

A Novel Single Nucleotide Polymorphism of Interleukin-10 Gene is Linked to Type 2 Diabetes Mellitus in Iraqi Patients with Toxoplasmosis^{(Conference Paper)#}

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Abstract

Type 2 diabetes mellitus (T2DM) is a chronic disorder that represents a serious health concern all over the globe, it is linked to Interleukin-10 (IL-10) single nucleotide polymorphisms (SNPs) at the promoter region. On the other hand, diabetes influences the cellular and humoral immunity predisposing the patient to a variety of opportunistic parasites one of them is *Toxoplasma gondii* (*T. gondii*), that may infect any nucleated cell, including pancreatic cells. The purpose of this research was to explore the association of IL-10 genetic polymorphisms with T2DM and toxoplasmosis among Iraqi patients with T2DM. Fifty-five and fifty-eight venous blood specimens were obtained from T2DM patients and age-matched non-diabetic persons, respectively. Sera had been tested for the presence of anti-*Toxoplasma* antibodies using the Enzyme Linked Immunosorbent Assay (ELISA) kits. Polymerase chain reaction (PCR) was performed by specific primers and the products were sequenced. A higher percentage of *T. gondii* infection was found in T2DM patients (52.1%) compared to 31.5% of non-diabetic persons. High frequency of the SNP at position -1091 among T2DM patients, which represents a novel finding. An interesting result, an increased risk of T2DM was observed in carriers of -1082 A/G variants, which was highly frequent among studied subjects. The carriers of both -1082 AG+GG and -1091 AG+GG of IL-10 genotypes had a synergistic effect on the risk for type 2 diabetes mellitus significantly.

Keywords: Interleukin-10, Type 2 diabetes mellitus, IL-10 gene polymorphism, *Toxoplasma gondii*.

شكل جديد للنوكليوتيدات المنفردة المتعددة الاشكال لجين إنترلوكين ١٠ يرتبط بمرض السكري من النوع ٢ في المرضى العراقيين المصابين بداء المقوسات (بحث مؤتمر) **

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الخلاصة

داء السكري من النوع الثاني (T2DM) هو اضطراب مزمن يمثل مصدر قلق صحي خطير في جميع أنحاء العالم، وهو مرتبط بتعدد أشكال النوكليوتيدات المفردة (SNPs) في المنطقة المروجة لانترلوكين ١٠ (IL-10). من ناحية أخرى، يؤثر مرض السكري على المناعة الخلوية والخلطية مما يجعل المريض عرضة لمجموعة متنوعة من الطفيليات الانتهازية، أحدها المقوسة الكوندية (*T. gondii*)، التي قد تصيب نواة أي خلية، بما في ذلك خلايا البنكرياس.

كان الغرض من هذا البحث هو تحديد الأنماط الجينية المختلفة وترددات نقل الأليل لانترلوكين ١٠ (عند موضع -1082) في نموذج من مرضى السكري النوع الثاني العراقيين المصابين بداء المقوسات. بالإضافة إلى استكشاف ارتباط تعدد الأشكال الجينية لانترلوكين ١٠ مع السكري من النوع الثاني وداء المقوسات بين المرضى العراقيين.

تم أخذ خمسة وخمسين وثمانية وخمسين عينة من الدم الوريدي من مرضى السكري النوع الثاني والأشخاص المتطابقين بالعمر من غير المصابين بالسكري، على التوالي. تم اختبار الأمصال من جميع العينات بحثاً عن وجود أجسام مضادة للتوكسوبلازما باستخدام المقاييس المتصاصية المناعية للأنزيم المرتبط (الآليزا). وتم استخدام تفاعل البلمرة التسلسلي (PCR) بواسطة بادئات محددة ومنتجات التفاعل تم تحديد تسلسلها في كوريا (ماكروجين).

تم العثور على نسبة أعلى من عدوى *T. gondii* في مرضى السكري النوع الثاني (52.1%) و (31.5%) في غير المصابين بالسكري. هناك تكرار عالي للتغاير في الموضع -1091 بين مرضى السكري النوع الثاني (T2DM)، وهذا يعتبر اكتشاف جديد. لوحظ نتيجة مثيرة للاهتمام، وهي وجود خطر متزايد من الإصابة بالسكري النوع الثاني في ناقلات -1082 A/G، والتي كانت عالية التكرار بين الأشخاص في الدراسة. وكان لحاملات كلا الطرز الوراثية -1082 AG+GG و -1091 AG+GG تأثير تآزري على خطر الإصابة بداء السكري من النوع ٢ بشكل ملحوظ.

هناك ارتباط كبير بين داء السكري من النوع الثاني وتعدد الأشكال للجين (IL-10) في المواضع A/G 1082 و A/G 1091. الكلمات المفتاحية: إنترلوكين ١٠، داء السكري من النوع ٢، تعدد الأشكال الجيني IL10، المقوسة الكوندية.

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Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic condition characterized by hyperglycemia caused by low insulin levels, insulin resistance, or both^(1, 2). According to the International Diabetes Federation (IDF), there are 463 million diabetic persons worldwide in 2019, with the number anticipated to rise to 700 million by 2045⁽³⁾. In Iraq, the reported prevalence of T2DM varies from 8.5 percent (IDF—age adjusted) to 13.9 percent⁽⁴⁾.

A minimal level of a persistent inflammation has been proven to have a significant impact on the onset and development of T2DM⁽⁵⁾. High levels of anti-inflammatory and pro-inflammatory cytokines, such as IL-10 and IL-6, have been found in the plasma of T2DM patients, and are therefore related with its complications^(6,7).

Interleukin-10 (IL-10) is a multifunctional regulatory cytokine that serves as a general inhibitor for both type 1 and type 2 helper T cell proliferation and cytokine responsiveness in an inflammatory response⁽⁸⁾. IL-10 shortage or aberrant expression may boost the inflammatory response to microbial insult, but it can also result in the development of a range of autoimmune disorders⁽⁹⁾.

Cytokine production has been demonstrated to be genetically controlled, with polymorphisms in the promoter region of cytokine genes determining lower or greater levels of production in response to certain stimuli. As a result, these polymorphisms may alter susceptibility to or severity of inflammatory disorders⁽¹⁰⁾. Three widespread single nucleotide polymorphisms (SNPs) have been found at the transcriptional start site in the 5' flanking region of IL-10; these SNPs are located at positions 1082, 819, and 592, respectively, relative to the translational start site⁽¹¹⁾. The IL-10 promoter region regulates transcription and includes SNPs linked to TDM2⁽¹¹⁻¹⁴⁾.

Environmental microorganisms have the capacity to produce low-grade inflammation, that may raise the risk for development of numerous metabolic disorders such as diabetes^(15,16), and may use IL-10's immunosuppressive potential to inhibit host immune response, resulting in prolonged infection⁽¹⁷⁾. Toxoplasmosis is a zoonotic infection caused by the *T. gondii* parasite, which is a common intracellular protozoa parasite^(18,19). This obligate intracellular parasite infects and replicates in any nucleated cell, including pancreatic cells⁽⁶⁾. *T. gondii* infection is normally handled by the immune system in immunocompetent persons and frequently goes unreported; nevertheless, it is life threatening in immunocompromised individuals^(20, 21). Diabetes, on the other hand, is a disease that affects cellular and humoral immunity, making the patient vulnerable to a range of opportunistic parasites, one of which is *T. gondii*⁽²²⁾.

This research intended to explore the relationship between IL-10 gene polymorphisms and T2DM and latent toxoplasmosis in Iraqi T2DM patients.

Patients and Methods

This study was conducted at the Specialized Center for Endocrinology and Diabetes/ Baghdad and the Iraqi national blood bank during the period from March 2021 until September 2021. The study was designed to be a retrospective study. Fifty-five and fifty-eight venous blood samples were collected from patients with T2DM with an age matched non-diabetic individuals, respectively. Fasting blood samples were obtained from all study participants. All patients were diagnosed of establishing T2DM on the basis of medical history and laboratory tests according to the criteria of the American Diabetes Association⁽²³⁾. Sera had been tested for the presence of anti-*toxoplasma* antibodies (IgM and IgG) using the Enzyme Linked Immunosorbent Assay (ELISA) specific kit supplied by ACON, USA⁽²⁴⁾. After extraction and purification of genomic DNA, PCR was performed using specific primers which were supplied by Alpha DNA company. Then PCR products underwent sequencing at Macrogen /Korea.

Informed consent had been obtained from all participants in the current study before sample collection, and the study have been revised and approved by the College of Pharmacy/ University of Baghdad, Research Ethics Committee application no. 3102020C.

Demographic data were collected from each participant by using predesigned questionnaire sheets.

Inclusion and exclusion criteria

Included cases were patients with T2DM from both sexes in aged ≥ 18 year. Excluded cases in this study were patients receiving toxoplasmosis treatment or providing incomplete information during completion of the questionnaire. In addition to patients providing treatment can affect the IL-10 level such as dexamethasone and vit D.

Genotyping

Genomic DNA was extracted and purified from whole blood samples using the *Easy Pure*® Blood DNA Kit (Catalog No.: EE121) according to the manufacturer's instructions. The genotyping of the IL10 gene SNPs -1082 A/G (rs1800896), and -1091 A/G (rs1263484331) was conducted by using polymerase chain reaction (PCR) technique. PCR was performed using the specific primers which were supplied by Alpha DNA Ltd (Canada) as lyophilized product of various picomoles concentration. Primer3 software was used to create the primers utilized in PCR amplification, which is a bioinformatics software available online for user to design PCR primers. The forward and reverse primers for IL-10 gene were F5/ -CTGGCTGCAACCCAACCTGGC-3 and R5/ -TCTTACCTATCCCTACTTCC-3, respectively. Each PCR reaction mixture comprises 4µl DNA template, 12.5µl Master mix EasyTaq® PCR SuperMix, 1 µl of each forward and reverse primers and 6.5µl nuclease free water to make a total volume of 25µl in each well. Following a preliminary denaturation at 94 C° for 2 minutes, 35 cycles of denaturation at 94 C° for 30 seconds and annealing at 4 C° for 30 seconds were performed, followed by a final extension at 72 C for 3 minutes. Annealing temperature was calculated according to the types of nucleotides within primers and a specific equation used for calculation of optimum annealing temperature⁽²⁵⁾. The PCR results were validated and seen using 1.0 percent agarose gel electrophoresis. UV transilluminator at 365nm and photographed.

Alignment analysis software

The computer application BioEdit Pro. version: 7.0.0, which is accessible on the website <http://www.mbio.ncsu.edu/bioedit/bioedit.html> was used to compare the sequence of the IL-10 gene in 113 samples with the sequence of the IL-10 gene available on website (<http://www.ncbi.nlm.nih.gov>).

Statistical analysis

Statistical Analysis System - version 9.1(SAS) was used to undertake data statistical analysis in order to determine the influence of various variables in research parameters. The least significant difference –LSD test (Analysis of Variation-ANOVA) was performed to compare means. The Chi-square test (0.05 and 0.01 probability) was employed to create a significant comparison between percentages^(26, 27).

Results

Participants were divided into four groups according to the results of anti-*Toxoplasma gondii* antibodies (IgG) seropositivity by ELISA method:

- Group 1: 30 patients having T2DM and have *T. gondii* infection.
- Group 2: 25 patients having T2DM and not have *T. gondii* infection.
- Group 3: 29 patients having *T. gondii* infection and not have DM.
- Group 4: 29 apparently healthy individuals not known to have DM nor *T. gondii* infection (referred to as the control group).

All DNA samples of patients and controls were undergone sequencing for IL-10 gene -1082 G/A (rs1800896). The results of the IL-10 gene sequencing revealed many SNPs, two of them namely, SNP -1082 A/G and SNP -1091 A/G, were undergone analysis (Table -1).

A significant difference in AG genotype frequency among studied groups in IL-10 SNPs at position -1091 (rs1263484331), and a non-significant difference in GG genotype frequency. While, a significant difference in G allele frequency between patients' group 1, group 2 and controls (Table -1). Non-significant difference in genotyping and allele carriage frequencies of IL-10 at locus -1082A/G (rs1800896) between all patients' groups and controls.

Table 1. Distribution of genotype frequencies of IL-10-1082 A/G and IL-10-1091 A/G polymorphisms.

Polymorphisms IL-10-1082 A/G and IL-10-1091 A/G							
	Controls n=29	Group1 n=30	Group2 n=25	Group3 n=29	P (OR)		
Genotype -1082	n (%)	n (%)	n (%)	n (%)	<u>0↔1</u>	<u>0↔2</u>	<u>0↔3</u>
AA	3 (10.3%)	0 (0.0%)	0 (0.0%)	4 (13.8%)	--(1)	--(1)	--(1)
AG	0 (0.0%)	3 (10.0%)	0 (0.0%)	0 (0.0%)	0.1 (49)	1 (7)	1 (0.78)
GG	27 (90.0%)	26 (89.7%)	25 (100.0%)	25 (86.2%)	0.237 (7.3)	0.24 (6.73)	1 (0.72)
Allele frequency							
A	6 (10.34%)	3(5%)	0 (0.0%)	8 (13.8%)	---(1)	---(1)	---(1)
G	52 (89.66%)	57 (95%)	50 (100.0%)	50 (86.2%)	0.318 (2.19)	0.0295* (12.5)	0.77 (0.72)
Genotype -1091							
AA	20 (68.9%)	14 (46.7%)	7 (28.0%)	25 (86.2)	---(1)	---(1)	---(1)
AG	6 (20.7%)	9 (30.0%)	14 (56.0%)	0 (0.0)	0.352 (2.14)	0.003** (6.66)	0.023* (0.062)
GG	3 (10.4%)	7 (23.3%)	4 (16.0%)	4 (13.8)	0.155 (3.33)	0.178 (3.8)	1(1.066)
Allele frequency							
A	46 (79%)	37 (62%)	28 (56%)	50 (86.2)	---(1)	---(1)	---(1)
G	12 (21%)	23 (38%)	22 (44%)	8 (13.8)	0.044* (1.19)	0.012* (3.01)	0.46 (0.613)

Note. 0: controls, 1: group1, 2: group 2, 3: group 3, p: Fischer exact p-value corresponding to genotype and allele frequency comparisons; (OR) odds ratios are age-adjusted, * (P≤0.05), ** (P≤0.01).

The results of the interaction analyses between the two SNPs of IL-10 gene -1082 A/G and -1091 A/G on the susceptibility to T2DM are shown in Tables - 2 and -3.

An increased risk for type 2 diabetes mellitus was observed in carriers of -1082A/G variants, while the

carriers of both -1082 AG+GG and -1091 AG+GG genotypes had a synergistic effect on the risk for type 2 diabetes mellitus significantly.

Table 2. The interaction between two single nucleotide polymorphisms (SNPs) at positions -1082 A/G and 1091 A/G of the interleukin 10 (IL-10) gene in different groups.

SNP of IL-10 gene		Group 1 No., P (OR)	Group 2 No., P (OR)	Group 3 No., P (OR)	Group 4 No., P (OR)
-1082 A/G	-1091A/G				
AA	AA	0, --- (referent)	0, ---(referent)	4, ---(referent)	3, --- (referent)
AG+GG	AA	14, 0.25(5.8)	7, 0.54 (3)	21, 1 (0.92)	17, ----(1)
AG+GG	AG+GG	16, 0.067 (12.1)	18, 0.054 (13.6)	4, 0.35 (0.33)	9, ----- (1)
Total		30	25	29	29

P= p fisher exact test, OR= odd ratio, * (P≤0.05), ** (P≤0.01).

Table 3. The interaction between two single nucleotide polymorphisms (SNPs) of the interleukin 10 (IL-10) gene at positions -1082 A/G and 1091 A/G in type 2 diabetes mellitus and non-diabetic subjects.

SNP of IL-10 gene		Patients with diabetes n=55	Non diabetic n=58	P (OR)
-1082 A/G	-1091A/G			
AA	AA	0	7	---- (1)
AG+GG	AA	21	38	0.087 (8.3)
AG+GG	AG+GG	34	13	0.0004** (38.3)

P= p fisher exact test, OR= odd ratio, * (P≤0.05), ** (P≤0.01).

Discussion

Despite substantial research, the specific pathogenic mechanism of DM remains unknown. However, the clear family aggregation tendency of T2DM, shows that genetic factors may play a significant role in its incidence and progression^(28, 29). Chronic low-grade inflammation leads to the pathophysiology and consequences of T2DM⁽³⁰⁾. There is epidemiological evidence that inflammatory biomarkers are key risk factors for the development of diabetes in the future⁽³¹⁾.

The results of the current study demonstrate that a highly significant association of IL-10 gene polymorphisms with the risk of developing T2DM, (p<0.01) (Table-3). Single nucleotide polymorphism at region -1082A/G, revealed that most of the studied individuals (patients and controls) had the mutant (GG) genotype, and the heterozygous (AG) genotype was observed in only 10 % of group1 subjects. In addition, there was higher GG genotyping and G allele frequencies in T2DM patients than non-diabetic individuals (Table-1). In Caucasians, the frequency of the high expression G

allele varies from 20 to 52 percent, whereas in Oriental Asians, it ranges from 21 to 84 percent⁽³²⁻³⁵⁾. Present study found that there was a non-significant difference in genotyping frequencies at the region -1082 of the IL-10 gene between different patients' groups and control group. However, there

was a significant association between IL-10 -1082A/G polymorphism and risk of T2DM. Risk ratio of developing T2DM was significantly higher for GG and AG genotypes compared to AA genotype. Odd ratios for GG genotype for group 1 and 2 were 7.26, 6.63 respectively, while odd ratios for AG genotype were 49 and 7 for group 1 and 2 respectively (Table 1). The present study's findings agreed with the findings of several earlier research^(6, 29, 32). Ayelign *et al.*, and Kolla *et al.*, who found that a significant increase in IL-10 -1082 GG genotype in Ethiopian and Indian patients with T2DM, respectively^(36,37). A meta-analysis of six case-control studies (1,835 patients and 2,257 controls, in different countries including Greece, Italy, India, China, Tunisia, and Turkiye) concerning IL-10 polymorphism at locus -1082 A/G found that a significant association between this polymorphism and T2DM under heterozygote comparison and the dominating genetic model was (GA/GG vs. AA: OR= 1.22, 95 percent CI = 1.05–1.41). In a stratified analysis by ethnicity, the IL-10 -1082 A/G polymorphism was linked with a substantially higher incidence of T2DM in Asian descendants under the dominant genetic model (GA/GG vs. AA: OR= 1.69, 95%CI = 1.21–2.38⁽³⁸⁻⁴¹⁾. Different genetic backgrounds and environmental exposures may contribute to this

ethnic disparity⁽⁴²⁾.

However, the results of the current study were in contrast with a number of studies^(33,43) that found no significant association between genotyping and T2DM. These contradictory results are most likely due to the small sample size and varied genetic backgrounds of the people. Larger-scale genomic investigations are needed to confirm these relations. Interleukin 10 is an anti-inflammatory cytokine. During an infection, it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for successful pathogen clearance but may cause tissue damage. As a consequence, IL10 may impede pathogen clearance while also improving immunopathology⁽⁴⁴⁾. In the current study, risk ratio of *T. gondii* infection was decrease in patients carrying AG, GG compared to AA genotypes of IL-10 SNP -1082 A/G; [(odd ratio (95%CI) = 0.78 (0.012 - 49.9) and 0.72 (0.15 - 3.55), respectively, (Table 1). Similarly, risk ratio was 0.72 for whom carrying G allele. As a result, -1082 A/G polymorphism may be considering as a protective factor against susceptibility for *T. gondii* infection.

Many studies concerned with evaluation of three SNPs of IL-10 at the promoter region namely: (rs1800872 -592C/A, rs1800871 -819C/T, and rs1800896 -1082 A/G), that have been found to regulate the expression of IL-10 cytokine level⁽¹¹⁾. According to data obtained and analyzed by current study, a novel finding that indicate a possible association of SNP -1091 A/G with increasing the risk of developing T2DM. No previous study had evaluated or deal with the polymorphism at position -1091 and its role in T2DM.

A significant difference in G allele frequency of IL-10 at position -1091 A/G between group 1 and controls. In addition, a significant difference in AG genotype and G allele carriage frequency between groups 2 and controls. Risk ratio of developing T2DM was higher for group 1 and 2 subjects with this polymorphism compared to controls. While, it was lower for group 3 subjects compared to controls (Table1).

The current study demonstrates that a significant difference in AG genotype frequency between patients' group 3 and controls. The risk ratio of developing toxoplasmosis or T2DM was less for patients with *T. gondii* latent infection (both group 1 and 3) whom carrying the mutant G allele compared to the wild type A allele. This indicate that the mutant G allele have protective properties against *T. gondii* infection. and there was decrease in risk for developing T2DM in case of the concomitant presence of toxoplasmosis and T2DM, Table (1). These findings were similar to those of previous SNP (-1082 A/G).

The results of the interaction between the two SNPs (-1082 and -1091) of IL-10 gene demonstrate that a highly significant association of these polymorphisms ($p < 0.01$) with the risk of developing

T2DM and a synergistic effect has been occurred when both polymorphisms present simultaneously (Table 2 and 3). It is widely known that IL-10 has both immunosuppressive and anti-angiogenic properties⁽⁴⁵⁾. The largest risk of T2DM was related with GG+AG IL10 gene genotypes at locations -1082 and -1091. (Table 3). A meta-analysis was performed in China includes 22 studies about -1082A/G polymorphism found that IL-10 has the ability to decrease inflammatory responses and may have anti-diabetic characteristics. IL-10, on the other hand, has anti-angiogenic effects and may limit microvasculature formation as well as enhance vascular complications of diabetes, and it is unlikely that a single IL-10 genetic variation can significantly contribute to its development⁽⁴²⁾.

The current study found an interesting high frequency of the mutant G allele and GG genotype of IL-10 SNP -1082 A/G among studied individuals. Novel SNP of IL-10 at position -1091 A/G that showed a significant association with increasing risk of the development of T2DM. Actually, a synergistic relationship has been occurred when both SNPs at positions -1082 (rs1800896) and -1091 (1263484331) present simultaneously ($P \leq 0.01$). In addition, a high significant risk of developing T2DM observed for G allele carriers compared to A allele carriers. Finally, patients with toxoplasmosis (non-diabetic) carrying G allele showed a decreasing in the risk of development T2DM and *T. gondii* infection. These patients carrying the mutant G allele may have a protective factor for toxoplasmosis.

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