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Serum Interleukin-6 and Gene Polymorphisms in Rheumatoid Arthritis Patients in Babylon Province, Iraq

Sarah H. Edin, Abdulsamie H. Alta'ee*, Sabah J. Al-Rubaie

College of Medicine, University of Babylon, P. Code 51002,Hilla P.O. Box 473, Iraq.

Abstract : Rheumatoid arthritis (RA) is a systemic chronic inflammatory, autoimmune disease depicted by persistent symmetric polyarthritis which commonly affects joints of hands and feet. Current study aims to examine the probable link between serum interleukin-6 (IL-6) levels and (-174 G/C) IL-6 gene promoter polymorphism in RA in Babylon Province. 60 patients with RA and 60 apparently healthy individuals were subjected topresent study. Measurement of serum IL-6 was assayed using commercially available ELISA kit. Disease severity score of RA patients was determined by use DAS-28. The polymorphism of (-174 G/C) IL-6 gene promoter was examined by the technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Present study find significant high levels of serum IL-6 and anti cyclic citrullinated peptide antibodies (ACCPA) in patients with RA in comparison with healthy controls .Genotype of (-174 G/C) IL-6 gene promoter polymorphism in RA patient were 80% GG,18.3% GC and 1.6% CC, whereas in healthy control were 98.3% GG, 1.6% GC and 0% CC. The elevated concentration of IL-6, and its positive association with DAS-28 may proposes a probable role ofIL-6 in RA pathogenesis. The polymorphisms of (-174G/C) IL-6 are also linked with the risk of RA, and the allele C has dramatically elevation in the susceptibility of RA in the population of Babylon.
Keyword: Rheumatoid arthritis, interleukin-6, (-174 G/C) IL-6 polymorphism, anti-cyclic citrullinated peptide antibodies, DAS-28.

Introduction

Rheumatoid arthritis(RA) is an inflammatory, autoimmune disease causes pain,joint stiffness and loss of function. Although there are several forms of arthritis, after osteoarthritis RA is the second most common and most serious forms of arthritis. RA may occur at any age and affects females more than male. Usually, it is common in persons over 30 years old. RA presents in a symmetrical manner i.e. affect the two sides of the body and is mainly affect hand joints. RA is reported to affect all body organs[1]. Immune system attacking joint causing irreversible and progressive damage. Pain of joint and swelling lead to disability and structural deformities, which cause limit movement in joint and muscle use. In turn, the strength and size of muscle decreases and the consequence abnormal forces on tendons cause deformity. Also, RA may causes a problems in heart, eyes, nerves and respiratory system. This disease initially affects synovial joints, lead to pain, deformity and finally functional limitation, lead to substantial morbidity and accelerated mortality and [2].The epidemiology of RA is 0.5-1% of the populations in the industrialized countries and females more frequently than males (3:1) [3-5]. The causes of the disease are unknown; however, several indirect evidence suggests that environmental factors play an important role. RA has already been regarded at the time of its description as a disease of the poor and lower levels of education and upbringing under adverse socioeconomic conditions thought to smoking may increase the risk and severity of RA [6].

Different immune modulators (effectors cells and cytokines) and marking pathways are involve in the RA pathophysiology[7]. The joint damage is result from complex interaction of immune modulators which begin at the synovial membrane and covers most IA structures. Synovitiscause by the local activation or influx, or together, of mononuclear cells (involving B, T, dendritic, plasma, mast cells and macrophages) as well as by angiogenesis [8].

Interleukin 6 (IL-6) might be a representative protein that includes redundancy and pleiotropic activity. The newly synthesize IL-6 share to vindication of host versus infectious agents and tissue injuries by causation hematopoietic and acute phase reactions as well as immunological responses. However, uncontrolled continual product of IL-6 might result in the state of many immune-mediated diseases [9].

It was discovered that IL-6 performs essential and multiple roles in inflammation and immune regulation, as well as oncogenesis[10]. In addition, it thought to be the main mediator for the progression of the several autoimmune and chronic inflammatory diseases involving RA [11].

RA is sustained by environmental and genetic factors. Several genes were implicated in the pathogenesis of RA [12]. The role of the variability of IL-6 production in the RA severity and pathogenesis is genetically determined [13] and its polymorphism of the -174 G/C promoter is linked with disease activity and susceptibility, andbecome a genetic risk factor [14].

Current study aims to estimate the concentrations of IL-6 and ACCPA, and to investigate their relationship with DAS-28in RA patients and healthy control. Also, this study aims evaluate the clinical significant of the polymorphism ofIL-6 -174 G/C promoter and their possible association with IL-6 levels in RA patients in Babylon Province, Iraq.

Materials and Methods

Ethical Issues

Current study is approved by the Committee of Ethics in Babylon General Directory of Health, Iraq. All persons subjected to current study was agreed to participate and signed an informed consent.

Patients

Sample size was determined according to sample size equation. Sixty patients (11 male and 49 female) with RA clinically diagnosis by specialist physician and according to the ACR 1987 and 2010 ACR / EULAR RA classification criteria[15]attended to out clinic of Merjan Teaching Medical City, Hilla City, Babylon Province, Iraq with mean age of (34.33±12.5) for males and (45.82±10.57) for females, as well as sixty (15males,45 females) with mean age of (35±3.16) for males and (40.30±7.57) for females who were apparently healthy control, were subjected to present study. The duration of current study was extended from September 2015 to July 2016.

The patients with RA were categorizedinto 2 groups according to the type of therapy:

1. Group 1 (untreated patients): Patients receiving only symptomatic drugs (non-steroidal anti-inflammatory drugs, analgesia and corticosteroid) and don't receiving disease modifying anti rheumatic drugs (DMARDs) like methotrexate, salazopyrine and biological therapy).

2. Group 2 (treated patients): Patients receiving DMARDs therapy in addition to symptomatic drugs.

Exclusion Criteria

Subject who suffered from any other chronic diseases, Subject who were smoker, pregnant women and obese.

InclusionCriteria

Patients with RA and in the age of 19-68 years.

Study Design

This study was designs as case control study.

Methods

Measurement ofDisease Activity Score (DAS-28)

DAS-28 was calculated as previously described by Van Riel[16].

Measurement of Serum IL-6

Determination of IL-6 concentration in the study groups was done using Bio Legend's ELISA MAX™ kit and according to manufacturer manual.

Measurementof Serum ACCP Antibodies

Determination of ACCPA concentration in the study group was done using Aeskulisa® ACCPA kit and according to manufacturer manual.

Measurement of Blood Rheumatoid Factor (RF)

The Spinreact® RF-latex agglutination test kit was used to measure RF in the study groups and according to manufacturer manual.

Measurement of Blood C-Reactive Protein (CRP)

CRP was determined using Agappy® latex-promoted nephelometry kit and according to manufacturer manual.

Determination of (-174) Promoter Codon Polymorphism of IL-6 Gene

The polymorphism of promoter region of IL-6 gene (-174 G/C) was examined bythe technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

DNA Isolation and IL-6 Genotyping

DNA was extracted from white blood cells by use Biospin Whole Blood Genomic DNA Extraction kit (Favorgen Taiwan).Enzymatic amplification was done by PCR using Master Taq polymerase enzyme and hybrid thermal cycler. Amplification of the promoter region (-174G/C) of the IL-6 gene was done as proposed by Pola et al. [17] using 2 primers (Promega®)forward primer:5'-GGAGTCACACACTCCACTG-3',and reverse primer: 5'-CTGATTGGAAACCTTATTAAG-3'. The reaction mixture of PCR (25 µl) consist of 12.5 µl, PCR Master Mix [10 × PCR buffer, 4 mM $MgCl_2$, 0.5 Taq DNA polymerase0.4 mMdNTPs (dTTP, dCTP, dATP anddGTP)], 1 µl of both primer, 3 µl of extracted DNA and 7.5 µl sterilized nuclease-free water. The reaction was performed with the following cycles:

One cycles95°C for 5 min.

Thirty five cycles of 1minfor denaturation at 95 °C.

One minfor annealing at 51.6 °C.

One minfor extension at 72 °C

Seven min final extension at 72 °C after completion of the cycles.

Then the amplification products weredigested with 5 units of Fast Digest (HSP92II) restriction enzymeat 37 °C for (1-4) hours.

Agarose gels electrophoresis was used asa standard method to identify, separate, and purify DNA fragments.

Results

Results of present study and other study were shown no difference between males and females in the most parameters involved. Therefore, categorization of participants did not depended on the gender.The persons participated in the current study were grouped into three groups: Group 1 (G1) (untreated patients) consist of 22 patients with RA (2 males and 20 females). Group 2 (G2) treated patients consist of 38 patients with RA (9 males and 29 females). Group 3 (G3) consist of 30 persons (7 males and 23 females) apparently healthy adults as control group. Mean age ± SD of persons participated in the present study was shown in Table 1.

Table 1: Number and Ageof Participants.

Groups	Total No.	Male No.	Female No.	Mean ±SD Age (Years) P value	
				Male	Female
Untreated Patients (G1)	22	2	20	39 ±11.31 0.704	43.6±11.58 0.285
Treated Patients (G2)	38	9	29	34.33±12.5 0.881	45.82±10.57 0.331
Healthy Controls (G3)	30	7	23	35±3.16	40.3±7.57

Table 2 shows the values of DAS-28 of patient groups with RA, Table 2don't shows DAS-28 for healthy control groups due to absence of any rheumatic manifestations. The comparison of DAS-28 among patient groups shown insignificant increase in DAS-28 of untreated RA patients than treated RA patients.

Table 2: Disease Activity Score (DAS-28) of RA Patients.

Groups	DAS-28		
	Mean	±SD	P value
Untreated Patient No. (22)	4.7045	1.5707	0.536
Treated Patient No. (38)	4.4421	1.5704	

The concentration of serum IL-6 and ACCPA of patient groups was found to be significantly elevated in untreated and treated RA patients when compared with control groups, as shown in Tables 3 and Table 4 respectively.

Table 3 Serum IL-6 Concentration of Patients and Healthy Control Groups.

IL-6 (pg/ml)	Untreated Patients	Treated Patients	Control
Mean	23.02	19.95	15.47
± SD	8.91	5.94	8.87
P	0.006	0.04	

Table 4 Serum ACCPA Concentration of Patients and Healthy Control Groups.

ACCPA (U/ml)	Untreated Patients	Treated Patients	Control
Mean	742.88	10.26	2.092
± SD	641.03	20.37	2.108
P	0.00000	0.018	

The Correlations among DAS-28 and IL-6 levels, IL-6 and ACCPA, and DAS-28 and ACCPA in untreated and in treated RA patients were investigated and found to be significant positive correlations.

RF of RA patients in this study was found to be positive in 83.32% and negative in 16.67 % in overall RA patients, whereas was negative in 99% of healthy control.

C-reactive protein (CRP) of patients with RA in the present study was found to be positive in 78.4% and negative in 21.6 % in overall patients. Positive CRP percentage in untreated male patients with RA found to be 50%, untreated females 90%, treated males 87% and treated females 86.6% patients with RA.

The Length of PCR product was 525 bp as shown in figure 1.

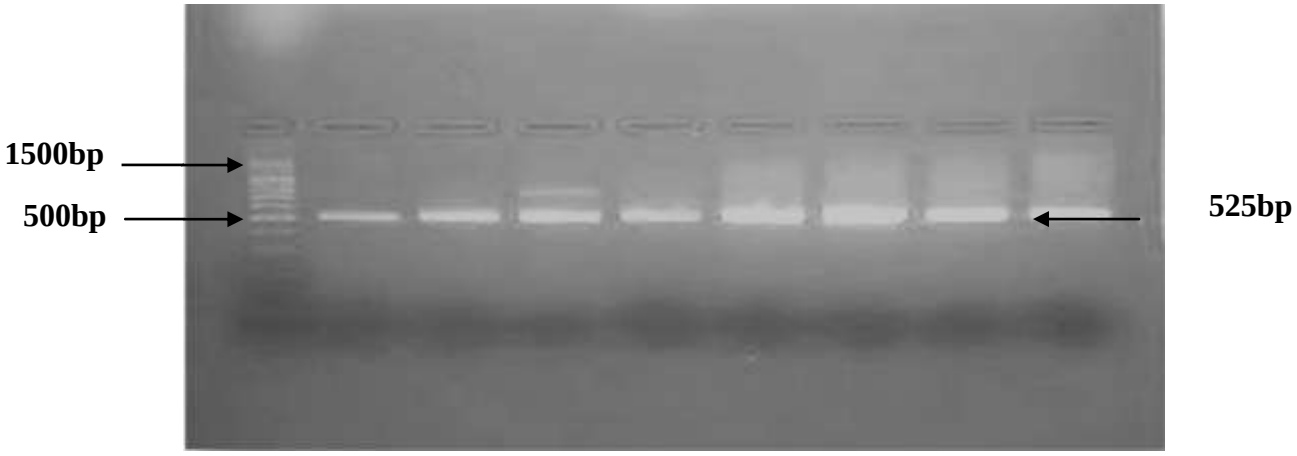

Figure 1 The PCR ProductElectropherogram.

The results of amplification and digestion by restriction enzyme *Hsp92II*of promoter codon -174 of IL-6 gene by PCR-RFLP assay were found to have two alleles (G and C) and three genotypes: CC with single band (loss of restriction site) and has molecular size of 327, 122 bp; and GG with two bands 327 and 169 bp;as well as GC with three bands 327, 169, and 122 bp, as shown in figure 2.

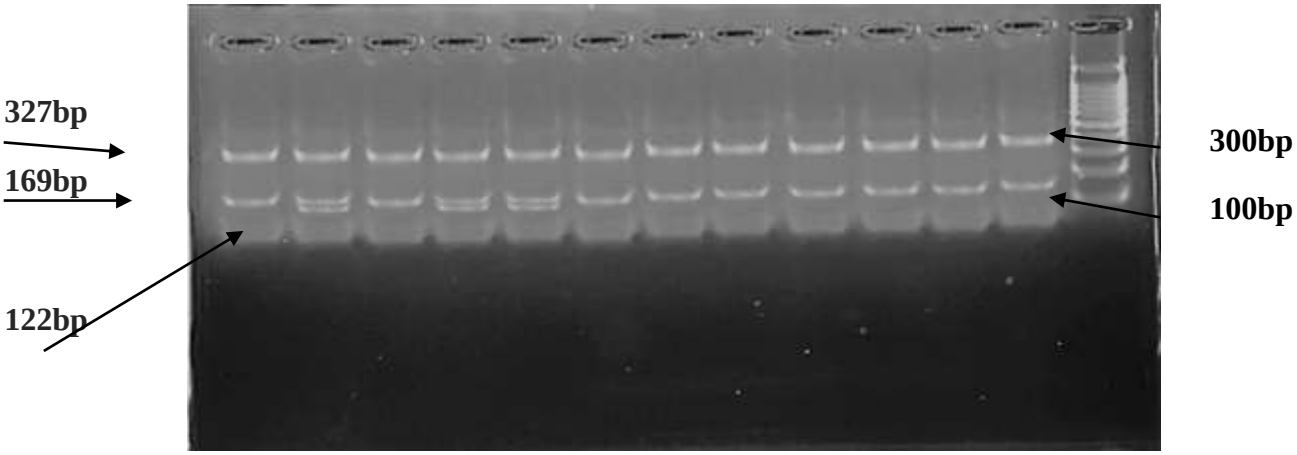

Figure 2 Amplification and Digestion by Restriction Enzyme of Promoter Codon -174 of IL-6 Gene.

The Genotype of (-174 G/C) IL-6 gene promoter polymorphism and its percentage in patient found to be 80% GG,18.3% GC and 1.6% CC, whereas in healthy control 98.3% GG, 1.6% GC and 0% CC.Relation between both of IL-6 levels and DAS-28 with genotyping of (-174 G/C) IL-6 gene promoter polymorphism in untreated and treated RA patients were found to be positive significant relation.

The relation between each of IL-6 levels and DAS-28 activity with genotyping for untreated RA patients were examined and shown positive significant relation (P =0.0009) and (P = 0.05)respectively. Whereas, the relation between ACCP antibody and genotyping in RA patients were investigated and shown trend toward negative relation (P = 0.44)

Discussion

Results of present is agreed with Yoshida *et al.*study that reported significantly highconcentrations of serum IL-6 in cases of RA in comparison with healthy individual [18].

After the revolution of TNF blockers that licensed to treat RA patients, and from the observations of animal study, IL-6 serum levels reduced by TNF blockader, suggesting a present of interrelationship between these cytokines. They reported that IL-6 attaches either to the receptor of membrane bound IL-6 or to a soluble receptor. IL-6 when attaches to the soluble receptor, it is not neutralized, rather it maypersist to signal across gp130. Antibodies of IL-6 receptor can inhibit this signaling process. Humanized antibody such as tocilizumab that attaches to membrane-bound IL-6to and soluble receptor, can inhibits the receptor complex and lead to prevent the IL-6 transmembrane signaling. They were concluded that IL-6 is a key pro-inflammatory cytokine and is mainly included in the pathogenesis of RA[19] and this is in agreement with present study suggestion.

The association between IL-6 and ACCP levels in the present study were investigated and shown significant positive correlation in untreated and treated patients groups. Result of present study is agreed with previous study aimed to examine the relationbetween IL-6, CRP, and ACCP antibodies in type 2 diabetes patients in age-effect study. They were found significant positive relation between serum IL-6 and ACCP antibodies in younger type 2 diabetes patients[20].Result of present study was disagreed with other study that aimed to investigate the correlationamong blood cytokines, acute phase reactants,autoantibodies, and disease activity in RA patients which was found that IL-6 is well correlated with CRP and ACCP is correlated with each of IL-1β, IL-2, IL-4, and IL-10[21].

The correlation between DAS-28 and serum IL-6in this study was positive and significant correlated for untreated patient and treated patients with RA. This result is agreed with Jin Chung *et al.* study [22]which shown that serum levels of IL-6 is decreased significantly after treatment in patients with high disease activity. They suggest a probable link for IL-6 in the pathogenesis of RA.

Present study was determined the alteration in IL-6 concentrations correlated with changes in DAS-28 before and after receiving therapy for patients with RA and with high disease activity.Other study was examined whether IL-6 concentration can change disease activity and found significant decrease in IL-6 levels of as DAS-28 decreased [23]. Although cross-sectional study didn't documented a significant relation between DAS-28 and IL-6 level, the alteration in the level of cytokines is well related with the clinical course of patients with high DAS-28. This result proposes that the levels of IL-6 could reflect the disease activity[24]. In the present study, IL-6 concentration is decreased by treatment. This finding may be consider as an example of dual impacts of IL-6.

Results of present study don't found an evidence for genetic correlation conferred by polymorphisms of (-174 G/C) IL-6 gene promoter with respect to susceptibility to RA and, there were 80% with GG IL-6 (-174 G/C) gene promoter polymorphism, 18.3% with GC and 1.6% with CC, while 98% of the control showed GG genotype with 1.6% with GC. Present study is agreed with Xiang Li *et al.* study [25] that carried out in Han population in Guangdong in China. Current study is disagreed with other Chinese study that conducted in Chinese Han population from Shandong Province, which investigated the (-174G/C, -597G/A, -572G/C) of IL-6 in promoter region and was reported after meta-analysis study that there is association between polymorphism of IL-6 promoter with RA. According to their results,they were proposed that rare IL-6 gene polymorphisms may correlate with RA capability in Han Chinese populations, and IL-6 may function a prime role in pathogenesis of RA, at least in the Chinese population [26].

Also, result of present study is agreed with another study carried out in Egypt by Gaber*et al.*[26] that design to determine the clinical significance of serum levels of IL-6 and (-174 G/C) IL-6 promoter polymorphism in RA patients. Results of Egyptian study were found sera IL-6 levels significantly elevated in patients with RA in comparison to control group with CC promoter polymorphism. Gaber*et al.* results were found that neither IL-6 nor DAS-28 would predict the IL-6 promoter polymorphism [27].However, additional investigation studies are needed to determine the validity of the association of (-174 G/C) IL-6 gene promoter polymorphism with RA.

Results of current study concerning allele association of (-174 G/C) IL-6gene promoter polymorphism of patients with RA were shown that the allele C is the disease allele when comparing between patients with

RA and healthy control, due to the finding of presence of high level of IL-6 in RA patients related with CC genotyping and GC genotyping, as well as normal level of IL-6 in RA patient and controls with genotyping GG. Therefore, current study concludes that the allele responsible for the disease is C allele which was associated with an increased IL-6 concentration.

Also, results of present study are agreed with results of other study carried out by Xiang Li *et al*. in population of Chinawhich was found that (-174G/C) IL-6 polymorphisms are associated with the RA risk, and the C allele of IL-6 promoter polymorphisms has dramatically elevate the susceptibility of RA in population of China[28].

Conclusions

The increasedconcentration of IL-6, and its positive relation with DAS-28 may propose a probablefunction ofIL-6 in the RA pathogenesis. Moreover, these biomarkers can be used as markersof disease activity in the diagnosis and treatment of RA.The polymorphismsof (-174G/C) IL-6 are also correlated with the RA risk, and the allele C of IL-6 promoter polymorphisms has dramatically rise the susceptibility of RA in Babylon population. This finding proposes that the (-174 G/C) IL-6 gene promoter polymorphism may be used as genetic marker to the onset and development of RA in Babylon population.

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Conflicts of Interest

There is no conflicts of interest in this study.

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