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Plasma TNF-α Elevation in Biologic Naive Rheumatoid Arthritis Patients Belonging to a Population with New Mutations in TLR4 and CYP51A1 genes without Association with Disease-Related Antibodies Levels

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ABSTRACT

In a system biology-based study, we previously reported that IL-6 and IL6R -specific m-RNA levels were elevated in leukocytes of patients with Rheumatoid arthritis (RA). Here, the association of toll-like receptor4 (TLR4) rs 141534085 and cytochrome P450 family 51 subfamily A member 1(CYP51A1) rs6 with tumor necrosis factor-α (TNF- α), rheumatoid factor (RF)- and Anti- cyclic citrullinated peptide (anti-CCP) antibody -positivity was investigated in almost the same subjects. Forty-six patients and 48 normal subjects were recruited in this study. The blood leucocytes TLR4 rs 141534085 and CYP51A1 rs6 -comprising DNA sequences were amplified by using tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) technique and the PCR products were checked by Sanger DNA sequencing method. ELISA method was used to determine plasma levels of TNF- α, anti-CCP antibody and RF positivity of plasma was evaluated through a latex agglutination test. The TNF- α level was significantly higher in the patient group than control subjects (p= 0.001). Moreover, we were not able to find any correlation between TNF-α levels and RF as well as anti-CCP antibodies when we used the K^2/ Fisher's exact test and Pearson test respectively. Our DNA sequencing data revealed the following new mutations in TLR4 rs141534085 comprising regions: A>T in position 1050, T>A in position 1052, and C>A in position 1054; and for CYP51A1 rs6 encompassing region, the new mutations were; G>A in position 21680, the T nucleotide was inserted in position 21762 and the G nucleotide was inserted in position 21763, G>T in position 21764. The data of this study showed that both TLR4 rs141534085 and CYP51A1 rs6 related DNA regions should be considered as hotspot areas in RA pathogenicity. Moreover, these data indicated that, TNF- α did not alter the production of anti-CCP and RF pathogenic antibodies in patients with long-term RA.

Keywords: Rheumatoid arthritis, TLR4, CYP51A1, TNF-α, mutation

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Introduction

Rheumatoid arthritis (RA) is a joint-targeting autoimmune disease whose global burden has increased during the last several decades and this increment will continue in the future (1). Thus, a much more comprehensive study may direct us to explore some much better tools in RA's early diagnosis and effective treatment. However, we have to consider some challenges that are hidden in the autoimmune nature of this chronic inflammatory disease. RA is a product of a well-established immune reaction that has been already triggered against heterogenous types of known and unknown autoantigens before the onset of disease (2). Another obstacle in the RA management is that this disease is not curable, thus the different types of a drug such as a corticosteroid and disease-modifying agents are obligatory used by patients for a life-long period. Thus, the drug-metabolizing enzyme may play some important roles in RA management, especially if we consider the hazardous effects of corticosteroid therapy on other drug metabolism such as methotrexate. On the other hand, we know that there are some nonmodifiable and modifiable risk factors for this disease. Nonmodifiable risk factors such as age, sex, and genetic background, the latter factor in particular, can govern modifiable factors such as infection (3-6). Accordingly, the exploration of a genetic variation such as single nucleotide polymorphism (SNP) in some key genes that play important roles in infection and metabolism, may help us to find some proper biomarkers to screen the people who are susceptible to RA before disease onset. Two of such genes are Toll-like receptor4 (TLR4) and cytochrome P450 family 51 subfamily A member 1 **(**CYP51A1).

The TLR-4 plays a key role in the innate type of immune response and binds specifically to bacterial lipopolysaccharide (LPS) and causes intracellular signaling which can lead to production of cytokines like TNF-α. Moreover, endogenous ligands such as heat shock proteins, hyaluronic acids, and fibronectin are able to stimulate this type of immune receptor. These ligands have been recognized as a vital biomolecule which are involved in the development of rheumatic diseases in the synovial joints of RA patients (7-11).

The CYP51A1 gene that encodes the lanosterol 14α-demethylase enzyme, is responsible for the regulation of cholesterol biosynthesis (12). Transcription of the lanosterol-14α-demethylase is repressed in TLR4-activated macrophages through histone deacetylases 1(HDAC1) activation via induction of type I interferon signaling pathway. This repression can result in the accumulation of lanosterol, an endogenous modulator of proinflammatory cytokines production (13). Macrophage and fibroblasts are main cells of synovial membrane which is currently considered a primary site of inflammation in RA patients. Moreover, high metabolic demands of these inflamed synovial cells as well as joint infiltrating leucocytes, can result in nutrient shortage and perpetuation of the inflammation in the diseased joints(14-16).These proinflammatory cytokines can alter the process of post-translational modifications of some self- protein including fibroblast- derived vimentin through citrullination which in turn can convert them to neo-antigen so-called citrullinated peptides(17).Until now, 53 of such proteins were introduced, such as vimentin and alpha enolase. These neoantigens induce the production of specified group of antibodies named anti-Cyclic Citrullinated Peptide (anti-CCP) (18). Furthermore, we have recently reported that the expression of IL-6 and IL-6 receptor (IL-6R) genes was upregulated at m-RNA level without change at protein levels in RA patients (19). These patients (except one) did not use any biologic drugs like Etanercept or Tocilizumab. It has been demonstrated that the neutralization of IL-6 by an anti-IL-6R antibody has a useful

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impact on autoimmune diseases such as RA. This blockage was at least as beneficial as blocking of TNF-α (20-21).

Thus, in the present study, we investigated the frequency of TLR4 rs141534085 and CYP51A1rs 6 and focused on TNF-α production in these biologic naive patients. Also, the correlation between TNF- α and anti-CCP and RF positivity was evaluated. We also investigated the RA comorbidity (heart disease and urinary tract infection (UTI)) and RA association with the employment/unemployment situations.

Materials and methods

Samples

The data generated in this study, derived from DNA and plasma samples which have been used in our IL-6/IL-6R gene expression analysis in a system-biology based study (20). In the present study, two further control subjects were recruited. As previously described, these specimens were prepared form 46 RA patients (35 women and 11 men) who were referred to the Rheumatology Department of Ayatollah Rouhani Hospital affiliated with Babol University of Medical Sciences (Babol, Iran). The patients were included in this study by an expert Rheumatologist based on the diagnostic criteria for RA, ACR/EULAR 2010 (American College of Rheumatology/European League Against Rheumatic 2010). The patients who suffered from coronavirus disease 2019 (COVID-19) or flu/ common cold were excluded from this study. The control group included 48 healthy people (30 women and 18 men) similar to patient subjects, in terms of age, sex, geographical location, and without history of autoimmune disorders and were negative for RF and c-reactive protein testes. A questionnaire was filled with the information of the groups including age, gender, disease duration, year of infection, diseases such as heart disease, urinary tract infection, occupation, and type of medicine used by the patients as previous report (19). The patients who were recruited in this study were heterogenous from the viewpoint of diseases' activity based on rheumatologist examination, without determination of disease activity index. This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences [Code of Ethics: IR.SBMU. RETECH. REC. 1400. 904], and the procedure was explained to all the recruited subjects who signed the written informed consent forms.

Measurement of plasma levels of RA- related antibodies and TNF-α

TNF-α, and anti-CCP antibody levels were measured with commercial ELISA kits and RF positivity was evaluated with RF-latex agglutination using the same EDTA-treated plasma samples. The kit for detecting of TNF-α was supplied by Diaclone (France) and the anti-CCP ELISA kit and RF latex were purchased from Pishgaman Sanjesh/Iran. And the optical density (OD) at 450 nm was read by ELISA reader, Stat Fax 2100(USA), and Synergy HTX, multi-mode reader (Biotek, USA) respectively.

DNA Extraction

Five mL of whole blood samples were drawn from patients and controls and collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and were kept at -80°C. Genomic DNA was extracted from the leukocyte by a blood DNA extraction kit (QIAGEN, Germany). The quality and quantity of extracted DNA were checked by using a nanodrop device and electrophoresis on 0.8% agarose gel.

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T- ARMS -PCR

SNPs of (rs141534085) TLR4 and (rs6) CYP51A1 were investigated through tetra-primer amplify-cation refractory mutation system polymerase chain reaction (T-ARMS-PCR). Specific primers were designed by SNAPGENE software (Table 1).

Table 1. Primer sequences.

Variant name	Primer	Primer sequence 5'-3'	Size of products
CYP51A1 (rs6)	Forward-inner	AGCCTGACCAACATGGATAAACCACA	177
	Reverse-inner	GGCTAATTTTTTGTATTTTAGTAGAGATAC	213
	Forward-outer	AAATGCAGAACATGATTAGCATTAAAGA	334
	Reverse-outer	TAATCTGTTTTTTTTTTTTTTGAGACGG	334
TLR4 (rs141534085)	Forward-inner	CATACTTAGACTACCTCGATGATAGTG	146
	Reverse-inner	CATTTGTCAAACAATTAAATAGTCCAT	120
	Forward-outer	AAAAGTTTGACAAATCTGCTCTAGAG	208
	Reverse-outer	TTAACTAATTCTAAATGTTGCCATCC	208

T-ARMS- PCR reaction mix with a total volume of 30 µL consisted of 2 µL of template DNA (concentration 50 ng/ml), 9 µL of nuclease-free water, and 15 µL of Taq DNA polymerase Master Mix PCR product with a concentration of 1x (Amplicon, Denmark) and 1 µL of the primer with a concentration of 10µM. PCR temperature program according to Tables 2 and 3 performed by Kaira thermocycler and the genotype of individuals were identified by the length of fragments following 2.5% agarose gel electrophoresis.

Table 2. T-ARMS- PCR program for *CYP51A1*.

	Temperature	Time	Cycle
Denaturation	95	5 min	1
Denaturation	94	40 sec	
Annealing	62	40 sec	35
Extension	72	1 min	
Final Extension	72	5 min	1

Table 3. T-ARMS- PCR program for *TLR4*.

	temperature	Time	Cycle
Denaturation	94	3 min	1
Denaturation	94	40 sec	
Annealing	55	40 sec	35
Extension	72	1 min	
Final Extension	72	5 min	1

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Statistical analysis

The Kolmogorov-Smirnov test was used to examine the normality of quantitative data. The chi-square (K^2) test was used to evaluate the relation between age, occupation, heart disease and urinary tract infections in healthy and RA groups. The TNF-α association with RF and anti-CCP positivity was determined by K^2- and Pearson test, respectively. Mann-Whitney U test was used to compare the mean of anti-CCP, TNF- α in two groups. Moreover, receiver operating characteristic (ROC) curve was drawn to determine cut-off-, sensitivity-, and specificity -value for TNF- α as a diagnostic marker for RA. A p-value < 0.05 was statistically considered as the significant level.

Results

Clinical and demographic data are presented in Table 4. The incidence rate of RA was higher in the female population and its ratio is almost 4 times. The mean age of patients who were referred to a rheumatologist was more than 50 years old (55.9±12.5489 y). The age of onset of RA was ranging between 17 to 68 years, with a mean of approximately 41.49 ± 11.99 y while disease duration ranged between 1- 43 years with a mean of 14.05 ±9.64 years. By studying the rate of urinary tract infection, it was observed that RA patients are more sensitive to this type of infection than control individuals (p=0.006). All of those patients had female gender. Moreover, 12.5% of all RA cases had heart disease. According to the data of the questionnaire, people who were not employed suffered from RA 9 times higher than employed people. The positivity of RF in patient group was 76%.

Table 4. Demographic and clinical data of subjects with RA and control group.

Variables	RA(n=46)	Control(n=48)	P- value
Sex ratio female/male	3.18	1.35	0.15
Age	55.9±12.5489	52.45±6.9	0.119
Onset age	41.49±11.99	-	-
Duration disease	14.05±9.64	-	-
Urinary infection ratio	17.4%	0	0.006
Coronary heart disease	12.5%	0	0.025
Employment/non employment	0.23	0.64	0.001
RF+	35(76%)	0(0%)	

As a result of this study, according to Figure 1, the mean of anti-CCP antibody was significantly higher in patients than control subjects, P< 0.0001. According to the kit's instruction, the positivity for anti-CCP antibody was >18 AU/mL, the borderline levels were 12-18 AU/mL and less than 12 AU/mL was considered as negative result.

To determine the frequency of different wild/mutated alleles of our two indicated genes, i.e., TLR4 rs141534085 and CYP51A1 rs6, we performed the T-ARMS-PCR. As illustrated in Figure 2, in our agarose electrophoresis profile, we observed the different types of PCR products, as we expected based on our primer designation. According to electrophoresis-based data, most of subjects in test and control –group, showed three bands in their electrophoresis profile for both genes whereas almost 1/3 of remaining subjects had two bands (Table 5).

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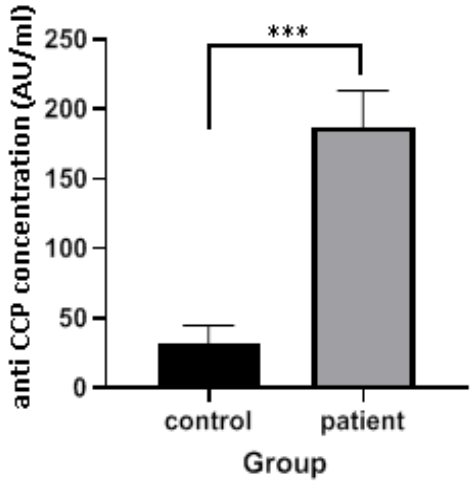


Fig. 1. Comparison of the plasma levels of anti-CCP in RA and control group. The patient's subjects exhibited a significant elevated level of anti-CCP antibody in comparison to normal control subjects. The data were expressed as mean and the bars indicating the standard deviation< 0.05 that was considered as statistically significant. *** p<0.0001.

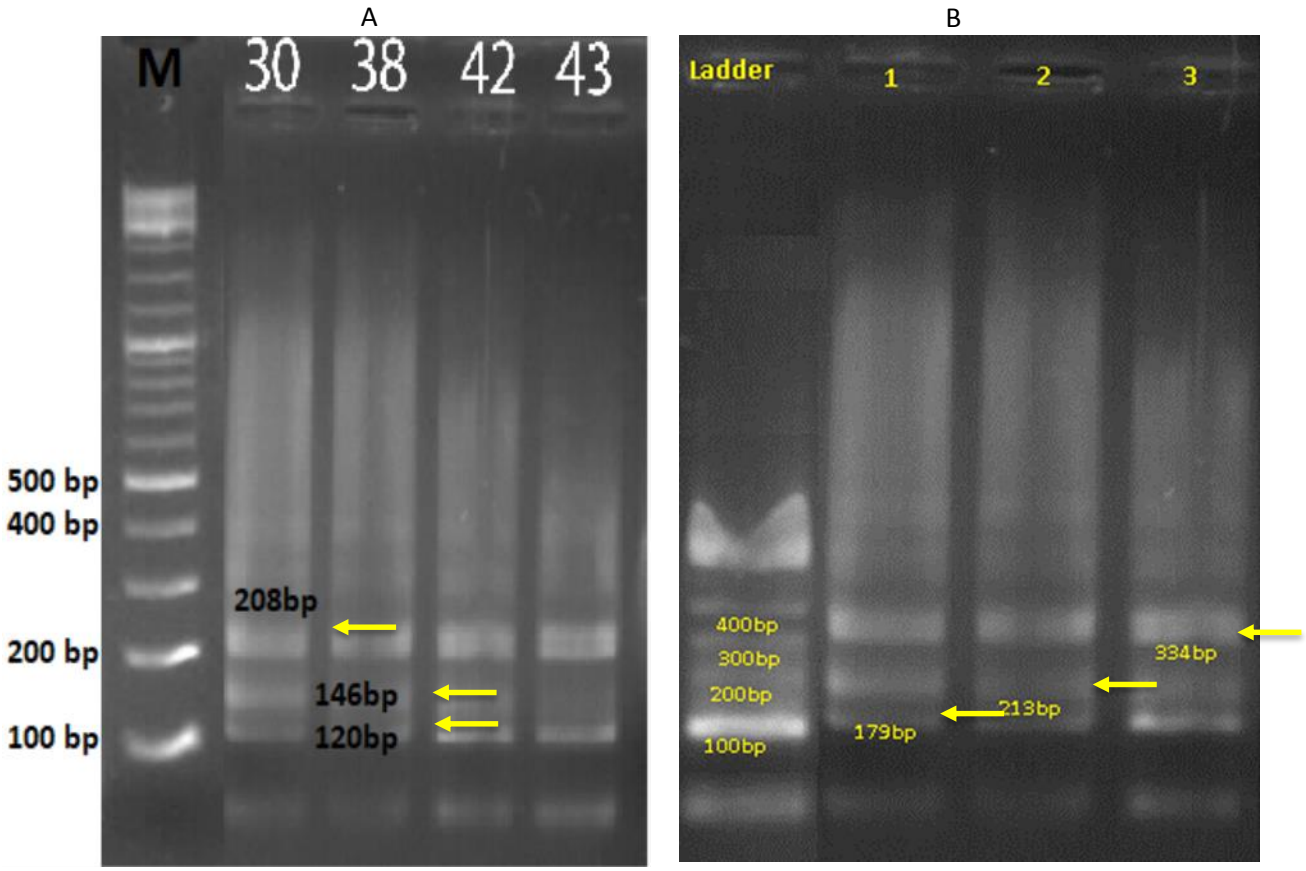


Fig. 2. Pattern of TLR4 and CYP51A1 T-ARMS-PCR products gel electrophoresis. A) TLR4 gene: three subjects (patient number: 30, 38 and 42) exhibited three bands with molecular size of 208,146 and 120 base pairs,whereas, the patient number 43 showed two bands with molecular size of 208 and 120 base pairs. Lane M shows the ladder of size maker. B) CYP51A1 gene: three patients (numbers 1 ,2 and 3) exhibited three bands with molecular size of 334,213 and 179 base pairs. The arrows show the indicated bands.

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Table 5. Number of indicated bands in agarose gel electrophoresis of T-ARMS-PCR products for TLR 4 andCYP51A1 genes in RA patients and healthy control subjects.

Gene	Number of bands	Patient group number (percentage)	Control group number (percentage)
TLR4	3	33(71.7%)	32(66.7%)
	2	13(28.3%)	16(34.3)
CYP51A1	3	31(66%)	34(77.3%)
	2	15(34%)	14(32.7%)

However, the TLR4 DNA sequencing of the whole amplicon which was amplified by this gene-specific forward and reverse outer primer was not able to confirm our favorite mutation i.e., TLR4 rs141534085 (A>G) in patient and control samples. Instead of this, we found the wide type allele in both groups as illustrated in Figure 3A. However, interestingly as it can be seen in Figure3B, during the DNA sequencing analysis, we realized that one of these patients exhibited three different types of point mutations in this area, appeared as follows: A>T in position 1050, T>A in position 1052 and C>A in position 1054. These newly discovered mutations have been already registered in the GenBank database. (https:// www. ncbi. nlm. nih. gov/ Genbank/ OR540493).

The age of this female patient was 63 who was suffering from RA for 15 years. She also suffered from heart disease and UTI. She was negative for blood TNF-α (5.7 ng/mL), and anti-CCP antibody but positive for RF tests.

Moreover, as can be seen in Figure 3C, surprisingly in this area of TLR4 gene in our second DNA sequencing analysis which DNA molecules were prepared from a 51-year-old female patient suffering from RA for one year, we found two other new mutations, A>T in position 1047, and at position 1051. She exhibited a deletion in an A- nucleotide. The blood test of this RF-positive and anti-CCP antibody-negative patient for TNF-α was negative. These newly discovered mutations have been already registered in the GenBank database https://www.ncbi.nlm.nih.gov/Genbank/OR540494).

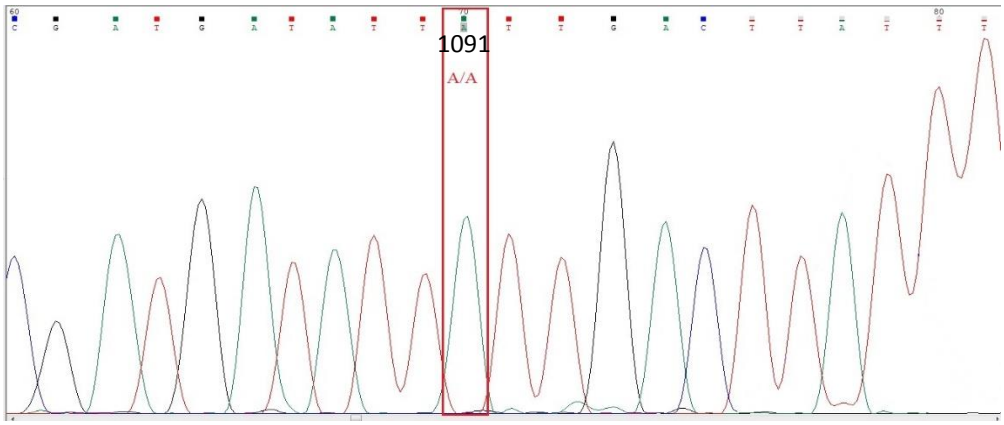
Similar to the TLR4 gene, PCR products for the CYP51A1 gene were also obtained from blood samples for 46 patients and 48 control subjects. Two of these products which appeared as 2 and 3 bands in agarose gel electrophoresis were sequenced through Sanger DNA sequencing (Figure 2B). Both patients had the wild-type allele at position 1091 (Figure 4A). However, we found that one of these patients exhibited six different types of point mutations in this area which appeared as follows: G>A in position 21680, T was inserted in position 21762 and G was inserted in position 21763, G>T in position 21764, and A was deleted in position 21815. These newly discovered mutations have been already registered in GenBank: (https://www.ncbi.nlm.nih.gov/nucore/OR420789).

The age of this female patient was 80 suffering from RA for 5 years. She also suffered from heart disease. She was negative for IL6, positive for blood TNF-α (99.8 pg. /ml) and sIL6R (3.1 ng/mL), and positive for anti-CCP antibody and RF tests. Surprisingly in this area of the CYP51A1 (rs6) gene in our second patient DNA sequencing analysis was prepared from a 57-year-old female patient suffering from RA for 25 years. We found three other new mutations, G>A in position 21679. She exhibited a deletion at

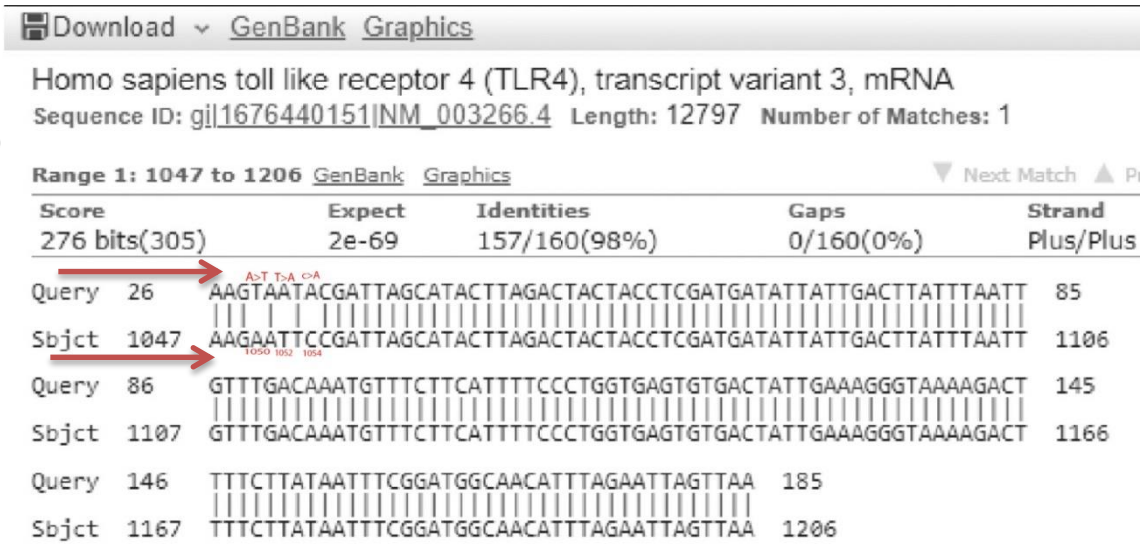
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A)



B)



C)

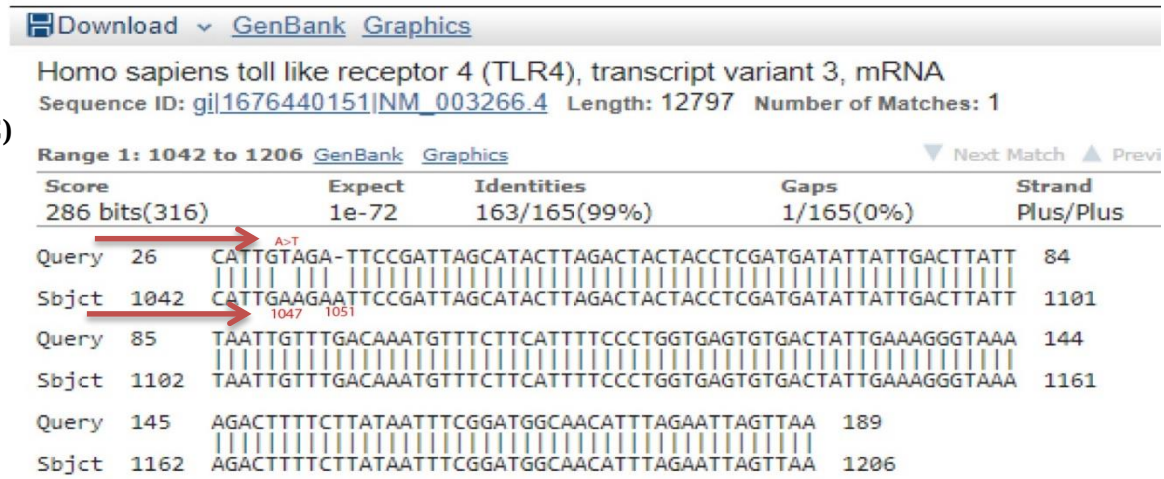
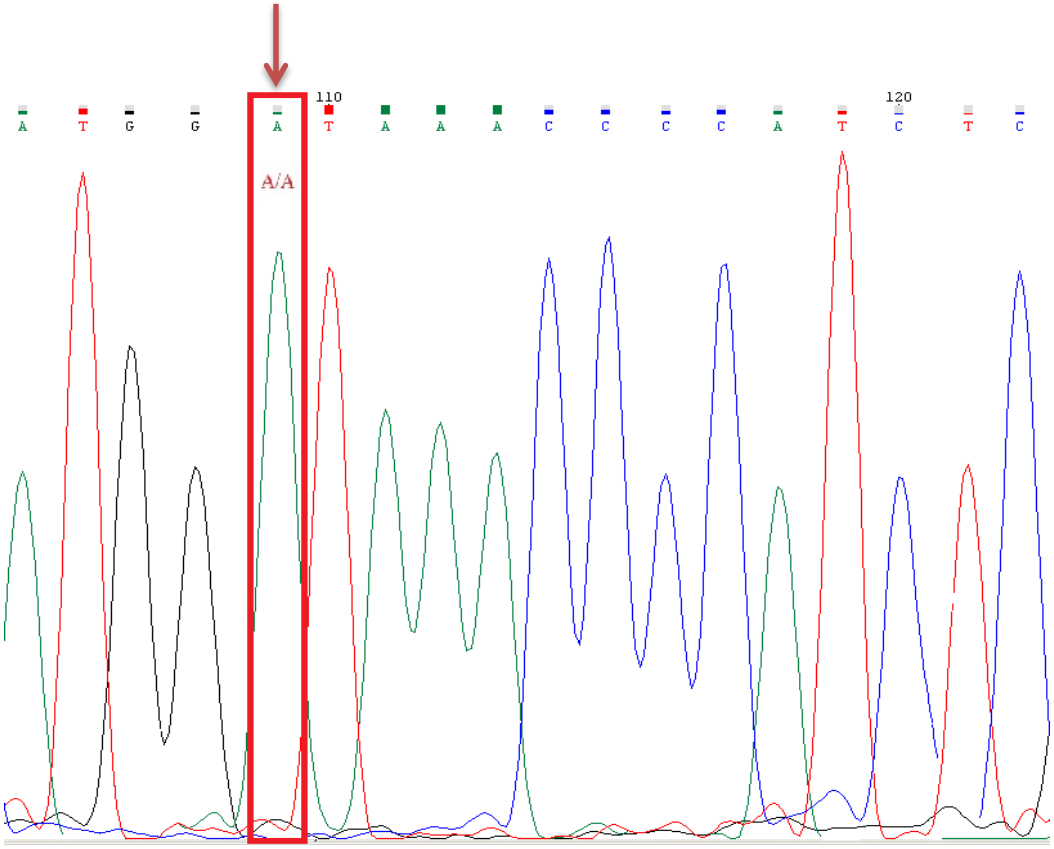


Fig. 3. A) Analysis of single nucleotide polymorphism TLR4 using Sanger sequencing. The arrow shows the A/A 1091 (wild type allele). B) Toll -like receptor-4 (TLR4) mutations in an RA patient (case 1). The upper arrow shows the different types of mutations: A>T, T>A and C>A, and the lower arrows indicate the position where these mutations happened, 1050, 1052 and 1054, respectively. C) Toll -like receptor-4 (TLR4) mutations in an RA patient (case2). The upper arrow shows the A>T mutation at position 1047 and the lower arrow indicates A-base deletion at position1051.

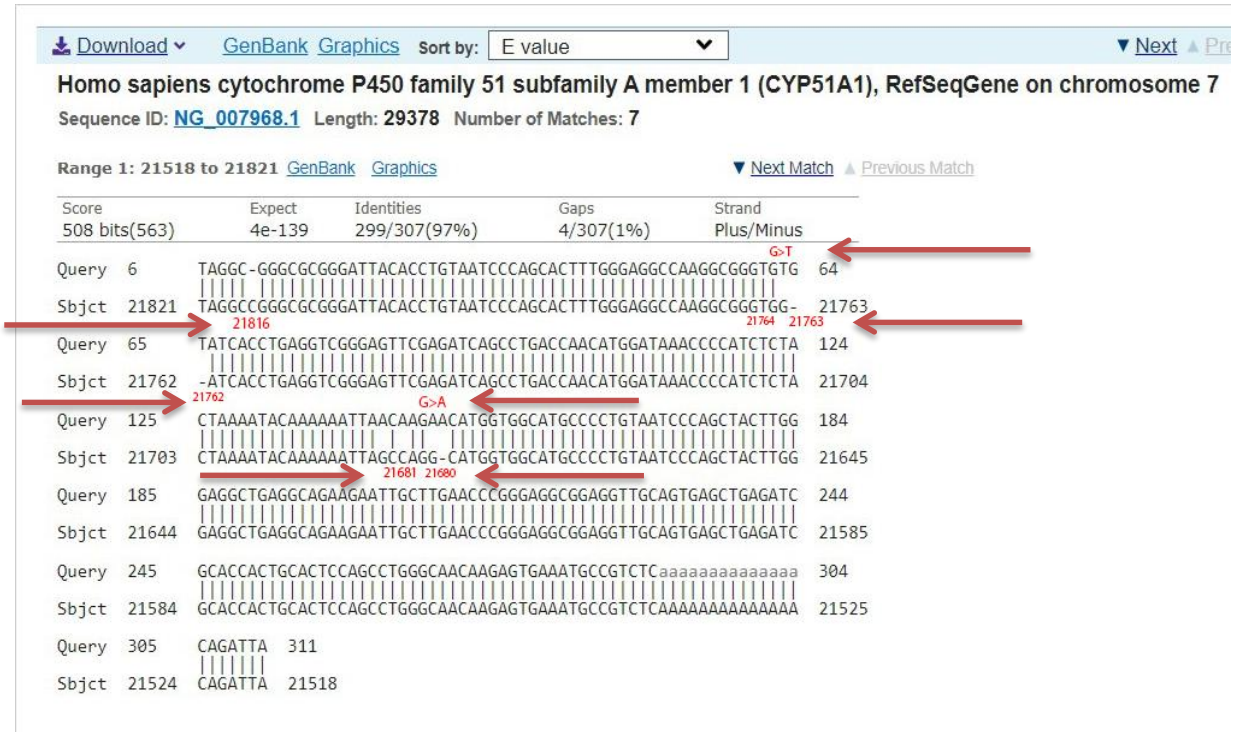
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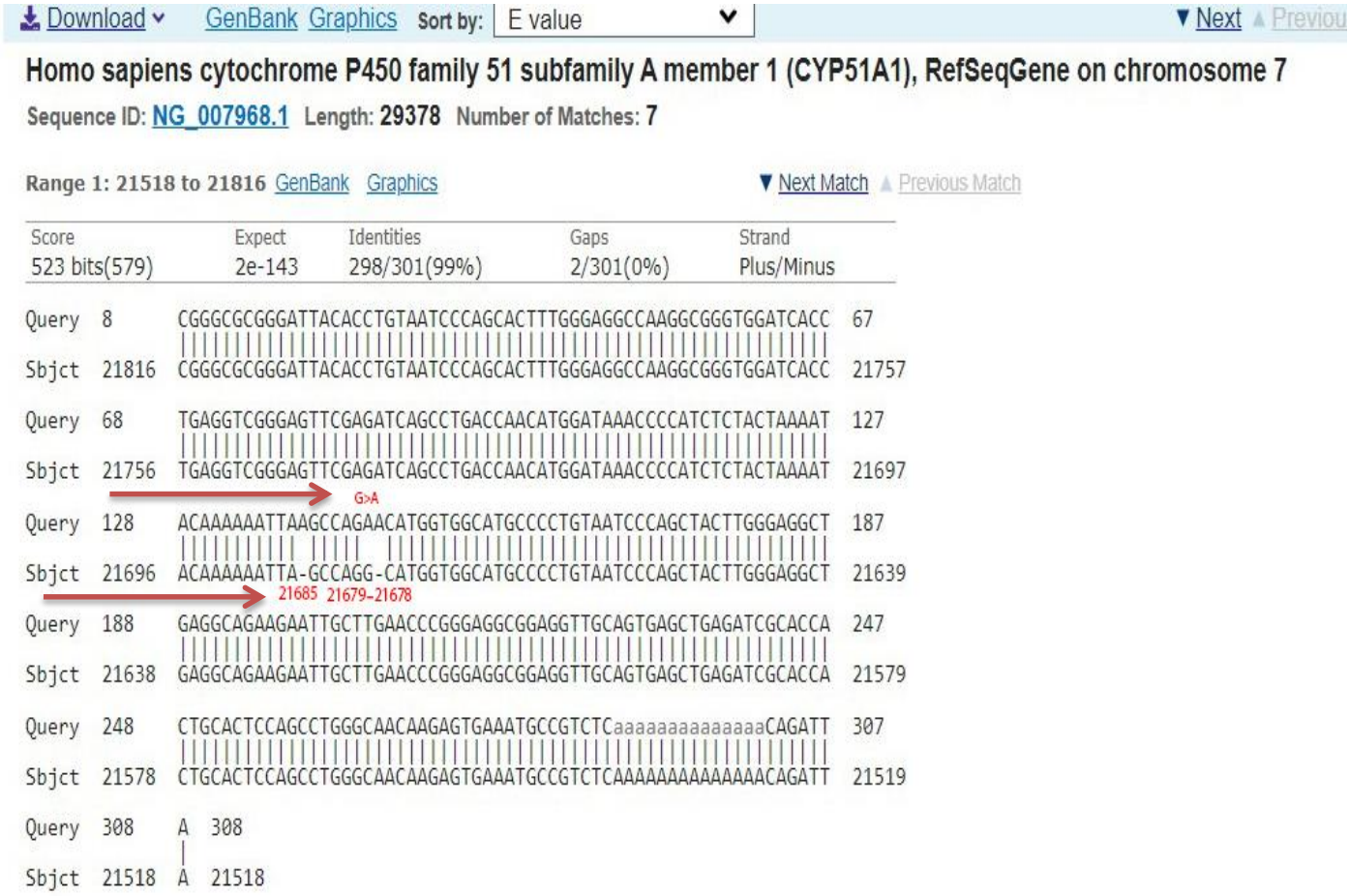


Fig. 4. A) Analysis of single nucleotide polymorphism CYP51A1 using sanger sequencing, A/A 109 indicative of a wild type allele B) CYP51A1 mutations in a patient with RA (case1). The upper arrow shows the different types of mutations; A insertion, G>A, T insertion, G insertion, G>T and C deletion, and the lower arrow indicates the position where these mutations happened, 21680, 21681, 21762, 21763, 21764 and 21816 respectively. C) CYP51A1 mutations in a patient with RA (case 2), the upper arrow the type of mutation; A insertion and G>A variation in CYP51A1 indicative positions 21678 A insertion, 21679 G>A and 21685 A insertion. and the lower arrow indicates the position where these mutations happened, 21678, 21685 and 21679, respectively.

positions 21678 and 21685 for adenine nucleotide. The blood test of this RF-negative and anti-CCP antibody-positive patient for TNF-α and IL-6 was negative, however, sIL-6R levels were significant, (4 ng/mL). She was also suffering from UTI. These newly discovered mutations have been already registered in the GenBank database (https: //www.ncbi.nlm.nih.gov/nucore/OR420790).

To evaluate the association of TLR4 and CYP51A1 polymorphisms with the master regulatory cytokine, i.e., TNF-α, the plasma TNF-α levels were compared between patients and control groups. As it can be seen in Figure 5, the mean of TNF-α was significantly higher in patients than in control subjects (P=0.001). ROC curve was also drawn for TNF-α in patient group (Figure 6) and its sensitivity, and specificity at concentration of 4.25pg/mL were 76% and 70%, respectively.

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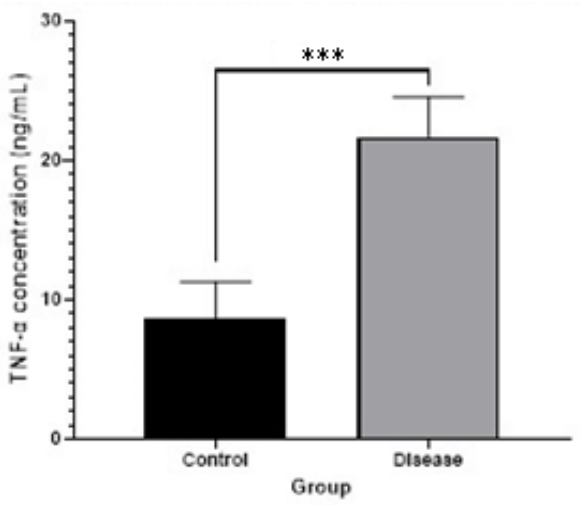


Fig. 5. Comparison of the plasma levels of TNF-α in RA and control group. The patient subjects exhibited a significant elevated level of TNF-α in comparison to normal controls. The data were expressed as mean and the bars indicate the standard deviation < 0.05 that was considered as statistically significant. *** P <0.001.

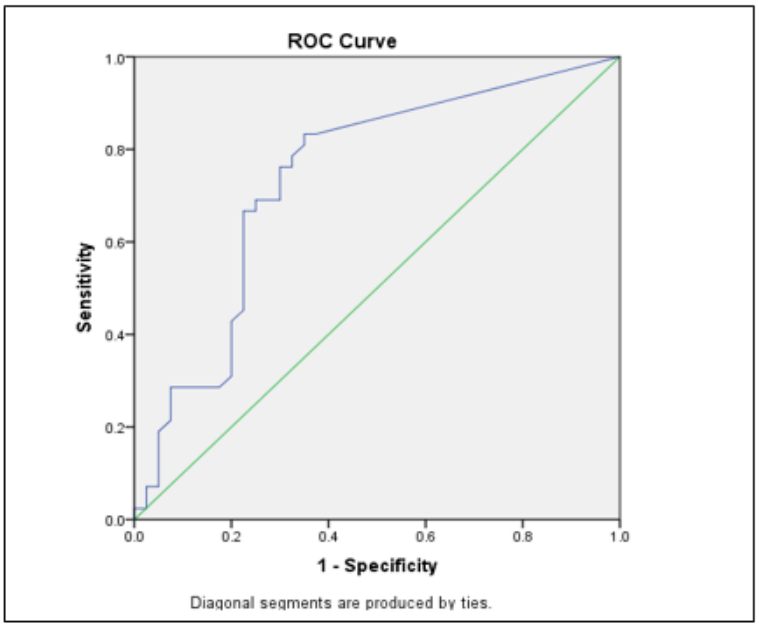


Fig. 6. ROC curve analysis. At concentrations of 4.25 pg/mL, the plasma TNF-α showed sensitivity, and specificity as 76% and 70%, respectively for RA.

Discussion

Clinical and demographic data are presented in Table 4. The incidence rate of RA was higher in the female population and its ratio is almost 4 times. The mean age of patients who were referred to a rheumatologist was more than 50 years old (55.9±12.5489 y). The age of onset of RA was ranging between 17 to 68 years, with a mean of approximately 41.49 ± 11.99 y while disease duration ranged between 1- 43

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However, the TLR4 DNA sequencing of the whole amplicon which was amplified by this gene-specific forward and reverse outer primer was not able to confirm our favorite mutation i.e., TLR4 rs141534085(A>G) in patient and control samples. Instead of this, we found the wide type allele in both groups as illustrated in Figure 3A. However, interestingly as it can be seen in Figure3B, during the DNA sequencing analysis, we realized that one of these patients exhibited three different types of point mutations in this area, appeared as follows: A>T in position 1050, T>A in position 1052 and C>A in position 1054. These newly discovered mutations have been already registered in the GenBank database. (https:// www. ncbi. nlm.nih.gov/Genbank/OR540493).

The age of this female patient was 63 who was suffering from RA for 15 years. She also suffered from heart disease and UTI. She was negative for blood TNF-α (5.7 ng/ml), and anti-CCP antibody but positive for RF tests.

Moreover, as can be seen in Figure 3C, surprisingly in this area of TLR4 gene in our second DNA sequencing analysis which DNA molecules were prepared from a 51-year-old female patient suffering from RA for one year, we found two other new mutations, A>T in position 1047, and at position 1051. She exhibited a deletion in an A- nucleotide. The blood test of this RF-positive and anti-CCP antibody-negative patient for TNF-α was negative. These newly discovered mutations have been already registered in the GenBank database https://www.ncbi.nlm.nih.gov/Genbank/OR540494).

Similar to the TLR4 gene, PCR products for the CYP51A1 gene were also obtained from blood samples for 46 patients and 48 control subjects. Two of these products which appeared as 2 and 3 bands in agarose gel electrophoresis were sequenced through Sanger DNA sequencing (Figure 2B). Both patients had the wild-type allele at position 1091 (Figure 4A). However, we found that one of these patients exhibited six different types of point mutations in this area which appeared as follows: G>A in position 21680, T was inserted in position 21762 and G was inserted in position 21763, G>T in position 21764, and A was deleted

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in position 21815. These newly discovered mutations have been already registered in GenBank: (https://www.ncbi.nlm.nih.gov/nucore/OR420789).

The age of this female patient was 80 suffering from RA for 5 years. She also suffered from heart disease. She was negative for IL6, positive for blood TNF-α (99.8 pg/mL) and sIL6R (3.1 ng/mL), and positive for anti-CCP antibody and RF tests. Surprisingly in this area of the CYP51A1 (rs6) gene in our second patient DNA sequencing analysis was prepared from a 57-year-old female patient suffering from RA for 25 years. We found three other new mutations, G>A in position 21679. She exhibited a deletion at positions 21678 and 21685 for adenine nucleotide. The blood test of this RF-negative and anti-CCP antibody-positive patient for TNF-α and IL-6 was negative, however, sIL-6R levels were significant, (4 ng/mL). She was also suffering from UTI. These newly discovered mutations have been already registered in the GenBank database (https://www.ncbi.nlm.nih.gov/nucore/OR420790).

To evaluate the association of TLR4 and CYP51A1 polymorphisms with the master regulatory cytokine, i.e., TNF-α, the plasma TNF-α levels were compared between patients and control groups. As it can be seen in Figure 5, the mean of TNF-α was significantly higher in patients than in control subjects (P=0.001). ROC curve was also drawn for TNF-α in patient group (Figure 6) and its sensitivity, and specificity at concentration of 4.25pg/mL were 76% and 70%, respectively.

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