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RELATIONSHIP BETWEEN THE TUMOR NECROSIS FACTOR-A AND ITS RECEPTOR GENE POLYMORPHISM AND TNF-A PLASMA LEVELS IN EGYPTIAN RHEUMATOID ARTHRITIS PATIENTS

Yousri M. Hussein [a], Samir Ab. Nassar [b] and Nahla H. El Shaer [b].

a- Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt and Head of Medical Laboratories Department, Faculty of Applied Medical Sciences, Taif University, Taif, KSA .

b- Zoology Department, Faculty of Science, Zagazig University, Zagazig, Egypt.

ABSTRACT

Rheumatoid arthritis (RA) it is a model of multigenic inflammatory disorder in which tumor necrosis factor-α (TNF-α) plays an important role. Genetic factors may be implicated in the susceptibility to disease initiation .Objective: The aim of this study is to elucidate putative association between the −308 G/A polymorphism in the promoter region of the TNF-α gene and 196M/R polymorphism of TNFRII genes and susceptibility to disease onset of RA. Methods: A total of 160 RA patients and a control group of 40 healthy subjects were available for the study. All patients fulfilled the American College of Rheumatology revised criteria for RA, Single-nucleotide polymorphisms (SNPs) at position −308 of TNF-α and 196 of TNFRII genes were determined using restriction fragment length polymorphism–polymerase chain

reaction (PCR–RFLP). The A allele of the −308 promoter polymorphism was more prevalent among the patients. 308GA genotype of TNF-α is associated with a significantly higher serum TNF-α level. TNFRII 196R allele more prevalent among the patients .Our findings suggest that the A allele of TNF-α and TNFRII 196M/R polymorphism are associated with RA susceptibility.

Keywords:

Rheumatoid arthritis (RA) , TNF-α, TNFRII, Gene polymorphism.

INTRODUCTION:

Rheumatoid arthritis (RA) is the most common chronic inflammatory joint disease, and it can leads to progressive joint destruction, deformity and severe disability. Early diagnosis of RA and timely initiation of disease-modifying antirheumatic drugs (DMARDs) are necessary to limit joint damage and optimize the functional outcome [1-2] . RA is a systemic autoimmune disease that affects approximately 1% of the entire world population. The etiology and pathogenesis of the disease is complex and remains unresolved [3-4]

TNF-α , an inflammatory cytokine that is released by activated monocytes , macrophages, and T lymphocytes, promotes inflammatory responses that are important in the pathogenesis of rheumatoid arthritis [5]. TNF-α binds to two receptors, type 1 TNF receptor and the type 2 TNF receptor , that are expressed on many types of the cells. The biological activity of TNF-α can be attenuated by soluble TNF receptors. Patients with rheumatoid arthritis have high concentrations of TNF-α in the synovial fluid. TNF-α is localized to the junction on the inflammatory pannus and healthy cartilage, and high synovial fluid TNF-α concentrations are associated with erosions of the bone [6]. TNF-α is related to the progression of RA, and TNF-α blockade has therapeutic potential to treat this disease. Both of the two

TNF-α receptors are expressed in the synovium and the expression levels are higher in RA synovium than normal [7].

Tumor necrosis factor receptor II (TNF-RII) has been extensively studied from the functional point of view; the major conclusions are that TNF-RII is implicated in the activation of the immune system locally in the joints and in the regulation of the cascade of events mediated by TNF-RI by controlling the binding of TNF- α to that receptor, thus resulting in a negative signalling message [8-9]. Both TNF-RI and TNF-RII are expressed in the synovial tissue and cartilage pannus junction in patients with rheumatoid arthritis (RA). Moreover, circulating levels of soluble TNF-RII were generally found to be elevated in serum and even more in synovial fluids of RA patients compared with patients with osteoarthritis and healthy subjects [10–11].

Members of the TNF superfamily play important roles in a number of autoimmune diseases, including RA .For example, increased concentrations of the soluble forms of several proteins of the TNF superfamily in the sera of RA patients have been directly correlated with disease pathology [12-13] . An accumulation of information reveals that TNF is the central cytokine in the pathogenesis of RA [14] playing a key role in the inflammation and joint damage. High levels of TNF are found in the synovial fluids of patients with RA. In vitro studies indicate that TNF plays a primary role in the cytokine cascade in RA , controlling the production of IL-1 and other pro-inflammatory cytokines, including IL-6 and IL-8 [15] .

Single-nucleotide polymorphisms (SNPs) are the most common genetic variations and occur at a frequency of approximately 1 in 1000 bp throughout the genome [16]. The −308 TNF SNP is a mutation that affects the promoter region of the TNF gene. The −308 TNF SNP involves the substitution of guanine (G) for adenine (A) [18-17] . Until now, several studies have reported association of the −308 TNF SNP and susceptibility to RA [19] . Genome scans have implicated 1p36 as a susceptibility locus for RA, and TNFRII is located within this locus

and consists of 10 exons and 9 introns [20] . 196M/R SNP consists of a single base substitution at codon 196 (ATG3AGG), which results in a nonconservative amino acid substitution (methionine [M] → arginine [R]) within the fourth extracellular domain of TNFRII [21] .The analysis of the SNP at position + 196 (rs 1061622) in exon 6 of the TNFRII gene was undertaken since this polymorphism occurs in the extracellular domain of the receptor and may affect in vivo receptor functioning. Indeed, it has been shown to be important for TNF- α binding [22].

SUBJECTS AND METHODS:

Patients and controls:

The study included 160 patient with RA. All fulfilled American College of Rheumatology criteria for RA .Their ages ranged from 27 to 65 years of mean ± SD (47.39 ± 9.3), Control group was 40 apparently healthy blood donors volunteers, they were age and ethnic origin matched with the patients, their ages ranged from 29 to 62 years of mean ± SD (49.35 ± 8.7). An informed written consent was obtained from all participants ,patient were recruited from inpatient and outpatient clinics of Rheumatology and Rehabilitation department of Zagazig University Hospitals, Egypt.

All patients were subjected to history taking especially for presenting symptoms, joint affection, together with history of medications. Locomotor examination were preformed to all patients including tender joint count (TJC) and swollen joint count (SJC). At assessment the disease activity was evaluated by clinical examination and laboratory tests (e.g., ESR, CRP, rheumatoid factor), X-rays of the hands and feet were obtained in all patients [23].

Biochemical analysis:

Blood samples were drawn from all subjects after an overnight fast. Sera were separated immediately and stored at _20 Cº. TNF- α were

measured using enzyme-linked immunosorbent assay (ELISA) kit. (RayBiotech, INC.). [24]

DNA extraction :

Genomic DNA was extracted from EDTA whole blood using a spin column method according to the protocol .DNA was stored at _20 Cº till the time of use (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany) .

Amplification of _308G/A promoter polymorphism of the TNF- α gene:

The subjects were genotyped for −308G/A promoter polymorphism of the TNF- α gene by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) as described previously.The region surrounding the polymorphism was amplified with following forward primers 5'-TTCCTGCATCCTGTCTGGAA and reverse primer 3'-TTCCTGCATCCTGTCTGGAA. PCR was performed at 93 Cº for 5 min, followed by 36 cycles at 93 Cº for 60 s, at 60 Cº for 60 s, and 72 Cº for 60 s. A final extension step was carried out at 72 Cº for 5 min. After RFLP analysis using enzyme BsmFI in 65 Cº for 3 h, striking on 2% agarose (Serva) with ethydium bromide and translucence by ultraviolet light, three genotypes were detected: GG (137 + 103 + 88 bp), GA (191 + 137 + 103 + 88 bp), and AA (191 + 137 bp) [25] .

Fig (1&2).

Amplification of TNF-RII polymorphism

The subjects were genotyped for TNF-RII exon 6 polymorphism by PCR–RFLP as described previously.The region surrounding the polymorphism was amplified with following forward primers 5'-ACT CTC CTA TCC TGC CTG CT-3'; and reverse primer 5'-TTC TGG AGT TGG CTG CGT GT-3. PCR was performed at 95 Cº for 5 min, followed by 35 cycles at 95 Cº for 60 s, at 57 Cº for 60 s, and 72 Cº for 60 s. A final extension step was carried out at 72 Cº for 5 min. After RFLP analysis using enzyme NlaIII in 65 Cº for 3 h, striking on 2% agarose with ethydium bromide and translucence by

ultraviolet light. The 242 bp PCR product was uncleaved in the 196R allele and cleaved into two fragments of 133 and 109 bp in the 196M allele [26] Fig (3&4).

Statistical analyses

All data were analyzed using SPSS, version 11 for windows. The results for continuous variables were expressed as means ± SD, comparison of genotypic and allelic frequencies between groups was performed by chi-square test to measure deviation between the observed and expected frequencies that was tested for significance . Odds ratio (ORs) at 95% confidence intervals (CIs) were calculated to asses the relative disease risk conferred by a specific allele and genotype . The means of the three-genotype groups were compared in a one-way analysis of variance (ANOVA). p-value of <0.05 was considered significant [27] .

RESULTS:

TNF-308 and TNFRII 196MR polymorphisms genotypes frequencies

The frequencies of the GG,GA, and AA genotypes of _308G/A polymorphism of the TNF- α gene (rs 1800629), were 92.5%, 5%, and 2.5% in controls, 75%, 21.25%, and 3.75% in Rheumatoid Arthritis patients. There was a significant difference in the genotypes frequencies polymorphism of the TNF- α promoter _308G/A between control and RA group ($p <0.05$) (Table 1). GA genotype was associated with a significantly increased risk of RA group as compared to control group (OR=3.78 , 95% CI(0.86-16.6) .

The frequencies of the MM, MR, RR genotypes of TNF-RII gene polymorphism were 50%, 45%, and 5% in control, and 31.25%, 52.5%,16.25% and in Rhematoid Arthritis patients . There was a significant difference in the genotypes frequencies polymorphism of the TNF-RII 196MR polymorphism between control and RA group ($p <0.05$) (Table 1). RR genotype was associated with a significantly

increased risk of RA group as compared to control group (OR=2.43, 95% CI(0.86-16.6)

TNF-308 and TNFRII 196MR polymorphisms allele frequencies :

The frequencies of the G and A alleles of −308G/A TNF- α gene were 95 % and 5% in control, 85.62% and 14.37% in RA patients. There was a significant difference in the allele frequencies polymorphism of the TNF- α promoter _308G/A polymorphism between control and RA group ($p < 0.05$) (Table 2). The A allele was associated with a significantly increased risk of (OR=1.00, 95% CI (0.03-29.08).

The frequencies of the M and R allele of TNF-RII gene polymorphism were 72.5%, and 27.5% in control , and 57.5 %,and 42.5 % in Rhematoid Arthritis patients, the R allele was associated with a significantly increased risk of (OR=3.19, 95% CI (1.11-9.14) .There was a significant difference in the allele frequencies polymorphism of the TNF-RII 196MR polymorphism between control and RA group ($p < 0.01$) . (Table 2)

Serum TNF-α level:

In control group, serum TNF-α level ranged from 18.90 to 42.10 pg/ml with a mean ± SD of 27.08 ± 6.77 pg/ml. In rheumatoid arthritis group, serum TNF-α level ranged from 17.50 to 59.50 pg/ml with a mean ± SD of 37.35±8.86 pg/ml Simple analysis of variance revealed that there is a significant difference in TNF-α serum level among control and RA groups ($T=6.85$, $p < 0.001$).
. (Table 3).

The relation between TNF-308 and TNFRII 196MR polymorphisms genotypes and TNF-α level :

For TNF-308:

The means ± SDs of serum level of TNF-α were 32.80±7.85 pg/ml, 33.97±7.6 pg/ml, and 38.59±8.96 pg/ml in GG, GA, AA rheumatoid arthritis patients respectively. There is significant

difference in TNF-α values among the all genotype GG, GA, and AA in RA patient (p=<0.01) . The post hoc comparisons by LSD showed that TNF-α values in the GG and GA genotype in RA group is significantly higher than AA , (P= 0.006 and 0.006 respectively) (Table 4).

For TNFRII 196MR :

The means ± SDs of serum level of TNFRII were 38.09±9.68 pg/ml, 37.94±8.73 pg/ml, and 34.06±7.03 pg/ml in MM, MR, RR rheumatoid arthritis patients respectively .There is no significant difference in TNF-α values among the all genotype MM, MR, and RR in RA patient . The post hoc comparisons by LSD showed that TNFRII values in the MR and RR genotype in RA group is significantly higher as compared to MM genotype , (P= 0.05 and 0.05 respectively) (Table 4).

Table 1 : The genotype frequencies of −308G/A promoter polymorphism of the TNF- α Gene and TNF-RII polymorphism among the controls and the RA patients

Polymorphism	Cases n = 160(%)	Controls n = 40 (%)	OR (95% CI)	X^2	P
TNF_308					<0.05
GG	120 (75)	37(92.5)	0.24 (0.07-0.83)	6.08	
GA	34 (21.25)	2 (5)	3.78 (0.86-16.6)		
AA	6 (3.75)	1 (2.5)	1.52 (0.17-12.99)		
TNFRII 196MR					<0.05
MM	50 (31.25)	20 (50)	0.46 (0.23-0.92)	6.46	
MR	84 (52.5)	18 (45)	1.5 (0.74-3.01)		
RR	26 (16.25)	2 (5)	2.34 (0.69-8.34)		

Table 2 : The allele frequencies of −308G/A promoter polymorphism of the TNF-α Gene and TNF-RII polymorphism among the controls and the RA patients

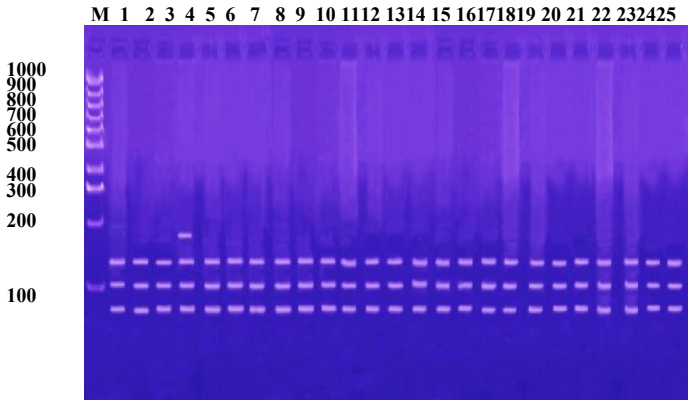
Polymorphism	RA	Controls	OR (95% CI)	X^2	P
TNF α −308					<0.05
G	274(85.62)	76 (95)	0.47 (0.19-1.14)	5.14	
A	46 (14.37)	4 (5)	1.00 (0.03-29.08)		
TNFRII 196MR					<0.01
M	184 (57.5)	58 (72.5)	0.55 (0.32-0.93)	6.02	
R	136 (42.5)	22 (27.5)	3.19 (1.11-9.14)		

Table 3 : levels of serum TNF-α in the studied groups

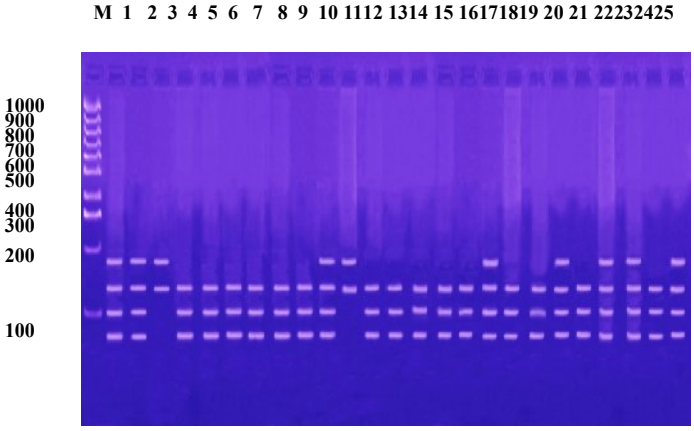
Group	n	Mean±SD for IL-10	Range Min-Max	T	P
Control	40	27.08±6.77	18.90-42.10	6.85	< 0.001
Rheumatoid Arthritis	160	37.35±8.86	17.50-59.50		

Table 4 :The levels of S TNF-α in the different genotypes in all the studied groups

Group	n	Mean±SD for s TNF-α	Range Min-Max	F	P
TNF_308					
GG	120	32.80±7.85	17.50-59.50	4.918	<0.01
GA	34	33.97±7.6	22.00-51.00		
AA	6	38.59±8.96	19.50-46.70		
TNFRII 196MR					
MM	50	38.09±9.68	17.50-59.50	2.16	0.118
MR	84	37.94±8.73	19.50-59.50		
RR	26	34.06±7.03	19.50-45.60		

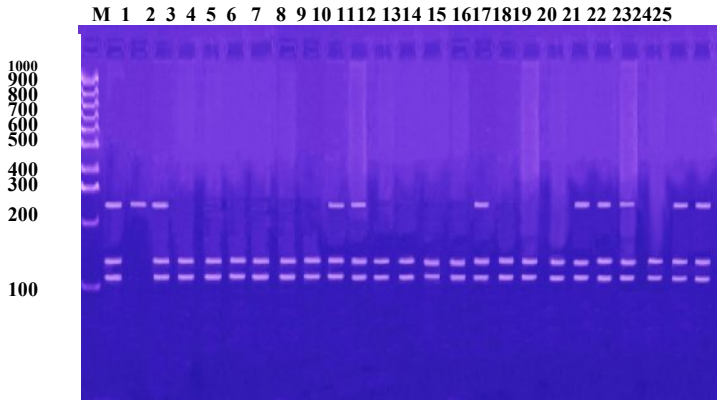


Fig,(1):control subjects 1→25

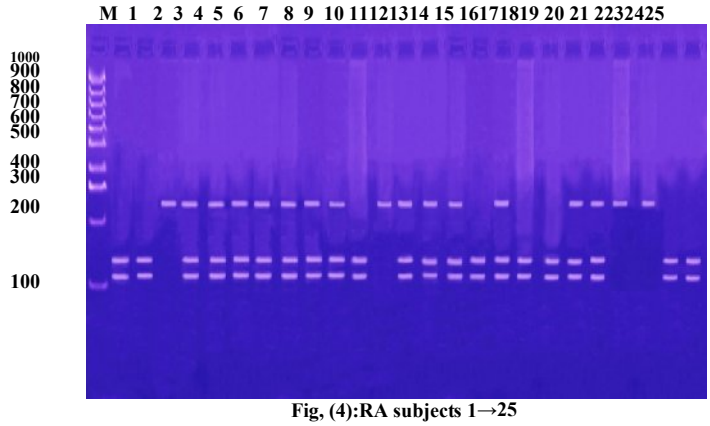


Fig, (2): RA subjects 1→25

Fig: (1) & (2) represent Agarose gel electrophoresis detection for TNF-α gene single nucleotide polymorphism at position _308G/A showing lanes of 191+137 bp fragment for AA homozygous genotype, 191+137+103+88 bp fragments for GA heterozygous genotype and lanes of 103+137+88 for GG genotype (100 bp Marker).



Fig,(3): control subjects 1→25



Fig, (4):RA subjects 1→25

Fig: (3) & (4) Agarose gel electrophoresis detection for TNFRII 196MR gene single nucleotide polymorphism showing lanes of 242 bp fragment for RR homozygous genotype, 242+133+109 bp fragments for MR heterozygous genotype and lanes of 133+109 for MM genotype (100 bp Marker).

DISCUSSION:

SNPs are highly abundant, stable, and distributed throughout the genome. These variations are associated with diversity in the population, individuality, susceptibility to diseases [28], and differential response to medical treatment [29] .

TNF and RA

Of all the SNPs that affect the TNF-α gene, the −308 TNF promoter polymorphism is probably the most relevant for increased TNF production, thus representing a genetic risk factor for RA predisposition [16] . Our results of the −308 TNF polymorphism in RA patients and controls demonstrated that the subjects GA genotype were significantly more likely to have susceptibility to RA (OR= 3.78 , P = 0.05) , this agree with Cuenca et al . 2001 [29] who reported that the frequency of the G/A −308 TNF polymorphism is higher in the healthy Caucasian population than in other ethnic groups such as Latin-American, Chinese, Japanese or black African populations. Although these frequency distribution differences remain for the G/A genotype the most significant reside in the A/A genotype , and Cvetkovic et al . [30] who demonstrated that RA patients GA heterozygous for the −308 TNF-α gene polymorphism had a more severe course of disease.

Vinasco et al. [31] found a significantly increased frequency of nodular disease in GA heterozygous patients. on the contrary Angelica et al. [32] demonstrate that −308 T2 (A) allele is associated with susceptibility to develop severe RA in Mexican , The −308 TNF SNP involves the substitution of guanine (G) for adenine (A), generating TNF1 and TNF2 (G/A or A/A genotypes) alleles [33] . The polymorphic TNF2 allele leads to a higher rate of TNF gene transcription than the wild-type TNF1 allele in vitro expression studies, and it has been linked to an increased susceptibility to a variety of illnesses [34-35].

Our results of the −308 TNF-α polymorphism in RA patients and controls demonstrated that the TNF A allele 14.37% and. 5% of individuals, respectively. The odds ratio (OR = 1, P = 0.05) suggests an association between the presence of the polymorphism and the disease, also Correa et al.[36] found that the TNF −308A allele was associated with an increased risk of RA. The influence of the TNF-α polymorphism on RA susceptibility or severity may depend on the differences in TNF-α synthesis. The production of TNF-α may be associated with the TNF-α promoter polymorphism. Nevertheless, the association between TNF-α production and the −308 G/A TNF-α promoter polymorphism remains unclear. Functional studies of the −308 G/A TNF-α polymorphism have produced conflicting results, with some investigators reporting higher transcription of the gene in the presence of the A allele, whereas others have not [37-38] .

However circulating TNF-α levels are regulated at different stages: gene transcription, post-transcription control of mRNA stability, cleavage of the membrane form to liberate the soluble form, and the expression of receptors [30], the present study we found a higher serum TNF- α level in the −308GG and −308GA genotype than the −308AA genotypes, this confirmed with Oregon-Romero et al. [39] who found that the −308GG genotype was associated with high TNF-α mRNA levels rheumatoid arthritis patients and healthy subjects, this result was not confirmed with other two studies Prasad et al. [40] who reported that 308GA and _308AA genotypes had significantly higher serum levels of TNF- α compared with 308GG genotype and Helmig et al. [41] who found that G-allele of TNF-α _308 is associated with a significantly higher TNF- α mRNA expression compared to the A-allele

Because early prescription of disease-modifying anti-rheumatic drugs may be more effective in controlling severe disease, early diagnosis and prediction of disease severity are important. Therefore, identification of other genetic markers associated with susceptibility to

or severity of RA is currently an important challenge. Moreover, studied polymorphism may be important in determining treatment responses to new anti-TNF-α therapy [42-43] .

In summary, we found a positive association of the A allele of the −308 promoter polymorphism of the TNF-α gene with more susceptibility of RA disease, also subjects GA genotype have high TNF-α serum level compared with AA genotype

TNFRII and RA:

It may be speculated that polymorphism within the TNFR genes could alter binding of ligands such as TNF-α or cleavage enzymes, thereby leading to an inappropriate inflammatory response due to excessive circulating TNFα, and hence contributing to RA susceptibility [44] .

Our results of the TNFRII polymorphism in RA patients and controls demonstrated that the TNFRII R allele 42.5% and 27.5% of individuals, respectively. The odds ratio (OR = 3.19, P = 0.009) this evaluate the possible association between presence of the TNFRII 196R allele and RA diagnosis and prognosis, also this result agree with Goëb et al.[45] their study shows that The TNFRII 196R allele appears to be significantly associated with RA.

The results of the present study support the hypothesis that there is an association between the TNFRII 196 M/R gene polymorphism and the functional severity of early RA [46] , however Recently, TNFR2 196R transfectants have been shown to be associated with higher production of interleukin-6 [47], which plays a crucial role in the pathogenesis of RA [48] , also agree with Cornelis et al. [49] and Shizozawa et al. [50] who draw attention to the gene encoding TNFRII as a strong candidate gene in RA pathogenesis , If we take into account the two independent observations from Europe demonstrating the association of 196R/R in familial RA and the functional relevance of 196R, it is fair to say that the TNFR2 196R/R

genotype may be a weak, but significant, risk factor in sporadic Japanese RA patients, as well. This result may also support the idea that the genes for susceptibility to systemic lupus erythematosus and RA are partially overlapping [51] .

Although the present findings revealed statistical significance between the TNFRII 196R allele and RA diagnosis, The frequencies of the TNFRII 196R allele observed in the present study are not statistically different from the previously reported frequencies in the UK and the French RA populations [52-53] ,in contrary Shibue et al .[54] reported that the tumor necrosis factor receptor II position 196 polymorphism (TNFR2 196M/R) was not significantly associated with rheumatoid arthritis (RA) in Japanese patients, in a study of 545 unrelated patients and 265 controls .

As previously reported by van der Helm-van Mil [55] and Glossop et al . [56] , we observed a lack of association between the TNFRII 196R allele and the functional severity of RA., which is in disagreement with the findings reported by Constantin [57] . There may be several explanations for these discrepancies, including the heterogeneity of the studied population, differences in the selected outcome criterion between studies, and the influence of treatment, notably with DMARDs and biologics, on outcome.

In conclusion, our findings suggest that TNFRII 196R allele may be associated with RA diagnosis, also the 308GA genotype of TNF-α is associated with a significantly higher serum TNF-α level compared to the 308GG genotype , also there is association of the A allele of the _308 promoter polymorphism with RA susceptibility.

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