ASSOCIATION OF PROGRAMMED CELL DEATH PROTEIN–1 (PD-1) GENE POLYMORPHISM AND SERUM LEVELS OF SOLUBLE PD-1 WITH TYPE 2 DIABETES MELLITUS

Aya Raed Rasheed 1*, Wafaa Ragee 1, Qasim S. Al-mayah 2

1. University of Technology, Iraq
2. Medical Research Unit, College of Medicine, Al-Nahrain University, Iraq
*Corresponding author E-mail: aya.alkatan@yahoo.com (Rasheed)

ABSTRACT

Type 2 diabetes mellitus (T2DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of fat and protein metabolism resulting from defects in insulin secretion, insulin action or both, and mediated in large part by the alteration in adaptive immunity. This study aimed to evaluate the role of programmed cell death protein – 1 (PD-1) gene polymorphism and serum level of soluble PD-1 in development of T2DM in Iraqi patients. Forty-five T2DM patients were recruited for this study. Other 45 apparently healthy subjects matched for age, gender and ethnic background for patients were also included as control group. Blood samples were collected from each participant, and DNA was extracted from leukocytes. The gene fragment corresponding the PD-1-538 G/A polymorphism was amplified with conventional PCR using specific primers. The genotyping was performed through restriction fragment length gene polymorphism (RFLP). Serum level of soluble PD-1 (sPD-1) was measured by enzyme linked immune sorbent assay (ELISA). There was no significant association between different genotypes of PD-1-538 G/A polymorphism. However, at allelic level, G allele was less frequent among patients than controls (28.89% versus 44.44%) with a significant difference. The median concentration of sPD-1 in patients was 53.12 pg/ml (range 18.24-312.89 pg/ml) compared to 63.83pg/ml (range 16.89-508.65 pg/ml) in controls with no significant difference. Median levels of sPD-1 in patients carrying GG, GA and AA genotypes were 51.67 pg/ml (range 18.24-160.41 pg/mL), 52.77 pg/ml (range 19.32-154.18 pg/mL) and 220.97 pg/ml (range 129.05-312.89 pg/mL) respectively. The GG genotype carriers differed significantly from AA genotype carriers, while there was no significant between AA genotype carriers and AG genotype carriers. These data suggest that A allele of PD-1-538 G/A might be a risk factor for development of T2DM. The GG genotype of this polymorphism associates with higher serum level of sPD-1 than other genotypes.

Keywords: Type 2 diabetes, gene polymorphism, programmed cell death

INTRODUCTION

Diabetes mellitus is a heterogeneous metabolic disorder characterized by the presence of hyperglycemia due to impairment of insulin secretion or/and defective insulin action. The burden of diabetes is rising rapidly
worldwide posing an enormous socioeconomic and health challenge \cite{1}. The number of people with diabetes is estimated to further increase from that of 415 million in 2015 to 642 million by 2040 \cite{2}. The etiology of T2DM is not well-understood, although associated health risk factors are recognized; for instance, a family history of diabetes, age over 45 years, ethnic background or race, ethnic background, insulin resistance syndrome, hypertension, obesity, and history of vascular disease such as stroke, abnormal cholesterol levels and history of gestational diabetes \cite{1}. Programmed cell death 1 (PD-1) is an immune-inhibitory factor belonging to the CD28/B7 family. It plays a vital role in regulating T cell activation and maintaining peripheral tolerance as a core costimulatory molecule. Recently, PD-1 has been widely studied as an immune checkpoint that is applied to the treatment of numerous advanced cancers \cite{3}. Accumulated studies showed that blockage of the interaction between PD-1 can help with better prognosis in various malignant tumors. However, autoimmune diabetes has been reported after receiving anti-PD-1 therapy for tumor in both mouse models and human cases \cite{4}. Studies had shown that low PD-1 might increase T cell proliferation and activation which lead to the destruction of beta cells, providing a possible mechanism for T1DM \cite{5}. Single nucleotide polymorphisms (SNPs) may have vital role in the transcription and/or translation of affected genes. Existing data pointed out that some PD-1 SNPs do associate with T2DM susceptibility in different populations \cite{6}. However, there is no study focus on the associations between PD-1 gene polymorphisms and T2DM in Iraqis population. Therefore, this study aimed to evaluate the role of programmed cell death protein – 1 (PD-1) gene polymorphism and serum level of soluble PD-1 in development of T2DM in Iraqi patients.

**MATERIAL AND METHODS**

**The Study Population**

Forty-five adult patients with clinically definite T2DM, diagnosed by a senior endocrinologist, were recruited from endocrinology department and outpatient clinic at Al-Imamain Al-Kadhumain Medical City during the period from November 2018 and January 2019. Other 45 family-unrelated, age- and sex-matched apparently healthy individuals who were attending the Medical City for regular checkup or to company their relative patients were recruited to be the control group.

The study was approved by the institutional review board of college of Applied Science /University of Technology. Each participant has signed a consent form explaining the aims and dimensions of the study.

**Sample Collection**

Five ml of venous blood were taken from all participants: 2 ml were placed in EDTA tubes and 3 ml blood were placed in gel tubes. Sera were separated by centrifugation and stored all the tubes at -20°C until be used.

**Molecular Assays**

A ready commercial kit (Promega/ USA) was used for DNA isolation according to the manufacturer’s protocol. Two primers were used to amplify the PD-1 gene corresponding the PD-1.1 polymorphism. These primers were forward: 5’- TTCTAGCCTCGCTTCGGTTA-3’ and reverse R: 5’-\text{CTCAACCCCACTCCATTCT-3’} with an expected fragment length of 552 bp. The PCR was carried out on the ABI 9600 (Hybaid/ England) in a total volume of 25 μl including 50 ng of genomic DNA, 1.5 μl of 10×PCR buffer, 0.3 μl of 10mMdNTPs, 0.25 μl of 10 pmol/μl of each primer, and 1.25 U of Taq DNA polymerase (Bioneer/Korea). Cycling parameters were as follows: 94°C for 2 min; 35 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The PCR products were...
subjected to enzymatic digestion with 5 U MspI (New England Biolabs LTD, Beijing, China) according to the manufacturer’s instructions. The genotypes of individuals were identified by length of digested fragments subsequent to 2.5% agarose gel electrophoresis stained by ethidium bromide. Allele discrimination was based on fragment size after digestion which was 227bp for G allele and 282bp for A allele.

**Serum Levels of Soluble Programmed Death 1(PD-1)**

A ready commercial ELISA kit (Cusabio/ China) was used to measure the serum level of soluble PD-1. The manufacturer’s instructions were followed precisely. The optical density (OD) was determined within 5 min using microplate reader at 450 nm. A standard curve (figure 1) was created between standards concentration (x-axis) and OD (y-axis). Furthermore, a regression equation was established according to which the ODs for samples were converted to concentrations.

Statistical Analysis

All statistical analyses were conducted using statistical package for social science (SPSS) software version 25.0. Continuous variables were expressed as mean± standard deviation (SD) and analyzed with Student t-test. Binomial variables were expressed as numbers and percentages and analyzed with Chi-square. The deviation of different genotypes from Hardy-Weinberg Equilibrium (HWE) was calculated online using https://www.easycalculation.com/health/hardy-weinberg-equilibrium-calculator.php websites. Binary logistic regression was used to calculate odds ratio (OR) and the corresponding 95% confidence intervals (CI) in order to assess the association between the polymorphism in PD-1 gene and the risk of T2DM. A P< 0.05 (exact two-sided) was accepted as the level of significant.

**RESULTS**

**Demographic Characteristics of the Study Population**

Mean age of patients was 55.2±10.67 years which did not differ significantly from that of controls (59.11±13.44 years). Likewise, there was no significant difference between patients and controls regarding BMI and the frequency of males and females. However, all patients had a positive family history of T2DM while none of the control group had such a history with highly significant difference (Table 1). HbA1c% per
se was far much higher in patients than controls (9.67±1.04% versus 4.77±0.49%) with a highly significant difference.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (45)</th>
<th>Controls (45)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean±SD</td>
<td>55.2±10.67</td>
<td>59.11±13.44</td>
<td>0.130</td>
</tr>
<tr>
<td>BMI, kg/m², mean±SD</td>
<td>27.06±5.8</td>
<td>24.73±6.9</td>
<td>0.224</td>
</tr>
<tr>
<td>Sex, No (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14(31.11%)</td>
<td>15(33.33%)</td>
<td>0.822</td>
</tr>
<tr>
<td>Female</td>
<td>31(68.89%)</td>
<td>30(66.67%)</td>
<td></td>
</tr>
<tr>
<td>Family History, No (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0(0%)</td>
<td>45(100%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>45(100%)</td>
<td>0(0%)</td>
<td></td>
</tr>
<tr>
<td>HbA1c%</td>
<td>9.67±1.04</td>
<td>4.77±0.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of disease, years</td>
<td>7.2± 5.11</td>
<td>...............</td>
<td>...........</td>
</tr>
</tbody>
</table>

BMI: body mass index, HbA1C%: Glycated hemoglobin

**PD-1-538 G/A polymorphism**

Conventional PCR was used to amplify PD-1 gene fragment corresponding the PD-1-538 G/A polymorphism using a specific pair of primers. The PCR products were stained with ethidium bromide and visualized under the UV transluminator. Restriction fragment length polymorphism was used for genotyping of the target SNP. According to this technique, PD-1-538 G/A polymorphism appeared in three genotypes AA, AG and GG (Figure 2). The frequencies of different genotypes and alleles of PD-1-538 G/A polymorphism are shown in table 4-2. The AA genotype showed higher frequency in T2DM patients (46.67%) than controls (24.44%) with no significant difference (p=0.062). In contrast, the GG genotype was less frequent among patients than controls (4.44% versus 13.33%), however, the difference did not reach the significant level (p=0.52). Genetic model analysis showed that the effect of this polymorphism (if any) is not through dominant model (p= 0.157), while in recessive model, the difference was significant (p= 0.03). At allelic level, G allele was less frequent among patients than controls (28.89% versus 44.44%) with a significant difference (p=0.031).
Figure 2: PD-1-538 G/A genotype patterns in diabetic patients after digestion with MpsI restriction enzyme, visualized under UV light after staining with ethidium bromide. Lanes 1, 2 and 11: AA genotypes, lanes 3, 6, 7, 8 and 10: GA genotypes, lanes 4, 5 and 9: GG genotypes, M: 100 bp molecular ladder.

Table 2: Genotypes and alleles of PD-1-538 G/A single nucleotide polymorphism in T2DM patients and controls

<table>
<thead>
<tr>
<th>PD-1-538 G/A</th>
<th>Patients (45)</th>
<th>Controls (45)</th>
<th>p-value</th>
<th>OR(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>21(46.67%)</td>
<td>11(24.44%)</td>
<td>0.062</td>
<td>1.0 Reference</td>
</tr>
<tr>
<td>AG</td>
<td>22(48.89%)</td>
<td>28(62.22%)</td>
<td>0.058</td>
<td>0.41(0.16-1.03)</td>
</tr>
<tr>
<td>GG</td>
<td>2(4.44%)</td>
<td>6(13.33%)</td>
<td>0.052</td>
<td>0.18(0.03-1.04)</td>
</tr>
<tr>
<td>HWE</td>
<td>0.203</td>
<td>0.081</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA+AG</td>
<td>43(95.56%)</td>
<td>39(76%)</td>
<td>0.157</td>
<td>1.0 Reference</td>
</tr>
<tr>
<td>GG</td>
<td>2(4.44%)</td>
<td>6(13.33%)</td>
<td></td>
<td>0.30 (0.06-1.59)</td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>21(46.67%)</td>
<td>11(24.44%)</td>
<td>0.030</td>
<td>1.0 Reference</td>
</tr>
<tr>
<td>AG+GG</td>
<td>24(53.33%)</td>
<td>34(75.56%)</td>
<td></td>
<td>0.37(0.15-0.91)</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>64(71.11%)</td>
<td>50(55.56%)</td>
<td>0.031</td>
<td>1.0 Reference</td>
</tr>
<tr>
<td>G</td>
<td>26(28.89%)</td>
<td>40(44.44%)</td>
<td></td>
<td>0.51(0.27-0.94)</td>
</tr>
</tbody>
</table>

OR: odds ratio, CI: confidence interval

Serum concentration of sPD-1

Data regarding serum level of sPD-1 were subjected to normality test and found to be non-normally distributed. Accordingly, Mann Whitney U-test was used to compare medians of these levels. The median concentration of sPD-1 in patients was 53.12pg/ml (range 18.24-312.89 pg/ml) compared to 63.83pg/ml (range 16.89-508.65pg/ml) in controls (Figure 3). Mann Whitney U-test revealed a significant difference between the two groups (p = 0.112).

Correlation between sPD-1 and other variables

Pearson’s correlation was used to examine the possible correlation between sPD-1 with each of disease duration and HbA1c. Figure 4-7 shows the correlation between sPD-1 and disease duration. There was weak positive correlation, however, it did not reach a significant level (r= 0.248, p= 0.101). Similarly, there was no significant correlation between sPD-1 and HbA1c% in diabetic patients (r= 0.107, p= 0.488) as shown in figure 3.
DISCUSSION

The present study showed a significant association of between G allele with the occurrence of T2DM (OR = 0.51, 95% CI (0.27-0.94). This implies that individuals carrying G allele will be at 0.51 less risk to develop T2DM compared with those carrying A allele. Different models showed that the recessive model was associated significantly with T2DM which implies that in order for allele G to demonstrate the protective trait, two alleles are required. Otherwise this trait will not appear. Very limited studies have been conducted regarding the role of this polymorphism with diabetes. In a similar study, Qian et al. \cite{7} investigated a total 166 Chinese patients with T1DM and 100 healthy subjects for the association of PD-1 and PD-L1 polymorphisms and the development of T1DM. The study revealed that PD-L1 but not PD-1 was significantly associated with T1DM. Other than diabetes, several studies had been committed to the association with PD-1-358 A/G and autoimmune disease and were found to be associated with systemic lupus erythematosus, ankylosing spondylitis, and allergic bronchial asthma \cite{8}. The most reasonable explanation for the significant association between GG genotype and the increase susceptibility for T2DM referred to the site of this polymorphism in the promoter region of PD-1 gene. It has been shown that this SNP resides in the putative binding site for upstream control element -2 (UCE-2) transcription regulator (GGCCG at position -610 to -606) on the gene \cite{9}. It seems that the presence of G allele in this position facilitate the binding of RNA polymerase with a subsequent increase in PD-1 protein production. Supporting this assumption is the study of Ishizaki et al. \cite{10} who demonstrated that haplotype with G allele is associated with high promoter activity and with the development of Japanese subacute sclerosing panencephalitis (SSPE). An interesting study was conducted on 1163 American patients with different malignancies who received PD-1 inhibitors for at least 6 years. The study identified 12 patients who developed new-onset insulin-dependent diabetes, while 9 patients experienced worsening of pre-existing T2DM. This form of diabetes is characterized by more rapid progression to severe insulin deficiency as compared with
spontaneous type 1 diabetes, frequently presents with diabetic ketoacidosis and does not appear to undergo remission\(^\text{[11]}\). Studies had shown that low PD-1 might increase T cell proliferation and activation which lead to the destruction of beta cells, providing a possible mechanism for T1DM. Lower PD-1 expression was proposed to have associations with the development of T1DM in mouse models\(^\text{[12]}\). The presence study revealed no significant difference in the serum level of sPD-1 between diabetic patients and controls. This result is partially agreed with the study of Shi \emph{et al.}\(^\text{[13]}\) who used flow cytometry to analyze the expression of PD-1 and PD-L1 on the peripheral lymphocytes from 125 diabetic patients and 48 healthy controls. The study revealed that lymphocytes from T2DM patients expressed higher PD-1 than that from healthy controls, while there was no significant difference between the two groups regarding serum level of sPD-1. In contrast, diabetic patients had significantly higher serum level of sPD-L1 than controls, and this level was significantly correlated with the level of IFN-γ. In another study, Jia \emph{et al.}\(^\text{[14]}\) compared the expression of PD-1 on lymphocyte from patients with T2DM and healthy controls. They showed that the percentage of T-lymphocytes carrying PD-1 was significantly higher in patients than controls. From these studies, it can be deduced that there is overexpression of PD-1 on T-lymphocyte in diabetic patients; however, the serum level of sPD-1 will not increase significantly. In contrast, Sun \emph{et al.}\(^\text{[15]}\) reported a downregulation in PD-1 expression on peripheral blood mononuclear cells (PBMCs) from diabetic patients compared with that from healthy controls. PD-1 is produced in two forms, namely the membrane-bound and soluble forms. The soluble form of molecules is usually produced by proteolytic cleavage of the membrane-bound form of the costimulatory proteins, or by translation of alternative spliced mRNA\(^\text{[16]}\). Four alternatively spliced PD-1 mRNA transcripts PD-1\text{△}ex2, PD-1\text{△}ex3, PD-1\text{△}ex2,3, and PD-1\text{△}ex2,3,4 were qualified apart from the full-length PD-1. These variants are generated by splicing out exon 2; exon 3; exons 2 and 3; and exons 2, 3, and 4, respectively. Differ from other transcripts, which do not have obvious biological functions, PD-1\text{△}ex3 is the soluble isoform of PD-1 and increases following the activation of PBMCs\(^\text{[17]}\). Recent studies showed that sPD-1 can stop the PD-1/PD-L1 pathway in regulating T-cell function during chronic infection, autoimmune diseases and antitumor immunity\(^\text{[18]}\). sPD-1 can link PD-1 ligands, block the interactions of PD-1/ligand, and enhance the cytotoxicity of tumor-specific CTLs\(^\text{[19]}\). In the current study, there was no significant correlation between sPD-1 with either the duration of diabetes or with HbA1C %. This result did not agree with the study of Sun \emph{et al.}\(^\text{[15]}\) who investigated 23 diabetic patients and 20 healthy donors for the correlation between PD-1 expression and some clinical and demographic data of the patients. The correlation analysis indicated that the PD-1 on NK cells has a positive correlation with insulin and diabetes duration. And an inverse correlation has been shown between the PD-1 expression on monocytes and BMI. This discrepancy between the two studies (the present study and Sun's study) can be explained by the fact that in the latter study, the PD-1 itself but not the sPD-1 was measured, while the current study depended on serum level of sPD-1. It is well-documented from many previous studies that there was no direct correlation between PD-1 expression and sPD-1\(^\text{[14,18]}\). It is reasonable that there is no correlation between duration of diabetes and HbA1C % on one hand and serum concentration of sPD-1 on the other hand because either duration of diabetes or HbA1C% cannot affect the detachment of extra cytoplasm part of PD-1 nor they influence the alternation splicing. Collectively, these data suggest that allele G of PD-1-538 G/A polymorphism has a protective role against T2DM may be through the upregulation of PD-1 gene.
transcription, and sPD-1 does not increase in diabetic patients; however, the expression of PD-1 on immune cells may do.

CONCLUSION

These data suggest that A allele of PD-1-538 G/A might be a risk factor for development of T2DM. The GG genotype of this polymorphism associates with higher serum level of sPD-1 than other genotypes.

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ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING: Self-funding

REFERENCES


