RESEARCH NOTE

Novel vitamin D 1α-hydroxylase gene mutations in a Chinese vitamin-D-dependent rickets type I patient

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Introduction

Hereditary rickets is classified into four types: X-linked hypophosphatemic vitamin-D-resistant rickets, autosomal dominant hypophosphatemic vitamin-D-resistant rickets, vitamin-D-dependent rickets type I, and vitamin-D-dependent rickets type II, and the genes responsible are *PHEX*, *FGF23*, *CYP27B1* and *VDR*, respectively (Clausmeyer *et al.* 2009; Gribaa *et al.* 2010; Malloy *et al.* 2010). Vitamin-D-dependent rickets type I (VDDR-I, MIM 264700), also known as vitamin D 1α-hydroxylase deficiency or pseudovitamin D deficiency rickets, is a rare autosomal recessive disorder characterized by the early onset of rickets with hypocalcemia. VDDR-I is caused by mutations of the 25-hydroxyvitamin D 1α-hydroxylase gene (*CYP27B1*, MIM 609506) (Wang *et al.* 1998; Kitanaka *et al.* 1998). We identified compound heterozygous mutations in *CYP27B1* gene in a Chinese VDDR-I patient. Both mutations are novel and include a small 11 nucleotide deletion (c.311-321delGGCCCGAGCGC, p.R104LfsX225) in exon 2 that alters the downstream reading frame and creates a premature TGA stop signal at codon 328, and a missense mutation (c.473T>C, p.L158P) in exon 3 causing the amino acid change L158P. This study confirms the relationship between mutations of the *CYP27B1* gene and the clinical findings of vitamin-D-dependent rickets type I.

Materials and methods

Informed consent, laboratory testing and X-ray examination

We collected peripheral venous blood samples, data of laboratory tests, photographs and radiographs from the Chinese VDDR-I patient and his parents with informed consent and approval by the China Medical University Institutional Review Board. Laboratory tests, including serum concentration of calcium, phosphorus, alkaline phosphatase, PTH, 25(OH)D and 1,25(OH)2D were done by routine methods. We made an X-ray examination of the patient's wrist both before and after treatment.

Detection of the mutant CYP27B1 gene

Genomic DNA was extracted from white blood cells by the standard sodium dodecyl sulphate–proteinase K–phenol/chloroform method. The nine coding exons and their flanking intronic sequences of the *CYP27B1* gene were PCR amplified and then, after purification, subjected to DNA sequencing using an automated ABI PRISM3730 DNA sequencer (Applied Biosystems, Foster City, USA). Putative mutations were confirmed by duplicate PCR amplification and sequencing of the affected exons directly from genomic DNA of the patient and his parents. The small deletion, c.311-321delGGCCCGAGCGC, was identified by cloning and sequencing. The amplicons of exon 2 (patient, his parents and 80 normal controls) were separated by electrophoresis in neutral 12% polyacrylamide gel directly and the allele fragments were detected with routine silver staining. Further, to confirm the mutation c.473T>C in exon 3, a *Pvu*I restriction site was introduced into the mutant 473C allele by using a mismatch primer. The genomic DNA of the patient and the controls was used as template with the mismatch primer 5′-GGTCGCAGACTACGTCGATC-3′ (the mismatched C and A are underlined) and the sense primer of exon 3 (5′-AGGAATTGGGCTAGGGTACA-3′) to create a *Pvu*I restriction site (***C***GATCG) (the C at

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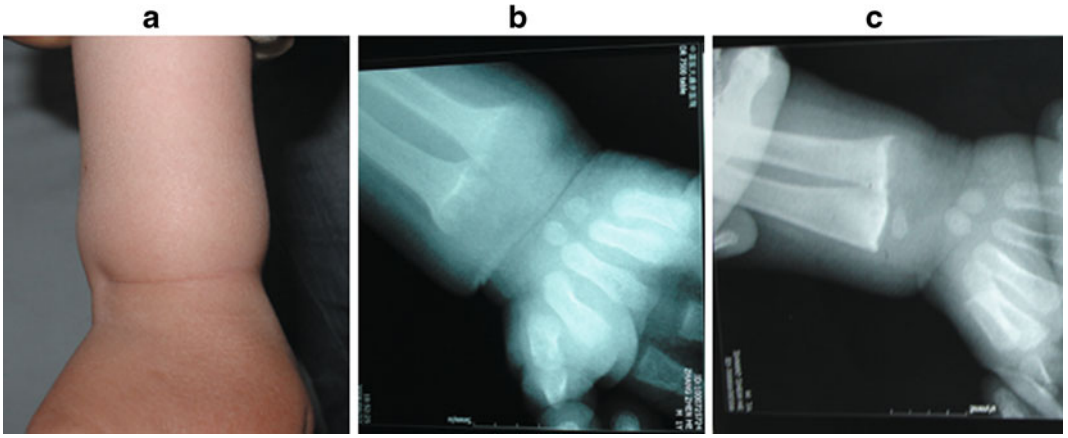

Figure 1. Photographs and radiographs of the Chinese VDDR-1 patient. (a) Photograph showing enlargement of the wrist, like wearing a bracelet. (b) Wrist radiograph before treatment presenting the vanishing of the zone of provisional calcification in distal metaphysis, rarefaction of bone trabecula and thinness of the cortical bone in the left ulna and radius. (c) Wrist radiograph after treatment showing reoccurrence of the zone of provisional calcification in distal metaphysis, the improvement of bone trabecula and thickening of the cortical bone.

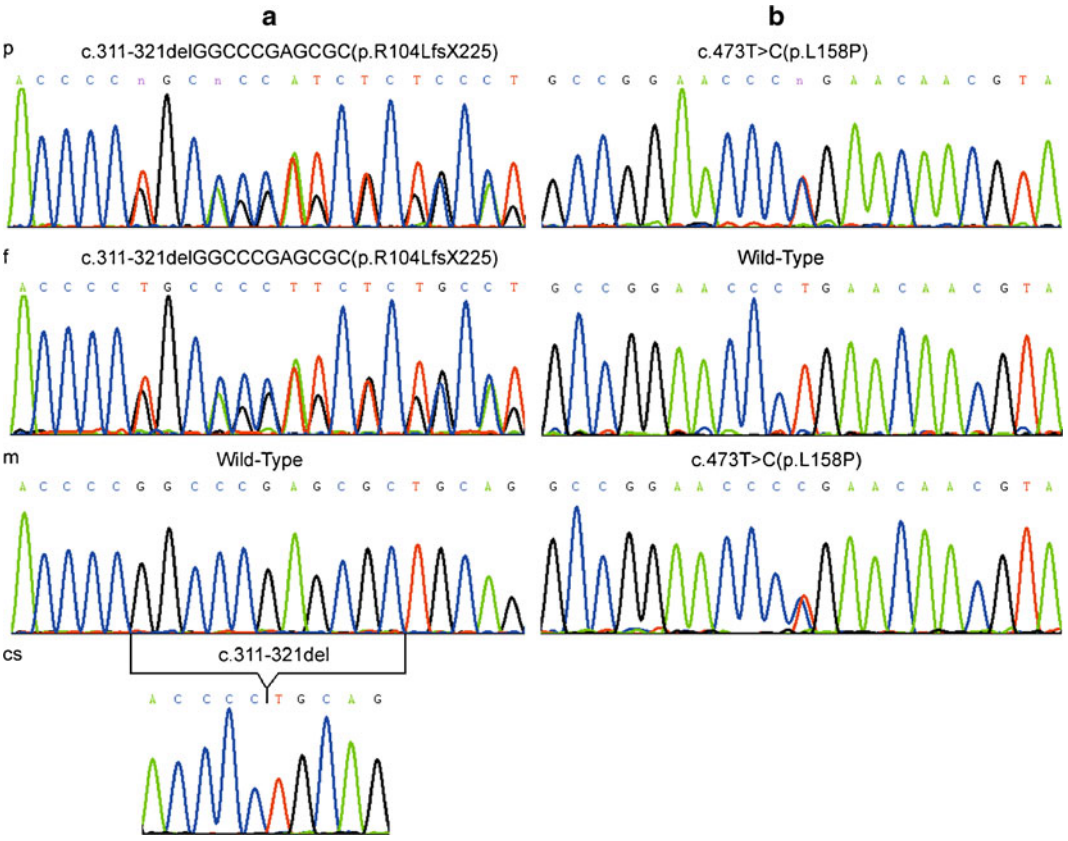

Figure 2. Novel compound heterozygous mutations in the Chinese VDDR-1 patient. Sequencing chromatogram showing the compound heterozygous small deletion c.311-321delGGCCCGAGCGC (p.R104LfsX225) and missense mutation c.473T>C (p.L158P). The deletion c.311-321delGGCCCGAGCGC (p.R104LfsX225) was from his father and the missense mutation c.473T>C (p.L158P) was from his mother. P, patient; m, mother; f, father; cs, cloning and sequencing; a, exon 2; b, exon 3.

nucleotide 473 is in bold italic) in the mutant 473C allele, but not in the normal 473T allele (TGATCG). The amplicons of the 378 bp were digested with restriction enzyme *Pvu*I and separated by electrophoresis in neutral 12% polyacrylamide gel and displayed by staining with silver.

Results

Clinical findings

The Chinese patient is a 13 month-old boy, and the diagnosis of 1α-hydroxylase deficiency was made on the basis of his clinical features and laboratory findings. He developed recurrent hypocalcemic seizures at 12 months. His physical findings included rachitic rosary, eversion of the costal margin, enlargement of the wrists and ankles and frontal bossing (figure 1a). Moreover, he had radiographic signs of rickets, including vanishing of the zone of provisional calcification in distal metaphysic, rarefaction of bone trabecula and thinness of cortical bone in left ulna and radius (figure 1b). Laboratory tests revealed hypocalcemia, increased serum concentrations of alkaline phosphatase, 25OHD and PTH, and greatly reduced concentrations of 1,25(OH)2D (data not shown). Subsequent treatments (2–6 months) with high doses of calcitriol, calcium and 1,25(OH)2D resulted in improvement or normalization of clinical, radiographic (figure 1c) and laboratory abnormalities. His parents were asymptomatic and, therefore, no laboratory tests or radiographic imaging were conducted.

Identification of novel CYP27B1 mutations

We searched for pathogenic mutations in the patient by direct sequencing of the PCR-amplified DNA fragments spanning nine exons of the *CYP27B1* gene. Sequencing revealed compound heterozygous mutations, a small deletion c.311-321delGGCCCGAGCGC (p.R104LfsX225) in the second exon, which alters the downstream reading frame from codon 104 and creates a premature TGA stop signal at codon 328, resulting in a severely truncated protein that might have no enzymatic activity; a missense mutation c.473T>C (p.L158P) in the third exon, substituting proline (P) for the highly conserved leucine (L) at position 158 of the 1α-hydroxylase, and both mutations were novel. Analysis of the family members showed that his father was heterozygous for the 11 nucleotide deletion in exon 2, and his mother was heterozygous for the c.473T>C mutation in exon 3 (figure 2). Fragment length polymorphism analysis with or without restriction endonuclease indicated that none of the mutations were present in 80 control individuals.

Discussion

Vitamin D is a biologically inactive prehormone molecule that has to be converted to its hormonally active form before it is biologically active. Vitamin D undergoes 25-hydroxylation in the liver, resulting in 25-hydroxyvitamin D (25OHD), then undergoes 1α-hydroxylation in the kidney by 25-hydroxyvitamin D 1α-hydroxylase, resulting in 1,25-dihydroxyvitamin D (1,25-(OH)2D), the hormonally active form of vitamin D (Miller and Portale 2000), which has an essential role in calcium metabolism, bone growth and cellular differentiation (Jones *et al.* 1998). The human 25-hydroxyvitamin D 1α-hydroxylase gene (*CYP27B1*) is on 12q13.3 and encodes 25-hydroxyvitamin D 1α-hydroxylase of 508 amino acids containing a ferredoxin-binding domain and a heme-binding domain. 25-Hydroxyvitamin D 1α-hydroxylase is a typical mitochondrial (type I) cytochrome P450 enzyme that functions as an oxidase, using electrons from reduced nicotinamide adenine dinucleotide phosphate and molecular oxygen.

To date, 39 different mutations in *CYP27B1* gene associated with VDDR-I have been reported, including those described here, 25 missense mutations, one nonsense mutation, six small deletions, three small insertions and four splice site mutations (Wang *et al.* 1998, 2002; Kitanaka *et al.* 1998, 1999; Yoshida *et al.* 1998; Smith *et al.* 1999; Kim *et al.* 2007). In this study, a novel small deletion and a novel missense mutation were found in different alleles. The c.311-321delGGCCCGAGCGC mutation was predicted to alter the downstream reading frame from codon 104 and create a premature TGA stop signal at codon 328, thus, the product lacked most of the wildtype amino acid residues and might have no 1α-hydroxylase activity. The L158 residue is highly conserved among mammals, all mammalian 1α-hydroxylase proteins with sequences available in the databases including human, rhesus, mouse, elephant, and opossum have leucine at position 158, which suggests a strong functional and structural constraint. The mutant L158P lie in the D-helix and might disrupt the 4-helix bundle consisting of the D, E, I and L helices, and thus it would cause a significant disruption in the P450 structure like the previously reported mutation D164N, which was localized in the same helix (Hasemann *et al.* 1995). Previous studies suggested mutations that disrupted the D, E, I and L 4-helix bundle might alter the general conformation of the enzyme and influence the functions of 1α-hydroxylase (Omdahl *et al.* 2001).

In summary, we have identified two novel mutations of the *CYP27B1* gene in a Chinese VDDR-I patient, a small deletion (c.311-321delGGCCCGAGCGC, p.R104LfsX225) and a missense mutation (c.473T>C, p.L158P). Further study of the p.L158P mutant would add to our understanding of the 1α-hydroxylase's function.

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References

Clausmeyer S., Hesse V., Clemens P. C., Engelbach M., Kreuzer M., Becker-Rose P. *et al.* 2009 Mutational analysis of the PHEX gene: novel point mutations and detection of large deletions by MLPA in patients with X-linked hypophosphatemic rickets. *Calcif. Tissue Int.* **85**, 211–220.

Gribaa M., Younes M., Bouyacoub Y., Korbaa W., Ben Charfeddine I., Touzi M. *et al.* 2010 An autosomal dominant hypophosphatemic rickets phenotype in a Tunisian family caused by a new FGF23 missense mutation. *J. Bone Miner. Metab.* **28**, 111–115.

Hasemann C. A., Kurumbail R. G., Boddupalli S. S., Peterson J. A. and Deisenhofer J. 1995 Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure* **3**, 41–62.

Jones G., Strugnell S. A. and DeLuca H. F. 1998 Current understanding of the molecular actions of vitamin D. *Physiol. Rev.* **78**, 1193–1231.

Kim C. J., Kaplan L. E., Perwad F., Huang N., Sharma A., Choi Y. *et al.* 2007 Vitamin D 1alpha-hydroxylase gene mutations in patients with 1alpha-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **92**, 3177–3182.

Kitanaka S., Takeyama K., Murayama A., Sato T., Okumura K., Nogami M. *et al.* 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.

Kitanaka S., Murayama A., Sakaki T., Inouye K., Seino Y., Fukumoto S. *et al.* 1999 No enzyme activity of 25-hydroxyvitamin D3 1alpha-hydroxylase gene product in pseudovitamin D deficiency rickets, including that with mild clinical manifestation. *J. Clin. Endocrinol. Metab.* **84**, 4111–4117.

Malloy P. J., Wang J., Srivastava T. and Feldman D. 2010 Hereditary 1,25-dihydroxyvitamin D-resistant rickets with alopecia resulting from a novel missense mutation in the DNA-binding domain of the vitamin D receptor. *Mol. Genet. Metab.* **99**, 72–79.

Miller W. L. and Portale A. A. 2000 Vitamin D 1 alpha-hydroxylase. *Trends Endocrinol. Metab.* **11**, 315–319.

Omdahl J. L., Bobrovnikova E. A., Choe S., Dwivedi P. P. and May B. K. 2001 Overview of regulatory cytochrome P450 enzymes of the vitamin D pathway. *Steroids* **66**, 381–389.

Smith S. J., Rucka A. K., Berry J. L., Davies M., Mylchreest S., Paterson C. R. *et al.* 1999 Novel mutations in the 1alpha-hydroxylase (P450c1) gene in three families with pseudovitamin D-deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages. *J. Bone Miner. Res.* **14**, 730–739.

Wang J. T., Lin C. J., Burridge S. M., Fu G. K., Labuda M., Portale A. A. and Miller W. L. 1998 Genetics of vitamin D 1alpha-hydroxylase deficiency in 17 families. *Am. J. Hum. Genet.* **63**, 1694–1702.

Wang X., Zhang M. Y., Miller W. L. and Portale A. A. 2002 Novel gene mutations in patients with 1alpha-hydroxylase deficiency that confer partial enzyme activity in vitro. *J. Clin. Endocrinol. Metab.* **87**, 2424–2430.

Yoshida T., Monkawa T., Tenenhouse H. S., Goodyer P., Shinki T., Suda T. *et al.* 1998 Two novel 1alpha-hydroxylase mutations in French-Canadians with vitamin D dependency rickets type I1. *Kidney Int.* **54**, 1437–1443.

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