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Association of genetic variation in tumor necrosis factor-α gene with susceptibility to rheumatoid arthritis in southern Iraq

Sara H. Jabbar
Department of Medical Lab Technology,
College of Health and Medical Technology, Southern Technical University,
Basra/Iraq
sara96941992@gmail.com

Khairallah A. S. Mohammed
Department of Medical Lab Technology,
College of Health and Medical Technology, Southern Technical University,
Basra/Iraq
dr.kmohammed@stu.edu.iq

Naael H. Ali
College of Medicine, University of Basra
Basra/Iraq
naaelhali66@gmail.com

***Abstract*—Background: Rheumatoid arthritis is the most commonly occurring systemic autoimmune disease caused by genetic, epigenetic, and environmental factors. It affects 0.5-1% of the worldwide population and increases in incidence with age. Tumor necrosis factor-α, major candidate gene in the pathogenesis of RA. Gene polymorphisms occurring in this pro-inflammatory cytokine may increase the risk of RA. This study aimed to evaluate the association between polymorphisms in TNF-α gene at location -308(rs1800629), -238(rs361525) and -376(1800750) (G/A) and RA susceptibility. The study also evaluated the effects of these polymorphisms on their corresponding gene levels in serum.**

Methods: This case-control study was conducted on 49 individuals (30 RA patients and 19 age- and sex-matched healthy volunteers as controls). Genomic DNA extracted from all participant blood, then amplified by PCR and genotyped by sequencing.Additionally, the serum level of TNF-α was quantified using an enzyme-linked immunosorbent assay (ELISA).

Results: The results showed that thehomozygosity for the *TNF-α -308 G* allele significantly act as a genetic factor associated with increased RA risk (p = 0.029, OR: 4.727, 95%CI: 1.175–19.016). The A allele of -238 and -376 polymorphisms may act as a possible risk factor for RA (OR: 7.63, 95%CI:0.409-14.07; OR:6.13, 95%CI:.0.32-11.214). However, no significant differences were observed.

Conclusion: The GG genotype ofTNF-α -308 SNP associated with increased rheumatoid arthritis risk.

***Keywords*—TNF-α, genetic polymorphism, rheumatoid arthritis, Susceptibility**

I. INTRODUCTION

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is defined by chronic synovial joint inflammation and subsequent synovial tissue proliferation, which eventually leads to cartilage and bone damage (Calabresi *et al.*, 2018).The global prevalence of RA is estimated to be between 0.5 and 1%., and the prevalence in females is four times that of males, with an increasing incidence with age (Okada *et al.*, 2019). In 2013, Alkazzaz(2013) reported an increase in RA incidence in Iraq from 1.60% in 2001 to 3.02% in 2011, while Al-Rawi*et al.* (1978) reported a 1% incidence rate in Iraq in 1978.

Although the pathogenic mechanism of RA is unknown (Yurkovich *et al.*, 2014), accumulating evidence has emphasized that tumor necrosis factor-α (TNF-α) is essential in the inflammatory course of this disease (Cope *et al.*, 1992; Choy & Panayi, 2001;Plenge *et al.*, 2007;Kokkonen *et al.*, 2010). TNF-α is secreted primarily by macrophages, but it can also be produced by lymphocytes, natural killer cells, and mast cells (Furst *et al.*, 2006). TNF-α is produced as a membrane-bound (memTNF) and a soluble (sTNF) protein. TNF-α converting enzyme (TACE) splits memTNF to produce sTNF. (Fragoso, 2014). Once released, it either binds to TNFR1 (p55) or TNFRII (p75) and triggers a cascade of proinflammatory cytokines that promotes adhesion molecule expression, boosts neutrophil activation, and acts as a costimulator for T cell activation and antibody production (Kalliolias & Ivashkiv, 2016); therefore, it has been a target for immunotherapy in RA patients (Fischer *et al.*, 2015). TNF is genetically localized to the short arm of chromosome 6 (6p21.3) within the HLA class III region, which codes for major immune response genes involved with RA susceptibility (Hachicha *et al.*, 2018).

A number of single nucleotide polymorphisms (SNPs) in the TNF-α gene have been linked to RA in patients (Jahid *et al.*, 2017). Among these, the G to A substitution at positions -238 (rs361525), -308 (rs1800629) and -376 (rs1800750) attracted widespread attention. SNPs can affect the behavior of coding protein. In fact, SNPs can result in altered gene

expression and thus affect gene function (Verweij, 1999;JHA *et al.*, 2019).

Several case-control studies were done to explore the connection of these polymorphisms with RA, but the conclusions from these reports are conflicting (Ates *et al*., 2008;Kobayashi *et al.*, 2009;Emonts *et al*., 2011;Hussein *et al.*, 2011;You *et al.*, 2013;Lagha *et al*., 2015;Domínguez-Pérez *et al*., 2017).The inconsistency could be due to a small sample size in some of the studies, ethnicity, or other factors**.** Since similar studies are still limited in Iraq, and to redouble these findings in Iraqi populations**,** our study was aimed to demonstrate the potential roles ofpreviously reported -238, -308 and -376 (G/A) polymorphisms in the TNF-α gene in susceptibility to RA andtheir association with TNF-α serum level in a sample of the southern Iraqi population.

II. METHODS

A. Study population

We conducted a case-control study consisting of 30 patients with RA (age range: 27-70 years; 9 males and 21 females), diagnosed according to 2010 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) RA classification criteria (Aletaha*et al*., 2010). Patients were recruited from the rheumatology unit in the Basra Teaching Hospital from November 2021 to March 2022. The control group consisted of 19 apparently healthy people without history of immunological diseases (age range: 27-57 years; 7 males and 12 females). The inclusion criteria were age above 18 years and without concomitant auto-immune disorder. Patients on corticosteroid therapy in the past 3 months and pregnant women were excluded. The complete history was reported, and a comprehensive questionnaire has been filled out. The questionnaire included questions about age, gender, region, height, weight, family history, duration of disease, and duration of morning stiffness. The ethics committees gave their approval to this study (No: 448/2021; Date: 03/11/2021), and all the participants signed their written informed consent for voluntary involvement in the study.

B. Clinical samples

All patients and controls provided blood samples in a K2-EDTA tube and gel tube.One tube was processed for molecular study, and the second tube was processed for immunological study.

C. Extraction of DNA

According to the manufacturer's instructions, genomic DNA was extracted from WBC using a commercially available kit (Promega, USA). Isolated DNA was confirmed for integrity and concentration using agarose gel electrophoresis and a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), and then kept at -20 °C until needed.

D. Molecular study (PCR amplification and sequence analysis)

The forward primer 5'-AACCAGCATTATGAGTCTC-'3 and revers primer 5'AACAACTGCCTTTATATGTC'3 (Pollo *et al.*, 2019) were used to amplify a 677-bp product in TNF-α promotor region by thermocycler (Applied Biosystem, US)**.** The PCR component consists of2 µl of DNA, 1 µl per primer, 12.5 µl master mix, and 8.5 µl of nuclease-free water**.** The following thermocycling program was used for PCR amplification: initial denaturation of 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec., annealing at 52°C for 45 sec., extension at 72°C for 1 min. Final extension of 8 minutes at 72°C. Then**,** theamplicon was sent for sequencing by the Sanger method using a DNA analyzer (Illumine, Macrogen firm, South Korea), and the obtained results were analyzed and aligned.

E. Immunological study

Circulating level of TNF-α in current study was measured by utilizing ELISA sandwich assay. SUNLOG ELISA kit (Cat No. SL1761Hu) was used according to the manufacturer's specifications. The absorbance was read at 450 nm wavelength.

F. Statistical analysis

The chi-square test or Fisher's exact test was used to examine the genotype and allele distributions of patients and controls. The odds ratio (OR) and the 95% confidence interval (CI) were calculated. The age and gender distributions of both groups were compared using the Pearson Chi-Square and t-test. The Mann-Whitney U test was used to discuss the difference in serum levels of TNF-α between patients and controls with different genotypes. The Statistical Package for Social Sciences (SPSS) Version 26 software was used. A P value of less than 0.05 was considered significant.

III. RESULTS

Table (1) shows the demographic and clinical characteristics. There was no statistical significance between the patient and control groups in terms of age or gender (p= 0.284 and p=0.655, respectively). Females were shown to have a much higher rate of RA than males (70% vs. 30%).

TABLE 1: DEMOGRAPHIC AND CLINICAL CHARACTERISTIC DATA OF THE STUDY SUBJECTS

Demographic characteristics		Patients	Healthy people	P-value
		n=30	n=19	
Age** years		48.27±12.457	46.93±9.157	0.284
Sex*	M	9 (30.0%)	7(36.8%)	0.655
	F	21(70.0%)	12(63.2%)	
Cigarette smoking*	Yes	5 (16.7%)	-	-
	No	25(83.3)	-	
Residency*	Urban	21 (70%)	-	-
	Rural	9 (30%)	-	

Clinical characteristics				
Disease durationyears**		9.87±4.974	-	-
Family history of RA*	**Yes**	10(33.3%)	-	-
	No	20(66.7%)	-	
Morning stiffness(minutes)**		15.73±22.806		-
Combined therapy*	**Yes**	21(70%)	-	-
	No	9(30%)	-	
BMI(kg/m2)**		29.0557±3.49616	-	-

Values are *number and percentage or **mean±SD.Abbreviations: *n*: number of cases; SD: standard deviation; M: male; F: female; RA: rheumatoid arthritis; BMI: Body Mass Index; DAS 28: disease activity score; bold text: significant p-value

Our study showed that none of the analyzed polymorphisms have mutant homozygous genotypes (AA). The GG genotype of TNF-α -308G/A SNP was found to be significantly related to RA risk at statistical levels ($p < 0.05$) (Table 2), (figure 1 and 2), where the GG genotype was more frequent in patients (86.7%) compared to controls (57.9%) (OR: 4.727; 95%CI: 1.175-19.016, p = 0.029), while the GA genotype was more frequent in controls (42.1%) compared to patients (13.3%) (OR: 0.211; 95%CI: 0.052-0.851). Similarly, the frequency of the G allele was also higher in patients than in controls (93.3% vs. 78.9%; OR: 3.733, 95%CI: 1.038-13.421), with a statistically meaningful difference(p = 0.04). The frequency of the A allele was higher in controls than in patients (21.1% vs. 6.7%; OR: 0.267, 95%CI: 0.074-0.963).

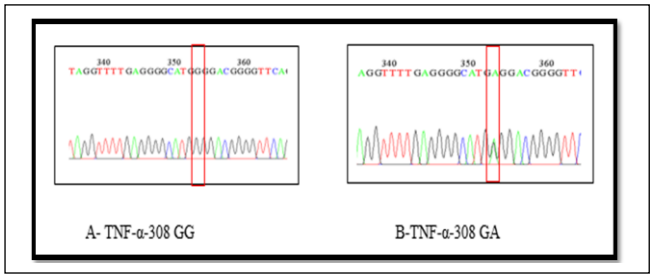

Figure 1: Sequence chromatograph results of TNF-α (-308G/A) SNP showing two genotypes.

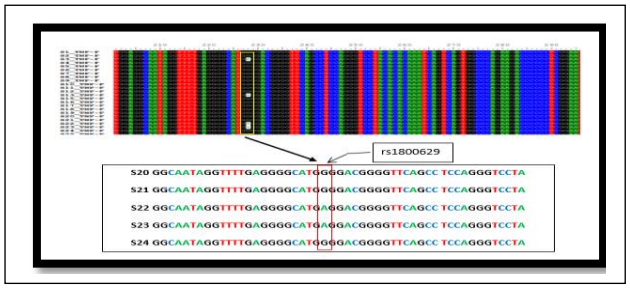

Figure2: The sequences alignment of TNF-α (-308G/A) SNP

The TNF-α -238 GG genotype was found in 83.3% of patients with RA and 100% of controls (OR: 0.118, 95%CI: 0.6-2.281, p = 0.157), while the GA genotype was found in 16.7% of patients and 0.0% of controls (OR: 8.411; 95%CI: 0.438-16.44) (Table 3). Concerning the allele frequencies, the G allele was higher in controls than in patients (100% vs. 91.7%; OR: 0.131, 95%CI: 0.7-2.44), whereas the A allele was higher in patients than in controls (8.3% vs. 0%; OR: 7.63, 95%CI :0.409–14.07).

The TNF-α -376 GG genotype was found in 86.7% of patients with RA and 100% of controls (OR: 151, 95%CI: 0.07-2.972, p = 0.213), while the GA genotype was detected in 13.3% of patients and 0.0% of controls (OR: 6.622, 95%CI: 0.336-13.351) (Table 4). Concerning the allele frequencies, the G allele was higher in controls than in patients (100% vs. 93.3%; OR: 0.163, 95%CI: 0.8-3.11), whereas the A allele was higher in patients than in controls (6.7% vs. 0%; OR: 6.13, 95%CI: 0.32-11.241).

The odd ratio of A allele for both SNPs suggests that it may act as a possible risk factor; however, the difference did not reach significant levels.

Circulating TNF-α levels were not significantly correlated with any SNP genotype, according to the research (Table 2 to 4).

TABLE 2: FREQUENCIES OF TNF-A (-308G/A) GENOTYPES/ALLELES AND THEIR CORRELATION WITH TNF-A CIRCULATING LEVELS BETWEEN PATIENT AND HEALTHY PEOPLE

SNP	**Molecular study**				**Immunological study**	
Genotype	**Patient vs. Healthy people**				**TNF-α serum level Median (pg/ml)**	
Allele						
-308G>A (rs1800629)	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**	**OR (95%CI)**	**P-value**	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**
GG	26(86.7)	11(57.9)	**4.727 (1.175-19.016)**	**0.029**	20.0	17.1
GA	4(13.3)	8(42.1)	**0.211 0.052-0.851**		22.15	14.8
AA	-	-	-	-	-	-
G	56(93.3)	30(78.9)	**3.733 (1.038-13.421)**	**0.04**		
A	4(6.7)	8(21.1)	**0.267 (0.074-0.963)**			
	P-value				0.446	0.186

TABLE 3: FREQUENCIES OF TNF-A (-238G/A) GENOTYPES/ALLELES AND THEIR CORRELATION WITH TNF-A CIRCULATING LEVELS BETWEEN PATIENT AND HEALTHY PEOPLE

SNP	**Molecular study**				**Immunological study**	
Genotype / Allele	**Patient vs. Healthy people**				**TNF-α serum level Median (pg/ml)**	
-238G>A (rs361525)	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**	**OR (95%CI)**	**P-value**	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**
GG	25(83.3)	19(100)	0.118 (0.006-2.281)	0.157	21.6	15.7
GA	5(16.7)	0(0)	8.411 (0.438-16.44)		15.7	
AA	-	-	-	-	-	
G	55(91.7)	38(100)	0.131 (0.7-2.44)	0.173		
A	5(8.3)	0(0)	7.63 (0.409-14.07)			
	P-value				0.266	

TABLE 4: FREQUENCIES OF TNF-A (-376G/A) GENOTYPES/ALLELES AND THEIR CORRELATION WITH TNF-A CIRCULATING LEVELS BETWEEN PATIENT AND HEALTHY PEOPLE

SNP	**Molecular study**				**Immunological study**	
Genotype / Allele	**Patient vs. Healthy people**				**TNF-α serum level Median (pg/ml)**	
-376G>A (rs1800750)	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**	**OR (95%CI)**	**P-value**	**Patient (n=30) N(%)**	**Healthy people (n=19) N(%)**
GG	26(86.7)	19(100)	0.151 (0.7-2.972)	0.213	21.75	15.7
GA	4(13.3)	0(0)	6.622 (0.336-13.351)		17.5	
AA	-	-	-	-	-	
G	56(93.3)	38(100)	0.163 (0.8-3.11)	0.228		
A	4(6.7)	0(0)	6.13 (0.32-11.214)			
	P-value				0.127	

IV. DISCUSSION

Tumor necrosis factor-α, a pro-inflammatory cytokine, has a key role in the pathogenesis of RA (Vassalli, 1992). More than 200 variants of this gene have been found, three of which are the most extensively studied and associated with the risk of suffering from many diseases, mainly ankylosing spondylitis, RA, psoriasis and inflammatory bowel disease(Membrive Jiménez *et al.,* 2021). The functional significance of a genetic variation could potentially change the levels of gene expression (Fragoso, 2014;Ramírez-Bello *et al*., 2013;El-Tahan *et al.*, 2016).

In the present investigation, three SNPs in the TNF-α promoter region (-308 G/A (rs1800629), -238 G/A (rs361525), and -376 G/A (rs1800750)) were analyzed for their association with RA risk. -308G/A SNP of the TNF-α gene has been extensively investigated in RA patients, but contradictory results have been reported. The results of the current study detect a higher frequency of -308 GG genotype in patients compared to controls (86.7% vs. 57.9%; OR:4.727, 95%CI: 1.175-19.016) with a significant difference (p = 0.029), whereas the GA genotype was higher in controls compared to patients (42.1% vs. 13.3%, OR:0.211, 95%CI: 1.038-13.421). It seems that RA susceptibility is linked to the G allele of -308 (OR:3.733, 95%CI: 1.038-13.421), while the A allele appears to be protective against the disease (OR:0.267, 95%CI: 0.074-0.963).

Our results agreed with the results of an Egyptian study that demonstrated a positive association between G allele and GG genotype of TNF-α -308 SNP and RA susceptibility (Mosaad *et al.*, 2011). The current study also agreed with a study in North Indian RA patients, which concluded that the A allele of the TNF-α -308 SNP was linked to protection rather than susceptibility *(*Gambhir *et al.,* 2010). Our results also correspond to those of Alanzy*et al.* (2018), found that none of the patients possessed the AA genotype.

Nevertheless, our findings disagreed with Mahmood *et al.* (2017), and Ates*et al*. (2008) reported that TNF-α -308 is not a genetic risk factor for RA susceptibility. There are no associations also found in Pakistani and Egyptian RA patients(Rizvi *et al*., 2019;Elsherbini *et al.,* 2021). Also, disagree(d) with studies that identified a significant association of AA genotype with RA (Alwaeli *et al.,* 2020;Dhabaan, 2017;Lagha *et al.*, 2015).

Concerning investigation of TNF-α -238 G/A genetic variant, the GG genotype was present in 90% of patients and 100% of controls, while GA genotype was only found in 10% of patients and not detected in any of the controls (0%).The current study's findings, however, revealed a non-significant link between TNF-α -238 G/A polymorphisms and RA susceptibility. Which in line with Hadinedoushan*et al*. (2016) study that was conducted in Iran on 90 RA patients, discovered no connection between RA and the TNF-α -238 G/A polymorphism. This result was also redouble in Mexican and Kashmiri RA patients (Cadena-Sandoval *et al.,* 2018;Shafia *et al.,* 2016).Nonetheless, the findings of some studies identified an association between -238 A allele and RA development (Schmeling *et al.,* 2006;Kazkaz *et al.,* 2006;JimÊnez-Morales *et al.,* 2009). In contrast, several investigations have found that the G allele increases the risk of RA (Rizvi *et al.,* 2019;Chen *et al.,* 2019). A meta-analysis of Caucasian juvenile idiopathic arthritis patients discovered

that the A allele of TNF-238 plays a protective role (Li *et al.*, 2018).

Regarding investigation of TNF-α -376 G/A, the results of the current study detect GG genotype in 86.7% of patients and 100% of controls. The GA genotype was detected in 13.3% of patients, while it was not detected in any of the controls (0%). TNF-α -376 G/A investigation in thisstudy resulted in the conclusion that this SNP was not associated with RA ($p>0.05$). Which was agreedwith a study completed on 383 RA patients that determined that there is no association between the TNF-α -376 alleles and RA susceptibility (Brinkman *et al.*, 1997). The same result was obtained by subsequent studies carried out on a different population(Mousa *et al.*, 2014;Cadena-Sandoval *et al.*, 2018).On the other hand, the Emonts*et al.* (2011) study discovered a significant relation between the TNF-α -376 G allele and the risk of RA.

The absence of A allele in TNF-α -238 SNP (0% vs. 8.3%) and TNF-α -376 SNP (0% vs. 6.7%) from controls compared to patients may suggest its relevance with the disease. However, these differences did not reach a significant level.

Regarding the relationship between different genotypes of SNPs and circulating levels of TNF-α, all investigated SNPs showed a non-significant association. Results were agreed with Marotte *et al. (2008)*results detected a non-significant difference for the TNF-α circulating level with respect to -308 genotypes (median 0.62 pg/ml for G/G versus 3.35 pg/ml for A/A or A/G; $P<0.05$). This result is also supported by subsequent studies that record a lack of association between the -308 and -238 genotypes and circulating levels of TNF-α (Alanzy *et al.*, 2018;Hadinedoushan *et al.*, 2016), and are contradictory to reports that show an association, such as (Cuchacovich *et al.*, 2006;Elahi *et al.*, 2009).

The discrepancy between prior studies and the current study may be due in part to the small number of participants and the patients' ethnic background, as stratification analysis revealed that various ethnicities would have different risk alleles (Chen *et al.*, 2019).

V. CONCLUSION

In a case-control study of RA patients and healthy people from Iraqhave performed an association study of the TNF-α promotors genetic variants, three SNPs (-308 G/A (rs1800629), -238 G/A (rs361525), and -376 G/A(rs1800750)), with the likelihood of RA. Currentdata showed an association between rs1800629 and RA risk. The GG genotype and G allele of rs1800629 exhibited an association with the risk of developing RA.

LIMITATION

The small sample size of this study may be related to the minimal number of patients who matched the inclusion criteria.

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