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Physiology and Anatomy

Elixir Physio. & Anatomy 88 (2015) 36372-36376

ISSN: 2229-712X

The Association of *Interleukin-6* Gene Polymorphism at -174G>C SNP in Iraqi Patients with type 2 Diabetes Mellitus

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ARTICLE INFO

Article history:
Received: 6 October 2015;
Received in revised form:
06 November 2015;
Accepted: 12 November 2015;

Keywords
T2DM,
IL-6 Gene,
PCR-RFLP.

ABSTRACT

Type 2 diabetes mellitus (T2DM) is multifactorial diseases caused by complex between genetic, lifestyle and environment risk factor; T2DM has also been recognized as an immune mediated disease leading to impaired insulin signaling and selective destruction of insulin producing beta cells in which cytokines play an important role.This study aimed to investigate the association of IL-6 gene polymorphism (-174G>C) 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) was carried out to detect polymorphism at the -174 position of IL-6 gene and determined the genotype for Iraqi population. The results revealed that 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. The results of the present study indicate that heterozygous GC genotype was associated with the incidence of T2DM.

Introduction

Type 2 diabetes mellitus is a common health problem which has reached epidemic proportions due to the rapidly increasing rates of this disease worldwide (IDF, 2013). Globally, the number of people with diabetes is estimated to be 346 million. This number is projected to grow to 552 million by the year 2030 (Whiting et al., 2011). Type 2 diabetes (T2DM) accounts for 90% of all diabetes cases. Therefore, the continuously growing epidemic reflects an increased incidence and prevalence of T2DM. 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). Several studies reported that cytokine imbalance is involved in pathogenesis of T2DM (Nosratabadi et al., 2009). IL-6, a major proinflammation cytokine expressed in several tissues such as, adipose tissue, muscles, immune cells and hypothalamus are associated with regulatory of energy balance in human (Wernstedt et al., 2004). Adipose tissue IL-6 expression accounts for 30% of systemic IL-6, and circulating IL-6 concentrations are positively correlated with obesity, impaired glucose tolerance, and insulin resistance (Bastard et al. 2002). Plasma IL-6 concentrations predict the development of T2DM (Vozarova et al. 2001). IL-6 gene located on chromosome 7p15-p21 in human this gene consists of five exons and four introns (Yasukawa et al. 1987). A high rate of plasma clearance of IL-6 suggested that concentration is regulated at the levels of transcription and translation (Mendoza-Carrera *et al.*, 2010). Gene polymorphisms play key roles in the regulation of cytokines expression (Kamali-Sarvestani *et al.*, 2005). Genetic polymorphisms in IL-6 gene can affect circulating levels of interleukin-6 (Stryjecki and Mutch, 2011) and have been associated with a modified risk of T2DM (Huth et al. 2006). The promoter region -174 of IL-6 gene has demonstrated to have a biological function. Fishman *et al.* (1998) showed that the G allele is associated with higher spontaneous IL-6 gene transcription activity than the C allele. Studies on single nucleotide polymorphisms (SNPs) in the promoter region of IL-6 gene in different populations worldwide suggested its possible role in T2DM susceptibility and there is evidence that the increase in IL-6 levels is associated not only with T2DM but also with impaired glucose tolerance and insulin resistance. IL-6 also has important effects on glucose (Kristiansen and Mandrup-Poulsen, 2005; Libby, 2002) and lipid metabolism (Nonogaki et al. 1995; Gerrit et al. 2003). For this reason, this study aimed to investigate the association of IL-6 gene polymorphism (-174G>C) with type 2 diabetes mellitus incidence in Iraqi population.

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 patients samples

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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 usind nanodrop. Also, the extracted DNA samples were electrophoresed on 1% agarose gel for checking.

Genotyping

Genotyping for IL-6 gene SNP (-174G>C) was carried out by using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) method. A 198 bp fragment of IL-6 gene was amplified by using the following primers: Forward: 5'-TGACTTCA GCTTTACTCTTTG-3' and Reverse: 5'-CTGATTGGAAACCTTATTA AG-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: *Taq*DNA 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: 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). After that PCR product of IL-6 gene (198bp) fragment digested with 2 unit of restriction enzyme *NlaIII* at 37°C for 3 hours (reaction volume 20 µl). Digested products were separated on (3%) agarose gel for 2:30 hours. Then, agarose gel is visualized under UV light using ultraviolet transilluminater. The 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 2) (Due to limitation of agarose gel in detection of fragments that are smaller than 50 bp, 30 bp fragment was invisible).

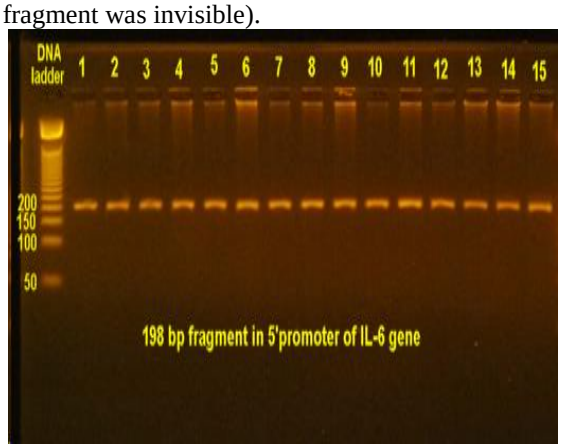

Figure 1. PCR product (198 bp) of targeted fragment flanking the -174C>G SNP (g.4880C>G, GenBank: *NG_011640.1*) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours. DNA ladder= 50 bp.

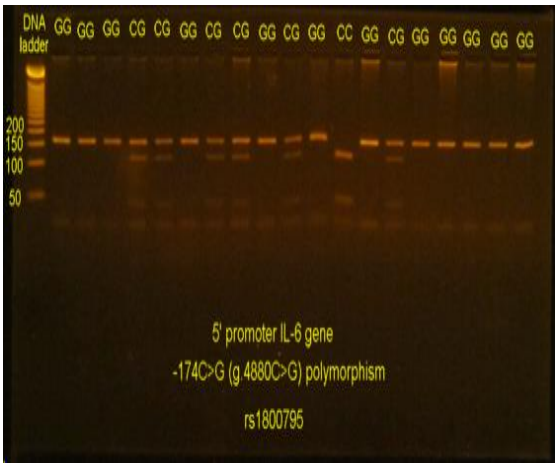

Figure 2. PCR product (198 bp fragment) of 5' promoter of IL-6 gene digested with NlaIII restriction enzyme and electrophoresed on 3% agrose. The genotypes are : CC (31, 45 and 122 bp), CG (31, 45, 122 and 167 bp) and GG(31 and 167 bp); DNA ladder: 50 bp, visualized under UV light.

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 (20 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).

Table1. Distribution of apparently healthy subjects and type 2 diabetes mellitus patients according to some parameters.

Parameters	*Control*[1] *n (%)*	*T2DM* [2] *n (%)*	*p-value*
Age (year)			
< 40	9 (18%)	4 (8%)	0.0355 *[3]
40-60	32 (64%)	31 (62%)	0.472 NS[4]
> 60	9 (18%)	15 (30%)	0.0391 *
Body mass index (BMI)			
18.5 - 25	9 (18%)	4 (8%)	0.0355 *
25.1 - 30	22 (44%)	23 (46%)	0.472 NS
> 30	19 (38%)	23 (46%)	0.052 *
Sex			
Male	31 (62%)	25 (50%)	0.0391 *
Female	19 (38%)	25 (50%)	0.0391 *
Family history			
Yes	12 (24%)	26 (52%)	0.0041 **[5]
No	38 (76%)	24 (48%)	0.0125 **
Smoking			
Yes	8 (16%)	5 (10%)	0.274 NS
No	42 (84%)	45 (90%)	0.274 NS
Hypertension			
Yes	10 (20%)	28 (56%)	0.0015 **
No	40 (80%)	22 (44%)	0.0001 **
Retinopathy			
Yes	3 (6%)	21 (42%)	0.0001 **
No	47 (94%)	29 (58%)	0.0001 **
Nephropathy			
Yes	1 (2%)	7 (14%)	0.0355 *
No	49 (98%)	43 (86%)	0.0355 *

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

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>C (- 174C>G) SNP (rs1800795) in *IL-6* gene (chromosome 7).

	Control[1]	**T2DM**[2]	X^2
Genotype frequency, n (%)			
CC	2 (4%)	2 (4%)	0.00 NS[3]
CG	11 (22%)	16 (32%)	4.29 *[4]
GG	37 (74%)	32 (64%)	4.29 *
Allele frequency, n (%)			
C	0.15	0.20	1.024 NS
G	0.85	0.80	1.024 NS

[1] apparently healthy subjects; [2] type 2 diabetes mellitus ; [3] no significant [4] significant at *0.05*.

Discussion

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 (Kubashek 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 ; Capurso 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 ; Terry et al., 2000 ; 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).

Acknowledgment

- Genetic Engineering and Biotechnology Institute for postgraduate / Baghdad University.
- the staff of the National Center for Diabetes / AL-Mustansiriya University for Cooperation
- Staff members of AL-Nahrain forensic DNA center for Training and Research/ AL-Nahrain University

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