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CYP3A4 mutation causes vitamin D–dependent rickets type 3

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Introduction
Vitamin D deficiency rickets is a childhood disorder associated with impaired growth and skeletal mineralization. In most cases, vitamin D deficiency is due to inadequate exposure to sunlight and/or insufficient dietary vitamin D (1). Vitamin D is biologically inactive and undergoes 2 enzymatic conversions to become fully active 1,25-dihydroxyvitamin D. The existence of rickets in areas with abundant sunlight has led to the identification of genetic forms of vitamin D–dependent rickets (VDDRs). Type 1 VDDRs are caused by defects in vitamin D activation, owing to mutations in the genes encoding either the renal 1-α hydroxylase (CYP27B1: VDDR-1A) (2) or the hepatic 25-hydroxylase (CYP2R1: VDDR-1B) (3, 4). Type 2 VDDRs are caused by defects in vitamin D receptor signaling due to mutations in the gene encoding the vitamin D receptor (VDR: VDDR-2A); or heterogeneous nuclear ribonucleoprotein C (HNRNPC: VDDR-2B) (5, 6), a VDR corepressor. Vitamin D homeostasis is also regulated by renal 24-hydroxylase (CYP24A1), an enzyme responsible for 24-oxidation of 1,25-dihydroxyvitamin D3 to inactive calcitriol (7) and degradation of 25-hydroxyvitamin D3 to inactive 24,25-dihydroxyvitamin D3 (8). Here we describe a third kind of VDDR (VDDR-3), in which a gain-of-function mutation in CYP3A4, which encodes a P450 enzyme that metabolizes many xenobiotics and drugs, leads to vitamin D deficiency through accelerated vitamin D metabolite inactivation.

Results and Discussion
Clinical and biochemical studies. We studied 2 unrelated females from non-consanguineous families. Both subjects were born full-term with normal birth weight and length. Proband 1.1 presented to medical attention prior to age 2 years with a history of bowed legs and unsteady gait noted at 20 months. Her pediatrician diagnosed rickets on the basis of bilateral genu varum, poor growth, and reduced serum calcium and phosphorus with elevated serum alkaline phosphatase and parathyroid hormone (Table 1). She was of European ancestry and lived in a sunny climate in Australia. At age 2.5 years she was referred for endocrine evaluation, at which time her height was 81.3 cm (6th percentile) and weight was 11.5 kg (35th percentile). Proband 2.1 was of Middle Eastern descent and did not walk until 4.5 years of age. She was treated for rickets in Jordan, Spain, and the Netherlands prior to evaluation in the United Kingdom at age 16 years, when she had a height of 154.3 cm (9th percentile). Wrist radiographs in both subjects showed features consistent with active rickets (Figure 1A). Both subjects had detectable serum vitamin D3, but low serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (Table 1), which increased only after administration of very large doses of vitamin D or calcitriol and declined rapidly thereafter. After oral administration of 50,000 IU of vitamin D3, proband 1.1 showed a normal increase in serum cholecalciferol, indicating normal absorption of vita-
Table 1. Biochemical analyses

<table>
<thead>
<tr>
<th>Normal range</th>
<th>Age</th>
<th>Basal calcium (mmol/l)</th>
<th>Phosphorus (mmol/l)</th>
<th>PTH (IU/l)</th>
<th>Total calcium (mmol/l)</th>
<th>Phosphorus (mmol/l)</th>
<th>25-(OH)D (nmol/l)</th>
<th>1,25-(OH)2D (pmol/l)</th>
<th>24,25-(OH)2D (nmol/l)</th>
<th>25-hydroxycholesterol in nmol/l (IU cholecalciferol daily)</th>
<th>Treatment to control rickets (IU cholecalciferol daily)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.2–2.8</td>
<td>12.1–18</td>
<td>1.6–6.9</td>
<td>130–450</td>
<td>75–160</td>
<td>151–730</td>
<td>208–682</td>
<td>22–60</td>
<td>0.74–3.59</td>
<td>25–125</td>
</tr>
<tr>
<td>Proband 1.1</td>
<td>27 mo</td>
<td>1.67</td>
<td>1.14</td>
<td>58.3</td>
<td>2,826</td>
<td>&lt;16</td>
<td>47</td>
<td>Proband 1.1 vitamin D response test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proband 2.1</td>
<td>16 yr</td>
<td>1.87</td>
<td>0.97</td>
<td>70.8</td>
<td>1,269</td>
<td>&lt;20</td>
<td>Proband 2.1</td>
<td>20 yr</td>
<td>6.0</td>
<td>18.8</td>
<td>679.2</td>
</tr>
</tbody>
</table>

Targeted sequencing identified no mutations in known VDDR1 genes; however, whole exome sequencing revealed a recurrent mutation in CYP3A4. Nucleotide sequences including 5 kb upstream of the transcription start site, as well as exons and exon-intron boundaries for the CYP2R1, CYP27B1, VDR (proband 1.1), and CYP24A1 genes were normal. Whole exome sequencing was performed in probands and parents, and the data were analyzed using models for X-linked, autosomal recessive, and dominant modes of inheritance. No potential candidate gene with 2 mutations was identified; however, each subject had several heterozygous variants potentially causing protein-function alterations. To identify de novo dominant variants, we assumed that disease-causing variants would be rare. Supplemental Table 1 (supplemental material available online with this article; https://doi.org/10.1172/JCI98680DS1) shows surviving candidate variants in the 2 subjects that were absent from their parents. Of these candidates, only CYP3A4 (GenBank NM_017460.5) showed a variant in both subjects, and remarkably, the subjects carried an identical heterozygous single nucleotide change (c.902T>C) that results in replacement of isoleucine by threonine at codon 301 (p.I301T). Sanger sequencing confirmed that both subjects but no available relatives, who were unaffected, carried the missense mutation, indicating the mutation was both recurrent and de novo (Figure 1B). This mutation was not present in public databases or in data from more than 3,000 exomes analyzed at CHOP. Isoleucine 301 is highly conserved (Figure 1C) and forms a critical portion of substrate recognition site 4 (SRS-4), one of 6 SRSs determining CYP3A4 substrate selectivity and product profile (10). SIFT (http://sift.jcvi.org/) and MutPred (http://mutpred.mutdb.org/) predict this change would damage function, but MutPred additionally predicts a possible novel catalytic function. To examine the possibility of a common genetic origin of the mutation, we determined CYP3A4 gene haplotypes in both families. No common haplotype was shared by the 2 mutation carriers, excluding the possibility of a common founder.

Functional assessment of the CYP3A4 p.I301T mutant. Because previous work had shown that p.I301 is a determinant of activity for CYP3A4 SRS-4 substrates (11), we hypothesized that threonine replacement of isoleucine 301 might increase oxidation of the vitamin D metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (12, 13). We tested this hypothesis by analyzing serum 4,25-dihydroxyvitamin D, the principal product of CYP3A4 metabolism of 25-hydroxyvitamin D (14, 15). In both subjects, the ratio of 4β,25-dihydroxyvitamin D to 25-dihydroxyvitamin D was markedly elevated to the range of values exhibited by patients taking rifampin (16), an inducer of CYP3A4 (Table 1). We next used a mammalian cell 2-hybrid expression system in which intracellular 1,25-dihydroxyvitamin D stimulates transcription of a luciferase reporter gene (4) to assess the ability of the mutant recombinant CYP3A4 protein to inactivate 1,25-dihydroxyvitamin D. Cells expressing mutant compared with WT recombinant CYP3A4 protein had decreased luciferase activity, indicating that p.I301T possessed greater ability to inactivate 1,25-dihydroxyvitamin D3 (Figure 2A) than WT (P < 0.01 for overall curves; post hoc multiple comparison–adjusted analyses confirmed significance at 0.03 ng/ml [mean difference: 0.13; 95% CI, 0.08–0.22], 0.1 ng/ml [mean difference: 0.72; 95% CI, 0.25–1.20], 0.3 ng/ml [mean difference: 2.03; 95% CI, 1.55–2.51], and 1 ng/ml [mean difference: 3.6; 95% CI, 3.1–4.1]; P < 0.01 for each). Moreover, p.I301T CYP3A4 was more active than CYP24A1, the principal inactivator of 1,25-dihydroxyvitamin D3 at 0.3 ng/ml (mean difference: 0.42; 95% CI, 0.06–0.77; P < 0.05). We also compared apparent kinetics data between enzymes, as measurements were performed using reporter activity in whole cells. The apparent catalytic efficiency
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terol, as this endogenous oxysterol is formed from cholesterol by CYP3A4 and is commonly used as a marker for CYP3A4 activity (17). Both subjects had normal circulating 4β-hydroxycholesterol (Table 1 and ref. 18). We next used a cell-based assay to assess p.I301T CYP3A4 activity for the alternative substrate luciferin IPA. Because the kidney cell line (HEK293T) does not express CYP3A4, we used it to create a cell-based assay. This assay directly measures the conversion of luciferin IPA to luciferin by CYP3A4 and is used to examine CYP3A4 induction (19). Enzymatic activity of both the WT and p.I301T mutant were inhibited by ketoconazole to levels indistinguishable from empty vector (Figure 2D: 1.1 ± 0.1 vs. 1.0 ± 0.1, n = 4 for each, P = 0.39). In contrast to the results for 1,25-dihydroxyvitamin D3 as a substrate, the p.I301T mutant had significantly decreased activity relative to the WT (Figure 2D: 30.0 ± 1.6 vs. 50.1 ± 1.6, n = 4 for each, P < 0.001) for luciferin IPA (19).

We identified a gain-of-function mutation in CYP3A4 in 2 unrelated children with severe rickets due to accelerated vitamin D metabolite inactivation. The observation of identical mutations in the patients, who do not share CYP3A4 haplotypes, makes a founder

Figure 1. CYP3A4 mutation causes VDDR. (A) Wrist radiographs of the 2 probands are consistent with those of untreated rickets despite high-dose supplementation. Wrist radiographs from these patients show the typical features of rickets: younger growing patients have bowing (green triangle), metaphyseal splaying (yellow triangle), and cupping (red triangle), as in proband 1.1 (P 1.1); older patients have metaphyseal lucency (blue triangle) and osteopenia (purple triangle), as in proband 2.1; features change as the role of calcification changes with age (supporting growth vs. maintaining bone integrity). (B) Family pedigrees are consistent with p.I301T mutations arising de novo. Family pedigrees of the 2 probands with Sanger sequencing results of the mutation region in CYP3A4 from all available family members. (C) Alignment of CYP3A4 protein sequence surrounding p.I301T mutation reveals high conservation of this residue across species. Isoleucine 301 is highly conserved in CYP3A4 across evolution and within the human 3A protein family.

\[ \frac{V_{\text{max(app)}}}{K_{\text{m(app)}}} \] of the WT CYP3A4 was 0.014, while that of the p.I301T CYP3A4 mutant was 0.14 and that of CYP24A1 was 0.08. Thus, p.I301T was nearly 10-fold more active than the WT and nearly twice as active as CYP24A1. Because both the WT and mutant CYP3A4 showed increased activity when cotransfected with a cDNA encoding P450 oxoreductase, which localizes to the ER, we believe that the c.I301T substitution does not prevent proper ER localization of CYP3A4.

Increased activity of CYP3A4 p.I301T in transfected cells is not due to increased recombinant protein abundance. To determine whether the increased in vitro activity of p.I301T mutant CYP3A4 might be due to increased protein abundance, we measured recombinant CYP3A4 proteins levels by semiquantitative immunoblotting. The size and abundance of the p.I301T mutant protein were similar to those of the WT (Figure 2, B and C).

CYP3A4 p.I301T does not have increased activity for non–vitamin D substrates. CYP3A4 has 6 substrate domains (10), and the replacement of isoleucine 301 by threonine is predicted to affect only SRS4 substrates. To assay the effect of p.I301T on non–vitamin D substrates, we first examined circulating 4β-hydroxycholesterol, as this endogenous oxysterol is formed from cholesterol by CYP3A4 and is commonly used as a marker for CYP3A4 activity (17). Both subjects had normal circulating 4β-hydroxycholesterol (Table 1 and ref. 18). We next used a cell-based assay to assess p.I301T CYP3A4 activity for the alternative substrate luciferin IPA. Because the kidney cell line (HEK293T) does not express CYP3A4, we used it to create a cell-based assay. This assay directly measures the conversion of luciferin IPA to luciferin by CYP3A4 and is used to examine CYP3A4 induction (19). Enzymatic activity of both the WT and p.I301T mutant were inhibited by ketoconazole to levels indistinguishable from empty vector (Figure 2D: 1.1 ± 0.1 vs. 1.0 ± 0.1, n = 4 for each, P = 0.39). In contrast to the results for 1,25-dihydroxyvitamin D3 as a substrate, the p.I301T mutant had significantly decreased activity relative to the WT (Figure 2D: 30.0 ± 1.6 vs. 50.1 ± 1.6, n = 4 for each, P < 0.001) for luciferin IPA (19).

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Although the impact of CYP3A4 on vitamin D homeostasis is limited under physiological conditions, under special circumstances CYP3A4 can have significant effects on vitamin D metabolism. For example, induction of CYP3A4 by anticonvulsants is associated with vitamin D deficiency (15). In addition, targeted induction of CYP3A4 by rifampin provides an alternative clearance mechanism for vitamin D metabolism unlikely and is most consistent with recurrent independent mutation events. CYP3A4 (Enzyme Commission [EC] 1.14.13.97) is the most abundant P450 in the liver and is also highly expressed in the intestine. CYP3A4 serves an important metabolic role in biotransforming a wide variety of compounds, including many drugs, steroids, xenobiotics, and carcinogens. Most relevant to this work, CYP3A4 can oxidize and inactivate the vitamin D metabolites 25-hydroxvitamin D and 1,25-dihydroxyvitamin D (15).

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that can normalize elevated vitamin D metabolites in patients who lack the native pathway for vitamin D oxidation due to loss-of-function mutations in CYP24A1 (16). Finally, common polymorphisms in CYP3A4 are associated with decreased bone density (20).

Several lines of evidence support our hypothesis that the p.I301T CYP3A4 mutation is the basis for a selective defect in vitamin D metabolism. First, the 2 affected subjects had elevated 4β,25-dihydroxyvitamin D/25-hydroxyvitamin D ratios but normal 4β-hydroxycholesterol. Second, molecular modeling and in vitro studies previously identified isoleucine 301 as an important determinant of substrate binding; and that replacing isoleucine 301 with another nonconservative residue, phenylalanine, results in a functional CYP3A4 enzyme (21). Third, our in vitro studies showed that p.I301T CYP3A4 significantly increased inactivation of 1,25-dihydroxyvitamin D3 but had no effect on clearance of substrate luciferin IPA. Although we did not measure other steroid hormones in the 2 patients, there was no clinical evidence of additional endocrine defects. Therefore, the genetic gain of function in CYP3A4 recapitulated the selective inactivation of vitamin D metabolites well described in patients taking drugs that induce CYP3A4 (21).

These observations lead us to propose that a gain-of-function mutation of CYP3A4 causes a distinct form of VDDR. In contrast to the previously described autosomal recessive forms of VDDR that result from defects in either synthesis of vitamin D metabolites or responsiveness to 1,25-dihydroxyvitamin D, this dominant form of VDDR is due to accelerated inactivation of vitamin D metabolites (schematic of VDDRs in Figure 3). Although nutritional vitamin D deficiency is the most common cause of childhood rickets, these 2 cases, representing the identification of a previously undescribed VDDR, emphasize the importance of considering accelerated vitamin D inactivation as a risk factor for vitamin D deficiency. There is renewed awareness of the importance of daily vitamin D prophylaxis for the prevention of vitamin D deficiency and rickets in infants and children, and our findings highlight the importance of genetic and induced variation in CYP3A4 activity as a modifier of the amount of vitamin D necessary to maintain vitamin D homeostasis.

### Methods

For further information, see Supplemental Methods.

**Biochemical and molecular analyses.** We measured serum and urine electrolytes, serum creatinine, and parathyroid hormone using routine methods. Measurements of vitamin D3, vitamin D2, 25-hydroxyvitamin D3, 25-hydroxyvitamin D2, 24,25-dihydroxyvitamin D, and 4β-hydroxycholesterol were performed by isotope dilution liquid chromatography–tandem mass spectrometry. We measured serum 1,25-dihydroxvitamin D3 and 4β,25-dihydroxyvitamin D using an ultrahigh resolution chromatographic separation procedure (15) that enables their complete separation.

We performed whole-exome sequencing of DNA from proband 1.1, proband 2.1, and their available relatives (Figure 1), as described previously (22). Analysis of candidate genes and CYP3A4 confirmation genotyping were performed by Sanger sequencing. Analysis of 1,25-dihydroxyvitamin D degradative activity of WT and mutant CYP3A4 recombinant proteins was performed using a mammalian 2-hybrid expression system in which activity of firefly luciferase is proportional to the concentration of intracellular 1,25-dihydroxyvitamin D (see Supplemental Methods and ref. 4). Activity against other substrates was assayed by measuring CYP3A4 conversion of luciferin IPA to the luminescent luciferin.

*Cell lines.* HEK293T cells were obtained from ATCC (catalog CRL-3216).

*In vitro assessment of expression and activity of CYP3A4.* Mutations were introduced by PCR site-directed mutagenesis into a full-length human CYP3A4 cDNA (provided by P.F. Hollenberg, University of Michigan; ref. 23).

*Statistics.* All assays were performed in biological quadruplicate. All statistical analyses were performed using GraphPad Prism 7. Sample means were compared using a t test or ANOVA with Tukey’s 2-tailed post hoc test as appropriate, with a P value of 0.05 defined as statistically significant. All data are presented as mean ± SEM.

*Study approval.* All studies were approved by the institutional review board of the Children’s Hospital of Philadelphia. Patients or parents provided written informed consent/assent prior to inclusion in the study.

### Author contributions

JDR prepared the manuscript and contributed to the study design and data analyses. DL and HH performed and interpreted the whole exome sequence analyses. LO performed assays and collected data. MKJ, NJS, PRE, HHH, CPR, KET, and TDT contributed to study design, collection of clinical and biochemical data, and characterization of the subjects’ phenotypes. MAL contributed to overall study design, data analysis, interpretation, and preparation of the manuscript. All authors reviewed and approved the final manuscript.

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4. Thacher TD, Fischer PR, Singh RJ, Roizen J, Levine MA. CYP2R1 mutations impair generation of 25-hydroxyvitamin D and cause an atyp-