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

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**Abstract:**

 The CTLA4 gene, a negative regulator of T cells, has been linked to a higher risk of autoimmune diseases. Using a case–control method, we looked at CTLA4 functional single-nucleotide polymorphisms (SNPs) for potential associations with Type 1 diabetes mellitus in an Iraqi children's population. We used the ARMS-PCR method to genotype +49AG (rs231775) variations in 60 obese children patients and 60 ethnically matched controls, all subjects were given anthropometric measurements and biochemical assays (fasting glucose, fasting insulin, and HbA1c). The glucose oxidase technique was 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 statistically significant difference in allele and genotype frequencies between patients and controls, according to CTLA4 +49AG analyses. In terms of the CT60 SNP, we discovered that AA cases had a highly frequency of A/A genotype than healthy participants, but a lower prevalence of A/G and G/G genotypes.

**Keywords: Diabetes mellitus type 1, CTLA4Polymorphism and obese children**

**INTRODUCTION**

 Type 1 diabetes is a chronic illness characterized by the body’s inability to produce insulin due to the autoimmune destruction of the beta cells in the pancreas. Most pediatric patients with diabetes have type 1 and a lifetime dependence on exogenous insulin (Marchini et al., 2018).

 T1D is a complex autoimmune disease characterized by T-cell-mediated destruction of the pancreatic island. Human leukocyte antigen (HLAs) account for about 60% of genetic susceptibility for the disease. About 20 non-HLA loci contributing to disease susceptibility have been identified. One among these is the CTLA-4 gene. CTLA-4 polymorphisms are associated with T1D in some but not all populations (Ihara et al., 2001).

Type 1 diabetes is a disease that involves many genes. The risk of a child developing type 1 diabetes is about 5% if the father has it, about 8% if a sibling has it, and about 3% if the mother has it. If one identical twin is affected there is about a 40% to 50% chance the other will be too. Some studies of heritability have estimated it at 80 to 86% (Marshall et al., 2009).

 More than 50 genes are associated with type 1 diabetes. Depending on locus or combination of loci, they can be dominant, recessive, or somewhere in between. The strongest gene, IDDM1, is located in the MHC Class II region on chromosome 6, at staining region 6p21. Certain variants of this gene increase the risk for decreased histocompatibility characteristic of type 1. Such variants include DRB1 0401, DRB1 0402, DRB1 0405, DQA 0301, DQB1 0302 and DQB1 0201, which are common in North Americans of European ancestry and in Europeans [Mack et al., 2009]. The cause of type 1 diabetes is unknown,( Piñero-Piloña, & Raskin, (2001)) but it is believed to involve a combination of genetic and environmental factors (Hirschhorn, 2003) The underlying mechanism involves an autoimmune destruction of the insulin producing beta cells in the pancreas (Pirot, 2008). Diabetes is diagnosed by testing the level of sugar or glycated hemoglobin (HbA1C) in the blood (Ghazanfari, et al 2010).Type 1 diabetes can be distinguished from type 2 by testing for the presence of autoantibodies (Palmer, 2005).

 Type 1 diabetes makes up an estimated 5–10% of all diabetes cases (Patterson et al., 2019). In fact, before the age of five, 80% of children with type 1 diabetes have multiple islet autoantibodies (Ziegler et al.,2013). Globally, 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, in addition around 5 million fatalities globally were attributed to diabetes in the age group of 20–99 years in every years (Cho, et al., 2018).

 HLAs account for about 60% of genetic susceptibility for the disease. About 20 non-HLA loci contributing to disease susceptibility have been identified. The function of only two non-HLA loci is known: the insulin gene and the cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene (Pociot, & McDermott, 2002).

 The CTLA-4 receptor is found on the surface of T cells. The T-cell attack can be turned off by stimulating the CTLA-4 receptor, which acts as an ‘off ’ switch In humans, the CTLA-4 protein is encoded by the CTLA-4 gene (Kristiansen et al., 2000).

Pro-inflammatory and anti-inflammatory cytokines are involved in the pathogenesis of autoimmune diseases, and an imbalance of Th1/Th2 cytokines is postulated in autoimmune them.3,4 Interferon (IFN)- is a multifunctional Th1 cytokine produced by helper T cells and natural killer cells, and acts as a key regulator of the immune system by enhancing activation of the pro-inflammatory nuclear transcription factor-kB (NF-kB) (Chatzigeorgiou et al., 2010).

 Many studies have shown that the CTLA-4 gene has an effect on how its product works, which can change the pathogenic pathways of autoimmune diseases, CTLA-4 stands for cytotoxic T lymphocyte-associated antigen-4, a cell surface protein that sets a precedent to T-cell activation. CTLA-4 gene polymorphisms have been researched extensively in relation to genetic vulnerability to autoimmune disorders, although results have been mixed in different groups (Kavvoura, & Ioannidis, (2005). The aim of our study was to investigate the frequency of CTLA-4 49A/G polymorphism in Iraqi children, and its susceptibility for development of T1D.

**Materials and Methods**

 **Study groups:**

 This study was tested on 60 children with diabetes World Health Organization used the endocrine hospital and Al-Qadsiya clinic from January 2021 to July 2021. Their ages ranged from one to sixteen years old. Patients included 30 boys and 30 girls (age range 5-7 years). The control group consisted of 40 healthy and disease-free children (Twenty boys and Twenty girls; age range 5-7 years) World Health Organization were age- and sex-matched with diabetics.

**The diagnostic & Principles criteria**

The basic criteria for the basic needs of children with diabetes are as follows:

(1) Fasting blood glucose with a minimum of 7.0 m.mol / L (6126 m.g / dL).

(2) Glucose 2 H after a meal at least 11.1 m.mol / L (200 m.g / dL).

(3) Glycosylated hemoglobin (HbA1c) at least 6.5.

(4) not taken insulin injection

Patients were divided according to HbA1c level (control <8 and poor control ≥ 8).

**The informed consent:**

 All sick and affected children and the control group, after obtaining informed consent from their families, underwent the following

(1) Detailed history evaluation.

(2) Complete general examination including anthropometric measurements.

**Laboratory investigations including:**

Estimation of fasting Glucose Pertaining Blood levels. Estimation of postprandial Glucose Pertaining Blood levels. Estimation of HbA1c.and Evaluation of thyroid functions by determining FT3, FT4, and TSH.

**Extraction, Purity and integrity of Total 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 determines by Nano drop spectrophotometer in a ratio of ~2.0 is generally accepted as “pure” for DNA.

**Polymerase Chain reaction ( PCR)**

 Primer Preparation Amplifcation refractory mutation system-polymerase chain reaction (ARMS-PCR) method was used to analyze the genotyping of CTLA4 (+49A/G) with the primers given in table (1) these primers were originally described by (Narooie et al., 2017), the primer was purchased from Bioneer, Korea as lyophilized product of different picomols concentrations, were dissolved in specific volumes of nuclease free water to obtain a concentration of 100 pmol/mcl stock solution. From which, diluted solution work solution was alsoprepared by adding 10µl of each stock solution primer to 90 µl of nuclease free water. This work solution was kept at -20 ˚C until further use.

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

|  |  |  |  |
| --- | --- | --- | --- |
| Primers | Type of primers | Sequence | Product size (bp) |
| CTLA-4 (+49A/G)Gene Polymorphism | **O-F** | **5-GTGGGTTCAAACACATTTCAAAGCTTCAGG-3** | 229 |
| **O-R** | **5-TCCATCTTCATGCTCCAAAAGTCTCACTC-3** |
| **Allele A** | **5-ACAGGAGAGTGCAGGGCCAGGTCCTAGT-3** | 162 |
|  **Allele G** | **5-GCACAAGGCTCAGCTGAACCTGGATG-3** | 120 |

 In a PCR thermal cycler, the conventional PCR was carried out in 0.2-ml tubes (Hybaid, Teddington, United Kingdom). The reaction mixture (50 µl) included reaction buffer (10 mM Tris-HCl, 2.5 mM MgCl2, and 50 mM KCl [pH 8.3]), a 200 M concentration of each deoxynucleoside triphosphate, 20 pmol of each primer, 500 ng of blood DNA, and 2.5 U of Taq DNA polymerase for amplification of the target sequence from DNA extracted from blood (Boehringer Mannheim, Mannheim, Germany). The amplification program consisted of 94°C for 4 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds, followed by 72°C for 7 minutes.

**Statistical analysis**

 The results of 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 was used and finally the Chi Square test.

**Results and Discussion**

Characteristics of Patients

 The CTLA-4 gene polymorphisms +49A/G (rs231775) were genotyped in 60 patients with Diabetic mellitus in this investigation. 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). 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 (Quijada et al., 2008) who was found Childhood obesity raises the risk of cardiovascular disease, and the Tg/HDL-C ratio may be a valuable indicator for children at risk of dyslipidemia, hypertension, and Metabolic syndrome.

Table 3: Biochemical characteristics of Lipid profile among study subjects

|  |  |  |  |
| --- | --- | --- | --- |
|  P value | Patient subjectsMean ±SD | Control subjectsMean ±SD | Parameter |
| - | 60(30/30) | 40(20/20) | No (M/F) |
| 0.56 | 9.83±2.76 | 10.35±2.6 | Age (y) |
| 0.41 | 186.12±25.89 | 168.71±23.86 | Cholesterol (mg/dl) |
| 0.033 | 124.39±27.82 | 116.78±34.73 | Triglycerides (mg/dl) |
| 0.231 | 25.00±6.00 | 24.00±7.54 | VLDL (mg/dl) |
| 0.21 | 88.56±24.00 | 92.67±25.91 | LDL (mg/dl) |
| 0.023 | 53.72±9.55 | 55.94±15.17 | HDL (mg/dl) |

 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). This result was agreement with (Önal et al., 2014) who was clarify that High HbA1c levels in obese children can be used as a screening tool to detect insulin sensitivity and resistance at an early stage.

|  |  |  |  |
| --- | --- | --- | --- |
|  P value | patient subjectsMean ±SD | Control subjectsMean ±SD | Parameter |
| 0.41 | 1.47±0.28 | 1.35±0.36 | HOMA-IR (units) |
| 0.033 | 8.5±0.93 | ±0.54.5 | HbA1C  |
| 0.021 | 25.00±6.00 | 55.78±7.54 | Insulin (Pmol/dl) |
| 0.21 | 258.35±24.00 | 92.67±25.91 | glucose (mg/dl) |

 Despite advances in diabetes technology and treatment, a majority of children and adolescents with type 1 diabetes (T1D) fail to meet hemoglobin A1c (HbA1c) targets. Among high-income nations, the United States has one of the highest mean HbA1c values, some of study refers there was a rise in the HbA1c between the fifth and sixth month postdiagnosis (Prahalad et al., 2021).some of study refers to the HbA1c trajectory after diagnosis can help to target interventions in the course of T1D and following the diagnosis of T1D, there is usually a decrease in HbA1c after starting insulin (Prahalad *et al*., 2019). Endogenous insulin production declines as T1D progresses, glucose control becomes more difficult, and HbA1c frequently rises.

Table 4 indicates that there are no significant differences in AST IU/L, ALP IU/L and ALT IU/L values between the group of patients and the healthy group,

|  |  |  |  |
| --- | --- | --- | --- |
|  P value | Patient subjectsMean ±SD | Control subjectsMean ±SD | Parameter |
| 0.81 | 25±6.29 | 29±3.73 | AST IU/L |
| 0.29 | 324.39±47.82 | 216.78±64.71 | ALP IU/L |
| 0.13 | 52.51±6.00 | 49.8±7.54 | ALT IU/L |

Some of study illustrated that individuals with T2DM have a higher rate of abnormal liver function tests than those without (Harris, 2005). The ALT was found to be elevated in 40.4 % of diabetics in this study, but the AST and ALP were only elevated in 17 % and 16 % of diabetics, respectively (Mandal et al., 2018).

 In the studied case control study, the CTLA-4 +49A>G genotype frequencies in Type 1 diabetes mellitus patients were detected with the following disposition: the frequencies of GG, GA and AA genotype were 13.3% (8/60), 48.4% (29/60) and 38.3% (23/60), respectively. The genotype distributions were in Hardy-Weinberg equilibrium. The distribution of CTLA-4 +49A>G genotypes and allele frequencies were analyzed with regard to clinic pathologic characteristics of diabetes mellitus patients. However, there were no significant differences between the presence of CTLA-4 +49A>G polymorphisms and Codominant, Dominant type, Recessive and additive (CEA and CYFRA21-1) in these cases (table 2).

Gene CTLA-4 (+49A/G) polymorphism has heightened risk of some diseases, such as autoimmune thyroiditis Graves' disease (Pastuszak et al., 2012) and hepatocellular carcinoma (Liu et al., 2015),thus are linked to polymorphism. As a result, a simple and reliable approach for detecting this polymorphism is required.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| value | Adjusted OR(95%CI) | Pvalue | Unadjusted OR(95% CI) | Controln=40 | Patientn=60 | CTLA-4 (+49A/G) Gene Polymorphism |
|  | Codominant |
|  | 10 | 23 | AA(Reference)  |
| 0.33 | 1.22(0.63-1.92) | 0.1 | 1.9(0.75-4.76) | 24 | 29 | AG |
| 0.35 | 1.32(0.73-1.92) | 0.48 | 1.72(0.47-6.28) | 6 |  8 | GG |
|  | Dominant |
| 0.37 | 1.42(0.71-1.87) | 0.16 | 1.86(0.76-4.51) | 30 | 37 | GG+AG |
|  | Recessive |
|  | 34 | 52 | AA+AG(Reference)  |
| 0.72 | 1.02(0.73-2.03) | 0.81 | 1.14(0.36-3.55) | 6 | 8 | GG |
|  | Additive |
| 0.41 | 1.46(0.65-2.11) | 0.52 | 1.22(0.66-2.66) | 36 | 45 | 2(GG)+AG |

 The CTLA-4 gene polymorphisms +49A/G have not been linked to diabetic mellitus . Table 3 displays the genotype and allele frequencies. the predominant genotype for the +49A/G single nucleotide polymorphism (SNP) in both groups 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 (Ueda et al., 2003). 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 with decreased mRNA levels (Megiorni et al., 2014).

 These results are also consistent with study (Gottenberg et al., 2007), who explained that there is no significant link between patients with diabetes and healthy controls when demonstrating a lack of association between CTLA-4 CT60 or +49A/G polymorphisms and Primary Sjögren syndrome among Caucasians.

 A representative T‐ARMS‐PCR electrophoretogram of CTLA-4 (+49A/G) Gene Polymorphism is shown in Figure 1. The AG genotype showed three bands: 229 bp, 162 bp, and 120 bp. The AA genotype showed two bands: 229 bp and 162 bp. The TT genotype showed two 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. The allele frequencies were 62.5% and 37.5% for the A allele and G allele, respectively. The distribution of genotypes was in Hardy‐Weinberg equilibrium (χ 2 = 0.0581; P > 0.05).



Electrophoretogram of T‐ARMS‐PCR results of CTLA-4 (+49A/G) Gene Polymorphism. Lane M is the DNA size standard marker; lane 1&5 is the products of the DNA fragment (GA genotype), with three bands at 229 bp, 162 bp and 120 bp; lanes 2, 6 and 7 are the products of the GG genotype, with two bands of 229 bp and 120 bp; lanes 3 and 4 are the results of AA genotype with two bands of 229 bp, and 162 bp.

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