THE ROLE OF ANTI-INFLAMMATORY INTERLEUKINE-4(-590C>T) AND PRO-INFLAMMATORY INTERLEUKINE-6(-174G>C) GENES POLYMORPHISMS WITH TYPE 2 DIABETES MELLITUS IN IRAQI PATIENTS

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ABSTRACT
Type 2 diabetes mellitus (T2DM) is a common health problem which has reached epidemic proportions due to the rapidly increasing rates of this disease worldwide. Unbalance between pro- and anti-inflammatory cytokines cause different diseases like Type 2 diabetes mellitus. This study aimed to verify the association of IL-4 (-590C>T) and IL-6 (-174G>C) genes polymorphisms with type 2 diabetes mellitus incidence in Iraqi population. Peripheral blood samples were collected from 50 diabetic patients and 50 apparently healthy individuals from both genders. DNA was extracted and Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) were carried out to detect polymorphism at the -590 and -174 position of IL-4 and IL-6 genes and determined the genotypes for Iraqi population. The results revealed that only CC genotype was found in all samples studied respect to IL-4 (-590C>T) polymorphism. the GG genotype in diabetic was significantly (p<0.05) lower than control group (64% versus 74%, respectively) while the GC genotype in diabetic was significantly (p<0.05) higher than control group (32% versus 22%, respectively), and there was no significant difference between diabetic and control group in the CC genotype with a non significant differences in either G or C allelic frequencies between diabetic patients and control group for IL-6 (-174G>C) polymorphism. The results of the present study indicate that heterozygous GC genotype was associated with the incidence of T2DM.

KEYWORD: T2DM, Cytokines, Genes Polymorphisms, PCR-RFLP.

INTRODUCTION
T2DM is a complex polygenic disorder in which common genetic variants interact with environmental factors to unmask the disease; therefore, type 2 diabetes are mainly developed when a diabetogenic lifestyle (i.e. excessive caloric intake, inadequate caloric expenditure, obesity) acts in conjunction with a susceptible genotype. Even though there is some disparity regarding the reasons for the development of type 2 diabetes. Most physicians and scientists agree that the major independent risk factors for developing the disease are obesity, family history (first-degree relative), ethnicity (some ethnic groups have higher prevalence of diabetes), history of previous impaired glucose tolerance or impaired fasting glycemia, hypertension or dyslipidemia, physical inactivity, history of gestational diabetes, low birth weight as a result of the in utero environment, polycystic ovarian syndrome leading to insulin resistance, and finally, decline in insulin secretion due to advancing age (Chen et al., 2011). Recently, T2DM has also been recognized as an immune-mediated disease leading to impaired insulin signaling and selective destruction of insulin-beta-producing cells in which cytokines play an important role (Pickup, 2004; Duncan et al., 2003; Rabinovitch and Suarez-Pinzon, 1998). Cytokines are a low-molecular-weight glycoprotein produced by immune as well as non-immune cells. Cytokines have been shown to regulate immunologic responses, hematopoietic development and cell-to-cell communication as well as host responses to infectious agents and inflammatory stimuli (Aggarwal et al., 1995; Meager, 1998). Under normal circumstances, cytokines are not detectable (or at low levels) in body fluids or tissues. Therefore, their presence at elevated levels of expression indicates activation of cytokine pathways associated with inflammation or disease progression (Dinarello and Wolff, 1993). Several studies reported that cytokine imbalance is involved in pathogenesis of T2DM (Nosratabadi et al., 2009). Traditionally cytokines have been also divided by their inflammatory activity into pro-inflammatory (e.g. IL-1, IL-6, TNF-β, TNF-α) and anti-inflammatory (e.g. IL-1Ra, IL-4, IL-10) subgroups. Cytokine mediated pro-inflammatory effectors functions may be inhibited by anti-
inflammatory cytokine or cytokine receptor specific antagonism (Dinarello et al., 1990; Callard et al., 1999). This study aimed to investigate the association of genes polymorphisms for IL-4 (-590 C>T) as an anti-inflammatory and IL-6 (-174G>C) as a pro-inflammatory with type 2 diabetes mellitus incidence in Iraqi patients.

MATERIAL AND METHODS

Subjects

This study includes 50 patients with type 2 diabetes; samples were collected from the National Center of Diabetes / Al-Mustansiriya University. The patients’ information consists of age, gender, genetic history, hypertension, retinopathy, nephropathy and smoking in addition to height and weight which are used to calculate the body mass index (BMI) and the control group includes 50 individuals who seem to be apparently healthy and whose fasting blood sugar range between (80-110 mg/dl). The same information on which 50 patient’s samples were based is considered for 50 controls. The patients treated with insulin have been excluded.

Blood sampling

Blood samples were collected by vein puncture from the patients and healthy controls. Then, 2.5 ml of blood was put in EDTA anticoagulant tubes and kept in refrigerator until the DNA was extracted.

DNA extraction

DNA was extracted from the whole blood samples by using the Wizard® Genomic DNA Purification Kit (Promega, USA). Both concentrations and purity of the extracted DNA samples were determined using nanodrop. Also, the extracted DNA samples were electrophoresed on 1% agarose gel for checking.

Genotyping

Genotyping for IL-4(-590C>T) and IL-6 (-174G>C) genes SNPs were carried out by using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method. A 195 bp fragment of IL-4 gene was amplified by using the following primers: forward 5’ TAAACCTTGGGAAGACAC TTGT-3’ and Reverse: 5’ TGGGGAAAGATAGAGTAATA-3’. while A 198 bp fragment of IL-6 gene was amplified by using the following primers: Forward: 5’ TGACTTCAGCTTTACTCTTTTG-3’ and Reverse: 5’CTGATTGGAAACCTTATTAGAGTT-3’. PCR was performed with a total volume of 25 μl. The reaction components consist of 12.5 μl of PCR pre Mix (promega) Ready-to-use: TaqDNA polymerase, dNTPs, MgCl2 and reaction buffer pH 8.5, 1 μl forward primer, 1 μl reverse primer, 3 μl DNA template and 7.5 μl free nuclease distilled water. The PCR thermo cycler was run with following program for : 95˚C for 5 min (initial denaturation) followed by 35 cycles of 95˚C for 30 s (denaturation), 53˚C for 30 s (annealing), 72˚C for 45 s (extension) and a final extension of 72˚C for 5 min. Then, PCR products are separated on 2% agarose gel with the present of (50-800bp) DNA ladder and visualized under UV light of transilluminater (figure1) and (figure2).

![Figure 1: PCR product (195 bp) of -590C>T SNP (g.4782C>T, GenBank: NG_023252.1) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours. DNA ladder= 50 bp, N= negative control.](image1)

![Figure 2: PCR product (198 bp) of targeted fragment flanking the -174C>G SNP (g.4880C>G, GenBank: NG_011640.1) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours. DNA ladder= 50 bp.](image2)
and 195bp). In this study only wild-type homozygous (CC) was found (figure 3).

Figure 3: PCR-RFLP analysis of AvaII digest of the PCR product that include -590C>T SNP (rs2243250) at position 4782 (NG_023252.1) of the IL-4 gene separated on a 3% agarose gel. DNA ladder= 50 bp, CC= wild-type homozygous.

The NlaIII enzyme cut the 198 bp PCR products into four fragments 167, 122, 45 and 31 bp in length. Fragments size of 122, 45 and 31 bp indicated the presence of a wild-type homozygous CC genotype, two 167 bp and 31 bp fragments displayed the presence of homozygous GG genotype and four fragments of 167, 122, 45 and 31 bp indicated the presence of heterozygous CG genotype (figure 4) (Due to limitation of agarose gel in detection of fragments that are smaller than 50 bp, 30 bp fragment was invisible).

Figure 4: PCR product (198 bp fragment) of 5’ promoter of IL-6 gene digested with NlaIII restriction enzyme and electrophoresed on 3% agarose. The genotypes are CC (31, 45 and 122 bp), CG (31, 45, 122 and 167 bp) and GG(31 and 167 bp); DNA ladder: 50 bp, visualized under UV light.

Statistical Analysis
The Statistical Analysis System (SAS) (2012) was used to compare between the characteristics of both patients group and apparently healthy subject group. Chi-square test was used to determine the significant differences between the study groups as related with genotype and allele frequencies.

RESULTS
The characteristics of the patients and the control groups are summarize in (table 1). There were three age groups (less than 40, 40-60 and more than 60 years old). The number of those with less than 40 years old in the control group was significantly (p<0.05) higher than in T2DM group (18 versus 8%, respectively). There was no significant difference between control group and T2DM group as related with the number within the age group 40-60 years old, while, the number of those with more than 60 years old in T2DM group was significantly (p<0.05) higher than in the control group (30 versus 18%, respectively). Within BMI of 18.5 to 25, the number was significantly (p<0.05) higher in the control group than in T2DM group (18 versus 8%, respectively). There was no significant difference between control group and T2DM group as related with the number within BMI of 25.1 to 30, while, the number of those with BMI of more than 30 was in T2DM group significantly (p<0.05) higher than in control group (46 versus 38%, respectively). WHO regards a BMI of less than 18.5 as underweight and may indicate malnutrition while a BMI greater than 25 is overweight and above 30 is considered obese depending on low BMI=Mass (kg)/ Height (m) (WHO, 2014). The BMI is used in a wide variety of contexts as a simple method to assess how much an individual’s body weight departs from what is normal or desirable. The present analysis of BMI showed a highest ratio in the control group was overweight (BMI, 25.1 to 30) while in T2DM group was 46% overweight (BMI, 25.1 to 30) and 46% obese (BMI, more than 30). The number of males was significantly (p<0.05) higher in the control group than in T2DM group (62 versus 50%, respectively). On the contrary, the number of females was significantly (p<0.05) higher in T2DM group than in the control group (50 versus 38%, respectively). The percentage of family history in the control group was significantly (p<0.01) lower than in T2DM group (24 versus 52%, respectively). There was no significant difference between the control group and T2DM group as related with smoking status. As related with hypertension, the percentage in the control group was significantly (p<0.01) lower than in T2DM group (24 versus 56%, respectively). Also, as related with retinopathy, the percentage in the control group was significantly (p<0.01) lower than in T2DM group (6 versus 42%, respectively). As related with nephropathy, the percentage was significantly (p<0.05) lower in control group than in T2DM group (2 versus 14%, respectively).
Table 1: Distribution of apparently healthy subjects and type 2 diabetes mellitus patients according to some parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control1 ( n (%) )</th>
<th>T2DM 2 ( n (%) )</th>
<th>( p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>( &lt;40 )</td>
<td>9 (18%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td></td>
<td>( 40-60 )</td>
<td>32 (64%)</td>
<td>31 (62%)</td>
</tr>
<tr>
<td></td>
<td>( &gt;60 )</td>
<td>9 (18%)</td>
<td>15 (30%)</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>( 18.5 - 25 )</td>
<td>9 (18%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td></td>
<td>( 25.1 - 30 )</td>
<td>22 (44%)</td>
<td>23 (46%)</td>
</tr>
<tr>
<td></td>
<td>( &gt;30 )</td>
<td>19 (38%)</td>
<td>23 (46%)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>31 (62%)</td>
<td>25 (50%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>19 (38%)</td>
<td>25 (50%)</td>
</tr>
<tr>
<td>Family history</td>
<td>Yes</td>
<td>12 (24%)</td>
<td>26 (52%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>38 (76%)</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes</td>
<td>8 (16%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>42 (84%)</td>
<td>45 (90%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Yes</td>
<td>10 (20%)</td>
<td>28 (56%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>40 (80%)</td>
<td>22 (44%)</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>Yes</td>
<td>3 (6%)</td>
<td>21 (42%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>47 (94%)</td>
<td>29 (58%)</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>Yes</td>
<td>1 (2%)</td>
<td>7 (14%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>49 (98%)</td>
<td>43 (86%)</td>
</tr>
</tbody>
</table>

1 apparently healthy subject; 2 type 2 diabetes mellitus; 3 p<0.05; 4 no significant; 5 p<0.01.

Our result show that only the wild-type homozygous CC genotype was found (100%) in all the samples studied respect to IL-4 -590C>T (4782, NG_023252.1), whereas CT and TT genotypes were not found. The distribution of genotype and allele frequency at -174C>G (4880, NG_011640.1) site of IL-6 gene is presented in (Table 2). As related with CC genotype frequency, there was no significant difference between the control group and T2DM group. The CG genotype was found in 32% of T2DM patients versus 22% in the control group and the difference was significant (p<0.05). In contrast, the GG genotype was found in 64% of T2DM patients which was significantly (p<0.05) lower than in the control group (74%). Both C and G allele frequencies were 0.15 and 0.85 in the control group and 0.20 and 0.80 in T2DM patients group, respectively.

Table 2: The genotype and allele frequencies of g.4880 C>G (-174C>G) SNP (rs1800795) in IL-6 gene (chromosome 7).

<table>
<thead>
<tr>
<th>Genotype, n (%)</th>
<th>Control1</th>
<th>T2DM2</th>
<th>( X2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>(4%)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CG</td>
<td>(22%)</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>GG</td>
<td>(74%)</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

Allele frequency, n (%)

<table>
<thead>
<tr>
<th>Allele</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.15</td>
</tr>
<tr>
<td>G</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Appareently healthy subjects; 2 type 2 diabetes mellitus ; 3 no significant 4significant at 0.05.

DISCUSSION

Interleukin 4 was discovered as a low molecular weight polypeptide (T cell-derived) of 153 amino acids, encoded by the IL-4 gene on chromosome 5q23.31. It is secreted by helper T cells (CD4) type 2 (Th2) lymphocytes, and natural killer (NK) T cells, and by cells of the innate immune system, including mast cells, basophils, and eosinophils (Voehringer et al., 2006). Interleukin 4 regulates proliferation, apoptosis, gene expression, and differentiation in many hematopoietic cells. In particular, it directs the Ig class switch to IgG1 and IgE and downregulates the production of T helper type 1 (Th1) cells (Ueta et al., 2007). IL-4 is suggested to protect human islets from cytotoxic damage induced by proinflammatory and Th1 cytokines. On the other hand, Arababadi et al. (2009) indicated that the Th2 cytokines, like IL-4, suppress the cellular immunity and may not play a clinical role in the etiology and genetics of diabetes. Mohammad (2010) found that the secretion of IL-4 can be affected by its polymorphism in – 590 site. Several studies reported that cytokine imbalance is involved in pathogenesis of T2DM (Nosratabadi et al., 2009). Researchers believe that T2DM is associated with immune system and in turn is related to the alteration of Th2 to Th1 immune response patterns (Skopinski et al., 2005). Gene polymorphisms play key roles in the regulation of cytokines expression for example substitution of C by T in -590 position of IL-4 gene causes reduced cytokine expression while TT form up-regulates the production of this cytokine (Kamali-Sarvestani et al., 2005). In this study, the gene polymorphism of IL-4 was analyzed in T2DM patients and the gene region that influences the expression of IL-4 gene was selected for the comparison between T2DM patients and apparently healthy controls. Only the wild-type homozygous CC genotype was found (100%) in all the samples studied, whereas CT and TT genotypes were not found. This result may be attributed to the small sample size or rare incidence of this SNP in the Iraqi population that requires a greater sample size for detecting the other genotypes. Ming-Yuh et al. (2009) found no relation between several promoter polymorphisms including – 590C>T SNP and T2DM patients without nephropathy. Maier et al. (2005) found no association of IL-4 polymorphisms with type 1 diabetes mellitus (T1DM) in white British population.
Several studies were done to study the relationship of IL-4 polymorphism with the onset of T2DM, while few studies were done on the IL-4 polymorphism relationship with the development of T2DM complications (Arababadi et al., 2009; Mohammed, 2010).

On the other hand, Bid et al. (2008) reported a significant relationship between IL-4 polymorphisms and T2DM in the north Indian population. They indicated that the genetic polymorphism of IL-4 may influence the initiation and progression of T2DM. In contrast, other researchers have found a negative relationship between –590C>T SNP (IL-4) and T2DM in Iranian patients (Arababadi et al., 2009). They attributed this result to the ethnic difference or due to technical errors in the sampling and sorting of cases. Moreover, Ho et al. (2010) reported a significant increase in the frequency of CT genotype of -590C>T SNP (IL-4) among Taiwanese cases with T2DM. El-Shabrawi et al. (2011) found a significant difference between Egyptian T2DM patients and the control as related with genotypes and alleles of –590C>T SNP of IL-4 gene. Similar results were obtained from Iranian population (Mohammed, 2010). El-Shabrawi et al. (2011) noted that the presence of the T allele of –590C>T SNP of IL-4 gene is associated with the risk of diabetic nephropathy compared to C allele and this result is contrary to the results of the present study. The discrepancy among the results mentioned above may be attributed to the difference in race and genetics from one population to another in addition to the small number of patients studied and the probable small effect of the mutation.

The single nucleotide polymorphism – 174C>G (rs1800795) is one of IL-6 functional polymorphisms in the promoter region, influences IL-6 gene transcription. It is located 174 nucleotides upstream of the major transcription initiation site of the IL-6 gene and the presence of either cytosine or guanine at this position gives rise to two different IL-6 alleles leading to three possible genotypes: CC, GC and GG. The genotype frequencies of polymorphisms are known to vary according to race or ethnicity (Zavaleta-Muniz et al., 2013). A study done on five ethnic groups from the European part of Russia and populations from twenty-four countries of Africa and Eurasia reported that the frequency of the -174G allele varied from 45-100% (Borinskaya et al., 2013). Vozarova et al. (2003) reported that GC genotype and G allele of –174C>G of IL-6 gene were associated with an increased risk of T2DM in native Americans and Caucasians. Vozarova et al. (2003) found in native Americans and Spanish Caucasians that G allele of –174C>G SNP of IL-6 gene to be associated with higher risk of T2DM. But this SNP was not linked with diabetes in Finnish Diabetes Prevention study (Kubaszek et al., 2003). In another study, non-diabetic subjects showed an association of CC genotype of –174C>G SNP of IL-6 gene with higher insulin sensitivity (Fernandez-Real et al., 2000; Kubaszek et al., 2003). In Finnish population, genotyping yielded a report of 26, 44, and 26% for GG, GC and CC genotypes, respectively (Kubashevk et al., 2003). European patients showed 37, 53 and 10% for GG, GC and CC genotypes, respectively (Brull et al., 2001). Illig et al. (2004) showed that GG genotype of –174C>G SNP of IL-6 gene to be associated with T2DM (p<0.01, OR=1.51). Stephens et al. (2004) stated that GG genotype of –174C>G SNP of IL-6 gene was associated with an increase in the risk of T2DM in British subjects compared to other genotypes. Also, they stated that CC and GC genotypes are protective against T2DM. Herbert et al. (2005) stated that GG genotype is protective for T2DM in American population. Qi et al. (2006) stated that GC genotype of –174C>G was not associated with risk of T2DM in American population. Helaly et al. (2013) observed a significant increase in CC genotype of –174C>G SNP of IL-6 gene in diabetic cases especially in cases with high insulin resistance and that CC genotype was associated with T2DM among Egyptian population. Kubaszek et al. (2003) established that CC genotype of –174C>G SNP of IL-6 gene is risky for T2DM than other genotypes and that GC genotype was found to be associated with insulin resistance in Finnish subjects. Gan et al. (2013) found that the ethnic variation in -174C>G polymorphisms of IL-6 gene in Malaysian population showed 4, 19 and 0% C allele frequencies in Malays, Indians and Chinese ethnic groups, respectively. Previous studies showed GC genotype frequencies of 0.2% for eastern Asians, 0% for Japanese, 0.6% for Koreans and 0.2% for southern Chinese (Juang-Hwa and Kim, 2012). Borinskaya et al. (2013) found that the frequency of -174G allele was 77% for southern regions of Italy and 58-59% for Germany. Saxena et al. (2014) found that CC genotype of -174C>G SNP of IL-6 gene was rare and GG genotype was most prevalent in Indian population and GC genotype was present in 14.48% controls and 21.6% T2DM cases. In humans, the IL-6 gene is located on short arm of chromosome 7 (7p21). It encodes for the proinflammatory cytokine, IL-6, secreted mainly by neutrophils, granulocytes and macrophage. The IL-6 is the main stimulant of the acute phase response; it stimulates T lymphocytes, differentiation of B lymphocytes and the production of C reactive protein (Groblewska et al., 2012; Erzen et al., 2007; Capruso et al., 2010). Various polymorphisms in the promoter region of the IL-6 gene was reported to influence IL-6 transcription (Laresgoiti-Servitje et al., 2010; Laresgoiti-Servitje and Gomez-Lopez, 2012; Arosio et al., 2004). Interleukin-6 is secreted by immune cells, adipose tissue and muscles and is able to accelerate or inhibit the inflammatory processes (Mohamed-Ali et al., 1997; Fried et al., 1998). The direct effect of IL-6 may be on glucose homeostasis and metabolism or it might act indirectly by action on adipocytes, pancreatic β-cells (Kristiansen and Mandrup-Poulsen, 2005). In humans, the gene for IL-6 maps to chromosome 7p15-p21. IL-6 mRNA expression and insulin resistance were found to have a significant correlation (Cardellini et al., 2005) and increased plasma IL-6 levels with higher risk of T2DM (Spranger et al., 2003; Qi et al., 2006), making it an
appealing candidate gene. One of the common polymorphisms in the IL-6 gene promoter (C-174G) was found to regulate transcription in response to inflammatory stimuli such as lipopolysaccharides or IL-1 (Fishman et al., 1998; Kubaszek et al., 2003). IL-6 promoter SNPs were considered as risk factors for T2DM development, as reported by other groups (Vozarova et al., 2003; Illig et al., 2004).

REFERENCES


