Abstract

Objective: Vitamin D dependent rickets type 1A (VDDR1A) is an autosomal recessive disorder caused by mutations in the 25OHD 1α-hydroxylase gene (CYB27B1). As it may be confused with nutritional rickets and hypophosphatemic rickets, genetic analysis is important for making a correct diagnosis.

Methods: We analysed genomic DNA from 11 patients from 8 different Turkish families. The patients were recruited for our studies if they presented with diagnosis of vitamin D dependent rickets.

Results: The mean age at diagnosis was 13.1 ±7.4 months. Seven patients had mild hypocalcemia at presentation while 4 patients had normal calcium levels. All patients underwent CYP27B1 gene analysis; The most prevalent mutation was the c.195 + 2T>G splice donor site mutation, affecting 5 out of 11 patients with VDDR1A. Two patients from the fourth family was compound heterozygous for c.195 +2T>G and c.195 +2 T>A in intron 1. Two patients from different families were homozygous for a previously reported duplication mutation in exon 8 (1319_1325dupCCCACC, Phe443Profs*24). One patient had homozygous splice site mutation in intron 7 (c.1215+2 T>A). And one patient had homozygous mutation in exon 9 (c.1474 C>T).

Conclusion: Intron 1 mutation was the most common mutation as in the previous studies, and all patients carrying that mutation were from same city of origin suggesting a “founder” or a “common ancestor” effect. VDDR1A should be definitely considered when a patient with signs of rickets has a normal 25-OH level or when there is unresponsiveness to vitamin D treatment.

What is already known on this topic?

Although vitamin D dependent rickets type 1A (VDDR1A) is a rare disease, it is relatively more common in Turkey. Intron-1 mutations have been reported only from Turkey so far. Intron-1 mutations have been reported to be associated with milder clinical findings. Clinical and laboratory findings can overlap with other types of rickets. Serum 1, 25-dihydroxyvitamin D (-OH2D) levels are usually known to be low with VDDR1A.

What this study adds?

In this study, we have observed that the patients with intron-1 mutations can present with clinical findings of variable severity. We have also emphasized that the concentrations of 1, 25-OH2D levels could be in inappropriately normal ranges in patients with VDDR1A and can lead to diagnostic confusion.

Introduction

Vitamin D (calciferol) comprises two biologically inactive, fat-soluble pro-hormones: ergocalciferol (vitamin D2), derived from ergosterol after UV light exposure; and cholecalciferol (vitamin D3), derived from animal tissues and 7-dehydrocholesterol, which is formed in human skin by the action of UV rays in sunlight (1).

Both forms need a two-step hydroxylation at 25th and 1st carbons for activation. The first step occurs in the liver, where vitamin D is hydroxylated to 25-hydroxyvitamin D (25-OHD) by the hepatic 25-hydroxylase. The second step occurs mainly in the kidney, where 25-OHD is hydroxylated by the mitochondrial vitamin D 1α-hydroxylase to the biologically active hormone 1, 25-dihydroxyvitamin D (1, 25-OH2D), which binds to its nuclear receptor and exerts its biological activities (1, 2, 3). The biologically active 1, 25-OH2D plays a central role in calcium homeostasis and bone metabolism, and also has a significant influence on cell proliferation and
differentiation of a variety of tissues (1, 3, 4). The renal synthesis of 1, 25-OH_{2}D from its precursor 25-OHD is a rate-limiting step and is tightly regulated by serum 1, 25-OH_{2}D, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), calcium and phosphate, with renal 1α-hydroxylase being stimulated by PTH, hypophosphatemia, or hypocalemia, and inhibited by FGF23 (4).

Four rare genetic errors of vitamin D metabolism that can cause rickets have been described. The first one involves 1α-hydroxylase deficiency, which is also described as vitamin D dependent rickets type 1A (VDDR1A). A selective mutation in CYP2R1 gene, which leads to 25-hydroxylase deficiency, called type 1B (VDDR1B). The second type involves a defective vitamin D receptor (VDR), resulting in vitamin D resistant rickets (VDDR), also known as vitamin D dependent rickets type 2A (VDDR2A). VDBR2B is an unusual form of rickets due to abnormal expression of a hormone response element-binding protein that interferes with normal function of vitamin D receptor (5-8).

VDDR1A is an autosomal recessive disorder caused by mutations in the 25-OHD 1α-hydroxylase gene (CYB27B1). CYB27B1 is composed of 9 exons and is approximately 5 Mb in size. The gene has been mapped to the chromosomal region 12q14.1 (9-12). Clinically, VDDR1A is characterized by hypotonia, muscle weakness, inability to walk, growth retardation, and radiographic findings of rickets. Typical laboratory findings are hypocalemia, elevated serum levels of alkaline phosphatase (ALP) and parathyroid hormone (PTH) with low or normal levels of 1, 25-OH_{2}D despite normal or increased concentrations of 25-OH_{2}D (9, 13). Patients with VDDR1 may present with aminoaciduria and hyperchloremic acidosis (3).

To date, over 100 patients with 78 mutations have been identified in the CYP27B1 gene in patients from multiple ethnic groups. These mutations span all exons of the gene and mostly include missense and nonsense changes, along with splice site changes, insertions, deletions, and duplications (14, hgmd, http://www.hgmd.cf.ac.uk/ac/index.php). Mutations in CYP27B1 lead to a loss of the 1α-hydroxylase activity and require treatment with calcitriol to normalize the clinical and laboratory abnormalities (15).

In the present study, we report 11 patients with VDDR1A from 8 unrelated Turkish families. The most prevalent mutation was the c.195 + 2T>G splice donor site mutation, affecting 5 out of 11 patients with VDDR1A. Clinical findings of patients were examined in detail and genotype-phenotype correlations were evaluated.

**Methods**

We analyzed genomic DNA of 11 patients from 8 different Turkish families. In five of these families parents were related. The study was approved by the University of Health Science Umraniye Training and Research Hospital Clinical Research Ethical Committee (approved number: 19/01/2018- 2926), and informed consent was obtained from patients/ families.

Eleven patients had the clinical findings of rickets including; X-bain deformity or bowed leg, chest roary, harrison’s groove, frontal bossing, widening of the wrist, growth retardation, hypotonia and inability to walk, hypocalemia seizure. Furthermore, the patients had biochemical features suggestive of rickets such as hypophosphatemia, hypo- or normocalcemia, elevated PTH and ALP, normal or high 25-OH_{2}D levels, and low or normal 1, 25-OH_{2}D levels. Wrist and knee radiographs of all patients demonstrated widened physes and metaphyseal cupping and fraying. Differentiation of nutritional rickets and VDDR1A was made by: normal/high 25-OH_{2}D levels, low/inappropriately normal 1, 25-OH_{2}D levels and recovery of clinical, biochemical and radiological findings of rickets after replacement of calcitriol. All patients received calcitriol and patients with hypocalemia received calcium replacement. Calcitriol was started at a dose of 1-1.5 mcg/day, twice daily. Subsequently the calcitriol dose was titrated according to the results of biochemical analyses. The aims of the treatment were to achieve normocalcemia, to maintain PTH levels within normal limits, and to avoid hypercalcemia.

**Targeted second generation sequence analysis**

DNA was isolated from a 200 ul peripheral blood sample using QIAamp DNA Blood Mini QIAcube Kit and QIAcube device (QIAGEN, Hilden, Germany). Then, the exons of the CYP27B1 gene were amplified for targeted sequencing. Amplification was controlled with agarose gel electrophoresis technique. Sequencing was carried out using Illumina MiSeq NGS System (Illumina Inc., San Diego, CA, USA) Miseq Reagent Kit V3 (600 cycles). The readings were aligned with hg19 genomic sequence and compared.

**Sanger Sequencing**

10 ml venous blood sample in EDTA tube was taken from each patient. DNA isolation was performed using the QIAamp DNA Mini QIAcube Kit from the peripheral blood. The Primer design was made to include CYP27B1 gene exons and close introns (Table 1). The products of PCR reaction [94 ° C -5 min, (95 ° C 30 sec -60 ° C 30 sec -72 ° C 30 sec) x 34, 72 ° C 5 min] with the primers, which were also shown in Table 1, were checked on a 2% agarose gel. After the amplification of correct gene regions, purification of PCR products was made by keeping 15 min at 37 °C (enzyme activation temperature) and 15 min at 80 °C (enzyme inactivation temperature) in the thermal cycler using ExoSAP enzyme. After purification, the primer and the cleaned template DNA were added to the PCR solution, which is called “The Big Dye Ready Reaction Mix”, and the PCR reaction was
started. The purification process was repeated after the PCR sequencing for the removal of uncoupled ddNTPs in the solution. Sanger sequencing of the purified samples was performed on the ABI 3130 XL (Applied Biosystems) capillary sequencing device. The obtained data were analysed by SeqScape analysis program.

**Data analysis**

Sequenced data was analyzed with the Genomize Variant Analysis Program and IGV (Integrative Genomics Viewer). The homozygote or compound heterozygote variants in the databases such as NCBI, HGMD, and Clinvar were primarily selected for data filtering. The effects of mutations on protein structures were tested with various in silico prediction tools, particularly Mutation Taster (16), Polyphen-2 (17), and SIFT (18).

**Statistical Analysis**

Statistical analysis was performed using IBM SPSS 21.0 for windows statistical software. The data were presented as mean ±SD (ranges).

**Results**

Among patients diagnosed with VDDR1A, 6 were male and 5 female, making up a total of 11 patients from 8 families. Clinical presentation and laboratory findings of the patients were summarized on Table 2. The mean age at diagnosis was 13.1 ±7.4 months. Seven patients had mild hypocalcemia at presentation while 4 patients had normal calcium levels. Five of eight families had consanguineous marriages. The two families that were not related were from the same city. All patients had clinical and laboratory features of rickets at the time of diagnosis. All patients had low levels of phosphorus whereas quite high levels of PTH and ALP levels. Five patients had fairly high levels of 25 OH D due to being formerly diagnosed with nutritional rickets and treated with vitamin D. Levels of 1, 25-OH2D, on the other hand, were normal in 3 patients. One patient was previously followed for hypophosphatemic rickets, and treated with calcitriol and phosphate. When he was diagnosed with VDDR1A, he had elevated PTH levels and typical radiological findings of rickets (Figure-1).

After the definite diagnosis all patients received calcitriol treatment. The duration of treatment with calcitriol ranged between 6 months and 7 years. Biochemical improvement with treatment occurred in a period ranging between 4 months and 12 months.

All patients underwent CYP27B1 gene analysis (Table 3). The most prevalent mutation was the c.195 + 2T>G splice donor site homozygous mutation, affecting 5 out of 11 patients with VDDR1A. Two patients from family-4 had compound heterozygous mutation for c.195 +2T>G and c.195 +2 T>A in intron 1. Two patients from different families had homozygous duplication mutation in exon 8 (1319_1325dupCCCACC, Phe443Profs*24), which was previously reported (Figure 2). A homozygous c.1215 +2T>A mutation in the splice donor site of intron-7 was found in one patient. And one patient was found to have a homozygous mutation in exon 9 (c.1474 C>T).

**Discussion**

In the present study, we report the clinical, biochemical, and genetic analysis of 11 patients with VDDR1A. We identified five previously reported mutations. The most prevalent mutation was the c.195 + 2T>G splice donor site mutation. Five patients from two different families had this mutation as homozygous and two patients from the same family had it as hemizygous as a part of compound heterozygous mutation. Durmaz E, et al (19) reported this mutation for the first time in a Turkish patient, and c.195 +2T>G homozygous mutation in intron 1 is present in a total of 20 patients including ours so far, all reported from Turkey (4, 9, 19). These patients were homozygous for the previously described splice donor site mutation c.195 +2T>G, where a thiamine is substituted for a guanine in the second nucleotide of intron 1. Since this mutation is common in Turkish patients and have not been reported in other ethnic groups, it may be unique representing a ‘founder’ or “common ancestor” effect, given the high rates of consanguinity. Although it has not been reported in other publications, all patients in the study by Tahir S, et al (9) were living in Diyarbakir or neighbouring provinces; all of our patients carrying that mutation were from Batman, which is geographically very close to Diyarbakir.

While Tahir S, et al (9) reported that patients with intron 1 mutation had a milder clinical presentation, Demir K, et al (4) reported that the most severe form of the disease occurred in a patient with intron 1 mutation. We could not identify any relationship between genotype and phenotype. All patients in the literature who had an intron 1 mutation had delayed walking and bowed legs at admission. While four of our patients also had the same complaint, another patient presented with hypotonia. Although 4 of 5 patients with intron 1 mutation had a height below -2 SD, patients with other mutations also had short stature.

We had only one patient presented with hypocalcaemic convulsion at the age of 11 months. Hypocalcaemic convulsion has also been reported rarely by other studies from Turkey. Tahir S, et al (9) reported hypocalcaemic convulsion in 5 of 22 patients; Demir K, et al (4) in 4 of 8 patients; and Durmaz E, et al (19) in 2 of 7 patients. Kim JC, et al (20) reported that 4 of 10 patients presented with hypocalcaemic convulsion. Edouard T, et al (21) reported that the admission symptom was hypocalcaemic convulsion in 4 of 21 pediatric patients. As these
patients had calcium levels that are in the lower limit of normal, hypocalcemic convulsion is not frequently encountered.

The clinical presentations of patients with VDDR1A could lead to a misdiagnosis of nutritional rickets or hypophosphatemic rickets. It can be differentiated from hypophosphatemic rickets by a high PTH level and nutritional rickets by a normal 25 OHD level. The hypophosphatemia in VDDR1A is a result of elevated PTH and renal excretion of phosphate. Clinical and laboratory features of VDDR1A are very similar to nutritional rickets; its differential diagnosis can be made by a low or inappropriately normal 1, 25-OH,3D level and unresponsiveness to vitamin D treatment. In our study, 6 patients also had been treated with vitamin D for a long time with the diagnosis of nutritional rickets, and they had extremely high 25-OHD levels. Four patients had normal calcium levels, and one of them had been followed with hypophosphatemic rickets. There were a few patients with normal 1, 25-OH,3D levels diagnosed with VDDR1A in the literature (4, 8). In fact, the expected 1, 25-OH,3D levels in 1α-hydroxylase deficiency are low, inappropriately normal 1, 25-OH,3D levels also indicate that the enzyme activity is insufficient. Recently, Nishikawa N, et al (22) reported that liver mitochondrial CYP27A1 can catalyse 1α-hydroxylation of 25-OHD. A small increase in serum 1, 25-OH,3D level has been observed in CYP27B1 knockout mice after given high dietary vitamin D, suggesting a conversion from 25-OHD to 1, 25-OH,3D by a non-CYP27B1 enzyme. Three of eleven patients of our study had normal 1, 25-OH,3D level. There was a history of high dose vitamin D intake in two of these three patients with normal 1, 25-OH,3D. In these patients, conversion from 25-OHD to 1, 25-OH,3D by a non-CYP27B1 enzyme may contribute to the normal 1, 25-OH,3D.

Maternal 1, 25-OH,3D does not cross the fetoplacental barrier (21, 23). 1, 25-OH,3D is increasing 2-3 fold in the first weeks of pregnancy where as maternal 25-OHD crosses the placental barrier. The rise in circulating 1, 25-OH,3D concentrations in the mother facilitates optimal in utero bone development by attaining a positive calcium balance (24). Edouard T, et al (21) reported that, unlike patients with severe vitamin D deficiency who can present within the first 6 months of age, none of the VDDR1A patients were symptomatic before the age of 6 months. Indeed, the infant who was diagnosed with VDDR1A at the age of 1 month had a low serum level of 1, 25-OH,3D and a positive CYP27B1 sequencing result but did not have any clinical or radiological signs of rickets (21). This indicates that 1,25-OH,3D is not critical for mineral ion homeostasis and growth plate mineralization in first months of life owing to in utero positive calcium balance in these patients. All patients in this study group were 6-month or older at admission.

Generally, a good response to treatment with alfalcaldiol or calcitriol (10- 400 ng/kg/day) is expected in cases with VDDR1A (4, 21). Calcitriol dose was tailored based on biochemical and clinical findings. Edouard T, et al (21) indicated short and long-term outcomes of calcitriol treatment in their patients. They started calcitriol treatment at a dose of 1.0 µg/day, given in two doses of 0.5 µg. Treatment with calcitriol resulted in the normalization of biochemical parameters within 3 months. The aims of the treatment were to achieve normocalcemia, to maintain PTH levels within normal limits, and to avoid hypercalciuria. Our patients did not reach their final height, and their treatment duration ranged between 6 months and 7 years. Improvement of biochemical parameters occurred at a somewhat later period, between 4 months and 12 months.

Study Limitations
The main limitation of our study is the relatively small number of patients.

Conclusion
Although VDDR1A is a rare disease, it is more common in Turkey where autosomal recessive disorders are common. In this study, we evaluated the genetic and clinical features of 11 patients with the diagnosis of VDDR1A. Intron 1 mutation was the most common mutation as in the previous studies, and all patients carrying that mutation were from same city of origin suggesting a “founder” or a “common ancestor” effect. As it may be confused with nutritional rickets and hypophosphatemic rickets, genetic analysis is important for making a correct diagnosis. VDDR1A should be considered when a patient with signs of rickets has a normal 25-OH level or when there is unresponsiveness to vitamin D treatment. We have also emphasized that the concentrations of 1, 25-OH,3D levels could be in normal ranges in patients with VDDR1A and can lead to diagnostic confusion.

Conflict of interest
The author declares that there is no conflict of interest.

Financial Disclosure: The author declared that this study has received no financial support.

Authorship Contributions

References

Figure 1: X-rays of this patient before (A) and at the 6th month of calcitriol treatment (B). A: abnormal cupping, widening and fraying of the metaphyses consistent with rickets. B: recovery of cupping and fraying, and a provisional calcification zone suggesting healing rickets.
**Figure 2:** A. Wild type sequence of exon 8 in CYP27B1 gene. B. Sequencing analysis of the CYP27B1 gene exon 8 showing the homozygous mutation (1319_1325dupCCCACCC, Phe443Profs*24).

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The patients were indicated with respective family number and the number of individual in that family. Patients 1.1, 1.2, 1.3, and 4.1 and 4.2 were siblings. SD: standart deviation, Ca: calcium, P: phosphate, PTH: parathyroid hormone, ALP: alkaline phosphatase, N: normal range

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