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Interleukin (IL)-10 Gene Polymorphisms Are Associated with Type 2 Diabetes With and Without Nephropathy: A Study of Patients from the Southeast Region of Iran

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Abstract—The impact of several environmental and genetic factors on diabetes and its complications is well documented. It has also been established that cytokines play a key role in the regulation of immune responses which have been shown to be important in the pathogenesis of diabetes. Studies showed that single-nucleotide polymorphisms within the −592 region of interleukin-10 (IL-10) are associated with the regulation of expression. In this study, we aimed to find polymorphisms of this region that may be associated to type 2 diabetic (T2D) patients with and without nephropathy. In this study, peripheral blood samples were collected from 100 T2D patients without nephropathy, 100 T2D patients with nephropathy, and 100 healthy controls. DNA was extracted, and a polymerase chain reaction-restriction fragment length polymorphism technique was performed to examine the polymorphisms within the −592 region of IL-10 gene. Our results showed a significant difference between the genotypes and alleles of the −592 region of IL-10 in nephropathic and non-nephropathic patients in comparison to the healthy controls. The differences between the two patient groups in relation to genotypes and alleles were not significant. Results of this study suggest that the functional gene polymorphism of IL-10 reported here may play an important role in the pathogenesis of diabetes, but it seems that these polymorphisms do not have an effect on the nephropathic complications of the disease.

KEY WORDS: IL-10; diabetes; nephropathy; polymorphism.

INTRODUCTION

The frequency of diabetes mellitus is increasing globally, and it is expected that this latent disorder will affect 200 million of people by 2010 and 300 million in 2025 [1]. Type 2, sometimes referred to as non-insulin dependent diabetes mellitus or adult-onset diabetes, is the most prevalent type of the diabetes [2]. Current studies showed that several genetic and environmental parameters are associated with type 2 diabetes (T2D) and its complications [3]. It has been suggested that diabetes is an immune-dependent disease in which the pattern of cytokine expression is changed [4]. As an example, in T2D peripheral blood monocytes produce inappropriate levels of inflammatory cytokines which...
may induce the associated pathology of the disease [5]. Subsequently, the cytokine/cytokine–receptor axis has been the subject of several studies to investigate their crucial roles in diabetes and its complications [3], and as a result, the important role of cytokine imbalance in T2D with and without nephropathy has been reported [6, 7]. Increased serum levels of inflammatory cytokines including interleukin (IL)-18 [8], IL-6 [9], IFN-γ [7], IL-17 [7], and TNF-α [9] have been documented in T2D and its nephropathic complications. Furthermore, the association of IL-10 in immunological disorders such as multiple sclerosis [10], systemic lupus erythematosus [11], nephrotic syndrome [12], graft rejection [13], asthma [14], and type 1 [15] diabetes is well established. The key roles of IL-10 as an inhibitory cytokine of autoimmunity and inflammation [16] raise questions concerning the impacts of this cytokine on the pathogenesis of other diseases including T2D and its nephropathic complications. Previous studies showed that the secretion of IL-10 can be affected by polymorphisms in its promoter region [17]. Therefore, this study was aimed to investigate the relation between the polymorphisms of the −592 region of IL-10 in T2D patients with and without nephropathy.

MATERIAL AND METHODS

Subject

Blood samples were collected from 100 type 2 diabetic patients without nephropathy, 100 T2D patients showing nephropathic complications, and 100 healthy controls. All the T2D patients were selected from the Rafsanjan population that had been referred to the Diabetes Clinic of the Ali Ebn Abitaleb Hospital. The T2D patients were selected based on peripheral blood glucose levels of more than 130 mg/dl, and nephropathy was assessed in diabetic patients based on proteinuria of at least 500 mg/24 h and glomerular filtration rates (GFR) of less than 25 ml/min [18]. GFR were calculated according to creatinine-based equations [19]. The patient and control groups were selected from within the Rafsanjan population with similar medical and demographic characteristics including duration of diabetes, sex, age, and socioeconomic status (Table 1). Assessments of socioeconomic conditions were measured based on the level of education and the monthly income of donors. Classifications for education were: a donor holding a diploma was considered weak, an undergraduate was considered moderate, and a postgraduate was considered high. Monthly income was classified as: under $250, weak; $250–1,000, moderate; and more than $1,000, high. Information regarding lipid levels, proteinuria, estimate of GFR, and drug therapy of patients is also listed in Table 1. Human ethical approval for this study was granted by the Ethical Committee of the Rafsanjan University of Medical Sciences. Consent forms were filled out by both patients and controls prior to blood collection. Previous studies showed that some factors such as infections [20–22], allergic conditions [23], and smoking [24, 25] were considered as bias factors in diabetes and nephropathy; hence, patients with these bias factors were eliminated from the study using a questionnaire.

Assays

Fasting blood sugar, urine albumin level, blood pressure, and clinical presentations were assessed three times during a period of 6 months for each patient and control group.

DNA Extraction

Peripheral blood was collected on EDTA, and genomic DNA was extracted using a commercial kit (Bioneer, Korea) following the manufacturer’s recommended procedures. Extracted DNA was aliquoted (for each patient sample) and stored at −20°C for further use.

Detection of Polymorphisms

The IL-10 gene polymorphism (within the gene promoter) was analyzed by PCR-RFLP method. PCR of the promoter region of the IL-10 gene was performed in a volume of 50 μL containing 5 μl of Taq DNA polymerase buffer (10×), 1.5 μl of MgCl₂ (stock concentration, 1.5 mM), 1 μl of each dNTP [(dATP, dCTP, dGTP, dTTP) at a stock concentration of 10 mM, 2 μl of each primer (stock concentration of 25 ng/μl), 1 μl of prepared DNA, and sterile double distilled water to a final volume of 50 μl. The sequence of the forward primer was 5′-GTAATXCTCTGTCGCC-3′, and the sequence of reverse primer was 5′-CATTCCAGAATA CAATGGG-3′. The amplification was performed using the following program: 1 cycle of 95°C for 5 min (denaturation), 50 s at 53°C for annealing, 72°C for 40 s (elongation) followed by 30 cycles of 95°C for 50 s, 50 s at 53°C for annealing, and 72°C for 40 s using a thermal cycler (C1000, Bio-Rad, USA). During the last 45 s of
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Table 1. Demographic, Socioeconomic Conditions, and Clinical Parameters of T2D Patients with and without Nephropathy and Controls

<table>
<thead>
<tr>
<th>Variant</th>
<th>Healthy control</th>
<th>Type 2 diabetic patients without nephropathy</th>
<th>Nephropathic type 2 diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40±7</td>
<td>40±9</td>
<td>40±6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>60 (60%)</td>
<td>59 (59%)</td>
<td>62 (62%)</td>
</tr>
<tr>
<td>Male</td>
<td>40 (40%)</td>
<td>41 (41%)</td>
<td>38 (38%)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>9±3</td>
<td></td>
<td>10±4</td>
</tr>
<tr>
<td>Socioeconomic status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>22 (22%)</td>
<td>21 (21%)</td>
<td>24 (24%)</td>
</tr>
<tr>
<td>Medium</td>
<td>47 (47%)</td>
<td>49 (49%)</td>
<td>46 (46%)</td>
</tr>
<tr>
<td>High</td>
<td>31 (31%)</td>
<td>30 (30%)</td>
<td>30 (30%)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60±7</td>
<td>50±7</td>
<td>50±9</td>
</tr>
<tr>
<td>Drug therapy</td>
<td></td>
<td>Metformin</td>
<td>Insulin</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>100±4</td>
<td>210±6</td>
<td>350±12</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>150±6</td>
<td>170±5.7</td>
<td>290±10</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>40±3</td>
<td>35±3</td>
<td>24±2</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>100±9</td>
<td>140±6</td>
<td>180±11</td>
</tr>
<tr>
<td>Glucose level (mg/dl)</td>
<td>95±105</td>
<td>140–190</td>
<td>160–205</td>
</tr>
<tr>
<td>Proteinuria (mg/dl)</td>
<td>25±1.5</td>
<td>36.6±3</td>
<td>899±50a</td>
</tr>
<tr>
<td>Estimated GFR</td>
<td>120±5</td>
<td>101±5</td>
<td>72±3b</td>
</tr>
</tbody>
</table>

*Significant difference in proteinuria \( (p<0.002, t\ test, case vs. control) \). Data are shown as mean±SE

RESULTS

Evaluation of polymorphisms within the −592 region of the IL-10 gene by Rsal restriction digestion showed that the prevalence of the C/C, A/C, and A/A genotypes was 60 (60%), 36 (36%), and 4 (4%) in non-nephropathic T2D patients, respectively (Table 2). Our results also revealed that the frequency of C/C, A/C, and A/A genotypes was 47 (47%), 47 (47%), and 6 (6%) in nephropathic T2D patients, respectively (Table 3). These values for the control group were 22 (22%), 55 (55%), and 23 (23%), respectively (Tables 2 and 3). Statistical analysis showed that the difference between diabetic (nephropathic and non-nephropathic) groups in comparison to the control group in relation to these genotypes was significant \( (p<0.001) \). Our results also showed that the frequency of C allele was 156 (78%), and the A allele was 44 (22%) in non-nephropathic T2D patients. The frequency of C and A alleles was 141 (70.5%) and 59 (29.5%), respectively, in T2D patients with nephropathy (Table 2). Evaluation of the control group showed that the frequency of the C allele was 99 (49.5%), and the A allele was 101 (50.5%) in this group (Tables 2 and 3). Statistical analysis regarding these alleles showed that the difference between patients (nephropathic and non-nephropathic) in comparison to the control group was also significant \( (p=0.001; \text{Tables 2 and 3}) \).

Statistical Analysis

Hardy–Weinberg equilibrium was assessed using the genotype data. Allele and genotype frequencies were calculated in patients and healthy controls by direct gene counting. Statistical analysis of the differences between groups was determined by the \( \chi^2 \) test using EPI 2000 and SPSS software version 13. A \( p \) value of less than 0.05 was considered significant.
Our results showed

<table>
<thead>
<tr>
<th>Condition</th>
<th>Patients</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>60 (60%)</td>
<td>22 (22%)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>A/C</td>
<td>36 (36%)</td>
<td>55 (55%)</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>4 (4%)</td>
<td>23 (23%)</td>
<td></td>
</tr>
<tr>
<td>Alleles, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>156 (78%)</td>
<td>99 (49.5%)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>A</td>
<td>44 (22%)</td>
<td>101 (50.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis showed that the frequency of the genotypes and alleles in T2D patients without nephropathy was not different from the T2D patients with nephropathy (p=0.27; Table 4). Our results showed that the difference between the groups in relation to age, sex, and socioeconomic conditions was not significant (Table 1).

DISCUSSION

The crucial role of cytokine networks in orientating immune responses is well documented [26]. Several factors such as infectious agents, hormonal conditions, and cytokine gene polymorphisms regulate expression and secretion of cytokines [27]. The main etiological cause of T2D and its inflammatory complications, such as nephropathy, has yet to be clarified. Some investigators suggested that immune-related factors play important roles in etiology and pathogenesis of T2D and its associated renal complications [28]. As clearly indicated in Table 1, both patients and control groups were demographically matched. Our findings indicated a significant difference between T2D patients with and without nephropathy when compared to healthy controls regarding both genotypes and alleles of the −592 region of the IL-10 gene. Our data revealed that there are no significant differences between the two T2D patient groups; therefore, based on the current results, it can be concluded that the polymorphisms described here are associated with T2D rather than its nephropathic complications in our studied population (southeast Iranian patients). In other words, T2D patients from the Rafsanjanese region show a significant correlation between their disease and the IL-10 −590 polymorphism.

In contradiction to our data presented here, Ezzidi et al. showed that the polymorphisms in −592 region of the IL-10 gene are not associated with nephropathy in T2D patients [29]. These findings supported earlier conclusions reported by Scarpelli et al., who had also shown that these polymorphisms are not associated with T2D [30]. On the other hand, Chang et al. reported a significant relationship between T2D without nephropathy and healthy controls regarding the −592 region of the IL-10 gene [31]. In addition, Mitraoui et al. reported that the IL-10 promoter polymorphisms of the −1082, −819, and −592 regions are associated with nephropathy complications in T2D [32]. Kolla et al. also demonstrated that the polymorphisms within the −1082 region of the IL-10 gene are associated with peripheral neuropathy in South Indian T2D patients [33].

The discrepancies between our results and those of other research groups may be due to differences in the populations that were studied; some of the groups are comprised of different races and genetic backgrounds. It seems that C/C genotype is associated with T2D in Rafsanjanese population, and probably, the C/C genotype can be considered as a risk factor for the population.
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Interestingly, our previous study on another anti-inflammatory cytokine (IL-4) showed that T2D patients with nephropathic complication, but not T2D without nephropathy, are significantly associated with IL-4 polymorphisms [34]. Therefore, based on the results of our current and previous studies, it may be concluded that the polymorphisms in IL-10 can affect the onset of T2D, but not nephropathic complication in Rafsanjani patients, while IL-4 polymorphisms may be correlated to nephropathy in the T2D patients.

In conclusion, nephropathic complications of T2D are very complex; apart from the myriad of cytokines discussed here and reported elsewhere, there is also an association with several environmental and other genetic factors. Significant studies are required to investigate all these parameters and how they influence each other before we can have a comprehensive understanding of the disorder.

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