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Association of Genetic Polymorphisms in a Sample of Iraqi Patients with Type1 Diabetes mellitus

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A B S T R A C T

Type 1diabetes (T1D) is an organ-specific autoimmunity characterized by the invasion of auto-reactive T lymphocytes to the cells of the Langerhans islets that secreted insulin and the production of autoantibodies versus pancreas antigens. 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 T1D patients and 30 healthy control from Iraqi population. The present local study demonstrated that *VDR* gene FoK-I, FF genotype (P = 0.003) ,Ff genotype (P = 0.002) and F, f alleles (P = 0.002) frequencies were significantly associated; *VDR gene* Bsm-IBB genotype ($P=1.9\times10^{-4}$), bb genotype ($P= 8.1\times10^{-4}$) and B, b alleles ($P=4.6\times10^{-6}$) was significantly associated .*CTLA-4*-1722(T>C) was non significant association while +49 (A>G) GG genotype (P=0.036) and AA genotype (P=0.004) shows significant association. These result suggest the involvement of *VDR* and *CTLA-4*gene in the genetic susceptibility to T1D. Interestingly FoK-I, Bsm-I and +49 (A>G) contributes to increasing the risk to the disease in our population. However, Further studies are require to confirmed this finding especially the *VDR* gene and*CTLA-4*-1722(T>C) gene investigated for the first time in Iraqi population.

Introduction

Type 1 Diabetes (T1D) is a chronic multifactorial disorder with a strong genetic component. It arises as a result of autoimmune destruction of pancreatic β-cells and the consequence is insufficient production of insulin. The spread of diabetes is increasing through the world (Kumar *et al*., 1995). Heredity plays an important role in determining who is more probably to develop type 1 diabetes. Biological parent are passed down the genes to offspring.

The risk of evolving T1D is determined by a complex interaction between various genes and environmental factors (Bakay *et al*., 2013). The *VDR* gene is related to T-cell-mediated autoimmune disease and influence susceptibility to T1D (Lee *et al*., 2012). It plays a central role in the pathogenesis and progression of T1D because vitamin D which is an intracellular hormone receptor do it action at a cellular level through attachment to the *VDR* gene, the *VDR* gene has considered a candidate gene for T1D (Lemos *et al*., 2008).

VDR gene located in the long (q) arm of chromosome 12 at position 13.11 and have eight coding exons and seven introns (Malloy *et al*., 2011).These polymorphisms in the *VDR* gene (FokI, BsmI and ApaI widely studied) have been suggested that they are linked with the increased susceptibility in different ethnic population to T1D. *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 mouseand in human. It located in the long (q) arm of chromosome 2 at position 33 and have four coding exons and three introns.(Brunet *et al*., 1987; Dariavach *et al*., 1988). Several single nucleotide polymorphisms (SNP) have been identified in the *CTLA-4* gene: −1661A/G and −1722C/T at the promoter, and the untranslated region of exon 1+49. Previous studies have shown the association of *CTLA-4* with T1D (Almasi *et al*., 2015).

The aim of this study is to determined 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 T1D in Iraqi population.

Materials and Methods

Sample Collection

Sixty human blood sample of patients with T1D were enrolled in this study and collected from Kadhimiya Teaching Hospital in Baghdad government, in addition to thirty human blood sample for healthy individual (control). There age ranged between 1 and 16 years and diagnosed with type 1 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 ℃ 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 0f Go Taq®Green Master Mix was provided by (Promega–USA), 1 µl of of each primer (10pmol), 1µl of DNA template and sterile distilled water was added to achieve a total volume of 25 µl.

The PCR reaction conditions carried out at 95 for 7 minutes followed by 35 cycles of 95°C for 45 seconds,(68°C to *VDR* gene and 56°C to *CTLA-4* gene) for 45 seconds, 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.

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 genetic polymorphism of *VDR* was determined at two positions FoK-I and Bsm-I, which were presented with three genotypes (FF, Ff, ff for FoK-I and BB, Bb, bb for Bsm-I) in T1D patients and controls. For VDR FoK-I, the FF genotype frequencies were higher in the patients than the controls (73.3%versus 40%, P = 0.003) while the Ff genotype frequencies were lower in the patients than the controls (21.6% versus 46.6%,P = 0.002) although the differences were for both genotype significant but FF as a risk factor for the disease and Ff as a protective factor. Both F and f alleles were significant (P=0.002),that F allele increased in patients (84.16 vs 63.3 %) and the f allele decreased (15.83 vs 36.6%) as shown in table (3).

For *VDR* Bsm-I, the BB genotype frequencies were lower in the patients than the control (24.6% versus 66.7%, P = 1.9×10^{-4}) while the bb genotype frequencies were higher in the patients than the controls (28% versus 0%,P = 8.1×10-4) although the differences were for both genotype statistically significant but bb considered as arisk factor and BB as a protective factor for the disease..Both B and b alleles were significantly (P=4.6 * 10^{-} 6),that B and b alleles increased in patients (48.3 vs 33.3 % and 51.7 vs 10.7% respectively) as shown in table (4).

The genetic polymorphism of *CTLA-4* gene was determined at two positions; -1722(T>C) and +49 (A>G), which were presented with three genotypes (TT, TC and CC for CTLA-4 -1722; GG, AG and AA for CTLA-4 +49) in Iraqi patients and controls. Out of the CTLA-4 genotypes observed at the two positions, only GG and AG genotypes at position +49 was observed with significant (P=0.036 and P=0.004 respectively); Decreased percentage frequency in T1D patients compare to controls (28 vs 53.3 %) for GG genotype and increased percentage frequency in T1D patients compare to controls (63.3 vs 30 %) for AG genotype, while the remained geneotypes fail to show a significant variation between patients and controls (Tables 5 and 6).

The cumulative evidence supports a strong genetic component associated with DM, epidemiological data showed wide differences in geographic prevalence with populations of European ancestry that having the highest presentation rate. T1D also has high concordance among the monozygotic twins (33% to 42%), and the disease runs strongly in families with siblings risk being approximately 10 times greater than in the general population; this is in clear contrast to the type 2 diabetes which is less genetic, where the sibling risk ratio is relatively medium. It is therefore important to understand the role of genes in Iraqi populations as there may be specific genetic susceptibility to T1D in these groups. The aim of the presented study was to evaluate the polymorphic sites in *VDR* and *CTLA-4* genes associated with type1 diabetes mellitus in a sample of Iraqi patients. This is, to our knowledge, the first study investigating whether -1722(T>C) of *CTLA-4* and FoK-I and Bsm-I of VDR are associated with T1D patients in Iraqi population.

The T1D patient's age ranged between (1-16) years, with median age of 8.5 years. The high frequency onset of T1D was all recorded at the child wood before the age of puberty. This age range was in agreement with the study of Wherrett *et al*., (2000) who found that T1D recorded at this child wood stage(9). In assessment of the results for the family history of diabetes in this study revealed that T1D for a parental history was 6.7% (4 out of 60),for the relatives was 18.3% (11 out of 60);The study by Fujisawa *et al*.(2004) reported that 22% with T1D have a parental history of diabetes compared to control 7% and this revealed that the family history in patients in T1D increased when compared with healthy controls (Fujisawa *et al*., 2004).

Table.1 The primers used 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'

Table.2 The alleles details

The Gene	The polymorphism	PCRproduct (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

Table.3 Observed numbers and percentage frequencies of VDR genotype and alleles at FoK-I position in T1D patients and controls.

Genotype or Allele	Patients (No.=60)		Controls (No.=30)		OR*	P value*
	No.	%	No.	%		
FF	44	73.3	12	40	4.12	**0.003**
Ff	13	21.6	14	46.6	0.32	**0.002**
ff	3	5	4	13.3	0.34	**N.S**
F	101	84.16	38	63.3	3.08	**0.002**
f	19	15.83	22	36.6	0.32	**0.002**

OR: odds ratio / P: probability

Table.4 Observed numbers and percentage frequencies of VDR genotype and alleles at Bsm-I position in T1D patients and controls.

Genotype or Allele	Patients (No.=57)		Controls (No.=30)		OR*	P value*
	No.	%	No.	%		
BB	14	24.6	20	66.7	0.16	**1.9×10^{-4}**
Bb	27	47.4	10	33.3	1.8	**N.S**
Bb	16	28.0	0	0.0	24.25	**8.1×10-4**
B	55	48.3	50	33.3	0.19	**4.6×10-6**
b	59	51.7	10	10.7	5.36	**4.6×10-6**

OR: odds ratio / P: propability

Table.5 Observed numbers and percentage frequencies of CTLA-4 genotype and alleles at -1722 position in T1D patients and controls.

Genotype or Allele	Patients (No.=60)		Controls (No.=30)		OR*	P value*
	No.	%	No.	%		
TT	57	95.0	30	100	-	**N.S**
TC	3	5.0	0	0.0	-	**N.S**
CC	0	0.0	0	0.0	-	**N.S**
T	117	97.0	60	100	-	**N.S**
C	3	2.5	0	0	-	**N.S**

OR: odds ratio / P: probability

Table.6 Observed numbers and percentage frequencies of CTLA-4genotype and alleles at +49 position in T1D patients and controls.

Genotype or Allele	Patients (No.=60)		Controls (No.=30)		OR*	P value*
	No.	%	No.	%		
GG	17	28	16	53.3	0.35	**0.036**
AG	38	63.3	9	30	4.03	**0.004**
AA	5	8.3	5	16.6	0.45	**N.S**
G	72	60	41	68.3	0.70	**N.S**
A	48	40	19	31.7	1.44	**N.S**

OR: odds ratio / P: propability

The present local study may be the first one demonstrating the relationship between the Diabetes and *VDR* gene polymorphisms in Iraqi population for both types. The FoK-I and BsmI shows significant association with T1D, that (FF, Ff, BB, bb) genotypes and (F,f,B,b) alleles respectively have significantly higher frequency in Iraqi population. The FF / bb considered as a risk facror and Ff /BB as protective factor for T1D patients. Iranian population also reported a similar finding for the VDR polymorphisms of FF allele (FoK-I) and Bb allele (BsmI) (Bonakdaran *et al*., 2012). Patients from Germany in Frankfurt also demonstrated that VDR FoK-I ff allele and Ff genotype were positively associated with the disease (Morán *et al*., 2015). Asian, Africans and Latino shared the findings of present study, in which B allele of Bsm-I demonstrated an association with the samples (patients),while the BB genotype increase the risk in Africans and the bb genotype in Asian and Latino (Qin *et al*., 2014). Asian researcher meta analysis proved that FoK-I is more associated with T1D in East Asian population while the BsmI is more associated with T1D in West Asian population and that was according to the classification by region geography (Wang *et al*., 2014). However, Finnish patients had no association with its allele or genotypes (Turpeinen *et al*., 2003).

The present study did not find any significant differences in CTLA-4 gene polymorphism at position -1722 between the T1D patients and controls ; an observation that also has been made in one recent study that involve this position(16).Analysis of CTLA-4 +49 genotypes in this study showed GG genotype (homozygous G allele) associated as a protective factor and AG genotype was associated with susceptibility to TID, while previous investigations showed that the homozygous genotype GG was associated with T1D as a risk factor in Egypt (Saleh *et al*., 2013), Croatian (Korolijan *et al*., 2009) and Japanese patients (Ide *et al*., 2004). In contrast, another studis by Egyptian (Kamel *et al*., 2014) and Japanese (Yanagawa *et al*., 1999) T1D patients demonstrated no significant association with alleles or genotypes of *CTLA-4* +49 polymorphism. Also an Iraqi study show the association of AG genotype with TID but did not show the GG genotype as a protective factor (21).

In conclusion, *VDR* SNPs Fok-I (2228570) and Bsm-I (rs1544410) ;*CTLA-4* SNP+49 (A>G)(rs231775), may have an effect on the occurrence of T1D in Iraqi population while *CTLA-4* SNP -1722(T>C) (rs733618) was not associated with it.

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