Alexandria Journal of Medicine

ISSN: 2090-5068 (Print) 2090-5076 (Online) Journal homepage: https://www.tandfonline.com/loi/tajm20

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To cite this article: Ali H. Ad'hiah, Aseel S. Mahmood, Abdul-Kareem A. Al-kazaz & Khadier K. Mayouf (2018) Gene expression and six single nucleotide polymorphisms of interleukin-6 in rheumatoid arthritis: A case-control study in Iraqi patients, Alexandria Journal of Medicine, 54:4, 639-645, DOI: 10.1016/j.ajme.2018.08.001

To link to this article: https://doi.org/10.1016/j.ajme.2018.08.001

Alexandria Journal of Medicine 54 (2018) 639–645

HOSTED BY

Contents lists available at ScienceDirect

Alexandria Journal of Medicine

journal homepage: http://www.elsevier.com/locate/ajme

Original Article

Gene expression and six single nucleotide polymorphisms of interleukin-6 in rheumatoid arthritis: A case-control study in Iraqi patients

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ARTICLE INFO

Article history:
Received 15 March 2018
Revised 6 June 2018
Accepted 16 August 2018
Available online 19 September 2018

Keywords:
Rheumatoid arthritis
Interleukin-6
Single nucleotide polymorphism
Gene expression
Etanercept

ABSTRACT

Background: Interleukin-6 (IL-6) plays a significant role in pathogenesis of rheumatoid arthritis (RA), but its single nucleotide polymorphisms (SNPs), as well as therapy may modulate such role.
Objectives: It was aimed to determine gene expression and six SNPs (rs1800796 C/G, rs7802307 A/C/T, rs7802308 A/T, rs36215814 A/G, rs184229712 A/G and rs867254801 C/G) of *IL6* in etanercept-treated Iraqi Arab RA patients.
Materials and methods: Fifty-one RA patients and 45 controls were enrolled, and the determinations were carried out by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Sanger's DNA sequencing. Disease activity and laboratory markers were considered in these evaluations, which were the first presentation in Iraqi patients.
Results: The ΔCt mean of *IL6* mRNA showed a significant increase in RA patients compared to control (9.084 ± 0.964 *vs.* 6.780 ± 2.240; $p = 0.0001$). In terms of a relative expression, the $2^{-\Delta\Delta Ct}$ means showed no significant variations between subgroups of patients distributed by clinical and laboratory findings, with the exception of C-reactive protein (CRP). CRP-positive patients showed a lower mean compared to CRP-negative patients (0.201 ± 0.109 *vs.* 0.312 ± 0.131; $p = 0.001$). Distributing patients by gender and duration of disease also revealed significant variations between male and female patients. With respect to SNPs, allele and genotype frequencies of four SNPs (rs1800796, rs7802307, rs184229712 and rs867254801) showed variations between patients and controls, while no differences were reported for rs7802308 and rs36215814 SNPs. In addition, *IL6* gene expression was significantly influenced by two SNP genotypes (rs36215814 GA and rs184229712 AG) compared to the corresponding GG genotypes.
Conclusion: Gene expression of *IL6* was down-regulated in RA patients, especially CRP-negative patients. Moreover, four SNPs of such cytokine may have a role in RA risk.

1. Introduction

Rheumatoid arthritis (RA) is a multifactorial and a heterogeneous autoimmune disease. Both genetic and environmental factors contribute to its etiology and pathogenesis.[1] Environment-gene interactions in RA are suggested to promote the immune system of patients to loss tolerance to self-proteins, especially those that contain a citrullinated peptide, which is produced by a post-translational modification.[2] This is followed by a localized inflammatory response that occurs in joints by mechanisms that may involve different humoral and cellular immune responses, in which microvascular and tissue-specific immunological components are involved. Initiation and perpetuation of synovitis is the outcome of these reactions. This is evident by a characterized hyperplasia in the synovium of RA patients.[3] Thus, innate and adaptive immune responses are dysregulated in RA patients, and cytokines are a major player in mediating these responses.

Abbreviations: ACCP, anti-cyclic citrullinated protein; CRP, C-reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; HWE, Hardy-Weinberg equilibrium; IL-6, interleukin-6; RA, rheumatoid arthritis; RF, rheumatoid factor; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SNP, single nucleotide polymorphism.

Peer review under responsibility of Alexandria University Faculty of Medicine.

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https://doi.org/10.1016/j.ajme.2018.08.001
2090-5068/

Cytokines play a significant role in etiopathogenesis of RA. They are probably responsible for inflammatory reactions and joint destruction that occur during the course of disease. One these cytokines is interleukin-6 (IL-6), which is a 26 kDa glycoprotein produced by various cells; including T and B cells, monocytes, fibroblasts, osteoblasts, keratinocytes, endothelial cells and mesangial cells. Immunologically, IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory properties and plays multiple roles in adaptive immunity.[4] In RA patients, IL-6 showed increased levels in serum and synovial fluid, and such increase was positively correlated with disease severity and joint destruction.[5] Synovitis is also promoted by IL-6 via inducing neovascularization, in which vascular endothelial growth factors mediate the proliferation and outcomes in inflammatory cell infiltration and hyperplasia of synovium.[6] Further evidence suggests that IL-6 is a mediator of joint erosion and causes bone resorption by inducing the formation of osteoclasts. Cartilage degeneration is also caused by IL-6 via matrix metalloproteinases produced by synovial cells and chondrocytes.[7] IL-6 is also responsible for mediating many of the inflammatory manifestations in RA patients; for instance, it induces hepatocytes to produce C-reactive protein (CRP).[4]

Tumor necrosis factor-α (TNF-α) is a further cytokine involved in pathogenesis of RA. In a clinical practice, TNF-α has been a target for immunotherapy, and etanercept is one of these therapies. Etanercept-treated RA patients have shown a more rapid improvement in disease activity and a slower rate of radiographic progression than methotrexate-treated patients.[8] However, it has also been reported that etanercept decreases IL-6 levels in RA patients.[9]

The diverse roles played by IL-6 in RA pathogenesis and severity have been further explored in terms of its single nucleotide polymorphisms (SNPs). Some studies examined the association between $IL6_{-174}$ SNP (G/C) and RA susceptibility and activity in different ethnic populations, but the results were subjected to ethnicity-associated variations and diverse findings were reported.[10–12] However, a meta-analysis showed that this SNP is significantly associated with an increased RA risk in an overall population analysis, but it was more pronounced in Asians.[13] A further SNP (−572 G/C) of *IL6* gene has also been associated with an increased RA risk in Chinese Hans patients. The SNP was suggested to exert an influence on the transcription rate of IL-6, and may influence its serum level in RA patients.[14] Additional findings indicated that $IL6_{-174}$ SNP is associated with severe joint radiographic damage, while $IL6_{-572}$ showed no such association.[15]

Accordingly, this study was conducted to assess gene expression and six SNPs (rs1800796 C/G, rs7802307 A/C/T, rs7802308 A/T, rs36215814 A/G, rs184229712 A/G and rs867254801 C/G) of IL-6 in etanercept-treated RA Iraqi Arab patients, with some emphases on disease activity, as well as clinical and laboratory markers of disease.

2. Materials and methods

2.1. Patients

The present case-control study was approved by the ethics committee at the Ministry of Health in Iraq. Fifty-one Iraqi Arab RA patients were enrolled during the period November 2015 – June 2016. Their age range was 20–63 years. The diagnosis was made by the consultant medical staff at the Rheumatology Unit (Baghdad Teaching Hospital). It was based on the revised diagnostic criteria established by the American College of Rheumatology (ACR), 2010. They included tender and swollen joint counts, erythrocyte sedimentation rate (ESR), CRP, anti-cyclic citrullinated protein (ACCP) antibodies, rheumatoid factors (RFs) and symptom duration.[16] The RA patients were under therapy for a period of 3–5 years. It was a single weekly subcutaneous dose of 25 mg of etanercept.

The patients were sub-grouped according to gender, duration of disease (<5, 5–10 and >10 years), and laboratory markers of RA. The latter included RFs, CRP (positive and negative) and ACCP antibodies (weak, 20.0–39.9; moderate, 40.0–59.9; and strong positive, ≥60.0 U/ml). A further sub-grouping of patients was based on ESR-disease activity score (DAS)-28.[17]

In addition to patients, 45 apparently healthy individuals (controls), matched patients for ethnicity (Iraqi Arabs), gender and age, were also enrolled in the study. Their age range was 25–52 years. The control individuals were also assessed for ESR, RFs and CRP.

2.2. Gene expression of IL6

The blood was collected from RA patients in EDTA tubes during their visit to the Rheumatology Unit to take their subcutaneous dose of etanercept (after seven days of previous dose and an hour before the next dose). The expression of *IL6* gene was determined by the reverse transcription quantitative polymerase chain reaction (RT-qPCR) method after isolation of total RNA. A ready-to-use reagent (TRIzolTM LS Reagent; Thermo Fischer Scientific; USA) was used to isolate total RNA, and the instructions of manufacturer were followed.

The isolated RNA was reversely transcribed to cDNA using the GoTaq® 1-Step RT-qPCR System kit (Promega, USA). Forward (5′-AATTCGGTACATCCTCGACGG-3′) and reverse (5′-GGTTGTTTTCTGC CAGTGCC-3′) oligonucleotide primers of *IL6* gene were adopted from a previous study, in which the forward and reverse primers of the housekeeping gene *GAPDH* (reference gene: *glyceraldehyde-3-phosphate dehydrogenase*) were also given.[18]

The reaction mix was adjusted to a final volume of 20 μl as suggested by the manufacturer, and included: 10 μl GoTaq® qPCR Master Mix (1X), 0.5 μl GoScript™ RT Mix for 1-Step RT-qPCR (1X), 2 μl of each primer (10 μM), 5 μl RNA (62–110 ng/μl), and 0.5 μl nuclease-free water. The mix was transferred to a real-time thermocycler (SaCycler; Sacace, Italy), which was programmed for the following optimized cycles: cDNA synthesis for15 minutes at 37 °C (one cycle), initial denaturation for 5 min at 95 °C (one cycle), 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 60 °C) and extension (30 s at 72 °C), and finally one cycle of melt curve at 65–90 °C.

The expression was given as $2^{-\Delta\Delta Ct}$, which represents the Relative Fold Change. Therefore, the results were expressed as a fold change in the expression level of a target gene that was normalized to endogenous control (housekeeping gene) and relative to a calibrator, which is the target gene in control subjects.[19]

2.3. IL6 gene SNPs

The Genomic DNA was extracted from EDTA blood using ReliaPrepTM Blood gDNA Miniprep System (Promega Corporation, USA), and after assessing purity and concentration, it was subjected to PCR amplification. Forward (5′-GGAGTCACACACTC CACCT-3′) and reverse (5′-CTGATTGGAAACCTTATTAAG-3′) primers of *IL6* were adopted from a previous study.[12] The PCR reaction was performed in a final volume of 25 μl, which included 12.5 μl GoTaq green Master mix, 0.75 μl of each primer (10 μM), 2 μl DNA sample (50 ng) and 9 μl nuclease-free distilled water. The PCR conditions were initial denaturation at 95 °C for 5 min (one cycle), followed by 35 cycles of denaturation at 95 °C (30 s), annealing at 60 °C (30 s) and extension at 72 °C (30 s), followed by a final extension at 72 °C for 7 min. The amplified PCR fragments were subjected to Sanger's sequencing using ABI3730XL automated DNA sequencer (Macrogen Corporation – Korea). The *IL6* gene SNPs (rs1800796, rs7802307, rs7802308, rs36215814, rs184229712 and rs867254801) and their genotypes were revealed by the

Geneious software version 10.2.2 after alignment with reference sequences in the Gene Bank.

2.4. Statistical analyses

Data of gene expression were given as mean ± standard deviation (SD), and significant differences between means were assessed by ANOVA (Analysis of Variance) followed by LSD (Least Significant Difference) or multiple range Duncan test. A probability that equals or less than 0.05 was considered significant. These analyses were carried out by using the statistical package SPSS version 13.0.

Allele frequencies of *IL6* SNPs were estimated by the direct gene counting method, while a significant departure from Hardy-Weinberg equilibrium (HWE) was estimated using HWE calculator for two alleles. Significant differences between the observed and expected genotype frequencies were assessed by Pearson's Chi-square test.[20]

Allele and genotypes of *IL6* SNPs were given as percentage frequencies, and significant differences between their distributions in RA patients and controls were assessed by Fisher's exact probability (p), which was corrected for the number of comparisons made at each locus (Bonferroni Correction). In addition, odds ratio (OR) and its 95% confidence interval were also estimated to define the association between allele or genotype and RA. The WINPEPI package version 11.36 was used to carry out these estimations.

3. Results

3.1. Basic characteristics of patients and controls

Clinical and laboratory characteristics (gender, age, duration of disease, DAS-28, RFs, CRP, ACCP antibodies and ESR) of RA patients and control are given in Table 1. It worth to mention, that control subjects were sero-negative for RFs and CRP, and their ESR mean was within the normal range (6.3 ± 2.9 mm/hour).

3.2. Gene expression of IL6

The ΔCt of *IL6* mRNA showed a significant increased mean in total, male and female RA patients compared to the corresponding means in controls (Table 2). However, when RA patients distributed according to their clinical and laboratory findings, no significant variation between the $2^{-\Delta\Delta Ct}$ means of their subgroups was observed, with the exception of CRP. Patients sero-positive for CRP showed a lower mean of $2^{-\Delta\Delta Ct}$ compared to sero-negative patients (Table 3). In addition, presenting $2^{-\Delta\Delta Ct}$ means in male and female patients distributed by duration of disease revealed significant differences. Female patients at durations of disease <5 and >10 years showed an increased mean of *IL6* mRNA folding expression compared to male patients. For the disease duration 5–10 years, an opposite observation was made; male patients showed a higher mean than female patients (Table 4).

3.3. IL6 gene SNPs

Six SNPs with polymorphic frequencies (rs1800796 C/G, rs7802307 A/C/T, rs7802308 A/T, rs36215814 A/G, rs184229712 A/G and rs867254801 C/G) were assigned in the DNA sequence of the PCR amplified region (527 bp) (Fig. 1). The genotype frequencies of these SNPs were in a good agreement with HWE in RA patients and controls, as there were no significant differences between the observed and expected genotype frequencies. However, comparing patients to controls revealed variations in four SNPs only (Table 5).

The first SNP was rs1800796 (C/G). It was observed with three genotypes in RA patients (CC, CG and GG) that had frequencies of 92.1, 5.9 and 2.0%, respectively. The corresponding *C* and *G* allele frequencies were 95.1 and 4.9%, respectively. Among controls, only the wild type genotype (CC) was found (100%). Although the mutant allele *G* was recorded in 4.9% of RA patients and none of the controls, no significant variation was observed between the two groups.

Table 1
Clinical and laboratory characteristics of rheumatoid arthritis patients and control.

Characteristic		Patients (N = 51)	Control (N = 45)
Gender: N (%)	Male	22 (43.1)	15 (33.3)
	Female	29 (56.9)	30 (66.7)
Age (Mean ± SD; years)		44.9 ± 10.7	41.3 ± 8.7
Duration of disease: N (%)	<5 years	14 (27.5)	–
	5–10 years	26 (51.0)	–
	>10 years	11 (21.5)	–
DAS-28: N (%)	Low	2 (3.9)	–
	Medium	29 (56.9)	–
	High	20 (39.2)	–
RF: N (%)	Positive	27 (52.9)	0.0
	Negative	24 (47.1)	100.0
CRP: N (%)	Positive	33 (64.7)	0.0
	Negative	18 (35.3)	100.0
ACCP: N (%)	Weak positive	24 (47.1)	NT
	Moderate positive	8 (15.7)	NT
	Strong positive	19 (37.2)	NT
ESR (Mean ± SD; mm/hour)		50.9 ± 29.5	6.3 ± 2.9

SD: Standard deviation, N: absolute number, DAS-28: Diseases activity score-28, RF: Rheumatoid factors, ACCP: Anti-cyclic citrullinated peptide, ESR: Erythrocyte sedimentation rate, NT: Not tested.

Table 2
The ΔCt means of *IL6* mRNA in rheumatoid arthritis patients and controls.

Groups		Number	ΔCt (Mean ± SD)	p-value
Total	Patients	51	9.084 ± 0.964	0.0001
	Controls	45	6.780 ± 2.240	
Males	Patients	22	9.181 ± 1.088	0.013
	Controls	15	7.793 ± 0.608	
Females	Patients	29	9.017 ± 0.888	0.0001
	Controls	30	6.273 ± 2.574	

SD: Standard deviation.

Table 3
Expression fold ($2^{-\Delta\Delta Ct}$) of *IL6* gene in rheumatoid arthritis patients.

Groups		Number	$2^{-\Delta\Delta Ct}$ (Mean ± SD)	p-value
Gender	Male	22	0.238 ± 0.154	NS
	Female	29	0.242 ± 0.107	
Duration of disease	<5 years	14	0.191 ± 0.067	NS
	5–10 years	26	0.257 ± 0.117	
	>10 years	11	0.262 ± 0.189	
DAS-28	Low	2	0.172 ± 0.098	NS
	Medium	29	0.229 ± 0.113	
	High	20	0.263 ± 0.152	
RFs	Positive	27	0.219 ± 0.119	NS
	Negative	24	0.263 ± 0.132	
CRP	Positive	33	0.201 ± 0.109	0.001
	Negative	18	0.312 ± 0.131	
ACCP	Weak positive	24	0.260 ± 0.146	NS
	Moderate positive	8	0.198 ± 0.110	
	Strong positive	19	0.232 ± 0.113	

SD: Standard deviationDAS-28: Diseases activity score-28, RFs: Rheumatoid factors, CRP: C-reactive protein, ACCP: Anti-cyclic citrullinated peptide, NS: Not significant.

Table 4
Expression fold ($2^{-\Delta\Delta Ct}$) of *IL6* mRNA in rheumatoid arthritis patients distributed by duration of disease and gender.

Duration of disease		Number	$2^{-\Delta\Delta Ct}$ (Mean ± SD)	*p*-value
<5 years	Males	6	0.162 ± 0.041	0.016
	Females	8	0.212 ± 0.076	
5–10 years	Males	9	0.319 ± 0.120	0.05
	Females	17	0.225 ± 0.107	
>10 years	Males	7	0.199 ± 0.195	0.008
	Females	4	0.372 ± 0.124	

SD: Standard deviation.

The AA genotype of the second SNP (rs7802307; A/C/T) showed a significant increased frequency in patients compared to controls (62.8 *vs.* 33.3%; $p = 0.004$; OR = 3.37; 95% CI = 1.47–7.74), and the difference remained significant after the correction probability ($pc = 0.02$). Such difference was more presented when the comparison was based on allele frequencies. The *T* allele showed a corrected significant decreased frequency in patients compared to controls (23.5 *vs.* 43.3%; $pc = 0.015$), while *A* allele showed a corrected significant increased frequency (76.5 *vs.* 56.7%; $pc = 0.015$). These findings suggest that *T* is a protective allele (OR = 0.40; 95% C.I. = 0.22–0.74), while *A* is a predisposing allele (OR = 2.49; 95% C.I. = 1.34–4.60).

The third SNP was rs184229712 (A/G), in which the homozygous genotype frequency of mutant allele (GG) showed a corrected significant increased frequency in patients compared to control (45.1*vs.* 26.7%; $pc = 0.003$; OR = 5.34; 95% C.I. = 1.94–14.66). In terms of allele frequencies, the wild *A* allele showed a corrected significant decreased frequency in patients compared to controls (23.4 *vs.* 56.7%; $pc = 0.0025$), while the mutant *G* allele showed a corrected significant increased frequency (67.6*vs.* 43.3%; $pc = 0.0025$). These findings suggest that *A* is a protective allele (OR = 0.37; 95% C.I. = 0.20–0.66), while *G* is a predisposing allele (OR = 2.73; 95% C.I. = 1.52–4.91).

The fourth SNP was rs867254801 (C/G). It was observed with three genotypes in RA patients (AA, AG and GG) and two genotypes in controls (AA and AG), which were correspondent to two alleles (*A* and *G*). Allele *A* showed a corrected significant decreased frequency in RA patients (63.7 *vs.* 80.0%; OR = 0.44; 95% C.I. = 0.23–0.84; $pc = 0.048$), while *G* allele showed a corrected significant

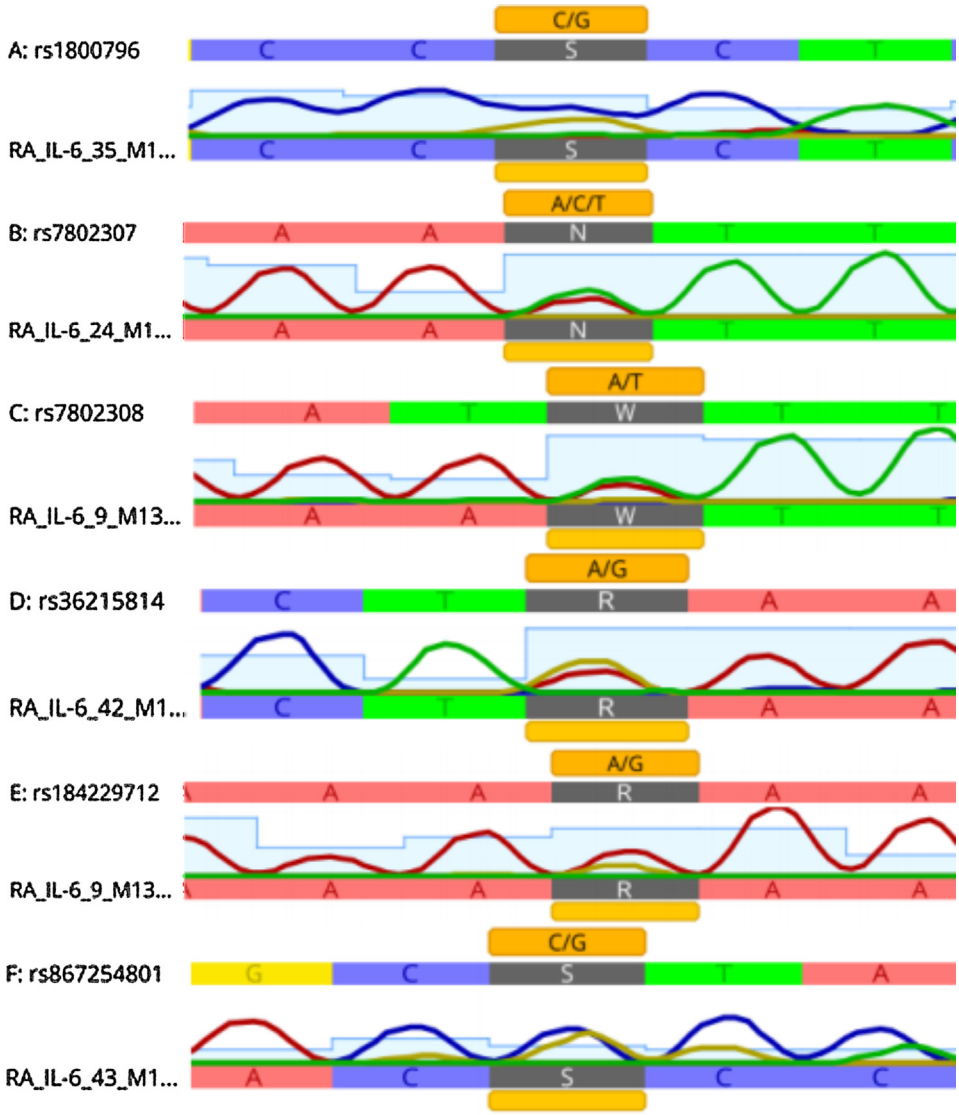

Fig. 1. DNA sequence chromatogram of six *IL6* gene SNPs (rs1800796 C/G, rs7802307 A/C/T, rs7802308 A/T, rs36215814 A/G, rs184229712 A/G and rs867254801 C/G) showing the heterozygous genotype of each SNP in rheumatoid arthritis patients (RA_IL_6). The reference sequences of SNPs are denoted with A, B, C, D, E and F, respectively.

Table 5
Allele and genotype frequencies of *IL6* gene single nucleotide polymorphisms in rheumatoid arthritis patients and control.

IL6 SNP allele and genotype	Percentage		OR	95% CI	*p*-value	Bonferroni Correction
	Patients (No. = 51)	Controls (No. = 45)				
rs1800796						
C	95.1	100.0	–	–	NS	NS
G	4.9	ND	–	–	NS	NS
CC	92.1	100.0	–	–	NS	NS
CG	5.9	ND	–	–	NS	NS
GG	2.0	ND	–	–	NS	NS
rs7802307						
A	76.5	56.7	2.49	1.34–4.60	0.003	0.015
T	23.5	43.3	0.40	0.22–0.74	0.003	0.015
AA	62.8	33.3	3.37	1.47–7.74	0.004	0.002
AT	27.5	46.7	0.43	0.19–1.00	0.04	NS
TT	9.8	20.0	0.43	0.14–1.39	NS	NS
rs7802308						
A	52.0	57.0	0.83	0.47–1.46	NS	NS
T	48.0	43.0	1.21	0.69–2.13	NS	NS
AA	27.5	33.3	0.76	0.32–1.80	NS	NS
AT	49.0	46.7	1.10	0.50–2.43	NS	NS
TT	23.5	20.0	1.23	0.47–3.23	NS	NS
rs36215814						
G	92.2	86.7	1.81	0.71–4.62	NS	NS
A	7.8	13.3	0.55	0.22–1.41	NS	NS
GG	84.3	73.3	1.95	0.72–5.27	NS	NS
GA	15.7	26.7	0.51	0.19–1.38	NS	NS
AA	ND	ND	–	–	NS	NS
rs184229712						
A	32.4	56.7	0.37	0.20–0.66	0.0005	0.0025
G	67.6	43.3	2.73	1.52–4.91	0.0005	0.0025
AA	9.8	26.7	0.30	0.10–0.92	0.029	NS
AG	45.1	60.0	0.55	0.25–1.22	NS	NS
GG	45.1	13.3	5.34	1.94–14.66	0.0006	0.003
rs867254801						
G	63.7	80.0	0.44	0.23–0.84	0.0095	0.048
A	36.3	20.0	2.28	1.19–4.37	0.0095	0.048
GG	35.3	60.0	0.36	0.16–0.83	0.013	NS
GA	56.9	40.0	1.98	0.88–4.42	NS	NS
AA	7.8	ND	–	–	NS	NS

SNP: Single nucleotide polymorphism, OR: Odds ratio; CI: Confidence interval; NS: Not significant; ND: Not detected.

increased frequency in patients (36.3 *vs*. 20.0%; OR = 2.28; 95% C.I. = 1.19–4.37; *pc* = 0.048).

Finally, allele and genotype frequencies of rs7802308 (A/T) and rs36215814 (A/G) SNPs showed no significant variations between patients and controls.

3.4. Impact of genotypes on IL6 expression

Out of the six investigated SNPs, two SNP genotypes impacted the expression of *IL6* gene in RA patients. The GA genotype of rs36215814 SNP was associated with a significant increased mean of $2^{-\Delta\Delta Ct}$ compared to GG genotype. The second SNP was rs184229712, in which the $2^{-\Delta\Delta Ct}$ mean was significantly increased in AG genotype compared to GG genotype (Table 6).

4. Discussion

The ΔCt of *IL6* mRNA showed a significant increased mean in total RA patients compared to controls, and a similar observation was made in male and female patients. Such findings suggest that *IL6* gene expression was down-regulated in RA patients. Assessing the $2^{-\Delta\Delta Ct}$ mean revealed that the relative expression was below one. When the clinical and laboratory characteristics of RA patients were considered (duration of disease, DAS-28, RFs, CRP and ACCP antibodies), no significant variation was observed between the $2^{-\Delta\Delta Ct}$ means of their subgroups, with the exception of CRP. Patients sero-positive for CRP showed a significantly lower mean of $2^{-\Delta\Delta Ct}$ compared to sero-negative patients. These results are not consistent with the general functional role of IL-6 in

Table 6
Impact of single nucleotide polymorphism genotypes on *IL6* expression in rheumatoid arthritis patients.

IL6 SNP	$2^{-\Delta\Delta Ct}$ (Mean ± SD)		
rs1800796	CC (0.242 ± 0.128)^{A}	CG (0.228 ± 0.140)^{A}	GG (0.137)
rs7802307	AA (0.235 ± 0.125)^{A}	AT (0.256 ± 0.132)^{A}	TT (0.224 ± 0.125)^{A}
rs7802308	AA (0.196 ± 0.082)^{A}	AT (0.251 ± 147)^{A}	TT (0.266 ± 0.121)^{A}
rs36215814	GG (0.216 ± 0.108)^{B}	GA (0.366 ± 0.155)^{A}	AA (Not detected)
rs184229712	AA (0.225 ± 0.116)^{AB}	AG (0.273 ± 0.136)^{A}	GG (0.154 ± 0.092)^{B}
rs867254801	GG (0.213 ± 0.119)^{A}	GA (0.246 ± 0.136)^{A}	AA (0.309 ± 0.061)^{A}

Similar superscript letters represent no significant difference between means of each SNP, while different superscript letter represent significant difference ($p \leq 0.05$) between means (horizontal comparison; multiple range Duncan test). SNP: Single nucleotide polymorphism, SD: Standard deviation.

pathophysiology of RA. IL-6 was found to be increased in serum and synovial fluid of RA patients, and such increase was correlated with RA activity. This statement further confirms the importance of conducting a correlation test between the expression fold of *IL6* gene and the DAS-28. Such test revealed a gradual increased expression as RA patients progressed from low to high DAS-28, but the difference was no significant. It has further been documented that this cytokine is connected with local inflammation and joint destruction in RA patients.[21] IL-6 also showed an increased expression in the inflamed synovial tissue of RA patients, and influenced the functions of macrophages and T and B lymphocytes and osteoclasts.[22] The pathological role of IL-6 was confirmed in mouse model of collagen-induced arthritis (CIA), and anti-IL-6R treatment was associated with amelioration of RA histopathological features (inflammatory synovitis and joint erosions) in these animals. Gene deficiency and blockade of IL-6 activity also reduced severity of arthritis in mouse model of RA.[23,24]

The discrepancy between the present results and the above mentioned studies might be ascribed to the therapy status of RA patients who were under the anti-TNF therapy etanercept, but such conclusion is limited because untreated patients were not included in the study. However, TNF inhibitors have been shown to reduce serum levels of IL-6.[25] It has been documented further that a pretreatment with etanercept in HIV patients may blunt activation of IL-6 and CRP expression induced by recombinant human IL-2.[26] In a further investigation, the influence of TNF-α on inflammation in CCl_4-induced rats treated with etanercept was studied. It was found that the expression of IL-6 was decreased as the concentration of TNF-α was blocked by increased doses of etanercept.[27] These findings clearly suggest the pathologic role of IL-6 in etiology of RA.

Such pathogenic-etiological role of IL-6 can be further understood when the gene polymorphism of such cytokine is determined. The present results demonstrated that GG genotype of *IL6* SNP rs184229712 (A/G) showed a significant increased frequency in RA patients compared to controls, and the associated OR was 5.34. Such difference was more pronounced when the comparison was based on allele frequencies, and *G* allele showed a corrected significant increased frequency (susceptibility allele), while *A* allele frequency was significantly decreased (protective allele). However, such SNP has not been investigated in RA, but other SNPs in vicinity of rs184229712 SNP has been studied and different results were reported. $IL6_{-174}$ (G/C) is among these SNPs, and studies have suggested its role in RA susceptibility and activity in RA patients of different ethnicities, and different results were reported. Such differences were related to the ethnicity of patients under investigation.[10–12] This SNP has also been subjected to a meta-analysis of different studies (European and Asian in origin), and its effect on risk of RA was suggested, especially in Asian patients.[13] Another *IL6* SNP ($IL6_{-572}$: G/C) has also been investigated and its association with RA susceptibility or severity has been suggested in patients of different ethnicities.[14,15]

The AG genotype of *IL6* rs184229712 SNP was observed to be significantly associated with an increased expression of *IL6* gene compared to GG genotype; therefore, it is suggested that such SNP may influence the gene expression of *IL6* and subsequently its serum level in RA patients. The $IL6_{-174}$ and $IL6_{-572}$ SNPs have also been suggested to exert an influence on the transcription rate of IL-6 protein, and may influence IL-6 serum level in RA patients.[14]

5. Conclusions

A down-regulation of *IL6* gene expression was suggested in RA patients, and it was possibly due to the effects of the anti-TNF therapy etanercept. Moreover, such expression was influenced by the genotypes of two *IL6* SNPs (rs36215814 and rs184229712). An association between four *IL6* SNPs (rs1800796, rs7802307, rs184229712 and rs867254801) and risk of RA is also suggested in Iraqi patients. However, the results could be more informative if two other groups of patients were investigated; untreated patients and patients under other types of therapies. The latter subgrouping of patients represents a limitation of present study. Moreover, further work in relation to other specific biological therapies (for instance, tocilizumab) versus the standard therapy methotrexate with follow up of patients can illustrate a better profile about the role of IL-6 in RA pathology and etiology.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that there is no conflict of interest.

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