

## Vitamin D Deficiency and Genetic Variations of *CYP2R1* Gene among Jordanian Patients

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**Abstract:** Reports show high prevalence of vitamin D deficiency among Jordanians. While the attention of researchers is focused on environmental factors and dressing styles in Jordan, we believe that more investigations are required on the contribution of genetic variations in vitamin D severity. Cytochrome P450 *CYP2R1* gene has been previously reported to play role in vitamin D deficiencies. By screening the entire coding sequence of the *CYP2R1* gene, here we investigated 58 patients (mean age 26.4±12.1 years) of varying severity levels of vitamin D deficiency. Findings showed the occurrence of one polymorphism in Exon 1 and two polymorphisms in Exons 3 and 4. The c.C177T (i.e., p.S59S; rs12794714) polymorphism was found with an allele frequency of 51.8% for C and 48.2% for T. Nearly 14 patients were homozygous C, 31 patients were heterozygous and 12 patients were homozygous for the polymorphism T. One non-synonymous heterozygous mutation c.G852A was reported in two patients (with mild and moderate severity) and is responsible for changing the amino acid Met to Ile (p.M284I). Also, a silent heterozygous mutation was found in two patients (c.C1059T or p.D353D). The patients displayed mild and insufficient vitamin D levels. The c.C177T polymorphism display some relationship with severity, however further investigation on a larger population size might provide more insights to the role of this genetic variation in severity of vitamin D deficiencies.

**Key words:** Vitamin D, *CYP2R1*, SNP, polymorphism, mutation

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### INTRODUCTION

Vitamin D is one of the fat soluble vitamins that plays important roles in bone development, muscle functions and regulation of the immune system (Li *et al.*, 2015). Cytochrome P450 enzymes in the kidney and liver are required for synthesis of the bioactive form of vitamin D. The microsomal *CYP2R1* protein was identified as genetic cause in classic symptoms of vitamin D deficiency (Cheng *et al.*, 2004). Genetic variations of the *CYP2R1* gene have been reported to play an important role in several diseases such as vitamin D deficiencies

(Slater *et al.*, 2015; Thacher *et al.*, 2015), asthma (Leung *et al.*, 2015) and prostate cancer (Shui *et al.*, 2015). Cheng *et al.* (2004) reported a mutation in exon 2 causing substitution of a proline for an evolutionarily conserved leucine at amino acid number 99 in the *CYP2R1* protein. This mutation eliminated vitamin D 25-hydroxylase enzyme activity. Also, Slater *et al.* (2015) identified a significant polymorphism (c.-1127T>C, rs10741657) in the 5'UTR of the *CYP2R1* gene that is associated with vitamin D levels below 30 ng mL<sup>-1</sup>.

Thacher *et al.* (2015) previously identified two mutations responsible for atypical form of vitamin D

deficiency, namely, L99P and a novel K242N. In silico analyses predicted that both substitutions would have deleterious effects on the variant proteins and in vitro studies showed that K242N and L99P had markedly reduced or complete loss of 25-hydroxylase activity, respectively.

Studies on CYP2R1 in mice showed direct relationship between obesity and decreased CYP2R1 mRNA expression in liver (Park *et al.*, 2015). These findings suggest a possible modifier role in genetic variants of CYP2R1 and obesity in vitamin D deficient patients.

## MATERIALS AND METHODS

**Sampling and recruitment of the patients:** All eligible patients attending Jordan University of Science and Technology (JUST) Health Center were invited to participate in this study. Written, informed assent and consent forms were sought from all patients and their parents/guardians, respectively. Participants were included in the study if they were Arab descent and the diagnosis of vitamin D deficiency has been confirmed based on documented vitamin D level ( $<40 \text{ ng mL}^{-1}$ ). Patients with vitamin D deficiency were classified into 3 groups: mild, moderate and severe. The severity of vitamin D levels in blood was evaluated according to review by Stroud *et al.* (2008). The normal vitamin D level in blood was above  $40 \text{ ng mL}^{-1}$ . Patients that displayed vitamin D levels below  $5 \text{ ng mL}^{-1}$  were considered severe. Patients that displayed vitamin D levels in the range of  $5-10 \text{ ng mL}^{-1}$  were considered moderate severity while those that displayed levels in the range of  $10-20 \text{ ng mL}^{-1}$  were considered mild. Patients with chronic disease were excluded from the study.

Another non-deficient vitamin D control individuals were invited from JUST Health Center to participate in the study. These individuals were recruited in order to assess the incidence of the polymorphisms of interest in a non-deficient vitamin D sample and to allow comparison with deficient vitamin D patients. These subjects were included in the study if they have sufficient vitamin D level ( $20-40 \text{ ng mL}^{-1}$ ) and with no history of chronic disease. The following demographics, clinical and medical data were obtained from patients and their files: age, height, weight, level of education, blood type, family history, diet and supplement information, habits and behaviors.

**DNA extraction:** Blood samples (5 mL) were collected from all included patients in EDTA tubes and stored at  $-80^\circ\text{C}$  until genotyped. DNA was extracted from blood samples using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's standard operating procedure.

**Sanger Sequencing of the CYP2R1 gene:** Primers were designed to cover the coding sequences of CYP2R1 (Accession ID: NM\_024514) plus at least 10 nucleotides in the intron region on both ends (Table 1). Primer extension sequencing was performed by GENEWIZ, Inc. (South Plainfield, NJ, USA) using Applied Biosystems Big Dye Version 3.1. Both forward and reverse strands were sequenced. The reactions were then run on Applied Biosystem's 3730x1 DNA analyzer.

**Data analysis:** The sequencing data were analyzed by GENEWIZ personnel using LasergeneSeqMan Software (DNASTAR, Madison, WI) to detect any mutations compared to the genomic DNA reference sequence. Chromatograms were also re-analyzed by authors using Chromas Pro Version 1.42 (Technelysium Pvt. Ltd., Australia). The comparisons of genotypes between different study groups were done using the Chi-square and Fisher exact tests where applicable. Statistical significance was set at  $p = 0.05$ . The IBM SPSS statistics Version 21 Software was used for all statistical tests.

## RESULTS AND DISCUSSION

About 58 patients with varying severity of vitamin D from local clinic were recruited in this study (Table 2). In total, 4 patients displayed severe levels, 33 patients displayed moderate levels and 13 patients displayed mild levels while 8 patients were vitamin D insufficient. The mean age of patients ( $\pm\text{SD}$ ) was  $26.4\pm 12.1$  years with a range of 11-73. Their mean BMI ( $\pm\text{SD}$ ) was  $22.9\pm 4.1$  with range 14-32.

Differences in diet and food consumption were also reported (Table 3). Consumption of liver showed improved vitamin D levels but vitamins and consumption of fish did not show clear improvement.

As shown in Table 4, behavioral patterns like application of sunscreen did not correlate with vitamin D levels with exception of dressing style (among women) that showed less severe vitamin D levels among western style dressing compared to conservative dressing styles in Fig. 1.

Table 1: Primers used to amplify the CYP2R1 gene

Amplicon	Primer	Product size (bp)
Exon 1	CAATGCCCTTGTGTCAACAT	686
	GGACTTCTCCCTTCCAGACC	
Exon 2	GGAGGGCACTCTGAACATTG	456
	ACAGCCTGAAAGGTCCTCAA	
Exon 3a	AGAACAGGACCCAACCATGT	598
	GCTGAGGTAGCTGAGGCTTT	
Exon 3b	CACCGATTTTCAGCACATGA	495
	TCGCAGGAGTTCCTAAAGAAAA	
Exon 4	GTTATCAGAGCACTGGCTACTG	764
	AGCCAGGGGTTCTCAAAGT	
Exon 5	GGGTCTGCTTGCTGAAGTG	691
	GGCAGATGGAGTCAAGAAGG	

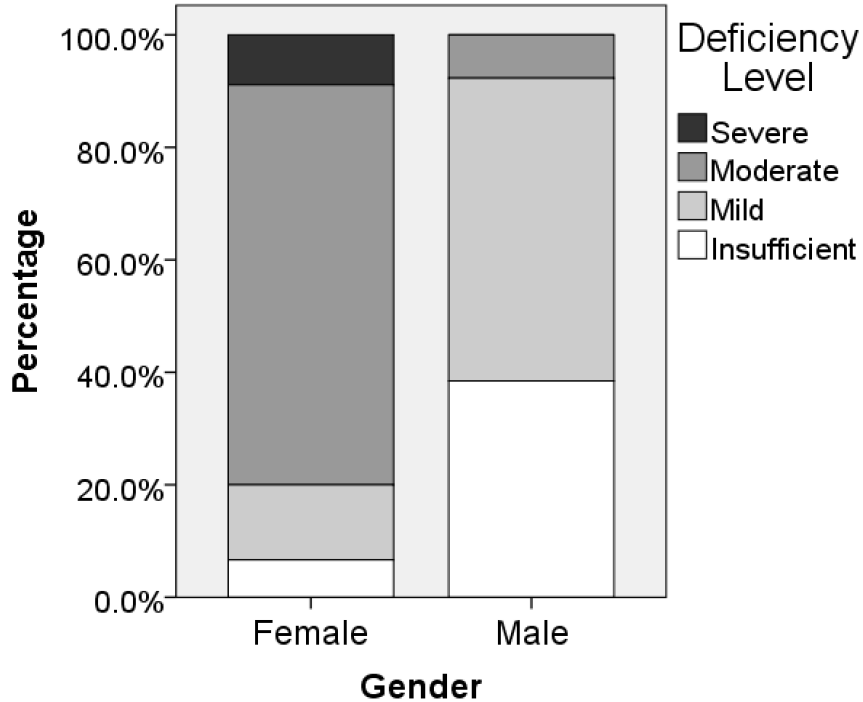


Fig. 1: Vitamin D deficiency level among 58 patients

Table 2: Clinical history for included patients (n = 58)

Characteristics	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 8)	Total
<b>Age</b>					
≤20	1	12	5	2	20
21-30	2	17	4	3	26
>30	1	4	4	3	12
<b>BMI</b>					
Mean±SD	20.5±2.9	21.9±3.1	25.5±5.4	24.5±3.8	22.9±4.1
<b>Family member with vitamin D deficiency</b>					
No	2	21	7	4	34
yes	2	12	6	4	24

In this study, DNA from 58 patients with varying severity was screened by direct automated Sanger sequencing of the *CYP2R1* gene for any possible variations among Jordanians. The *CYP2R1* gene is located on chromosome 11p15.2 and consists of 5 Exons. Here, six amplicons were used to cover the sequencing of all 5 Exons. Our findings showed the occurrence of one polymorphism in Exon 1 and two polymorphisms in Exons 3 and 4 (as shown in Table 5 and Fig. 2). The c.C177T polymorphism was found with an allele frequency of 51.8% for C and 48.2% for T. The genotype distribution of this SNP was as follow: 14 patients were homozygous C, 31 patients were heterozygous and 12 patients were homozygous T. The c.C177T (i.e., p.S59S ) is a silent mutation that does not change the codon for another amino acid. In Exon 3, one non-synonomous mutation

Table 3: Diet and supplement information for participants

Diet information	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 8)	Total
<b>Milk or milk product</b>					
No	0	10	2	1	13
Yes	4	23	11	7	45
<b>Specify milk product</b>					
Cow milk	0	9	4	3	16
Powder	0	3	3	2	8
Cow milk and powder	3	9	4	2	18
All	1	2	0	0	3
<b>Milk supported with vit. D</b>					
Yes	3	4	3	1	11
<b>How often to drink milk</b>					
Daily	2	9	3	2	16
Once a month or more	0	6	6	4	16
Rarely	1	8	2	1	12
Never	1	10	2	1	14
<b>Fish consumption</b>					
Once a month or more	3	15	7	7	32
Rarely	1	12	6	1	20
Never	0	6	0	0	6
<b>Liver consumption</b>					
Once a month or more	1	8	6	4	19
Never or rarely	3	25	7	4	39
<b>Multivitamin in 6 months</b>					
No	4	28	13	6	51
Yes	0	5	0	2	7
<b>Multivitamin type</b>					
B12	0	3	0	1	4
B complex	0	1	0	0	1
Ca and vit. D	0	0	0	1	1
Folic acid	0	1	0	0	1

c.G852A was reported in two patients and it is responsible for changing the amino acid Met to Ile (p.M284I). This

mutation was heterozygous in both patients which displayed mild and moderate vitamin D levels. In addition,

Table 4: Habits and behaviors among included patients

Behaviour	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 8)	Total
<b>Sun exposure</b>					
<7 h	1	21	11	7	40
7-14 h	2	6	2	1	11
>14	0	6	0	0	6
Never	1	0	0	0	1
<b>Sunscreen</b>					
No	4	15	10	6	35
Yes	0	18	3	2	23
<b>Sunscreen (how often)</b>					
Rarely	0	2	0	0	2
If needed	0	2	0	1	3
Daily	0	14	3	1	18
<b>Sunscreen applied on</b>					
Face	0	13	3	0	16
Hand	0	1	0	0	1
Face and hand	0	3	0	2	5
Whole part of body	0	1	0	0	1
<b>Dressing style</b>					
Showed only their hands and faces	4	29	6	3	42
Western style	0	3	7	5	15

in Exon 4, a silent heterozygous mutation was found in two patients (c.C1059T or p.D353D) who displayed mild and insufficient vitamin D levels. The minor allele frequency for both mutations was 1.7%.

There was no significant relationship between the genetic variations and vitamin D levels. However, the polymorphism c.C177T has been associated with more severe levels of vitamin D in patients (Fig. 3).

Recently, Elkum *et al.* (2014) reported that two mutations in CYP2R1 including our reported c.C177T were exclusive to Arabs. The study included populations of Arabs, South Asians and Southeast Asians living in Kuwait.

Studies on CYP2R1 in mice showed direct relationship between obesity and CYP2R1 expression (Park *et al.* 2015). In that report, hepatic mRNA levels of 25-hydroxylases (CYP2R1, CYP27A1 and CYP2J3) were lower in the obese group (31, 30 and 48% lower, respectively). Renal 1 $\alpha$ -hydroxylase (CYP27B1) mRNA levels were higher and 24-hydroxylase (CYP24) mRNA

Table 5: Distribution of genetic variations in CYP2R1 gene among included patients

SNP	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 7)	Total (n = 57)
<b>c.c177t p.S59S</b>					
C/C	1 (25%)	4 (12.12%)	5 (38.46%)	4 (57.14%)	14
C/T	1 (25%)	22 (66.67%)	7 (53.85%)	1 (14.29%)	31
T/T	2 (50%)	7 (21.21%)	1(7.69%)	2 (28.57%)	12
<b>c.g852a p.M284I</b>					
G/G	4 (100%)	32 (96.97%)	12 (92.31%)	8 (100%)	56
G/A	0 (0%)	1 (3.03%)	1(7.69%)	0 (0%)	2
<b>c.c1059t p.D353D</b>					
C/C	4 (100%)	33 (100%)	12 (92.31%)	7 (87.25%)	56
C/T	0 (0%)	0 (0%)	1(7.69%)	1(12.5%)	2

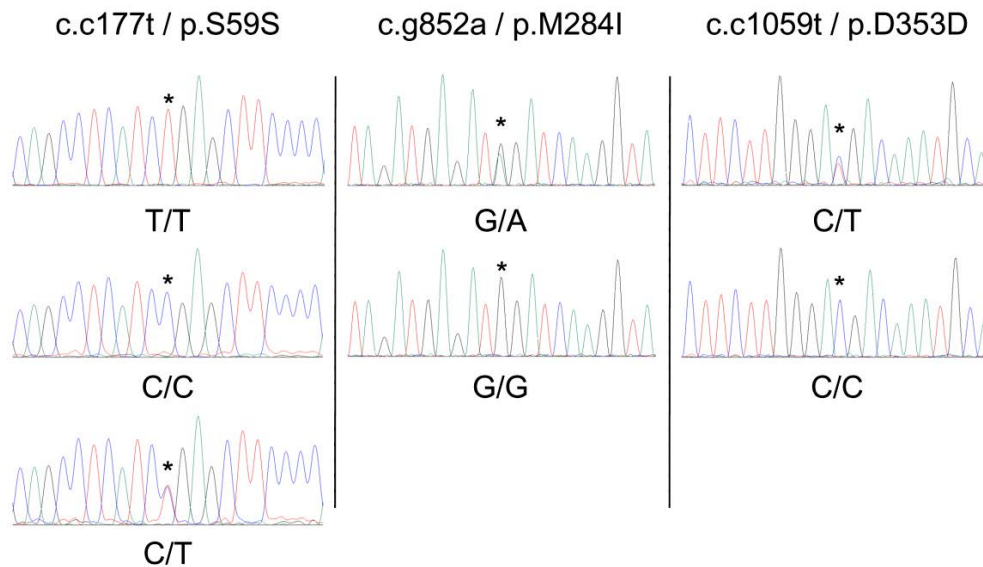


Fig. 2: Representative chromatograms of the genotypes identified in CYP2R1 gene

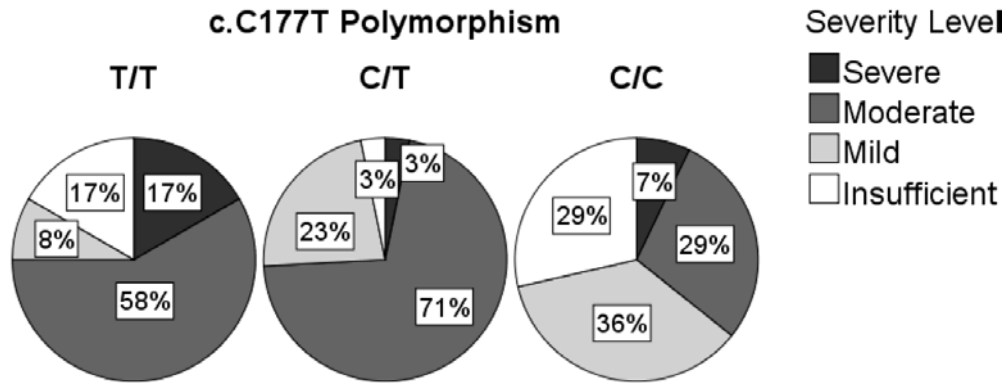


Fig. 3: Distribution of c.C177T polymorphism among included patients

levels were lower in the obese group. Nissen *et al.* (2015) showed that CYP2R1 polymorphism can be genetic determinants of vitamin D levels after consumption of vitamin D fortified bread and milk.

### CONCLUSION

The cytochrome *CYP2R1* gene have been previously reported to play role in some vitamin D deficiency cases. The information about CYP2R1 genotypes among Arab descent is scarce. Here we identify three genetic variations among vitamin D patients; one polymorphism and two mutations. The c.C177T polymorphism display some relationship with severity, however further investigation on a larger population size might provide more insights to the role of this genetic variation in severity of vitamin D deficiencies.

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### REFERENCES

Cheng, J.B., M.A. Levine, N.H. Bell, D.J. Mangelsdorf and D.W. Russell, 2004. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc. Natl. Acad. Sci. USA*, 101: 7711-7715.

Elkum, N., F. Alkayal, F. Noronha, M.M. Ali and M. Melhem *et al.*, 2014. Vitamin D insufficiency in Arabs and South Asians positively associates with polymorphisms in GC and *CYP2R1* genes. *PloS One*, Vol. 9, 10.1371/journal.pone.0113102.

Leung, T.F., S.S. Wang, M.F. Tang, A.P.S. Kong and H.Y. Sy *et al.*, 2015. Childhood asthma and spirometric indices are associated with polymorphic markers of two vitamin D 25-hydroxylase genes. *Pediatr. Allergy Immunol.*, 26: 375-382.

Li, Y.C., Y. Chen and J. Du, 2015. Critical roles of intestinal epithelial vitamin D receptor signaling in controlling gut mucosal inflammation. *J. Steroid Biochem. Mol. Biol.*, 148: 179-183.

Nissen, J., U. Vogel, H.G. Ravn, E.W. Andersen and K.H. Madsen *et al.*, 2015. Common variants in CYP2R1 and GC genes are both determinants of serum 25-hydroxyvitamin D concentrations after UVB irradiation and after consumption of vitamin D3-fortified bread and milk during winter in Denmark. *Am. J. Clin. Nutr.*, 101: 218-227.

Park, J.M., C.Y. Park and S.N. Han, 2015. High fat diet-induced obesity alters vitamin D metabolizing enzyme expression in mice. *BioFactors*, 41: 175-182.

Shui, I.M., A.M. Mondul, S. Lindstrom, K.K. Tsilidis and R.C. Travis *et al.*, 2015. Circulating vitamin D, vitamin D-related genetic variation and risk of fatal prostate cancer in the national cancer institute breast and prostate cancer cohort consortium. *Cancer*, 121: 1949-1956.

Slater, N.A., M.L. Rager, D.E. Havrda and A.F. Harralson, 2015. Genetic variation in CYP2R1 and GC genes associated with vitamin D deficiency status. *J. Pharm. Pract.*, 1: 1-6.

Stroud, M.L., S. Stilgoe, V.E. Stott, O. Alhajian and K. Salman, 2008. Vitamin D: A review. *Aust. Family Physician*, 37: 1002-1005.

Thacher, T.D., P.R. Fischer, R.J. Singh, J. Roizen and M.A. Levine, 2015. CYP2R1 mutations impair generation of 25-hydroxyvitamin D and cause an atypical form of vitamin D deficiency. *J. Clin. Endocrinol. Metab.*, 100: E1005-E1013.