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ORIGINAL ARTICLE

Mutation update and long-term outcome after treatment with active vitamin D_3 in Chinese patients with pseudovitamin D-deficiency rickets (PDDR)

Y. Chi[1] · J. Sun[1] · L. Pang[1] · R. Jiajue[1] · Y. Jiang[1] · O. Wang[1] · M. Li[1] · X. Xing[1] · Y. Hu[1] · X. Zhou[1] · X. Meng[1] · W. Xia[1]

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Abstract

Summary Pseudovitamin D-deficiency rickets is a rare disease which is caused by CYP27B1. In this study, we identified 9 mutations in 7 PDDR patients. In addition, we observed the response to long-term treatment of calcitriol in 15 Chinese patients with PDDR, which showed that the biochemical abnormalities had been corrected satisfactorily after 1-year treatment.

Introduction Pseudovitamin D-deficiency rickets is a rare autosomal recessive disorder resulting from a defect in 25-hydroxyvitamin D 1α-hydroxylase, which is encoded by CYP27B1. The purpose of this study was to identify the CYP27B1 mutations and investigate the response to long-term treatment of calcitriol in Chinese patients with PDDR.

Methods We investigated CYP27B1 mutations in seven individuals from six separate families. To investigate the response to long-term (13 years) treatment with calcitriol in PDDR patients, we additionally collected clinical data of eight families from our previous report and analyzed their biochemical parameter and radiographic changes during the treatment.

Results Nine different mutations were identified: two novel missense mutations (G194R, R259L), three novel and one reported deletion mutations (c1442delA, c1504delA, c311-321del, and c. 48-60del), two novel nonsense mutations (c.85G>T, c.580G>T), and a reported insertion mutation (c1325-1332insCCCACCC). The statistical analysis revealed that parathyroid hormone (PTH) and ALP significantly decreased after 6-month and 1-year treatment with calcitriol respectively. Urine calcium was measured in all the patients without kidney stones being documented. After 6-year treatment, the radiographic abnormalities had also been improved. Two patients who had reached their final height are both with short stature (height Z-score below − 2.0).

Conclusions We identified seven novel mutations of CYP27B1 gene in seven Chinese PDDR families. Our findings revealed after 1-year treatment of active vitamin D_3, PTH and ALP significantly decreased. The correction of the biochemical abnormalities had not improved the final height satisfactorily.

Keywords Calcitriol · CYP27B1 · Mutation · Pseudovitamin D-deficiency rickets (PDDR) · Treatment

Introduction

The secosteroid hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is essential for proper maintenance of calcium and phosphate homeostasis [1]. Vitamin D must undergo two successive hydroxylation reactions before acquiring hormonal activity. In the liver, an hydroxyl group is first added onto carbon 25 by the liver cytochrome P450 enzyme, vitamin D-25 hydroxylase (CYP27), resulting in 25-hydroxyvitamin D [25(OH)D] with minimal activity [2]. Secondly, in kidneys, the 25-hydroxyvitamin D-1α hydroxylase (CYP27B1) drives the addition of a hydroxyl group on carbon 1, leading to the formation of $1,25(OH)_2D_3$, the active hormonal form of vitamin D_3, which is a rate-limiting step and is tightly regulated by serum $1,25(OH)_2D_3$, parathyroid hormone (PTH), FGF23, calcium, and phosphate [3, 4]. Although 1α-hydroxylase activity occurs mainly in the kidney, it is also found in extrarenal tissues such as skin, placenta, colon, pancreas, lymph node, and macrophages [5].

Pseudovitamin D-deficiency rickets (PDDR; OMIM 264700; also referred to as vitamin D-dependent rickets type I) is a rare autosomal recessive disorder caused by mutations of the CYP27B1 gene, characterized by the early-onset and a

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✉ W. Xia
xiaweibo8301@163.com

[1] Department of Endocrinology, Key Laboratory of Endocrinology, Ministry of Health, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Shuaifuyuan No. 1, Wangfujing, Dongcheng District, Beijing 100730, China

severe syndrome of rickets [6]. Patients with PDDR develop growth retardation, hypotonia, weakness, and sometimes accompanied by hypocalcemic seizures in early infancy [7–9]. Apart from low circulating levels of $1,25(OH)_2D_3$, biochemical features include hypocalcemia, secondary hyperparathyroidism, and normal to elevated levels of 25-hydroxyvitamin D (25OH D) [10].

To date, approximately 100 patients with PDDR have been reported in the literature [9, 11, 12]. The causative gene CYP27B1 encoding vitamin D 1α-hydroxylase (NM_000785) was mapped to chromosome 12q13.3, which consists of nine exons spanning 4859 bases. Various types of mutations in the CYP27B1 gene have been identified, including missense and nonsense mutations, deletions, insertions, and splice-site mutations [9]. In our previous study [9], we described nine novel mutations and correlated activity reduction of 1α-hydroxylase in eight Chinese families. To our best knowledge, 48 mutations in the CYP27B1 gene have been identified so far [9, 13–15]. 1α-hydroxylase, a member of the mitochondrial cytochrome P450 enzyme family, shows a remarkable conservation of the topology and tertiary structure. Erdem Durmaz' et al. analyzed seven patients from four Turkish families with PDDR and showed there is a good genotype-phenotype correlation in PDDR [16]. They found that some patients may recover from the loss of CYP27B1 function, probably due to 1α-hydroxylase activity exerted by a non-CYP27B1 enzyme [16].

Physiological doses of 1α(OH) vitamin D_3 (1α-OHD) or $1,25(OH)_2D_3$ induce reversal of all the clinical, biochemical, and radiological abnormalities related to PDDR [17, 18]. In Dardenne O's study [19], by feeding 1α-OHase mutant animal models with high-calcium diet, the PDDR phenotype could be rescued, while bone growth remained impaired, implicating the high-calcium diet does not appear as effective as $1,25(OH)_2D_3$. Edouard T et al.'s study showed that treatment with calcitriol started in infancy results in short- and long-term correction of the clinical and laboratory abnormalities without serious adverse events [20]. PDDR, as a rare disease, makes it difficult to perform large-scale studies of clinical response to treatment by active vitamin D. It still remains unclear whether treatment response by active vitamin D is related with the genotype of PDDR patients.

In the current study, we analyzed the CYP27B1 gene mutations in seven Chinese patients diagnosed with PDDR by sequence analysis. In order to further investigate the response to short-term treatment with calcitriol, we additionally analyzed the clinical data of eight PDDR patients reported in our previous study [9].

Subjects and methods

Subjects

From 2010 to 2011, we recruited six pedigrees (seven patients) with 1α-hydroxylase deficiency from the outpatient clinic at Department of Endocrinology, Peking Union Medical College Hospital (PUMCH), China. Four male and three female patients aged from 3 to 20 years old were investigated. All the subjects were from Han ethnic group and from nonconsanguineous parents.

This study was approved by the Department of Scientific Research at Peking Union Medical College Hospital. Written informed consent was obtained from the patients and their family members included in this study.

Biochemical assessment

Fasted whole blood samples were placed at room temperature for 30 min and then centrifuged at 3000 r/min for 3 min to separate the serum for analysis. Reference ranges were obtained from the central laboratory of PUMCH and were all age/sex/ethnically appropriate. Serum 25-hydroxyvitamin D and serum intact PTH levels were assayed by an automated Roche electrochemiluminescence system (E 170; Roche Diagnostics, Basel, Switzerland). Serum $1,25(OH)_2D_3$ levels were determined by a 1,25-dihydroxyvitamin D 125I RIA kit (Diasorin, USA). Both fasted spot urine and full-day urine collections were obtained. Assays were performed according to the manufacturer's protocol.

Imaging techniques

Radiographic studies were performed in the Department of Radiology at the Peking Union Medical College Hospital. Plain X-ray of the lower extremities was performed to detect bone deformities.

DNA sequence analysis of CYP27B1

Genomic DNA was extracted from peripheral leukocytes of seven patients and some of their parents and siblings, using a QIAamp DNA Blood Mini Kit (50) (Qiagen, Germany). All nine exons and exon-intron boundaries of the CYP27B1 gene were amplified by PCR, with eight pairs of primers designed using software Oligo 7.0 (Supplemental Table). Taq DNA polymerase (Takara, Japan) and its standard buffer were used in all reactions under the following conditions: initial denaturation at 95 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 50–58 °C for 30 s, and 72 °C for 50 s. The amplified products were sequenced by an automated sequencer (ABI3730XL) according to the manufacturer's protocol. Sequence alignment was performed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information database. We used an in silico prediction method to analyze the identified variants (http://genetics.bwh.harvard.edu/pph).

Long-term treatment of calcitriol

Except for the seven patients recruited in the current study, we also retrospectively analyzed the clinical data of another eight patients from our previous study [9]. In all 15 patients, clinical data were collected at the date of diagnosis, 3 and 6 months and 1 and 2 years after treatment with calcitriol. After starting calcitriol treatment in newly diagnosed children, serum levels of calcium, phosphate, alkaline phosphatase, and PTH as well as 24-h urinary calcium and phosphate were measured at each time point during the treatment.

Statistical analysis

Data were presented as median. All statistical analyses were conducted using Numpy 1.14 and Scipy 1.0. Wilcoxon's rank-sum test was conducted to compare differences between pairwise groups. Spearman rank correlation coefficients were calculated to describe the association between change of height after calcitriol treatment and duration of treatment as well as age at diagnosis. *P* values less than 0.05 were considered significant.

Results

Clinical characteristics of the seven newly recruited patients with PDDR

Clinical features of the seven subjects on admission are summarized in Table 1 and Fig. 1. The initial symptoms of these patients all appeared within the first 2 years of life. The height of these patients was all below the third percentile for age. Baseline biochemical parameters are summarized in Table 2. The diagnosis of pseudovitamin D-deficiency rickets (PDDR) was based on the clinical manifestations and laboratory findings. Except for patient 7, all the other patients responded well to treatment with calcium and 1,25-$(OH)_2D_3$ supplementation.

Patient 1 (Fig. 2a), a 3-year-old boy, presented with leg deformity and skelalgia at the age of 1 year and was unable to walk steadily until 16 months of age. He had been administered with calcium occasionally before coming to our clinic. Physical examination showed short stature, waddling gait, slightly thinning hair, skull deformities, and sparse and irregularly arranged teeth. He also presented with pectus carinatum, prominent rachitic rosary, enlargement of wrists and ankles, genu varum, and saber-like bowed legs. Laboratory data showed hypocalcemia, normal serum phosphorus, elevated ALP and PTH levels, normal $25(OH)D_3$, and low $1,25(OH)_2D_3$ concentration.

Patient 2 (Fig. 2b), a 5-year-old boy, had suffered from diarrhea frequently until 2 months of age. He developed recurrent tetany cramps and frequently sweating at the age of 8 months, without measurement of serum calcium. He began teething at 10 months of age with sparse teeth and was unable to walk steadily until 17 months of age. He was found to be hypocalcemic and developed bowing of legs at the age of 1 year and had been administered with calcium since then. He was easy to catch a cold. Physical examination showed microsomia, murky coarse sparse teeth, rachitic rosary, enlargement of wrists and ankles, and genu varum. Laboratory data showed normal serum calcium and phosphorus, elevated ALP and PTH levels, normal $25(OH)D_3$, and $1,25(OH)_2D_3$ concentration.

Patient 3 (Fig. 2c), a 3-year-old girl, with normal teething and walking time, developed walking difficulty at the age of 24 months. She was unwilling to stand and walk before coming to our clinic. Physical examination showed short stature, thick hair, enlarged wrists and ankles, slight pectus carinatum, and rachitic rosary. Laboratory data showed normal serum calcium and hypophosphatemia, elevated ALP and PTH levels, low $1,25(OH)_2D_3$, and normal $25(OH)D_3$ concentration.

Patient 4 (Fig. 2d), an 11-year-old girl, was able to walk at the age of 11 months. She developed lower extremity deformities and was diagnosed with "hypophosphatemic rickets" at the age of 17 months. She had been treated with phosphate and active vitamin D supplementation since then. She changed teeth by the age of 8 years without eruption of permanent teeth. She presented with skelalgia, muscle weakness,

Table 1 Clinical characteristics of the seven PDDR patients

Patient	Gender	Birth	Age at consult	Height (percentage)	Growth retardation	Walking difficulty	Leg pain	Leg deformity	Bracelet of wrist	Rachitic rosary
1	M	Jan. 2007	3 years 7 months	< 3%	+	+	+	Saber-like bowed legs	+	+
2	M	Oct. 2005	4 years 5 months	< 3%	+	+	+	Genu varum	+	+
3	F	Oct. 2008	2 years 5 months	< 3%	+	+	+	Slight genu varum	+	+
4	F	Oct. 2000	10 years 10 months	< 3%	+	+	+	Genu valgum	+	+
5	M	Sep. 1998	12 years 6 months	< 3%	+	+	+	Genu varum	+	+
6	F	Sep. 1991	19 years 6 months	< 3%	+	+	+	Genu valgum	+	+
7	M	Jan. 2003	8 years 6 months	< 3%	+	+	+	Saber-like bowed legs	+	+

M denotes male, *F* denotes female

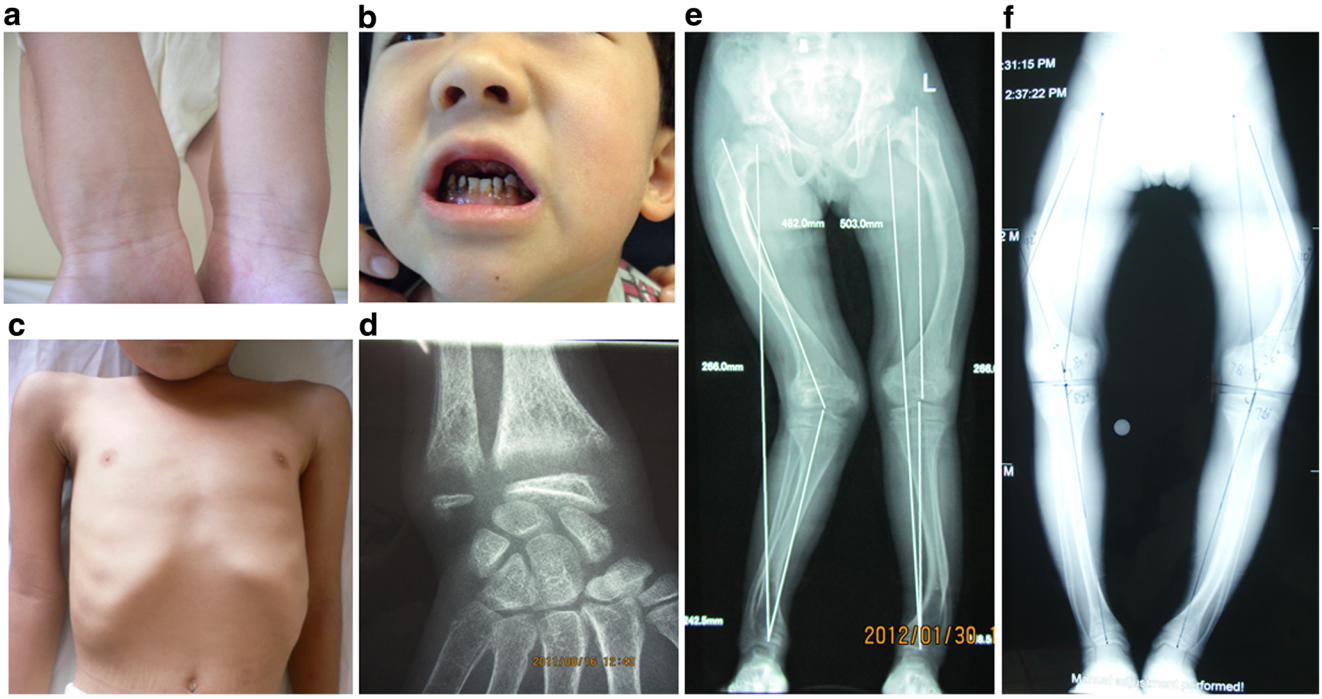

Fig. 1 The clinical characteristics and X-ray features of PDDR patients. **a** Patient 4 had enlargement of wrists. **b** Patient 3 had odontodysplasia, with murky gray and sparse teeth. **c** Patient 4 showed rachitic rosary. **d** Patient 4 showed cupping and fraying of the metaphyseal region of wrists. **e** Patient 4 presented with genu valgum. **f** Patient 5 presented with genu varum

lower extremities numbness, and tetany cramps on admission. Physical examination showed microsomia, swaying gait, muscle weakness, sparse teeth, rachitic rosary, and enlargement of wrists and ankles. Laboratory data showed hypocalcemia, normal serum phosphorus, and elevated ALP and PTH levels.

Patient 5 (Fig. 2e), a 13-year-old boy, presented with pillow bald at the age of 6 months, teething at 10 months, and was not able to walk steadily until 16 months of age. He developed hypocalcemic seizures and muscle stiffness and had been treated with calcium supplementation since 8 months of age. Physical examination showed short stature, waddling gait, irregularly arranged murky teeth, and genu varum. Laboratory data showed hypocalcemia, normal serum phosphorus, and elevated ALP and PTH levels. X-ray showed genu varum and cupping and fraying of the metaphyseal region of wrists, consistent with the features of rickets.

Patient 6 (Fig. 2e), sister of patient 5, 20 years old, could not walk steadily until 15 months of age and developed tetany cramps at the age of 8 years. She presented with scoliosis at the age of 5 years, which aggravated progressively, and took scoliosis surgery at the age of 19 years. She walked into our clinic with the help of crutch. Physical examination showed scoliosis, murky gray and dense teeth, rachitic rosary, enlargement of wrists and ankles, and genu valgum. Laboratory data

Table 2 Baseline biochemical parameters of the seven PDDR patients

Patient	Serum Ca (mmol/L)	Serum P (mmol/L)	Serum ALP (U/L)	Serum PTH (pg/mL)	Serum $1,25(OH)_2D_3$ (pg/ml)	Serum $25(OH)D_3$ (ng/mL)	Glycosuria	Amino aciduria	Proteinuria	24-h urine Ca (mmol)	24-h urine P (mmol)
1	1.29↓	1.50	800↑	363↑	5.82↓	43.4	-	-	-	0.11	4.88
2[a]	2.21	1.24	675↑	225↑	51.7	21.2	-	NA	-	0.33	3.51
3	2.18	0.70↓	1859↑	608↑	7.06↓	22.2	-	+	TRACE	0.18	4.21
4	1.96↓	1.08↓	1128↑	526↑	NA	NA	NA	NA	NA	0.50	NA
5	1.56↓	1.41	2396↑	732↑	59.0[b]↑	19.7	-	±	-	0.14	NA
6	2.32	1.12	115	205↑	22.8	63.4↑	-	NA	0.3 g/L	0.28	7.2
7	1.68↓	1.18	1844↑	781↑	5.01↓	21.1	-	NA	TRACE-(+)	0.06	6.15
Normal range	2.13–2.70	1.09–1.87	42–390	12–65	14.1–56.5	8–50	-	-	< 0.25 g/L		

NA, not available; *ALP*, alkaline phosphatase; *PTH*, parathyroid hormone

[a] Patient 2 has been receiving treatment with calcium and vitamin D

[b] Measured after alpha calcitriol treatment

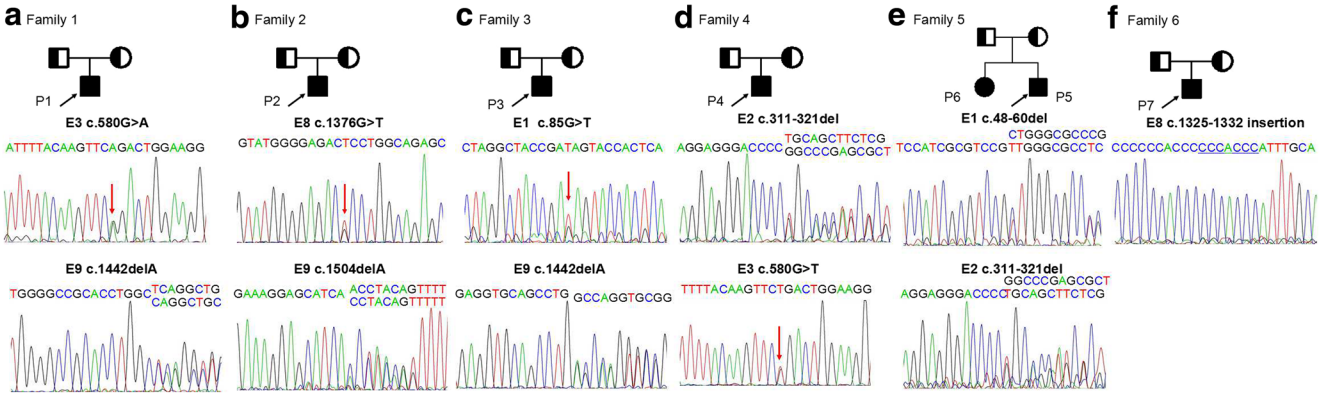

Fig. 2 **a**–**f** Sequence analysis of human CYP27B1 gene and family pedigrees. All nine exons and exon-intron boundaries of the CYP27B1 gene were amplified by PCR, and then directly sequenced. Mutations found in six Chinese families with PDDR are shown. The affected probands are indicated by black arrows. Compounds heterozygotes are indicated by solid circles (females) or squares (males), and heterozygotes are indicated by half solid circles or squares. The missense mutations are indicated by red arrows at the positions of nucleotide substitutions. The insertion mutation is shown by the blue dash underlying the nucleotides

showed normal serum calcium, phosphorus, ALP, $1,25(OH)_2D_3$, elevated ALP, and $25(OH)D_3$ concentration.

Patient 7 (Fig. 2f), an 8-year-old boy, was not able to walk until 3 years old. He was diagnosed as vitamin D-dependent rickets at 2 years old and had been treated with active vitamin D and calcium occasionally. Physical examination showed cephalus quadratus, enlarged wrists and ankles, costal margin valgus, rachitic rosary, Harrison's groove, and genu varum. X-ray showed decreased bone mineral density, thinning cortical bone, cupping and fraying of the metaphyseal region, and saber-like deformity of tibia and fibula bones.

Sequence analysis of the CYP27B1 gene

In the genetic analysis, nine mutations were found in the CYP27B1 gene (Table 3). Seven novel mutations were identified: two missense mutations (c.580G>A, c.1376G>T), three deletions (c1504delA, c311-321del, c.1442delA), and two nonsense (c.85G>T, c.580G>T) mutations. No identical mutations were detected in the 50 unrelated control samples (data not shown).

Patient 1 was compound heterozygous for two novel mutations (Fig. 2a). The missense mutation c.580G>A results in the substitution of glycine to arginine at codon 194. The deletion c.1442delA, which was also found in patient 3, eliminated the normal termination signal and allowed the translation of additional 31 base pairs downstream.

Patient 2 was also compound heterozygous for two novel mutations (Fig. 2b). The missense mutation c.1376G>T in exon 8 results in the substitution of arginine to leucine at codon 459. The deletion c.1504delA in exon 9, which altered the reading frame after codon 502, also resulted in the translation of additional 31 base pairs downstream.

Patient 3 was compound heterozygous for two novel mutations c.1442delA and c.85G>T (Fig. 2c). The deletion c.1442delA was also found in patient 1. The novel nonsense mutation c.85G>T (E29X) in exon 1 altered the reading frame downstream of codon 29, which resulted in a truncated protein containing 29 amino acids.

Table 3 Nine CYP27B1 mutations in seven Chinese patients with PDDR

Mutation	Nucleotide change	Amino acid change	Mutation type	Exon no.	Patient no.
1	c.48-60delCTGGGCGC CCGAG	Frameshift after 16L	Deletion	1	5, 6
2[a]	c.85G>T	E29X	Nonsense mutation	1	3
3[a]	c.311-321delGGCCCGAGCGC	Frameshift after 104R	Deletion	2	4, 5, 6
4[a]	c.580G>T	G194X	Nonsense mutation	3	4
5[a]	c.580G>A	G194R	Missense mutation	3	1
6	c.1325-1332 ins CCCACCC	Frameshift after 441H	Insertion	8	7
7[a]	c.1376G>T	R459L	Missense mutation	8	2
8[a]	c.1442delA	Frameshift after 481E	Deletion	9	1,3
9[a]	c.1504delA	Frameshift after 502N	Deletion	9	2

[a] Novel mutations found in this study

Patient 4 was also compound heterozygous for two novel mutations c.311-321delGGCCCGAGCGC in exon 2 and c.580G>T (G194X) in exon 3 (Fig. 2d). The deletion c.311-321del, which was also found in patients 5 and 6, caused the frameshift after 104R and resulted in a truncated protein containing 36 amino acids.

Patients 5 and 6 were compound heterozygous for a reported mutation c.48-60delCTGGGCGCCCGAG and a novel mutation c.311-321del (Fig. 2e), as found in patient 4. The deletion c.48-60del altered the reading frame downstream of codon 16.

Patient 7 was homozygous for a known c.1325-1332 ins CCCACCC insertion mutation in exon 8 (Fig. 2f), which altered the reading frame downstream of codon 441 and created a premature TGA stop signal at codon 446.

Clinical outcome after long-term treatment with active vitamin D_3

All the 15 patients had been given active vitamin D and calcium supplementation after diagnosis. The dosage of active vitamin D for each patient was adjusted according to their biochemical parameters during the treatment. The aims of the treatment were to achieve normocalcemia, to maintain PTH levels within normal limits, and to avoid hypercalciuria. We measured serum PTH, ALP, urine calcium, and urine phosphate from diagnosis to 13 years after treatment. At the time of the pretreatment evaluation, 11 of the 15 patients had hypocalcemia and 12 out of 15 had high alkaline phosphatase levels (on average threefold above the upper limit of the reference range). Baseline PTH levels, documented in 14 patients, were all high (on average sevenfold above the upper limit of the assay specific reference range), indicating secondary hyperparathyroidism.

The statistical analysis revealed that PTH and ALP significantly decreased after 6-month and 1-year treatment with calcitriol respectively and maintained within normal range during the follow-up period (Fig. 3). Urine calcium was measured in all the 15 patients (median 1.85 mmol/24 h, range 0.06–10.88 mmol/24 h) without kidney stones or nephrocalcinosis being documented. The height change of the patients is positively related to the duration of the treatment ($r = 0.772$, $p = 0.009$) during the first 2 years (data not shown). Two patients had reached their final height during the treatment, and all of them had short stature (height Z-score at or below − 2.0). X-ray showed that after 6-year treatment by calcitriol, the cupping and fraying of the metaphyseal region had been significantly improved, while the decrease of bone mineral density could still be observed (Fig. 3a, b). Figure 3c showed saber-like deformity of the tibia and fibula had not been changed after 8-year treatment by calcitriol.

Discussion

In the present study, we reported six new Chinese kindreds with 1-αhydroxylase deficiency and identified nine different mutations including seven novel mutations of CYP27B1 in these families. All of the patients were proven to have mutations in both alleles. To date, a total of 54 mutations of CYP27B1 have been identified including the seven novel mutations found in this study. In our previous and current study, we found 17 mutations in 15 Chinese PDDR patients [9], which are mostly distributed in five exons: E1 (17.6%), E2 (11.8%), E3 (11.8%), E8 (29.4%), E9 (23.5%). Most mutations associated with PDDR lead to a total loss of 1α hydroxylase activity when expressed in vitro [21].

The CYP27B1 gene consists of nine exons, encoding a 509-residue protein, with the initiation codon located in exon 1. Yamamoto et al. constructed a three-dimensional structure of CYP27B1 by the homology modeling technique, thus enabled us to predict the function of other mutation residues responsible for PDDR [22]. The three-dimensional (3D) structure of CYP27B1 contains 17 helices and 6 β-strands [22, 23]. The transient N-terminal region and the following region of CYP27B1 (Met1-Ala38) were not constructed because the coordinates of the corresponding region of CYP2C5 were not determined [22].

The c.48-60del mutation, a 13-bp deletion located in exon 1, has been found in patient 5 and his sister, which has also been found in our previous study (patient 7) [9]. This mutation resulted in a truncated protein containing only 21 amino acids. Patients 5 and 6 also carried another deletion mutation, c.311-321del, which created a premature TGA stop signal at codon 328, forming a truncated protein with 181 amino acids reduced. These two deletions may explain the severe manifestations in these two patients. Both of them presented with hypocalcemic seizures and had murky teeth. Patient 6 presented with scoliosis and undertook scoliosis surgery. Patient 5 had been treated with calcium supplementation since 8 months, which may partially explain why he did not develop with scoliosis.

The novel nonsense mutation c.85G>T (E29X), which was found in patient 3, resulted in a truncated protein containing 29 amino acids. This mutation changes the first structure of 1α-hydroxylase which is supposed to abolish the enzyme activity. The other mutation found in patient 3 is c.1442delA, also been found in patient 1, which generated an abnormal protein containing 540 base pairs (extended by 31 nucleotides), which changes the structure of a β-sheet that binds substrate [18, 19]. Both of the mutations are supposed to reduce the activity of 1α-hydroxylase significantly and may explain the relatively high PTH and ALP levels in patient 3.

Another novel nonsense mutation c.580G>T (G194X), found in patient 4, resulted in an early termination of signal at codon 194 instead of codon 509 and produced a truncated

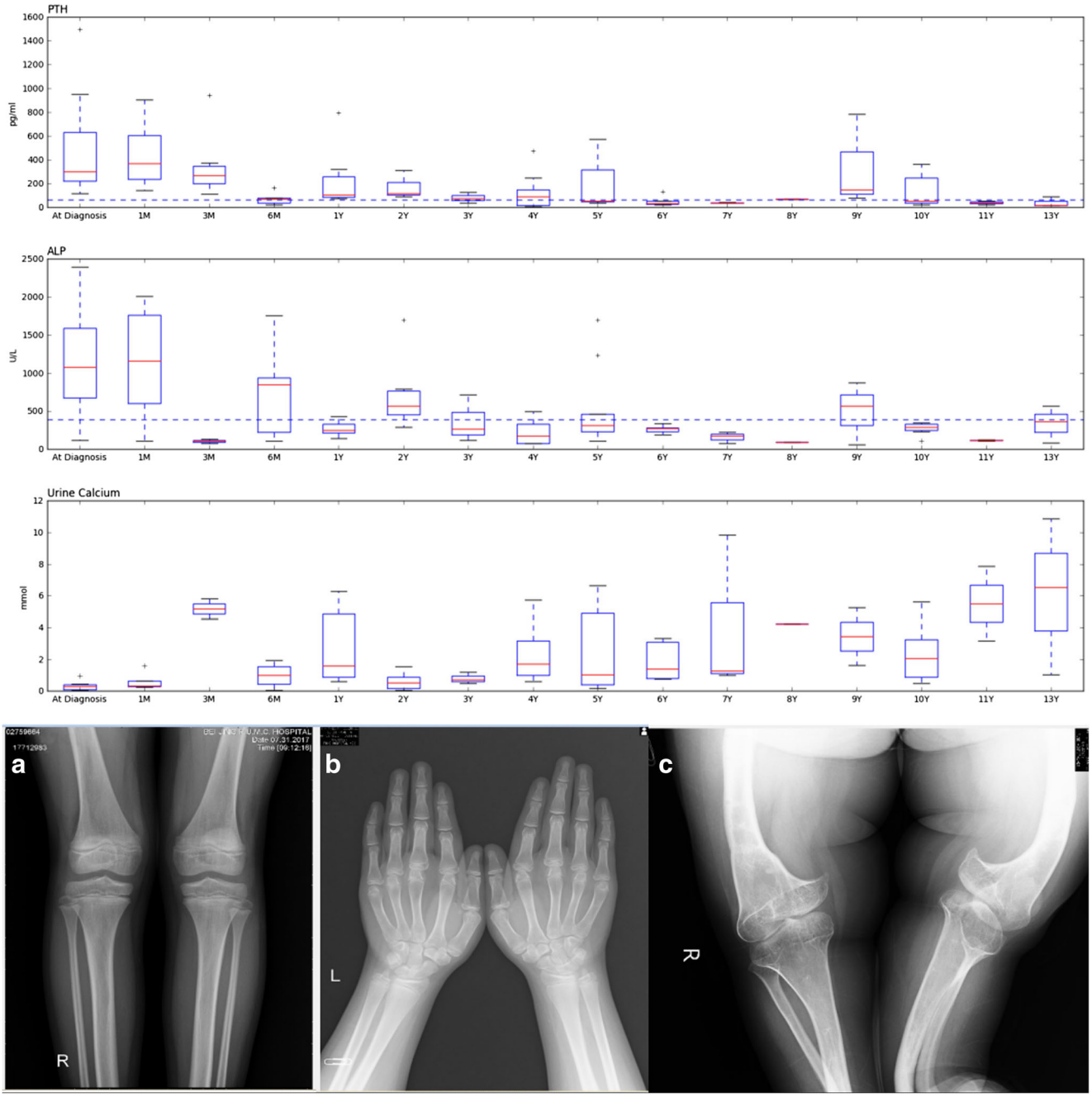

Fig. 3 PTH (pg/mL), ALP (U/L), and urine calcium (mmol/24 h) levels of the 15 PDDR patients during the 13 years of treatment with calcitriol. X-ray showed that after 6-year treatment by calcitriol, the cupping and fraying of the metaphyseal region had been significantly improved, while the decrease of bone mineral density could still be observed in patients 3 and 7 recruited in this study (**a**, **b**). Saber-like deformity of the tibia and fibula had not been changed after 8-year treatment by calcitriol (**c**)

protein with 315 amino acids reduced. This mutation changes the first structure of the enzyme, which is supposed to abolish the enzyme activities too. The deletion c.311-321del was found in patient 4 and family 5 who are from two unrelated families. This may implicate that the c.311-321del would probably be another common mutation in Chinese Han race. Patient 4 was relatively severe both in clinical manifestations and biochemical parameters, which is consistent with the genotype.

The insertion c.1325-1332insCCCACCC, found in patient 7, has been reported in many ethnic groups. The 7-bp insertion in exon 8 altered the reading frame after codon 441 and disrupted the heme-binding domain. This mutation has also been found in our previous study [9]. Our findings further

confirmed that this insertion is a common mutation distributed widely. The novel missense mutation c.580G>A (G194R) found in patient 1 locates in E helix, which plays an important role in protein folding. The deletion c.1442delA was found in both patients 1 and 3, who are from two unrelated families. It implicates that the c.1442delA may be another mutation hot spot in Chinese Han race.

The missense mutation c.1376G>T (R459L) was found in patient 2. The heme is sandwiched between the L helix including its N-terminal loop and the I helix. The sulfide of C455 provides the axial ligand at the fifth coordination site of the heme iron [22]. The mutant R459L locates in the L helix and is just four residues away from the thiolate cysteine 455, which is also highly conserved. Wang JT et al. reported a similar mutant R453C, which disrupts a salt bridge that interacts with the heme propionate and influences heme binding [21]. Another novel deletion c.1504delA was also found in patient 2, which altered the reading frame after codon 502, and generated an abnormal protein containing 540 base pairs. This deletion, similar with c.1442delA, changes the structure of a β-sheet that binds substrate.

Patient 1 and patient 2 are both compound heterozygous for a missense mutation and a deletion. Interestingly, both of the deletion (c.1442delA and c.1504delA) resulted in an extended protein containing 540 base pairs. It seems that these two patients share the same onset of age and similar levels of PTH and ALP. While compared with patients 3, 4, 5 and 7, who carried either nonsense mutation or deletion/insertion, their PTH and ALP are relatively lower, elucidating the correlation between phenotype and genotype. However, bone deformities, $25(OH)D_3$, and $1,25(OH)_2D_3$ concentration are not consistent with the genotype, suggesting that some other endogenous or exogenous factors and intake of calcium and vitamin D metabolites may influence the 1α-hydroxylase activity as well.

In the present study, after 1-year treatment with calcitriol, serum ALP and PTH significantly decreased to nearly normal range. While in Edouard T's study [20], treatment with calcitriol resulted in the normalization of serum levels of calcium, phosphate, alkaline phosphatase, and PTH within 3 months. The correction delay of these parameters in our study could possibly be explained by the difference of initial calcitriol dosage. Their treatment was started on an outpatient basis at a dose of 1.0μg/day, given in two doses of 0.5 μg. However, in our study, the median initiation daily calcitriol dose was 0.5 μg/day (range 0.25–1.5 μg).

In the long-term follow-up period, ALP and PTH maintained within normal range and no kidney stones had been observed, which implicated that the dosage used in our study could correct the biochemical abnormalities as well as avoiding hypercalciuria. Edouard T et al. observed that lumbar spine areal BMD also normalized within 3 months, which further elucidated that even having low levels of $1,25(OH)_2D_3$ during childhood is compatible with achieving a normal peak bone mass [20, 24]. After the correction of the biochemical parameters, the radiographic abnormalities had also been improved. Height change of the patients is positively related to the duration of treatment in the first 2 years. Two patient with final height Z-score below −2.0 both had received calcitriol after puberty, which implicated the importance for early diagnosis and calcitriol treatment. Our findings were consistent with Edouard T et al.'s study [20].

There are some limitations in our study. We have not conducted the functional analysis of the mutant 1α-hydroxylase activity in the newly recruited patients. The treatment response of our patients varied with treatment history and was significantly associated with the age at which calcitriol treatment was started. The height change data during the follow-up were not available for further analysis of the response to long-term treatment.

Conclusion

In conclusion, we described seven novel mutations of the CYP27B1 gene in seven Chinese families with PDDR in addition to two known mutations, which updated the database of CYP27B1 gene mutations. The mutations c.311-321del and c.1442delA would probably be the hot spot mutations in Chinese Han race. Our findings revealed after 1-year treatment of active vitamin D_3, PTH and ALP significantly decreased. The correction of the biochemical abnormalities had not improved the final height satisfactorily. Further studies are needed to determine whether the response to treatment was correlated with genotype.

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Compliance with ethical standards

This study was approved by the Department of Scientific Research at Peking Union Medical College Hospital. Written informed consent was obtained from the patients and their family members included in this study.

Conflicts of interest None.

References

1. Bikle DD (2010) Vitamin D: newly discovered actions require reconsideration of physiologic requirements. Trends Endocrinol Metab 21:375–384
2. Ponchon G, Kennan AL, Deluca HF (1969) "Activation" of vitamin D by the liver. J Clin Invest 48:2032–2037
3. Prosser DE, Jones G (2004) Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 29:664–673
4. Sakaki T, Kagawa N, Yamamoto K, Inouye K (2005) Metabolism of vitamin D3 by cytochromes P450. Front Biosci 10:119–134
5. Zehnder D, Bland R, Williams MC, et al. 2001 Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. J Clin Endocrinol Metab 86(2):888-894
6. Michael F, Holick (2006) Resurrection of vitamin D deficiency and rickets. J Clin Invest 116(8):2062–2072
7. Kitanaka S, Takeyama K, Murayama A, Sato T, Okumura K, Nogami M, Hasegawa Y, Niimi H, Yanagisawa J, Tanaka T, Kato S (1998) Inactivating mutations in the 25-hydroxyvitamin D3 1alpha-hydroxylase gene in patients with pseudovitamin D-deficiency rickets. N Engl J Med 338:653–661
8. Kim CJ, Kaplan LE, Perwad F, Huang N, Sharma A, Choi Y, Miller WL, Portale AA (2007) Vitamin D 1alpha-hydroxylase gene mutations in patients with 1alpha-hydroxylase deficiency. J Clin Endocrinol Metab 92:3177–3182
9. Ningyi C, Weibo X, Hua S, Li P et al (2012) Novel mutations of CYP27B1 gene lead to reduced activity of 1α-hydroxylase in Chinese patients. Bone 51:563–569
10. Scriver CR, Reade TM, Deluca HF, Hamstra AJ (1978) Serum 1, 25-dihydroxyvitamin D levels in normal subjects and in patients with hereditary rickets or bone disease. N Engl J Med 299:976–979
11. Wang X, Zhang MY, Miller WL, Portale AA (2002) Novel gene mutations in patients with 1alpha-hydroxylase deficiency that confer partial enzyme activity in vitro. J Clin Endocrinol Metab 87:2424–2430
12. Kim CJ, Kaplan LE, Perward F, Huang N, Sharma A, Choi Y, Miller WL, Portale AA (2007) Vitamin D 1α-hydroxylase gene mutations in patients with 1α-hydroxylase deficiency. J Clin Endocrinol Metab 92:3177–3182
13. Kim CJ, Kaplan LE, Perwad F, Huang N, Sharma A, Choi Y, Miller WL, Portale AA (2007) Vitamin D 1alpha-hydroxylase gene mutations in patients with 1alpha-hydroxylase deficiency. J Clin Endocrinol Metab 92:3177–3182
14. Alzahrani Ali S, Zou M, Baitei EY (2010) A novel G102E mutation of CYP27B1 in a large family with vitamin D-dependent rickets type 1. J Clin Endocrinol Metab 95:4176–4183
15. E D, Zou M, Al-Rijjal RA, Bircan I, Akçurin S, Meyer B, Shi Y (2012) Clinical and genetic analysis of patients with vitamin D-dependent rickets type 1A. Clin Endocrinol 77(3):363–369
16. Durmaz E, Zou M, Al-Rijjal RA, Bircan I, Akçurin S, Meyer B, Shi Y (2012) Clinical and genetic analysis of patients with vitamin D-dependent rickets type 1A. Clin Endocrinol (Oxf) 77(3):363–369
17. Fraser D, Kooh SW, Kind HP, Holick MF, Tanaka Y, Deluca HF (1973) Pathogenesis of hereditary vitamin D-dependent rickets. An inborn error of vitamin D metabolism involving defective conversion of 25-hydroxyvitamin D to 1α,25-dihydroxyvitamin D. N Engl J Med 289:817–822
18. Porcu L, Meloni A, Casula L, Asunis I, Marini MG, Cao A, Moi P (2002) A novel splicing defect (IVS6+1G>T) in a patient with pseudovitamin D deficiency rickets. J Endocrinol Investig 25: 557–560
19. Anderson PH, Hendrix I, Sawyer RK, Zarrinkalam R, Manavis J, Sarvestani GT, May BK, Morris HA (2008) Co-expression of CYP27B1 enzyme with the 1.5kb CYP27B1promoter-luciferase transgene in the mouse. Mol Cell Endocrinol 285:1–9
20. Edouard T, Alos N, Chabot G, Roughley P, Glorieux FH, Rauch F (2011) Short- and long-term outcome of patients with pseudovitamin D deficiency rickets treated with calcitriol. J Clin Endocrinol Metab 96(1):82–89
21. Wang JT, Lin CJ, Burridge SM, Fu GK, Labuda M, Portale AA, Miller WL (1998) Genetics of vitamin D 1alpha-hydroxylase deficiency in 17 families. Am J Hum Genet 63(6):1694–1702
22. Yamamotoa K, Masunoa H, Sawada N, Sakaki K, Inouye K, Ishiguro M, Yamada S 2004 Homology modeling of human 25 hydroxyvitamin D3 1α hydroxylase(CYP27B1) based on the crystal structure of rabbit CYP2C5 J Steroid Biochem Mol Biol 89–90: 167–171
23. Yamamoto K, Uchida E, Urushino N et al (2005) Identification of the amino acid residue of CYP27B1 responsible for binding of 25-hydroxyvitamin D3 whose mutation causes vitamin D-dependent rickets type 1. J Biol Chem 280:30511–30516
24. Adams JS, Kantorovich V, Wu C, Javanbakht M, Hollis BW (1999) Resolution of vitamin D insufficiency in osteopenic patients results in rapid recovery of bone mineral density. J Clin Endocrinol Metab 84:2729–2730