MOLECULAR CHARACTERIZATION OF NTERLEUKIN-4 AND INTERLEUKIN-6 IN PATIENTS WITH TYPE-2 DIABETES MELLITUS IN IRAQ

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ABSTRACT

This study aimed to investigate the association of polymorphisms of Interleukin-4 (IL-4) and Interleukin-6 (IL-6) genes to the susceptibility and severity of type-2 diabetes mellitus (T2DM) and to evaluate the serum levels of these interleukins in patients with T2DM. A number of 90 individuals from Wasit province, Iraq inclusive of 64 patients with T2DM and 26 healthy individuals with normal fasting blood sugar as a control group. Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of IL-4 and IL-6 in sera from patients with T2DM. The allele-specific polymerase chain reaction (PCR) technique was used to investigate the polymorphism of the interleukins: *IL4* -590C/T, (rs2243250) and *IL6* -174G/C, (rs1800795) genes polymorphisms. Results of this study showed that IL-4 concentrations had a non-significant difference ($p = >0.052$) between patients with T2DM (154.48 pg/ml ± 7.00) with the control group (151.49 pg/ml ± 6.21). Patients with T2DM revealed an elevated serum level of IL-6 (637.1 pg/ml ± 355.9) compared to control group (266.3 pg/ml ± 128.8). *IL4* −590 C/T displayed that C allele is a major allele in the studied group. The CC genotype has an association with T2DM and this genotype increases the probability of contracting the T2DM with an odds ratio (OR) = 1.2222. The genetic polymorphism of *IL6* −174G/C patients with T2DM showed a significant frequency (chi-square = 0.601, $p = 0.001$) of *IL-6* G/C genotype (31.25%) in comparison with controls (23%). *IL6* −174G/C displayed that G allele is a major allele in the studied group. As a conclusion, the levels of IL-4 may not be associated with T2DM, whereas, the elevated levels of IL-6 may be related with T2DM. The study implicates that functional gene polymorphisms of IL-4 and IL-6 will provide additional information related to the pathogenesis of diabetes mellitus.

Pencirian molekul Interleukin-4 dan Interleukin-6 dalam kalangan pesakit dengan diabetes mellitus jenis-2 di Iraq

ABSTRAK

Kajian ini bertujuan untuk mengkaji hubungan antara polimorfisme gen Interleukin-4 (IL-4) dan Interleukin-6 (IL-6) dengan sensitiviti dan keparahan diabetes mellitus jenis-2 (T2DM) dan menilai aras interleukin dalam serologi pesakit T2DM. Kajian ini melibatkan 90 individu dari daerah Wasit, Iraq yang melibatkan 64 pesakit T2DM dan 26 peserta sihat sebagai kumpulan kawalan. Asai imunosorben berkaitan enzim (ELISA) digunakan bagi mengukur tahap IL-4 dan IL-6 dalam serum yang diperolehi daripada pesakit T2DM. Teknik tindak balas berantai polimerase (PCR) alel spesifik digunakan bagi mengkaji polimorfisme interleukin: polimorfisme gen *IL4* -590C/T, (rs2243250) dan *IL6* -174G/C, (rs1800795). Keputusan kajian menunjukkan perbezaan aras IL-4 yang tidak signifikan (p = >0.052) apabila dibandingkan antara pesakit T2DM (154.48 pg/ml ± 7.00) dengan kumpulan kawalan (151.49 pg/m ± 6.21). Walau bagaimanapun, aras serum IL-6 dalam pesakit T2DM menunjukkan peningkatan (637.1 pg/ml ± 355.9) berbanding kawalan (266.3 pg/ml ± 128.8). *IL4* −590C/T menunjukkan alel C adalah alel utama dalam kumpulan yang dikaji. Genotip CC mempunyai perkaitan dengan T2DM dan genotip ini meningkatkan kebarangkalian mendapat T2DM dengan nisbah ganjil (OR) = 1.2222. Polimorfisme gen *IL6* −174G/C dengan T2DM menunjukkan kekerapan signifikan (khi kuasa dua = 0.601, p = 0.001) genotip *IL-6* G/C (31.25%) berbanding dengan kumpulan kawalan (23%). *IL6* −174G/C menunjukkan alel G adalah alel utama dalam kumpulan yang dikaji. Kesimpulannya, aras IL-4 berkemungkinan tidak mempunyai perkaitan dengan T2DM, manakala, peningkatan aras IL-6 menunjukkan wujud kemungkinan perkaitan dengan T2DM. Kajian ini menunjukkan implikasi bahawa polimorfisme gen berfungsi IL-4 dan IL-6 akan memberikan informasi tambahan yang berkaitan dengan patogenesis diabetes mellitus.

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DM	Diabetes Mellitus	PKC	Protein Kinase-C
T1DM	Type-1 Diabetes Mellitus	ATP	Adenosine triphosphate
T2DM	Type-2 Diabetes Mellitus	IRS	Insulin-receptor signaling
hs-CRP	High sensitivity C reactive protein	Ser/Thr	Increased Serine and threonine phosphates
Th1	T-helper type-1 cells	PTP1B	protein tyrosine phosphatase-1B
Th2	T-helper type-2 cells	PI3K	Decrease in Phosphoinositide 3-kinases
Treg	T regulatory cells	GLUT-4	glucose-transporter type-4
IL-4	Interleukin 4	SFA	Saturated fatty-acids
IL-6	Interleukin 6	Ang-II	Angiotensin-II
IL-1Ra	Interleukin-1 family	BMI	Body-mass index or
IGT	Impaired glucose tolerance	HPHC	Harmful and potentially harmful constituents
SNP	single nucleotide polymorphisms	*TCF7L2*	transcription factor 7-like 2
IDDM	Insulin –dependent diabetes mellitus	*CAPN10*	Calpain 10
β-cell	beta-cell	*PPARG*	The peroxisome proliferator-activated receptor gamma
GDM	Gestational diabetes mellitus	*IRS-1* and *IRS-2*	Insulin receptor substrate *IRS-1* and *IRS-2*
HPL	Human Placental Lactogen	PCOS	Polycystic ovary syndrome

PRLR	The Prolactin Receptor	*KCNJ11*	Potassium voltage-gated channel subfamily J member 11
TNF->	Tumor necrosis factor->	*WFS-1*	Wolframin ER Transmembrane Glycoprotein
MODY	Maturity onset diabetes of the young	AMP	Adenosine monophosphate
HIV	human immunodeficiency virus	*HNF1A*	Hepatocyte nuclear factor 1 homeobox A
AIDS	Acquired immunodeficiency syndrome	*HNF1B*	Hepatocyte nuclear factor 1B
CVD	Cardio vascular disorders	*HNF4A*	Hepatocyte nuclear factor 4 alpha
DR	Diabetic retinopathy	*HHEX*	hematopoietically expressed homeobox
DN	Diabetic nephropathy	*SLC30A8*	Solute carrier family 30 (zinc transporter), member 8
NASH	Nonalcoholic steatohepatitis	*CDKN2A/B*	Cyclin dependent kinase 4 inhibitor 2A/B
HCC	Hepatocellular carcinoma	*IGF2BP2*	insulin like growth factor 2 mRNA binding protein 2
GWAS	Genome-wide association studies	WTCCC	Wellcome Trust Case Control Consortium
MC4R	melanocortin 4 receptor	IGF2	insulin-like growth factor 2
FTO	Fat mass and obesity-associated protein	*HMGA2*	high mobility group AT-hook 2
CDKAL1	CDK5 regulatory subunit associated protein 1 like 1	*NOTCH2 ADAM30*	Notch 2-ADAM metallopeptidase domain 30
KCNQ11	potassium voltage gated channel, KQT like subfamily, member 1	HDL	High density lipoprotein
IR	insulin resistance	GCKR	Glucokinase Regulator

SUR1/SUR2A/SUR2B	Sulfonylurea receptor-1, 2A, 2B	LIF	leukemia inhibitory factor
WBCs	White blood cells	OSM	oncostatin M
(TNF)-α	tumor necrosis factor-α	CNTF	ciliary neurotrophic factor
(TGF)-β	transforming growth factor-β	CT-1	cardiotrophin-1
ROS	Reactive oxygen species	AMPK	5'AMP-activated protein kinase
TNFR-1	Tumor necrosis factor receptor 1	STAT3	Signal transducer and activator of transcription
IgG	Immunoglobulin G	GM-CSF	Granulocyte macrophage colony-stimulating factor
IgE	Immunoglobulin E	IFNγ	Interferon-γ
SOCS3	Suppressor of cytokine signaling 3	OR	odds ratio
SOCS1	Suppressor Of Cytokine Signaling 1	Tag	TA-ging snip
TLR	Toll-like receptors	IgA	Immunoglobulin of the class A
EDTA	Ethylenediamine tetraacetic acid	DPS	Diabetes Prevention Study
ELISA	enzyme-linked immunosorbent assay technique	IDT	Integrated DNA Technologies
OD	optical density	CI	Confidence intervals
PCR	polymerase chain reaction	IDT	Integrated DNA Technologies
RR	relative risk	MHC	Major histocompatibility complex.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

This chapter provides an overview of type-2 diabetes mellitus (T2DM). Type 2 diabetes mellitus has been described as a serious epidemic. Diabetes was known to depend on immunity. Cytokines are a major part of the immune system since cytokines have an important role in the ability to detect patients at high risk with other types of diseases. On the other hand, some types of these cytokines, whether pro- and/or anti-inflammatory, have the ability to induce insulin resistance and block glucose uptake by insulin. Interleukin-4 and Interleukin-6 may be among the most important types of cytokines associated with type 2 diabetes. Both may play an important role in the pathophysiology of type 2 diabetes.

In addition, the development taking place in type 2 diabetes may be influenced by disturbances in the production of cytokines genes through the formation of the single nucleotide polymorphisms (SNP). Thus, there may be a possible relationship between some cytokine genetic forms and the development of type 2 diabetes. For all of this, the levels of serum interleukin 4 and interleukin 6 in the blood of patients with type 2 diabetes and the extent of the association of polymorphism in the genes of interleukin-4 and interleukin-6 should be recognized by the susceptibility and severity of type 2 diabetes is of great importance on identifying individuals at risk of developing this disease at the molecular level.

1.2 Background of the study

Recently, diabetes is classified as an epidemic disease due to its worldwide spread in varying proportions (Nuhiar, Salman and AL-Rekaby, 2018). Diabetes mellitus (DM) is defined as a heterogeneous metabolic issue brought about by hyperglycemia derived from either insulin activity insufficiency or impaired insulin secretion or both (Punthakee, Goldenberg and Katz, 2018), which alters carbohydrate, protein, and fat metabolism. The absence of insulin secretion can be either through the inability of the β-Langerhans islet cells of the pancreas in producing insulin or due to the defects that arise in insulin uptake in the peripheral tissue (Al-Goblan, Al-Alfi, and Khan, 2014). Generally, there are two types of diabetes which include Type 1 diabetes mellitus (T1DM) commonly known as insulin-dependent diabetes mellitus, and Type 2 diabetes mellitus (T2DM) known as non-insulin-dependent diabetes mellitus (Wu et al., 2014).

At present, the prevalence of diabetes continues to rise especially amongst people between the age of 20–79 years having the disease predicted to rise to 642 million by 2040 globally (Huang et al., 2018). To date, the mortality rate of diabetes has increased to 1.5 million people making diabetes the 1st leading cause of death in the world (Ates, 2018). At a local setting, the prevalence of the disease in Iraq in 2012 was 10.9% depicting a serious rise in the number of T2DM patients (Hussain et al., 2018). Because of this, T2DM has now become an important health concern with known causes of disability and premature death, mainly through chronic complications (Chukwuani et al., 2016).

Due to the abovementioned reasons, studying factors that contribute to the late diagnosis of diabetes and whether these determinants are sex-associated have become a necessity. Thus, identifying risk factors at the earliest stage must be prioritized (Malenica et al., 2017), which include several genetic and environmental parameters that are readily known to be associated with T2DM. Furthermore, DM is an immune-dependent disease, in which, the pattern of cytokine expression is changed (Banerjee and Saxena, 2014; Cieślak et al., 2015), therefore, pro-inflammatory cytokines cause damage to pancreatic islet cells resulting in pro-inflammatory and protective cytokines imbalance (Cieślak et al., 2015).

Current studies are suggesting the possibility of inflammation being an important contributor to diabetes. This is due to the fact that inflammation can provoke changes in diabetes predominantly at the cellular level, altering the functionality of tissues and cells demonstrating reactions of the inflammation including regulators, mediators, fibrinogen hs-CRP high sensitivity C reactive protein (Malenica et al., 2017). There is

about 200 cytokines are recognized to date, differentiated as products derived from Th1 (T-helper type-1 cells), Th2 and Th17 cells as well as T regulatory cells (Treg) (Galavi et al., 2016).

Studies have shown that these cytokines and their receptors are already demonstrating their capability to detect high risks patients affected with other types of diseases. Research has indicated that certain pro and/or anti-inflammatory cytokines are capable of inducing insulin resistance and obstructing the glucose uptake of insulin (Helaly et al., 2013).

Additionally, resistin, leptin, and adiponectin which belong to the adipocytokine family, together with some chemokine/cytokines and interleukins may be involved in causing T2DM. These families are active mediators in glucose metabolism and inflammation/disease (Saxena and Modi, 2014). One family member of the cytokines is Interleukin-4 (IL-4) which is a typical cytokine of T helper type-2 (Th2) cells, could inhibit effect on the inflammation, decrease the production of pro-inflammatory cytokines and reduce the destructive enzymes through monocytes (Wei et al., 2017) and also plays a crucial role in the pathophysiology of T2DM. For instance, the gene for IL-4Ra, known to contain several polymorphisms, IL-1Ra, and IL-4, has been proposed to be involved in events causing T2DM (Banerjee and Saxena, 2014).

In addition to Interleukin-4 (IL-4), Interleukin-6 (IL-6) is a proinflammatory mediator cytokine biosynthesized by T-lymphocytes, macrophages, adipocytes and different sources, for example, endothelial cells, fibroblasts, and skeletal muscles. (Galavi et al., 2016). On the other hand, IL-6 is liable for some assignments, for

example, controlling the activation and differentiation of T-lymphocyte responses and proinflammatory responses and also plays a role in the pathogenesis of autoimmune and inflammatory diseases, in the regulation of body weight, and in lipid metabolism (Galavi et al., 2016).

To start an inflammatory response, this cytokine must tie with its receptor complex, including the Interleukin 6 receptor (IL-6R) and two molecules of glycoprotein 130 (gp130); the latter plays a co-receptor role (Galavi et al., 2016). Several studies have revealed that IL-6 increases postprandial, in parallel to glucose and insulin levels, in the interstitial fluid of subcutaneous adipose tissue that may result in impairment of glucose tolerance (IGT), indicating a potential role of this cytokine in its etiology (Ashif et al., 2017).

In this study, to investigate the association between T2DM and interleukins, the concentrations of interleukin-4 and interleukin-6 from sera of patients with T2DM were determined using ELISA assay. Also during this study, the target gene for *IL4* −590C/T Single nucleotide polymorphisms (SNP) (rs2243250) and *IL6* −174G/C Single nucleotide polymorphisms (SNP) (rs1800795) was investigated using the allele-specific polymerase chain reaction (PCR) technique. Furthermore, the association of polymorphisms of interleukin-4 and interleukin-6 genes to the susceptibility and severity of type 2 diabetes mellitus was investigated by testing the polymorphism of IL-4 and IL-6 using allele-specific polymerase chain reaction (PCR). Assessment of the correlation between IL-4 and IL-6 concentrations and genetic analysis has been done. The IL-4 and IL-6 concentrations in this study had related the association between

T2DM and these interleukins. Depth association has been displayed by polymorphism analysis.

1.3 Problem statement

One of the most widely recognized maladies on the planet is type-2 diabetes mellitus. More than 415 million people worldwide suffer from complications of the disease, making it one of the most important health problems in the world (Al-janaby, Al-ani and Rasheed, 2018). At the local level, an increase in the prevalence of this disease has been observed, which has reached epidemic proportions in Iraq. According to a study conducted in 2007, it is estimated that the number of infected with this disease is about two million people or 7.43% of the total Iraqi population (Ali et al., 2018). In a survey of adults in Basra, southern Iraq, one out of five people were diagnosed with diabetes (Mansour and Douri, 2015).

The increased acute immune response and pro-inflammatory cytokines were detected in diabetics in 1997 (Shih et al., 2013). Since then, emerging evidence has shown that T2DM is a chronic inflammatory disease in which various stimuli, such as genetic or fatal metabolic pre-programming, over-nutrition or increased age, can increase levels of cytokines expressed (Banerjee and Saxena, 2014).

Selected beta-cells that produce insulin and cytokines are destroyed and insulin signaling damaged by T2DM causing T2DM to be categorized as an immune-mediated disease (Banerjee and Saxena, 2012). Some of the pro and/or anti-inflammatory cytokines have been observed that can interfere with insulin-sensitive glucose uptake

and stimulate insulin resistance (Saxena and Modi, 2014). Individuals who progress to type 2 diabetes display features of low-grade inflammation years in advance of disease onset. This low-grade inflammation has been proposed to be involved in the pathogenetic processes causing type 2 diabetes. IL-6 has in addition to its immunoregulatory actions been proposed to affect glucose homeostasis and metabolism directly and indirectly by action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic β-cells, and neuroendocrine cells (Kristiansen and Mandrup-Poulsen, 2005).

Studies have shown that Th1 and Th2 cells had a key functional role in regulating the inflammatory process, although they are activated later than macrophages in inflammation (Martinez et al., 2008; Lumeng, Bodzin and Saltiel, 2007). To control antibody responses, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Kahn, Hull and Utzschneider, 2006).

Macrophages are the most common inflammatory cells in adipose tissue in obese people, ranging from 5–10% in lean people to up to 50% in obese people (Weisberg et al., 2003; Boutens and Stienstra, 2016). Adipose tissue macrophages are divided into two groups based on their cytokine synthesis and activation conditions: proinflammatory M1 (classically activated macrophage) and anti-inflammatory M2 (alternatively activated macrophage). TNF-, IL-6 and IL-1 are proinflammatory cytokines released by M1 macrophages, which contribute to local and systemic inflammation (Lumeng, Bodzin and Saltiel, 2007).

Th1 cells could secret IFN-γ to promote M1 polarization and enhance its pro-inflammatory functions by inducing the release of IL-1, IL-6, and TNF-α. In contrast, Th2 cells could produce anti-inflammatory IL-4 and IL-13 to skew the differentiation of macrophage towards M2. Therefore, Th1 and Th2 responses, which are closely related to M1/M2 polarization, may also have a critical role in obesity and diabetes (Xia, Rao and Zhong, 2017).

The risk of developing T2DM elevates when the production of cytokine genes is disturbed with the formation of the single nucleotide polymorphisms (SNP). Studies carried out recently concluded that there is a possibility polymorphism recent studies suggested that there may be a possible relationship between some cytokine gene polymorphisms and the development of type 2 diabetes (Ates, 2018). The production of many cytokines is under genetic control and polymorphisms have been identified within a large number of these genes (Banerjee and Saxena, 2014).

Therefore, this study aims to investigate the possible relationship between some cytokines (interleukin-4 and interleukin-6) and type 2 diabetes. On the other hand, the discovery of the link between some genetic forms of these interleukins and the development of type 2 diabetes in the Iraqi population will be of clinical significance as indicators of T2DM susceptibility. Thus, this study of cytokine genes will help develop prognostic markers to identify individuals at risk at the molecular level.

1.4 Research Questions

This section will present the thesis questions:

1- What are the levels of Interleukin-4 (IL-4) and Interleukin-6 (IL-6) in sera from patients with type 2 diabetes mellitus?

2- What are the genotypes of interleukins to investigate the association of polymorphisms of Interleukin-4 and Interleukin-6 genes to the susceptibility of type 2 diabetes mellitus?

3- What is the correlation between gene polymorphism of nterleukin-4 and interleukin-6; allele frequency; genotype frequency and their levels in sera?

1.5 Research Objectives

This section will present the objectives of our study:

1. To determine serum levels of Interleukin-4 and Interleukin-6 of patients with type 2 diabetes mellitus among cases from Wasit province-Iraq.
2. To investigate the association of polymorphisms of interleukin-4 and Interleukin-6 genes to the susceptibility of type 2 diabetes mellitus of the study groups.
3. To correlate the relationship between the polymorphism of these interleukins genes and their levels in sera.

We can specify the objectives above as follows:

1. To measure the levels of interleukin-4 and interleukin-6 in sera from patients with type-2 diabetes mellitus by Enzyme-linked immunosorbent assay (ELISA)
2. To investigate the polymorphism of interleukin-4 and interleukin -6 by detection of the *IL4* −590C/T gene polymorphism (rs2243250) and the *IL6* −174 G/C gene polymorphism (rs1800795) using the allele-specific PCR technique.

3. To determine the interleukin-6 *IL6* −174G/C SNP genotype and interleukin-4 *IL4* −590C/T genotype in an allele-specific PCR including the G and C alleles, C and T alleles.

1.6 Significance of the study

Diabetes has grown to be a great concern as one of the major causes of morbidity and mortality among younger and middle-aged people (Saxena, Srivastava and Banerjee, 2013). Furthermore, it has been reported that T2DM accounts for 90% of these cases due to the influence of genetic, environmental and dietary factors (Vana et al., 2019).

Moreover, several studies have shown that alteration in cytokine gene expression has a considerable impact on one's obesity, insulin sensitivity, and risk of T2DM (Oguntibeju, 2019). In this, single nucleotide polymorphisms (SNPs) that occur in the cytokine genes especially in their regulatory regions have been observed to affect the

levels of cytokine expression (Gupta et al., 2015). Additionally, SNPs have also been implicated to demonstrate different levels of variation amongst different ethnic groups with regards to the pathogenesis of the diabetes disease (Banerjee and Saxena, 2014).

In line with these views, genotypic variants of SNPs, as well as their associations to the gene to gene interactions, can be used as indicators of T2DM susceptibility in different populations, thereby contributing to the enhancement of predicting individuals with a higher risk (Banerjee and Saxena, 2014).

In the search of the relation between T2DM and the genotypic interaction between SNPs located in one cytokine gene or several genes, a study needs to be carried out to investigate polymorphisms of Interleukin-4 (IL-4) and Interleukin-6 (IL-6). Polymorphisms related to IL-4 and IL-6 genotypes may be considered as a risk factor for T2DM among Iraqi subjects which could have a direct impact on the management and counseling of families. Functional gene polymorphisms of IL-4 and IL-6 will provide data related to the pathogenesis of DM which could be proven useful to individuals at risk to observe preventive steps in avoiding or impeding the onset of the disease.

1.7 Scope of the study

This study will be conducted to study diabetes as an immunological disease to investigate the association of *IL-6* gene polymorphism (-174G/C) and *IL-4* polymorphism (-590C/T) with T2DM incidence in Iraqi population of Wasit city. To reach the objectives of this study, experiments will be carried out through the adoption

of basic standards in the methods of work advocated in the associated studies. Finally will be used Statistical Package for the Social Sciences (SPSS) in data analysis.