Original Article

Association of PD-1 and PDL-1 Gene Polymorphisms in Type 2 Diabetes with Toxoplasmosis

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ABSTRACT

Objective: To investigate the relationship between PD1 and PDL-1 gen polymorphisms with incidence toxoplasmosis in type 2 diabetes disease (T2DM).

Study Design: Case control study

Place and Duration of Study: This study was conducted at the Al-Iamamain Al-Kadhumain Teaching Hospital, Baghdad Iraq from 1st December 2018 to 30th April 2019.

Methods: One hundred and eighty patients with Type 2 diabetes mellitus (T2DM) aged 31-79 years, attending the diabetes were enrolled. A control group of 163 healthy subjects was also included. The PCR products of the SNPs were sequenced using the Big Dye Terminator method and compared to sequences in GenBank.

Results: Extracted DNA were used for molecular study for determination of the (PD-1.5 C/T rs 2227981) and the (PDL-1 G\C\A rs1970000) gene polymorphisms by conventional PCR method using specific primer. Sequencing of PCR products revealed three genotypes for the PD-1.5 C/T(CC, CT, TT). Regarding PD-1 showed no significant difference in each CT and CC genotypes between case and control groups while TT genotype showed significant difference between them. As well as related to PDL-1 showed significant difference between case and control while, CC showed non-significant between case and control groups. A allele significantly higher frequency in controls than cases. In contrast, C allele non-significantly higher frequency in cases than controls

Conclusion: There is a significant association between the phenotypic polymorphism of the (PD-1.5 C/T gene rs2227981) Toxoplasmosis and type 2 diabetes for the groups in the current study, as it was found that people who carry the genotype TT are more likely to develop type 2 diabetes than people who carry the genotype CC.

Key Words: PD-1, PDL-1, Polymorphisms, Toxoplasmosis, Type 2 Diabetes

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INTRODUCTION

Diabetes mellitus is a significant global health issue affecting 9% of adults, while Toxoplasma gondii, a protozoan parasite, infects about one-third of the population and is among the most effective human parasites. T. gondii is recognized by the CDC as a significant neglected parasitic infection due to its severity and prevalence, primarily transmitted through contaminated food, water, or soil, with cats being the only definitive hosts.[1-3]

Diabetic patients may be more vulnerable to T. gondii infection due to their weakened immune systems, and chronic toxoplasmosis could be a potential risk factor for type 2 diabetes.[4-6] The text discusses the relationship between (T2DM) and immune dysfunction, suggesting that T2DM may resemble a chronic infection and highlighting the need for further research on its impact on infections like toxoplasmosis, particularly in regions like Iraq and the Middle East.[7-9]

Programmed death-1 (PD-1) is a co-inhibitory receptor that plays a crucial role in regulating T lymphocyte activity and promoting immune tolerance, which can allow tumor cells to evade the immune system. This study focuses on the relationship between PD-1 and PD-L1 polymorphisms and their potential impact on susceptibility to type 2 diabetes (T2DM) and toxoplasmosis. Specifically, it examines two single nucleotide polymorphisms (SNPs): PD-1 rs2227981 C > T and PD-L1 rs1970000 G/C/A, as no prior research has explored this correlation in the context of these conditions.

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METHODS

180 patients (ages 31 to 79) with Type 2 diabetes mellitus (T2DM) who visited the diabetes division at the Al-Iamamain Al-Kadhumain Teaching Hospital in Baghdad, Iraq, between December 2018 and April 2019 were included in this study. Venous blood samples of five ml were collected from patients and healthy individuals at a time of clinical diagnosis and (ELISA and molecular diagnosis) were performed as described in the following sections; all samples divided in two tubes 3 ml of the blood used for immunological diagnosis (ELISA)and 2 ml blood which placed in sterilized EDTA tube for molecular diagnosis.

ELISA T. gondi–IgG: Using ELISA kits supported by the Bioactiva Company in Germany, the sera of all samples (control and patient) were tested for the presence of specific IgG antibodies of Toxoplasma gondii and the test was connected in accordance with the manufacturer's instructions.

Genomic DNA isolation from whole blood: Using a commercial blood DNA purification system (AccuPrep® Genomic DNA Extraction Kit, Bioneer, Korea), genomic DNA was extracted from whole blood samples of the study groups in accordance with the manufacturer's instructions. This was done in order to magnify the (PD-1. Gene polymorphisms: 5 C/T (rs 2227981) and (PDL 1G\C\A) (rs1970000) using a conventional PCR method using a specific primer (Table 1) and green master mix (Promega). The PCR conditions used (Table 2).

The Big Dye Terminator method [Macrogen, Korea] was used to directly sequence all of the PCR products of the SNPs under study. The obtained sequences were compared to the standard Gen Bank sequences.

All statistical analyses were conducted utilizing SPSS-24. P value ≤0.05 was accepted as significant. Diverse genotypes from Hardy-Weinberg Equilibrium (HWE) were employed, and the association between the diverse genotypes and alleles of polymorphisms with the risk of PD-1.5 and PDL-1 was surveyed by calculating the odds ratio (OR) and comparing 95% CI using binary logistic regression.

RESULTS

Table No. 1: SNPs with specific primers

Genes	SNP	Location	Primers (5`- 3`)	PCR Products
PD-1	C/T (rs 2227981)	Exon 5	AGACGGAGTATGCCACCATT CACTGTGGGCATTGAGACAT	333bp
PDL-1	G\C\A (rs1970000)	Intron 4	AATGGCTTGTTGTCCAGAGATG GTACCACATGGAGTGGCTGC	553bp

Table NO. 2: PCR Condition of PD1 and PDL-1

Step	Temperature (c°)		Time		No. of cycles	
	PD1	PDL-1	PD1	PDL-1	PD1	PDL-1
Initial Denaturation	95	95	3 min	10 min	1	1
Denaturation	95	60	15 sec	30 sec	35	37
Annealing	64	72	30 sec	3 min	-	-
Extension	72	95	15 sec	30 sec	-	-
Final extension	72	72	10 min	10 min	1	1

There were 117 (34%) specimens of sera participants have been founded Type 2 diabetes mellitus with toxoplasmosis (T2DMT), 63 (18%) samples have Type 2 diabetes mellitus (T2DM), 55(16%) cases have positive control group (PC) and 108 samples (32 %) were regarded as negative control group (NC) without any infections (Table 3). Gel electrophoresis of PD-1.5 C/T and PDL-1 G\C\A polymorphism PCR product is shown in (Figs 1-2). The fragment length was 333 bp and 553pb sequencing of PCR product revealed three genotypes for the PD-1.5 C/T and PDL-1 G\C\A polymorphism in individuals and healthy subjects (Figs.3-4). The distribution of different genotypes of PD1.5 C/T polymorphism in participants and controls was in a good agreement with HWE.

the table 4 displays an analysis of genotyping and allele frequency between non-diabetic individuals (NC) and Type 2 Diabetic Mellitus (T2DM) patients. The CT genotype was more prevalent in the NC group, while the TT genotype was significantly less frequent. There was no discernible difference in the CC genotype between the two groups. At the allelic level, the T allele was more common among T2DM patients, indicating a significant difference, while the C allele was more frequent in the NC group, though this difference was not significant. In the T2DMT andT2DM groups, The CT genotype was more common in T2DMT patients, while the TT genotype was significantly less common. The CC genotype showed no significant difference between the two groups. Notably, the T allele was more prevalent in diabetic patients than in those with both diabetes and T2DMT, indicating a substantial genetic disparity. However, the C allele frequency did not show a significant difference, suggesting complex interactions between diabetes and T2DMT. In the T2DMT and PC group, In the PC group, the CT

genotype was marginally more common., while the T allele was more common in T2DMT patients. However, none of the observed differences were statistically significant. The study concluded that both groups exhibit similar genetic characteristics, as indicated by various markers, with p-values suggesting no substantial differences in allele frequencies. The CT, TT, and CC genes had the same frequency in both the PC and NC groups (67%, 8%, and 25%) and there was no significant difference between them (P=0.748, 0849, and 0679).The T allele was found at the same frequency in PC and NC, with no significant difference. Also, the frequency of the C allele was equal in the PC and NC groups at 58%, with no significant difference.

The analysis focuses on the frequency of genetic types in non-diabetic controls (NC) and type 2 diabetes mellitus (T2DM) patients(Table 5). The AA genotype is significantly more prevalent in the NC group, while the AC genotype is more common in T2DM patients. The study also shows that the A allele occurs more frequently in NC individuals compared to T2DM patients, indicating a potential association with diabetes risk. Conversely, the C allele is more prevalent among T2DM patients, although the difference is not statistically significant. Overall, the findings highlight important genetic variations linked to diabetes. The AC genotype was more prevalent in T2DMT patients (66%) compared to T2DM patients (33%), However, there was no statistically significant change. The CC genotype was less common in T2DMT (17%) compared to T2DM (50%), also lacking significance. The AA genotype was equally present in both groups (17%). At the allelic level, the A allele was more frequent in T2DMT (50% vs. 33%), while the C allele was more common in T2DM (67% vs. 50%), with the latter showing a statistically significant difference. This research explores the distribution of genotypes AC, CC, and AA among patients with T2DMT compared to a control group. The findings indicate that while the AC genotype was more frequent in T2DMT patients (66%) than in controls (50%), The CC genotype did not significantly differ between the two groups. Similarly, the CC genotype was slightly more prevalent in T2DMT patients (17%) than controls (12%), but again, not significantly. The AA genotype was more common in the control group (38%) compared to T2DMT patients (17%), nearing significance. Overall, no significant allelic differences were found. In PC and NC groups no significant no significant variations in genotype and allele frequencies between the regarding recurrence rates.in genotype and allele frequencies between the regarding recurrence rates.

Table No. 3: Levels of IgG antibodies (IU/ml) for all study groups

Groups	(No. of Samples)	(%)	(Mean ± SD)	(Confidence Interval for Mean)	
				Lower Bound	Upper Bound
Type 2 diabetes mellitus with Toxoplasmosis (T2DMT)	117/180	65.0	22.9±6.42	21.71	24.06
Type 2 diabetes mellitus (T2DM)	63/180	35.0	5.5±1.51	5.17	5.93
Positive control (PC)	55/163	16.0	14.3±8.73	11.90	16.62
Negative control (NC)	108/163	32.	3.0±1.98	2.64	3.39

Table No. 4: Genotypes and alleles of PD-1.5 rs2227981 C/T single nucleotide polymorphism in patients with Type 2 diabetes mellitus and Toxoplasmosis and controls

	T2DM	NC	P value	OR	CI
CC	6 (25%)	6 (25%)	0.083	1.0	Reference
CT	9 (37.5%)	16 (67%)	0.59	6.0	0.932-38.629
TT	9 (37.5%)	2 (8%)	0.029	7.0	1.221-40.124
Allele					
T	27 (57%)	20 (43%)	0.002	0.125	0.032-0.485
C	21 (43%)	28 (57%)	0.322	0.563	0.179-1.765
	T2DMT	T2DM	P value	OR	CI
CC	3 (25%)	3 (25%)	0.087	1.0	Reference
CT	7 (58%)	2 (17%)	0.274	0.286	0.030-2.692
TT	2 (17%)	7 (58%)	0.027	0.082	0.009-0.753
Allele					
T	11 (46%)	16 (67%)	0.006	12.250	1.788-83.946
C	13 (54%)	8 (33%)	0.195	3.50	0.505-24.270
	T2DMT	PC	P value	OR	CI
CC	3 (25%)	3 (25%)	0.642	1.0	Reference
CT	9 (58%)	8 (67%)	0.398	3.333	0.204-54.532

TT	2 (17%)	1 (8%)	0.689	1.714	0.123-54.532
Allele					
T	11 (52%)	10 (48%)	0.407	0.438	0.061-3.160
C	13 (48%)	14 (52%)	0.863	0.875	0.191-3.999
	PC	NC	P value	OR	CI
CC	3 (25%)	3 (25%)	0.679	1.0	Reference
CT	8 (67%)	8 (67%)	0.748	0.60	0.027-13.582
TT	1 (8%)	1 (8%)	0.848	1.333	0.069-25.912
Allele					
T	10 (42%)	10 (42%)	1.0	1.0	0.112-8.947
C	14 (58%)	14 (58%)	1.0	1.0	0.224-4.468

Table 5: Genotypes and alleles of PDL-1 rs1970000 G\C\A single nucleotide polymorphism in patients with Type 2 diabetes mellitus and Toxoplasmosis and controls

	T2DM	**NC**	**P value**	**OR**	**CI**
AA	4 (17%)	9 (56%)	0.040	1.0	Reference
AC	12 (50%)	5 (31%)	0.027	9.0	1.285-63.025
CC	8 (33%)	2 (13%)	0.592	1.667	0.257-10.792
Allele					
A	20 (42%)	23 (72%)	0.010	5.40	1.421-20.518
C	28 (56%)	9 (28%)	0.506	0.60	0.132-2.724
	T2DMT	T2DM	P value	OR	CI
AA	2 (17%)	2 (17%)	0.214	1.0	Reference
AC	8 (66%)	4 (33%)	0.395	0.333	0.027-4.186
CC	2 (17%)	6 (50%)	0.079	0.167	1.153-31.228
Allele					
A	12 (50%)	8 (33%)	0.456	2.0	0.320-12.510
C	12 (50%)	15 (67%)	0.027	6.0	1.153-31.228
	T2DMT	PC	P value	OR	CI
AA	2 (17%)	3 (38%)	0.054	1.0	Reference
AC	8 (66%)	4 (50%)	0.224	6.0	0.335-107.420
CC	2 (17%)	1 (12%)	0.392	0.250	0.010-5.985
Allele					
A	12 (50%)	10 (62.5%)	0.211	3.0	0.525-15.159
C	12 (50%)	6 (37.5%)	1.0	1.0	0.125-7.995
	PC	NC	P value	OR	CI
AA	3 (38%)	6 (76%)	0.290	1.0	Reference
AC	4 (50%)	1 (12%)	0.661	2.0	0.090-44.350
CC	1 (12%)	1 (12%)	0.442	0.250	0.007-8.560
Allele					
A	10 (52.5%)	13 (81%)	0.063	8.0	0.725-88.23
C	6 (37.5%)	3 (19%)	0.343	4.0	0.211-75.66

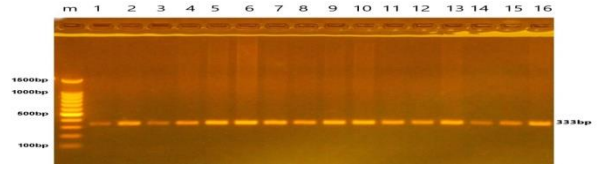

Figure No. 1: Electrophoresis of acarose gel (2%), 75 volts for two hours, which shows the results of a reaction test. Conventional polymerase chain reaction enzyme (PD-1) (333 bp) for all study groups for a specific fraction and size

M: L(100-2000bp). *4-1 Group of patients with type 2 diabetes and toxoplasmosis gondii (T2DMT). *8-5 Group of patients with type 2 diabetes only (T2DM). *12-9 Positive control group (PC). *13-16 negative control group (NC).

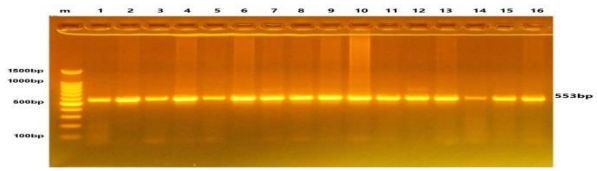

Figure No. 2: Electrophoresis of acarose gel (2%), 75 volts for two hours, which shows the results of a reaction test. Conventional polymerase chain reaction enzyme (PDL-1) (553 bp) for all study groups for a specific fraction and size

M: L(100-2000bp) . *4-1 Group of patients with type 2 diabetes and toxoplasmosis gondii (T2DMT). *8-5 Group of patients with type 2 diabetes only (T2DM). *12-9 Positive control group(PC). *13-16 negative control group(NC).

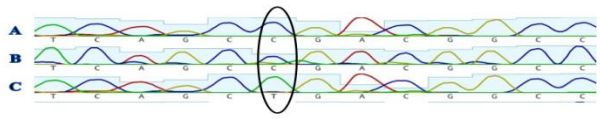

Figure No. 3: Chromatograph of DNA sequencing for PD-1.5 C/T polymorphism. A: wild-type homozygous genotype (CC), B: Heterozygous genotype (CT), C: mutant homozygous genotype (TT)

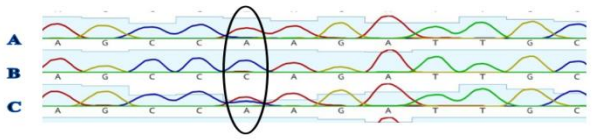

Figure No. 4: Chromatograph of DNA sequencing for PDL-1 G\C\A polymorphism. A: wild-type homozygous genotype (AA), B: mutant homozygous genotype (CC), C: Heterozygous genotype (AC)

DISCUSSION

Studies investigating the connection between toxoplasmosis and type 2 diabetes and the PD1.5 gene variation at rs2227981 are not yet accessible, therefore, the results of the current study were compared with similar studies on the same gene and mutation. The current study's findings were discovered to be nearly identical to those of a study carried out by Hashim[10]. where the frequency of the T allele (41.25%) in patients with cutaneous leishmaniasis was higher than its frequency (26.5%) in healthy individuals. In the current investigation, there was a noteworthy correlation between TT genotypes and allele T were both higher significantly in type 2 diabetes patient and control of PD-1.5 SNP.

Almost similar results were obtained by Hou[11], who found that there is a significant association of PD-1.5 with chronic HBV where the frequency of PD-1.5 TT genotype and allele T were both significantly higher in spontaneously recovered group than chronic HBV infection group.

In another study conducted by Sarvari et al[12], significant differences were found in the genetic patterns and alleles between a group of patients suffering from chronic Hepatitis C virus (HCV) compared to a matched group of recovered individuals and a healthy control group. Mojtahedi et al[13] demonstrated an association between the C/T PD-1.5 polymorphism and the development of colon cancer among the Iranian population. Conversely, The PD-1.5 polymorphism was not linked to breast cancer, according to a study by Haghshenas et al[14].

On the other hand, the present study was designed to assess the possible association of PDL-1SNPs with T2DM and toxoplasmosis. According to the result of sequencing revealed there was significant association between AC genotypes and allele A were both higher significantly in T2DM patient and control of PDL-1, revealed there was significant association between CC genotypes and allele C were both higher significantly in T2DMT and T2DM. There are no available studies on the role of the PDL-1 gene variant rs1970000 in Behcet's disease on one hand and Type 2 diabetes on the other hand. A single study conducted by Meng et al[15] investigated this variant in a chronic autoimmune disease, Behcet's Disease, and the results of this study showed no association between the PDL-1 gene variant and Behcet's Disease among the Han Chinese population..

CONCLUSION

There is a significant association between the phenotypic polymorphism of the (PD-1.5 C/T gene rs2227981) Toxoplasmosis and type 2 diabetes for the groups in the current study, as it was found that people who carry the genotype TT they are more likely to develop type 2 diabetes than people who carry the genotype CC.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Farah E. Mohammed
Drafting or Revising Critically:	Ali N. Yaseen, Muhammed A. H. Aldabagh
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

Conflict of Interest: The study has no conflict of interest to declare by any author.

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