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The Effect of TNF-Alpha Gene Polymorphisms At -376 G/A, -806 C/T, and -1031 T/C on The Likelihood of Becoming a Non-Responder to Etanercept in A Sample of Iraqi Rheumatoid Arthritis Patients.

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Abstract

Tumor necrosis factor-alpha (TNF-α) antagonists' therapy are expensive and has a non-responsive rate between 30% to 40% in rheumatoid arthritis patients. Genetic variation plays a vital role in the responsiveness to this type of therapy.The aim of this study is to investigate if the presence of genetic polymorphism in the TNF-α gene promoter region at locations -376 G/A (rs1800750), -806 C/T (rs4248158), and -1031 T/C (rs1799964) affects rheumatoid arthritis patient's tendency to be a non-responder to etanercept.

Eighty RA patients on etanercept (ETN) for at least six months were recruited from the Rheumatology Unit at Baghdad Teaching Hospital. Based on The European League Against Rheumatism response (EULAR) criteria, patients were divided into two groups: responders and non-responders. After polymerase chain reaction amplification of their DNA, the amplified DNA was sequenced by Sanger method to determine the polymorphisms at the positions -376G/A, -806 C/T, and -1031T/C.

The results of this study found that equally Phi correlation and binary logistic regression analysis revealed a non-significant association for all genotypes in the three polymorphic sites with the tendency for being non-responder. Moreover, there was no significant difference in TNF-α mean level or the change in disease activity score for 28 joints (DAS28) after six months of etanercept therapy between all genotypes for each polymorphic site.

The present study concludes that there was no correlation between the polymorphisms in the TNF-α promoter region at -376G/A, -806 C/T, and -1031T/C with the tendency for being non-responder to ETN.

Key words: Rheumatoid arthritis, Etanercept, Genetic polymorphism, Response.

تأثير تعدد الأشكال الجينية في عامل نخر الورم الفا بالمواقع -٣٧٦ G/A , -٨٠٦ C/T و -١٠٣١ T/C على الميل لعدم الاستجابة للأيتانرسبت في عينة من مرضى التهاب المفاصل الرثوي في العراق

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الخلاصة

العلاجات المضادة لعامل نخر الورم (TNF-α) هي علاجات باهظة الثمن ،وتصاحب استخدامها نسبة عالية من عدم استجابة قد تصل ما بين ٣٠٪ إلى ٤٠٪ في مرضى التهاب المفاصل الرثوي . يلعب التباين الجيني دورًا حيويًا في تحديد نسبة الاستجابة لهذا النوع من العلاج.

هدف الدراسة هو لمعرفة ما إذا كان هناك وجود لتعدد الأشكال الجيني في المنطقة المحفزة لجين عامل نخر الورم الفا في المواقع - ٣٧٦ G/A (rs1800750)و -٨٠٦ C/T(rs4248158) و -١٠٣١T/C(rs1799964) في مرضى التهاب المفاصل الرثوي بالعراق وهل هناك تأثير لهذا التباين الجيني على ميل المريض في أن يكون غير مستجيب لعقار الايتانرسبت. تم تسجيل ثمانين مريضًا من مرض التهاب المفاصل الرثوي والمستخدمين لعلاج الايتانرسبت لمدة ستة أشهر على الأقل من وحدة علاج المفاصل في مستشفى بغداد التعليمي. وبناءً على معايير استجابة الرابطة الأوروبية لمكافحة الروماتيزم (EULAR) ، تم تقسيم مرضى التهاب المفاصل الرثوي إلى مجموعتين: المستجيبين وغير المستجيبين. وبعد تضخيم الحمض النووي الخاص بهم باستخدام تقنية تفاعل البلمرة المتسلسل ، تم اجراء تسلسل للحمض النووي المتضخم للمرضى المشاركين بأستخدام تقنية سانغر لتحديد تعدد الأشكال الجيني في المواضع -٣٧٦ G/A و -٨٠٦ C/T و -١٠٣١ T/C.اهم نتائج الدراسة كانت كشف كل من معامل الارتباط فاي وتحليل الانحدار اللوجستي الثنائي عن عدم وجود ارتباط ذو دلالة احصائية لجميع الأنماط الجينية للمواقع الثلاثة مع ميل المريض لعدم الاستجابة لعقار الايتانرسبت.كذلك لم يكن لتغيير الانماط الجينية لأي من المواقع الثلاثة تأثير ذو دلالة احصائية على مقدار معدل مستوى معامل نخر المرض الفا في المريض ولا على مقدار التغيير الحاصل لنتيجة نشاط المرض لـ ٢٨ مفصل خلال ستة اشهر من العلاج.

ملخص هذه الدراسه اوضح انه لم يكن هناك ارتباط بين تعدد الأشكال الجيني في المنطقة المحفزة لعامل نخر الورم الفا في المواقع - G/A٣٧٦ و -٨٠٦ C/T و -١٠٣١ T/C مع قابلية مريض التهاب المفاصل الرثوي إلى عدم الاستجابة لـلأيتانرسبت.

الكلمات المفتاحية : التهاب المفاصل الرثوي , الايتانرسبت , تعدد الاشكال الجيني , الاستجابة.

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Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune joints disease. It is defined by progressive symmetric inflammation of the afflicted joints, which results in cartilage damage, bone erosion, and disability[1].

One influential proinflammatory cytokine that plays a critical role in controlling the inflammatory response and mediating RA pathogenesis is tumor necrosis factor-alpha (TNF-α) [2]. Consequently, extensive research on TNF-α induced inflammatory processes has resulted in the conception of TNF-α antagonist medications to treat many inflammatory autoimmune diseases, including RA[3]. They are produced from either a recombinant TNF-α receptor, like etanercept (ETN), or a monoclonal antibody against TNF-α like infliximab and adalimumab[3].

Nowadays, TNF-α antagonists therapy has been widely used and is quite successful in treating RA[4]. However ,They are prohibitively expensive, may induce some undesirable side effects, and not all RA patients respond well to these medications[5].In fact, between 30% to 40% of people with active RA do not respond successfully to TNF-α antagonists[6].

The genetic disparity is a significant factor influencing TNF-α expression, and consequently, the response to TNF-α antagonists since the promoter region of the TNF-α gene is highly polymorphic [7].

TNF-α antagonists are not all equally effective in all patients. As a result, being able to identify which TNF-α antagonist would be the most effective and should be taken initially would be tremendously beneficial in terms of reducing the time required to ensure an effective therapy, which has been repeatedly proved to be a significant factor in achieving long-lasting disease remission[8].

Etanercept , a recombinant human TNF-α receptor (p75) fusion protein that suppresses TNF-α competitively[9], is safe and effective in patients with active RA[10].

As with other TNF-α antagonists, the clinical response to ETN is variable, and one of the significant determinants of the response is genetic variability in the TNF-α gene[7].

As a result, polymorphism testing aids in distinguishing patients with a high response rate from those with an insufficient response or even non-responsive [11]. This information may be beneficial for some patients who concerned about their exposure to side effects or the associated high cost of biological therapy [11].

Several research articles published in the last few years have revealed conflicting outcomes about a possible association between TNF-α antagonists' response and polymorphisms in the TNF-α gene at several gene locations [12–14].

Due to the racial and ethnic variation in pharmacogenetics, which occurs as a result of the different in allele frequencies between populations, polymorphism analysis across populations is crucial for determining the genetic variables that may affect ETN responsiveness. [15].

Earlier investigations did not examine the effect of single nucleotide polymorphisms (SNPs) in the TNF-α promoter region on the tendency to being non-responder to ETN in Iraqi RA patients; thus, the current study sought to determine whether the presence of SNPs in the promoter region of the TNF-α gene at positions -376 G/A (rs1800750), -806 C/T (rs4248158) and -1031 T/C (rs1799964) may affect a patient's proclivity to be a non-responder.

Patients and Methods

This research article was a part of a large observational cross-sectional study conducted between October 12, 2020, and August 8, 2021. The study recruited a sample of eighty Iraqi RA patients with established RA according to the revised 2010 American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) Classification Criteria for RA[16].

The patients were recruited from the Rheumatology Unit of Baghdad Teaching Hospital in Baghdad, Iraq. This unit provides services to a wide range of communities in Iraq, including rural, urban, and inner-city districts from numerous governorates.

The Scientific and Ethical Committee of College of Pharmacy- University of Baghdad and Rheumatology Medical Department at Baghdad Teaching Hospital approved ethical permission with the number (RECACPUB-3102020B) on October 3, 2020. Furthermore, written consent was obtained from all participants.

Patients selection

Ninety-seven patients with active RA received ETN alone as a singular therapy during the study and met the inclusion criteria listed below. However, only eighty-six patients agreed to participate in this study, and only eighty completed the full requirements.

The inclusion criteria:

The patients must have been diagnosed with RA using the 2010 ACR/EULAR RA classification criteria[16]. Also, patients with high disease activity according to disease activity score based on 28 joints and ESR (DAS28-ESR)[17] which is calculated as follows:

DAS28 = 0.56 * sqrt(tender28) + 0.28 * sqrt(swollen28) + 0.70 * ln (ESR) + 0.014 * patient global health.

In order to be included, the DAS28-ESR should be more than 5.1 at baseline. Additionally, patients had to take ETN subcutaneously and consistently for at least six months before enrollment, with no history of missed doses.

The exclusion criteria:

- Patient who has used ETN for less than six months.
- Patients who use additional disease-modifying anti-rheumatic drugs (DMARDs) with ETN.
- Patients with co-existent other connective tissue diseases
- Patient with inadequate data like missed laboratory data or initial DAS28.

Patients classification:

As presented in Figure 1, the patients were divided into two groups according to EULAR response criteria[17], which are based on the clinical response as determined by (DAS28) [18] following at least six continuous months of ETN treatment.

When the DAS28 value after six months was reduced from a high value of ≥ 5.1 to a value less than 5.1 and with a change in DAS28 of greater than 0.6, the patient was classified as an ETN responder. On the contrary, the patient has categorized as a non-responder if the DAS28 value did not fall below 5.1 or if the change in the DAS28 was less than 0.6.

Accordingly, the patients are distributed regarding their responses into two groups. The first group (group A) contained forty-one RA patients who responded clinically to ETN.

The second group (group B) contained thirty-nine RA patients who failed to respond to ETN.

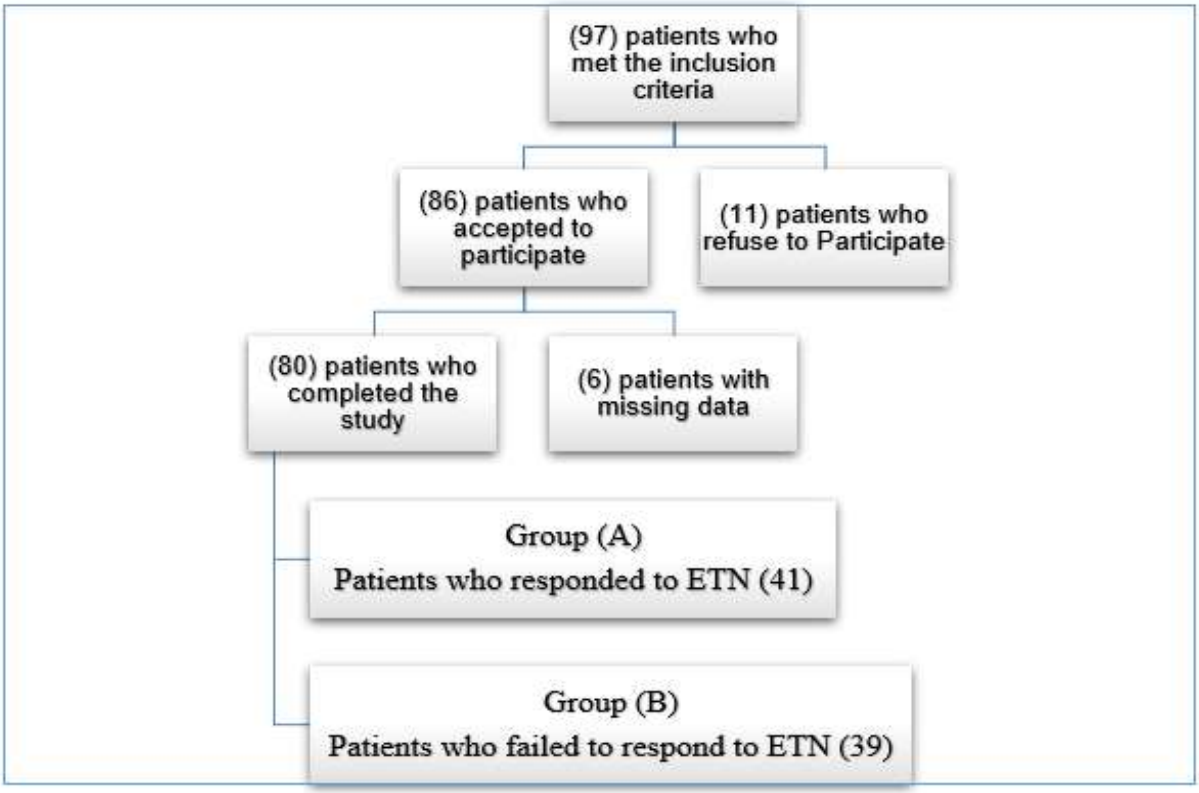

Figure 1. Flow diagram for the study participants.

Data collection

Demographic data (age, weight, disease duration, tender and swollen joints, and the visual analog scale (VAS) for the patients and the evaluator) were obtained via direct patient interviews utilizing a patient information chart designed explicitly for this study.

Sample collection and preparation

Five milliliters of venous blood were obtained from each patient's forearm vein. Then, two milliliters of blood were transformed into an ethylene diamine tetraacetic acid (EDTA) tube for DNA extraction. At the same time, the remaining three milliliters of blood were put into a gel tube and centrifuged for ten minutes (4000 rpm).

The remainder of the serum was collected in an Eppendorf tube and stored at (-20 C°) until all samples were obtained. TNF-α was then measured using the ELIZA approach.

Measurement of Serum Tumor Necrosis Factor-Alpha (TNF-α)

The serum TNF-α level was determined using a Cusabio ELISA kit (Wuhan, China; Cat. No. CSB-E04740h). This assay employs the quantitative sandwich enzyme immunoassay technique[19].

DNA extraction:

The "Promega ReliaPrepTM Blood gDNA Miniprep System" for Genomic DNA enables the straightforward purification of DNA from blood samples. Enzymatic amplification was performed using PCR and a hybrid thermal cycler.

Quantitation of DNA

The "The Quantus™ Fluorometer" was used to determine the concentration of extracted DNA and thus the sample's quality for downstream applications. (199 µl) diluted Quantifluor Dye was combined with 1 µl of DNA, and then DNA concentration readings were determined during a 5-minutes incubation at room temperature.

The primer:

The TNF-α gene DNA sequences were obtained from the NCBI GenBank database. The Primer Premier 3 software was used to create PCR primers (Table 1), with melting temperatures ranging from (58 to 62ºC), primer lengths ranging from (18 to 23) nucleotides, and PCR amplicon lengths ranging from (800 to 1000) base pairs.

Table 1. The sequences, annealing temperature and size of the primers used in the study

Primer Name	Sequence	Annealing Temp. (°C)	Product size (bp)
TNF-α_1-F	5`-TGTAAAACGACGGCCAGTCTCAGAGAGCTTCAGGGATA-3`	60	966
TNF-α_1-R	5`-CAGGAAACAGCTATGACCGGGACACACAAGCATCAA-3`		

TNF-α_1-F: the forward primer. TNF-α_1-R: the reverse primer.

Primer preparation

The forward and reverse primers used in this study were given in lyophilized form by the Macrogen Company. The primers were then dissolved in nuclease-free water to provide a stock solution with the highest concentration of (100 pmol/µl) that can be kept in the freezer at 20 °C. After that, a working solution for these primers was made by combining 10 µl of primer stock solution with 90 µl of nuclease-free water to yield a solution comprising (10 pmol /µl).

Primer optimization and PCR amplifications

To determine the optimal annealing temperature for primers, the DNA template was amplified using the identical primer pair (Forward) (Reverse) at annealing temperatures of 55, 58, 60, 63, and 65°C respectively. The best annealing temperature for the primer was 60°C as seen in Figure 2. PCR amplifications were performed with 20µl volumes containing 10µl GoTaq Green Master Mix (2X); 1µl for each primer (10pmol); 6µl nuclease-free water and 2µl of template DNA. PCR cycling was performed with PCR Express (Thermal Cycler, BioRad, USA) with the following temperature program: firstly, DNA denaturation occurred at 94ºC for 4 min followed by 30 cycles of denaturation at 94°C for only 30 sec; after that, annealing at 60°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation of 7 min at 72ºC was included, followed by a 10 min incubation at 4ºC to stop the reactions.

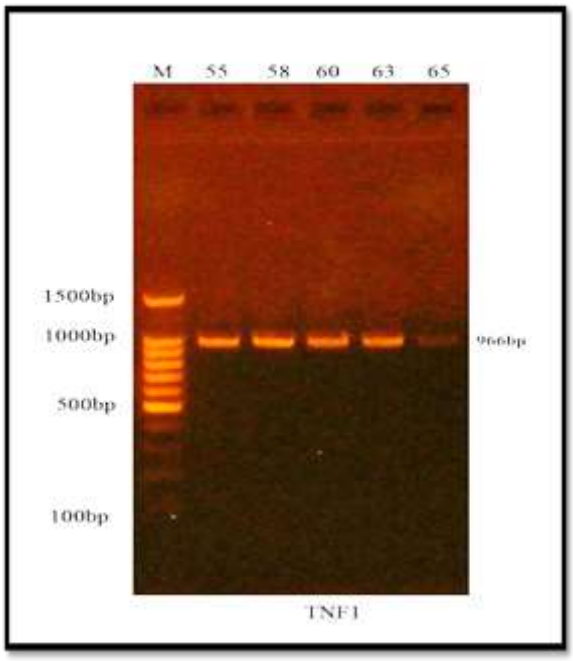

Figure 2. Primer optimization at annealing temperatures of 55, 58, 60, 63, and 65°C.

PCR products sequencing

PCR product was sequenced by Sanger method of sequencing using DNA analyzer (ABI3730XL) (Macrogen Corporation – Korea). The results were obtained by email and analyzed with the use of Geneious Prime software (V 2021.1.1) (Biomatters Ltd., Auckland, New Zealand; www.geneious.com).

Statistical analysis

Data were investigated using the SPSS for Windows 26.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 7.04 (California, USA). Continuous variables were stated as mean ± SEM of the values. Allele and genotypes are presented as percentages and frequencies. A probability that equals or less than 0.05 was considered significant. A Shapiro–Wilk test was used to test the normality of the results. The unpaired t-test was used for normally distributed data to determine if there is a significant difference in demographic characteristics and parameters between responding and nonresponding groups. A one-way analysis of variance (ANOVA) test was used to analyze the difference between the means of more than two groups. Then, a post-hoc analysis was used whenever a significant difference between three sample means was revealed by (ANOVA). The Chi-square test or Fisher's exact test was used to test group differences of proportions. Fisher's exact test was used if one of the expected values in a 2 x 2 comparison is < 5. Phi correlation coefficient (phi) was used to measure the correlation between each genotype and the likelihood of being non-responsive. The binary logistic regression analysis was used to estimate the relationship between TNF-α level and the effect of genetic polymorphism on the likelihood of becoming a non-responder.

Results

Demographic data and clinical characteristics parameters of the study groups.

Table 2 summarizes the demographic characteristics of the study groups. In the current study, patients were matched. However, clinically, there was a significant difference in baseline DAS28 values between responder and non-responders (P. Value <0.001). Significant variations in DAS28 were also observed after six months of ETN administration. In addition, after six months of ETN medication, TNF-α levels were likewise shown to differ considerably between responders and non-responders.

Table 2. Demographic data and clinical characteristics parameters of the study groups.

Category		Responders group n=41	Non-responders group n=39	P-Value
Age(years)		49.46±10.54	51.18±11.95	0.497 [a]
Gender	**Male n (%)**	6 (14.6)	4 (10.3)	0.55 [c]
	Female n (%)	35 (85.4)	35(89.7)	0.55 [b]
Weight(kg)		80.59±14.49	78.03 ±12.82	0.40 [a]
Disease duration (years)		10.10± 6.82	8.31±3.73	0.15
TNF-α (pg/mL)		78.63±34.1	113.35 ±54.54	0.001* [a]
Baseline DAS28		5.58± 0.343	6.06 ± 0.38	<0.001* [a]
DAS28 after 6 months		3.34 ± 0.82	5.73 ± 0.54	<0.001* [a]

Results are reported as means ±SD or frequency (percentage). TNF-α: tumor necrosis factor-alpha. DAS28: disease activity score in 28 joints. [a]: Independent 2 sample t-test. [b]: Chi-square test. [C]: Fisher-exact test

DNA concentration (µg/ml)

The extracted DNA concentration in all samples was found in a range of 20-30 µg/ml.

PCR amplification Result

The amplification of the TNF-α gene-specific region of human samples was presented in Figure 3. The amplification of the TNF-α gene-specific region of human samples was fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide (Eth.Br).

Figure 3. Human samples' amplification of the TNF-α gene-specific region. The sample was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-80 resemble 966bp PCR products.

Sanger sequences data analysis

Analysis of TNF-α (-376 G/A) (rs1800750) SNP

Figure 4 shows the analysis of (rs1800750) SNP of the TNF-α gene using Sanger sequencing. Single "G" peak indicative of a (G) homozygous allele. The presence of the "G" and "A" peaks is indicative of the (G/A) heterozygous allele

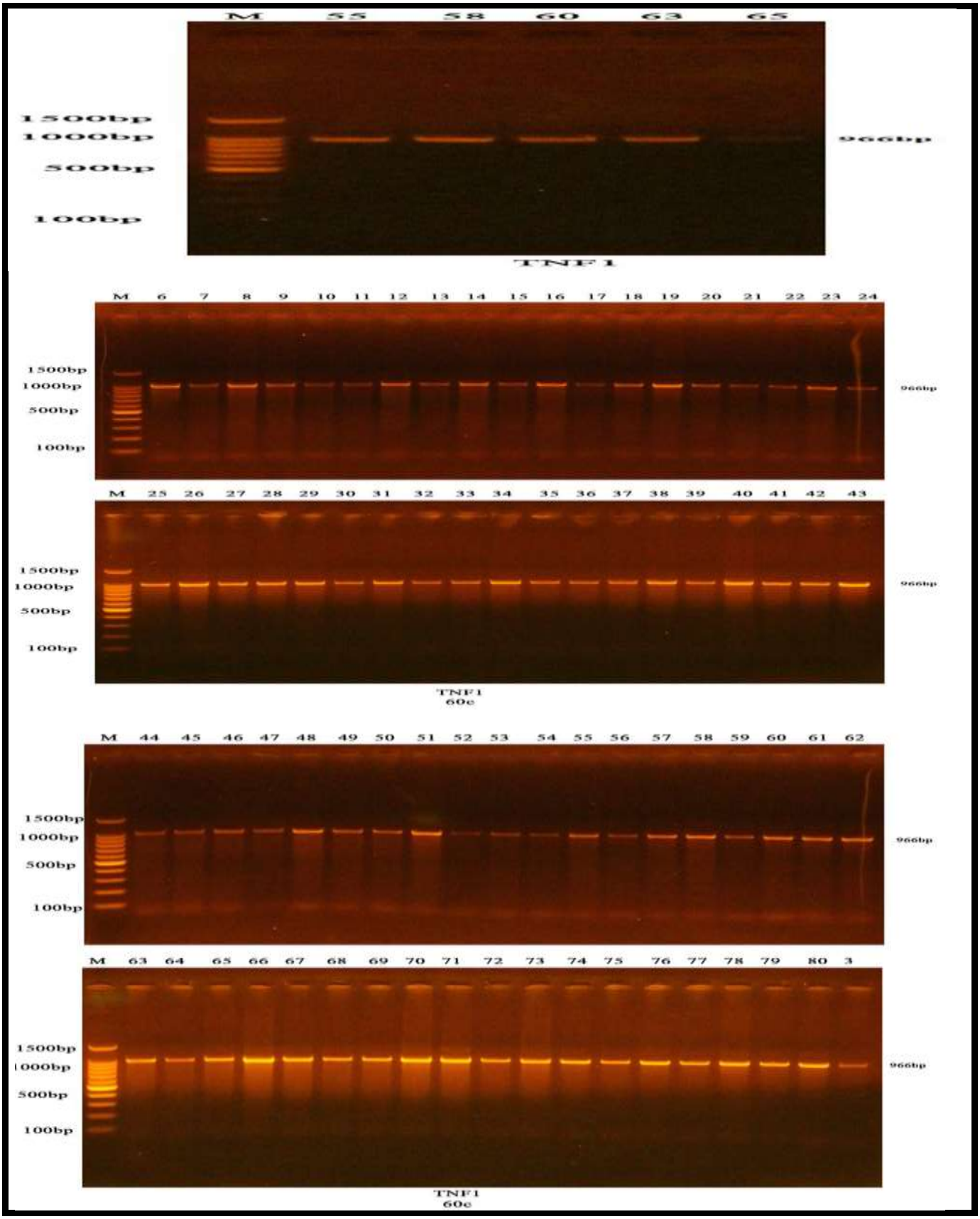

Figure 3. Human samples' amplification of the TNF-*α* gene-specific region. The sample was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-80 resemble 966bp PCR products.

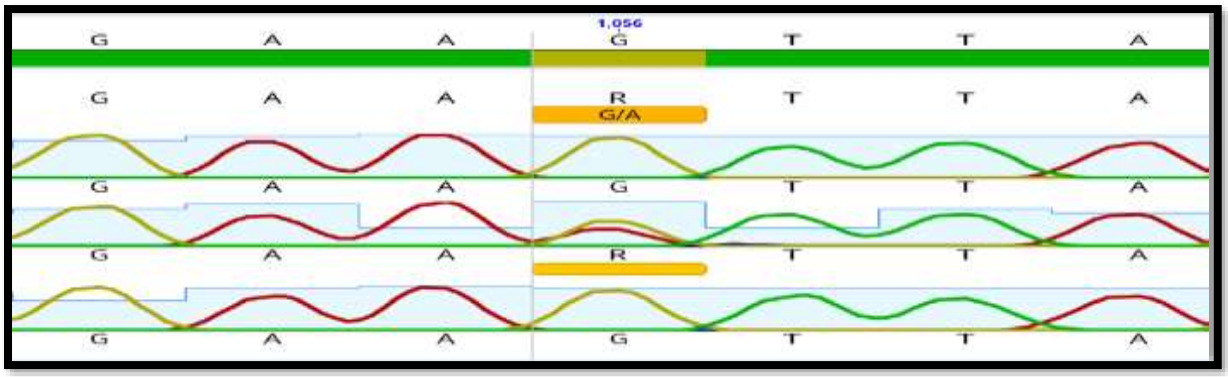

Figure 4 Analysis of rs1800750 SNP of TNF-α gene using Sanger sequencing.

Analysis of TNF-α (- 806C/T) (rs4248158) SNP

Figure 5 highlights the analysis of (rs4248158) SNP of the TNF-α gene using Sanger sequencing. Single "C" peak indicative of a (C) homozygous allele. Presence of the "C" and "T" peaks indicative of (C/T) heterozygous allele.

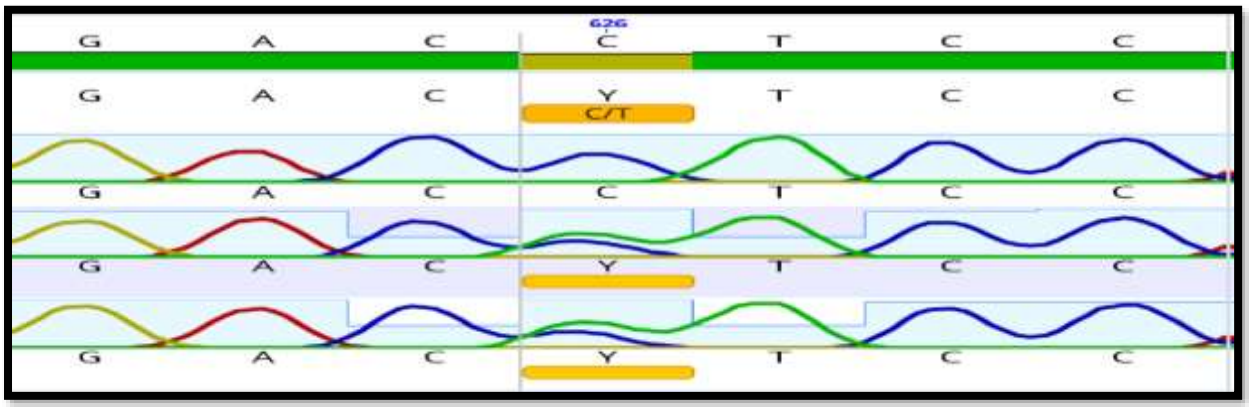

Figure 5. Analysis of rs4248158 SNP of TNF-α gene using Sanger sequencing.

Analysis of TNF-α (-1031 T/C) (rs1799964) SNP

Analysis of rs1799964 SNP of TNF-α gene using Sanger sequencing is presented in Figure 6. Single "T" peak indicative of a T homozygous allele. Single "C" peak indicative of a (C) homozygous allele. The presence of the "T" and "C" peaks is indicative of the (T/C) heterozygous allele.

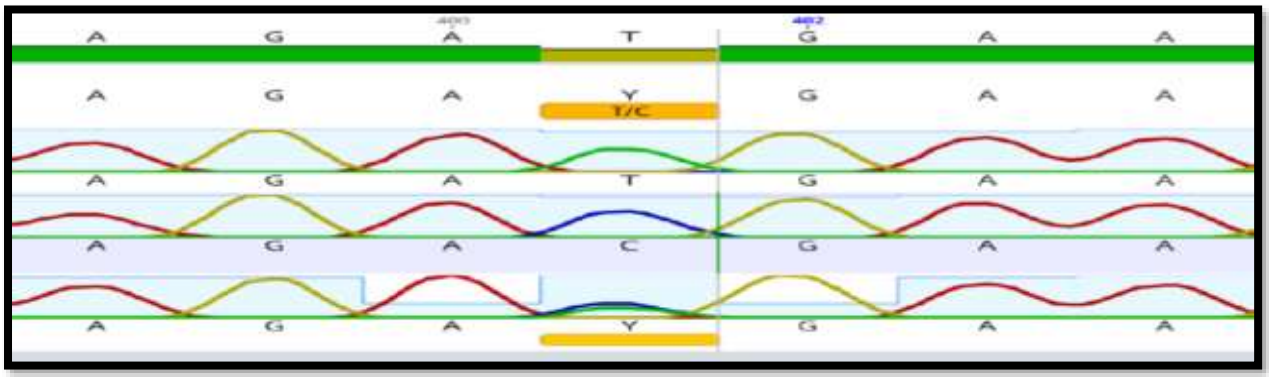

Figure 6. Analysis of rs1799964 SNP of TNF gene using Sanger sequencing.

Prevalence of genotypes polymorphism

Table 3 highlights the high proportions of GG genotypes of -376 G/A. The TT genotype was the most prevalent in more than half of the patients with -1031T/C. Almost three-quarters of patients had the CC genotype for the -806 C/T variant. Table 3 also shows the allele frequency for all the SNPs.

Table 3. Genotypes and alleles frequencies of TNF-α -1031 T/C, -806 C/T and -376 G/A gene polymorphisms in RA patients. N=80 patients

Genotypes				
-1031T/C	**Genetic variant**	**CC**	**TC**	**TT**
	No. (%)	3(3.75)	22(27.5)	55(68.75)
	Allele	**T**	**C**	
	No. (%)	77(96.25)	25(31.25)	
-806 C/T	**Genetic variant**	**CC**	**CT**	
	No. (%)	72(90)	8(10)	
	Allele	**C**	**T**	
	No. (%)	80(100)	8(10)	
-376 G/A	**Genetic variant**	**GA**	**GG**	
	No. (%)	4(5)	76(95)	
	Allele	G	A	
	No. (%)	80(100)	4 (5)	

Regarding the difference in alleles frequencies between the responders and non-responders, the results show no significant difference for all three polymorphic sites, as seen in Table 4. Additionally, the results of this study indicated that there was no significant difference in the distribution of all genotypes for all three sites in the TNF-α promoter region between responders and non-responders (Table 4).

Table 4. Difference in genotype frequencies of TNF-α -1031 T/C, -806 C/T and -376 G/A gene polymorphisms between responders and non-responders.

Genotypes		**Responders group (n=41) No. (%)**	**Non-responders group (n=39) No. (%)**	**P. Value**
- 1031 T/C	**CC**	2 (4.9)	1 (2.6)	**0.58**
	TC	12 (29.3)	10 (25.6)	**0.71**
	TT	27 (65.9)	28 (71.8)	**0.56**
	T	39 (95.1)	38(97.4)	**0.97**
	C	14 (34.1)	11 (28.2)	**0.40**
-806 C/T	**CC**	35 (85.4)	37 (94.9)	**0.15**
	CT	6 (14.6)	2 (5.1)	**0.15**
	C	41(100)	39(100)	**1**
	T	6(14.6)	2(5.1)	**0.17**
-376 G/A	**GA**	2 (4.9)	2 (5.1)	**0.95**
	GG	39 (95.1)	37 (94.9)	**0.95**
	G	41 (100)	39 (100)	**1**
	A	2 (4.9)	2(5.1)	**1**

A Chi-square test or Fisher exact test was used to identify the statistical difference between the groups.

Association between genotypes and the likelihood of being non-responder

Table 5 shows the binary logistic regression analysis results, which are non-significant for all genotypes of the three SNPs. This indicates that changing the genotype from the wild type to another polymorphic genotype cannot predict the tendency for being non-responder.

Table 5: Binary logistic regression analysis of genotypes to predict the tendency of being non-responder for ETN.

Parameter	**Coefficient**	**OR**	**P-Value**	**95% CI.**	
				Lower	**Upper**
- 1031 T/C	0.35	1.42	0.27	0.75	2.67
-806 C/T	-0.88	0.41	0.10	0.14	1.21
-376 G/A	0.73	2.08	0.36	0.43	10.07

Similarly, by using Phi- correlation all the demonstrated genotypes were either correlated positively or negatively with the tendency to be a non-responder, but none were statistically significant as seen in Table 6.

Table 6. Correlation between each genotype and the likelihood of being a non-responder.

Genotypes		phi-coefficient	P. Value
- 1031 T/C	**CC**	**-0.061**	**0.58**
	TC	**-0.041**	**0.71**
	TT	**0.064**	**0.56**
-806 C/T	**CC**	**0.189**	**0.09**
	CT	**-0.189**	**0.09**
-376 G/A	**GA**	**0.006**	**0.95**
	GG	**-0.006**	**0.95**

phi-correlation coefficient was used to find the correlation between each genotype and the likelihood of being a non-responder.

The Correlations between the Genotypes and The Difference in DAS28 Over Six Months . As shown in Table 7, most of the differences in DAS28 after six months of ETN treatment were not statistically significant.

Table 7. Association of the change in DAS28 over six months between different genotypes in -1031 T/C, -806 C/T and -376 G/A TNF-α genotypes polymorphism.

Genotypes					
-1031T/C	**Genetic variant**	**CC**	**TC**	**TT**	**P. Value**
	ΔDAS28	1.71±1.24	1.59±1.31	1.19±1.18	**0.37** [a]
-806 C/T	**Genetic variant**	**CC**	**CT**		
	ΔDAS28	1.22 ± 0.14	1.38 ± 0.54		**0.72** [b]
-376 G/A	**Genetic variant**	**GA**	**GG**		
	ΔDAS28	1.29 ± 0.13	0.73± 0.48		**0.36** [b]

Results are reported as means ±SD, ΔDAS28: the change in disease activity score of 28 joints over six months, [a] = one-way ANOVA used to find the statistical difference, b = unpaired t-test used to find the statistical difference.

TNF-α Level in Different Genotypes

After six months of continuous ETN therapy, there was no significant difference in TNF-α level between all genotypes of each SNP, as reported in Table 8.

Table 8. TNF-α level in different genotypes

Genotypes					
-1031T/C	**Genetic variant**	**CC**	**TC**	**TT**	**P. Value**
	TNF-α	121.3 ±63.57	80.14±40.06	99.95±48.93	0.17 [a]
-806 C/T	**Genetic variant**	**CC**	**CT**		
	TNF-α	93.51 ± 5.67	111.1 ± 16.79		0.30 [b]
-376 G/A	**Genetic variant**	**GA**	**GG**		
	TNF-α	98.03± 15.86	95.35±5.61		0.91[b]

Results are reported as means ±SD, [a] = one-way ANOVA used to find the statistical difference, [b] = unpaired t-test used to find the statistical difference.

Discussion

ETN has been proven to promote remission, reduce disease activity, and delay clinical and radiological disease progression in patients with RA. This has resulted in significant improvements in symptoms, function, and quality of life [(20)].

As well known, the effectiveness of ETN fluctuates considerably amongst RA patients, with around one-third of patients failed to clinically respond [(21)].

As a result, early identification of RA patients who will not respond to TNF-α antagonists, including ETN, enables a swift switch to alternative biological medications, thus increasing the patient's likelihood of promptly attaining treatment goals[(22)].

Studying the effect of genetic variation in response to ETN is essential because, in contrast to other variables that can influence and modify the ETN response, genetic determinants will remain consistent throughout a patient's lifetime [(23)].

Numerous SNPs were studied in the promoter regions of the TNF-α gene for their potential to alter the production of TNF-α or other cytokines, hence influencing the susceptibility or severity of RA[(24,25)].

Regarding the study's demographic characteristics, the results were equivalent in terms of the mean of age and duration of RA disease to those of another Iraqi study that examined beliefs about medications among a sample of Iraqi RA patients[(26)]. In terms of

male-to-female ratio variation, the findings were comparable to other Iraqi studies that confirm a high female-to-male ratio in RA illness[27–29].

The results of this study identified three SNPs in the promoter region of the TNF-α gene in a sample of eighty RA patients treated with ETN: -376 G/A, -806 C/T, and -1031 C/T.

Three earlier studies in Iraq[27,30,31] investigated the association between a polymorphism in the promoter region of TNF-α and RA in a cohort of Iraqi patients. However, no study has evaluated the relationship between these SNPs and ETN responsiveness. Furthermore, previous studies focused on only one or two SNPs, in contrast to the current study.

Many studies have examined the effect of SNP combination in the TNF-α gene on the response to TNF-α antagonists [32].

For instance, the SNPs -857C > T, -308G > A, -238G > A, and +489G > A in the TNF-α gene and their association to therapeutic efficacy were evaluated in retrospective study that involve 58 Greek RA patients taking infliximab[33].

Similarly, an association study of three TNF-α related SNPs (-308G/A, -238G/A, and -857C/T) was conducted in Poland and involve 280 RA patients of Caucasian origin who treated with TNF-α inhibitors for at least 6 months [34].

Additionally, meta-analyses have been conducted to examine the relationship between a variety of SNPs and TNF-inhibitor responsiveness, including the -308 G/A polymorphism, the -857 C/T polymorphism, and the -238 G/A polymorphism [3,35].Nonetheless, the current study was the only study that investigated the association between three SNPs -376 G/A, -806 C/T, and -1031 C/T on ETN responsiveness.

Concerning the prevalence of -1031T/C genotypes, the current study found that more than 68 % of patients have the TT genotype. While the CC genotype had the lowest prevalence 3.75%. Moreover, 97.5 %of patients possessed the T gene, while only 30% possessed the C allele.

Similarly, in the 1000 Genomes Database, the -1031 T/C promoter polymorphism has a reported minor C allele frequency of 22%.

Also, the results of this study are similar to those of Saudi patients with diffuse large B-cell lymphoma[36], patients with Behçet's disease in Western Algeria [37], and Japanese patients with Crohn's disease[38], all of whom had a high frequency of the TT genotype and a low frequency of the CC genotype.

Recent pieces of evidence have highlighted the associations between the -1031 T/C polymorphism and immune-mediated illnesses such as RA [38,39]. It can thus be reasonably assumed that the polymorphism in this site may influence the response to ETN.

However, the current study found no statistically significant correlation between ETN responsiveness and polymorphism in -1031T/C. No previous studies investigated the effect of-1031T/C on response to ETN in RA patients nevertheless, the -1031 T/T genotype was predictive of a favorable response to TNF-α antagonists in Chinese Han ankylosing spondylitis patients[40].

Likewise, a case-control study which performed in Spain and include 109 patients with psoriasis indicated that those with the TNF-α -1031 TT genotype responded better to infliximab[41].

Along with the small sample size, the current study's inconclusive results may be due to the use of ETN in RA, whereas prior trials involved infliximab or adalimumab in diseases other than RA. Moreover, ethnicity differences play a significant role in the pharmacogenetic investigations since allele frequencies in the populations studied may vary[42].

Regarding the prevalence of -806 C/T genotypes, the CC genotype was detected in 90% of RA patients enrolled in the current investigation. Though, the T allele was present in only 11.25 % of patients, the C allele was present in all patients, and there was no statistically significant difference in the availability of these genotypes or alleles between the responders and non-responders.

Although the number of studies investigating the role of -806C/T in various conditions is limited, the results are consistent with those of a recent study examining the putative role of TNF-α gene polymorphisms in patients with South Indian systemic lupus erythematosus (SLE)[43].

Also, These values correlate favorably with results from a South German Caucasian cohort research [44].

Furthermore, the findings are comparable to a case-control study conducted in Taiwan that looked at the connections between SNPs in the Promoter Region of the TNF-α and susceptibility to Nasopharyngeal Carcinoma[45].

The current study is the first to investigate the role of -806 C/T in RA patients' responsiveness to ETN. The findings clearly demonstrated a non-significant connection between any genotype and a proclivity for non-response to ETN. Unfortunately, no comparable study exists to which the findings may be compared. Nonetheless, the only comparable result was from a study in South Indian systemic lupus erythematosus (SLE) patients, which discovered no correlation between the - 806C/T genotypes and the tendency to develop SLE disease [43]. This result may suggest that this polymorphic site plays a minor role in autoimmune disease and, consequently, in response to medications used to treat these diseases.

In the case of -376 G/A genotypes, the current study discovered that 95 % of patients possessed the GG genotype while heterozygotes GA were found in 5% of the cases, and there were no homozygotes AA genotypes. Likewise, the A allele was only found in

5% of RA patients; however, the dominant G allele existed in all patients.

The findings are similar to those of a Turkish study that examined the association of TNF-α (-376 G/A) polymorphisms in Turkish tuberculosis patients[46].

Also, the result was parallel to an Iranian study which examined -376 G/A TNF-α gene polymorphisms in Iranian celiac patients to healthy controls[47].

While various experimental studies which tried to address the relation between TNF-α polymorphisms and its expression, and the mechanisms controlling its expressions in many cell types and diseases highlights that the TNF -376G/A SNPs may have functional significance and may influence TNF-α gene expression levels[41,48,49]; however, the TNF-α -376 G/A polymorphisms have received scant attention in RA patients, and their consequence on increased susceptibility to RA is uncertain [50–52].

For instance, the polymorphism -376 G/A was not associated with an increased risk of RA in Egyptian RA patients[53] or Mexican patients[54]. Correspondingly, the study of Kang et al. [55] found that -376G/A was not polymorphic in Korean RA patients.

The current study's finding failed to find a significant correlation between TNF-α (-376 G/A) polymorphisms and response to ETN in Iraqi RA patients.

No earlier studies have examined the connection between the -376 G/A and the response to ETN or even other TNF-α antagonists, this made the comparison with other studies in applicable.

The most plausible reason for the result mentioned above, in addition to the study's small sample size, is that the polymorphism at -376 G/A was very marginally associated with RA[50–52] , despite its effect on TNF-α gene expression[41,48,49].

The DAS28 score is the most often used outcome measure in investigating therapeutic response in RA [56]. Concerning the associations between various genotypes and DAS28 change after six months of continuous ETN treatment, the current study could not confirm a significant change in DAS28 for all three sites. Although he uses different response measures in his study, Kang et al.[55] found a non-significant change in ACR20 or ACR70 linked with -1031T/C and -863C/A SNPs in a sample of Korean RA patients.

The discrepancy between the current study and other studies in the lack of a significant change in DAS28 might be related to the vulnerability of subjective outcome scores to heterogeneity, such as DAS28, are vulnerable to heterogeneity depending on the reporting clinician. Moreover, It is generally established that measuring joint tenderness and patient perceptions of RA disease activity are two areas that can be challenging because there is no objective metric to validate clinical assessment[56].

In addition to the differences in research design, sex ratios, sample size clinical outcomes assessed (DAS28ESR vs. DAS28CRP), anti-TNF medicine utilized, and concomitant use of disease-modifying antirheumatic therapies (DMARDs) with the TNF-α antagonist in some studies.

Regarding the difference in TNF levels between different genotypes, the current study results indicated that, polymorphism did not cause any significant difference in all three sites.

Although no prior research has explored the association between these variants and TNF-α levels in RA patients after taking ETN therapy, one study analyzed the -1031 T/C polymorphism and TNF-α levels in patients with the acute coronary syndrome[57]. The results indicated that TC carriers had significantly greater TNF-α levels than TT homozygotes[57]. In contrast, another investigation discovered that carriers of the uncommon TNF-α -1031 C allele tended to have a more significant blood TNF-α level in chronic obstructive pulmonary disease patients[58].

In the current study, the CC of carriers the -1031 T/C had the highest mean level of TNF-α but not significantly different from TT and TC carriers. Also, TT carriers have a slightly higher but not statistically significant than TC carriers.

Regarding the -376 G/A and -806 C/T polymorphisms, the current investigation found that the polymorphic genotypes of these SNPs (-376 GA, -806 CT) had a slightly higher level of TNF, but the difference was not statistically significant.

Two investigations indicated that the A allele of the -376 G/A is associated with higher TNF-α production and transcription[59,60]. However, this contrasts with Musa et al., who discovered no correlation between the -376 G/A genotypes and TNF-α levels in Egyptian patients with RA[53]. On the other hand, Katkam et al.[43] found no association between -806 C/T polymorphisms with the TNF-α level in a patient with SLE.

Limitations

The current study has several limitations. First, this study had a small sample size, which could be attributed to the small number of patients who used only ETN and met all other inclusion criteria.

Second, because this study was conducted in a single center, caution is required when generalizing the findings to all Iraqi rheumatoid arthritis patients. However, the chosen center treated patients from a variety of Iraqi governorates.

Moreover, the study was conducted during the COVID-19 era, and the overall number of patients included was limited due to repeated curfews that resulted in the loss of several patients as a result of their inability to attend the rheumatology units and obtained their medications.

Although the study excludes smokers and patients taking ETN in combination with another DMARD to rule out any response effect that could bias the

results, one significant weakness is that the researchers focused exclusively on polymorphisms in the TNF-α promoter region, and ignored polymorphisms in other regions that could alter the response to ETN.

Furthermore, because most of the patients were already on ETN when they were enrolled, the baseline level of TNF- was not measured. The author relies on the difference in levels across genotypes to determine whether certain genotypes are associated with high levels of TNF-α, which may lead to non-responsiveness to ETN.

Finally, the authors were unable to conduct a more powerful prospective cohort study by selecting only patients with specific genotypes and following them for six months due to the small number of patients who received only ETN, insufficient financial resources, and the lengthy time frame required to recruit and conduct this type of study.

Conclusions

This study has revealed that patients who do not respond to ETN have a higher TNF-α level than respondents. Sanger sequencing of the TNF-α gene promoter region revealed three polymorphic sites -1031 T/C, -806 C/T, and -376 G/A. The polymorphisms at these three locations did not affect the likelihood of Iraqi RA patients being non-responder. Since the significance of these SNPs in RA patients' response to ETN has not been established in the current study, and no previous study has inspected the effect of these SNPs in other populations. Therefore, it is essential to examine this polymorphism in a larger group of populations with diverse ethnic backgrounds to confirm or refute the results of this study.

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