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Research Article

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Association of Certain Vitamin D Metabolizing Enzymes (CYP2R1 and CYP27B1) Genes Polymorphisms with Type 1 Diabetes Mellitus in Jordanian Patients

Wiam Y Khalil[1], Yacoub M Irshaid[1]*, Nahla M Khawaja[2], Mohammed S El-khateeb[2] and Kamel M Ajlouni[2]

[1]*Department of Pharmacology, Faculty of Medicine, the University of Jordan*

[2]*The National Center for Diabetes, Endocrinology, and Genetics, Amman 11942, Jordan*

***Corresponding author:**
Yacoub M Irshaid, Department of Pharmacology, Faculty of Medicine, The University of Jordan, Amman, 11942, Jordan, Phone: +962 777818284; Fax: +962 6 5300820; Email: y.irshaid@ju.edu.jo

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Abstract

Objectives: *Type 1 diabetes mellitus (T1DM) is a multifactorial disorder, where environmental and genetic factors may interact and contribute to its pathogenesis. Several T1DM susceptibility genetic loci have been identified using genome-wide screens. Among those are loci located in the genes of vitamin D metabolizing enzymes; CYP2R1 (rs10741657 and rs12794714) and CYP27B1 (rs10877012). The aim of this study was to investigate the association of polymorphisms in CYP2R1 (rs10741657 and rs12794714) and CYP27B1 (rs10877012) genes with T1DM.*

Methods: *The study was performed on 123 T1DM patients and 123 non-diabetic control subjects. It was approved by the "Institutional Review Board" of the National Center for Diabetes, Endocrinology, and Genetics (NCDEG), Amman, Jordan. Polymorphisms in rs10741657, rs12794714 and rs10877012 were studied by genotyping using polymerase chain reaction - restricted fragment length polymorphism (PCR-RFLP) assay method.*

Results: *A significant association was found between rs12794714 AA genotype and T1DM. The allele was more frequent in T1DM patients (0.524) than in control subjects (0.407) (P-value = 0.0089). No significant association was found between T1DM and rs10741657 and rs10877012 polymorphisms. However, The AA-GG haplotype of CYP2R1 rs12794714 - rs10741657 maintained the association between rs12794714 AA genotype and T1DM. The association between rs12794714 AA genotype and T1DM was enhanced in the presence of CYP27B1 rs10877012 CC genotype (odds ratio increased from 2.71 to 4.27, P = 0.0003).*

Conclusion: *Our study revealed an association between CYP2R1 rs12794714 AA genotype and T1DM. This association was enhanced in the presence of CYP27B1 rs10877012 CC genotype.*

Keywords: CYP2R1, CYP27B1, rs10741657, rs12794714, rs10877012, Type 1 Diabetes.

Introduction

The hallmark of type 1 diabetes mellitus (T1DM) is selective beta cell destruction and severe or absolute insulin deficiency, leading to impaired glucose homeostasis. The most common form of T1DM is the immune-mediated [1]. T1DM afflicts millions of people worldwide and its incidence rate is increasing over the decades across the globe, including Jordan [2, 3]. The rise in the incidence of type 1 diabetes worldwide was too rapid to be explained by increased transmission of the high risk alleles from one generation to another, supporting the etiological role of environmental factors [4]. The obvious variation in the prevalence of T1DM around the globe, and the rapid increase in its incidence rate gave a clue that the etiology and pathogenesis of T1DM is unlikely to be a result of a single factor or event, but rather a combination of genetic and environmental factors that contribute, together, to the progression of the disease [4]. Viral infection, intestinal bacterial microbiome, and vitamin D have been found to be associated with T1DM. The exact mechanism by which vitamin D plays a role in T1DM pathophysiology is not yet clears [5-12].

The active form of vitamin D, 1, 25-dihydroxy vitamin D_3 [1, 25$(OH)_2$ D], is involved in bone mineral homeostasis and many other diverse physiological functions including effects on growth of cancer cells and protection against some autoimmune diseases [13]. The active form of vitamin D is the ligand for vitamin D receptor (VDR) located in the DNA, where it binds to vitamin D response elements (VDREs). There are thousands of VDERs responsible for the regulation of hundreds of genes which are cell-specific [14].

Vitamin D is converted to its active form through two enzymatic steps. First, the conversion of vitamin D3 to 25(OH) vitamin D in the liver mainly by the enzyme CYP2R1 , which is considered to play the major role in such hydroxylation having the highest affinity

and specificity for vitamin D . Second, the hydroxylation of 25(OH) vitamin D to the active form 1, 25(OH) 2D, by the enzyme CYP27B1 mainly in the kidney [13, 14].

CYP2R1 gene spans on around 15 kb of DNA on human chromosome 11 at location 11p15.2 and contains five exons separated by four introns [15]. CYP2R1 is primarily expressed in the liver where the hydroxylation of vitamin D to 25(OH) D mainly occurs [16]. Single nucleotide polymorphisms (SNPs) in CYP2R1 have been implicated to play a role in the variation of vitamin D status as well as in T1DM risk. Among these polymorphisms are the rs10741657 and rs12794714, which have been the focus of the recent studies. The rs10741657 is a 5' near gene SNP mapped to a 2-kb mRNA transcript of CYP2R1 in chromosome 11 where an A/G variation takes place [17]. The rs12794714 is a synonymous (no effect on the encoded amino acid, Serine) single nucleotide polymorphism with an allele variation A/G at exon 1 of CYP2R1 gene in chromosome 11 [17]. So far, the polymorphism rs12794714 has not been extensively studied to be implicated in T1DM susceptibility. Its first analysis in this context was in 2007 in the German population [18].

CYP27B1 gene is localized in chromosome 12 and encodes its protein in the inner mitochondrial membrane where the 1-alpha hydroxylation of vitamin D takes place [17]. CYP27B1 is expressed mainly in the kidney, but also in some extra-renal tissue such as the pancreas [19]. CYP27B1 gene spans approximately 6 kb and consists of 9 exons and 8 introns with a promoter region which possesses some polymorphic sites that were suggested to be implicated in T1DM risk [20]. The polymorphism rs10877012 (-1260 C>A) is a 5' near gene SNP at the -1260 bp to the promoter region of the enzyme CYP27B1 gene.

The association between vitamin D metabolizing enzymes gene polymorphism with T1DM among various studies has been conflicting. This kind of association has not been studied in Jordan. Therefore, we designed this study to investigate the association of the polymorphisms rs10741657 and rs12794714 in CYP2R1 gene and rs10877012 in CYP27B1, with T1DM susceptibility in a sample of Jordanian patients.

Subjects and Methods

Subjects

One hundred and twenty three cases of T1DM and 123 of non-diabetic subjects (controls) participated in this study. They were recruited from the NCDEG, Amman, Jordan. The age of all participants ranged from 1 to 40 years at the time of taking the blood sample. The study was performed in accordance with the Helsinki Declaration. The study protocol was approved by The NCDEG Institutional Review Board. A written and signed consent form had been obtained from each participant (or from parents of children) prior to blood sample and data collection.

Genotyping

A minimum of 3 ml of venous blood were collected from each subject using ethylendiaminetetraacetic acid (EDTA)-containing tubes and stored at 4ºC for an immediate DNA extraction. Genomic DNA was extracted from patients' lymphocytes according to manufacturer protocol using commercial DNA extraction kit and stored at -20°C.

All the three SNPs (of rs10741657, rs12794714 and rs10877012) were studied using the restriction fragment length polymorphism (RFLP) technique and polymerase chain reaction (PCR) with the forward and reverse primers that are complementary to either side of the polymorphic site in the genome sequence. The primers sequences used for all SNPs are listed in (table 1), along with the restriction enzymes used and their cut alleles [18, 21, 22]. A negative control sample was prepared in each PCR run to ensure that the samples, materials used and working environment were free of any DNA contamination. Samples with already known genotypes had undergone RFLP with each run as positive control samples to ensure that the enzymes cut the PCR products properly.

Table 1: Primers sequences in PCR and restriction enzymes in RFLP

		PCR product length (bp)	Restriction enzyme	Cut allele
rs10741657 (18, 21)	Forward primer: 5'-GCCCTGGAAGACTCATTTTG-3'	287 bp	MnlI	G
	Reverse primer: 5'-GGGAAGAGCAATGACATGGA-3'			
rs12794714 (18)	Forward primer: 5'- GGAAGCTTTGGAGAGCTGAA -3'	303 bp	FokI	G
	Reverse primer: 5'-GCCATAAGTCCAACCAGGAA-3'			
rs10877012 (22)	Forward primer: 5'-TTCAATTCCAGAACTTCAGAGC-3'	298 bp	TfiI	C
	Reverse primer: 5'-AACATAGTCGAACTGTCTCTAC-3'			

Statistical Analysis

The odds ratios for differences between proportions were calculated using the online "2x2 contingency tables with odds ratios, etc" calculator at http://vassarstats.net/odds2x2.html. This method also calculates the 95% confidence interval (CI) of the odds ratio, the Pearson Chi-square statistic, and the p value. The difference between proportions was considered significant when the 95% CI did not include 1 and if the p value was less than 0.05. The differences between minor allele frequencies among various populations were evaluated by the Z test using the online Z test calculator for 2 proportions at http://www.socscistatistics.com/tests/ztest/.

Results

CYP2R1 genotypes polymorphisms and allele frequencies and association with T1DM

rs12794714

The genotypes GG and GA were more frequent among controls than diabetic patients, but this difference did not reach statistical significance. However, a higher number of the genotype AA carriers was found in diabetic patients compared to controls (p = 0.0015). The AA genotype carriers appeared to be 2.71-fold more susceptible to T1DM than GG carriers (OR= 2.71, 95% CI= 1.45-5.08, P-value=0.0015). In addition, the combination of GG+GA genotypes was also more frequent in controls (85.4%) compared to diabetics (68.3%), P value = 0.0015. In regard to the allele frequencies, the G allele has been found in a significantly higher frequency in the control group (59.3%) than in diabetics (47.6%) (P value = 0.0088). The A allele has been found in a significantly higher frequency in the diabetics (52.4%) than in controls (40.7%), P value = 0.0088. These results are shown in (table 2).

Table 2: rs12794714 Genotypes and allele frequencies among diabetics and controls

Genotypes	Genotypes count (%)		Odds ratio (95% CIa)	Pearson χ2 statistic	P-value
	Diabetics(n=123)	Controls (n=123)			
GG	33 (26.8 %)	41 (33.3 %)	0.73 (0.42-1.27)	1.24	0.265
GA	51 (41.5 %)	64 (52.0 %)	0.65 (0.40-1.08)	2.76	0.097
AA	39 (31.7 %)	18 (14.6 %)	2.71 (1.45-5.08)	10.07	0.0015
Total	123 (100%)	123 (100%)	---	---	----
GG+GA	84 (68.3%)	105 (85.4%)	0.37 (0.20-0.69)	10.07	0.0015
AA+GA	90 (73.2%)	82 (66.7%)	1.36 (0.79-2.36)	1.24	0.265
Alleles	Alleles count (%)				
	Diabetic (n=246)	Controls (n=246)			
G	117 (47.6%)	146 (59.3%)	0.62 (0.44-0.89	6.87	0.0088
A	129 (52.4%)	100 (40.7%)	1.61 (1.13-2.30)	6.87	0.0088
Total	246 (100%)	246 (100%)	---	---	---

[a] = 95% Confidence interval

rs10741657

No significant differences were found in the frequencies of genotypes and alleles among diabetic patients compared to control subjects (data not shown).

CYP27B1genotypes polymorphisms and allele frequencies and association with T1DM

rs10877012

No significant differences were found in the frequencies of genotypes and alleles among diabetic patients compared to control subjects (data not shown).

Genotype frequencies of rs12794714 in the presence of genotypes of rs10741657 or rs10877012 and association with T1DM: The CYP2R1 rs12794714 - rs10741657 haplotypes

The AA-GG haplotype frequency was higher among diabetics (30.1%) compared to controls (14.6%); odds ratio 2.51, 95% CI 1.34-4.72, p = 0.004. The rs10741657 GG genotype retained the significant association of rs12794714 AA genotype with T1DM. No significant association was demonstrated with the other haplotypes (table 3).

Table 3: Genotypes frequencies of rs12794714 in the presence of genotypes of rs10741657 or rs10877012, in diabetics and controls

Genotypes*	Genotypes count (%)		Odds ratio (95% CIa)	Pearson χ2 statistic	P-value
	Diabetics	Controls			
rs12794714 - rs10741657					
GG – GA	11 (8.9)	19 (15.4)	0.54 (0.24-1.18)	2.43	0.119
GA - GG	28 (22.8)	35 (28.5)	0.74 (0.42-1.32)	1.05	0.306
GA - GA	23 (18.7)	29 (23.6)	0.75 (0.40-1.38)	0.88	0.348
AA - GG	37 (30.1)	18 (14.6)	2.51 (1.34-4.72)	8.45	0.004
rs12794714 – rs10877012					
GG - CC	18 (14.6)	28 (22.8)	0.58 (0.30-1.12	2.67	0.102
GG - AA	15 (12.2)	11 (8.9)	1.41 (0.62-3.22)	0.69	0.406

GA - CC	31 (25.2)	40 (32.5)	0.70 (0.40-1.22)	1.60	0.206
GA - AA	17 (13.8)	23 (18.7)	0.70 (0.35-1.38)	1.07	0.301
AA - CC	31 (25.2)	9 (7.3)	4.27 (1.94-9.42)	14.45	0.0001

a = 95% Confidence interval
*Other combinations are not listed because of inadequate numbers for statistical analysis.

The CYP2R1 rs12794714 AA genotype in the presence of CYP27B1 rs10877012 CC genotype

The AA-CC combination frequency was higher among diabetics (25.2%) compared to controls (7.3%); odds ratio 4.27, 95% CI 1.94-9.42, p = 0.0001. The rs10877012 CC genotype apparently enhanced the association of rs12794714 AA genotype with T1DM. No significant association was demonstrated with the other genotypes of CYP27B1 (table 3).

The minor allele frequencies of rs 12794714 (A) of CYP2R1 in some populations

These are presented in (table 4). The minor allele (A) frequency in Jordanians is similar to some Arab, Caucasian and some Chinese populations, but is significantly different from Lebanese, South East Asians, and Han Chinese subjects [18, 23-29].

Table 4: Minor allele frequencies of rs 12794714 of CYP2R1 in some poulations

Population	Sample size (n)	Sample properties	References	Minor allele frequency	Z score	P value
Jordanian	123	Non-diabetic patients less than 40 years	Current Study	0.407	---	---
Jordanian	123	Type 1 diabetic patients less than 40 years	Current Study	0.524	- 2.62	**0.0088**
Lebanese	172	Elderly patients	23	0.500	- 2.25	0.0244
Arab	907	Random sample from multiethnic origin across the State of Kuwait	24	0.420	- 0.41	0.6892
South Asian	489			0.450	- 1.23	0.2187
Sout East Asians	153			0.250	3.88	0.0001
European	16,125	Genome-wide association study on individuals of European descent drawn from 5 epidemiological cohorts	25	0.43	- 0.74	0.4593
United states	156	Non-Hispanic white men and women randomly selected from 3 vitamin D studies in the osteoporotic research center at Creighton University	26	0.43	- 0.55	0.5823
German	133	Type 1 diabetic patients	18	0.460	- 1.19	0.2340*
					1.49	0.1368**
Chinese	1,199	Unrelated employees and retired workers of a factory in Dali, Yunnan province	27	0.350	1.77	0.0767
Chinese in Singapore	497	Middle-aged and elderly subjects	28	0.360	1.35	0.1770
Chinese	506	Han Chinese children from Harbin, Northeastern china	29	0.55	- 4.05	0.0000

*when compared to nondiabetic Jordanians, **when compared to diabetic Jordanians

Discussion

In this study, we examined the association of some polymorphisms in the genes of vitamin D metabolizing enzymes; CYP2R1 (rs12794714, and rs10741657) and CYP27B1 (rs10877012) with T1DM in 123 patients in comparison to 123 non-diabetic control subjects.

Our study has revealed a significant association between the polymorphism rs12794714 AA genotype and not rs10741657 of CYP2R1 gene withT1DM. Also we did not observe an association between CYP27B1 (rs10877012) and T1DM. A case-control study in German population reported an association between the variant G allele of rs10741657 with T1DM [18]. This study did not find an association between rs12794714 and T1DM. Bailey et al, 2007 studied patients with T1DM and controls, in addition to a fully independent collection of families from Great Britain. They found that the common allele C of CYP27B1 rs10877012 was associated with increased risk for T1DM development in both the case-control and the family studies [30]. Cooper, et al [31] reported, using both a case-control and a family-based design in a British population, an association of both rs12794714, and rs10741657 SNPs and T1DM.

They also confirmed an association between T1DM and CYP27B1 rs10877102.

Our results concerning rs10741657 and rs10877012 lack of association with T1DM are not in agreement with an Egyptian study, which showed that rs10741657 GG and rs10877012 CC genotypes increased the risk of T1DM [21]. In another study from Egypt, rs10877012 CC genotype and the C allele of CYP27B1 was found to be more frequent in T1DM compared to healthy controls [32]. A polish study reported on a lack of association between CYP27B1 rs10877012, C (-1260) A and T1DM [22].

However, in our study, the rs10877012 CC genotype enhanced the association of rs12794714 AA genotype with T1DM (odds ratio increased from 2.71 to 4.27, table 3).

These variations in results among the various studies could be attributed to ethnic differences, differences in study design, and the numbers of study samples, and differences in the contribution of environmental factors to the pathogenesis of T1DM.

The minor allele frequency of rs12794714 (A) in our study which was found to be 0.407 was of the same order of magnitude of what reported in Arabs and Caucasians from Europe and the United States, as well as south Asian populations [18, 24-26]. Our Frequency was higher than that reported from Chinese studies, but the difference did not reach statistical significance [27, 28]. However, this frequency was significantly different from Lebanese, South East Asians and Han Chinese Children [23, 24, 29].

In conclusion, we observed a significant association between CYP2R1 rs12794714AA genotype and T1DM in Jordanians. This association was enhanced in the presence of CC genotype of CYP27B1 rs10877012CC genotype.

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None of the authors have any conflict of interest.

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