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Genotyping Study of CTLA-4 Gene Polymorphism (Rs2319775) In A Sample of Type2 Diabetes Mellitus With And Without Hypertensive Iraqi Patients

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الخلاصة
الخلفية سكري البالغين او ما يعرف بالسكري من النوع الثاني(T2DM) هي حالة تتميز بارتفاع مستويات السكر في الدم. وتتميز باضطراب الايض لكل من الكربوهيدرات والدهون والبروتينات نتيجة التغير في افراز الانسولين او تداخله او كليهما. خلال العقد الماضي مرض السكري وصل الى مستويات وبائية في العراق مناظرا للارتفاع العالمي بإصابات السكري.
الجزء العملي وقد أجريت هذه الدراسة كدراسة للحالات والشواهد التي ترصد التغيرات الجينية المرتبطة بمرضى السكري من النوع الثاني والمصاحب لمرض ارتفاع ضغط الدم.
المواد وطريقة يتكون البحث من مجموعتين رئيستين الاولى تتكون من 100 مريض ينقسمون الى مجموعتين فرعيتين الاولى تتكون من 50 مريض لديهم سكري من النوع الثاني مع ارتفاع الضغط والثانية سكري من النوع الثاني بدون ارتفاع الضغط اما مجموعة الاصحاء فتتكون من 100 شخص. تم استخدام الطرق العادية في تقدير مستويات الدهون بينما تم استخدام تقنية الT-ARMS-PCR
النتائج بمعادلة هاردي-وينبرغ G اليل هو الاكثر وجودا في مجاميع مرضى السكري مع ارتفاع الضغط او بدونه بنسبة (0.51% & 0.52%) اكثر من وجوده في مجموعة الاصحاء(0.425 %).الطراز العرقي GG لديه تأثير ملحوظ بقيمة الP بنسبة اقل من 0.0001 والOR= 22.85 في موديل السائد المشترك، بينما الGA كانت له الOR=3.5 .
الاستنتاج وجدنا أن النمط الجيني GG و AGهما أكثر شيوعا في مجموعات المرضى من النمط الجيني للأصحاء، مما يشير إلى أنه عامل خطر له ارتباط بمرض السكري للنوع الثاني.
الهدف كان لتحديد الارتباط بين النمط الجيني والنمط الظاهري في السكان العراقيين القائمين على التسجيل لدراسات الحالات والشواهد المرتبطة بالتعدد الأشكال الجينية CTLA-4 مع مرض السكري من النوع الثاني

Abstract
Background
The background Adult-onset diabetes, also known as type 2 diabetes mellitus (T2DM), is a condition characterized by elevated blood sugar levels. It's marked by disruptions in carbohydrate, lipid, and protein metabolism due to irregular in insulin secretion, intervention, or both. Over the last decade, diabetes has reached epidemic levels in Iraq, mirroring global rises in the incidence of T2DM.

Method This study was done as a case-control study to replicate these genetic associations in patients with T2DM and hypertension.

Material and Methods Verification of genetic interactions with T2DM and HTN was done as a case-control study. A total of 100 subjects were studied (50 T2DM+HTN and 50 T2DM-HTN), with a control group of 100 subjects. Traditional methods were used to determine the levels of lipid profile. *CTLA-4* genes were genotyped using tetra-primer ARMS PCR technique.

Result in Hardy–Weinberg Equation the G allele is more common in patients with T2DM with hypertension and T2DM without hypertension (0.51 % and 0.52 %, respectively) than in controls (0.425 %).GG genotype has significant risk factor that had significant P value of about <0.0001 and OR is 22.85 in codominant model, while the OR of the AG is 3.5.

Conclusions We found that the GG genotype is more common in patient groups than the GA genotype, indicating that it is a risk factor for T2DM disease.

Aim this research was to establish the genotype–phenotype correlation in the registry-based Iraqi population and the association of *CTLA-4* gene polymorphism with T2DM in a case-control study.

Keywords: Type 2 diabetes; *CTLA-4*; Genotype; Polymorphism; Hypertension; T-ARMS-PCR

Introduction

Diabetes has achieved epidemic status in Iraq over the last decade, with a significant (115 %) rise from 19.58/1000 in 2000 to 42.27/1000 in 2015. This is consistent with global trends for the prevalence of diabetes mellitus[1].

Cardiovascular diseases (CVD) such as hypertension (HTN), stroke, and end-stage renal disease are all linked to type 2 diabetes mellitus (T2DM). Diabetes and hypertension are sometimes found together, and the two diseases exacerbate each other [2] and increase the cause of morbidity and death[3]. Type 2 diabetes epidemiology is affected by genetic and environmental factors. Genetic factors have an impact after being exposed to an obesogenic environment marked by sedentary behavior and excessive sugar and fat intake [4] [5]. T2DM is caused by a group of genes known as regulatory genes. These genes' resistance varies depending on where they are on the chromosome. Because of the interaction of environmental factors with these genes that contribute to the onset of the condition, identifying the genetic laborer trigger associated with T2DM is challenging. initiation[6]. The most signs and symptoms of DM are typically included: polyuria, polydipsia, polyphagia, weight loss, blurring vision, increased susceptibility to illness, weakness, neuropathy, itchiness[7]. The CTLA-4 a part of the superfamily of immunoglobulins and encodes a protein that communicates to T cells an inhibitory signal. There is a V domain, a transmembrane domain, and a cytoplasmic tail in the protein. Alternate versions of transcriptional splices have been characterised, coding various isoforms. The membrane-bound isoform acts as a disulfide-bond entangled homodimer, whereas the soluble isoform behaves as just a monomer[8].One of the most basic immunosuppressive cytokines is cytotoxic T-lymphocyte antigen 4 (*CTLA-4*), which is predominantly expressed on activated T cells[9].*CTLA-4* gene encode a CTLA-4 protein receptor the gene *CTLA-4* is found in the long. arm of chromosome 2 at position of 33.2 (2q33.2) and consists of four exons and three introns[10], comprises more than 100 polymorphic sites[11] and is converted into a peptide of 233 amino acids. The CTLA-4 protein consists of a signal peptide and a main chain (the first 35 amino acids). The *CTLA-4* gene identifies three excellently polymorphic markers amongst which, *CTLA-4* +49A/G has been extensively studied as well as its relation with vulnerability to some human diseases such as Gravis disease, recurrent pregnancy loss RPL, type 2 diabetes mellitus and autoimmune thyroid [12].

Change of A-to-G at exon 1 position 49 (+49A/G (rs231775) that occurs in alanine threonine substitution at codon 17 of the fusion protein[13] , contributes to the production of a faulty receptor and impairs the inhibition activity of *CTLA-4* on the activation of lymphocyte T cells[14]. Expression of gene products can be impaired by genetic variations[15].

Tetra primer-amplification refractory mutation system PCR (T-ARMS-PCR) is a contractor PCR modulation since one of the amplicons is equipped to provide a polymorphic unit of nucleotide of contractor PCR in the pattern at its 3' position[16].ARMS-PCR is widely used to identify SNPs as an allele-based PCR or PCR amplification of specific alleles(PASA) or AS-PCR. In the PCR technique, the key enzyme is *Taq* polymerase, which is unable to prolong after a nucleotide, and thus if the 3' nucleotide in the primer fits the sequence, the replication of a PCR unmatched output takes place alone. The approach can be combined at the very same time to categories up to 20 SNPs. In use, inserted unparalleled at the 3' is necessary for a rigorous assessment to a minus 3 base position slightly lighter primer bounding. ARMS moderate and low-cost treatment for SNP identification each for a single tube or in different tubes. This is a professional approach done of great precision and accuracy [17]–[19].

In recent days, the ARMS PCR has been one of the important techniques in the diagnosis of genetic disorder. The process of restriction digestion is not 100 percent effective and therefore not all sequences of the restriction site are available. Thus, in all forms of mutation or polymorphism, restriction digestion may not valid. The approach is intended specifically for SNP genotyping, such approach could distinguish homozygous and heterozygous, it's indeed simple, secure, low precise & quick[20].

Materials and Methods

Study Design and Population adult

This research included 200 participants in a case–control study. This study had two main groups: a main group of 100 patients split into two subgroups (one group of 50 T2DM patients with hypertension disease, and another group of 50 T2DM patients without hypertension disease), and a second group of 100 healthy individuals gathered from colleagues, relatives, and people who go to the hospital for checkups. The patient and control groups were balanced in terms of age and gender. In the patients group, there were (51 females and 49 males) and in the control group, there were (59 females and 41 males). During the period of August 2020 to January 2021, all patients groups were randomly selected from the Diabetic Center at AL-Sadder Teaching Hospital in An Najaf governorate. The research was carried out in the department of biochemistry at the University of Kufa College of pharmacy. The Tetra Amplification Refractory Mutation System PCR (ARMS-PCR) technique was used to determine the relationship of SNP rs231775 of the cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) genes in T2DM with and without hypertension disease.

Collecting samples

Fasting blood samples overnight 8-10 hours were obtained from all T2DM as well as healthy subjects, 2mml of blood was taken from them by vein puncture and collecting in EDTA tube and saved by freezing at -20˚.

DNA Extraction and Quantification of genomic nucleic acid

The genomic DNA (gDNA) was extracted from whole blood according instruction of corporation (Promega; USA). Typically, its concentration and purity were the first thing one need to hear about a DNA harvested. Both were measured by the calculation of ultraviolet light absorption. Dependent on the wavelength, DNA more or less intensely absorbs UV. A Quantus spectrophotometer (Promega; USA) was used, which takes 260/280 nm wavelength measurements**.**

Tetra-Primer Amplification Refractory Mutation System PCR (T-ARMS-PCR)

The A updated T-ARMS PCR system was adapted previously to the T-ARMS PCR in which a nested PCR result was introduced. This nested PCR would act as a T-ARMS PCR DNA template. The *CTLA-4* gene genotyping technique was used to evaluate the tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) technique. It depends of Taq polymerase and four primers in brief as in (Table.1)

Table 1 Primers of T-ARMS PCR for detection of rs 231775 of *CTLA-4* (+49 A/G)

Target gene		Sequence (5'-3')	Tm (°C)	Product size
Outer	F	5'-GTGGGTTCAAACACATTTCAAAGCTTCAGG-3'	56	226 bp
	R	5'-TCCATCTTCATGCTCCAAAAGTCTCACTC-3'		
Allele G	F G	5'-GCACAAGGCTCAGCTGAACCTGGATG-3'	56	117 bp
Allele A	R A	5'-ACAGGAGAGTGCAGGGCCAGGTCCTAGT-3'	56	162 bp

For a final reaction volume of 25 µl, the ratio of the outer primer to the genotype-specific primer is 1:10. The reaction state consisted of preheating for 5 min at 94 °C followed by 35 denaturation cycles for 30 s at 94 °C, annealing for 30 s at 56 °C, extension for 60 s at 72 °C, and final extension for 5 min at 72 °C. Scoring was achieved by operating the PCR products for 40 minutes at 3-5 volts/cm on a 2 percent agarose gel electrophoresis[19]. In comparison to molecular size markers, the genotypes are distinguished by verifying the amplicon sizes.

Statistical Analysis Statistical analysis

The presented data were analyzed by SPSS v.20.0 software (PASW Statistics, Journal Pre-Proof Journal Pre-proof 8 SPSS Inc., Chicago, IL, USA). All clinical parameters and demographic were exhibited as mean ±SD and analyzed using t-test and Chi-square ($\chi 2$) test. Hardy-Weinberg equilibrium was done by SNP-Analyzer version 1.15 ga easy analysis ($p>0.05$) using the frequencies of intended gene for healthy individuals. Genotyping calculations between healthy subjects and patients was performed using odds ratio (OR) and 95% confidence intervals (95% CI). P value less than 0.05 was considered significant in comparison assessment**.**

Results

Table 2 measurement of human body and its proportions and lipid profile of T2DM with and without hypertension disease

Parameter	Group	No.	Mean ± SD	Min-Max	P-value
Age	control[a]	100	48.2.3±6.2	35-62	0.114
	T2DM-HTN [b]	50	46.5±5.4	35-58	0.825
	T2DM+HTN [c]	50	46.8±5.6	34-60	0.184
BMI(kg/m^2)	control	100	26.9±4.5	19.7-39.68	0.637
	T2DM-HTN [b]	50	27.7±3.2	21.7-40.26	0.512
	T2DM+HTN [c]	50	27.4±3.2	21.9-37.39	0.230
Cholesterol (mg/Dl)	control	100	154.3±20.9	113-183.8	0.0000[a]
	T2DM-HTN [b]	50	238.3±34.2	178.77-303.90	0.0000[b]

	T2DM+HTN [c]	50	216.9±40.5	119.06-305.77	0.0010[c]
TG (mg/dL)	Control [a]	100	117.2±18.7	82.63-143.98	0.0000[a]
	T2DM-HT N[b]	50	230.4±41.3	154.81-297.58	0.0000[b]
	T2DM+HTN [c]	50	213.3±57.2	82.63-297.58	0.0230[c]
HDL-c (mg/dL)	Control [a]	100	71.9±5.7	61.04-88.48	0.0000[a]
	T2DM-HTN [b]	50	49.0±6.3	35.34-64.98	0.0000[b]
	T2DM+HTN [c]	50	53.3±11.6	35.34-84.14	0.0050[c]
VLDL-c (mg/dL)	Control [a]	100	23.4±3.7	16.53-28.80	0.0000[a]
	T2DM-HTN [b]	50	46.1±8.2	30.96-59.52	0.0000[b]
	T2DM+HTN [c]	50	42.6±11.4	16.53-59.52	0.0230[c]
LDL-c (mg/dL)	Control [a]	100	59.0±22.0	16.15-95.01	0.0000[a]
	T2DM-HTN [b]	50	143.2±36.4	71.16-209.02	0.0000[b]
	T2DM+HTN [c]	50	120.9±39.5	16.15-202.21	0.0000[c]

a= Comparison between control and (T2DM-HT) groups, b= Comparison between control and (T2DM+HT) group, c= Comparison between (T2DM-HT) and (T2DM+HT), SD:standerd deviation, P-Value: Probability, TG: trichleceride, HDLc: High Density Lipoprotein Binding Cholesterol, VLDLc: Very Low Density Lipoproteins Binding Cholesterol, LDLc: Low Density Lipoprotein Binding Cholesterol.

This study included a total of 200 randomly unrelated Iraqi subjects; 50 patients with T2DM and 50 patients T2DM with hypertension and 100 controls in T2DM patients (T2DM and 50 patients T2DM with hypertension) and controls, the average age [(Mean±standard deviation (SD)] was (45.77 ± 5.42; 46.03 ± 5.62), 47.78 ± 7.43 years respectively. the age and BMI is non-significant in this study as it shown in the (Table 2).and all lipid profile show significant p-value <0.05.Subsequent to PCR, the genotyping of *CTLA-4* gene polymorphism was made by utilizing T-ARMS-PCR technique.

Table 3 Frequencies of different polymorphism Genotyping of the re231775 (A>G) polymorphism in the groups of the study

Genotype	Healthy control=100	Patients		
		T2DM+HTN NO=50	T2DM-HTN NO=50	Total NO=100
AA	58	5	17	22
AG	39	34	18	52
GG	3	11	15	26
Total	100	50	50	100

(Table 3) shown the abundance of multiple genotypes of polymorphism of rs231775 for healthy controls and patients with type 2 diabetes mellitus including T2DM with hypertension (T2DM+HTN) and T2DM without hypertension (T2DM-HTN). In this study the most type available is the (wild type) homozygote (AA) which is more frequent in control group than others genotypes of patients groups; while (AG) and (GG) genotype is more frequent in patients group than the control.

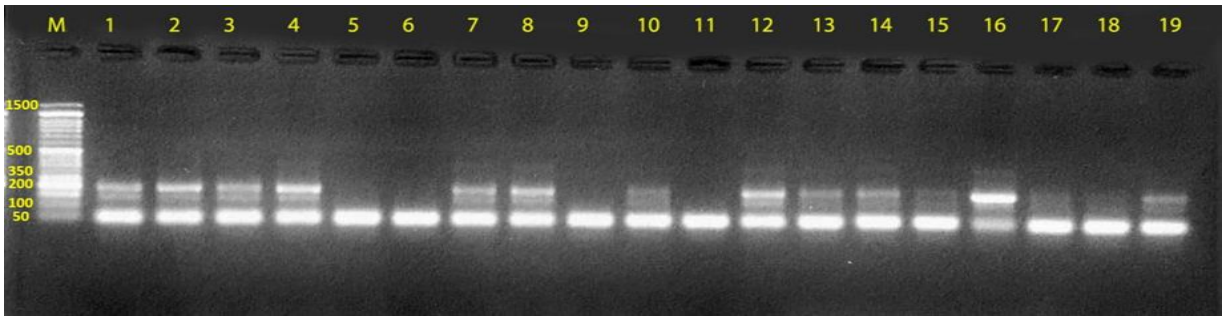

Figure 1 Detection of rs231775 (+49A/G) SNP of gene homo sapiens using T-ARMS PCR technique

Table 4 Results of genotypes bands in Agarose gel electrophoresis of the Tetra-primer PCR Assay Products of rs231775 SNP

Genotype		Bands	Size(bp)
Wild type	AA	2	226,162
Heterozygous	AG	3	226,162,117
Homozygous	GG	2	226,117

The bands and it size of three groups of genotypes of rs231775 SNP in electrophoresis are shown in the (Figure 1) and the (Table 4)

Table 5 *CTLA-4* SNP rs2319775 (Ala946Thr) analysis of genotype that show the expected and observed HWE values for control samples, T2DM with hypertension and T2DM without hypertension

Genotype of control					
Genotype of Control	Observed	Expected	Difference	X^2	P value
AA Reference	58	60.06	2.02	1.4	0.11
AG Heterozygote	39	34.87	4.13		
GG Recessive	3	5.06	2.06		
Genotype of T2DM with hypertension					
AA Reference	5	9.68	4.68	7.22	0.02
AG Heterozygote	34	24.64	9.36		
GG Recessive	11	15.68	4.68		
Genotype of DM without hypertension					
AA Reference	17	13.52	3.48	3.88	0.015
AG Heterozygote	18	24.96	6.96		
GG Recessive	15	11.52	3.48		

Chi-square ($\chi 2$) test was used. P-value is significant at <0.05.

The (Table 5shown the HWE rapprochement that consistent between the observed and expected genotype frequencies in the polymorphism was accomplished based on the $\chi 2$ test for all groups, which is consistent in genotype of control group as the P-value is more than 0.05, while its not consistent in genotype of both T2DM with hypertension group and T2DM without hypertension group due to P value which is less than 0.05. the frequency of the alleles of *CTLA-4* SNP re231775 have some differences, it appear that the A allele in both groups of patients

T2DM with hypertension and T2DM without hypertension(0.49% & 0.48%) respectively, while it (0.575%)in control group.in the other hand the G allele in both groups of patients T2DM with hypertension and T2DM without hypertension are (0.51% & 0.52%) in both patient groups respectively and (0.425%)in control group, That mean the G allele is more frequent in patients groups than other.

Table 6 Genotype of CTLA-4 SNP rs1231775 (+49A/G) in the studied groups of both the T2DM with hypertension and patients of T2DM without hypertension

SNP rs1231775	T2DM-HTN=50	T2DM+HTN=50	OR (CI 95%)	P- value
Codominant				
AA (Wild type)	17	5	1.00	0.001
AG	18	34	6.4 2.03 to 20.3	
GG	15	11	2.5 0.7 to 8.8	0.09
Dominant				
AG+GG	33	45	4.64 1.55 to 13.84	0.006
Over dominant				
AA+GG	32	16	1.00	0.001
AG	18	34	3.78 1.65 to 8.65	
Recessive				
AA+AG (Wild type)	35	39	1.00	0.14
GG	15	11	0.66 0.2670 to 1.6220	
Additive				
2GG+AG	48	56	3.96 1.36 to 11.5	0.01

The (Table 6) shown the analysis of homozygote genotype (GG) in this study is not significant alone in Codominant model and recessive models when compare betweenT2DM+HTN and T2DM-HTN, but when together with (AG)[AG+GG] increase the risk 4.1 in dominant model and in [2GG+AG] 3.9 folds in additive model. Heterozygous genotype (AG) the OR=6.4, (95%CI:2.03 to 20.3), P= 0.01 in codominant and OR=3.78, (95% CI: 1.65 to 8.65), P= 0.01 in over dominant that mean the presence of heterotypic will increase the risk about 6.4 and 3.7 folds respectively.

Table 7 Genotype of CTLA-4 SNP rs1231775 (+49A/G) in the studied groups of both the control and all patients of T2DM with and without hypertension

SNP rs231775	Control=100	Total patients=100	OR (CI 95%)	P- value
Codominant				
AA (Wild type)	58	22	1.00	0.0001
AG	39	52	3.5 1.85 to 6.68	
GG	3	26	22.85 6.28 to 83.17	0.0001
Dominant				
AG+GG	42	78	4.89 2.64 to 9.1	0.0001
Over dominant				
AA+GG	61	48	1.00	0.0657
AG	39	52	1.69 0.97 to 2.97	
Recessive				
AA+AG (Wild type)	97	74	1.00	0.0001
GG	3	26	11.4 3.3 to 38.976	
Additive				
2GG+G	45	104	6.1 3.34 to 11.13	0.0001

In this study the (Table 7) shown the significant result of the homozygote genotype (GG) the OR=22.85, (95%CI: 6.28 to 83.7), P= 0.0001 in Codominant model while have OR=11.4, (95%:3.3 to 38.976) , P= 0.0001 in recessive model that mean increased risk of T2DM+HTN about 22.8 and 11.4 folds respectively. Heterozygous genotype (AG) the OR=3.5, (95%CI: 1.85 to 6.68), P= 0.0001 in codominant model that increase the risk about 3.5 fold.(AG+GG) and (2GG+AG) will be more significant and increase the risk to 4.8 and 6.1 folds respectively.

Discussion

Following communicable disorders, cardiovascular disease, cancer, and accidents, diabetes mellitus is now the sixth leading cause of death worldwide in the upper - middle income countries and the ninth in the lower-middle countries and the fourth in Iraqi patients[21] So there is great interest of diabetes mellitus disease because it is among the most common chronic diseases that affects human health, and its occurrence progressively growing throughout the globe[22].

CTLA-4 is a key regulatory factor in the interaction between T cells and antigen-presenting cells, and it has been identified on chromosome 2q33[20[24]. T2DM is a metabolic condition in which the pancreas develops insufficient amount of insulin to satisfy the body's requirements or may be due to defect in insulin receptor. T2DM has also been suggested as an innate immune system disorder[25]. *CTLA-4* dysfunction has been linked to autoimmune disorders such as type 1 diabetes T1DM, Graves' disease, and asthma [26][27].*CTLA-4* gene +49A>G (exon 1) polymorphisms have been genotyped by a previously described (T-ARMS-PCR) tetra-primer amplification refractory mutation system polymerase chain reaction (PCR)[28][29]. Some

exploratory studies have recently looked at possibilities between *CTLA-4* gene polymorphisms and the much more common type 2 diabetes (T2DM)[30]. It is involved in antigen-specific apoptosis and pancreatic cell death, which is a common symptom of occurrence of T2DM. The results of SNP appeared deviated from HWE when analyzed as a case-control genetic association of the groups of T2DM with and without hypertension, here the Chi square showed results equal to (7.22& 3.88)respectively with significant p value of 0.05, while the control group is consistence with HWE with of p value less than (0.05) and Chi square is 1.4 and this result as same with (Zixian *et al*; 2013)[27] .

Conclusions

The rs231775 SNP in *CTLA-4* gene is a risk factor for the development of HTN in type 2 diabetic patients. Carriers of the genotypes AG and GG have a chance to have HTN 3.5 and 22.8 times respectively relative to those of the wild genotype (AA).

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