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Evaluation of CTLA-4 (+ 49A/G) polymorphism association with rheumatoid arthritis in Egyptian patients

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Abstract

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an inhibitory molecule that has an essential role in T-cell homeostasis and self-tolerance because of its inhibitory signals. Genetic polymorphisms in the CTLA-4 gene have been associated with several autoimmune diseases. We aimed to assess the association between the CTLA-4 +49 A/G polymorphism (rs231775) and rheumatoid arthritis (RA) in Egyptian RA patients. The study included 104 RA patients and 81 apparently healthy control individuals. The polymorphism was assessed using restriction fragment-length polymorphism analysis. Genotype distribution was compared between patients and controls under different models of inheritance. Under the codominant model, RA patients showed a higher frequency of AG and GG genotypes compared to the control subjects (p=0.0092). Under the dominant model, RA patients showed a higher frequency of AG and GG genotypes grouped together compared to control subjects (p=0.0026). Under the over-dominant model, the AG genotype was more frequent in RA patients compared to control subjects (p= 0.0395). No association was observed between CTLA-4 polymorphism rs231775 and RA using the recessive model (p=0.1356). A significant association was observed between carrying the G allele and the presence of RA (p=0.0032). In conclusion, our findings showed a positive association between the CTLA-4 gene +49 A>G polymorphism and RA. However, discrepancies in literature reflect both ethnic variability in CTLA-4 gene polymorphisms as well as the complex pathogenesis of RA.

Keywords: CTLA-4; rheumatoid arthritis; polymorphism

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Introduction

Rheumatoid arthritis (RA) is an inflammatory condition marked by persistent autoimmune systemic inflammation that damages joints over time and may result in permanent disability.[1] Worldwide prevalence of RA has been estimated to be 460 cases per 100,000 population (i.e., 0.46%).[2] Although joint inflammation is the main feature of RA, many extra-articular manifestations and comorbidities are likely to occur e.g., rheumatoid nodules,

vasculitis, pulmonary, cardiovascular, gastrointestinal, neurological, hematologic, and renal illness.[3]

The pathogenesis of RA involves a complicated network of many immune cells and cytokines that promote the proliferation of synovial cells and the damage of bone and cartilage. T cells play a fundamental role in RA pathogenesis where local antigens can cause local T cell proliferation in the joint, due to both the high concentration of cluster of differentiation 4 (CD4)+ memory T cells in the joint as well as the increase of CD4+ clones in the synovial tissue. The role of T-cells in the immunopathogenesis of RA is highlighted by the effectiveness of blocking the CD80/CD86 to CD28 co-stimulation using cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) immunoglobulins.[4] RA is a result of both genetic and environmental factors. Genetic studies have identified more than 100 polymorphisms conferring disease risk.[5]

CTLA-4 is an inhibitory molecule that belongs to the immunoglobulin superfamily. CD80 and CD86, found on the surface of antigen-presenting cells, can bind to either CD28 resulting in a costimulatory response to T cells, or bind to CTLA-4 resulting in an inhibitory response. CTLA-4 is an essential regulator of T-cell homeostasis and self-tolerance because of its inhibitory signals. [6] The CTLA-4 gene is localized on chromosome 2q33 and consists of four exons. Genetic polymorphisms in the CTLA-4 gene were found to be associated with different autoimmune diseases e.g., ankylosing spondylitis, [7] systemic lupus erythematosus,[8] psoriasis[9], type 1 diabetes[10] and Sjögren's syndrome.[11] In this study, we aimed to examine the association between the CTLA-4 +49 A/G polymorphism (rs231775) and rheumatoid arthritis in Egyptian RA patients.

Materials and Methods

Study subjects

The study was conducted in the Rheumatology Department, Ain Shams University Hospitals. The study included 104 patients with RA diagnosed according to the American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) 2010 classification criteria. [12] The study population was recruited from the inpatient rheumatology unit and outpatient rheumatology clinic. The control group included 81 apparently healthy control individuals matched in age and sex.

Assessment of disease activity score

Patients were evaluated for disease severity using the disease activity score 28 (DAS28). [13] The score was calculated using the number of tender joints, the number of swollen joints, the value of c-reactive protein (CRP) [or erythrocyte sedimentation rate (ESR)], and the patient's own sense of health. According to their DAS28, patients in the study were classified into 4 groups: remission (DAS<2.6), low activity (DAS28 2.6 – ≤3.2), moderate activity (DAS28 >3.2–≤5.1), and severe activity (DAS28 > 5.1).

Blood samples

Three blood samples were withdrawn from each subject under complete aseptic conditions. The first blood sample (2 ml) was collected into a gel vacutainer tube, the tube was centrifuged at 4000 rpm for 20 minutes then serum was used to assess CRP, rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (anti-CCP).

The second blood sample (2 ml) was collected in an EDTA tube and stored at -20˚C until genotyped for rs231775 single nucleotide polymorphism using the polymerase chain reaction (PCR) amplification and restriction fragment-length polymorphism analysis (RFLP).

The last blood sample (2 ml) was collected on a 3.2% sodium citrate vacutainer tube to assess the ESR.

Assessment of CRP, anti-CCP, RF, and ESR

Levels of both CRP and RF were measured using Cobas 6000 analyzer, Roche diagnostics, Switzerland, while anti-CCP levels were measured using Cobas e411 analyzer, Roche diagnostics, Switzerland. Finally, ESR was determined using the Westergren method.

Genotyping analysis

The assay of CTLA-4 rs231775 single nucleotide polymorphism was done by PCR amplification

and restriction fragment-length polymorphism analysis.

DNA extraction

Genomic DNA was extracted from whole blood using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Briefly, 20 µl of Qiagen protease were added to 200 µl of whole blood and vortex mixed followed by the addition of 200 µl of lysis solution and vortex mixed. Samples were then incubated at 56°C for 10 minutes (for cells to completely lyse). After incubation, 200 µl of ethanol were added to the mixture and mixed by pipetting, followed by transfer of the mixture to a spin column. The spin column was centrifuged then washed twice and finally, DNA was eluted in 200 ul of elution buffer and the purified DNA was stored at -20°C till amplification.

Amplification

Amplification was performed using the forward and reverse primers shown in Table 1 on a thermocycler (Biometra TProfessional thermal cycler, Biometra, Germany). Primers were supplied by Applied Biosystems, USA. The amplification mixture contained 1ug of DNA template, 12.5 µl of TaqMan Universal master mix (Applied Biosystems, USA), and 1 µl of each primer, and the volume completed to 25 µl by nuclease-free water. The amplification protocol included initial denaturation for 4 minutes at 95°C followed by thirty five cycles of: denaturation at 95°C for 30 seconds, annealing at 56°C for 40 seconds and extension at 72°C for 50 seconds, followed by a final extension of 10 minutes at 72°C.

RFLP and Gel electrophoresis

The amplified products were subjected to digestion with the restriction enzyme BbvI (New England Biolabs, USA). The digestion reaction consisted of 2.5 µl NE buffer (New England Biolabs, USA), 1 µg DNA, and 1 µl of the restriction enzyme, and the volume completed to 25 µl by nuclease-free water. Digestion was done at 37°C for 20 minutes followed by incubation at 60°C for 5 minutes to deactivate the enzyme.

An aliquot of the digested product was loaded on 2% agarose gel (stained with ethidium bromide) and electrophoresis was done at 100 volts for 30 minutes. The presence of a single band of 402 bp indicated an AA genotype. Cleavage of the amplified product indicated the presence of a G allele; the presence of two bands of 241bp and 161 bp indicated a GG genotype while the presence of 3 bands of 402bp, 241bp and 161bp indicated an AG genotype (Figure 1).

Statistical analysis

Analyses were performed using the SPSS Statistics program, version 20 (SPSS, USA). Data are presented as mean and standard deviation (SD) for parametric data, median and interquartile range (IQR) for non-parametric data, and as number and percentage for categorical data. Departure from the Hardy–Weinberg equilibrium was assessed using $\chi 2$ goodness-of-fit test. Differences between groups were evaluated using the Chi-square test, Student's t-test, Mann-Whitney test, and Kruskal Wallis test. Odds ratios (OR) with a 95% confidence interval (CI)was used to assess the association of the CTLA-4 gene polymorphisms with RA. A p-value of <0.05 was considered statistically significant.

Table 1. Sequence of used primers.

	Sequence (5'-3')
Forward primer	CCACGGCTTCCTTTCTCGTA
Reverse primer	ATCACTGCCTTTGACTGCTGA

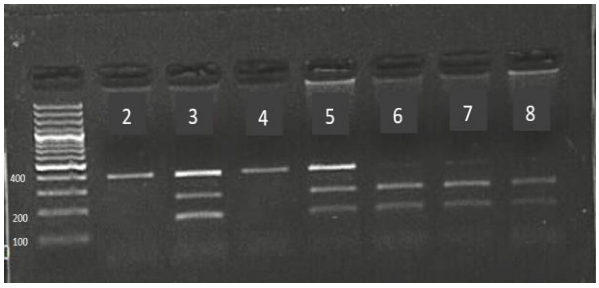

Figure 1. Agarose gel 2% electrophoresis of restriction products. Lane 1:100 bp ladder, lanes 2 and 4: AA genotype (402bp band), lanes 3 and 5: AG genotype (402bp, 241bp and 161bp bands), lanes 6-8: GG genotype (241bp and 161bp bands).

Results

Clinical and laboratory data

Clinical and laboratory data of the recruited RA patients and controls are shown in Table 2. The mean age for RA patients was 47.6±9.4 years with an average age at disease onset of 38.6±5.9 years. Females represented 83.7% of the patients while males represented 16.3%. As for the control subjects, the mean age was 46.9±12.1 with females representing 87.7% and males representing 12.3%.

Levels of CRP were higher in RA patients compared to controls with a median of 10.4 mg/L in patients versus a median of 1.4 mg/L in controls. ESR levels were also higher in patients with a median of 47 mm/h versus a median of 11 mm/h in controls. RF was positive in 88 patients while 83 patients were positive for anti-CCP. Regarding disease activity as assessed by DAS28, 15 patients were in remission at the time of the study while 36 patients had low disease activity, 13 patients had moderate disease activity and 40 patients had severe disease activity.

Table 2. Clinical and demographic characteristics of study subjects.

	RA patients (n= 104)	Controls (n= 81)	*p*-value
Age in years, mean±SD	47.6±9.4	46.9±12.1	NS*
Age of disease onset in years, mean±SD	38.6±5.9	-	
Gender			
Male, n (%)	17 (16.3%)	10 (12.3%)	NS§
Female, n (%)	87 (83.7%)	71 (87.7%)	
CRP mg/L, median (IQR)	10.4 (6-22)	1.4 (0.7-2.7)	<0.001#
ESR mm/h, median (IQR)	47 (29-62)	11 (7.5-13)	<0.001#
Positive RF, n (%)	88 (84.6%)	-	
Positive Anti-CCP, n (%)	83 (79.8%)	-	
DAS 28			
Remission, n (%)	15 (14.4%)		
Low, n (%)	36 (34.6%)	-	
Moderate, n (%)	13 (12.5%)		
Severe, n (%)	40 (38.5%)		

*Student's t test, § Chi-square test, # Mann-Whitney test. Anti-CCP: antibody to cyclic citrullinated peptides; CRP: c-reactive protein; DAS: disease activity score 28; ESR: erythrocyte sedimentation rate; n: number; RA: rheumatoid arthritis; RF: rheumatoid factor. $P > 0.05$ is not significant (NS).

Genotype distribution

The analysis of CTLA-4 rs231775 (+49 A/G) gene polymorphism showed significant differences in distribution between RA patients and controls under different models of inheritance (Table 3). Genotype frequencies for rs231775 polymorphism hold the Hardy-Weinberg equilibrium in the studied population (p> 0.05).

Under the codominant model, RA patients showed a significantly higher frequency of AG (51%) and GG genotypes (14.4%) compared to the control subjects (35.8% for the AG genotype and 7.4% for the GG genotype) (p=0.0092). To assess the association between rs231775 polymorphism and the risk of RA, the AA genotype was considered as a reference and the AG genotype and GG genotype were compared to the reference. The association of the AG genotype had an odds ratio of 2.335 (CI:1.245-4.379) while the association of the GG genotype had an odds ratio of 3.194 (CI:1.126-9.062).

Similarly, a significant association was observed with AG and GG genotypes, added together, under the dominant model with an odds ratio of 2.483 (CI:1.366–4.511). RA patients showed a significantly higher frequency of AG and GG genotypes grouped together (65.4%) compared to control subjects (43.2%)

(*p*=0.0026). The dominant model assumes that having either one or two copies of the G allele increases the risk.[14]

The recessive model assumes that two copies of the G allele are required to alter the risk.[14] Thus, individuals having the GG genotype were compared to individuals having genotypes AG and AA grouped together. No significant association was observed between CTLA-4 polymorphism rs231775 and RA using the recessive model (*p*=0.1356).

Under the over-dominant model (i.e., heterozygote advantage, i.e., maintenance of polymorphism by overdominance, i.e., heterozygous individuals have higher fitness than homozygous ones or heterozygotes usually have a fitness advantage because of deleterious effects in homozygotes[15]), AG genotype frequency was significantly higher in patients with RA (51%) compared to the control subjects (35.8%) (*p*= 0.0395) with an odds ratio of 1.863 (1.028-3.379).

Table 3. Distribution of Genotype Frequency in study subjects.

Model	RA patients (n=104)	Controls (n=81)	*p*-value*	OR (95%CI)
Codominant				
AA, n (%)	36 (34.6%)	46 (56.8%)		Reference
AG, n (%)	53 (51%)	29 (35.8%)	0.0092	2.335 (1.245-4.379)
GG, n (%)	15 (14.4%)	6 (7.4%)		3.194 (1.126-9.062)
Dominant				
AA, n (%)	36 (34.6%)	46 (56.8%)	0.0026	Reference
AG+GG, n (%)	68 (65.4%)	35 (43.2%)		2.483 (1.366-4.511)
Recessive				
AA+AG, n (%)	89 (85.6%)	75 (92.6%)	NS	Reference
GG, n (%)	15 (14.4%)	6 (7.4%)		2.107 (0.7784-5.702)
Over dominant				
AA+GG, n (%)	51 (49%)	52 (64.2%)	0.0395	Reference
AG, n (%)	53 (51%)	29 (35.8%)		1.863 (1.028-3.379)

*Chi-square test. n: number; OR: Odds ratio; CI: Confidence interval; RA: rheumatoid arthritis.
P > 0.05 is not significant (NS).

Allele distribution

Comparing allele frequency between RA patients and controls revealed an increase in the frequency of the G allele among RA patients (39.9%) compared to the control group (25.3%) (*p*=0.0032). A significant association was observed between the G allele and the presence of RA with an odds ratio of 1.960 (CI:1.249–3.073) (Table 4).

Table 4. Distribution of Allele Frequency in RA Patients and Controls.

Allele	RA patients (n=104)	Controls (n=81)	*p*-value*	OR (95%CI)
A, n (%)	125 (60.1%)	121 (74.7%)	0.0032	Reference
G, n (%)	83 (39.9%)	41 (25.3%)		1.960 (1.249-3.073)

*Chi-square test. n: number; OR: Odds ratio; CI: Confidence interval. **P* ≤ 0.05 is significant.

Clinical characteristics in different genotypes

To examine whether having a specific +49A/G CTLA-4 genotype affects clinical or laboratory findings in RA patients, different parameters were compared between RA patients having the AA genotype, RA patients having the AG genotype, and RA patients having the GG genotype. Comparative analysis of different

studied parameters between different genotypes revealed no significant association between the CTLA-4 genotype and either CRP level (p=0.6842), ESR level (p=0.0756), RF positivity (p=0.2729), anti-CCP positivity (p=0.4783) or disease activity assessed by DAS28 score (p=0.2179) (Table 5).

Table 5. Comparison of studied parameters between different +49A>G CTLA-4 genotypes in RA patients.

	AA (n=36)	AG (n=53)	GG (n=15)	*p*-value
CRP mg/L, median (IQR)	10.9 (2.6-25.1)	9.1 (6.1-21.2)	11.2 (9.0-26.8)	NS*
ESR mm/h, median (IQR)	34.5 (17-61)	52 (36.5-67.5)	47 (36-61)	NS*
Positive RF, n (%)	33 (91.6%)	42 (79.2%)	13 (86.7%)	NS#
Positive Anti-CCP, n (%)	31 (86.1%)	41 (77.4%)	11 (73.3%)	NS#
DAS 28				
Remission, n (%)	4 (11.1%)	7 (13.2%)	4 (26.7%)	NS#
Low, n (%)	10 (27.8%)	25 (47.2%)	3 (20%)	
Moderate, n (%)	5 (13.9%)	5 (9.4%)	3 (20%)	
Severe, n (%)	17 (47.2%)	16 (30.2%)	5 (33.3%)	

* Kruskal-Wallis test, # Chi-square test, $P > 0.05$ is not significant (NS).

Discussion

Rheumatoid arthritis is a multifactorial autoimmune inflammatory disease brought on by interactions between both environmental and genetic variables. Environmental factors include smoking, infections, dietary factors, exposure to pollutants, high birth weight, and other factors. Genetic factors represent 50% to 60% of the risk of developing RA and include the HLA-DRB1 gene which is the main genetic locus that confers RA susceptibility. Other genetic factors include genetic polymorphisms including SNPs outside the HLA locus.[16]

The immunopathogenesis of RA is orchestrated by T cells, B cells, and several inflammatory cytokines. CTLA-4 plays a significant part in peripheral tolerance and in prevention of autoimmune illness through inhibition of activated T cells and regulatory T cells. CTLA-4 is constitutively expressed on T reg cells and is expressed on effector T cells 24-36 hours after stimulation. Thus, CTLA-4 polymorphisms have been the focus of a study related to autoimmune diseases.[17]

The present study assessed the association between the presence of +49 A>G polymorphism of the CTLA-4 gene and rheumatoid arthritis. The polymorphism was assessed in a total of 104 RA patients and 81 controls by PCR-RFLP technique. We identified an association between the AG and GG genotypes and susceptibility to RA in the codominant, dominant, and over dominant genetic models. We also identified an association between carrying the G allele and susceptibility to RA. The frequencies of the AG genotype, the GG genotype and the G allele in RA patients were significantly higher than those in control individuals. The association between the polymorphism in CTLA-4 and risk of RA can be explained by the effect that this polymorphism confers on the inhibitory function of CTLA-4. The gene that encodes CTLA-4 is located on band 33 of the long arm of chromosome 2 (2q33). The gene includes 4 exons (i.e., exon 1-exon 4) that encode the peptide leader domain, ligand binding domain, transmembrane domain, and intracytoplasmic tail.[18] The +49 A/G gene polymorphism is located in exon 1 of the gene. In this polymorphism, the adenine base is substituted by a guanine base resulting in the translation of an alanine amino acid instead of a threonine amino acid at the 17th codon of the leader peptide. [19] The GG genotype has been found to result in decreased inhibitory function of CTLA4 on the proliferation of T cells after exposure to stimulation by an allogenic cell line.[20] Moreover, the +49G polymorphism was found to result in

incomplete glycosylation of the leader peptide thus altering the molecule's processing in the endoplasmic reticulum and resulting in lower surface levels of CTLA-4 in transfected cells. Less CTLA-4 expression results in fewer B7-CTLA4 complexes, which in turn results in a less effective CTLA4- mediated T cell suppression and a considerable increase in T cell proliferation.[21]

Several studies have assessed the link between +49 A>G mutation and RA, however, the results are conflicting in different populations. A study in Iraq by Zayed et al.,[22] found that the GG and AG genotypes were more common in RA patients compared to controls with an odd ratio of increased risk of 2.09 (1.13-3.86) and 1.69 (1.10-2.59) respectively for the genotypes. The authors reported that the G allele was associated with RA with an odds ratio of 1.96 (1.43-2.67). Another study also done in Iraq, reported an association of the G allele to RA with an odds ratio of 1.38 (1.04-1.77).[23] Similarly, in a study done in China, Tang et al., 2013[24] found that the GG genotype was more common in RA patients than controls with an odd ratio of increased risk of 1.37 (1.12-1.67) and that the G allele was associated with increased susceptibility to RA with an odd ratio of 1.17 (1.04-1.30). Another study by Sameem et al., 2015[25] assessed the association between the +49 A>G polymorphism and RA in a group of Pakistani RA patients and stated that the GG genotype was found to increase the risk of RA with an odds ratio of 3.0186 (1.6774-5.4322) while carrying the G allele was associated with increased susceptibility to RA with an odd ratio of 2.637 (1.744-3.959). Moreover, Muñoz-Valle et al., 2010,[26] reported that the AG and GG genotypes were more common in RA patients compared to controls with an odd ratio of increased risk of 1.87 (1.19-2.93) and 1.86 (1.05-3.28) respectively together with the association of the G allele with an odds ratio of 1.45 (1.09-1.92) in a group of Mexican RA patients. Furthermore, in Egypt, Elshazli et al., 2015[27] found that the AG and GG genotypes were more frequent in RA patients compared to controls and reported an association of the G allele to RA with an odds ratio of 1.93 (1.29-2.89).

However other studies reported the absence of association between the +49 A>G polymorphism and RA disease in Pakistan,[28] Iran,[29] Slovakia,[30] and Poland.[31]

Two meta-analyses highlighted the different distribution of genotype and allele frequency of the +49 A>G polymorphism in RA patients in different populations. The meta-analysis done by Li et al., 2014,[32] included 27 studies and the meta-analysis done by Zhou et al., 2021, included 66 studies.[33]

Discrepancies between a positive association and a lack of association in different populations can be attributed to either: causes related to the study design e.g., sample size, sample selection bias (ascertainment bias) and differences in the molecular methodology used for genotyping, or causes related to genetic background e.g., ethnic differences or linkage disequilibrium. The G allele of the +49 A>G polymorphism may not be a direct causative allele for RA, but it is more likely to be in linkage disequilibrium with another causative allele of another polymorphism. Maps of linkage disequilibrium do vary among populations that differ in gene history (i.e., linkage disequilibrium is different between ethnic and racial groups).[34]

In the present study, RA patients showed higher CRP and ESR levels compared to control subjects. However, upon examining the association between different genotypic frequencies and different clinical and laboratory parameters in RA patients, we found no significant association. CRP levels and ESR levels did not differ between RA patients having the AA genotype, the AG genotype, or the GG genotype. Also, in our study, the +49 A>G genotype was not associated with either RF status, anti-CCP status, or disease activity by DAS 28. In line with our study results, several studies demonstrated the lack of association between different genotypes and clinical parameters. Zayed et al., 2022,[22] and Louthrenoo et al., 2021,[35] reported a lack of association between the +49 A>G genotype and either the RF status or the anti-CCP status. Elshazli et al., 2015,[27] reported a lack of association of the genotype with RF status and anti-CCP status while an association was observed regarding DAS28 disease activity.

Muñoz-Valle et al.[26] reported a lack of association with ESR, RF, and DAS 28 while CRP levels were higher in RA patients with the GG genotype. The reason behind the lack of association observed in our study as well as others is most probably because the clinical and laboratory findings of RA change over the course of the disease depending on disease flare-up and treatment efficiency.

AA genotype was the most common +49 A>G genotype in control individuals of the present study with a frequency of 56.8% followed by the AG genotype (35.8%) then the GG genotype (7.4%). Similar frequencies were reported by other studies involving Egyptian controls. Elshazli et al., 2015,[27] reported a frequency of 58.2% for the AA genotype, 36.9% for the AG genotype, and 4.9% for the GG genotype in control subjects in their study assessing the association between +49 A>G polymorphism and rheumatoid arthritis. Saleh et al., 2013,[36] and Kamel et al., 2014,[37] examined the association between +49 A>G genotype and type 1 diabetes and found that the AA genotype was the most frequent in their study controls with a frequency of 53% and 53.9%, respectively followed by the AG genotype with a frequency of 39% and 40.7%, respectively while 8% and 5.4% of the studied controls had the GG genotype, respectively.

In conclusion, our findings showed a positive association between the +49 A>G polymorphism of the CTLA-4 gene and the risk of RA. Nevertheless, ethnic variability exists in CTLA-4 gene polymorphisms and discrepancies in literature reflect the complex pathogenesis of RA that includes environmental factors in addition to the genetic component. Cross-genotyping analysis assessing the presence of multiple polymorphisms in the CTLA-4 gene or in more than one gene can help reveal more about the association of polymorphisms with the development of RA.

Author Contributions

RH and NE recruited patients, collected the samples, and performed the clinical assessment. DR and RS performed the laboratory work, statistical analysis and analyzed the data. DR and RM drafted the paper. RM and NE revised the paper critically. All authors contributed significantly to the study's conception, design, and final approval of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was approved by the Ethical Committee, Faculty of Medicine, Ain Shams University, (Approval number: R202/2022).

Informed consent

Written informed consent was provided by all subjects participating in the study.

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