Role of the Allelic Polymorphisms of a Pleiotropic Cytokine (IL-6) as Genetic Factors in Iraqi Type 2 Diabetes Mellitus Patients

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Abstract- Type 2 diabetes mellitus may happen under different causes, which may be detected by interactions between environment and genes. In this study we have explored the possible role of two single nucleotide polymorphisms in the 174 and 597 nucleotide positions at the promoter region of IL-6 gene as predictor for type 2 diabetes mellitus. The study population was included 120 Iraqi patients with type 2 diabetes mellitus. The distribution frequency of patients according to gender revealed that the higher rates of patients were in male than female with a significant difference ($P<0.05$) between them. The estimated prevalence of type 2 diabetes mellitus increased in the third age group (47-56 years) but without a significant difference in comparison with the other groups. The results of serum sugar, creatinine, urea, cholesterol, triglyceride, and LDL estimation were showed significantly ($p<0.05$) higher serum levels when compared to their ranges in the controls, while the serum HDL levels in patients were lower than in controls but without a significant difference. Finally, the present study had found that the genotype distribution among Iraqi patients with type 2 diabetes mellitus was either GG or GC in the region of promoter of IL-6 gene at the nucleotide in the 174 position (-174G/C), with a significant difference between the allelic distribution of SNP in the regulatory promoter region of IL-6 gene of diabetic patients (GG: n= 101, 84.17%; GC: n=19, 15.83%; CC: n=0) and controls (GG: n= 49, 98%; GC: n=1, 2%; CC: n=0). The -597G/A single nucleotide polymorphism of IL-6 gene had no significant association with type 2 diabetes mellitus among Iraqi study population. That means the SNP in the regulatory promoter region of IL-6 gene (-174G/C) may plays role in the prediction and progression of type 2 diabetes mellitus among Iraqi study population.

INTRODUCTION

Diabetes mellitus are conditions which characterized by high levels of sugar (glucose) in the blood. Type 2 diabetes is a progressive disease where caused by two conditions: either the islet β-cell of pancreas does not produce sufficient amount of the insulin hormone or the body cells have become resistance to this hormone action (Huang *et al.*, 2006).

Type 2 diabetes represents a hug health problem, affecting high percentage of people worldwide among countries (developed and developing) and caused high rates of morbidity and mortality (Wu *et al.*, 2014).

There is a strong association between type 2 diabetes and many risk factors, such as, hypertension, obesity, and dyslipidaemia (referred to as metabolic syndrome) (Taskinen,2003; Bassuk& Manson, 2008; Chan *et al.*, 2009).

Lifestyle, many environmental factor, and genetic components have an essential role in type 2 diabetes pathogenesis (Das & Elbein, 2006).

Diabetes is relating with developing long-term complications, like ischemic heart disease, vascular disease, retinopathy, nephropathy neuropathies, and chronic kidney disease (Betônico *et al.*, 2016; Serhiyenko & Serhiyenko, 2018).

Many studies recorded that inflammation and its responses play a major role in development of type 2 diabetes (Wellen & Hotamisligil, 2005; Zozulinska and Wierusz-Wysocka, 2006; Hotamisligil and Erbay, 2008; Donath and Shoelson , 2011; Goldfine and Shoelson, 2017).

The inflammatory cytokines (especially Interleukin-6 (IL-6) and tumor necrosis factor (TNF)) have been found in the blood of type 2 diabetes patients and related to its compli-cations. The cytokines caused the secretion of C-reactive protein (CRP) as acute-phase protein (the most commonly used inflammation marker) from the hepatocytes to begin the immune response. In the cases of type 2 diabetes patients, inflammation markers and its increasing concentrations are strongly associated with this disease incidence (IL-6, plasminogen activator inhibitor 1, CRP, and white blood cells) (Ridker *et al.*, 1997; Kumar and Clark, 2009; Emerging Risk Factors Collaboration *et al.*, 2010).

IL-6 has been described as a good prognostic marker in type 2 diabetes patients and it implicated in the development of inflammation, β-cell dysfunction and the resistance to insulin (Akbari and Hassan-Zadeh, 2018).

Gene of IL-6 is located on 7p21chromosome, and involved 5 axons and 4 introns. IL-6 is produced by monocytes, adipocytes, fibroblasts, and mesangial cells of kidney (Abbas and Lichtman, 2003).

IL-6 secretion converts B-lymphocytes to antibody-producing cells and plays very important role in the production of CRP (II'yasova *et al.*, 2008; Yvan-Charvet *et al.*, 2009). IL-6 cause proliferation of kidney mesangial cells in stages of glomerulopathy (Horii *et al.*, 1993).

The biological functions of IL-6 is numerous (pleiotropic) and varying between its contribution in host defense (hematopoietic and immune activities) and its ability to induce the acute phase response (which mean it has anti-inflammatory and pro-inflammatory actions) (Schett, 2018).

The production of inflammatory cytokines is under control of genes by many of single nucleotide polymorphisms (SNPs) within these genes. These (SNPs) may be found in the exons, introns (non-coding regions) or in the region of gene promoter (Bidwell *et al.*, 1999).

IL-6 gene has four genetic variants (polymorphisms) in the flanking or promoter region (634 C/G, -174 G/C, -572 G/C, and -597 G/A) (Rao *et al.*, 2007). Two of these polymorphisms (-174 G /C, and -597 G/A) had a strong effect on plasma level of IL-6 because they controlled the level of IL-6 gene transcription (Brull *et al.*, 2001).

Many studies had explored the associations between the genetic variants of IL-6 gene and arthritis (Fishman *et al.*, 1998), cardiovascular disease (Rao *et al.*, 2005), and diabetic renal disease (Kitamura *et al.*, 2002)

The aim of this study was to assess the diagnostic prevalence and prognostic significance of two genetic variants (-174 G/C, and -597 G/A)) in the flanking or promoter region of IL-6 gene in Iraqi patients with type 2 diabetes mellitus.

MATERIALS AND METHODS

This study was carried out in Alsader Teaching Hospital in Al-Najaf province and laboratory of molecular biology in the Department of Biology in the Faculty of

Science-University of Kufa, during the period from June 2017 through September 2018.

2. Study and Control Subjects

a) Study group: this group consists from 120 patients. They were admitted to Alsader Specialized Center of diabetes. All the patients selected for the present study were having type 2 diabetes mellitus (Blood samples were obtained as part of the routine clinical protocol).

b) Control group: It consists from 50 healthy individuals; all were without any inflammatory disorders or clinical manifestation of any disease.

3. Collection of Blood Samples

Four ml of venous blood was collected from patients and control.

a) Two ml was allowed to clot at room temperature then centrifuged at 3000 rpm for 5 minutes the serum was used freshly for the biochemical tests of fasting sugar, creatinine, urea, cholesterol, triglyceride, HDL and LDL.

b) Two ml was collected in EDTA tubes and store at ˉ20°C until used for PCR test.

4. Biochemical Tests

Fasting sugar, creatinine, urea, cholesterol, triglyceride, HDL and LDL tests were done by using Kits, which are products of BIOLABO REAGENT (Maizy, France). LDL level was measured by the formula (Friedewald et al., 1972): LDL = TC - HDL - TG/5.0 (mg/dL).

5. DNA extraction and Polymerase Chain Reaction (PCR):

a) Genomic DNA extraction: DNA extraction was done by using protocol from Genomic DNA Mini Kit (Geneaid Biotech. Ltd., Taiwan Company, Cat. No. GB100, LOT. No. TJ21207), which designed for purifying genomic DNA from frozen samples of blood.

b) Polymerase Chain Reaction (PCR): PCR amplification was achieved in 20 µl tube of PreMix PCR Reaction Mixture (Bioneer Corporation, USA) which containing 5 µl of extracted DNA (template), 2 µl reaction buffer, 1 unit DNA polymerase, 2 µl dNTPs, 2 µl loading-dye and stabilizer, and 2µl of each primer (that variant for each specific SNP) (2 µl forward and 2 µl reverse). Distilled water was added to the final volume of 20 µl.

Amplification was performed in a thermal cycler (Multigene OptiMax Labnet International/USA) programmed for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 66°C for 45 seconds, and extension at 72°C for 45 seconds, preceded by an initial denaturation of 10 min at 95°C. Final extension was for 7 min at 72°C.

1. SNP (−174 G/C) in the promoter region of gene IL-6: A sequence of SNP in the 174 nucleotide at the promoter or flanking region of IL-6 gene (−174 G/C) was amplified by using polymerase chain reaction amplification of specific alleles which called amplification refractory mutation system (ARMS-PCR). ARMS-PCR was used to amplify the both alleles (G and C) of the promoter region in IL-6 gene in two PCR tubes for a single reaction; one for the wild type (WT) or normal sequence (N) and the other for the mutant one(M). This facilitate the determination of IL-6 genotyping (homo/ heterozygosity)

The sequences of primers (synthesized by AccuOligo® Bioneer Corporation .USA) were published previously (Chua *et al.*, 2009). The primers pairs that used to amplify the sequence of SNP in the 174 nucleotide at the promoter or flanking region of IL-6 gene are:

Forward Primer (G): 5'-GCACTTTTCCCCCTAGTTGTGTCTTACG -3'

Forward Primer (C): 5'-ATGACGACCTAAGCTTTACTTTTCCCCCTAGTTGTGT

CTTGAC-3' (the PCR product for the primers are 121 bp & 136 bp respectively)
Reverse Primer: 5'-ATAAATCTTTGTTG GAGGGTGAGG-3'
2. SNP (−597 G/A) in the promoter region: A sequence of SNP in the 597 nucleotide at the flanking region of IL-6 gene (−597 G/A) was amplified by using primer-pairs synthesized by AccuOligo® Bioneer Corporation/ USA. The sequences of primers were published previously (Hongmei *et al.*, 2016). The primers-pairs sequences are:
Forward Primer: 5'- CTCCTCTAAGTGGG CTGAAG -3'
Reverse Primer: 5'- CAAGCCTGGGATTAT GAAGA -3'
Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has been used. The 412-bp PCR product was digested overnight with one unit of RsaI restriction enzyme (Source: *Rhodopseudomonas sphaeroides* Rf.) at 37°C (synthesized by Fermentas Corporation, USA, Cat. No. ER1124). After the digestion, we had one of these results for each sample:

1) Two 412-bp fragments for allele G.
2) 236 and 176-bp for allele A (Mutant homozygous AA).
3) 236 and 176-bp and 412-bp for heterozygous genotype (GA).

Finally, the gel electrophoresis method was done according to Sambrook and Russell (2001), and 5 µl of each samples was loaded onto 2% agarose gel.

6. Statistical Analysis: Statistical analyses of the results were achieved with the help of Statistical Package for Social Science software (SPSS, version 17) and Microsoft office Excel 2010. Data are reported as (range or percentage) mean± standard deviation (SD). The test of Chi square has been adopted for the statistical analysis of association among the study variables studied (P value was considered significant at level less than 0.05). Data are reported as percentages and mean± standard deviation (SD).

RESULTS

The study population was included 120 Iraqi patients with type 2 diabetes mellitus. The assessment of study population according to gender revealed that the higher rates of patients were in male (62%) than female (38%). There was a significant difference (P< 0.05) between them.

Assessment of age presentation of patients revealed that 19(15.83%) in age group 27-36 years, 28(23.33%) in age group 37-46 years, 30(25%) in age group 47-56 years, 23(19.17%) in age group 57-66 years, and 20 (16.67%) in age group 67-76 years. Their ages ranged from 27 to 76 years, with a mean age of 46.5±9.24 years. The estimated incidence of type 2 diabetes increased in the third age group but without a significant difference in comparison with the other groups.

The results of serum fasting sugar, creatinine, urea, cholesterol, triglyceride, HDL and LDL (Table 1) were showed (except HDL) a significantly (p<0.05) higher serum Fasting sugar, creatinine, urea, cholesterol, triglyceride, and LDL levels when compared to the normal ranges in the blood of patients and controls, while the serum HDL in patients was lower than in controls but without a significant difference.

Table 1. Biochemical parameters (Fasting sugar, creatinine, urea, cholesterol, triglyceride, HDL, and LDL tests of diabetic patients and controls.

Parameter	Diabetic patients	Controls
Fasting sugar (mg/dl)	193±8.4*	98±4.1*
Creatinine (mg/dl)	5.751±1.7*	1.1±1.02
Urea (mg/dl)	83.26 ± 7.3 *	39.25±2.6

Cholesterol (mg/dl)	193.65 ± 4.26*	145.08±1.17
Triglyceride (mg/dl)	209.63 ± 4.2*	113.72±1.06
HDL (mg/dl)	38.66 ± 3.7	40.25±6.3
LDL (mg/dl)	192.45 ± 9.2 *	102.13±5.2

*p< 0.05 significant (values were expressed as mean± SD).

The results of molecular experiences showed that:

1. SNP (−174 G/C) in the promoter region of IL-6 gene:

a) Healthy controls: 49(98%) healthy persons had contained an amplified product in the PCR tubes for the normal sequence, and one (2%) has an amplified product in both PCR tubes; for the mutant and normal sequence (Table 2 & Figure 1).

b) Diabetic patients: The genotypic distribution among patients (120) was: 101 (84.17%) had an amplified product in the in the PCR tubes for the normal sequence which indicating to the normal homozygous genotype, 19 (15.83%) contain an amplified product in both normal and mutant PCR tubes; assigning those individual to heterozygous genotype. While there was no patient had amplified product in the mutant PCR tubes indicating to the homozygous genotype (Table 2 & Figure 1), which mean that Iraqi patients with type 2 diabetes mellitus has either GG or GC genotype in the region of promoter of IL-6 gene (with a significant difference with control persons ($p<0.05$)).

Table 2. Genotypic distribution of the polymorphic variants in the promoter region of IL-6 gene (-174 G/C and-597 G/A).

Polymorphic Variants	Subjects	PCR results			Total
		N	M	N&M	
-174 G/C	Healthy controls	49 (98%)	0	1(2%)	50 (29.41%)
	Diabetic patients	101 (84.17%)	0	19(15.83%)*	120(70.59%)
	Total	**150(88.2%)**	**0**	**20(11.7%)**	**170 (100%)**
-597 G/A	Healthy controls	50 (100%)	0	0	50 (29.41%)
	Diabetic patients	120 (100%)	0	0	120(70.59%)
	Total	**170 (100%)**	**0**	**0**	**170 (100%)**

Keys: N= Normal homozygous individuals, M= Mutant homozygous individuals, N&M= Heterozygous individuals (Normal and Mutant alleles); values were expressed as number of participants and their percentages.

2. SNP (−597 G/A) in the promoter region of IL-6 gene:

a) Healthy controls: all the 50 healthy persons had found as homozygous wild (GG, normal alleles) (Table 2).

b) Diabetic patients: All the 120 patients had found as homozygous wild (GG, normal alleles) (there was no significant difference between patients and control persons ($p>0.05$)) (Table 2).

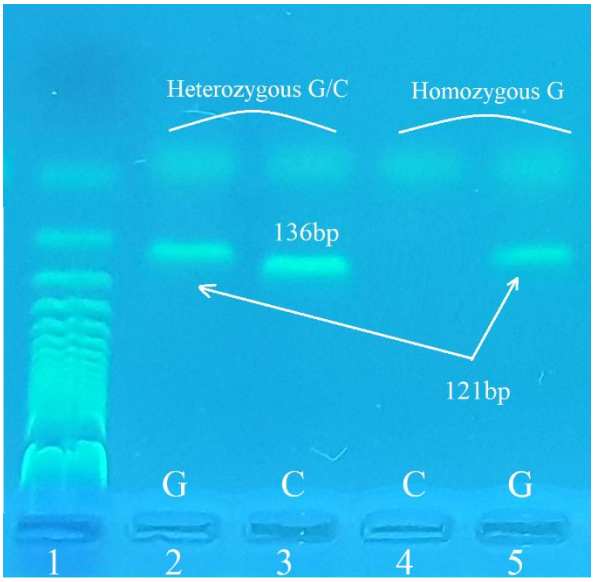

Figure 1. PCR amplified products of IL-6 promoter polymorphism (-174 G/C). Lane1: 50 bp DNA ladder. Lane 2&3: Heterozygous G/C (121 and 136 bp). Lane 4&5: Homozygous G (121 bp).

DISCUSSION

Type 2 diabetes is a complex disease that may happen under different causes, which may be detected by interactions between genetic and environmental factors. While the environmental factors, obesity and activity state, are well studied, identification of the genetic causes has been a challenge. However, recent years have seen the explosion of genetic variants in danger and protection T2D due to the technical evolution that allowed for genome-wide and next-generation sequencing studies (Prasad & Groop, 2015).

In this study we have explored the possible role of IL-6 genetic polymorphisms as predictor for type 2 diabetes and severity of pro-inflammatory responses.

The study population was included 120 Type 2 diabetes patients. The distribution frequency of the patients according to gender revealed that the higher rates of patients were in male than female with a significant difference ($P< 0.05$) between them).

Recently, male sex has been reported as a risk factor for the development of type 2 diabetes (Tracey *et al.*, 2016).

Obesity in combination with a parent's history of diabetes may be particularly dangerous in men as it is observed that these two factors increase the risk of type 2 diabetes in men (Wikner *et al.*, 2013).

Men tend to have more visceral and hepatic adipose tissue than women, whereas women have more subcutaneous fat. In contrast to visceral fat, subcutaneous fat is associated with improved insulin sensitivity and is therefore protective against type 2 diabetes (Geer& Shen, 2009).

Thus, the fact that men are diagnosed with diabetes at a lower BMI can be explained by the fact that men have more visceral fat for a particular body mass index than women and therefore a relatively higher risk of developing type 2 diabetes than body mass index (Logue *et al.*, 2011 ; Nordström *et al.*, 2016).

Central obesity has also been found to be a greater risk factor for glucose intolerance, insulin resistance, metabolic disorders than body mass index (Wiklund *et al.*, 2008 ; Chandra *et al.*, 2014).

The estimated incidence of type 2 diabetes increased in the third age group(47-56 years) but without a significant difference in comparison with the other groups.

The incidence of diabetes increases with age until about 65 years, after which it appears that, the rate of infection and spread has stopped. As a result, elderly people with diabetes may have either an accident (diagnosed after age 65) or long-term diabetes with onset in middle age or before (Selvin *et al.*, 2006).

Finally, the present study had investigated the role of two genetic polymorphisms in the promoter region of IL-6 genes in incidence

and prevalence of type 2 diabetes mellitus patients.

Several studies have documented the association of genetic polymorphisms at cytokine genes with the development and severity of various inflammatory diseases (Shu *et al.*, 2000; Hoffmanna *et al.*, 2002; Spriewald *et al.*, 2005).

In this study, the genotype distribution among Iraqi patients with type 2 diabetes mellitus was either GG or GC in the region of promoter of IL-6 gene at the 174 nucleotide (-174G/C), with a significant difference between the allelic distribution of SNP in the regulatory promoter region of IL-6 gene of diabetic patients and controls. That means the SNP in the regulatory promoter region of IL-6 gene (-174G/C) may plays role in the prediction and progression of type 2 diabetes mellitus among Iraqi study population.

The [-597G/A] gene polymorphisms had no significant association with type 2 diabetes mellitus among Iraqi study population.

Many studies had reported that the C substitution in174 position (GC and CC genotype) is strongly associated with cardiovascular disease (Aker *et al.*, 2009), higher diastolic blood pressure and left ventricular thickness (Losito *et al.*, 2003), and impaired renal function in type 2 diabetic patients (Ng *et al.*, 2008).

Feng *et al.* (2017) explained that heterogeneity among studies (by a meta-analysis may have possible reasons: the difference in the features of research populations, methods of diagnosis, methods of allele's investigation, and environment.

The levels of inflammatory markers such as C-reactive protein, salic acid, and interleukin-6 (IL-6) are independent predictors of the future development of diabetes (Pickup *et al.*, 2000).

Another studies had found that a polymorphism (a G/C change in position 174) affect plasma levels of IL-6 (Humphries *et al.*, 2001; Popko *et al.*, 2010) and in a report by Duncan and his colleagues (2003), IL-6 was strongly linked to glucose levels and was a strong indicator of diabetes in individuals at risk.

Mirza and his colleagues (2011) demonstrated that IL-6 levels were significantly and positively affected by diabetes

In a study on the possibility of whether SNP in the regulatory promoter region of IL-6 gene plays a role in T2DM in the population of northern India, Saxena et al. (2014) indicate that IL-6 gene polymorphisms play a prominent role in T2DM sensitivity.

Kubaszek *et al.* (2003) explored the role of IL-6 gene polymorphism in the development of obesity and type 2 diabetes showed that the CC genotype had more insulin resistance, high serum glucose concentration, and BMI is slightly higher than the subjects that carry G allele.

The study conducted by Möhlig *et al.* (2004) in the German population showed an increased likelihood of developing Type 2 diabetes in the CC genotype.

On the other hand, Fishman and his colleagues (1998) discovered that the C allele (in CC genotype) caused lower expression of IL-6 gene as compared with the allele G (in GG and GC genotype because that substitution had an ability to create binding site for a transcriptional repressor (of gene expression). Also, another study in the Finnish population has failed to show any direct association between the polymorphisms and susceptibility to diabetes. However, it has been shown that the CC genotype significantly increases the risk of diabetes in patients with a diagnosis of glucose intolerance (Fernández-Real *et al.*, 2000).

CONCLUSION

In this study, The genotype distribution among Iraqi patients with type 2 diabetes mellitus was either GG or GC in the promoter region of IL-6 gene at the nucleotide in the 174 position. That means The C substitution in -174 position at the promoter promoter region of IL-6 gene (-174G/C) may plays role in the prediction and progression of type 2 diabetes mellitus, and it is associated with type 2 diabetes mellitus long-term complications like impaired renal function and dyslipidemia. While The -597G/A single nucleotide polymorphism of IL-6 gene had no significant association with type 2 diabetes mellitus among Iraqi study population.

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