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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-synonomous 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

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^{-1}.

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

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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 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 ng mL^{-1}. Patients that displayed vitamin D levels below 5 ng mL^{-1} were considered severe. Patients that displayed vitamin D levels in the range of 5-10 ng mL^{-1} were considered moderate severity while those that displayed levels in the range of 10-20 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 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°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 3730×l 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 (±SD) was 26.4±12.1 years with a range of 11-73. Their mean BMI (±SD) was 22.9±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
	AGCCAGGGGTTCTCAAAAGT	
Exon 5	GGGTTCTGCTTGCTGAAGTG	691
	GGCAGATGGAGTCAAGAAGG	

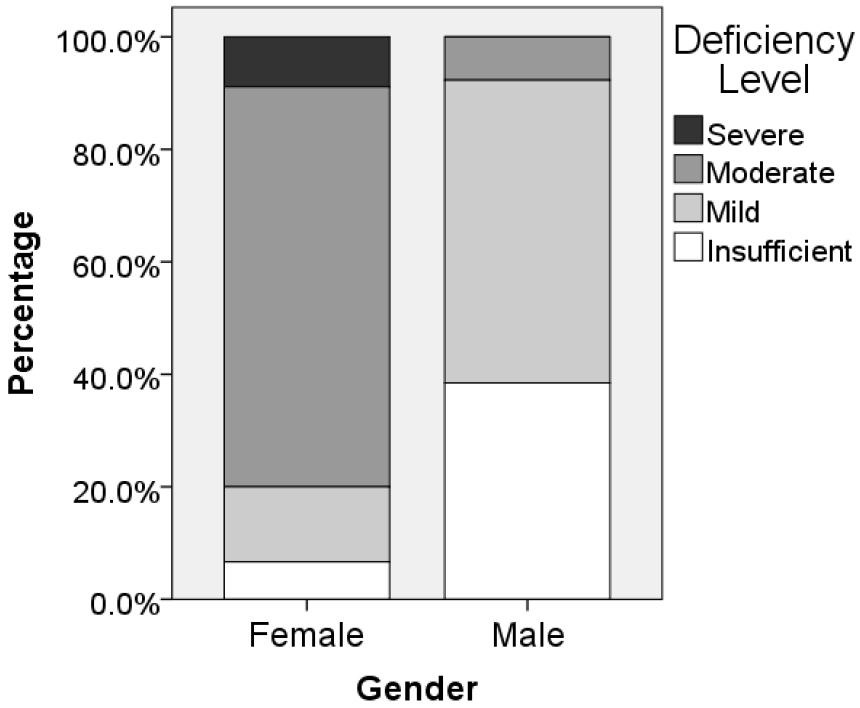

Fig. 1: Vitamin D deficiency level among 58 patients

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

Characteristics	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 8)	Total
Age					
≤20	1	12	5	2	20
21-30	2	17	4	3	26
>30	1	4	4	3	12
BMI					
Mean±SD	20.5±2.9	21.9±3.1	25.5±5.4	24.5±3.8	22.9±4.1
Family member with vitamin D deficiency					
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
Milk or milk product					
No	0	10	2	1	13
Yes	4	23	11	7	45
Specify milk product					
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
Milk supported with vit. D					
Yes	3	4	3	1	11
How often to drink milk					
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
Fish consumption					
Once a month or more	3	15	7	7	32
Rarely	1	12	6	1	20
Never	0	6	0	0	6
Liver consumption					
Once a month or more	1	8	6	4	19
Never or rarely	3	25	7	4	39
Multivitamin in 6 months					
No	4	28	13	6	51
Yes	0	5	0	2	7
Multivitamin type					
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, 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 1α-hydroxylase (CYP27B1) mRNA levels were higher and 24-hydroxylase (CYP24) mRNA

Table 4: Habits and behaviors among included patients

Behaviour	Severe (n = 4)	Moderate (n = 33)	Mild (n = 13)	Insufficient (n = 8)	Total
Sun exposure					
<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
Sunscreen					
No	4	15	10	6	35
Yes	0	18	3	2	23
Sunscreen (how often)					
Rarely	0	2	0	0	2
If needed	0	2	0	1	3
Daily	0	14	3	1	18
Sunscreen applied on					
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
Dressing style					
Showed only their hands and faces	4	29	6	3	42
Western style	0	3	7	5	15

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)
c.c177t p.S59S					
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
c.g852a p.M284I					
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
c.c1059t p.D353D					
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

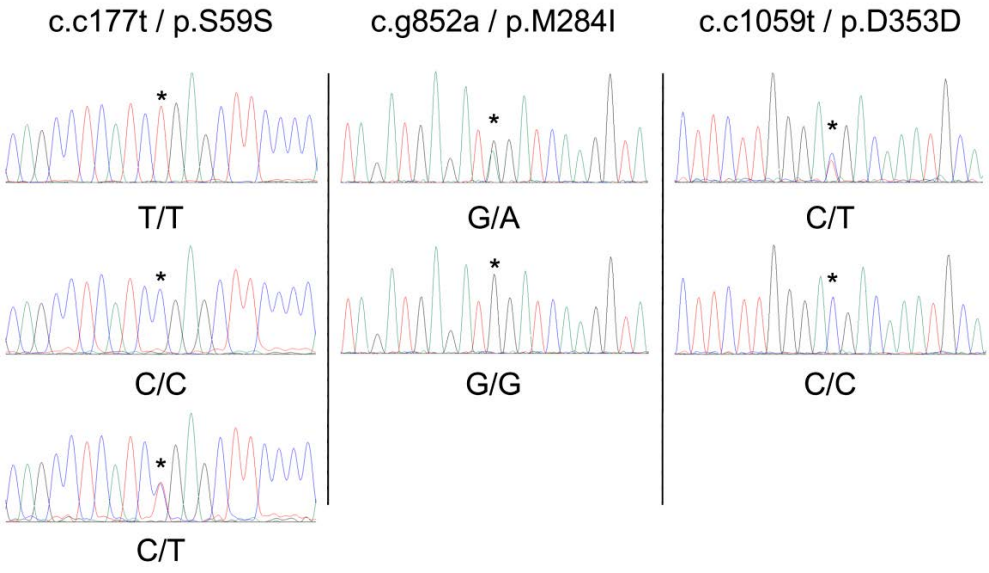

Fig. 2: Representative chromatograms of the genotypes identified in *CYP2R1* gene

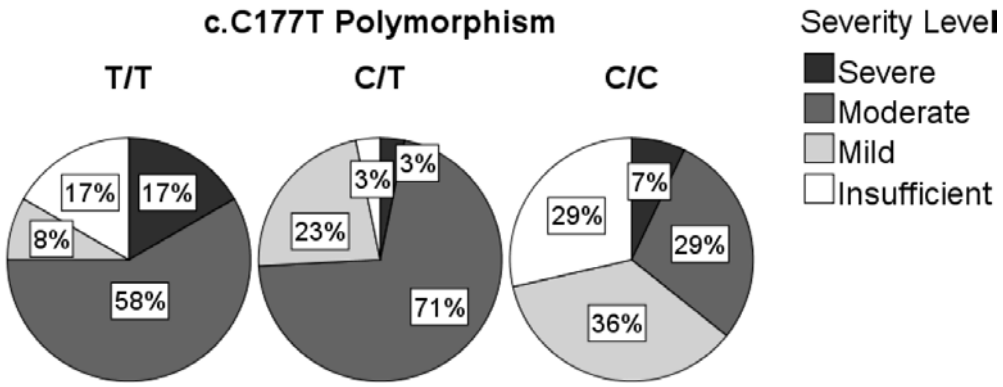

Fig. 3: Distribution of c.C177T polymorphism among included patients

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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