VITAMIN D plays an important role not only in the maintenance of mineral homeostasis but also in the modulation of osteoblast differentiation [1]. Native vitamin D is produced from food intake or synthesized in the skin in response to ultraviolet light. Vitamin D is then sequentially activated by two hydroxylases: 25-hydroxylase converts native vitamin D into 25-hydroxyvitamin D (25-OHD) in the liver and 1-alpha-hydroxylase (1α-OHase) converts 25-OHD into 1,25(OH)2D in the kidney. Inactivation of vitamin D also occurs in the liver by 24-hydroxylase which converts 25-OHD and 1,25(OH)2D into 24,25(OH)2D and 1,24,25(OH)3D, respectively, in order to avoid vitamin D toxicity and to maintain the concentration of active vitamin D within the physiological range. These types of hydroxylation occur under hormonal control in relation to mineral metabolism, especially calcium (Ca) metabolism [2].

Serum Ca level is maintained by PTH and 1,25(OH)2D primarily to avoid hypocalcemia. PTH secretion is under the control of Ca-sensing receptors on the surface of the parathyroid gland. The dose-response curve of serum PTH level to serum Ca has an inverted sigmoid shape, which allows an immediate response to hypocalcemia [3]. When serum Ca decreases below the lower limit, parathyroid hormone secretion is up-regulated through the inactivation of

Urinary calcium to creatinine ratio: a potential marker of secondary hyperparathyroidism in patients with vitamin D-dependent rickets type 1A

Kentaro Miyai1), 2), Toshikazu Onishi2), 3), Kenichi Kashimada2), 4) and Yukihiro Hasegawa1)

1) Division of Endocrinology and Metabolism, Tokyo Metropolitan Children’s Medical Center, Tokyo 183-8561, Japan
2) Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Tokyo 113-8510, Japan
3) Department of Pediatrics, Kinki Central Hospital, Hyogo 664-8533, Japan
4) Department of Pediatrics, Tsuchiura General Hospital, Ibaraki 300-0053, Japan

Abstract. Patients with vitamin D-dependent rickets type 1A (VDDR1A) are usually treated with alfacalcidol, an analog of vitamin D. Around puberty, an increased dose of alfacalcidol is recommended for these patients to avoid hypocalcemia and secondary hyperparathyroidism. However, no indicators of secondary hyperparathyroidism except for PTH are presently known. The aim of this study is to evaluate whether urinary calcium to creatinine ratio (U-Ca/Cr) is useful as a biomarker of secondary hyperparathyroidism in VDDR1A patients in order to determine the proper dose of alfacalcidol. Two brothers with VDDR1A were recruited who had null mutations of CYP27B1 which encodes 1-alpha-hydroxylase of vitamin D. We investigated the relationship between U-Ca/Cr and intact-PTH around puberty when the brothers showed hypocalcemia with secondary hyperparathyroidism. The results were compared to those of five patients with vitamin D deficiency (VDD). As a result, high intact-PTH levels were observed when U-Ca/Cr decreased to less than 0.1 (mg/mg) in both VDDR1A brothers. This relationship was also observed in the VDD patients. However, it is necessary to take into account body calcium status, either in depletion or in excess, to accurately evaluate the relationship between U-Ca/Cr and secondary hyperparathyroidism. First, low U-Ca/Cr was detected in situations with calcium depletion without hyperparathyroidism in the VDDR1A patients. Second, high U-Ca/Cr with hyperparathyroidism could be detected theoretically in a condition of excess calcium supply. In conclusion, a U-Ca/Cr ratio of less than 0.1 (mg/mg) in VDDR1A patients is useful to accurately evaluate calcium depletion and secondary hyperparathyroidism.

Keywords: Urinary calcium to creatinine ratio, Secondary hyperparathyroidism, Vitamin D-dependent rickets type 1A, Vitamin D deficiency, Growth spurt

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Ca-sensing receptors. Secreted PTH increases serum Ca levels via enhancement of reabsorption in the proximal tubule of kidneys, bone resorption, and absorption in the intestines through activation of 1α-OHase of vitamin D.

Vitamin D-dependent rickets type 1A (VDDR1A) (OMIM #264700) is a rare autosomal recessive disease. The gene responsible for VDDR1A has been identified as CYP27B1, located on chromosome 12q13.1-13.3 [4], which encodes 1α-OHase of 25-OHD. Patients with VDDR1A are recognized by symptoms related to hypocalcemia such as hypotonia, rickets, and seizures. To prevent such hypocalcemic events, patients with VDDR1A are usually treated with alfacalcidol (1-hydroxycholecalciferol), a vitamin D analog, which is converted to 1,25(OH)2D by 25-hydroxylase in the liver. To achieve normocalcemia and to avoid hypercalciuria, the dose of alfacalcidol should be adjusted based on serum Ca, intact-PTH, and urinary calcium to creatinine ratio (U-Ca/Cr) levels. But, to date, there has been only one report on the dose of calcitriol (1,25-dihydroxycholecalciferol), another analog of vitamin D, in VDDR1A patients during adolescence [5].

Here we report two brothers with VDDR1A who have compound heterozygous mutations of the CYP27B1 gene. The two mutations, previously reported [4, 6], are a missense mutation and a frameshift mutation, both of which are regarded as null function mutations. Around puberty, the brothers were treated with three to four times the dose of alfacalcidol (0.06-0.1µg/kg/day) compared to the dose used in childhood (0.02-0.03µg/kg/day) to avoid hypocalcemia and secondary hyperparathyroidism. We examined the relationship between U-Ca/Cr and intact-PTH in the brothers. Furthermore, we examined whether a similar relationship is observed in patients with vitamin D deficiency (VDD) diagnosed after the age of two years.

Materials and Methods

Patients

The patients included two brothers diagnosed as having VDDR1A, and five VDD patients whose ages at diagnosis were between 2.4 and 15.1 years. All patients were from our hospital. Because most children with VDD are diagnosed and treated before age two, when urinary creatinine secretion is low and U-Ca/Cr is highly variable, we excluded VDD patients below the age of two years. All study participants provided their informed consent, and the study was approved by our local review board.

Blood and urinary samples and biochemical measurements

Blood and urinary samples of the VDDR1A brothers were obtained every two months as occasional specimens more than two hours after meal. Laboratory data of the VDDR1A brothers were collected from prepubertal period to pubertal period (from age 9 to 15 years of the elder brother and from age 7 to 13 years of the younger brother), and those of the VDD patients were collected during two to four years after diagnosis.

Serum intact-PTH was measured by ECLIA (SRL, Inc., Japan), and serum Ca, phosphate (P), alkaline phosphatase (ALP), urinary calcium and urinary creatinine were measured by standard colorimetric methods. U-Ca/Cr was calculated as urinary calcium (mg/dL)/urinary creatinine (mg/dL).

Sequence analyses of the CYP27B1 gene

Total genomic DNA was isolated from peripheral blood using the QIAamp DNA blood midi kit (QIAGEN Inc., Tokyo, Japan). Coding regions including the exon-intron boundaries of the CYP27B1 gene were amplified by PCR using primers listed in Table 1. PCR products were directly sequenced by Operon Biotechnologies, Inc., Japan. Mutational nucleotide sequences were compared to the human gene mutation database website (http://www.biobase-international.com/product/hgmd).

Statistical Analyses

Statistical analyses were performed using SPSS version 20 software. Pearson’s correlation coefficient between intact-PTH and U-Ca/Cr was calculated, and p values less than 0.05 were considered significant.
U-Ca/Cr in detecting secondary HPT

The younger brother was brought to the hospital because of retardation of motor development at the age of 15 months. He showed hypotonia, and blood examination showed hypocalcemia (6.2 mg/dL), hypophosphatemia (3.1 mg/dL), and increased high-sensitive PTH level (2000 pg/mL, normal range: 160-520 pg/mL). Because his elder brother was diagnosed as having PHP at that time, he was also diagnosed as having PHP and was treated with alfacalcidol. When he was 10 years old (subsequent records showed that this was one and a half year before the start of a growth spurt), blood examination showed hypocalcemia (4.8 mg/dL), hyperphosphatemia (7.3 mg/dL) and high ALP (2939 U/L) with low U-Ca/Cr (0.03 mg/mg) and high %TRP (98%) (Fig. 1B). The dose of alfacalcidol was increased from 0.02 µg/kg/day to 0.03 µg/kg/day until adolescence (Fig. 1A). When he was 11 years old (subsequent records showed that this was the beginning of a growth spurt), routine blood examination showed hypocalcemia (6.7 mg/dL) with hyperphosphatemia (6.7 mg/dL) with low U-Ca/Cr (0.00 mg/mg) and high tubular reabsorption of phosphorus (%TRP) (95%). The dose of alfacalcidol was then increased to 0.04 µg/kg/day. Hypocalcemia improved soon after the dose was increased, but 6 months later, blood examination showed hypocalcemia (7.3 mg/dL) with hyperphosphatemia (7.2 mg/dL) with low U-Ca/Cr (0.01 mg/mg) and high %TRP (96%) again. The dose of alfacalcidol was then increased to 0.09 µg/kg/day and was maintained at that level for about one and a half years. When he was 13 years old, the dose of alfacalcidol was gradually decreased because U-Ca/Cr was high (0.44 mg/mg). After the age of 15 years the dose of alfacalcidol was maintained at 0.02 µg/kg/day.

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mations [4, 6], and the brothers were diagnosed as having vitamin D-dependent rickets type 1A (VDDR1A).

**Relationship between intact-PTH and U-Ca/Cr**

To find a predictor of secondary hyperparathyroidism, we investigated the relationships between intact-PTH and U-Ca/Cr. When U-Ca/Cr decreased to less than 0.1 (mg/mg), intact-PTH tended to increase above the upper limit in both brothers (Fig. 3A, B). We were unable to compare the data with other VDDR1A patients because VDDR1A is rare. Instead, to test whether a similar relation was present in other patients related to vitamin D metabolism, we also investigated five patients who were diagnosed as having VDD after the age of two years old. Their clinical data at diagnosis are shown in Table 2. Each of the five patients had an unbalanced diet at diagnosis. Intact-PTH and U-Ca/Cr had a reverse sigmoid relationship (Fig. 4A) that could be made more linear in a log-log plot (Fig. 4B). When the scatter diagrams of the VDDR1A brothers were displayed together with those of the VDD patients in a log-log plot, the two sets of data overlapped (Fig. 4C). It should be noted that U-Ca/Cr less than 0.1 (mg/mg) with normal intact-PTH levels, and U-Ca/Cr more than 0.1 (mg/mg) with high intact-PTH levels were also observed (Fig. 4C).

**Genomic analyses of CYP27B1 gene**

We examined the brothers and their father for CYP27B1 gene mutations after obtaining written informed consent. Though the brothers were diagnosed as having PHP and their serum P levels were high in spite of low serum calcium levels around the age of puberty, several observations (that is, their age of first appearance with hypocalcemia and hypophosphatemia, the demand of alfacalcidol at puberty, the high PTH levels with normal to low 1,25(OH)2D levels (14.7-31.3 pg/mL; reference range 20-70 pg/mL), and the normalized PTH levels by alfacalcidol), led us to conclude that their condition was consistent with 1α-OHase deficiency and not with PHP.

Genomic analyses revealed that the brothers had the same compound heterozygote of missense mutation of c.320G>A (p.R107H) in exon 2, and frameshift mutation of c.1319_1325dupCCCACCC in exon 8, and that their father was heterozygous for the latter (Fig. 2). These mutations were previously reported as null function mutations [4, 6], and the brothers were diagnosed as having vitamin D-dependent rickets type 1A (VDDR1A).
U-Ca/Cr in detecting secondary HPT

Fig. 3  Relationships between intact-PTH and U-Ca/Cr in two VDDR1A brothers
(A) Elder brother (from 9 to 15 years old) and (B) younger brother (from 7 to 13 years old). Black lines show the regression lines. Vertical dashed lines show U-Ca/Cr=0.1 (mg/mg) and transverse dashed lines show the upper limit of the normal range of intact-PTH (70 pg/mL).

Table 2  Clinical data of five patients with vitamin D deficiency

<table>
<thead>
<tr>
<th>No.</th>
<th>Age at Dx</th>
<th>Sex</th>
<th>Factor</th>
<th>Serum Ca (mg/dL)</th>
<th>Serum P (mg/dL)</th>
<th>ALP (U/L)</th>
<th>Intact-PTH (ng/mL)</th>
<th>U-Ca/Cr (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4y</td>
<td>F</td>
<td>vegetarian</td>
<td>7.8</td>
<td>3.6</td>
<td>2812</td>
<td>742.6</td>
<td>0.006</td>
</tr>
<tr>
<td>2</td>
<td>3y</td>
<td>F</td>
<td>unbalanced diet</td>
<td>7.2</td>
<td>3.6</td>
<td>5064</td>
<td>906</td>
<td>0.012</td>
</tr>
<tr>
<td>3</td>
<td>10y</td>
<td>F</td>
<td>unbalanced diet</td>
<td>9.6</td>
<td>4.8</td>
<td>1177</td>
<td>96.4</td>
<td>0.046</td>
</tr>
<tr>
<td>4</td>
<td>10.5y</td>
<td>M</td>
<td>unbalanced diet</td>
<td>8.8</td>
<td>5.0</td>
<td>1130</td>
<td>149.4</td>
<td>0.029</td>
</tr>
<tr>
<td>5</td>
<td>15.1y</td>
<td>M</td>
<td>unbalanced diet</td>
<td>9.3</td>
<td>5.1</td>
<td>750</td>
<td>119.9</td>
<td>0.031</td>
</tr>
</tbody>
</table>

The age of diagnosis (Dx) for all patients was more than two years old.

Fig. 4  Relationships between intact-PTH and U-Ca/Cr in VDD patients and VDDR1A brothers
(A, B) VDD patients data shown on linear (A) and log-log (B) scales. (C) Superposition of VDD patients data (gray symbols) and VDDR1A brothers data (black symbols). Vertical dashed lines show U-Ca/Cr=0.1 (mg/mg), and transverse lines show an upper limit level of the normal range of intact-PTH (70 pg/mL).
Discussion

We found that a U-Ca/Cr ratio less than 0.1 (mg/mg) was useful for detecting secondary hyperparathyroidism in VDDR1A and VDD patients. It is difficult to predict an increase of PTH secretion from the levels of serum Ca because dynamic changes in PTH secretion occur as a result of minute changes in serum Ca [7], and because normocalcemic secondary hyperparathyroidism can occur as a result of compensation by PTH. Theoretically, a decrease in U-Ca/Cr precedes a decrease in serum Ca levels as a result of Ca reabsorption in the kidneys. Therefore, U-Ca/Cr could be a sensitive indicator of secondary hyperparathyroidism. While a high U-Ca/Cr ratio is often used as an indicator of hypercalciuria leading to nephrocalcinosis and nephrolithiasis [8-10], the present study is the first to establish a lower limit for this ratio.

A low U-Ca/Cr was found to be related to secondary hyperparathyroidism in both the VDDR1A brothers and the VDD patients. In the five VDD patients, intact-PTH rose sharply at U-Ca/Cr ratios below 0.1 (mg/mg) (Fig. 4A), in agreement with a previous report on the relationship between intact-PTH and serum ionized calcium [11]. On the other hand, the relationships between intact-PTH and U-Ca/Cr in the two brothers were more linear (Fig. 3A, B). This is because the range of intact-PTH was kept much lower in the two brothers than in the VDD patients as a result of therapeutic intervention.

U-Ca/Cr might be more sensitive than PTH for calcium depletion in the body in VDDR1A patients. Indeed, U-Ca/Cr ratios less than 0.1 (mg/mg) were not always related with high serum intact-PTH levels in the VDDR1A brothers (Fig. 3A, B and 4C). Such data were often obtained just before and after secondary hyperparathyroidism became overt. Therefore, it would be suggested that when calcium depletion begins to occur, the reabsorption of calcium in the kidneys, which is independent of PTH signaling [12], precedes the mobilization of calcium from the bone to maintain serum Ca levels within normal range. It would be also suggested that the reabsorption lasts until net calcium in the body, especially in the bone, is satisfied. From these viewpoints, U-Ca/Cr would not always reflect secondary hyperparathyroidism but could serve as an indicator of calcium depletion in the body. Furthermore, U-Ca/Cr may be beneficial in detecting calcium depletion in patients with hypoparathyroidism who do not properly secrete PTH in response to hypocalcemia, and in patients with PHP who do not properly respond to PTH.

The relationship between U-Ca/Cr and PTH might not be identical in VDDR1A and VDD. On one hand, because the lack of alfacalcidol could be directly linked to decreased active vitamin D in VDDR1A patients, serum Ca level could be maintained mainly by reabsorption in the kidneys, which depends mostly on PTH. Thus, U-Ca/Cr would be low in situations with slightly short active vitamin D. On the other hand, although we could not detect such situations in our VDD patients, in the initial stage of VDD it is theoretically possible that serum Ca level could be maintained by increased absorption of calcium in the intestines and bone resorption through increased active vitamin D, leading to relatively high urinary calcium excretion. Therefore, it might be difficult to detect the initial stage of secondary hyperparathyroidism in VDD patients only by U-Ca/Cr.

U-Ca/Cr levels could also be influenced by the supply of calcium. When a calcium supply is not sufficient, U-Ca/Cr could be low with or without hyperparathyroidism in both VDDR1A patients and VDD patients. Conversely, when a calcium supply by oral intake or by excess active vitamin D transiently exceeds the storage capacity in the bone, U-Ca/Cr could be more than 0.1 (mg/mg) even when intact-PTH levels are high in both VDDR1A patients and VDD patients. Therefore, it is necessary to assess U-Ca/Cr and other parameters such as serum Ca and intact-PTH, in light of the supply of calcium, in order to accurately evaluate calcium depletion and secondary hyperparathyroidism.

The dose of alfacalcidol (µg/kg) that the VDDR1A brothers were administered around puberty might reflect the physiological levels of active vitamin D in normal adolescents, because the mutations that the brothers have are considered as null function mutations and because the growth rate of the brothers appeared to be normal. Indeed, in normal subjects the level of 1,25(OH)2D showed a sharp increase in early pubertal stage followed by subsequent decrease in late pubertal stage [13]. A previous report showed that the dose of calcitriol (µg/day) given to VDDR1A patients was increased in pubertal period [5], though it is unclear whether the dose per body weight was also increased. We found that the amount of active vitamin D (i.e., alfacalcidol (µg/kg)) required during puberty is more than the amounts required in the periods before and after puberty.

It is possible that the episodes of hypocalcemia of
the brothers around puberty were caused by depletion of oral calcium intake, and could have been corrected with calcium supplementation. Indeed, the demands for calcium intake and bone mineral content per height increase starting from 9 years of age and peak at 13-14 years of age [14]. During this period calcium requirements would be more than 1000 mg/day [14]. As the brothers exhibited hypocalcemia around puberty, we cannot exclude the possibility that they had a deficiency of calcium intake.

It is unclear why blood examinations showed hyperphosphatemia together with hypocalcemia and high intact-PTH levels (104-141 pg/ml) around puberty in the VDDR1A brothers. One possibility is that the dose of alfalcacidol that they were given was not enough, resulting in a condition resembling the early phase of vitamin D deficiency. Previous reports showed that vitamin D deficient rickets patients exhibit hypocalcemia with normal to elevated phosphate levels in the early phase [15, 16]. Another possibility is that the dose of active vitamin D that was needed to maintain serum phosphate within the normal range was less than the dose needed to maintain serum calcium, because the brothers’ %TRP was high when they were hyperphosphatemic and hypocalcemic simultaneously. It is well known that when patients with vitamin D deficiency who show hypocalcemia concomitant with hyperphosphatemia were often misdiagnosed as pseudohypoparathyroidism type 2 [17, 18], which is defined by normal cyclic AMP production without phosphaturia by PTH. When the deficiency of vitamin D is not severe, the effect of vitamin D action on increasing serum phosphate might be stronger than the phosphaturic effect of PTH.

In conclusion, measuring U-Ca/Cr is a simple and useful method of detecting body calcium depletion in VDDR1A patients. U-Ca/Cr should be evaluated with intact-PTH and serum Ca as well as calcium supply to diagnose calcium depletion and secondary hyperparathyroidism precisely.

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

References


