Changes in bone metabolic parameters following oral calcium supplementation in an adult patient with vitamin D-dependent rickets type 2A

Yuka Kinoshita1), Nobuaki Ito1), Noriko Makita1), Masaomi Nangaku1) and Seiji Fukumoto2)

1) Division of Nephrology & Endocrinology, Department of Medicine, the University of Tokyo Hospital, Tokyo, Japan
2) Fujii Memorial Institute of Medical Sciences, Tokushima University, Tokushima, Japan

Abstract. Vitamin D-dependent rickets type 2A (VDDR2A) is a rare inherited disorder with decreased tissue responsiveness to 1,25-dihydroxyvitamin D \( [1,25(\text{OH})_2\text{D}] \), caused by loss-of-function mutations in the vitamin D receptor (VDR) gene. Approximately 50 types of mutations have been identified so far that change amino acids in either the N-terminal DNA binding domain (DBD) or the C-terminal ligand binding domain (LBD) of the VDR protein. The degree of responsiveness to 1,25(\text{OH})_2\text{D} varies between patients with VDDR2A, which may depend on their residual VDR function. In this report, we describe a female patient with VDDR2A caused by an early stop codon (R30X) in the VDR gene that resulted in a severely truncated VDR protein. She developed alopecia and bowed legs within a year after birth and was diagnosed with rickets at the age of 2. She had been treated with active vitamin D and oral calcium supplementation until 22 years of age, when she developed secondary hyperparathyroidism and high bone turnover. The genetic diagnosis of VDDR2A promoted the discontinuation of active vitamin D treatment in favor of monotherapy with oral calcium supplementation. We observed amelioration of the secondary hyperparathyroidism and normalization of bone metabolic parameters within 6 years.

Key words: Rickets, Vitamin D receptor, Calcium, Hyperparathyroidism

RICKETS is a childhood disease characterized by skeletal deformities, such as bowed legs and short stature, caused by impaired mineralization of bone matrix and disorganized growth plates. The equivalent disease in adults is osteomalacia, which commonly causes back and hip pain, muscle weakness, and bone fragility [1]. Rickets and osteomalacia are typically caused by a lack of sufficient vitamin D, calcium, or phosphate [1, 2]. Nutritional deficiency is the most common cause of rickets worldwide [3]; however, there are also genetic etiologies that can result in abnormal calcium and phosphate metabolism [4, 5]. Vitamin D-dependent rickets type 2A (VDDR2A) (OMIM#277440), also known as hereditary vitamin D-resistant rickets (HVDRR), is a rare inherited disorder with decreased tissue responsiveness to 1,25-dihydroxyvitamin D \( [1,25(\text{OH})_2\text{D}] \) caused by loss-of-function mutations in the vitamin D receptor (VDR) gene [6, 7].

VDR is one of several nuclear transcription factors composed of an N-terminal DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) [8]. VDR is nearly ubiquitously expressed in cells and regulates as much as 3% of all human genes [9]. VDR forms a heterodimer with the retinoid X receptor (RXR) in the nucleus and regulates transcription of target genes by binding to their promoter regions [10-12]. Although vitamin D is essential for calcium and bone homeostasis, \textit{in vivo} studies using total or tissue-specific VDR knockout or VDR transgenic animals have suggested more diverse functions of the VDR-vitamin D system in the body [9].

In patients with VDDR2A, approximately 50 kinds of mutations have been identified that change amino acids in both the DBD and the LBD of the VDR protein [7]. The degree of responsiveness to 1,25(\text{OH})_2\text{D} varies between patients, and some may respond to high doses of active vitamin D. Additionally, some patients develop total or partial alopecia in childhood.
An association between disease severity and alopecia has been described, and patients with mutations in the N-terminal DBD are more likely to develop total alopecia [13, 14]. Although a high-calcium rescue diet has been shown to normalize serum calcium levels and prevent the development of secondary hyperparathyroidism and skeletal abnormalities in VDR-null mice, it was not able to reverse alopecia [15].

In this report, we describe an adult patient with VDDR2A caused by an early stop codon in the VDR gene. When genetic diagnosis clarified the etiology of her rickets at the age of 22, we speculated that active vitamin D treatment was ineffective in correcting her hypocalcemia because VDR function may have been completely lost. Therefore, we discontinued active vitamin D treatment and started to treat her with oral calcium supplementation alone. Significant changes in her bone metabolic parameters were observed over 6 years.

**Subjects and Methods**

**Subjects**

A 22-year-old female patient was introduced to our hospital in order to clarify the etiology of her hypocalcemia and childhood rickets. She was born by cesarean section at 38 weeks. She had no family history of rickets, but her parents were second cousins. Her birth weight was 3,000 g, and her birth length was 48 cm. She developed alopecia at the age of 10 months and bowed legs at the age of 14 months. She was diagnosed with rickets at the age of 2 when her body height was 74.2 cm (-3.3 SD) and her body weight was 8.2 kg (-2.6 SD). A bone radiograph demonstrated bowed legs, widened growth plates, and cupping and fraying of metaphyses, which are compatible with a diagnosis of rickets. Laboratory data showed hypocalcemia, hypophosphatemia, and elevated serum 1,25(OH)_{2}D.

**Analysis of the VDR gene**

This study was approved by the institutional review board of the University of Tokyo and written informed consent was obtained from the patient.

Genomic DNA was extracted from the peripheral blood of the patient using the QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). Coding exons and

| Table 1 | Laboratory data and bone mineral density of our patient |
|---|---|---|---|---|---|---|
| Age (years) | 22 | 23 | 24 | 25 | 26 | 28 |
| BAP (µg/L, 2.9-14.5) | 97.5 | 41.4 | 37.3 | 36.1 | 25.6 | 13.0 |
| TRACP-5b (mU/dL, 120-420) | 1,490 | 870 | NR | 662 | 666 | 423 |
| 1,25(OH)_{2}D (pg/mL, 20-60) | 1,290 | 128 | 88.9 | NR | NR | 91.8 |
| Aldosterone (pg/mL, 35.7-240) | 160 | NR | 356 | 346 | NR | 323 |
| Plasma renin activity (ng/mL/hr, 0.3-2.9) | 5.2 | NR | 5.8 | 6.5 | NR | 3.3 |
| K (mmol/L, 3.6-4.8) | 3.6 | 3.8 | 3.4 | 3.2 | 3.8 | 3.8 |
| eGFR (mL/min/1.73m²) | 165.4 | 150.5 | 156.8 | 136.2 | 120.0 | 105.9 |
| BMD L2-L4 (g/cm³) | 0.918 | NR | NR | 1.135 | NR | 1.289 |

Units and reference ranges are shown in parentheses after items; NR, not recorded; * The unit and the reference range of BAP at this point is U/L and 9.6-35.4.
exon-intron junctions of the \textit{VDR} gene were amplified by PCR using the SapphireAmp® Fast PCR Master Mix (TAKARA BIO, Shiga, Japan). PCR conditions were as follows: 1 minute at 94 °C, followed by 35 cycles of 5 seconds at 98 °C, 5 seconds at 58 °C, and 5 seconds at 72 °C, with a final extension for 3 minutes at 72 °C. Primer sequences can be obtained upon request. PCR products were purified using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Duren, Germany) and were directly sequenced with dye-deoxy termination cycle sequencing using the same primers as those used for the PCR reaction.

\textbf{Analysis of the VDR mRNA}

RNA was extracted from the peripheral blood of the patient, along with the normal controls, using the NucleoSpin® RNA Blood (Macherey-Nagel, Duren, Germany), and it was transcribed to cDNA using the PrimeScript® RT Master Mix (TAKARA BIO, Shiga, Japan). A 150 base-pair segment of \textit{VDR} cDNA and a 100 base-pair segment of \textit{GAPDH} cDNA were amplified using the SapphireAmp® Fast PCR Master Mix (TAKARA BIO, Shiga, Japan). The primer sets were 5′-tcctcttgggaagcctttgg-3′ and 5′-gttccggtcaagtctcag-3′ for \textit{VDR} and 5′-tggcaccgtcaaggctgaga-3′ and 5′-ccagcategeccacttgat-3′ for \textit{GAPDH}. Conventional PCR conditions were as follows: 1 minute at 94 °C, followed by 35 cycles of 5 seconds at 98 °C, 5 seconds at 58 °C, and 5 seconds at 72 °C, with a final extension for 3 minutes at 72 °C. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide, and then visualized with UV light. For quantitative analysis of \textit{VDR} mRNA, real-time PCR was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific K.K., Kanagawa, Japan) using SYBR Premix Ex Taq II (TAKARA BIO, Shiga, Japan) and the same primers as those used for the conventional RT-PCR. The expression levels of \textit{VDR} and \textit{GAPDH} mRNA were interpolated from the standard curves.

\textbf{Results}

\textbf{Analysis of the VDR gene and VDR mRNA}

We identified a homozygous nonsense mutation in the \textit{VDR} gene of the patient (c.88C>T, p.R30X) (Fig. 1A). This mutation has previously been reported in patients with VDDR2A [17-19]. RT-PCR detected \textit{VDR} mRNA in peripheral blood of the patient and controls (Fig. 1B). The expression level of \textit{VDR} mRNA in peripheral blood of the patient was higher than that in controls (Table 2).

![Fig. 1](image)

**Fig. 1** A) The patient encoded a homozygous nonsense mutation in the \textit{VDR} gene (c.88C>T, p.R30X). B) RT-PCR showed \textit{VDR} mRNA in peripheral blood of the patient and controls. MM, molecular marker; G, GAPDH; V, VDR.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Real-time PCR analysis of \textit{VDR} mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 1</td>
</tr>
<tr>
<td>Ct value of \textit{GAPDH} $^#$</td>
<td>20.6 ± 0.0</td>
</tr>
<tr>
<td>Ct value of \textit{VDR} $^#$</td>
<td>29.5 ± 0.2</td>
</tr>
<tr>
<td>\textit{VDR}/\textit{GAPDH} mRNA $^*$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^\#$ The Ct values of \textit{GAPDH} and \textit{VDR} are shown as means±SD (N=2). $^*$ The \textit{VDR}/\textit{GAPDH} mRNA is shown as the ratio of each value to that of Control 1.
Clinical course

Due to an early premature stop codon in the VDR gene of the patient, we speculated that her VDR function was completely lost and that active vitamin D would be ineffective in correcting her hypocalcemia. Therefore, we decided to treat her with oral calcium alone. As she had initially been treated with calcium lactate (3.5 g/day), alfacalcidol (54 µg/day), and calcitriol (18 µg/day), we stopped both the alfacalcidol and calcitriol and increased the dose of calcium lactate to 5 g/day. Since then, she has been treated with 5-6 g/day calcium lactate. Fig. 2 and Table 1 show changes in laboratory data and BMD over 6 years. Her serum calcium and phosphate levels fluctuated around the lower limit of the reference range, with an overall upward trend. Her serum ALP and intact PTH levels were significantly reduced over 6 years and nearly normalized at the age of 28 (Fig. 2). Coincidental with the improvement in secondary hyperparathyroidism, an ultrasound revealed a reduction in size of her parathyroid glands. Urine analyses with random urine samples showed that the urine calcium/creatinine ratio (uCa/Cr) and the ratio of tubular maximum reabsorption of phosphate to GFR (TmP/GFR) moved in parallel with serum calcium and serum phosphate, respectively (Fig. 3).

Fig. 2  The clinical course of our patient
The dotted area represents the reference range of serum corrected calcium (cCa) and phosphate (P). Serum cCa and P levels fluctuated around the lower limit of the reference range with an overall upward trend. Concurrently, serum ALP and intact PTH levels were significantly reduced over 6 years.

Fig. 3  Serum and urine parameters of calcium (Ca) and phosphate (P) metabolism in our patient
The serum corrected Ca (cCa) and urine Ca/creatinine ratio (uCa/Cr) moved in parallel with an upward trend (upper panel). As intact PTH decreased, serum P and the ratio of tubular maximum reabsorption of phosphate to GFR (TmP/GFR) moved in parallel, with an upward trend (lower panel).
Both the bone formation marker, bone alkaline phosphatase (BAP), and the bone resorption marker, tartrate-resistant acid phosphatase 5b (TRACP-5b), were extremely elevated at the age of 22. These gradually decreased nearly to the reference range, with a concurrent increase in BMD (Table 1). Serum 1,25(OH)\textsubscript{2}D declined after the cessation of active vitamin D treatment but remained higher than normal, and the estimated glomerular filtration rate (eGFR) gradually decreased over 6 years.

The patient displayed elevated plasma renin activity and serum aldosterone, along with occasional mild hypokalemia, which suggested upregulation of the renin-angiotensin-aldosterone system (Table 1). However, an ambulatory blood pressure monitoring (ABPM) showed no signs of systemic hypertension and an echocardiogram did not detect left ventricular hypertrophy.

**Discussion**

We present here an adult patient with VDDR2A whose bone metabolic parameters had been observed over 6 years. *In vivo* analyses have shown that a high calcium diet can improve calcium homeostasis and skeletal abnormalities in *VDR*-null mice [15, 20]; however, little is known about its effects on bone metabolism in adult patients with VDDR2A. The *VDR*-null mice that were placed on the high calcium, high phosphate, and high lactose rescue diet maintained normal calcium and phosphate levels and did not develop rickets [15]. Although a rescue diet could not prevent alopecia in *VDR*-null mice, PTH levels and parathyroid glandular size of *VDR*-null mice on the rescue diet were not different from those of wild-type littermates [15]. Oral calcium supplementation ameliorated secondary hyperparathyroidism in our patient and nearly normalized her bone metabolism over 6 years. The clinical course of our patient and a 3-year-old patient with a homozygous R73X mutation [22] suggest that monotherapy with oral calcium supplementation should be considered in patients with deleterious *VDR* mutations. Urine analyses with random urine samples from our patient showed high urine calcium excretion relative to serum calcium. This relative hypercalciuria, which has also been shown in *VDR*-null mice on a rescue diet [23], is probably due to disturbed calcium reabsorption in the distal tubules, which are mainly controlled by PTH and 1,25(OH)\textsubscript{2}D [24].

In addition to our case, three different families with R30X mutations in the *VDR* gene have been reported [17-19]. The first case is a 12-year-old boy in Brazil whose asymptomatic parents were first cousins [17]. The second case is a 2-year-old boy from nonconsanguineous French-Canadian parents [18]. The third case is a 2-year-old girl with a mother from Indonesia and a father from the Czech Republic [19]. The first two cases are homozygous for the R30X mutation, and the last is heterozygous for the R30X mutation and a 3-bp in-frame deletion that results in the deletion of lysine 246. All three patients developed total alopecia within a few months after birth, and showed severe early-onset rickets. They also presented with secondary hyperparathyroidism at referral. They were treated with 3 g of elemental calcium and 3-30 µg calcitriol, which partially improved their laboratory data and bone deformities. However, their alopecia could not be reversed.

The loss of function of VDR in our patient seems to be the direct result of a truncated VDR protein and not secondary to nonsense-mediated mRNA decay. It has been reported that some nonsense mutations result in the total absence or greatly reduced amounts of *VDR* mRNA [25], but northern blot analysis has shown a normal amount of *VDR* mRNA in patients with R30X mutations [18]. We confirmed the presence of *VDR* mRNA in our patient by RT-PCR and real-time PCR using total RNA extracted from peripheral blood. The stability of *VDR* mRNA with a nonsense mutation seems to depend on its location, and R30X in the *VDR* gene may escape mRNA decay.

The VDR-vitamin D system plays an important role in osteoclastogenesis. *In vitro* analyses have shown that 1,25(OH)\textsubscript{2}D stimulates RANKL expression through VDR in osteoblastic cells, which in turn increases osteoclastogenesis [26]. However, co-cultures of osteoblasts and osteoclast progenitors from *VDR*-null mice demonstrated that other factors, such
as PTH and IL-1 alospha, could stimulate RANKL expression in the absence of a functional VDR-vitamin D system [27]. Therefore, we hypothesize that the high turnover bone phenotype of our patient at referral was a result of excessive RANKL activation caused by secondary hyperparathyroidism. Interestingly, the VDR-vitamin D system seems to be essential in post-natal hair cycling [28]. Alopecia is reported in some patients with VDDR2A, especially those with deleterious mutations, but not in patients with vitamin D-dependent rickets type 1, which is caused by 25(OH)D 1-alpha-hydroxylase deficiency [29, 30]. The exact mechanism of alopecia in VDR-null mice and patients with VDDR2A is not clear, but previous studies have suggested ligand-independent actions of VDR in skin, which may involve the canonical Wnt signaling pathway [31].

Serum FGF23 levels in our patient were consistently below the limit of detection, and her serum phosphate levels fluctuated around the lower limit of the reference range. A previous study of a patient with R73X mutation also showed suppressed levels of serum FGF23 [22]. Thus, we hypothesize that the hypophosphatemia in our patient is not FGF23-related and is caused by a lack of 1,25(OH)2D responsiveness and secondary hyperparathyroidism, respectively. Since her serum phosphate levels and TmP/GFR changed in parallel, PTH, which is a negative regulator of TmP/GFR, seems to be the major determinant of serum phosphate levels in our patient (Fig. 3). Considering the fact that VDR-null mice also displayed undetectable FGF23 levels [32, 33], and 1,25(OH)2D stimulates FGF23 expression [34-36], the VDR-vitamin D system definitively plays an important role in FGF23 regulation. It has been shown that FGF23-null mice display hyperphosphatemia, hypercalcemia, elevated serum 1,25(OH)2D, and vascular calcification of the kidneys [37]. However, double-mutant mice lacking both FGF23 and VDR were normophosphatemic, normocalcemic, and showed no ectopic calcifications when fed a high calcium diet [38]. Therefore, adverse effects of FGF23-deficiency are primarily caused by excessive actions of 1,25(OH)2D, which we do not expect to occur in patients with VDDR2A.

In vitro studies suggest that VDR downregulates the transcription of renin by binding to the promoter region of the REN gene, and studies using VDR-null mice and 1-alpha-hydroxylase-deficient mice show high plasma renin activity and increased renal renin mRNA [39, 40]. Moreover, cardiac hypertrophy and fibrosis were detected in VDR-null mice regardless of coexisting hypertension [41]. A previous analysis of seventeen patients with VDDR2A aged 6 to 36 years showed normal renin activity and aldosterone levels, without hypertension or gross heart abnormalities [42]. These patients had mutations in the VDR gene that caused alopecia; fifteen of them had a nonsense mutation in exon 8 (Y295X), and two of them had a missense mutation in exon 2 (G33D) [42]. Further studies are necessary to clarify the regulation of the renin-angiotensin-aldosterone system in patients with VDDR2A. Until then, we will closely monitor for future cardiovascular complications in our patient although she is currently normotensive and does not have cardiac hypertrophy.

There are two limitations of our study. First, we were unable to perform genetic analysis of the patient’s parents. Therefore, we could not rule out the possibility of uniparental disomy of chromosome 12, which has been reported in a previous case [22]. However, the fact that her parents are second cousins favors the presence of a homozygous R30X mutation. Second, very low serum 25(OH)D levels at referral raises the possibility of concurrent vitamin D deficiency. While VDR is considered to be essential for 1,25(OH)2D to exert most of its functions, VDR-independent 1,25(OH)2D actions, such as anti-proliferative effects in tumor cells, have been reported [43]. Therefore, we could not rule out the possibility that vitamin D deficiency might adversely affect the patient’s bone metabolism in a VDR-independent manner. It has been shown that the CYP27B1 gene encoding 25(OH)D 1-alpha-hydroxylase was downregulated by VDR and upregulated by PTH [12, 44]. Therefore, increased 25(OH)D 1-alpha-hydroxylase activity may result in increased conversion from 25(OH)D to 1,25(OH)2D and subsequently lower 25(OH)D levels in patients with VDDR2A, regardless of their vitamin D status.

In conclusion, we describe a patient with VDDR2A who developed secondary hyperparathyroidism and high bone turnover in adulthood. Although the R30X mutation found in this case resulted in a severely truncated VDR protein, sufficient amounts of oral calcium supplementation ameliorated her secondary hyperparathyroidism and nearly normalized her bone metabolic parameters.
Disclosure

None of the authors have any potential conflicts of interest associated with this research.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

References