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Correlations of Interleukin-6 (IL-6) (G174C and G572C) gene polymorphisms and sera levels with risk of Coronary Artery Disease in Thi – Qar province : A Case-Control Study

Taqwa Kadhem Jawad
Dept.of Biology / College of *Science/*
University of Thi-Qar
Iraq
taqwa.jaw.bio@sci.utq.edu.iq

Rasha Salih Nuhiar
Dept.of Biology / College of *Science/*
University of Thi-Qar
Iraq
rasha06_bio@sci.utq.edu.iq

***Abstract*— The present study was carried out in the labs of Technical Institute –Al-Nasiriyah /Community Health Department and Nasiriyah Heart Center in Thi- Qar province, during the period of research was extended from January to July 2022 . The aim of study was to determination polymorphisms of IL-6 gene in patients with Coronary Artery Disease and measuring its levels in serum using a technique enzyme-linked immune sorbent adsorptive (ELISA). The study included a total of 100 Iraqi patients with coronary artery disease(CAD) and healthy control group their age between 3-85 years. DNA has been isolated and PCR was performed by using primers specific for genotypes of IL-6 gene (572 C/G and 174 C/G), the results showed that gel electrophoresis for amplified IL-6 gene of CAD patients, gel electrophoresis of 167bp (IL-6 572C/G) PCR products and 198bp PCR product(IL-6 174C/G). Frequency of genotypes for the IL6 gene showed a high frequency of GG genotype compared to the GC and CC genotypes in all study groups , the frequency of G and C allele in IL6 gene was 95 % and 5 %, respectively with significant differences between genotypes in various groups, in the patient group (90 %) had GC genotype, (10%) were heterozygote and had (0%) had CC genotype compare with control group, (100%) had GC genotype, (0%) were heterozygote and had (0%) had CC genotype. The immunological study showed that there was a significant increase of concentrations of (IL-6) in patients compared to the healthy control group. Thus it seems There is no correlation between IL-6 gene polymorphisms and the risk of coronary artery disease in Thi- Qar province and the Serological Assay (ELISA) showed a significant increase in the concentration of IL-6 in patients with coronary artery disease compared with the healthy control group.**

***Keywords*— Coronary Artery Disease , polymorphism , Interleukin-6 (IL-6), promoter region of Interleukin-6 (IL-6) gene .**

I. INTRODUCTION

Coronary artery disease, namely coronary atherosclerotic heart disease[1].Coronary artery disease (CAD) is a disease that affects many middle-aged and elderly people and is usually accompanied by hypertension, diabetes mellitus and dyslipidaemia[2]. Coronary artery disease (CAD) continues to be the most common type of cardiovascular diseases and the major cause of death in both genders[3] [4]. The complex process of atherosclerosis begins early in life and is thought to initiate with dysfunction of endothelial cells that line the coronary arteries; these cells are no longer able to appropriately regulate vascular tone (narrowing or constriction of the vessels). There are several factors that contribute to coronary artery disease. However, these factors vary from one person to another. Genetics is considered to be one of the factors influencing the development of CAD. Some studies have reported 50 risk points in the human genome that can influence CAD development[5] Many studies have examined the effect of cytokines polymorphisms such as IL-6 , IL-10 , IL-18 , TNF-a and interferon-Y and evaluate its influence on coronary artery disease. [6] [7] [8] [9] [10] [11] Several types of immune cells, including macrophages, T and B lymphocytes also accumulate in the arterial walls and are involved in the development of atherosclerotic disorders through cytokines and other mediators[12] . genetic variations in the IL-6 gene and its receptor gene (IL-6R) induced different immune responses and susceptibility to CAD[13]. The human IL-6 gene is located on chromosome 7p21, consisted of five exons and four introns. IL-6 functional promoter single nucleotide polymorphism (SNP), has been identified previously and may be associated with elevated IL-6 levels . Elevated IL-6 levels are related to higher mortality rate in people with cardiovascular diseases [14].There are three single nucleotide polymorphisms (SNPs), 174 G>C (rs1800795), 572 G>C (rs1800796), and 597 G/A (rs1800797), have been widely

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investigated because of their association with the risk of various diseases[15].

II. Materials and Methods

A. Samples Collection

This study was performed on 100 Iraqi patients with coronary artery disease (CAD), who attended to Nasiriyah Heart Center and healthy control group in the period from the January to July 2022. Blood samples were collected by venipuncture from 61 patients and 39 controls (Five milliliters of venous blood) were drawn by disposable syringe under aseptic technique. Each blood sample was divided into two parts (Three milliliters were put directly in a sterile tube containing EDTA for DNA extraction and Two milliliters were placed in a sterile plane tube(without EDTA) and allowed to clot, then serum was separated by centrifugation at 4000 rpm for 15 minutes. The serum was stored at -20 C˚ freezing . These sera (61 patients and 39 controls) were used for estimating the concentration of interleukin-6 (IL-6).

B. Molecular Study

- **DNA Extraction**

DNA was extracted from blood samples according to the leaflet attached with DNA Extraction Kit (Geneaid /Thailand) :

Which includes the following steps:

DNA Template and Polymerase Chain Reaction (PCR) :

PCR technique was used to amplify the IL-6 gene according to [16] and used the following Primers for the PCR technique. Primers (forward and reverse) as the table(1)the kit provide by Solarbio science and tecnology company.

Table(1): Oligonucleotide Primer Sequences used for Amplification of IL-6 (174 G/C) gene.

Primers			Primer sequences
Primers of IL 6	174 G/C	F	5′TGACTTCAGCTTTACTCTTTGT -3′
		R	5′ CTGATTGGAAACCTTATTAAG 3′

Primers (F,R),D.W and DNA were mixed in master mix tube (20µl)as shown in table(2)

Table (2): PCR reaction for amplification of IL-6 (174 G/C) gene:

Materials	Volume
Master Mix	25 µl
Primer Forward	0.5 Mm
Primer Reverse	0.5 Mm
DNA	15 µl
D.W	-
Total	50 µl

The master mix tube put in the thermal cycle according to table (3). The primer pairs were included in the PCR for simultaneous amplification of fragments in **IL-6 (174 G/C)** gene.

Table (3): PCR condition for amplification of IL-6 (174 G/C) gene :

No of steps	Steps	Temperature	Time	No . of cycle
1	Denaturation 1	94	3 min	1
2	Denaturation 2	95	30 sec	30
3	Annealing	60	60 sec	
4	Extension 1	72	60 sec	
5	Final Extension 2	72	5 min	1

The products were transfered by the electrophoresis apparatus. The condition of electrophoresis was (1.5% agarose concentration), stained with ethidium Bromide (0.5µl/ml). under voltage 105 V and 400 mA for 40 minutes..

Table(4): Oligonucleotide Primer Sequences used for Amplification of IL-6 (572 C/G) gene.

Primers			Primer sequences
Primers of IL 6	572 C/G	F	5 GGAGACGCCTTGAAGTAACTGC 3
		R	5 AGTTTCCTCTGACTCCATCGCAG 3

Primers (F,R),D.W and DNA were mixed in master mix tube (20µl)as shown in table(5)

Table (5): PCR reaction for amplification of IL-6 (572 C/G) gene:

Materials	Volume
Master Mix	25 µl
Primer Forward	0.5 Mm
Primer Reverse	0.5 µM
DNA	15 µl
D.W	-
Total	50 µl

The master mix tube put in the thermal cycle according to table (6). The primer pairs were included in the PCR for simultaneous amplification of fragments in **IL-6 (572 C/G)** gene.

Table (6): PCR condition for amplification of IL-6 (572 C/G) gene :

No of steps	Steps	Temperature	Time	No . of cycle
1	Denaturation 1	94	3 min	1
2	Denaturation 2	95	30 sec	30
3	Annealing	60	60 sec	
4	Extension 1	72	60 sec	
5	Final Extension 2	72	5 min	1

The products were transferred by the electrophoresis apparatus. The condition of electrophoresis was (1.5%

agarose concentration), stained with ethidium Bromide (0.5µl/ml). under voltage 105 V and 400 mA for 40 minutes..

- **Product Analysis**

The products were transferred by the electrophoresis apparatus by dissolving (1.2) of the Agarose gel in 60 mL of the TBE to become the final concentration (2%) and Visualization of amplified product was conducted by U.V transilluminator (302nm). The size of amplified product was determined according to ladder marker and imaged by digital camera.

C. Immunilogical Study

- **Determination of serum levels of IL-6**

The sera of 88 patients and controls were assessed for the level of IL-6, by means of ELISA (technique enzyme-linked immune Sorbent adsorptive) kit are employing the quantitative sandwich that were based on similar principles according to the Bioassay Technology laboratory company.

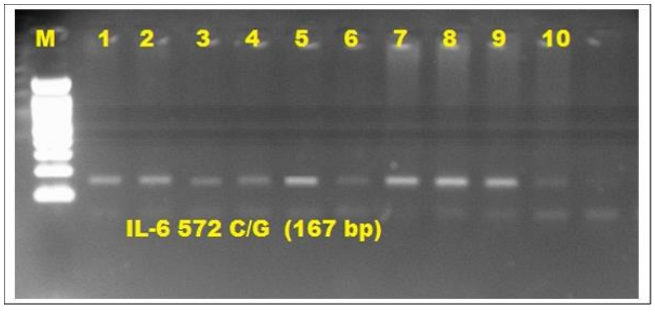
Two milliliters were placed in a sterile plane tube and allowed to clot, then serum was separated by centrifugation at 4000 rpm for 15 minutes. The serum was stored at -20 C° freezing . These sera (61 patients and 27 healthy controls) were used for estimating The concentration of interleukin-6 .

III. Result

A. Molecular Studies :

- **Gel Electrophoresis for Amplified IL-6(572 C/G and 174 C/G) Gene Polymorphism of CRD Patients :**

The results of the current study have shown that an agarose gel electrophoresis for amplified IL-6 gene of CAD patients, gel electrophoresis of 167bp (IL-6 572C/G PCR products and 198bp PCR product(IL-6 174C/G). The electrophoresis conditions were: agarose 1.5% stained with Ethidium Bromide and run for 40 minutes under voltage of 105 V and 400 mA, figure (1) and (2).



Figure(1):gel electrophoresis of 167bp products (IL-6 572 C/G).

M: molecular ladeer (100bp), lanes 1-10 positive. The electrophoresis conditions were : agarose 1.5% stained with Ethidium Bromide, run for 40 minutes under voltage 105 V and400 Ma.

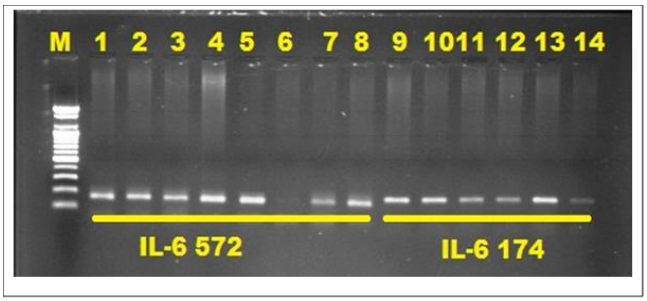


Figure (2): gel electrophoresis of 167bp (IL-6 572 C/G) PCR oroducts and 198bp PCR product (IL-6 174C/G). M: molecular ladder (100bp), lanes1-5:

IL-6 572, line 6: control negative, lanes 9-14: IL-6 174. The electrophoresis conditions were: agarose 1.5% stained with Ethidium Bromide, run for 40 minutes under voltage 105 V and 400

- **Frequency of genotypes for the IL6 gene samples of CAD patients and healthy controls:**

The current study have shown a high frequency of GG genotype compared to the GC and CC genotypes in all study groups, table (7). showed the percentage of G allele was higher than C allele. The frequency of G and C allele in IL6 gene was 95 % and 5 %, respectively with significant differences between genotypes in various groups (P<0.05). In the patient group, 9/10 (90 %) had GG genotype, 1/10 (10%) were GC genotype, On the other hand control group showed 10/10 (100%) GC genotype, 0/10 (0%) were heterozygote and had 0/10 (0%) had CC genotype.

Table (7): Distribution of genotypes for the IL6 gene samples of CRD patients and healthy Control.

Alleles	No. (%)	
G	19 (95)	
C	1 (5)	
Genotypes	**Patients**	**Control**
GG	9/10(90)	10/10(100)
GC	1/10(10)	0/10 (0)
CC		0/10(0) 0/10(0)
Odds ratio		3.3158
95 % CI:		0.1200 - 91.6070

B. Immunological study:

- **Serum IL-6 Concentration of healthy and CAD Patients :**

The results of this study have shown there was a significant increase (P<0.05) of concentrations of (IL-6) as seen in table (8) as the rate of concentration of IL-6 concentration (80.886 pg/ml) for patients compared with the healthy control (54.932 pg/ml) with a significant difference (0.014)

Table (8): Serum IL-6 Concentration of healthy and CAD Patients :

Group Statistics							
Parameters	Group	N	Mean	Std. Deviation	T	Df	P. Value
IL6 pg / ml	Patients	61	80.886	51.555	2.508	86	0.014
	Healthy	27	54.932	22.291			

(P<0.05) df : degree of freedom

IV. DISCUSTION

A. *Molecular study:*

Cardiovascular disease is a complex genetic disease influenced by both genetic and environmental factors and characterized by high blood pressure, Age, gender, lifestyle (sedated life, obesity, excessive alcohol consumption, salty diet, stress) and metabolic diseases such as diabetes mellitus are important factors influencing the development of hypertension. In this study, we investigated the effects of polymorphisms of the IL-6 gene -174 G/C and -572 G/C and their possible effects on the etiology of patients with cardiovascular disease.

Previous studies reported association between polymorphisms of IL-6 gene -174 G/C or -572 G/C or both polymorphisms and hypertension In Iraqi population whereas some other studies showed no relationship [16] [17]. A study of [18], investigate increased IL-6 concentration lead to vascular resistance and hypertension in consequence of endothelial dysfunction, also, noted the IL-6 stimulates release of acute phase reactants such as CRP, amyloid A, fibrinogen, TNF-a and IL-ip, it is known that elevated CRP concentration in the blood enhanced hypertension development by inflammation. A recent study [19], have shown both IL-6-174 G/C and -572 G/C polymorphisms were associated with elevated plasma CRP levels, and elevated IL-6 or CRP concentration or both were found to be associated with hypertension. The study by alomino-Morales *et al* [20], have reported that genes for proinflammatory cytokines such as IL-6 and TNFα are recognized as triggers of systemic and local manifestations of rheumatoid arthritis. Contribution of the IL-6-174 GG genotype to the development of acute endothelial dysfunction by endothelium-dependent vasodilation in rheumatoid arthritis patients.

B. *Immunological Study*

The present study investigated the concentration of pro-inflammatory IL-6 increased significantly in CVD patients than control group. The current study agreed with study of[21], their study have shown that a patient with CVD have high concentration of IL-6. Elevated circulating levels as well as intracardiac IL-6 levels in patients with congestive heart failure (CHF). IL-6 may contribute to the development of myocardial damage and dysfunction in chronic heart failure syndrome due to various causes.As a cause of congestive heart failure in cardiomyopathies, myocarditis, allograft rejection, and left ventricular assist device (LVADs) cases, circulating IL-6 levels correlate with the severity of left ventricular dysfunction and are strongly predictive of subsequent clinical outcome [22]. Also, the study of[23], have agreed with the present study, have shown the IL-6 receptor increase above normal in patients with CVD, also showed in mice experiment blockade IL-6 receptor through genetic deletion was associated with reduced cardiac hypertrophy and fibrosis after angiotensin II stimulation. The study of[24] and study of[25], have shown most patients with CVD have long-term high level of IL-6 and CRP, and concluded in humans, consistent with early data in the primary prevention of atherosclerotic events, biomarkers of inflammation including hs-CRP, IL-6, and GDF15 predict incident heart failure, exacerbating outcomes among those with predominant heart failure, as well as Reverse heart remodeling.

The study of[26], showed IL-6 was increased significantly in CVD patients and their study concluded the CVD is associated with chronic, low-level inflammation, including elevations in circulating pro-inflammatory cytokines as IL-10 and acute phase reactant CRP. It is difficult to say whether inflammation is a cause or a consequence of certain disorders, but evidence suggests that it may play a major role in the pathogenesis of cardiovascular disease and other chronic diseases. In contrast the study of[27] showed the serum IL-6 levels were not affected in the infective endocarditis (IE) process. Further understanding of the role of serum cytokine concentrations in the diagnosis, treatment, monitoring, and prognosis of IE may be valuable under the condition of suspected diagnosis, especially when pathogens cannot be detected in blood cultures. A marked increase in IL-6 and IL-10 is provoked by direct exercise and exertion anti-inflammatory effects by inhibiting TNF-a and stimulating IL-6 [28].

A study of[29], have suggested an important issue related to the development of atherosclerosis in patients with rheumatoid arthritis is the search for the causes of endothelial dysfunction. In this context, proinflammatory cytokines such as IL-6 has been linked to carotid Intima-Media Thickness. The high level of IL-6 is closely related to heart disease, and this is what most recent studies have recorded in patients with Covid-19.

V. CONCLUSION

There is no correlation between IL-6 gene polymorphisms and the risk of coronary artery disease in Thi- Qar province and the serological Assay (ELISA) have shown a significant increase in the concentration of IL-6 in case group with coronary artery disease compared with the control group.

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