Iraqi Journal of Biotechnology, May 2019, Vol. 18, No. 2, 126-134

The Association of Vitamin D Deficiency and Insufficiency with Genetic Polymorphism (*CYP27B1* SNP *rs10877012*) in Iraqi Samples

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Received: January 16, 2019 / **Accepted:** April 7, 2019

Abstract: Vitamin D comprises a group of fat-soluble secosteroid found naturally only in a few foods, responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, in addition to other biological effects.In humans, the two majorphysiologically forms are vitamin D2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D2 is obtained from dairy products whereas Vitamin D3 is produced in the skin after exposure to ultraviolet light. Vitamin D from the diet or skin synthesis is biologically inactive; enzymatic conversion (hydroxylation) in the liver and kidney is required for activation.Blood Sample were collected 100 individual to determine (test) vitamin D state and these sample divided into three group, the first group 80 sample, were collected randomly from people have normal vitamin D level, and 20 sample as control samples which were divide into two group, 11 sample from people with normal level of vitamin and 9 patient have deficiency in their level of vitamin D. These samples were tested using ELISA to determine the level of 25(OH)D. Genomic DNA was extracted from these samples and analyzed using real time PCR. The results from ELISA groups were 29% sample with vitamin D deficiency in normal people (they did not have any idea about their vitamin D level) 44 ± 24.06^{B}. The negative control was 67.55 ± 21.85^{A} and the positive control was 9.85 ± 5.19^{C}. These results show revealed the relation between the active form of vitamin D enzyme and *CYP27B1 gene which* is associated with the deficiency of vitamin D state. The genetic analysis of *CYP27B1* gene polymorphism, the results from comparison between positive control and healthy group showed the TG and GG genotypes frequencies have significant association with vit. D deficiency (*P*= 0.002; OR= 16.3; CI 95%= 0.2-9.7) and (*P*=0.02 ; OR= 11.8 ; CI 95%= 0.1-10.2) respectively, while the G allele frequency was significantly associated with positive control group (*P*= 0.004; OR= 1; CI 95%= 0.5-4.2). The results of comparison between the negative control and healthy group showed no association in both genotype and allele frequencies. From these results we conclude the immunological test of plasma 25(OH)D is a useful marker to indicate the risk of clinicaldeficiency and insufficiency, while the genetic analysis we suggest it is associated with the deficiency and insufficient of vitamin D level but the study wasn't performed with the enough number of samples to prove this aim.

Keywords: Vitamin D Genetic Polymorphism, CYP 27B.

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Introduction:

Vitamin D is a steroid hormone involved in the active intestinal absorption of calcium and in the regulation of its homeostasis, it has two isomers: Vitamin D2 and Vitamin D3(1). Vitamin D2 is obtained from dairy products whereas Vitamin D3 is produced in the skin after exposure to ultraviolet light (2).

In the liver, Vitamin D is hydroxylated at its carbon 25 to form 25-OH Vitamin D. This metabolite is the predominant circulating form of Vitamin D and is considered to be an

accurate indicator of the general Vitamin D status of an individual (1,3).

Accurate monitoring of total 25-OH vitamin D level is critical in clinical settings. Vitamin D deficient patients who are prescribed a daily Vitamin D supplement should regularly monitor their serum or plasma Vitamin D levels in order to reach an optimal level and prevent their 25-OH Vitamin D concentrations from reaching excessive levels that are considered toxic (1).

Deficiency of vitamin D one of the most underestimated health problems in the world. Low serum level of 25(OH)D, the principal form of circulating vitamin D, is the main marker of vitamin D deficiency (4) .

Vitamin D plays an important role in bone mineralisation and other metabolic processes in the human body such as Ca and phosphate homeostasis and skeletal growth (5,6).

Some of diseases linked to vitamin D levels, for example, causes rickets in children, leading to skeletal abnormalities, short stature, delayed development or failure to thrive(7).

In adults, low values of vitamin D are associated with osteomalacia, osteopenia, osteoporosis and subsequent risk of fractures (5).

The final level of active vitamin D3 is dependent on both environmental factors, such as sun exposure and diet, and in additionally on genetic factors such as the *CYP27B1* gene. CYP27B1 converts 25-hydroxyvitamin D3 to active 1,25-dihydroxyvitamin D3, also known as calcitriol. It's Playing a vital role in calcium homeostasis and bone growth (8,9). *CYP27B1* is mapped to chromosome 12q14.1 (the long q arm of chromosome 12 at position (14.1) (NCBI: NC_000012.12 57762334 – 57767193, complement) and contain functional polymorphism (SNP: rs10877012).

The aim of this study is to Investigate the relationship between vitamin D deficiency and insufficiency with serum level of active form of it, 25 (OH) D enzyme and study the association between *CYP27B1*gene polymorphism with vitamin D deficiency, insufficiency and active form of VTD [25 (OH) D enzyme] .

Materials and Methods:

This study was completed from September 2017 to April 2018 in Baghdad, Iraq. One hundred sample were collected from Baghdad-Abdulmajed hospital laboratory and from other different laboratories in Baghdad also. As a total study samples consist from 80 randomly male and female apparently normal group (healthy cases), and 20 sample subjected as a control sample divided into two group including 9 of it has insufficient amount of vitamin D, and 11 of it as a normal amount of vitamin D.

Venous blood samples (5ml) were collected from all participating individuals and divided in to two portions: first, for DNA extraction and second, for immunological test. The serum levels of 25-hydroxyvitamin D3 (25OHD3) were measured in all patients and controls samples by Enzyme-Linked Immunosorbent Assay (ELISA) using Vitamin D level ELIZA kit (Bioactive diagnostic/Germany). Total genomic DNA was isolated from the whole frozen blood by using the

gSYNC Genomic DNA Extraction kit (Geneadi /Korea.) DNA purity and concentration were determined using a spectrophotometer (Nanodrop).

Genotyping for CYP27B1 rs10877012 (SNP) was performed using TaqMan SNP Genotyping Assay (Biometra) and the ABI7500 Fast Real-Time thermo cycler, according to manufacturer's recommendation in kits used, which are, real time PCR Master mix WizPureTM qPCR Master (PROBE) (Wizbiosolution company/ Korea), and the Primer and Probe (ALPA DNA company/ Columbia-America).

The amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermo cycling.

Oligonucleotide primers and probes sequence showing in the (Table 1).

Table (1): Oligonucleotide primers and probes used for the amplification of CYP27B1-A gene (rs10877012) polymorphism.

Primer and probes	Sequence (5'- 3')	Length	Reference
Forward	5′-AACAGAGAGAGGGCCTGTCT-3′,	20	(19)
Reverse	5′-GGGAGTAAGGAGCAGAGAGGTAAA-3′	24	
Vic probe	5′-CTGTGGGAGATTCTTTTAT-3′,	19	
Fam probe	5′-TGTGGGAGATTATTTTAT-3′.	18	

Reaction profiles were optimized according to the manufacturer's instructions in kits used, which are, real time PCR Master mix WizPureTM qPCR Master (PROBE) (Wizbiosolution company/Korea), and the Primer and Probe (ALPA DNA company/Columbia-America).

Statistical package for the Social Sciences (SPSS), version 17.1 forwindows software (SPSS Inst. Inc., Chicago, USA) was used for statisticalanalysis. The data normally distributed and were expressed as mean ± standard deviation (SD). Student ṡ t-test was performed to analyze the statisticalsignificance of difference between control and total VTD deficiency and insufficient patients. One-wayanalysis of variance (ANOVA) test was used to compare the parameters amonggroups. A difference among groups was defined to be statistically significant ifthe corresponding P-value was than 0.05 (10,11).

Data of alleles and genotypes of (rs10877012) were presented asfrequencies and percentage by using simple statistical parameter. Chi-squaretest was used to compare frequencies in this study. The odd ratio (ORs) and 95%confidence interval (95%CI) were used to evaluate the potential associationsbetween genetic polymorphism of CYP27B1 gene (rs10877012) and its risk on deficiency and insufficient level of VTD patients. P value for all tests was considered significant if ≤ 0.05 (12).

Results and Discussion:

General Characteristics of the study groups:

The total number of samples used in this study were 100 samples, 20

samples were control including 9 that had insufficient amount of vitamin D, and 11 of them with normal amount of vitamin D. The other 80 samples were collected form random patients, **7** of these 80 sample were indicated as error samples. The statistical analysis show in (Table 2), gave the (mean ±SD) to healthycases group, positive control group, and negative group. The comparison between (mean ±SD) of healthy cases (n = 93) gave (44.76 ± 24.06^{A}), but positive control sample (n=9) gave (9.85 ± 5.19^{c}), while negative control (n=11) gave (67.55 ± 21.85^{B}), comparing these results with other study (13) , give mean ± SE of the VTD insufficient group and of sufficient group. The results of their study are not statistically significant which mean they are more specific compared with the results of this study, therefore the size of the samples were not enough to be achieve any statistical significance.

Table (2): Statistic analysis of immunological ELISA test:

Group	Mean ± Standard Error (cells/cu.mm .blood)
Positive control N.9	9.85 ± 5.19^{c}
Negative control N.9	67.55 ± 21.85^{B}
Healthy cases No. 73	44.76 ± 24.06^{A}

To show the moral difference between (Mean ± Standard Error) of each group of ELISA test result, we displayed each of it as a Duncan[a] analysis showed in (Table 3).

Table (3): Duncan[a] table. Means for groups in homogeneous subsets are displayed, uses Harmonic Mean Sample Size = 9.000.

Groups	Subset for alpha = 0.05		
	1	2	3
Positive control n=9	9.85^{C}		
Negative control n=9			
Healthy cases n=9		44.76^{B}	
			67.55^{A}
Sig.	1.000	1.000	1.000

The data of all samples of immunological test (ELISA) analysis the active form of vitamin D ([25 (OH) D enzyme]) level concentration associated with the deficiency and insufficient of vitamin D shown in the (Table 4), it showed that 29% of all samples have lower levels of vitamin D (n=27 of 93 samples, marked in (Table 4).

This result showed the spread of this serious state of VTD in Iraqi population not low.

Table (4): Show the result of vitamin D level patient samples:

Sample No.	Vitamin D level	Sample No.	Vitamin D level	Sample No.	Vitamin D level	Sample No.	Vitamin D level	Sample No.	Vitamin D level	Sample No.	Vitamin D level	Sample No.	Vitamin D level
1	26.8	16	36.1	31	42.5	46	97.2	61	86.74	76	78.98	91	8
2	35.92	17	15.94	32	7.13	47	62.54	62	12.62	77	100.3	92	51.7
3	82.31	18	47.79	33	77.67	48	111.6	63	32.23	78	54.03	93	16.5
4	55.89	19	39.05	34	18.58	49	40.76	64	2.03	79	74.16	94	4.2
5	57.64	20	45.49	35	39.02	50	81.04	65	35.06	80	84.43	95	9.8
6	74.58	21	27.73	36	62.91	51	57.22	66	54.55	81	55.81	96	8.9
7	65.37	22	26.07	37	75.91			67	82.76	82	47.74	97	17.9
8	63.99	23	29.84	38	23.49	53	64.69	68	45.85	83	62	98	9.1
9	35.64	24	54.88	39	73.58	54	69.29	69	58.33			99	12.4
10	65.28	25	27.73			55	51.7	70	28.66	85	8	NA	NA
11	77.52	26	35.55	41	46.79	56	74.91	71	75.31	86	13.7	NA	NA
12	37.39	27	60.7			57	92.92	72	68.38	87	20.3	NA	NA
13	82.77	28	35.86	43	62.85			73	51.72	88	8.4	NA	NA
14	32.79	29	67.75	44	66.53	59	6.65	74	30.34			NA	NA
15	21.83	30	44.75	45	95.56	60	76.36	75	82.23	90	1.87	NA	NA

Genetic Analysis of *CYP27B1*gene polymorphism (rs10877012) SNP:

Genomic DNA was extracted from blood samples of healthy and VTD deficiency control samples and insufficient patients, the purity of DNA solutions were recommended and suitable for further genetic analysis by using real time PCR technique as mentioned by (14).

The *CYP27B1* gene polymorphism (rs10877012) SNP was successfully amplified by real time PCR (qPCR) technique, The result of amplified product was detected with the use of fluorescent dyes. The result revealed was significantly associated with VTD deficiency (O.R 95% CI, P=0.05) in control.

In this study the result as showed in (Table 4), twenty one out from 73, showed a significant association between rs10877012 in *CYP27B1* gene and increased prevalence of VTD deficiency. This study agrees with Al Anouti1 *et al*., (13).

The real time PCR results showed the 5' untranslated region, SNP (rs10877012) in *CYP27B1*, which was presented with three genotypes (TT, GT, GG).

The highest frequency in the positive control group(n=9) was GT genotype which was found in n=5 with frequency (55.6%) followed by TT genotype was found in n=2 with frequency (22.2%) and GG genotype found in n=2 with frequency (22.2%).

The highest frequency in the Healthy group (n= 80) was TT genotype which was found in n=59 with frequency (80.8%) followed by GT genotype found in n=9 with frequency (12.4%) and GG genotype found in n=5 with frequency (6.8%).

Table (5): The percentage of genotype appear groups indicate in comparison of the Genotype and Allele of *CYP27B1* gene polymorphism rs10877012 between Control group and Patient group.

Genotype	Wild (TT)	Heterozygote (GT)	Mutant (GG)
% of Healthy cases (n=73)	80.8 %	12.3 %	6.8 %
% of Negative control (n=9)	100 %	0 %	0 %
% of Positive control(n=11)	22.2 %	55.5 %	22.2 %

For (rs10877012), the odd ratio (95% CI) and P value were calculated by a comparison between the positive control individuals and healthy cases group and the results obtained are shown in (Table 6). The wild type TT genotype and wild type T allele were taken as references. The odd ratio and CI for GT of the (rs10877012) was 16.3 (0.2-9.7) with (P=0.002), (p>0.05).

The difference from the control was statistically significant. However, GG revealed a statistically also significant with an odd ratio was 11.8 (0.1-10,2) and (P=0.02). The O.R of G allele was 0.1 (0.5-4.2), P= 0.004 with significant comparing with the control (p> 0.05). Show in (Table 6).

The highest frequency in the negative control group (n=11) was TT genotype which was found in n=11 with frequency (100%) followed by GT genotype found in n=0 with frequency (0.0%) and GG genotype found in n=0 with frequency (0.0%). The highest frequency in the Healthy group was TT genotype which was found in n=59 with frequency (80.8%) followed by GT genotype found in n=9 with frequency (12.4%) and GG genotype found in n=5 with frequency (6.8%).

For (rs10877012), the odd ratio (95% CI) and P value were calculated by a comparison between the negative control individuals and healthy cases group and the results obtained are shown in Table(7).

In Table (7), the wild type TT genotype and wild type T allele were taken as references. The odd ratio and the CI for GT of the (rs10877012) was 3.6 (0.1- 67.6) with (P=0.3), (p>0.05). The difference from the control was statistically significant. However, GG genotype was also statistically significant with an odd ratio 11.8 (0.1-10.2) and (P=0.02). The O.R of G allele was 0.1 (0.5-4.2), P=0.004. with no significant difference from the control (p> 0.05). As shown in (Table 7). Our results indicated that the haplotype of SNP rs10877012 in CYP27B1 could be associated with low vit D levels, specifically T allele from rs10877012 in CYP27B1 are more common in people affected with vit D deficiency (13).

An association between reduced 25(OH) D3 levels and rs10877012 in CYP27B1 promoter polymorphism leading to reduced 1,25 (OH)2 D3 levels among the British population (14,15).

The variant that we have studied here, rs10877012 in the 5'region, reduce the activity of CYP27B1 leading to decreased the level of 1,25 (OH)2 D3 and our results support this finding, as rs10877012 was associated with increased risk of VTD insufficiency. Specifically the GT+TT genotype of rs10877012 (13).

CYP27B1 also known as 1α-hydroxylase is the enzyme responsible for the hydroxylation of 25 (OH) D3 to form 1,25(OH)2D[12]. This enzyme

carries out the final reaction to convert VTD to its active form, 1,25 (OH)2 D3 in the kidneys (16,17). This could have important implications for predicting the VTD status and further direct prognosis for a number of clinically significant diseases such as cancer and Type 2 diabetes in subjects with these haplotypes (13).

Studies of genetic variants that specifically affect 25 (OH) D3 concentrations can provide another route to interpret the underlying cause of VTD insufficiency(18). Therefore in the present prospective study, we examined the association of genetic polymorphism of the metabolizing enzymes, rs10877012 in CYP27B1 gene with VTD status among a representative sample of some Iraqi population. Specifically, we have investigated SNP; rs10877012 in CYP27B1.

Table (6): Comparison of the Genotype and Allele of *CYP27B1* gene polymorphism rs10877012 between positive control group and healthy cases group (Blood).

rs10877012 **polymorphism**	**Frequencies (%)**		**P value**	**Odd ratio (95% CI)**
	Control Positive control (n=9)	**Patient (n=73)**		
TT	22.2% (n=2)	80.8% (n=59)	---	1.00 (Reference)
TG	55.6% (n=5)	12.4% (n=9)	0.002	16.3 (0.2-9.7)
GG	22.2% (n=2)	6.8% (n=5)	0.02	11.8 (0.1-10.2)
T	50.0% (9)	86.9% (127)	---	1.00 (Reference)
G	50.0% (9)	(19)13.1	0.004	0.1 (0.5-4.2)

Table (7): Comparison of the Genotype and Allele of CYP27B1 gene polymorphism rs10877012 between negative control group and healthy cases group (Blood).

rs10877012 **polymorphism**	**Frequencies (%)**		**P value**	**Odd ratio (95% CI)**
	Control negative control (n=11)	**Patient (n=73)**		
TT	100.0% (n=11)	80.8% (n=59)	---	1.00 (Reference)
TG	0.0% (n=0)	12.4% (n=9)	0.3	3.6 (0.1-67.6)
GG	0.0% (n=0)	6.8% (n=5)	0.6	2.1 (0.1-41.3)
T	100.0 (22)	86.9% (127)	---	1.00 (Reference)
G	0.0 (0)	(19) 13.1	0.1	6.8 (0.4-11.8)

rs10877012 of CYP27B1 was amplified in DNA solution extracted from each blood samples of VTD deficiency and insufficient patients and controls sample both with normal and VTD deficiency level. Real time polymerase chain reaction was performed in three-steps under optimal amplification conditions by using specific probe and primer.

Depending on the groups of ELISA test and groups of genotyping analysis of real time PCR, three genotypes found in the samples were genetically analysis, (TT) wild genotype, (GT) heterozygote genotype, and (GG) mutant genotype. The percentage of these genotyping was mention in (Table 5), the percentage of mutant genotype healthy cases 6.8 % and percentage of mutant genotype positive control 22.2 % show there is an association between VTD deficiency, insufficient with the genotype selected (CYP27B1

rs10877012), but this appear it isn't significant study comparing with other studies(12) to achieve the aim of investigate association between active form of VTD and the genotype (CYP27B1 rs10877012) because the number of samples collected in this study was not enough due to the short period of study preforming and the cost also.

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