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Review

CYP2R1 mutations causing vitamin D-deficiency rickets

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ABSTRACT

CYP2R1 is the principal hepatic 25-hydroxylase responsible for the hydroxylation of parent vitamin D to 25-hydroxyvitamin D [25(OH)D]. Serum concentrations of 25(OH)D reflect vitamin D status, because 25(OH)D is the major circulating metabolite of vitamin D. The 1α-hydroxylation of 25(OH)D in the kidney by CYP27B1 generates the fully active vitamin D metabolite, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D). The human *CYP2R1* gene, located at 11p15.2, has five exons, coding for an enzyme with 501 amino acids. In *Cyp2r1*−/− knockout mice, serum 25(OH)D levels were reduced by more than 50% compared wild-type mice. Genetic polymorphisms of *CYP2R1* account for some of the individual variability of circulating 25(OH)D values in the population. We review the evidence that inactivating mutations in *CYP2R1* can lead to a novel form of vitamin D-deficiency rickets resulting from impaired 25-hydroxylation of vitamin D.

We sequenced the promoter, exons and intron-exon flanking regions of the *CYP2R1* gene in members of 12 Nigerian families with rickets in more than one family member. We found missense mutations (L99P and K242N) in affected members of 2 of 12 families. The L99P mutation had previously been reported as a homozygous defect in an unrelated child of Nigerian origin with rickets. *In silico* analyses predicted impaired CYP2R1 folding or reduced interaction with substrate vitamin D by L99P and K242N mutations, respectively. *In vitro* studies of the mutant CYP2R1 proteins in HEK293 cells confirmed normal expression levels but completely absent or markedly reduced 25-hydroxylase activity by the L99P and K242N mutations, respectively. Heterozygous subjects had more moderate biochemical and clinical features of vitamin D deficiency than homozygous subjects. After an oral bolus dose of 50,000 IU of vitamin D_2 or vitamin D_3, heterozygous subjects had lower increases in serum 25(OH)D than control subjects, and homozygous subjects had minimal increases, supporting a semidominant inheritance of these mutations. No *CYP2R1* mutations were found in 27 Nigerian children with sporadic rickets, a cohort of 50 unrelated Nigerian subjects, or in 628 unrelated subjects in the 1000 Genomes Project.

We conclude that mutations in *CYP2R1* are responsible for an atypical form of vitamin D-deficiency rickets, which has been classified as vitamin D dependent rickets type 1B (VDDR1B, MIM 600081).

Contents

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1. CYP2R1 and vitamin D 25-hydroxylation

Vitamin D, produced in the skin after exposure to ultraviolet B irradiation as vitamin D_3 (cholecalciferol) or ingested in the diet as vitamin D_2 (ergocalciferol) or D_3, is hydroxylated in the C-25 position primarily by the hepatic cytochrome P450 enzyme CYP2R1, to generate 25(OH)D. This results in production of 25(OH)D. Serum concentrations of 25(OH)D reflect vitamin D status, because 25(OH)D is the principal circulating form of vitamin D. It is the intermediate metabolite that serves as the reservoir for regulated production of metabolically active 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] by CYP27B1 in the kidney.

Although there are a variety of enzymes that possess 25-hydroxylase activity, current evidence indicates that CYP2R1 is the principal 25-hydroxylase in humans [1,2]. In humans, CYP3A4 and CYP27A1 can also function as vitamin D 25-hydroxylases, but appear to have a lesser role. For example, CYP27A1 lacks specificity for 25-hydroxylation and also hydroxylates the C-26 and C-27 positions. Vitamin D metabolism and 25(OH)D levels are normal in humans and mice that lack functional CYP27A1 [3,4]. On the other hand, *Cyp2r1*$^{-/-}$ knockout mice retain some residual 25-hydroxylase activity and show an approximately 50% reduction in serum 25(OH)D concentrations compared with wild-type mice at various ages [1].

The human *CYP2R1* gene is located on chromosome 11 at 11p15.2 (geneID:120227) and comprises 5 exons coding for an enzyme with 501 amino acids, produced in hepatic microsomes. The CYP2R1 amino acid sequence is highly conserved across species. For example, 89% of the amino acids of the CYP2R1 protein are identical in humans and mice [5]. The *CYP2R1* gene has been characterized by many single nucleotide polymorphisms (SNPs) that are relatively common in the population. Synonymous SNPs do not affect the protein sequence, while nonsynonymous SNPs alter the amino acid sequence of the protein.

In genome-wide association studies, at least seven SNPs of *CYP2R1* explain some of the variation in population 25(OH)D levels [6]. A genome-wide association study of DNA from over 30,000 white individuals of European descent examined the association of various SNPs of genes in the vitamin D pathway with 25(OH)D levels [7]. There was a highly significant association of the rs10741657 SNP of *CYP2R1* with 25(OH)D levels, providing evidence that CYP2R1 has a crucial role in 25(OH)D production. However, the rs10741657 SNP maps to a non-coding region 5'-UTR. Other studies have failed to find an association between *CYP2R1* SNPs and 25(OH)D levels [8]. The effects of *CYP2R1* SNPs on serum 25(OH)D concentrations may vary between populations.

Although the primary site of vitamin D 25-hydroxylation by CYP2R1 is in the liver, human *CYP2R1* expression has been found in human testes, brain, and other tissues [9]. It has been proposed that impaired testicular *CYP2R1* expression could result in significantly reduced circulating 25(OH)D and reduced bone mineral density [10,11].

2. CYP2R1 and rickets

Nutritional rickets is the most common form of bone disease in growing children, and is usually a manifestation of vitamin D deficiency due to inadequate dietary intake of vitamin D and/or insufficient sunlight exposure. Nutritional rickets is highly prevalent in the Middle East, Africa, and Indian subcontinent, and in immigrants to other countries from these regions [12,13]. In nutritional rickets, 25(OH)D levels are low, generally below 12 ng/ml (30 nmol/L). As a result, there is inadequate production of the active hormone $1,25(OH)_2D$, which leads to reduced intestinal absorption of calcium and secondary hyperparathyroidism with resultant hypophosphatemia. Inadequate calcium and phosphate impair bone mineralization, while hypophosphatemia impairs growth plate homeostasis, leading to rickets.

Nutritional rickets can also be caused by inadequate dietary calcium, particularly in tropical regions [14–16]. In Nigeria, the onset of calcium deficiency rickets typically occurs between 1 and 5 years of age, and 15% of cases have multiple affected family members [17]. The risk of nutritional rickets is a function of both calcium intake and vitamin D status. Although rickets can result from vitamin D deficiency or calcium deficiency, more commonly these two conditions interact to increase the risk of developing rickets [12,13,15,18]. Clinical rickets develops when the availability of calcium and/or phosphate is inadequate for mineralization of growing bones.

A possible genetic defect in 25-hydroxylation as a cause of rickets was first reported in 1994 in two brothers of Nigerian descent [19]. Both had rickets associated with low 25(OH)D levels at baseline. Their 25(OH)D concentrations were restored to the normal range with relatively high doses of vitamin D (4000 IU/d). With more modest doses of vitamin D (1000 IU/d), their 25(OH)D concentrations were relatively low. Both had normal absorption of vitamin D. The authors concluded that these children likely had an inherited defect in the conversion of vitamin D to 25(OH)D.

Lymphocytes had been collected and stored for the older sibling in that report. DNA was extracted, and the *CYP2R1* and *CYP27A1* genes were amplified and sequenced. The Nigerian boy was homozygous for a transition mutation of thymine to cytosine (c.296T > C) in exon 2 of the CYP2R1 gene [20]. This mutation substitutes proline for leucine at position 99 of the encoded protein (L99P). No mutations were found in the *CYP27A1* gene.

Genomic DNA from 50 unaffected African-American subjects and 50 Nigerian subjects had no L99P mutation of *CYP2R1* [20], indicating that it is relatively uncommon. Leucine is a conserved amino acid in the CYP2R1 of humans, mouse, rat, and puffer fish. Furthermore, *in silico* analysis with bioinformatics software predicted that replacement of leucine would impair CYP2R1 folding. These findings suggest that the L99P substitution is a mutation rather than polymorphism.

In vitro studies with HEK293 cells transfected with wild type *CYP2R1* DNA and incubated with radiolabelled vitamin D_3 as substrate produced 25(OH)D, but cells transfected with *CYP2R1* having the L99P mutation did not produce detectable 25(OH)D [20]. Further evidence of impaired function of CYP2R1 with the L99P mutation was obtained *in vitro* using reporter systems in which luciferase activity is increased by fully active metabolites of vitamin D [20]. Transfected cells expressing the wild type CYP2R1 enzyme showed increased luciferase activity in these assays, indicating robust 25-hydroxylation of the vitamin D substrate. By contrast, cells transfected with CYP2R1 expressing the L99P mutation showed no luciferase activity, consistent with absence of 25-hydroxylase function. To determine if mutant CYP2R1 was active at higher substrate concentrations, transfected cells were incubated with progressively higher concentrations of substrate vitamin D_3 or vitamin D_2. Cells transfected with wild type CYP2R1 showed progressively greater luciferase transcription with higher vitamin D concentrations, but cells transfected with mutant CYP2R1 showed impaired VDR activation similar to control CMV transfected cells.

3. *CYP2R1* mutations in Nigerian children with rickets

Because the first case of a *CYP2R1* mutation was described in a Nigerian child, and nutritional rickets is highly prevalent in Nigeria, we examined whether the L99P mutation was present in Nigerian children with rickets [21]. We extracted DNA from 39 Nigerian children with rickets: 27 with sporadic rickets and 12 index cases with one or more first-degree relatives having a history