of rickets. J Pediatr. 1994;124:929–32.
2. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci USA. 2004;101:7711–5.
3. Levine MA, Dang A, Ding C, Fischer PR, Singh R, Thacher T. Tropical rickets in Nigeria: mutation of the CYP2R1 gene encoding vitamin D 25-hydroxylase as a cause of vitamin D dependent rickets. Bone. 2007;40:S60–1.
4. Thacher TD, Fischer PR, Singh RJ, Roizen J, Levine MA. CYP2R1 mutations impair generation of 25-hydroxyvitamin D and cause an atypical form of vitamin D deficiency. J Clin Endocrinol Metab. 2015;100:E1005–13.
5. Al Mutair AN, Nasrat GH, Russell DW. Mutation of the CYP2R1 vitamin D 25-hydroxylase in a Saudi Arabian family with severe vitamin D deficiency. J Clin Endocrinol Metab. 2012;97:E2022–5.
6. Kaufmann M, Gallagher JC, Peacock M, et al. Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24, 25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD. J Clin Endocrinol Metab. 2014;99:2567–74.
7. Thacher TD, Fischer PR, Pettifor JM. Vitamin D treatment in calcium-deficiency rickets: a randomised controlled trial. Arch Dis Child. 2014;99:807–11.
8. Fraser D, Kooh SW, Scriver CR. Hyperparathyroidism as the cause of hyperaminoaciduria and phosphaturia in human vitamin D deficiency. Pediatr Res. 1967;1:425–35.
9. Garabedian M, Vainsel M, Mallet E, et al. Circulating vitamin D metabolite concentrations in children with nutritional rickets. J Pediatr. 1983;103:381–6.
10. Markestad T, Halvorsen S, Halvorsen KS, Aksnes L, Aarskog D. Plasma concentrations of vitamin D metabolites before and during treatment of vitamin D deficiency rickets in children. Acta Paediatr Scand. 1984;73:225–31.
11. Eastwood JB, de Wardener HE, Gray RW, Lemann JL. Normal plasma-1,25-(OH)2-vitamin-D concentrations in nutritional osteomalacia. Lancet. 1979;1:1377–8.
12. Chesney RW, Zimmerman J, Hamstra A, DeLuca HF, Mazees RB. Vitamin D metabolite concentrations in vitamin D deficiency. Are calcitriol levels normal. Am J Dis Child. 1981;135:1025–8.
13. Kruse K. Pathophysiology of calcium metabolism in children with vitamin D-deficiency rickets. J Pediatr. 1995;126:736–41.
14. Arnaud C, Rasmussen H, Anast C. Further studies on the interrelationship between parathyroid hormone and vitamin D. J Clin Invest. 1966;45:1955–64.
15. Portale AA, Perwad F, Miller WL. Rickets due to hereditary abnormalities of vitamin D synthesis or action. In: Glorieux F, Pettifor JM, Jüppner H, editors. Pediatric bone. 2nd ed. Oxford: Elsevier; 2012. p. 679–98.
16. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev. 1998;78:1193–231.
17. Jones G, Prosser DE, Kaufmann M. Cytochrome P450-mediated metabolism of vitamin D. J Lipid Res. 2014;55:13–31.
18. Zhu JG, Ochalek JT, Kaufmann M, Jones G, Deluca HF. CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. Proc Natl Acad Sci USA. 2013;110:15650–5.
19. Zhu J, de Luca HF. Vitamin D 25-hydroxylase—four decades of searching, are we there yet? Arch Biochem Biophys. 2012;523:30–6.
20. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. J Biol Chem. 2003;278:38084–93.
21. Bordier P, Rasmussen H, Marie P, Miravet L, Gueris J, Ryckwaert A. Vitamin D metabolites and bone mineralization in man. J Clin Endocrinol Metab. 1978;46:284–94.
22. Tosson H, Rose SR. Absence of mutation in coding regions of CYP2R1 gene in apparent autosomal dominant vitamin D 25-hydroxylase deficiency rickets. J Clin Endocrinol Metab. 2012;97:E796–801.
23. Kaufmann M, Lee SM, Pike JW, Jones G. A high-calcium and phosphate rescue diet and VDR-expressing transgenes normalize serum vitamin D metabolite profiles and renal Cyp27b1 and Cyp24a1 expression in VDR null mice. Endocrinology. 2015;156:4388–97.
24. St-Arnaud R. CYP24A1: structure, function, and physiological role. In: Feldman D, Pike JW, Adams JS, editors. Vitamin D. 3rd ed. London: Academic Press, Elsevier; 2011. p. 43–56.