Genotype and phenotype characteristics in 22 patients with Vitamin D Dependent Rickets Type I (VDDR-I)

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OBJECTIVES

Vitamin D Dependent Rickets Type I (VDDR-I) is an autosomal recessive disorder caused by mutations in the 25-Hydroxyvitamin D_3 1-alpha-hydroxylase gene (*CYP27B1*). Mutations in *CYP27B1* disrupt or lead to a total loss of the 1-alpha hydroxylase activity and require treatment with physiological doses of calcitriol. In this study we aim to evaluate the genotype and phenotype characteristics of a large cohort of VDDR-I patients.

PATIENTS AND METHODS

Patients who were admitted to our tertiary paediatric endocrine centre with the clinical (bowed legs, rachitic rosary, frontal bossing, caput quadratum, widening of wrist, Harrison's groove and developmental delay), biochemical (low serum calcium, low serum phosphorus, elevated alkaline phosphatase (ALP), elevated parathyroid hormone (PTH)) and radiological features (decreased bone density, epiphyseal broadening, cupping, fraying, greenstick fracture, chest rosaries and bowed long bone) were recruited.
Differentiation of nutritional and VDDR1 was made by;
•Normal/high 25 (OH) vitamin D,
•Low/undetectable 1,25 dihydroxyvitamin D (when applicable),
•Recovery of clinical, biochemical and radiological findings of rickets after replacement of 1,25 dihydroxyvitamin D (calcitriol).
Patients were referred from Diyarbakır, a south-eastern city of Turkey, and neighbouring cities. Although the frequency of vitamin D deficient rickets in this region is not known, climate of the region is well-known as sunny, therefore provides sufficient sunlight for individual vitamin D synthesis.

CYP27B1 gene sequencing
Informed consent was taken from all patients, or their parents for genetic testing. Genomic DNA was extracted from peripheral blood leukocytes using a standard procedure. Primers were designed to cover all 9 exons of the CYP27B1 gene, including their flanking intronic regions and splice sites. The online available Primer 3 [7, 8] software was used for this. The gene was amplified through Polymerase Chain reaction (PCR), and the products were run on agarose gel to check for size of products, and purity. The amplified products were first purified to remove primers and dNTPs, and then sequenced using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, California) kit in two separate reactions using the forward and reverse primers. The sequences were compared to a reference sequencing using the Sequencher 5.3 software [9]. Any deviations from the reference sequence were checked in the dbSNP database, 1000 Genomes Project and in Exome Variant Server, to check whether the changes are novel or known. The variants were subsequently sequenced in the patient's parents and other unaffected siblings of the patients where DNA was available.
In silico analysis of the novel variants was performed using online available protein prediction software's PolyPhen2, Mutation Taster, SiFT and Provean. These tools predict the impact of an amino acid substitution on the structure and function of the protein. These prediction programs also give information on the conservation of the amino acid sequence across species.
PSIPRED was used to predict the effect of the mutation on CYP27B1 proteins secondary structure (alpha helices, beta sheets and coils) from the primary sequence. PSIPRED incorporates two feed-forward neural networks and performs an analysis on results obtained from PSI-BLAST. Any mutations leading to a change in the secondary structure of the protein are considered to be damaging.

Mutation analysis for the *CYP27B1* gene was performed on 22 patients with the diagnosis of VDDR1. We identified 2 previously reported mutations, and 3 novel changes in our cohort. Overall all VDDR1 patients were homozygous for a mutation, with the exception of one, who had a compound heterozygote mutation. From the 36 unaffected family members, 28 had identical heterozygous mutations detected in their family, and 8 had no mutations.
The online protein prediction software predicted the damaging effects of the missense mutations. The novel P192K>E mutation was predicted to be damaging and disease causing, with PSIPRED predicting a change to the secondary structure of the protein. Similarly the p.197G>D mutation was also predicted to be damaging and disease causing.

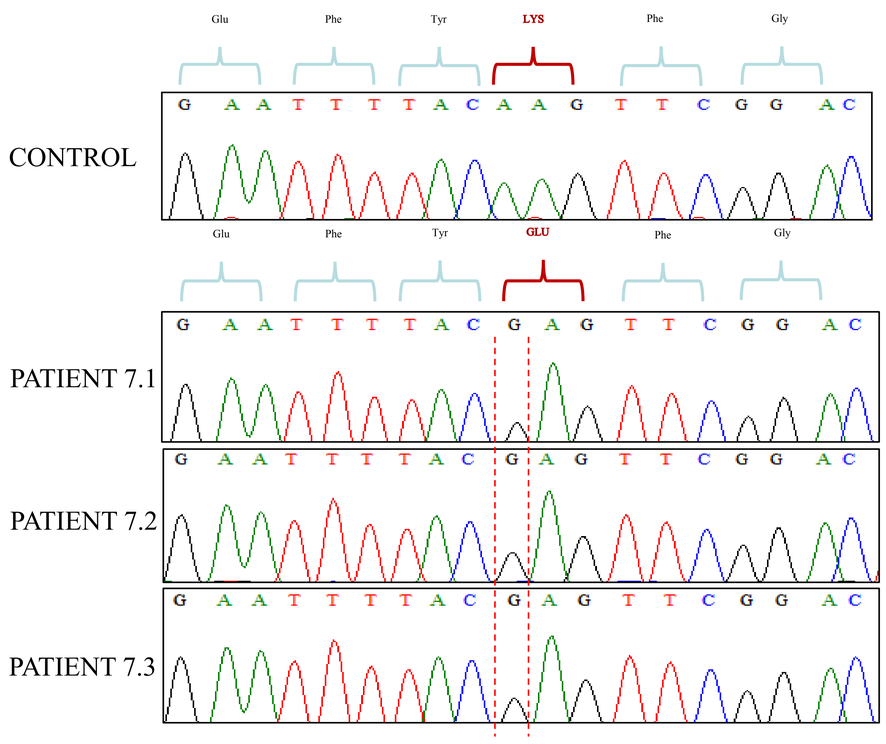

Figure 1: Sequencing results for the 3 patients in family 7 with the novel p.192K>E mutation and a control. The normal protein at position 192 is lysince, however this is substituted to glutamic acid in the 3 patients. Highlighted by the dashed line is the base pair changed in the patients.

RESULTS

Number of patients diagnosed with VDDR-I was 22 (11 female). At the time of presentation all patients had full clinical, radiological and biochemical features of rickets. The mean age of the diagnosis was 1.9±0.6 years. Median age of diagnosis was not statistically different among patients with various mutations (p=0.525). At their first visit to our clinic majority of patients (63.6%) had a height-SDS lower than -2 SD. Detailed presenting complaints, auxological and biochemical characteristics for 22 VDDR-I patients are summarized in Table 1.

Table 2. Characteristics of the mutations detected in 22 VDDR-I patients

P&F No.	Exon/intron	DNA description	Protein description	Type	Zygosity	Mutation status of unaffected family members	Evidences for pathogenicity
1.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Parents, 4 unaffected siblings and 1 cousin were carriers, 2 siblings and 5 cousins were normal for reference allele	Described previously. Functional analysis: Retention of 130 bp of intron 1 results in a frame shift and a premature stop codon. The end protein is truncated and unlikely to have any enzymatic activity.
1.2	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM		
2.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Parents are carrier	
2.2	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM		
3.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Parents are carrier	
4.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Father is carrier	
5.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Parents are carriers	
5.2	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM		
5.3	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM		
6.1	Intron 1	c.195 + 2T>G	FS after p.65Q	SS	HM	Parents are carriers	
7.1	Exon 3	c.574A>G	p.192K>E	MS	HM	Parents are carriers	Novel mutation
7.2	Exon 3	c.574A>G	p.192K>E	MS	HM		Computational analysis:
7.3	Exon 3	c.574A>G	p.192K>E	MS	HM		PolyPhen2: Probably damaging, Mutation Taster: Disease causing, SiFT: Damaging, Provean: Deleterious, PSIPRED: changes secondary structure
8.1	Exon 3	c.574A>G	p.192K>E	MS	HM	Father is carrier, unaffected sibling normal for reference	
8.2	Exon 3	c.574A>G	p.192K>E	MS	HM		
9.1	Exon 4	c.590G>A	p.197G>D	MS	HM	Parents are carriers	Novel mutation
9.2	Exon 4	c.590G>A	p.197G>D	MS	HM		Computational analysis:
10.1	Exon 4	c.590G>A	p.197G>D	MS	HM	Mother carrier	PolyPhen2: Possibly damaging,
10.2	Exon 4	c.590G>A	p.197G>D	MS	HM		Mutation Taster: Disease causing, SiFT: Damaging, Provean: Deleterious, PSIPRED: Changes secondary structure
11.1	Exon 8	1319–1325dupCCCACCC	Phe443Profs*24	Dup	HM	Parents are carriers	Previously reported seven-nucleotide duplication. Frame shift mutation which creates a premature stop signal at codon 446, and a protein without enzymatic activity.
12.1	Exon 3/ Exon 8	c.574A>G / 1319–1325dupCCCACCC	p.192K>E/ Phe443Profs*24	MS, Dup	CHT	Parents are carriers	
13.1	Exon 1	c.171_171delG	p.L58Cfs*20	Del	HM	Parents are carriers	Novel mutation Computational analysis: Mutation Taster predicts the deletion of a G nucleotide at position 171 to be disease causing.

P&F No:Patient and family number, FS: Frame shift, SS: Splice site, MS: Missense, Dup: Duplication, Del: Deletion, HM: Homozygous, CHT: Compound heterozygous

All 10 patients from family 1 to 6 were homozygous for the previously described splice donor site mutation, c.195 + 2T>G, where a thiamine is substituted for guanine in the second nucleotide of intron 1. The parents and 7 other unaffected family members (siblings and first cousins) from family 1 were found to be heterozygous carriers of the c.195 + 2T>G mutation. Asymptomatic parents of patients from family 2 to 6 were also found to be heterozygous carriers.
Five patients from family 7 and 8 were found to be homozygous for a novel missense p.192K>E (c.574A>G) mutation, substituting amino acid lysine for glutamic acid at protein position 192. Parents of the patients were heterozygous carriers.
The 4 patients from family 9 and 10 were homozygous for the novel p.197G>D (c.590G>A) mutation. The mutation substitutes the amino acid glycine with aspartic acid at protein position 197. Patient 11.1 was found to be homozygous for the previously reported 7 bp duplication mutation 1319–1325dupCCCACCC (Phe443Profs*24) in exon 8. This is a frame shift mutation which creates a premature stop signal at codon 446, and results in a truncated protein without enzymatic activity.
Patient 12.1 was compound heterozygote for the novel p.192K>E and the previously described 1319–1325dupCCCACCC mutations. This patient is the first cousin of the 3 affected members from family 7 who had p.192K>E variant in the homozygous state. The two fathers from both families are brothers and heterozygous carriers for the p.192K>E mutation. The mother of the patient 12.1 was heterozygous for the 1319–1325dupCCCACCC mutation.
In patient 13.1 we observed a novel single base pair deletion mutation at position c.171_171delG. Predicted to be disease causing by mutation taster, this change causes a frame shift from protein 58L and downstream, leading to an early stop. The difference between the wild type and mutated sequence as predicted by mutation taster is shown in Table 3. This truncated protein is likely to be devoid of any enzymatic activity.

Table 3: Prediction on the effect of the novel c.171_171delG mutation on the protein. Mutation shifts the reading frame and inserts other amino acids (bold/underlined) before leading to an early stop

Wild-type protein sequence

MTQTLKYASR	VFHRVRWAPE	LGASLGYREY	HSARRSLADI	PGPSTPSFLA	ELFCKGGLSR
LHELQVQGAA	HFGPVWLASF	GTVRTVYVAA	PALVEELLRQ	EGPRPERCSF	SPWTEHRRCR
QRACGLLTAE	GEEWQRLRSL	LAPLLLRPQA	AARYAGTLNN	VVCDLVRRLR	RQRGRGTGPP
ALVRDVAGEF	YKFGLEGIAA	VLLGSRLGCL	EAQVPPDTET	FIRAVGSVFV	STLLTMAMPH
WLRHLVPGPW	GRLCRDWDQM	FAFAQRHVER	REAEAAMRNG	GQPEKDLESG	AHLTHFLFRE
ELPAQSILGN	VTELLLAGVD	TVSNTLSWAL	YELSRHPEVQ	TALHSEITAA	LSPGSSAYPS
ATVLSQLPLL	KAVVKEVLRL	YPVVPGNSRV	PDKDIHVGDY	IIPKNTLVTL	CHYATSRDPA
QFPEPNSFRP	ARWLGEGPTP	HPFASLPFGF	GKRSCMGRRL	AELELQMALA	QILTHFEVQP
EPGAAPVRPK	TRTVLVPERS	INLQFLDR*			

Mutated protein sequence

MTQTLKYASR	VFHRVRWAPE	LGASLGYREY	HSARRSLADI	PGPSTPSFLA	ELFCKGG**CRG**
YTSCRCRAPR	**TSGRCG***				

P&F No.	F/M	Age at Diagnosis*	Current Age*	Consanguinity	Height cm (SDS)	Weight (kg)	Ca (N:2.1-2.7) (mmol/l)	P (N: 1-2.1 mmol/l)	ALP (N:145-673 U/L)	PTH (N:12-88 pg/ml)	UCCR (N:<0.2)	25 OHD (N:30-80 ng/ml)	Calcitriol dose (pg/kg)	Presenting complaints
1.1	M	3.0	7.4	Yes	74.2 (-5.5)	9.2	1.7	0.7	1705	756	0.1	48.7	81.5	Recurrent pneumonia, delay in walking
1.2	F	2.2	6.3	Yes	77 (-4.03)	8.5	1.9	0.6	3153	435	0.1	36.3	117.6	Delay in walking, hypotonia
2.1	M	2.1	3.5	Yes	73.5 (-3.74)	10.3	1.7	0.8	1245	348	0.1	154.4	48.5	Delay in dentition, bowed legs
2.2	F	1.5	7.8	Yes	69.3 (-3.9)	8.4	2.3	0.9	996	372	0.2	136.6	44.6	Delay in dentition, hypotonia
3.1	M	2 .2	4.8	Yes	75.7 (-4.3)	10.1	1.5	1.4	1873	678	0.1	27.4	49.5	Hypotonia, delay in walking
4.1	M	1.3	3.2	No?	69.5 (-3.2)	8.9	2.1	1.3	2499	148.1	0.1	46.6	56.2	Delay in walking, hypotonia
5.1	F	1.9	4.2	Yes	78.7 (-2.28)	9.20	2.0	2.0	2036	1423	0.1	58.1	108.7	Bowed legs, history of VDDR sibling
5.2	M	1.7	14.5	Yes	119.7 (-2.6)	24.7	1.8	2.1	642	643.7	0.1	47.2	30.4	Bowed legs,delay in walking, hypotonia
5.3	F	1.8	11.4	Yes	74 (-3.1)	10.0	2.1	0.7	1099	610	0.1	71.2	75.0	History of VDDR sibling
6.1	F	0.8	12.9	Yes	127.5 (-3.79)	32.0	1.4	0.7	1586	473	0.02	37.0	31.3	Seizures, widening of wrist
7.1	M	0.7	12.5	Yes	101.7 (0.32)	32.0	2.3	1.3	1540	485	0.1	37.3	7.8	Seizures, history of VDDR siblings
7.2	F	3.0	11.0	Yes	127 (0,35)	23.0	1.9	0.9	940	247	0.02	27.1	21.7	Seizures, delay in walking, pathologic bone fracture
7.3	M	2.0	13.0	Yes	129 (-1.93)	25.0	1.2	1.0	2536	448	0.03	58.3	20.0	Seizures, delay in walking, pathologic bone fracture, bowed legs
8.1	M	3.0	10.8	Yes	127.5 (-1.74)	35.2	1.6	0.6	1780	783	0.1	103	28.5	Delay in walking, bowed legs
8.2	M	2.1	8.5	Yes	108.8 (-0.35)	20.7	1.8	0.8	1287	560	0.1	89.3	48.3	History of VDDR sibling, bowed legs
9.1	F	1.7	6.5	Yes	68 (-4.8)	8.8	1.6	0.7	1319	1690	0.1	53.8	42.6	Hypotonia, delay in walking
9.2	F	0.8	5.2	Yes	70 (-0.43)	9.3	1.8	1.4	1757	1794	0.1	100.0	13.4	Hypotonia, history of VDDR sibling
10.1	F	2.2	6.3	Yes	77 (-4.03)	8.5	1.9	0.7	3153	435	0.1	36.3	117.6	Delay in walking, hypotonia
10.2	M	2.2	5.0	Yes	78 (-3.9)	8.9	2.1	0.6	3382	590.2	0.04	115	NA	Delay in walking, hypotonia, poor appetite
11.1	F	1.7	1.8	Yes	65 (-5.7)	5.9	1.8	0.8	4123	680	0.1	65.0	42.3	Hypotonia, developmental delay, delay in dentition, dilated cardiomyopathy
12.1	M	1.7	3.3	Yes	78.3 (-2.01)	10.9	2.2	0.9	1601	649.5	0.1	31.9	22.9	Bowed legs
13.1	F	2.6	8.5	Yes	87.1 (-1.12)	12.1	2.1	1.4	1382	975	0.1	45.8	41.3	Seizures, delay in walking, pathologic bone fracture, X-bine deformity

TREATMENT AND FOLLOW UP

All patients had been treated with an active form of vitamin D (calcitriol) and calcium supplement as required. Mean dose for calcitriol to attain clinical, biochemical and radiological recovery was 48.1±29.7 (Range: 7.8-117.6) pg/kg/day. The clinical, biochemical and radiological improvement was observed in all patients after initial commencement of calcitriol therapy. However, on long-term follow up a remarkable lack of compliance was observed which resulted in failure to sustain the biochemical and radiological recovery. In addition, the dose range for calcitriol was quite different among patients (Table 1). However, analysis of dose vs. mutation relationship revealed no statistically significant difference between the median calcitriol doses used in patients with different mutation (p=0.287).

CONCLUSION

In the study evaluating the clinical, biochemical and molecular genetics characteristics of 22 patients with the diagnosis of VDDR1, we identified three novel and two previously described variants in *CYP27B1* gene. A marked phenotypical diversity was observed among families that carried identical mutations suggested phenotypical heterogeneity perhaps due to some other modifier genes or epigenetic factors.

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