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Genetic Polymorphism in TNF-α Promoter Region: Its Association with Severity and Susceptibility to Rheumatoid Arthritis in Iraqi Patients with Active Disease

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Abstract

To study the prevalence of rs1799964 (-1031 T/C) and rs361525 (- 238 G/A) single nucleotide polymorphisms (SNPs) and their effect on the disease activity, severity, and cytokines production in newly diagnosed Iraqi rheumatoid arthritis patients.

Sixty-three patients were diagnosed by a specialist physician while attending the rheumatology unit and twenty control were participated. The inflammatory markers were measured and polymerase chain reaction (PCR) amplification and sequencing were performed to demonstrate tumor necrosis factor alpha (TNF-α) SNPs.

Regarding (-1031 T/C) SNP, the TT genotype and allele C were significantly present in the controls, and the TC genotype was distributed significantly in the patients. The TT genotype was mostly distributed in the mild-moderate group, while TC genotype and allele C were significantly distributed in the severe group. Disease activity score in 28 joint (DAS28), TNF-α, interleukin-1 beta (IL-1), anti-citrullinated protein/peptide antibody (ACPA) were significantly associated with this SNP. While non-significant difference were appeared in the analysis of -238 G/A SNP.

The presence of the TC genotype and C allele of **(**-1031 T/C**)** was associated with susceptibility to RA. Also, the TC genotype and C allele were associated with more severe disease. Also, TT genotype was associated with less severe disease. Furthermore, an associated between -1031 T/C and the inflammatory markers and DAS28 were reported.

Key words: Rheumatoid arthritis, SNPs, TNF-α, \–238 G/A, -1031T/C.

تعدد الأشكال الجيني في المنطقة المحفزة لعامل نخر الورم الفا: و علاقته مع شدة مرض التهاب المفاصل الروماتويدي وقابلية الأصابة به لدى المرضى العراقيين المصابين بالتهاب المفاصل الروماتويدي النشط

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الخلاصة

دراسة معدل انتشار (C / T -1031) rs1799964 و (A / G - 238) rs361525 وتأثيرهما على نشاط المرض وشدته وإنتاج السيتوكينات في مرضى التهاب المفاصل الروماتويدي العراقيين المشخصين حديثًا.

تم تشخيص ٦٣ مريضاً من قبل طبيب متخصص أثناء حضورهم وحدة أمراض الروماتيزم وشارك عشرون شخص سليم. تم قياس علامات الالتهاب وتم إجراء تضخيم وتسلسل البلمرة للكشف عن تعدد اشكال النيوكليوتيدات المفردة في عامل نخر الورم الفا.

فيما يتعلق ب تعدد اشكال النيوكليوتيدات المفردة (C / T ١٠٣١-) ، كان النمط الوراثي TT والأليل C موجودًا بشكل كبير في عناصر التحكم ، وتم توزيع النمط الجيني CT بشكل كبير في المرضى. تم توزيع النمط الوراثي TT في الغالب في المجموعة الخفيفة والمتوسطة ، بينما تم توزيع النمط الوراثي **CT** والأليل **C** بشكل كبير في المجموعة الشديدة. ارتبطت DAS28 و TNF-α و IL-1 و ACPA بشكل كبير مع تعدد اشكال النيوكليوتيدات المفردة هذا. بينما ظهر اختلاف غير مهم في تحاليل (G / A ٢٣٨-).

ان وجود النمط الجيني CT وأليل C لـ (C / T ١٠٣١-) كان مرتبطا مع القابلية للإصابة بـالتهاب المفاصل الروماتويدي. بينما ارتبط النمط الجيني CT والأليل C بمرض أكثر حدة. ، أيضا ارتبط النمط الجيني TT مع مرض أقل حدة. علاوة على ذلك ، تم الإبلاغ عن ارتباط بين - ١٠٣١ (C / T ١٠٣١-) وعلامات الالتهاب وDAS28.

الكلمات المفتاحية : التهاب المفاصل الروماتويدي ،SNPs ، TNF-α ، (G / A ٢٣٨-) ، (C / T ١٠٣١-)

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease, characterized by chronicity, systemic inflammation, and joint damage. Women are more prone to have the disease than men with a 2-3:1 ratio [(1)]. The prevalence of RA globally is (0.2-1.2) , however , because of the diversity in ethnicity, weather and socioeconomic level, the frequency of RA is different from country to country [(2)]. The prevalence in the Iraqi population is about (1%) [(3)]. Many comorbidities were developed in RA patients for example permanent function impairment develops with progressive inflammation [(4,5)].

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The primary goal of treatment is to minimize the disease activity and to reach a clinical remission state [6]. Almost all RA patients experience symptoms such as weakness, a modest increase in temperature, anemia, and an elevation in inflammatory markers [7].

Despite that RA has an unknown etiology, both hereditary and environmental factors may predispose to RA [8]. In fact, genetics has a critical role in developing RA. In addition it affects the severity of the disease and also the benefit of the treatment [9].

Rheumatoid arthritis is characterized by polygenicity and heterogeneity. Human leukocyte antigen (HLA), specifically the HLA-DRB1 alleles, (carrying the shared epitope sequence) is the primary gene that accounts for about (40%) of the genetic of RA [10]. It was reported that the shared epitope is highly associated with a rheumatoid patient with a positive anti-citrullinated protein/peptide antibody (ACPA) [11]. Additionally, cytokine genes are an important player in the development of RA [12]. Cytokines are the major contributor predisposing autoimmune response and are the cornerstone in RA pathophysiology [13].

From these, tumor necrosis factor-alpha (TNF-α) has a pivotal role in the pathophysiology of RA. It's involved in apoptosis and regulation of the immune system. Also patients with RA show chronic increase in TNF-α levels both in joint and blood [12]. Actually, TNF-α is the major contributor to synoviocytes activation which consequently release different cytokines [13]. Another cytokine besides TNF that is thought to have a significant role in progressive RA is IL-1. Also, the pro-inflammatory cytokine (IL-6) has a pivotal role in the development and progression of RA. It was reported that SNPs in these cytokines affect the disease severity, susceptibility and treatment response [12].

The genetic polymorphism of cytokines affects the disease course, susceptibility, and benefit from medication [12]. Since about (60%) of TNF-α level differences among RA patients have been explained by genetics. Polymorphism in TNF-α takes major attention in genetic studies [14].

The gene of TNF is situated in the HLA region on the sixth chromosome. The promoter region in TNF gene display multiple single nucleotide polymorphism [15] as rs1799964, rs1800630, rs1799724, rs1800750, rs1800629, rs361525, rs4248158, rs4248160, and rs4248161 [16,17].

Multiple studies and research were published in the last 20 years regarding the association between TNF-α polymorphisms and the severity and susceptibility to RA, the data showed conflicting and controversial results [18–21]. Such controversial findings can be explained by racial and ethnic differences [22]. Therefore, investigation of polymorphism in the various ethnic group is critical to determine the genetic differences that may affect disease severity and susceptibility [23].

This study was designed to determine the prevalence of rs1799964 (-1031 T/C) and rs361525 (- 238 G/A) SNPs and their effect on the disease activity and cytokines production in newly diagnosed Iraqi rheumatoid arthritis patients.

Patients and Methods

Study design

Eighty-three participants were enrolled in the study. Those are classified into three groups, the first contains thirty-two mild-moderate RA patients, the second includes thirty-one RA patients with severe disease and the last group contains twenty healthy adults as controls. All patients were confirmed to be newly diagnosed with RA according to the American College of Rheumatology (ACR)/ European League against Rheumatism (EULAR) Classification Criteria. The 2010 ACR criteria give score for the involved joints, RF, ACPA, CRP, ESR and disease duration [24]. The diagnosis was made by a specialist physician while attending a rheumatology unit during the period from January 2021 to December 2021. The controls were randomly selected; they are healthy and are age and sex-matched to the selected patients. Ethical approval with the number (RECAUBCP-2692020) on 29/9/2020 was obtained. Written consent was obtained from all the participants.

Inclusion criteria

All patients who enrolled in this study were newly diagnosed with RA according to the 2010 EULAR classification criteria [24]. And the disease activity will depend on the measurement of the Disease Activity Score in 28 Joint (DAS28) [25].

Exclusion criteria

1. Patients using DMARDs even they are newly diagnosed.
2. Patients using a high-dose steroid.

Clinical evaluation

The classification of the patient group was made based on measuring DAS28 according to the following formula.

$$\mathbf{DAS28} = (\mathbf{0.56} \times \sqrt{\mathbf{SJC}} + (\mathbf{0.28} \times \sqrt{\mathbf{SJC}} + ((\mathbf{0.7} \times \mathbf{LN}(\mathbf{ESR})) + (\mathbf{0.014} \times \mathbf{VAS})$$

TJC: tender joint count, SJC: swelling joint count, ESR; erythrocyte sedimentation rate, VAS: visual analog score.

The result of the score is interpreted as followed: a score of >5.1 indicates highly active disease, >3.2 and ≤ 5.1 means moderate activity, ≥2.6 and ≤3.2 low disease activity, and <2.6 means remission [25]. The ESR estimation can be performed by the modified Westergren method using a whole blood sample anticoagulated with EDTA. Serum TNF-α and Interleukin-1 beta (IL-1 beta) levels were

determined using a commercial kit obtained from Cusabio, using the sandwich ELISA method.

Genotyping

Promega ReliaPrep TM Blood gDNA Miniprep System was used for genomic DNA extraction from the blood sample. The concentration of the extracted DNA was determined using "The Quantus™ Fluorometer" to ensure the sample quality [26]. Primer for PCR was designed using Primer Premier 3 software. These primers (Table 1) were supplied by Macrogen Company in a lyophilized form. The genotyping reaction in PCR was performed by a thermal cycler device (Thermo Fisher Scientific, USA) using a master mix from Promega, USA.

Table 1. Primers

Primer	Sequence	Annealing Temp	Product size (bp)
TNF-F	5`- TGTAAAACGACGGCCAGTGCTTCAGGGATATGTGATGG-3`	60(°C)	1008
TNF-R	5`- CAGGAAACAGCTATGACCCTTCTGTCTCGGTTTCTTCTC-3`	60(°C)	1008

TNF-F: forward primer, TNF-R: reverse primer, bp: base pair

Primer Optimization

The perfect temperature required for primers annealing was determined by amplification of the DNA template, at multiple annealing temperatures (55, 58, 60, 63, and 65°C) and it was found to be 63°C (Figure 1) and the amplification step was confirmed by applying agarose gel electrophoresis Promega, USA. (Figure 2).

Sanger method was performed for sequencing the PCR product by DNA analyzer (ABI3730XL) (Macrogen Corporation – Korea). Geneious Prime software (V 2021.1.1) (www.geneious.com; Biomatters Ltd., New Zealand) was used for data generation [26].

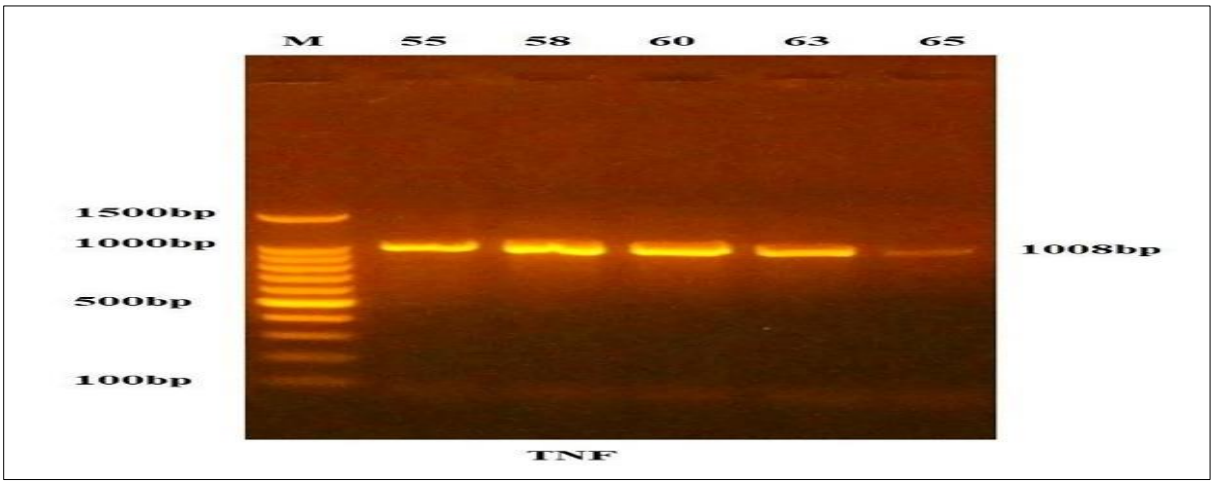

Figure 1. Optimization of TNF primer

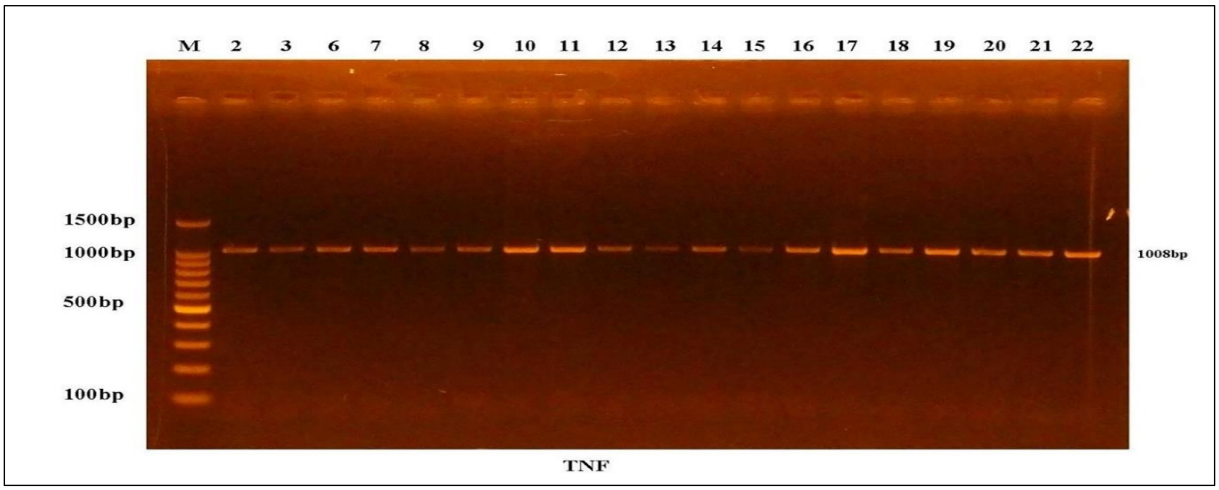

Figure 2. Gel electrophoresis of the amplified DNA

Statistical analysis

The statistics of the result were calculated employing SPSS for Windows 26.0 software (SPSS, Inc., Chicago, IL, USA). Continuous parameters were shown as mean ± standard deviation (SD) while discrete variables were presented as numbers and frequencies. The Shapiro Wilk test is used to test the normality of data. For discrete variables, Chi-square or Fisher's exact test was used to test the group difference. For groups that showed normal distribution, an unpaired independent sample T-test is used to test the mean differences of two independent samples; while more than two groups were analyzed by one-way ANOVA. Linear analyses were used to find the correlations between categorical and continuous data. Finally, phi-coefficient was used to determine the correlations between two categorical variables. A probability that equals or less than 0.05 was considered significant, less than 0.01considerd highly significant and less than 0.001considerd very highly significant.

Results

Demographic and inflammatory markers Characteristics for the Patients and Controls

The Demographic data for all study groups are presented in (Table 2). While the results of the inflammatory markers are listed in (Table 3). It showed a significant increase in ESR in the mild-moderate and the severe groups when compared to the controls, also a significant increase in ESR was detected in the patient with severe RA in comparison with the mild-moderate RA patients.

The ACPA titer has a significant increase in the mild-moderate group comparing to the control group, and a significant increase in a patient with severe RA compared to the controls and in the severe RA patients compared to the mild-moderate RA patients.

Interleukin-1 beta and TNF-α levels were significantly increased in patients with mild-moderate and severe RA compared to the controls, and a significantly increased in the severe RA patients compared to the mild-moderate RA patients.

Table 2. Demographic data for the patients and controls.

Group Parameter	Mild-moderate disease	Sever disease	Control	P - value
Age (year)	33.4±7.3	34.9±6.7	32.1±5.6	0.809[a]
Female (no: %)	28: 87.5 %	27: 87.1 %	17: 85 %	0.965[b]
Male (no: %)	4: 12.5 %	4: 12.9 %	3: 15%	0.965[b]
Smoking (no: %)	3: 9.4 %	2: 6.5 %	2: 8.7 %	0.298[b]

Continuous variables were presented as Mean ± Standard deviation; and discrete variables as numbers and frequencies, no: number of the patients. [a]: one-way ANOVA [b]: Chi-square.

Table 3. Results of the inflammatory markers for all participants.

Markers	Control	Mil-Mod	Sever	P- value cont/mil	P- value cont/sev	P- value severe/ mild
ESR (mm/h)	11.6 ± 3.7	20.3 ± 9.1	34.8 ± 10.7	<0.0001 ⁂	<0.0001 ⁂	<0.0001 ⁂
ACPA(IU/ml)	9.6 ± 2.5	12.1 ± 4.8	17.6 ± 5.6	0.042*	<0.0001 ⁂	<0.0001 ⁂
IL-1beta pg/ml	890 ± 256	2280 ± 633	2685 ± 939	<0.0001 ⁂	<0.0001 ⁂	0.048 *
TNF-α pg/ml	60 ± 25	191 ± 55	247 ± 124	<0.0001 ⁂	<0.0001 ⁂	0.02 *

Variables are shown as Mean ± Standard deviation. The Analysis was made by Independent 2 sample t-test. * = significance difference (P<0.05), ⁂ = very highly significant (P<0.001) difference. ESR: erythrocyte sedimentation rate, IU: international unit, ACPA: anti-citrullinated protein/peptide antibody, IL-1: interleukin-1, TNF-α: tumor necrosis factor alpha, mm/h millimeter per hour, pg/ml: pictogram per milliliter, cont: controls, sev: sever, mil: mild-moderate.

Result of genetic polymorphisms.

Result of PCR Amplification.

The amplified region from the TNF-α gene of all participant was fractionated by agarose gel electrophoresis [(26)] (Figure 2).

Analysis of TNF-α (– 1031 T/C) SNP

The (–1031 T/C) SNP was analyzed by the Sanger method of sequencing (Figure 3). The finding of a single T peak means [T] homozygous allele. While the presence of only a C peak suggests a [C] homozygous allele. And the presence of both T and C peaks indicates [T/C] heterozygous allele.

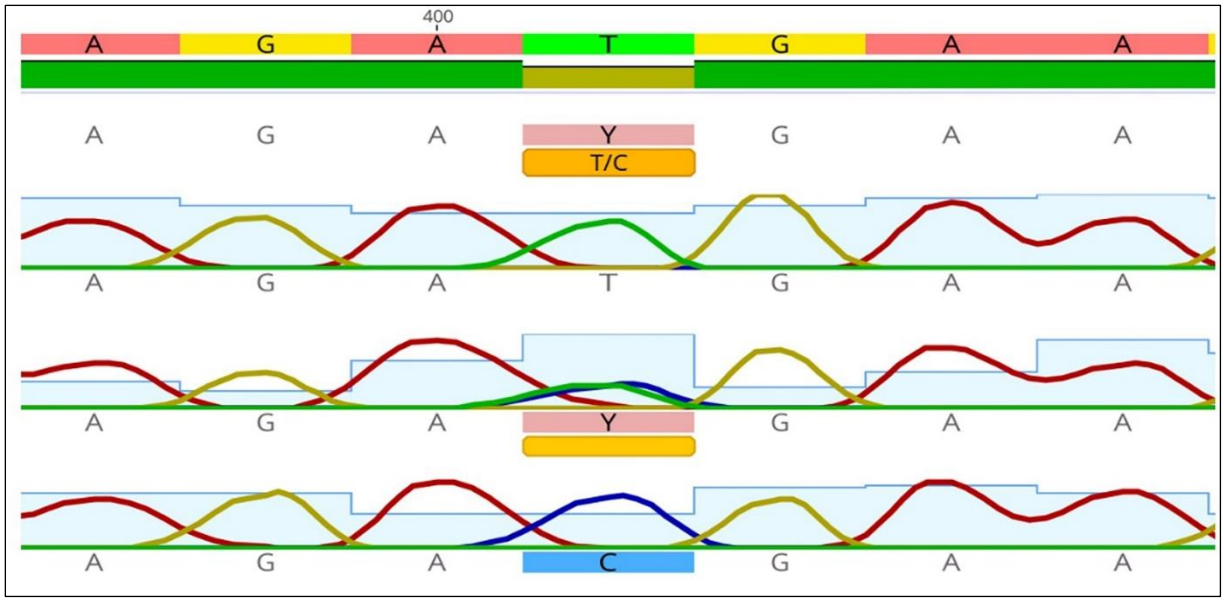

Figure3. Analysis of rs1799964 SNP of TNF gene. Single "T" peak indicative of a T homozygous allele. Single "C" peak indicative of a C homozygous allele. Presence of the "T" and "C" peak indicative of T/C heterozygous allele

The prevalence of (-1031) T/C SNP in all study participants.

The prevalence of (-1031) T/C SNP was shown in (Table 4). The TT genotype of (-1031) SNP was highly significantly distributed within the control group compared to the patient groups, whereas the CT genotype was distributed significantly in the patient group compared to the control, but the CC genotype and the T allele have a non-significant difference between the patients and the controls. Also, the allele C was highly significantly present in the patient group.

When comparing the mild-moderate RA group with the severe RA group, we found a significant difference in the distribution of TT genotype, mostly distributed in the mild-moderate group, while CT genotype was significantly distributed in the severe RA group. However, the CC genotype and the T allele were shown a non-significant difference between the two groups. Also, the allele C was significantly present in the severe group compared to the mild-moderate group.

Table 4. Prevalence of (-1031) T/C SNP in all study participants

Genotypes	Mild-moderate (n=32) No. (%)	Severe (n=31) No. (%)	P - value severe versus mild-moderate	All patients (n=63) No. (%)	Control (n=20) No. (%)	P - value patient versus controls
CC	2(6.25)	2(6.45)	0.1[a]	4(7.94)	0(0.0)	0.56[a]
CT	11(34.38)	19(61.29)	0.032*[b]	30(47.62)	4(20)	0.037*[a]
TT¤	19(59.37)	10(32.25)	0.031*[b]	29(46.03)	16(80)	0.009*[a]
Alleles						
C	13(40.62)	21(67.74)	0.031*[b]	34(53.97)	4(20)	0.009*[a]
T	30(93.57)	29(93.54)	1[b]	59 (93.65)	20(100)	0.56[a]

*: significance difference (P<0.05) and *: highly significant (P<0.01) difference, [a]: Fisher's exact test, [b]: Chi-square test, ¤: wild genotype

Association between rs1799964 (-1031) T/C SNP and the likelihood of severe disease.

The severity of RA measured by DAS28 had a positive significant association with the TT and TC genotypes whereas a non-significant correlation was observed with the C genotype (Table 5).

Table 5. Association between (-1031) T/C SNP and DAS 28 in RA patients.

Genotype		Phi coefficient	P-Value
-1031 T/C	**TT ¤**	-0.27	0.031 *
	TC	0.26	0.032 *
	CC	0.04	0.97

Phi-coefficient analyses was used for analysis, *: significance difference (P<0.05), ¤: wild genotype.

Association between (-1031) T/C SNP and clinical parameters in RA patients

The associations between (-1031) T/C SNP and the clinical parameters in RA patients were determined using Liner regression analysis (Tables 6).

The ACPA titer showed a very highly significant association with (-1031) T/C in both the severe and the mild groups. Also, the association between (-1031) T/C and TNF-α level was found to be highly significant in the mild-moderate group and significant in the severe groups. Non-significand associations were found between (-1031) T/C SNP and ESR in the mild-moderate meanwhile the severe groups were significantly associated with this SNP. The association with IL-1beta level was found to be significant within the severe group, and non-significant within the mild-moderate group.

Analysis of TNF-α (– 238 G/A) SNP

The (– 238 G/A) SNP was analyzed by the Sanger method of sequencing (Figure 4). The finding of a single G peak means [G] homozygous allele. While the presence of only A peak suggests a [A] homozygous allele. And the presence of both G and A peaks indicates [G/A] heterozygous allele.

Table 6. Association between rs1799964 (-1031) T/C SNP and inflammatory markers.

Group	Marker	Regression Coefficient	P-Value
Mild-moderate RA	ACPA titer	0.49	0.004 ⁑
Sever RA		0.16	<0.001 ⁂
Mild-moderate RA	TNF-α	0.46	0.008 ⁑
Sever RA		0.38	0.031*
Mild-moderate RA	ESR	-0.20	0.25
Sever RA		0.57	0.001 ⁑
Mild-moderate RA	IL-1beta	-0.01	0.92
Sever RA		036	0.041*

Linear regression analyses was used to find the correlations, *: significance difference (P<0.05), ⁑: highly significant difference (P<0.01), ⁂ = very highly significant (P<0.001) difference, ESR: erythrocyte sedimentation rate, ACPA: anti-citrullinated protein/peptide antibody, IL-1: interleukin-1, TNF-α: tumor necrosis factor alpha.

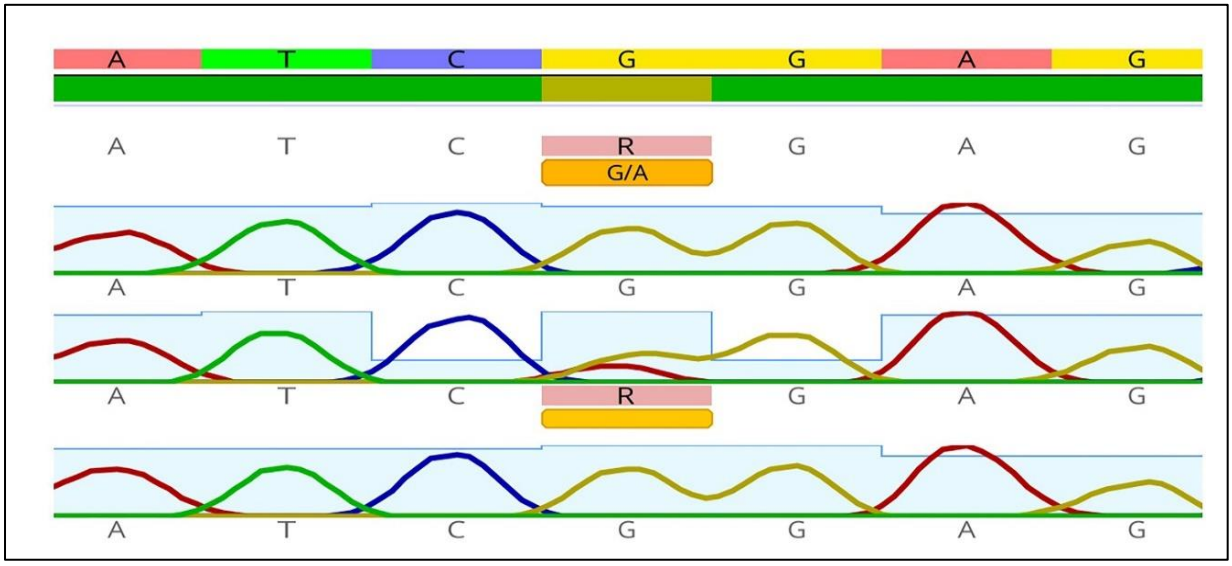

Figure 4. Analysis of rs3615254 SNP of TNF gene . Sigle "G" peak indicative of a G homozygous allele . Single " A" peak indicative of A homozygous allele . Presence of the " G " and " A" peak indicative of G/A heterozygous allele

The prevalence of (-238) G/A SNP in all study participants

A non-significant difference in the prevalence of GG, AA, and GA genotypes and the distribution of both G and A alleles of the (-1031) T/C SNP in all the study groups (Table 7).

Table 7. Prevalence of (-238) G/A SNP in all study participants.

Genetic variant	Mild-moderate (n=32) No. (%)	Severe (n=31) No. (%)	P value Severe versus mild-moderate	All patients (n=63) No. (%)	Control (n=20) No. (%)	P value patient versus controls
GG¤	31(96.88)	29(93.55)	0.61	60(95.24)	18(90)	0.59
GA	1(3.12)	2(6.45)	0.61	3(4.76)	2(10)	0.59
Allele						
G	32(100)	31(100)	1	63(100)	20(100)	1
A	1(3.12)	2(6.45)	0.61	3(4.76)	2(10)	0.59

Analyses was made by Fisher's exact test ¤: wild genotype.

Association between (-238) G/A SNP with DAS 28 and the clinical parameters in RA patients.

The associations of (-238) G/A SNP with DAS 28 and the clinical parameters in all patients' groups were found to be non-significand (Table8 and Table 9).

Table 8. Association between (-238) G/A SNP and DAS 28 in RA patients.

Genotype		Phi coefficient	P-Value
-238 G/A	**GG ¤**	0.07	0.53
	GA	-0.07	0.53

Phi coefficient analyses was used to find the correlations ¤: wild genotype.

Table 9. Association between (-238) G/A SNP and the inflammatory markers.

Group	Marker	Regression Coefficient	P-Value
Mild-moderate RA	ACPA titer	-0.29	0.10
Sever RA		-0.10	0.56
Mild-moderate RA	TNF-α	-0.004	0.98
Sever RA		-0.16	0.32
Mild-moderate RA	ESR	-0.20	0.25
Sever RA		-0.05	0.75
Mild-moderate RA	IL-1beta	-0.37	0.84
Sever RA		-0.15	0.40

Linear regression analyses was used to find the correlations ESR: erythrocyte sedimentation rate, ACPA: anti citrullinated protein/peptide antibody, IL-1: interleukin-1, TNF-α: tumor necrosis factor alpha.

Discussion

The current research studied the association between SNPs in the TNF-α promoter region and the severity of RA and also determine the prevalence of those genes in RA patients in a sample of 63 newly diagnosed RA patients (32 with mild-moderate and 31 with severe disease) and 20 healthy controls.

We found that the level of inflammatory markers (ESR, ACPA, IL-1beta, TNF-α, hsCRP, RF) was elevated in patients with RA, those results were similar and consistent with previous studies [26–31].

According to our knowledge, the prevalence of rs1799964 (-1031) T/C SNP in Iraqi RA patients and its association with disease activity and severity was not studied.

The current study found that the TT genotype of rs1799964 (-1031) T/C SNP was found in about 46% of patients and 80% of controls (a highly significant difference). While the CT genotype was found only in 20% of controls and in 47% of patients (significant difference). However, the CC genotype was found only in the patient group (6%).

The T allele was non-significantly distributed between the controls (100%) and the patients (93%), whereas the C allele was found in 20% of controls and 54% of the patients (significant difference).These results indicate the presence of the CC genotype may increase the risk of getting RA while the TT genotype may decrease that risk. These results were in accordance with the

previously reported increase in TNF- α production in the CC genotype [32].

Some previous studies conducted in Iraq have studied the association between SNP in TNF-α and RA. However, they examined different SNPs other than -1031T/C genes [26, 33].

Globally these results were agreed by a study on Irish and British populations that showed an increased prevalence of -1031 SNP in RA patients [34]. While a case-control study in Egypt and a study in the Tunisian population found a non-significant difference in the prevalence of rs1799964 (-1031) T/C SNP between RA patients and controls [35,36]. The difference between these studies and the current study could be attributed to the differences in the geographical area of patients (differences in the ethnicity of study participants). Another explanation is due to the small sample size which is only 35 in the Egyptian study.

Also, this study found the TT genotype of rs1799964 (-1031) T/C SNP was found in about 60% of the mild-moderate RA patients and only in 32.25 % of the severe RA patients (significant difference). While the CT genotype was distributed 34.38% in the mild-moderate RA group and 32.29 % in the severe RA group). However the CC genotype was distributed equally between the two groups (6%). Also, the T allele was distributed equally in the two groups (93%), whereas the C allele was found in 40.62% of the mild-moderate RA group and 67.74 % in the severe RA group.

These findings may indicate that the presence of the TT genotype in RA patients might be associated with less severe disease. These results were contradictory to the Tunisian study that showed that the TT genotype increased in patients with no remission (63%) compared to patients with remission (21%) and the CT genotype was found mostly in the remission group (71%) and only 27% in the non-remission group. However, the distribution of the CC genotype was similar to the present study [36].

This difference in result could be attributed to the difference in the criteria of groups of the patient studied, the Tunisian study measured a remission versus non-remission patients after treatment by contrast, the current study exclude treated patient. More over the difference could be attributed to difference in the ethnicity between the two populations.

The current study also demonstrates that rs1799964 (-1031) T/C SNP was significantly associated with disease activity (measured by DAS 28). Karray F et al., 2011 study, showed that TNF -1031 C allele was associated with RA patients in the remission state [36], while Zaghlol et al., 2019 study, found a non-significant association between RA activity measured by Sharp score [35]. However, both studies measured different criteria with a different study design from the present study.

Multiple studies focused on the associations between TNF-α gene polymorphism and DAS28, however, they study SNP other than (-1031) T/C. a study in the Italian population show an association between GG genotype TNFa-238G/A and the severity of disease [37], while no association was found in Syrian and French population when measuring TNFa-308 G/A SNP [38]. A study that includes 172 RA Egyptians found that the GG genotype of TNFa-308 G/A is associated with disease severity and the AA genotype with disease susceptibility [39].

Also, the present study indicates a significant association between rs1799964 (-1031) T/C SNP and the inflammatory markers (ACPA titer, TNF-α) within each patient group, however, IL-1 beta and ESR were associated with (-1031) T/C SNP only in the sever group. Zaghlol et al., found a non-significant association between (-1031) T/C SNP and ACPA titer, TNF-α, however, there is no data for this study [35], and Barton 2004, doesn't find an association between rs1799964 (-1031) T/C SNP and the severity of inflammatory polyarthritis by comparing erosive and non-erosive disease [40]. According to my knowledge, no other study investigates the effect of SNP in TNF -1031 on the inflammatory markers in RA patients. A study in Turkish people with Sjögren's syndrome showed an association between TNF-α level and rs1799964 (-1031) T/C SNP [41]. Another study found an association between TNF-α level and rs1799964 (-1031) T/C SNP in patients with diabetic nephropathy [42]. This may be explained by the increase in TNF-α production in (-1031) T/C SNP [43–45].

Regarding rs3615254 (-238) G/A SNP, the result found that there was no difference in the distribution of GG genotype between the patients and the control group, its presence in 90% of the patients and 95% of the controls. Also, the GA genotype was distributed non-significantly between the patient and the controls (10% of controls and 4% of patients).

Previous Iraqi study in Al Najaf province investigating rs3615254 (-238) G/A SNP in the Iraqi population [46]. Their results were controversial to the present study; there was a significant difference between patients and control. The later could also be attributed to ethnic diversity of patients from all of Iraq compared to the study conducted on patients from Al-Najaf province only.

However, multiple global previous studies found conflicting results about the rs3615254 (-238) G/A SNP. In one of them, 97% of patients and 87% had the GG genotype and 2% of patients and 12% of control contained the GA genotype in 130 RA patients and 169 controls of Mexican nationality [21]. Similar findings were shown in a case-control study in Netherland, which includes 283 RA patients and 116 controls

which found 92% and 95% GG genotype in patients and the controls respectively, and the GA genotype was 8% and 9.5% in patients and the controls respectively [18]. Additional similar results were founded in a study in the Chinese population that includes 452 RA patients and 373 controls [47] and in the Caucasian study that includes 376 RA patients and 463 controls [19]. A controversial result was obtained from an Indian study that found only 34% of GG genotype in patients and 32% in control while the GA genotype was present in 66% and 68% of patients and controls respectively [48].

Additionally, the current study showed that the G allele was found in all patients and controls while the A allele presents only in about 5% of patients and 2% of controls. Similar findings were obtained in some previous studies [18,21]. But a study in India showed that the A allele was present in 49% of patients and 68% of controls. However the G allele was distributed in all patients similarly to the current study [48]. The small sample size in our study and the ethnic differences could be an accepTable explanation for that difference. In agreement with our explanation, a meta-analysis that includes 1386 RA patients and 1535 healthy individuals showed different findings across ethnicities [49].

Also, the present study found a non-significant difference in the distribution of the GG (97% and 93%) and the GA (3% and 6%) genotypes in the mild-moderate and severe groups respectively, and in both A (2% and 6%) and G (100% and 100%) alleles in the mild-moderate and sever groups respectively. These results agreed with the Mexican study, however, the GA genotype doesn't present in any patient from the severe group [21]. A controversial finding was shown in al Najaf's study [46]. An accepTable explanation related to the fact that the current study investigates newly diagnosed RA patient only and depend on DAS 28 in the stratification of the patients, but the previous study classify the patient according to the rheumatic nodule and joint changes. Also, the number of patients in each group of a Najaf study was small which could affect the statistical findings.

The current study indicates that rs3615254 (-238) G/A SNP does not affect disease activity and severity in each group individually. This result was in accordance with a previous study in Czech, that found a non-significant association between rs3615254 (-238) G/A SNP and RA severity, however, they use erosion and non-erosion criteria to stratify severity [40]. While the Caucasian study found an association with severity [19], however, the later study stratified the patients according to their need for anti TNF- therapy not according to DAS 28, and involved patients treated for a long period. By contrast, the present study stratified the patients according to DAS 28 and involved only the newly diagnosed RA patients.

Lastly, the present study doesn't find any association between (-238) G/A SNP and the inflammatory markers in both groups. Despite the numerous studies that investigate (-238) G/A SNP in RA [18,21], apparently no one compared its association with these common inflammatory markers. However, the association was studied in other SNPs, like the Egyptian study that found a non-significant association between ACPA titer, TNF-α lever, ESR, and hs-CRP level and (-1031) T/C, (-308) G/A and LTA252 SNP in RA patients [35]. On the other hand, this association was also studied by Georgescu A et al in a prospective study for sepsis susceptibility in Romania as they reported that TNF-α level was higher in GA genotype compared to the AA genotype [50].

Conclusion

This study concluded that the CT genotype and the C allele of TNF -1031 T/C were associated with susceptibility to RA. As well as the TT genotype of TNF -1031 T/C was associated with a lower risk for RA. Moreover the CT genotype and the C allele of TNF -1031 T/C were associated with more severe disease. Whereas the TT genotype of TNF -1031 T/C was associated with less severe disease. In addition, rs1799964 (-1031) T/C SNP was associated with the inflammatory markers and DAS28.

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Conflicts of Interest

The authors declare that there is no conflict of interest

Ethics Statements

This study was approved by the Ethical and Scientific Committee of the College of Pharmacy at the University of Baghdad, Iraq with Ethical approval with the (RECAUBCP-2692020) on 29/9/2020.

Author Contribution

The authors confirm contribution to the paper as follows: study design: Zainab M. Hashim; data collection Khalid Abdulhussein; analysis and interpretation of results: Zainab M. Hashim, Khalid Abdulhussein; draft manuscript preparation: Zainab M. Hashim, Khalid Abdulhussein. All authors reviewed the results and approved the final version of the manuscript.

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