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Proinflammatory cytokine IL-6 -174G/C (rs1800795) gene polymorphism among patients with chronic renal failure

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Key words: Interleukin-6; gene polymorphism; hemodialysis; renal transplantation.

Abstract. Interleukin-6 polymorphism is regarded as an aggravating factor in the development and progression of renal disease. IL-6-174G/C (rs1800795) genotypes are associated with increased risk of cardiovascular events in patients receiving hemodialysis. The aim of this study was to determine the relation between IL-6 gene polymorphism in patients with chronic kidney disease, in an Iraqi Kurdish population. IL-6 polymorphism at -174G/C (rs1800795) was assessed in 108 patients with chronic kidney disease (54 on hemodialysis and 54 renal transplanted) and 54 healthy subjects. The mean ages were 46.1, 36.8, and 40.2 for the hemodialysis patients, renal transplanted and healthy subjects, respectively. In the hemodialysis patients, GC intermediate producers were found at a double-fold higher risk of progression of the disease. Allele frequency G was 0.66 and 0.74 for the patients and the control group, respectively. While for renal transplanted patients, GG high producers were lower than the control group. GC genotype was one-and-half-fold higher for the renal transplanted patients than the control group. Levels of serum IL-6 increased significantly in GG and GC genotypes between both patient groups ($P<0.001$). However, there was no significant difference between the patient and control groups in terms of their CC genotype ($P>0.05$). These findings support the hypothesis of the impact of IL-6 on the progression of chronic renal failure. The IL-6 -174G/C (rs1800795) polymorphism predicts the inflammatory status of the progressed disease, and has a significant effect on the level of pro-inflammatory cytokine IL-6 in end-stage renal disease patients.

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Polimorfismo del gen de la citosina IL-6 pro inflamatoria en pacientes con insuficiencia renal crónica.

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Palabras clave: IL-6; polimorfismo génico; hemodiálisis; trasplante renal.

Resumen. El polimorfismo de la interleucina-6 se considera un factor agravante en el desarrollo y la progresión de la enfermedad renal. Los genotipos de IL-6-174G / C se asocian con un mayor riesgo de eventos cardiovasculares en pacientes que reciben hemodiálisis. En este estudio se determinó la relación entre el polimorfismo del gen de la IL-6 y enfermedad renal crónica, en una población kurda iraquí. El polimorfismo de IL-6 a -174G/C se evaluó en 108 pacientes con enfermedad renal crónica (54 en hemodiálisis y 54 con trasplante renal) y en 54 sujetos sanos. En los pacientes en hemodiálisis, se encontró que los productores intermedios de GC tenían el doble de riesgo en la progresión de la enfermedad. La frecuencia alélica G fue de 0,66 y 0,74 para los pacientes y el grupo control, respectivamente. Mientras que, para los pacientes trasplantados renales, los productores con GG alto fueron menores que en el grupo de control. El genotipo de GC fue una vez y media más alto para los pacientes trasplantados renales que para los individuos sanos. El nivel de IL-6 sérica aumentó significativamente en los genotipos GG y GC entre ambos grupos de pacientes (P<0,001). Sin embargo, no hubo diferencias significativas entre los grupos de pacientes y de control en términos de su genotipo CC (P>0,05). Estos hallazgos apoyan la hipótesis del impacto de la IL-6 en la progresión de la insuficiencia renal crónica. El polimorfismo IL-6 (-174G/C) predice el estado inflamatorio de la enfermedad progresiva, y este polimorfismo tiene un efecto significativo en el nivel de la citoquina pro inflamatoria IL-6 en pacientes con enfermedad renal en etapa terminal.

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INTRODUCTION

Renal replacement therapy in both forms, renal transplantation (RT) and hemodialysis (HD), has become quite prevalent, such that more than one million patients with end-stage renal disease (ESRD) undergo this type of therapy worldwide every year (1). More than forty years ago; however, ESRD patients were not cured through dialysis because it was associated with numerous complications such as atherosclerotic cardiovascular disease (CVD) and metabolic disorders (2,3). Currently, although there are remarkable technological improvements in dialysis and there is an enhanced focus on promoting small solute clearance, a large number of ESRD patients experience premature death as a result of CVD (4).

One of the characteristics of ESRD is a state of chronic inflammation, which is caused by endothelial dysfunction, oxidative stress, wasting, and vascular calcification (5, 6). In fact, robust predictors of outcome in patients with ESRD include a wide range of inflammatory biomarkers such as white blood cell count, interleukin IL-6,TNF, IL-1 and C-reactive protein (CRP). Also, research

has revealed that it is not clearly known why the risk for chronic inflammation increases in ESRD patients (7, 8). Pro and anti-inflammatory cytokines are known to play a direct role in end-organ damage (9). It is reported that a large number of patients with ESRD experience chronic inflammation as a result of different factors, such as genetic factors like polymorphism (10, 11). According to the reports of previous studies, development of acute kidney injury (AKI) in different clinical settings, such as sepsis or post-cardiac surgery, development of chronic kidney disease (CKD) and ESRD, are significantly influenced by pro and anti-inflammatory cytokines such as (IL-6) and their functionally relevant gene polymorphism (12, 13).

According to research studies, IL-6, which plays a critical role in the inflammatory cascade, is related to hypertension, promotes procoagulant changes and regulates a wide range of biological activities such as causing dyslipidemia (14). In addition, research has indicated that a poor outcome in ESRD patients and increased mortality are associated with plasma IL-6 (15). Moreover, hypertension and as a result ESRD, can be caused by high production of IL-6 (16).

Human IL-6 gene, which is located on chromosome 7p21, consists of five exons and four introns. According to the genotype, different levels of secreted protein are associated with the polymorphism of the IL-6 gene. In the promoter region, IL-6 possesses some polymorphisms including -634 C/G, -174 G/C, -572 G/C, and -597 G/A (17). Different studies have examined the association between the three functional proteins in the IL-6 gene (-634C/G, -174G/C, and -572C/G) and the risk of various diseases (18, 19). By changing protein expression and gene regulation, the gene polymorphisms -174G/C, and -572C/G may affect CVD susceptibility expression (20). There are conflicting results regarding the association between IL-6 -174 G/C (rs1800795) polymorphism and CKD. Moreover, variations in transcription may be caused by polymorphic variants in the promoter region of the IL-6 gene (21).

Studies have revealed that the –174 G→C promoter region polymorphism of the IL-6 gene is functionally relevant. According to transient transfection experiments that have been carried out through reporter gene constructs containing both allele, C allele leads to suppression of IL-6 transcription in response to endotoxin or IL-1 in HeLa cells (22). Increased circulating levels of IL-6 are reported to be associated with various diseases in patients with ESRD, including hypertension, malnutrition, atherosclerosis, cardiovascular events and left ventricular hypertrophy (13, 23, 24).

MATERIAL AND METHODS

One hundred and eight patients with CKD participated in the present study. They were divided into two groups of patients: on hemodialysis (HD) and renal transplanted (RT) patients (each consisting of 54 patients). In addition, there was a control group consisting of 54 healthy subjects. The required data were collected using questionnaire, which was completed through direct interviews with the participants. Seven mL of blood samples were taken from all participants and put in two different tubes. Of those, 2-3 mL for a gel tube to obtain serum, to be used to determine serum concentrations of IL-6 (in pg/mL), by ELISA, according to the manufacturer's protocol. The remaining 2 mL of blood were put into EDTA tubes to analyze cytokine gene polymorphism by amplification refractory mutation system (ARMS) PCR. The genomic DNA was isolated and extracted from the venous blood of the studied samples according to the manufacture's protocol. The primer sequences were as follows IL-6 generic primer, 5´-GCC TCA GAG ACA TCA CCA GTC C-3´, IL-6 (G) Allele Primer 5´-CCC CTA GTT GTG TCT TGC G-3´, and IL-6 (C) Allele Primer 5´-CCC CTA GTT GTG TCT TGC C-3´.

The PCR reaction was carried out in thermal cycler (PX2) with the following program. The samples were placed in a 20 μL reaction volume containing 40 ng genomic DNA, 1.5 mM dNTPs, 25 mM $MgCl_2$, 1 μL of 10 pmol of each primer, and 0.4 units of Taq polymerase (Fermentas, Maryland, USA) in 1X Reaction Buffer. Cycling conditions were as follows: 1 minute at 95ºC, followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 58°C, 40 seconds at 72˚C, followed by 20 cycles of 20 seconds at 95˚C, 50 seconds at 54°C and 50 seconds at 72°C, with 5 minutes at 72˚C as the final extension. The amplified products were analyzed on a 2% agarose gel.

Statistical analysis

Statistical Package for the Social Sciences (V. 19.0) (SPSS Inc., Chicago, IL, USA) was used to analyze the data collected in this study. Variables were expressed as mean ± SD for their normal distribution. A p-value of under 0.05 was considered to be statistically significant. Intergroup comparisons were carried out through ANOVA for categorical variables and to examine the variables and serum cytokine concentration. For IL-6 gene polymorphism, the allele was counted by direct allele counting. Genotype and allele frequencies were compared between the groups using Chi-square (χ^2) test of independent variable with 2 x 2 contingency tables and the z statistics.

RESULTS

The mean ages of the HD and RT patients were 46.1 and 36.8 years, respectively. Half of the HD patients were males and half were females, while the ratio was different in the RT patients with 55.6% males and 44.4% females. Both of the studied groups had a normal body mass index (BMI) (BMI>25). Smoking is considered as a risk factor for renal failure. There were 27.8% and 20.4% smokers among the HD and RT patients, respectively. Diabetes is also reported to be related with renal failure. The number of patients with diabetes in the HD group was twice than those in the RT group. Another factor that is thought to participate in the progression of end stage renal disease is hypertension. In this regard, it was observed that 50% of the HD patients had hypertension, while only 16.7% of the RT patients had high blood pressure. Nearly both of the studied groups showed the same rate of family history for the disease (Table I).

According to the genotypes, high producers (GG) were found higher in the control group 53.7%, while high producers GC were found in the HD patients, and it was

TABLE I
SHOWED THE DEMOGRAPHIC DISTRIBUTION OF THE STUDIED GROUP

Characters	Hemodialysis N=54	Renal Transplanted N=54
Age (Mean± SD)	46.1±1.6	36.8±2.8
Sex:		
Male	27/54(50%)	30/54(55.6%)
Female	27/54(50%)	24/54(44.4%)
BMI (Mean± S.D)	24.83±0.5	24.17±1.0
Smokers	15/54(27.8%)	11/54(20.4%)
Diabetes	14/54(26%)	7/54(13%)
Hypertension	25/54(50%)	9/54(16.7%)
Family History	11/54(20.4%)	13/54(24.1%)

nearly twice higher in the control group (OR=1.96), which means a higher susceptibility of this genotype carriers to get the disease. In addition, the CC was found to be equal in the HD patients and the control group. Allele frequency G was 0.66 and 0.74 for the HD and control groups, respectively. The frequency of C allele was 0.34 for the HD patients and 0.26 for the control group (Table II), and the gel electrophoresis of PCR products for patients' genotyping is shown in (Fig. 1).

Renal transplanted patients had 40.7% of GG genotype, while this rate was greater at 53.7% in the control group. However, GC genotype was found higher in the RT patients (51.9%) compared to the control group (40.7%), and the RT patients were about 1.5 fold more at risk to get progress the disease (OR=1.56). Allele frequencies were 0.67 and 0.74 for G allele of the RT and control groups, respectively, and the rate of C allele were respectively 0.33 and 0.26 for them (Table III).

Table IV and Fig 2 showed the concentration of IL-6 pg/mL of the studied groups. There was a significant difference between the HD and RT patients when compared with control group (P<0.001). Regarding the genotypes and the level of IL-6 in the patients and the control group, the results showed different concentrations. Genotype GG producers had a higher level of IL-6 in both patient groups than the control group (P<0.0001). However, the GC producer showed a significant increased level of the IL-6 in the HD and RT patients when compared to the control group (P<0.001). With regard to the CC genotype, no significant differences were found between the studied groups (P>0.05).

Fig 1. IL-6 (174G/C) product of ARMS-PCR on agarose gel (2%) amplicon size (230bp): M: 100bp size DNA ladder.

TABLE II
IL-6 GENOTYPES AND ALLELE FREQUENCY OF THE HD AND HEALTHY SUBJECTS.

IL-6 G/C	Hemodialysis	Control	OR (CI 95%)	P
No. of genotypes	N=54	N=54		
GG	20 (37%)	29 (53.7%)	0.507(0.235-1.094)	P>0.05
GC	31 (57.4%)	22 (40.7%)	1.96(0.912-4.214)	P>0.05
CC	3 (5.6%)	3 (5.6%)	1	P= 1
Allele frequency				
G	0.66	0.74		
C	0.34	0.26		
$\chi 2$	4.067	0.199		
P value	0.04	0.65		

TABLE III
IL-6 GENOTYPES AND ALLELE FREQUENCY OF THE RT AND HEALTHY SUBJECTS

IL-6 G/C	Renal Transplanted	Control	OR (CI 95%)	P
No. of genotypes	N=54	N=54		
GG	22 (40.7%)	29 (53.7%)	0.592(0.276-1.270)	P>0.05
GC	28 (51.9%)	22 (40.7%)	1.566(0.731-3.353)	P>0.05
CC	4 (7.4%)	3 (5.6%)	1.36(0.289-6.388)	P>0.05
Allele frequency				
G	0.67	0.74		
C	0.33	0.26		
$\chi 2$	1.50	0.199		
P value	0.22	0.65		

TABLE IV
CONCENTRATION OF SERUM IL-6 pg/mL (MEAN ± SD) IN THE STUDIED GROUPS REGARDING THEIR GENOTYPES

Cytokine		Hemodialysis (54)	Renal Transplanted (54)	Control (54)	P
IL-6 (pg/mL)		58.48[a] ± 31.55	45.99[a] ± 31.27	24.56[b] ± 2.42	<0.001
IL-6 (pg/mL)	GG	60.22[a] ± 32.53	43.26[a] ± 27.7	24.56[b] ± 15.31	<0.0001
	GC	55.5[a] ± 28.68	43.63[a] ± 28.32	25.06[b] ± 16.27	<0.001
	CC	47.91 ± 23.43	40.97 ± 23.61	28.44 ± 10.52	>0.05

Different letters shows difference between means of row.

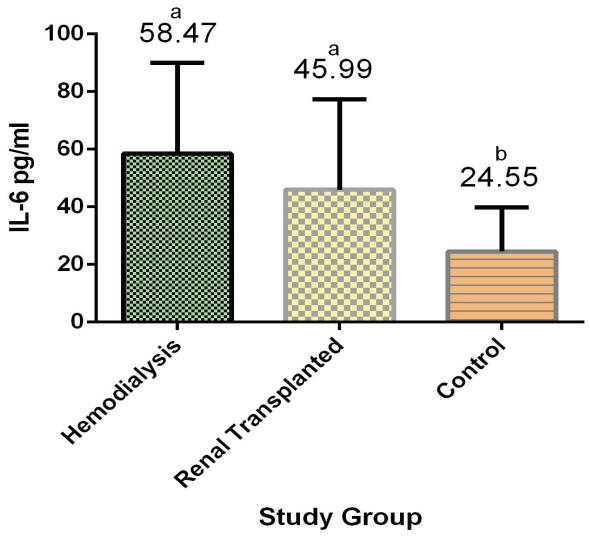

Fig 2. Concentration of IL-6 pg/mL in the patients and healthy subjects.

TABLE V
IL-6 GENOTYPES AND DIABETIC NEPHROPATHY OF HD AND RT PATIENTS.

Genotypes of	Hemodialysis with Diabetic Nephropathy N=14(%)	Renal Transplanted with Diabetic Nephropathy N=7(%)	p
GG	8/14(57.14%)	5/7(71.4%)	0.52
GC	5/14(35.72%)	2/7(28.6%)	0.6
CC	1/14(7.14%)	0/7(0%)	0.68
Allele Frequency			
G allele	0.75	0.86	
C allele	0.25	0.14	

Results of genotyping for the patients with diabetic nephropathy were as follows; high producers GG were higher in both patients group, which were 57.14% and 71.4% for HD and RT, respectively. While GC producers were 35.72% for HD and 28.6% for RT patients. However, CC or low producers was 7.14% in HD patients, and there was no CC genotype in RT patients (Table V).

DISCUSSION

Research has shown that appropriate regulation of the immune response fails during chronic inflammatory conditions, which in turn leads to disease progression. In these conditions, chronic inflammation, deleterious tissue damage and development of autoimmunity, are affected by cytokines (25). The reason for the increased risk for chronic inflammation in patients with ESRD is complex (7). The impaired function of neutrophils and T cells and the impaired immune response in uremia in ESRD patients translate into increased infection risk (26). While defensive responses are impaired, the immune system is also under constant stimulation as a result of the process of dialysis itself (27).

Genetic factors are highly significant and have substantial impact on IL-6 expression, which is best depicted by the identifying functional polymorphisms within the IL-6 promoter. In the promoter region, -174G/C (rs1800795) polymorphism of the IL-6 gene, is the first and the most frequently studied polymorphism because it leads to enhanced IL- 6 expression due to its functional significance from the transcriptional start site (28). Moreover, it has been reported that susceptibility to ESRD is related with these polymorphisms (29), which supports the findings of the present study. The results of the current study revealed a significant difference between the healthy subjects and CKD patients with regard to the distribution of allele of IL-6 gene. In agreement with the results of the current study, IL-6 gene polymorphisms are reported by several studies to be associated with the risk of cardiovascular events in HD patients (30-32). Moreover, studies have considered IL-6 polymorphism as an aggravating factor in development and progression of renal disease (32, 33). In the present study, it was found that IL-6 -174GC genotype led to an increased risk of cardiovascular events in patients who had received HD. In conflict, to our study no association between IL-6-174G/C polymorphism and progression of ESRD patients was reported by previous studies (33-35). This discrepancy may be attributed to differences in sample size and ethnicities. Therefore, the association between IL-6- 174G/C polymorphism and risk of cardiovascular events in patients on HD needs to be taken into account in further studies.

According to recent studies, the plasma levels of IL-6 protein and the transcriptional

activity of the IL-6 gene are associated with a single G/C base-exchange polymorphism sited at -174 promoter region (30). Similar to the results of the present study, different studies have indicated that homozygotes for allele G and G/C heterozygotes have higher IL-6 gene transcriptional activity, higher plasma IL-6 levels, and higher IL-6 inducible responses than subjects homozygous for allele C (22, 36).

Studies have also indicated that plasma levels in dialytic patients are abnormally elevated, particularly for IL-6, and revealed that polymorphism of the IL-6 gene -174G/C is associated with ESRD, high blood pressure and coronary artery disease in healthy men (22, 37). Subjects who are homozygous for the G allele or heterozygous for G/C at position -174, as indicated by research studies, have higher plasma IL-6 levels and higher IL-6 gene transcriptional activity compared to individuals who are homozygous for the C allele (22, 12). Moreover, another study revealed that the CC genotype is associated with cardiovascular events in HD patients (35). Research has revealed that IL-6 can stimulate intracellular adhesion molecules, that lead to endothelial cell damage and enhance the attachment and diapedesis of leukocytes across endothelial cells (38). It is also indicated that elevated levels of IL-6 can stimulate vascular smooth muscle cell growth, which in turn increases endothelial synthesis of plasminogen activator inhibitor and finally, lead to progressive fistula stenosis in HD patients with ESRD (35). Interlukin-6 levels drop immediately after transplantation and during acute rejection (AR) (39). Both IL-6 and sIL-6R are regarded as important prognostic markers of clinical outcome in patients with ESRD. Serum IL-6 levels are reported to be elevated before treatment in ESRD patients (40). This elevation in IL-6 levels has been attributed to impaired excretion due to reduced kidney function (15), although ESRD patients are reported to have increased IL-6 mRNA in their peripheral blood mononuclear cells (41). In addition, elevated serum IL-6 and sIL-6R levels in the beginning of treatment are powerful predictors of mortality in patients with HD and these changes may be an indication for the systemic inflammatory status of a patient, and often correspond to elevations in C-reactive protein (42, 43). Research has shown that high levels of IL-6 are prognostic of cardiovascular risk (44) and contribute to dialysis-associated malnutrition (45, 46), which are adverse outcomes of hemodialysis (7). A clinical study of ESRD patients reported serum creatinine as a major determinant of plasma IL-6 levels (47). Although decreased elimination may be a major cause of increased IL-6 levels in patients with ESRD, increased cytokine generation may play a role, too. In fact, as renal function declines, increased IL-6 levels may be caused by both fluid overload and congestive heart failure (CHF) (48). Takahashi et al. (49) reported that HD leads to an increase in blood mononuclear cell IL-6 mRNA expression and plasma IL-6 levels. Also, Caglar et al., (50) showed that circulating IL-6 levels rises after HD, which provides evidence of HD-induced delayed inflammatory response. It is also reported that several HD-related factors contribute to the generation of IL-6 and/or increase the inflammatory effect of IL-6, particularly, the use of bioincompatible membranes and nonsterile dialysate (7). Finally, since a large number of cytokine encoding genes are also expressed in adipocytes, it is estimated that the adipose tissue may account for as much as 20% of systemic IL-6 concentrations (51). Therefore, visceral adiposity may be another cause for elevated IL-6 levels in patients with ESRD (52).

Research has also shown that this cytokine may have an important role in the pathogenesis of diabetic nephropathy (28) and the diabetic patients without CKD and healthy patients were not reported to be significantly different regarding the distribution of alleles (53). Research has shown a significantly high frequency of GG genotype in the diabetic patients with CKD compared

to the healthy subjects, which indicates that significant protection against kidney disease is provided by both GC and CC genotypes, and GG genotype is highly susceptible for the progression of kidney dysfunction (54). A study done by Senthilkumar *et al.* (55) has found that IL-6 level increases in diabetic patients with kidney disease compared to diabetic patients without complications. According to these observations, progression of kidney dysfunction in diabetic patients may be caused by polymorphism of the IL-6 gene. Benefits of IL-6 include improved quality of life, patient survival, and healthcare cost (56). Moreover, IL-6 has long been introduced as a pro-inflammatory cytokine which is associated with renal allograft rejection. IL-6 levels are low in the serum and urine of healthy individuals, while renal transplant recipients have high levels of IL-6 in their serum and urine (57).

Similarly to our results, a study found that in CKD patients in a population, to be a male, to be older than 55 years, and have hypertension, obesity and diabetes, increase the risk of developing CKD (58).

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