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Association of CTLA-4 (+49A/G) gene Polymorphism with type 1 diabetes mellitus in Iraqi children

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ABSTRACT

CTLA4 is a regulator gene for T cells and relates to autoimmune diseases. By using a case-control method, CTLA4 functional single-nucleotide polymorphisms for potential associations with Type 1 diabetes mellitus in an Iraqi children's population. ARMS-PCR method is used for genotyping +49AG (rs231775) variations in 60 obese children and 60 ethnically matched controls; all measured subjects were (fasting glucose, fasting insulin, and HbA1c). The glucose oxidase method is used to determine plasma glucose levels. The amounts of insulin in the blood were determined using a radioimmunoassay (RIA); Insulin resistance was measured using the HOMA-IR index. A HOMA-IR cut-off level of 2.5 was acceptable. There was no significant difference in allele and genotype frequencies between the groups, according to CTLA4 +49AG analyses. In conclusion, AA cases had a high frequency of A/A genotype than healthy participants but lower rates of A/G and G/G genotypes.

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Introduction

Type 1 diabetes is a chronic disease that includes the inability to produce insulin because of the destruction of the beta cell in the pancreas due to autoimmune reactions. Most children with type 1 diabetes have depended on exogenous insulin during their lifetime (1,2).

Autoimmune reactions to Type 1 diabetes include the destruction of the pancreatic island due to T-cell activity. Human leukocyte antigen (HLAs) was 60% of genetic susceptibility in Type 1 diabetes. Nearly 20 non-HLA loci contributing to disease susceptibility are identified, one of which is the CTLA-4 gene. CTLA-4 polymorphisms are associated with Type 1 diabetes (2,3) in some cases.

T 1 D is a disease that is related to many genes. The risk of a child developing T1D is 5% when the father has T1D, but it is 8% when the sibling has T1D and 3% when the mother has T1D. When one identical twin has T1D, (40-50) % will be too. Some reports showed that T1D was estimated at (80-86) % (4).

There are 50 genes or more associated with type 1 diabetes. They can be dominant depending on the combination of loci or locus. IDDM1 is the most potent gene placed in the MHC Class II region on the sixth chromosome. Some variants of IDDM1 decreased histocompatibility characteristics of type 1. Some variants, such as DRB1 (0401, 0402, 0405), and DQA (0301, 0302, 0201), are prevalent in Europeans and North Americans of European ancestry(5). Type 1 diabetes occurs due to several causes of genetic factors and environmental factors (6-8). The autoimmune destruction of the beta-cell causes type 1 diabetes (9). Diabetes is detected depending on sugar or glycated hemoglobin concentrations(10). The physician can distinguish between Type 1 diabetes and type 2 diabetes depending on the autoantibodies(11).

Type 1 diabetes cases have (5–10%) of all diabetes cases. In fact, before the age of five, 80% of children with type 1 D had multiple islet autoantibodies (12), and 451 million persons (aged 18–99 years) were predicted to have diabetes in 2017. By 2045, these numbers were predicted to reach 693 million. Nearly half of all people living with diabetes (49.7 %) are expected to be undiagnosed; around 5 million

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fatalities globally were attributed to diabetes in (20–99) years every year (13).

HLAs account for over 60% of disease susceptibility genetics. There are around 20 non-HLA regions relating to illness vulnerability. The function of only two non-HLA loci is known: the insulin gene and the cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene (14).

The CTLA-4 receptor is found on the surface of T cells. The CTLA-4 receptor works as an 'off' switch for the T-cell onslaught. The CTLA-4 gene encodes the CTLA-4 protein(15).

Autoimmune disorders are caused by an overabundance of Th1/Th2 cytokines. Inflammatory nuclear transcription factor-kB (NF-kB) is an interferon (IFN)-like cytokine generated by helper T cells and natural killer cells (16).

Many studies have demonstrated that the CTLA-4 gene affects how its product functions, which has the potential to alter the pathogenic pathways of autoimmune diseases. CTLA-4 is an abbreviation for cytotoxic T lymphocyte-associated antigen-4, a cellular protein that is a precursor to T-cell activation and is linked to autoimmune diseases. In recent years, CTLA-4 gene polymorphisms have been intensively investigated in connection to a genetic sensitivity to autoimmune illnesses, with varying degrees of success in various research groups (17,18). This research aimed to determine the prevalence of the CTLA-4 49A/G polymorphisms in Iraqi children and the relationship between this Polymorphism and the development of Type 1 diabetes.

Materials and Methods

Study groups

Sixty children with diabetes were used for making this study in Al-Qadisiyah special clinic from January 2021 to July 2021. Their ages ranged (from 1 to 16) years old. The patients included (30 boys and 30 girls). The control group consisted of 40 healthy children (diabetes-free) (20 boys and 20 girls); aged (5-7 years).

The diagnostic & Principles criteria

The used basic diagnostic for children with diabetes are included:

(i) Fasting blood glucose.

(ii) Glucose 2 H after a meal.

(iii) Glycosylated hemoglobin (HbA1c)

(iv) Not taken insulin injection.

The informed consent

After obtaining consent from their families, all affected children and the control group underwent the following

(i) Detailed history evaluation.

(ii) Complete the general examination, including anthropometric measurements.

Laboratory investigations

Estimation of fasting Glucose Pertaining and postprandial are done; estimation of HbA1c and thyroid functions by determining FT3, FT4, and TSH.

Extraction, Purity, and integrity of DNA

Extraction of the total DNA from blood samples using Wizard® Plus SV Minipreps DNA Purification Systems (Promega/USA) and assessment of the integrity of the DNA samples will determine by gel electrophoresis, and its Purity determined by Nanodrop spectrophotometer in a ratio of ~2.0 is generally accepted as "pure" for DNA.

Polymerase Chain reaction (PCR)

The Primer ARMS-PCR preparation is used for genotyping CTLA4 (+49A/G) using the primers listed in table (1). These primers were initially described in previous research (19), and the primers were purchased from Bioneer, Korea, as lyophilized products of different picomols concentrations, which were dissolved in specific volumes of nuclease-free water to obtain a level of 100 pmol/mcl standard solutions (see Table 1) for further information. In addition, a diluted solution work solution was made by mixing 10 mL of each stock solution primer with 90 mL of nuclease-free water to make 90 mL. This solution was kept at -20 degrees Celsius until it was needed again.

The traditional PCR was carried out in 0.2-ml tubes in a PCR thermal cycler, and the results were analyzed (Hybaid, Teddington, United Kingdom). The target sequence from blood DNA was amplified using a 50-l reaction mixture that contained reaction buffer (Tris-HCl, MgCl2, and KCl), deoxynucleoside triphosphate, primers, blood DNA, and Taq DNA polymerase (Boehringer company, Germany). It was decided to

use (94°C- four minutes), followed by 30 cycles of (94°C-half minute), (56°C-half minute), (72°C-one minute), (72°C-seven minutes), (94°C- four minutes).

Table 1. Illustrates the primers used to amplify the CTLA-4 (+49A/G) Gene Polymorphism

GENE	TYPE OF PRIMERS	SEQUENCE	PRODUCT SIZE (BP)
CTLA-4 (+49A/G) GENE POLY MORPHISM	O-F	5-TGGGTTCAAACACATTTCAAAGCTTCAGG--3	٢٢٩
	O-R	5-TCCATCTTCATGCTCCAAAAGTCTCACTC-3	٢٢٩
	Allele A	5-ACAGGAGAGTGCAGGGCCAGGTCCTAGT-3	162
	Allele G	5-GCACAAGGCTCAGCTGAACCTGGATG-3	120

Statistical analysis

Continuous measurements with average SD (minimum-maximum) and batch measurement outputs (٪) are presented. Significance was estimated at a significance level of 5%, and in order to achieve the importance of continuous study factors between the two groups - group analysis between groups) on the metric parameters of the student "t" test and the Chi-Square test.

Results

IN THIS INVESTIGATION, the CTLA-4 gene polymorphisms +49A/G (rs231775) were genotyped in 60 patients with Diabetic Mellitus. The case group (50 % both male and females) had a mean age of 9.83±2.76 years, while the control group (50 % both male and females) had a mean age of 10.35±2.6; the results of the study showed that there were no significant differences in blood measurements for each of the patients when compared with the control ones in the Triglycerides (mg/dl), VLDL (mg/dl) and LDL (mg/dl), except Triglycerides (mg/dl) and HDL (mg/dl). (Table 2).

On the other hand, when comparing type 1 diabetes mellitus children with healthy groups when analyzing glycemic parameters, we found that there were no significant differences in each of the HOMA-IR and glucose (mg/dl); we also found significant differences between the two groups for each HbA1C and Insulin levels (Pmol/dl) (Table 3).

Table 2. Biochemical characteristics of Lipid profile among study subjects

Parameter	The control Mean ±SD	The patient Mean ±SD	P value
No (M/F)	40(20/20)	60(30/30)	-
Age (y)	10.35±2.6	9.83±2.76	0.56
Cholesterol	168.71±23.86	186.12±25.89	٠,٤١
Triglycerides	116.78±34.73	124.39±27.82	0.033
VLDL	24.00±7.54	25.00±6.00	0.231
LDL	92.67±25.91	88.56±24.00	0.21
HDL	55.94±15.17	53.72±9.55	0.023

Table 3. Mean and Standard deviation of Glycemic Parameters between Patent and control

Parameter	The Control Mean ±SD	The patient Mean ±SD	P value
HOMA-IR (units)	1.35±0.36	1.47±0.28	٠,٤١
HbA1C	±0.5٤,٥	8.5±0.93	0.033
Insulin (Pmol/dl)	55.78±7.54	25.00±6.00	0.021
glucose (mg/dl)	92.67±25.91	258.35±24.00	0.21

However, there are no significant differences in AST IU/L, ALP IU/L, and ALT IU/L values between the patients and the healthy group (Table 4). In the studied case-control study, the CTLA-4 +49A>G genotype frequencies in T1D were detected with the frequencies of GG, GA, and AA genotype was 13.3% (8/60), 48.4% (29/60), and 38.3% (23/60), respectively (Table 4).

The CTLA-4 gene polymorphisms +49A/G have not been linked to diabetic Mellitus. Polymorphism of T-ARMS-PCR electrophoretogram of *CTLA-4 (+49A/G)* was represented in Figure 1. The AG genotype showed (3) bands: 229 bp, 162 bp, and 120 bp. The AA genotype showed (2) bands: 229 bp and 162 bp. The TT genotype showed (2) bands: 229 bp and 120bp. Among the 60 patients, 8 samples (13.3%) were the GG genotype, 29 samples (48.4%) were the AG genotype, and 23 samples (38.3%) were the AA genotype. An allele and G allele have 62.5% and 37.5%, respectively. The distribution of genotypes was in Hardy-Weinberg equilibrium ($\chi^2 = 0.0581$; $P > 0.05$).

Table 4. Displays the genotype and allele frequencies, the predominant genotype for the +49A/G single nucleotide polymorphism (SNP) in both groups was AA.

CTLA-4 (+49A/G) Polymorphism	Patient n=60	Control n=40	Unadjusted OR (95% CI)	P value	Adjusted OR (95%CI)	P value
Codominant						
AA (Reference)	23	10				
AG	29	24	1.9 (0.75-4.76)	0.1	1.22 (0.63-1.92)	0.33
GG	8	6	1.72 (0.47-6.28)	0.48	1.32 (0.73-1.92)	0.35
Dominant						
GG+AG	37	30	1.86 (0.76-4.51)	0.16	1.42 (0.71-1.87)	0.37
Recessive						
AA+AG(Reference)	52	34				
GG	8	6	1.14 (0.36-3.55)	0.81	1.02 (0.73-2.03)	0.72
Additive						
2(GG)+AG	45	36	1.22 (0.66-2.66)	٠,٥٢	1.46 (0.65-2.11)	٠,٤١

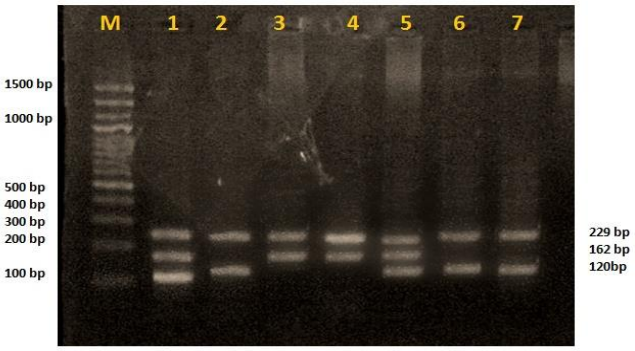

Figure 1. Electrophoresis of T-ARMS-PCR results of CTLA-4 (+49A/G) Polymorphism**.** Lane M is marker; lane (1-5) is DNA fragment (GA genotype), with (3) bands at 229 bp, 162 bp, and 120 bp; lanes 2, 6, and 7 are GG genotype, with two bands of 229 bp and 120 bp; lanes 3 and 4 are AA genotype with (2) bands of 229 bp and 162 bp.

Discussion

The CTLA-4 gene polymorphisms +49A/G (rs231775) were genotyped in 60 diabetic patients. The case group (50%) was 9.832.76 years old, while the control group (50%) was 10.352.6. However, the study found no significant differences in Triglycerides (mg/dl), VLDL (mg/dl), and LDL (mg/dl) measurements between patients and controls, cholesterol levels in children are influenced by three major factors, a diet that is unhealthy, heavy in fats obesity and family history of high cholesterol, especially if one or both parents have high cholesterol, table 3, these result were agreement with(20) who was found Childhood obesity raises the risk of cardiovascular disease. The Tg/HDL-C ratio may be a valuable indicator for children at risk of dyslipidemia, hypertension, and Metabolic syndrome.

However, when comparing Type 1 diabetes children to healthy controls, we found no significant differences in HOMA-IR or glucose (mg/dl) but significant differences in HbA1C and Insulin (Pmol/dl).

Although there have been significant advancements in diabetes technology and treatment, many children with type 1 diabetes do not achieve the hemoglobin A1c (HbA1c) standards established. The USA has the highest mean HbA1c values among high-income countries, and according to certain studies, there was an increase in the HbA1c during the fifth and sixth months after diagnosis (21). Some research suggests that tracking HbA1c trajectory after diagnosis might aid in targeting treatments along the course of T1D. After the diagnosis of T1D, there is often a drop in HbA1c after initiating insulin therapy (21). As type 1 diabetes worsens, endogenous insulin production decreases, glucose management becomes more complex, and HbA1c increases.

Some of the studies illustrated that individuals with T2DM have a higher rate of abnormal liver function tests than those without(22). The ALT was elevated in 40.4 % of diabetics in this study, but the AST and ALP were only elevated in 17 % and 16 % of diabetics, respectively(23).

In T1D patients, the CTLA-4 +49A>G genetic markers frequencies were found to be 13.3 percent (8/60), 48.4 percent (29/60), and 38.3 percent (23/60), respectively. The genotype distributions were in Hardy-Weinberg equilibrium, and the prevalence of CTLA-4 +49A>G genotypes and allelic were analyzed concerning the clinicopathologic features of diabetes mellitus patients. However, there were no significant differences between CTLA-4 +49A>G variants and the presence of Codominant, Dominant type, Recessive and additive (CEA and CYFRA21-1) polymorphisms in these patients.

Gene CTLA-4 (+49A/G) polymorphism has a heightened risk of some diseases, such as autoimmune thyroiditis, Graves' disease(24), and hepatocellular carcinoma, thus are linked to Polymorphism. As a

result, a reliable and straightforward approach to detecting this Polymorphism is required.

Diabetic Mellitus has not been linked to CTLA-4 polymorphism +49A/G. The predominant genotype for the +49A/G single nucleotide polymorphism was AA. A G to A transition at position 49 (+49A/G) of exon 1 results in an alanine to threonine amino acid substitution at codon 17 in the leader peptide (A17T), while a C to T transition at position 60 (CT60) occurs in the 3'-untranslated region(25). The G allele of +49A/G has been linked to a higher risk of autoimmune disorders; studies of CTLA-4 polymorphisms should include haplotype analysis. The CT60 G variant has been linked to an increased risk of autoimmune disorders, and a functional approach revealed that the CT60 G allele is related to decreased mRNA levels(26).

These results are consistent with a study (27), which found no significant link between diabetes cases and healthy controls. There is no association among Caucasians between CTLA-4 CT60 or +49A/G and Sjögren syndrome.

Conclusion

An allele and G allele frequencies were 62.5% and 37.5%, respectively. The genotypes distribution was in Hardy-Weinberg equilibrium ($\chi^2 = 0.0581$; $P > 0.05$).

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