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Association of Genetic Polymorphisms in a Sample of Iraqi Patients with Type2 Diabetes Mellitus

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Abstract: Type 2 Diabetes (T2D) is a progressive condition that is characterized by high blood sugar (hyperglycemia), with T2D the body either resist the effect of insulin or does not produce enough insulin. In order to underline the role of the genes involved in this study, we investigate ,using PCR-RFLP for *CTLA-4* gene , PCR-RFLP and sequencing for *VDR* gene. Polymorphisms of two single nucleotide polymorphisms (SNPs) belonging to both genes in 60 T2D patients and 30 healthy control from Iraqi population. The present local study demonstrated that *VDR* FoK-I, FF genotype (P = 0.04) and F,f alleles (P=0.07) frequencies were significantly associated while *VDR* Bsm-I was significantly non-associated. *CTLA-4* -1722(T>C) and +49 (A>G) shows non-significant association. These result suggest the involvement of *VDR* gene in the genetic susceptibility to T2D. Interestingly FoK-I, contributes to increasing the risk to the disease in our population. However, Further studies are require to confirmed this finding.

Key words: Type 2 Diabetes, VDR gene ,CTLA-4 gene, polymorphisms.

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Introduction

Type2 Diabetes (T2D):(formerly non insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes) is a progressive condition that is characterized by high blood sugar (hyperglycemia).With T2D the body either resist the effect of insulin or does not produce enough insulin (1). Scientists believed that genetic susceptibility and environmental factors are the most suitable triggers of type2 diabetes (2,3). The diabetes cases involved many genes, considered a contributor to an increased chanced of becoming type 2 diabetes (4). Studies was showed that vitamin D could play a central role in the developed of T2D too. Epidemiological data illustrated a reduction in vitamin D defect in a population at risk for T2D compared with subjects not at risk. So the *VDR* gene considered as a important candidate gene for T2D, because vitamin D do its action through the *VDR*. Genetic alterations of the *VDR* [Vitamin D (1, 25- dihydroxy vitamin

D3) receptor] gene may cause to defects in gene activation or changes in the protein structure of the *VDR*, both of which could affect the cellular functions of vitamin D. (5). *VDR* gene located in the long (q) arm of chromosome 12 at position 13.11 and have eight coding exons and seven introns (6).

CTLA4 or *CTLA-4* (cytotoxic T-lymphocyte-associated protein 4), also known as CD152 (cluster of differentiation 152), is a protein receptor that is important as an immune checkpoint, downregulates the immune system. *CTLA4* is located on the surface of T cells, and acts as an "off" turning when bound to CD80 or CD86 on the surface of antigen presenting cells. The *CTLA-4* gene encoded the *CTLA-4* protein in mouse and in human. It located in the long (q) arm of chromosome 2 at position 33 and have four coding exons and three introns.(7,8).Another studies have showed an association of *CTLA4* alanine-17 with T2D (9,10).CTLA4 considered a key regulatory element in the T cell/antigen-presenting cell interaction (11). Because *CTLA4* mediates antigen-specific apoptosis and advanced β-cell failure is a typical characteristic of type 2 diabetes, *CTLA4* may be a candidate gene to confer sensitivity also to T2D (12). The aim of this study is to determine whether polymorphisms [FoK-I , Bsm-I,-1722(T>C) and +49(A>G)] of the genes (*VDR* and *CTLA-4*) respectively contribute to the development of T2D in Iraqi population.

Materials and Methods

Sample collection

Sixty human blood sample of patients with T2D were enrolled in this study and collected from Kadhimiya Teaching Hospital in Baghdad government during the period from the first November 2014 to the end of February 2015, in addition to thirty human blood sample for healthy individual (control).There age ranged between 20 and 77 years and diagnosed with type 2 DM according to World Health Organization criteria (pancreatic beta-cell destruction as the primary cause of diabetes, and tendency to ketoacidosis). All the volunteer were informed about the aim of this investigation. A questionnaire was filled including the time of onset of diabetes ,the history of the family and the geographical origin.

SNPs Genotyping

Approximately (3-5) ml venous blood samples were collected in sterile EDTA tubes by sterile syringe, The DNA was extracted from blood samples by using wizard genomic DNA purification kit (Promega-USA) and stored at -20 °C until use.

Genomic DNA was amplified using PCR technique with primers for each SNPs and genes as shown in Table1.The total volume was 25 µl containing: 12.5 µl of Go Taq®Green Master Mix was provided by (Promega–USA), 1 µl of each primer (10pmol), 1µl of DNA template and sterile distilled water was added to achieve a total volume of 25 µl.

Table 1 : The primers uesed in PCR technique for genotyping the SNPs of *VDR/CTLA-4* gene and their details

Primers	Sequence
Fok-1-F	5- AGCTGGCCCTGGCACTGACTCTGGCTCT-3,
Fok1-R	5- ATGGAAACACCTTGCTTCTTCTCCCTC -3.
Bsm-I-F	5- CAACCAAGACTACAAGTACCGCGTCAGTGA-3,
Bsm-I-R	5- CAACCAAGACTACAAGTACCGCGTCAGTGA-3,
-1722C>T -F	5’CAAGCTTTGTCCTGTGACCA3’
-1722C>T -R	5’AAGCGCCAACAAGCATAAC3’
+49A>G -F	5'-AAGGCTCAGCTGAACCTGGT-3'
+49A>G -R	5'CTGCTGAAACAAATGAAACCC3'

The PCR reaction conditions carried out at Initial Denaturation (95 for 7 minutes)followed by 35 cycles of Denaturation (95°C for 45 seconds), Annealing (68°C to *VDR* gene and 56°C to *CTLA-4* gene for 45 seconds) , Extension (72°C for 1 minute) and the final extension (72°C for 7 minute) for both primers. Next the PCR product digested with Restriction enzyme, the PCR products of the all subjects of *FoK-I* were digested by *FoK-I* restriction enzyme (Biolabs NEW England, R0109S) , for one hour at 37°C, the PCR products of the all subjects of *Bsm-I* were digested by *HhaI* restriction enzyme (*Promega USA* ,R644A) , for one hour at 37°C , The PCR products of the all subjects of -1722(**T>C**) were digested by an *ApeK-I* restriction enzyme (Biolab New England, R0643S) , for 15 minute at 75°C and The PCR products of the all subjects of +49(**A>G**) were digested by an *BstEII* restriction enzyme (Promega USA, R664A) , for 1 hours minute at 60°C. The digested DNA fragments were separated in a 3% of Agarose and then stained with ethidium bromide and visualized under UV illumination and photographed. The digested alleles yielded the fragments listed in Table 2.

Table 2: The Alleles details

The Gene	The polymorphism	PCR product (bp)	Digested alleles	Restricted fragment (bp)
VDR	*FoK-I*	270bp	F,f	196,69
VDR	*Bsm-I*	820bp	B,b	650,175
CTLA-4	-1722C>T	398bp	T,C	266,132
CTLA-4	+49A>G	152bp	G,A	152,131

Statistical Analysis

Allele frequencies of *VDR* and *CTLA-4* genes was calculated by direct gene counting method, while significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles, which is available free online at http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-3-alleles.html. Hardy Weinberg equilibrium is the expected frequencies of genotypes if mating is non-assortative and there are no mutations from one allele to another. When there are two alleles for a particular gene; A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB and BB are p^2, 2pq and q^2, respectively. Significant differences between the observed and expected frequencies were assessed by Pearson's Chi-square test.

Results and Discussion

The genotype FF of *VDR* gene at *Fok-I* position demonstrated a significant (P=0.04) increased percentage frequency in T2D patients (65%) compared to controls (40%). The F allele was increased (80.83 vs 63.3 %), while f allele was decreased (19.16 vs 36.6%) in patients (Table3). However, in *Bsm-I* ,neither genotypes (BB, Bb and bb) nor alleles (F and f) demonstrated a significant difference between patients and controls (Table 4) .While the genetic polymorphism of *CTLA-4* was determined at -1722 and +49 , which were presented with three genotypes (TT,TC and CC for -1722 and GG,AG and AA for +49) in T2D patients and controls. However, none of these genotypes or their corresponding alleles showed a significant variation between patients and controls (Tables 5 and 6).

Table 3: Observed numbers and percentag frequencies of *VDR* genotype and alleles at *FoK-I* position in T2D patients and controls

Genotype or Allele	Patients (No.=60)		Controls (No.=30)		P value
	No.	%	No.	%	
FF	39	65	12	40	**0.04**
Ff	19	31.6	14	46.6	**N.S**
ff	2	3.3	4	13.3	**N.S**
F	97	80.83	38	63.3	**0.017**
f	23	19.16	22	36.6	**0.017**

Table 4: Observed numbers and percentage frequencies of *VDR* genotype and alleles at *Bsm-I* position in T2D patients and controls

Genotype or Allele	Patients (No.=57)		Controls (No.=30)		P value
	No.	%	No.	%	
BB	31	51.7	20	66.7	**N.S**
Bb	23	38.3	10	33.3	**N.S**
bb	6	10.0	0	0.0	**N.S**
B	85	70.8	50	33.3	**N.S**
b	35	29.2	10	10.7	**N.S**

Table 5: Observed numbers and percentag frequencies of *CTLA-4* genotype and alleles at -1722 position in T2D patients and controls

Genotype or Allele	Patients (No.=53)		Controls (No.=30)		P value
	No.	%	No.	%	
TT	52	98.1	30	100	**N.S**
TC	1	1.9	0	0	**N.S**
CC	0	0	0	0	**N.S**
T	105	99.1	60	100	**N.S**
C	1	1	0	0	**N.S**

Table 6: Observed numbers and percentag frequencies of *CTLA-4* genotype and alleles at +49 position in T2D patients and controls

Genotype or Allele	Patients (No.=60)		Controls (No.=30)		P value
	No.	%	No.	%	
GG	21	35	16	53.3	**N.S**
AG	25	41.6	9	30	**N.S**
AA	14	23.3	5	16.6	**N.S**
G	67	55.83	41	68.3	**N.S**
A	53	44.17	19	31.7	**N.S**

The observations among other populations around the world, showed that T2D has a strong genetic component, as evidenced by a high concordance rate (70%) in monozygotic twins and the over 40% risk of having T2D for an offspring of a T2D patient. In contrast to simple monogenic diseases, the pathophysiology of T2D involves an interaction of several common genetic risk factors with environmental factors. Also, the vast majority of T2D-related risk alleles have been find through genome-wide association studies, the most conducted in European Descendants and Asian populations (13). It is therefore important to understand the role of genes in Iraqi populations as there may be specific genetic susceptibility to T2D in these groups. The aim of the presented study was to evaluate the polymorphic sites in *VDR* and *CTLA-4* genes associated with type2 diabetes mellitus in a sample of Iraqi patients. This is, to our knowledge, the first study investigating whether +49 (A>G) and -1722(T>C) of *CTLA-4* and *FoK-I* and *Bsm-I* of VDR are associated with T2D patients in Iraqi population. The results showed, a significant association between the presence of the *VDR Fok-I* F allele and the T2D was observed, and at the same time that the *VDR Fok-I* f allele was more frequent among control individual. These finding suggest a protective role for the f allele in opposition to the role of F allele , which seems to be a predisposing factor to T2D in Iraqi population. In addition, the results seem to reinforce the association of the FF genotype with the susceptibility to the T2D .Also the presented study did not find any significant differences in *VDR* gene polymorphism at position Bsm-I between the patients and the controls, an observation that has been corroborated by a analysis study by Mackawy and Badawi, (2014) (14) ,in which the FF genotype association and the *Bsm-I* genotypes and allele non association with T2D is clearly obtained, but this result in contrast with the data from Morocco (5) that have the ff allele associated with T2D. However, further studies failed to show association between this polymorphisms and T2D in Polish (15) and Asian meta analysis study (16). The results of *CTLA-4* suggest that the +49 genetic polymorphism is not involved in susceptibillity to or ;protection against T2D in Iraqi patients. similary, Uzer *et al* . (2010) (17) and .RAU *et al* .(2001) (18) reported no significant differences in the frequency of +49 genotypes and alleles in Turkish and Caucasian T2D patients respectively. The result for the -1722(T>C) with T2D were the first investigation in world wide. In conclusion, *VDR* SNP *Fok-I* (2228570), may have an effect on the occurrence of T2D in Iraqi population while *VDR* the SNP *Bsm-I* (rs1544410), and *CTLA-4* SNP -1722(T>C) (rs733618) and SNP +49 (A>G)(rs231775)were not associated with it.

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