CrossMark

ORIGINAL ARTICLE

Tumor necrosis factor-α -308 polymorphism in North Indian rheumatoid arthritis patients and association with mRNA and serum TNF-α

Mohd Jahid[1] · Rehan-Ul-Haq[2] · Puja Kumari Jha[1] · Diwesh Chawla[3] ·
Rajnish Avasthi[4] · Rafat Sultana Ahmed[1]

Abstract Rheumatoid arthritis (RA) is a severely disabling chronic autoimmune disorder that leads to progressive inflammation of the joints and surrounding tissues. TNF-α, a potent proinflammatory cytokine, plays a pivotal role in the pathogenesis of RA. The endogenous formation of TNF-α may be influenced by TNF-α promoter polymorphisms. Hence, the present study was designed to explore any possible association between genetic polymorphism of TNF-α -308 G/A, messenger RNA (mRNA) expression, serum levels of TNF-α, and inflammatory markers in North Indian RA patients. A total of 214 controls and 187 RA patients were recruited according to the revised American College of Rheumatology 2010 criteria. TNF-α -308 G/A genetic polymorphism within promoter region was analyzed by using PCR-RFLP. Levels of inflammatory markers and serum TNF-α were estimated by ELISA. The mRNA expression of TNF-α gene was measured by quantitative real-time PCR. Higher levels of autoantibodies (RF and anti-CCP) were present in RA patients as compared to controls. We found a positive and significant correlation of circulating TNF-α levels with RF ($r = 0.18$), anti-CCP ($r = 0.16$), and mRNA expression of TNF-α gene ($r = 0.57$) in RA patients. The mRNA expression levels of TNF-α was 4.5-fold higher in patients with RA as compared to controls. The heterozygous mutant variants (G/A) and homozygous mutant variants (A/A) were found to be significantly associated with RA as compared to control (OR = 1.52 and 3.02, respectively). Our observations illustrated a significant association of allele -308 A TNF-α with progression of RA. Significant and positive correlation of TNF-α levels with mRNA expression and inflammatory marker levels suggests that serum TNF-α may be a susceptibility marker for RA.

Keywords Inflammatory markers · Serum TNF-α and mRNA expression · TNF-α promoter polymorphism

✉ Rafat Sultana Ahmed
rafatnizam@rediffmail.com

Mohd Jahid
zahidkhan780@gmail.com

Rehan-Ul-Haq
docrehan1975@gmail.com

Puja Kumari Jha
Pujajha26@gmail.com

Diwesh Chawla
diweshchawla@yahoo.co.in

Rajnish Avasthi
rajnishavasthi@yahoo.co.in

1 Department of Biochemistry, University College of Medical Sciences and GTB Hospital (University of Delhi), Dilshad Garden, Delhi 110095, India

2 Department of Orthopedics, University College of Medical Sciences and GTB Hospital (University of Delhi), Dilshad Garden, Delhi 110095, India

3 Central Research Laboratory, University College of Medical Sciences and GTB Hospital (University of Delhi), Dilshad Garden, Delhi 110095, India

4 Department of Medicine, University College of Medical Sciences and GTB Hospital (University of Delhi), Dilshad Garden, Delhi 110095, India

Introduction

Rheumatoid arthritis (RA) is one of the most common autoimmune disorders of unknown etiology and it is a destructive arthropathy. It is characterized by inflammation of synovial membrane and erosion of bones and cartilages which lead to the progressive destruction of joints. The prevalence rate of RA is about 0.5–1% worldwide [1]. In India, it is about 0.8%, almost equal to the world prevalence rate [2]. HLA-DRB1 gene is the major genetic susceptibility locus for RA, and it has been confirmed by linkage as well as association studies of this gene [3]. The non-HLA genes have also been implicated in the susceptibility of RA, e.g., PTPN22, TNFAIP3, STAT4, TRAF1/C5, IL2RB, AFF3, CD40, CTLA4, MMEL1, and PADI4 [4].

The other genetic factors that might be associated with the susceptibility or progression of RA are cytokine genes [5]. Cytokines are small molecular weight regulatory proteins and act as intercellular mediators in the production and control of immune and inflammatory responses [6]. Tumor necrosis factor-alpha (TNF-α) plays a pivotal role in the pathogenesis of RA. TNF-α, a potent proinflammatory cytokine, is the prime mediator of inflammation, which is upregulated in the joints of RA patients, and its blood concentration is correlated with the disease activity [7]. TNF-α is secreted by different types of cells, but mainly by macrophages [8] and is reported to stimulate cytokine production and enhances the expression of adhesion molecules to increase the neutrophil activation [9]. It also regulates many important cellular processes such as proliferation, differentiation, growth, and the immune responses [10].

Gene encoding TNF-α is located in the class III region of the major histocompatibility complex between the HLA-B and HLA-DR genes on chromosome 6 [11]. TNF-α is reported to have contradictory roles associated with genetic polymorphisms in the genes regulating its production and the polymorphisms in the TNF locus [12]. It has been reported that transgenic mice that overexpress the human TNF gene develop polyarthritis similar to RA [13]. It has been reported that anti-TNF therapy considerably improves the arthritis in these patients [14]. TNF-α activates the chondrocytes and induces proteolytic and metalloproteinase enzymes which lead to cartilage destruction. On activation of osteoclasts, there is bone resorption and destruction of subchondral bone [15]. Thus, TNF-α may be a susceptibility gene for RA.

There are many promoter polymorphisms of TNF-α relative to the transcription start site, but the one most studied is TNF-α -308 (G/A) gene polymorphism. The genetic variant guanine (G) defines the common variant (wild), and the presence of adenine (A) defines the less common variant (mutant). In this SNP, a guanine is substituted by an adenine. The endogenous formation of serum TNF-α may be influenced by affecting the messenger RNA (mRNA) and protein expression levels by TNF-α promoter polymorphisms.

Numerous studies have addressed the issue of TNF-α gene promoter SNPs in RA patients from various ethnic populations but some contradictory results emerged. Polymorphism of the TNF-α gene within the promoter region -308 (G/A) has been studied worldwide but the reports are conflicting. To the best of our knowledge, there are no studies available regarding -308 (G/A) polymorphism reporting the association with inflammatory markers, mRNA, and protein expression levels in North Indian RA patients. Hence, the present study was designed to explore any possible association between genetic polymorphism of TNF-α -308 (G/A), gene expression, serum cytokine levels, and inflammatory markers in North Indian RA patients.

Material and methods

Recruitment of subjects The present case-control study was carried out at the Department of Biochemistry, UCMS and GTB Hospital, Dilshad Garden, Delhi. After obtaining the informed consent of subjects, a total number of 187 cases, according to the revised American College of Rheumatology (ACR, 2010) (Aletaha et al. 2010) guidelines were recruited, and it was ensured that patients were not on any treatment including NSAIDS, DMARDs, and anti-TNF-α drugs prior to recruitment. A total number of 214 healthy controls were also enrolled by voluntary participation for the study. Subjects who are smokers, alcoholic, diabetic, and having endocrine disorders, acute infections, and COPD with emphysema, and pregnant women were excluded from the study. All the subjects included in the study were selected from the same ethnic group of North India. The study was approved by the Institutional Ethical Committee for Human Research (IEC-HR).

Laboratory assessment

Sample collection About 5.0 ml of peripheral venous blood was collected in fasting state (12 h) from the patients and controls in plain and EDTA vials for the estimation of biochemical parameters specific to RA, serum cytokine levels, genotyping, and gene expression study. Blood serum was freshly separated and stored in aliquots at −80 °C before cytokine determination.

Diagnostic and inflammatory markers of RA Erythrocyte sedimentation rate (ESR) was measured by the modified Westergren method. Rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (anti-CCP), and C-reactive protein (CRP) were measured by standard ELISA kits (Omega Diagnostics Ltd., Alva, UK) using a microplate reader (Thermo Scientific, Hudson, NH, USA) as per the

manufacturer's instructions. All standards, RA samples, and control samples were run in duplicate.

Cytokine assay TNF-α was quantified by standard ELISA kits (Omega Diagnostics Ltd., Alva, UK) using a microplate reader (Thermo Scientific, Hudson, NH, USA) as per manufacturer's protocol. All standards, RA samples, and control samples were run in duplicate.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for screening of TNF-α promoter polymorphism

DNA extraction Genomic DNA was extracted using DNA extraction kit Prolab (Zymo research, USA). The quality of the extracted DNA was assessed by agarose gel electrophoresis and quantified by NanoDrop (Thermo Scientific). The extracted DNA was stored at −20 °C for the analysis of polymorphisms. PCR was performed for TNF-α gene by PCR-RFLP method using the Thermocycler (Eppendorf AG, Hamburg, Germany). Amplification of TNF-α -308 promoter was performed by PCR using the forward primer F:5′AGGCAATAGGTTTTGAGGGCCAT-3′ and reverse primer R:5′-TCCTCCCTGCTCCGATTCCG-3′. PCR conditions included denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 35 s, annealing at 54 °C for 45 s and extension at 72 °C for 1 min. The PCR was completed with a final extension step of 7 min at 72 °C. The PCR product of 107 bp was size-matched on 2% agarose gel (Lonza, Switzerland) stained with ethidium bromide bromide (Sigma Aldrich, USA). The PCR product of 107 bp was digested by using 1.5 units of restriction nuclease, NcoI enzyme (Fermentas, Germany), and incubated at 37 °C for 16 h. The restriction pattern was observed on 4% agarose gel electrophoresis, and the bands were visualized in UVP DIGI DOC IT Gel Documentation System (UVP, Upland, CA, USA) under UV light after staining with ethidium bromide.

Electrophoretic restriction patterns of TNF-α genotype In TNF-α -308 polymorphism after digestion, the resulting fragments 87 + 20 bp were diagnostic for wild-type genotype (GG). The remaining uncut resulting in a single 107-bp fragment represents the homozygous mutant genotype (A/A) whereas heterozygous mutant (G/A) produced all the three bands (107 + 87 + 20 bp). The genotypic analysis was repeated at least 20% of the samples to rule out genotypic error.

RNA extraction and reverse transcription to study the TNF-α gene expression RNA extraction was done by Trizol method as per manufacturer's protocol. Total RNA was extracted by using Trizol (Invitrogen, Thermo Fisher Scientific, USA).

Quantification of RNA The total RNA was quantified using NanoDrop (Thermo Fisher Scientific, USA) by measuring the optical density value at 260/280 and the concentration in ng/µL. The extracted RNA had an optical density 260/280 ratio between 1.9 and 2.0. The integrity of the extracted RNA was corroborated by agarose gel electrophoresis. The samples with low quality and degraded RNA were excluded from the study. Complementary DNA (cDNA) synthesis was done by using Maxima First Strand cDNA Synthesis kit (Verso, Thermo fisher scientific, USA) according to manufacturer's protocol.

Quantitative real-time polymerase chain reaction The experiment was conducted to measure the expression levels of mRNA of cytokine gene TNF-α. The reactions were performed on a quantitative real-time PCR thermo cycler (LC480-Roche, Basel, Switzerland) by using fluorescent dye SYBR green (Thermo Fisher Scientific, USA) to assess expression levels of mRNA of TNF-α gene. Reaction for the real-time PCR consisted of 3.0 µl of template cDNA, 12.5 µl of pyrostart master mix SYBR Green Master Mix (2X), and 0.5 µM of forward and reverse primers. A duplicate of each sample along with no template control wells were set aside for PCR amplification. The primer sequence for target gene (TNF-α) and constitutive genes (β-actin and 18s) were as follows: TNF-α, F:5′CTT CTC CTT CCT GAT CGT GG3′ and R:5′GCT GGT TAT CTC TCA GCT CCA3′; β-actin, F:5′GCA CCA CAC CTT CTA CAA TG3′ and R:5′TGC TTG CTG ATC CAC ATC TG3′; and 18s, F:5′CGG AGG TTC GAA GAC GAT CAG ATA3′ and R:5′TTG GTT TCC CGG AAG CTG CC3′. The PCR thermoprofile was initial denaturation for 3 min at 95 °C, 39 cycles of 20 s at 94 °C denaturing, 30 s at 58 °C, 30 s at 72 °C extension, and final extension for 3 min. Cumulative fluorescence was measured at the end of the extension phase of each cycle. The product of amplification was confirmed for specification by melt curve analysis. Throughout thermal cycling, the emission of fluorescence from each sample was recorded and LC480 Roche software processed the fluorescence data to produce threshold cycle (C_t) value. The constitutive genes β-actin and 18s were used for internal normalization. The expression levels of mRNA of TNF-α gene were quantified by ΔC_t value. ΔC_t value explains the expression levels as the lower the ΔC_t value, the higher the expression levels. ΔC_t value was calculated as C_t value (target gene) − C_t value (constitutive gene). To examine the expression pattern, relative quantification was done. The fold change or relative expression level was calculated by $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Statistical analysis was carried out using standard statistical methods by SPSS software (version 17.0). Demographic data

and routine biochemical parameters between the study groups were analyzed using one-way analysis of variance (ANOVA). Association of inflammatory markers with TNF-α polymorphism among RA patients was analyzed using ANOVA followed by post hoc Tukey's test. Data were expressed as mean ± standard deviation. Pearson's correlation analysis was done in RA subjects to determine the association of inflammatory markers and levels of serum protein TNF-α and of mRNA expression of TNF-α gene. Concerning the evaluation of TNF-α gene polymorphisms, the significance of differences from Hardy–Weinberg equilibrium (HWE) was tested using the χ^2 test. The frequency of the TNF-α SNPs among RA patients was found compatible with Hardy–Weinberg equilibrium ($p > 0.05$). The power of the sample size for the SNP at the 5% significance level and 80% power were calculated by using genetic power calculator, and the sample size was set accordingly. The comparison of the prevalence of different genotypes in different subject groups, the odds ratios, and relative risk (RR) at 95% confidence interval was computed by using logistic regression analysis. Etiological fraction was calculated by the formula (1-RR/RR). p values less than 5% were considered significant.

Results

Characteristics of the study subjects The demographic profile, clinical characteristics, and biochemical parameters of the control subjects and RA patients are presented in Table 1. There was no significant difference in the age or sex distribution between the study groups. Regarding the clinical characteristics, the average number of painful joints was found to be 14.5 ± 2.7 in RA patients. Rheumatoid nodules were observed in 13 RA patients. Seventy percent of patients reported fatigue and anemia was observed in 5.9% of patients with RA. The average time of morning stiffness was around 62 ± 17.6 min in RA patients. Among the lipid profile, the serum levels of total cholesterol, VLDL, LDL, and triglycerides were higher and levels of HDL were significantly lower in RA patients as compared to controls. Serum levels of ALT, ALP, and albumin were also significantly higher in RA patients as compared to control subjects. Hb was significantly lower in patients with RA as compared to controls.

Serum inflammatory markers, serum TNF-α, and mRNA expression levels The levels of serum inflammatory markers in the studied subjects are presented in Table 2. We found significantly higher levels of autoantibodies (RF and anti-CCP) in RA patients as compared to control subjects which confirmed the positive test for the patients enrolled. Anti-CCP has good sensitivity and superior specificity to RA. The acute phase reactants serum CRP, ESR, and TNF-α were also significantly higher in RA subjects as compared to control

Table 1 Demographic, clinical, and biochemical parameters among control subjects and RA patients

	Control subjects (214)	RA patients (187)
Age (years)	38.4 ± 10.1	37.6 ± 9.1
Sex (male/female)	30/184	22/165
Duration of disease (months)	0	6.8 ± 2.9
Number of painful joints	0	14.5 ± 2.7
Rheumatoid nodules	0	(13) 6.9%
Fatigue	0	(131) 70%
Anemia	0	(11) 5.9%
Morning stiffness (min)	0	62 ± 17.6
Cutaneous vasculitis	0	0
Systemic vasculitis	0	0
Ocular manifestations	0	0
Cardiac manifestations	0	0
Urea (mg/dL)	23.6 ± 6.1	24.7 ± 6.0
Creatinine (mg/dL)	0.8 ± 0.18	0.9 ± 0.3
Uric acid (mg/dL)	3.98 ± 0.95	6.5 ± 2.1[a]
Conj bil (mg/dL)	0.36 ± 0.27	0.32 ± 0.57
Total bil (mg/dL)	0.64 ± 0.20	0.63 ± 0.34
ALT (U/L)	30.78 ± 9.1	34.17 ± 9.5[a]
ALP (U/L)	57.4 ± 14.1	155.9 ± 12.4[a]
Protein (mg/dL)	7.3 ± 0.69	7.4 ± 0.77
Albumin (mg/dL)	3.92 ± 0.67	4.18 ± 0.76[a]
TC (mg/dL)	167.9 ± 15.24	229.47 ± 20.76[a]
HDL (mg/dL)	50.0 ± 7.55	35.63 ± 3.85[a]
VLDL (mg/dL)	19.92 ± 5.52	30.43 ± 3.62[a]
LDL (mg/dL)	90.74 ± 13.3	146.5 ± 12.16[a]
TG (mg/dL)	101.56 ± 17.36	158.71 ± 10.35[a]
Hb (g/dL)	12.5 ± 1.47	10.61 ± 1.82[a]

Data are presented as mean ± SD. Comparison between the groups was performed with one-way ANOVA

ALT alanine aminotransferase, *ALP* alkaline phosphatase, *TC* total cholesterol, *HDL* high density lipid, *VLDL* very low density lipid, *LDL* low density lipid, *TG* triglycerides, *Hb* hemoglobin

[a] $p < 0.01$, RA subjects vs. healthy subjects

Table 2 Diagnostic and inflammatory markers among control subjects and RA patients

	Control subjects	RA patients
ESR (mm/h)	6.56 ± 3.43	36.46 ± 11.25[a]
RF (U/ml)	58.3 ± 17.5	127.3 ± 21.3[a]
Anti-CCP (U/ml)	0.6 ± .7	17.8 ± 8.3[a]
CRP (mg/L)	0.5 ± 0.29	17.86 ± 7.10[a]
TNF-α (pg/ml)	15.54 ± 6.6	36.7 ± 10.91[a]

Data are presented as mean ± SD. Comparison between the groups was performed with one-way ANOVA

ESR erythrocyte sedimentation rate, *RF* rheumatoid factor, *anti-CCP* anti-cyclic citrullinated peptide, *CRP* C-reactive protein

[a] $p < 0.001$, RA subjects vs. healthy Controls

subjects. The mRNA expression levels of TNF-α assayed by quantitative real-time PCR was 4.5-fold higher (constitutive gene, β-actin) and 4.2-fold higher (constitutive gene, 18s) in RA patients as compared to control subjects.

Correlation of inflammatory markers and the mRNA expression and circulating serum protein levels of TNF-α in RA patients Correlation analysis was carried out to evaluate the association of serum TNF-α levels with inflammatory markers and fold change of mRNA in RA patients. We found positive correlation of circulating TNF-α levels with ESR and CRP, positive and significant ($p < 0.05$) correlation with RF ($r = 0.18$), and anti-CCP ($r = 0.16$) in RA patients. To find out the dependence of mRNA expression of TNF-α with serum TNF-α levels, we carried out the Pearson's correlation and found significant positive correlation between mRNA expression with serum TNF-α levels ($r = 0.57$) in RA patients (Table 3).

TNF-α -308 G/A promoter gene polymorphism The genotype and allele frequency distribution of SNP TNF-α -308 G/A are presented in Table 4. The frequency of -308 A was higher in RA as compared to controls. In TNF-α -308 G/A promoter polymorphism, wild-type genotype (GG) was found to be significantly lower in the RA group as compared to control subjects. Both the heterozygous mutant variants (G/A) and homozygous mutant variants (AA) were significantly associated with RA (OR = 1.52, $p < 0.05$) and (OR = 3.02, $p < 0.05$), respectively. For evaluation of association at the genotype level, the mutant groups (GA + AA) were combined together and logistic regression analysis was carried out. The association still persisted (OR = 1.64, $p < 0.05$) (Table 5).

Association of inflammatory markers with TNF-α genetic variants among RA patients The levels of inflammatory marker serum TNF-α, RF, anti-CCP, CRP, and ESR were not significantly associated with the genetic variants of TNF-α -308 G/A promoter polymorphism in RA patients (Table 6).

Discussion

Rheumatoid arthritis is a severely disabling chronic autoimmune disorder that leads to progressive inflammation of the joints and surrounding tissues resulting in erosion of bones and cartilages. Genome-wide association studies exhibit several polymorphisms associated with susceptibility to RA [16]. There are a number of different inflammatory cells, such as macrophages and T lymphocytes in inflammatory synovitis. These cells communicate via a network of small molecular regulatory proteins known as cytokines. Some of these exert

proinflammatory actions and the others provide anti-inflammatory or immunoregulatory effects. In normal physiology, these cytokines are maintained in balance; however, in RA, the balance shifts in favor of the proinflammatory cytokines [17]. TNF-α is a pleomorphic cytokine and intervenes a wide variety of effector functions that are of prime importance in the pathogenesis of RA such as endothelial cell activation and chemokine amplification [18]. Hence, quantification/assessment of cytokine gene and serum protein expression is essential to understand the autoimmunity [19].

The present study was undertaken in order to investigate any possible association between genetic polymorphism of TNF-α -308 G/A, gene expression, serum protein TNF-α, and inflammatory markers in North Indian RA patients. A total of 401 subjects (187 RA cases and 214 control subjects) were recruited for the study. In RA cases, there were 22 males and 165 females (M/F = 0.133) and the control group consisted of 30 males and 184 females (M/F = 0.163). Lipid profile was found to be deranged in RA patients similar to earlier reports [20, 21] (Table 1). Dyslipidemia commonly occurs in RA patients and may be the secondary impact of chronic inflammation as observed in these patients. The patients with RA are considered to be genetically predisposed to RA development related to deranged lipid profile.

Rheumatoid factor is the first well-known immunological and biochemical marker of RA [22] and plays a pivotal role in the differential diagnosis of polyarthritis. In healthy subjects, the prevalence rate of RF is less than 5%, and in RA patients, this increases to 70–90% [23]. In our study, the serum levels of RF were significantly higher in RA patients than in controls and were positively and significantly correlated with the serum levels of TNF-α indicating a clear association with higher levels of disease activity [24]. The serum levels of anti-CCP were significantly higher in RA patients than in controls and may be associated with higher inflammatory activity, poorer radiologic outcome [25], and higher frequency of extra-articular manifestations such as painful joints and rheumatoid nodules as observed in our study (Table 1). Similar result has been reported in Kashmiri population [26]. Increased levels of RF and a-CCP are associated with persistent joint inflammation [27]. C-reactive protein (CRP) is an acute-phase protein synthesized by the hepatocytes in response to stimulation by proinflammatory cytokines [28] and is used to discriminate systemic inflammatory disorders such as RA from non-inflammatory diseases such as osteoarthritis. The observed significantly high CRP levels (Table 2) in RA patients compared to controls are correlated with disease activity [29]. Erythrocyte sedimentation rate is an acute-phase reactant and is extensively used to monitor active RA, and it is higher in these patients. As expected, we found significantly higher ESR in RA patients as compared to healthy controls [30]. TNF-α is the major proinflammatory cytokine involved in immunopathogenesis [31] and mediates the subchondral

Table 3 Correlation of TNF-α with inflammatory markers and the mRNA expression levels of TNF-α gene among RA patients

	ESR	RF	Anti-CCP	CRP	Fold change (β-actin)	Fold change (18s)
TNF-α levels (r)	0.067	0.18[b]	0.16[a]	0.07	0.57[a]	0.79[a]

[a] Correlation is significant at the 0.01 level (2-tailed)

[b] Correlation is significant at the 0.05 level (2-tailed)

erosion process in RA [32]. In the present study, the serum levels of TNF-α were significantly higher in RA patients than in controls (Table 2). The mRNA expression levels of TNF-α were higher in patients with RA in comparison to control subjects (4.5-fold higher with reference to constitutive gene β-actin and 4.2-fold higher with reference to constitutive gene 18s). Our results are in agreement with earlier reports in Mexican population [33].

The levels of TNF-α were positively correlated with the levels of ESR and CRP and positively and significantly correlated with the levels of RF, anti-CCP, and mRNA expression levels of TNF-α among RA patients (Table 3). The distribution of polymorphic alleles was according to the Hardy–Weinberg equilibrium for both the groups ($p > 0.05$). We observed a significant difference in genotype and allelic frequencies in the TNF-α -308 G/A promoter gene polymorphism between RA patients and controls. The allele -308 "A" may lead to the development of RA as indicated by the odds ratios and relative risk (RR = 1.44, $p < 0.05$) (Tables 4 and 5). The etiological fraction calculated was 0.29 (E.F. = 0.29). The levels of RF, anti-CCP, CRP, ESR, and serum TNF-α were not found to be significantly associated with the different genotypic variants of TNF-α -308 G/A (Table 6).

A thorough survey of literature revealed that although there are many studies which have reported the association of genetic polymorphisms of the TNF-α -308 G/A gene with susceptibility to RA, the results are contradictory for different ethnic populations. A study carried out in Argentinean population reported that TNF-α -308 G/A was associated neither with susceptibility to RA nor with the variables related to the course and outcome of the disease [34]. Another study carried out by Gambhir et al. 2010 found that TNF -308 A was less prevalent among RA patients in comparison to control

subjects [35]. It has been reported that the frequency of allele "A" was higher in RA patients than in controls (15 vs. 5.8%, respectively) in a cohort study of Punjab population indicating that it may increase susceptibility to RA [36]. Our results are in agreement with this report as we have also observed that the frequency of allele "A" is higher in RA patients than in controls (24 vs. 17%). In Tunisian population, -308 A allele and -308 A/A genotype were positively associated with erosion in RA [37], whereas in Han Chinese population from Hunan, susceptibility to RA was increased in patients with "G" allele, especially in the female carrier of genotypes and TNF-α -308 A allele might play a positive role in reduction of RA risk in males [38]. Polymorphisms in cytokine genes are highly influenced by ethnicity. It is well known that frequency and distribution of gene polymorphisms in India are substantially different from other populations and ethnic groups.

Our results show that genotype TNF-α -308 A/A was higher in RA patients than in controls, indicating its role in disease development. The single nucleotide polymorphism in TNF-α promoter region may modulate mRNA expression and circulating levels of TNF-α production, which in turn may have an impact on inflammatory responses and disease expression. The possible mechanism may be that the allele "A" of promoter region positively activates gene expression and increases the circulating serum levels of TNF-α as observed in our study and further confirmed by the positive and significant correlation of serum TNF-α and mRNA expression levels in RA patients (Table 3).

In conclusion, we found significant association of -308 G/A TNF-α polymorphism with susceptibility/risk of RA. Further, the serum TNF-α levels were positively and significantly correlated with the levels of autoantibodies (RF and anti-CCP) and mRNA expression levels of TNF-α in RA patients. To the best of our knowledge, we report here for the first time the association between TNF-α -308 G/A SNP,

Table 4 Percent distribution of allelic and genotypic frequency of TNF-α -308 polymorphism among the study groups

	Control subjects ($N = 214$)	RA Patients ($N = 187$)
GG	68.7%	57.5%
GA	29%	36.9%
AA	2.3%	5.35%
G allele	83%	76%
A allele	17%	24%

Table 5 Odds ratio for the presence of different genotypes in TNF-α -308 polymorphism between control subjects and RA patients

Genotype	Odds ratio	95% confidence interval	p value
GA vs. GG	1.52	1.00 to 2.33	0.04
AA vs. GG	3.02	1.02 to 8.95	0.04
AA + GA vs. GG	1.64	1.09 to 2.47	0.02

Table 6 Association of different genotypes with levels of serum TNF-α, RF, anti-CCP, CRP, and ESR among RA patients

Genotypes	N	TNF-α	RF	Anti-CCP	CRP	ESR
GG	108	35.5 ± 11.00	125.52 ± 25.4	18.02 ± 7.86	17.25 ± 7.12	36.28 ± 11.02
GA	69	36.3 ± 12.01	129.49 ± 13.9	17.09 ± 9.19	18.68 ± 6.94	36.26 ± 11.75
AA	10	43.1 ± 1.2	127.35 ± 21.28	21.16 ± 7.43	18.97 ± 7.1	39.8 ± 10.65

Data are presented as mean ± SD, one-way ANOVA followed by post hoc Tukey's test

[a] $p < 0.05$ was performed in RA patients

mRNA expression, serum TNF-α levels, and inflammatory markers in North Indian RA patients.

Compliance with ethical standards

Disclosures None.

References

1. Gibofsky A (2012) Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. Am J Manag Care 18(13 Suppl):S295–S302

2. Malaviya AN, Kapoor SK, Singh RR, Kumar A, Pande I (1993) Prevalence of rheumatoid arthritis in the adult Indian population. Rheumatol Int 13(4):131–134

3. Yamamoto K, Okada Y, Suzuki A, Kochi Y (2015) Genetic studies of rheumatoid arthritis. Proc Jpn Acad Ser B Phys Biol Sci 91(8):410

4. Barton A, Worthington J (2009) Genetic susceptibility to rheumatoid arthritis: an emerging picture. Arthritis Care Res 61(10):1441–1446

5. Shafia S, Dilafroze SFA, Rasool R, Javeed S, Shah ZA (2014) Rheumatoid arthritis and genetic variations in cytokine genes: a population-based study in Kashmir Valley. Immunol Investig 43(4):349–359.

6. Harrison P, Pointon J, Chapman K, Roddam A, Wordsworth B (2008) Interleukin-1 promoter region polymorphism role in rheumatoid arthritis: a meta-analysis of IL-1B-511A/G variant reveals association with rheumatoid arthritis. Rheumatology 47(12):1768–1770

7. Barton A, Platt H, Salway F, Symmons D, Barrett E, Bukhari M, Lunt M, Zeggini E, Eyre S, Hinks A (2004) Polymorphisms in the tumour necrosis factor gene are not associated with severity of inflammatory polyarthritis. Ann Rheum Dis 63(3):280–284

8. Aguillón JC, Cruzat A, Aravena O, Salazar L, Llanos C, Cuchacovich M (2006) Could single-nucleotide polymorphisms (SNPs) affecting the tumour necrosis factor promoter be considered as part of rheumatoid arthritis evolution? Immunobiology 211(1): 75–84

9. Song GG, Bae S-C, Kim J-H, Lee YH (2014) Association between TNF-α promoter –308 A/G polymorphism and rheumatoid arthritis: a meta-analysis. Rheumatol Int 34(4):465–471

10. Hayashi K, Piras V, Tabata S, Tomita M, Selvarajoo K (2013) A systems biology approach to suppress TNF-induced proinflammatory gene expressions. Cell Commun Signal 11(1):84

11. Zhang B-B, Liu X-Z, Sun J, Yin Y-W, Sun Q-Q (2013) Association between TNF α gene polymorphisms and the risk of duodenal ulcer: a meta-analysis. PLoS One 8(2):e57167

12. Elahi MM, Asotra K, Matata BM, Mastana SS (2009) Tumor necrosis factor alpha− 308 gene locus promoter polymorphism: an analysis of association with health and disease. Biochim Biophys Acta (BBA) - Mol Basis Dis 1792(3):163–172

13. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, Kollias G (1991) Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J 10(13):4025

14. Feldmann M, Maini RN (2003) TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. Nat Med 9(10):1245–1250

15. Mariaselvam CM, Sofiane S, Boukouaci W, Fortier C, Charron D, Krishnamoorthy R, Tamouza R, Negi VS (2014) TNF alpha promoter polymorphism may confer susceptibility to rheumatoid arthritis and influence TNF alpha production but not the clinical phenotype and treatment response. J Mol Biomark Diagn 5(6):1

16. Nakano K, Boyle DL, Firestein GS (2013) Regulation of DNA methylation in rheumatoid arthritis synoviocytes. J Immunol 190(3):1297–1303

17. Kay J, Calabrese L (2004) The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. Rheumatology-Oxford 43(3):iii2

18. Rodríguez-Rodríguez L, González-Juanatey C, Palomino-Morales R, Vázquez-Rodríguez TR, Miranda-Filloy JA, Fernández-Gutiérrez B, Llorca J, Martin J, González-Gay MA (2011) TNFA − 308 (rs1800629) polymorphism is associated with a higher risk of cardiovascular disease in patients with rheumatoid arthritis. Atherosclerosis 216(1):125–130

19. Ramos-Payán R, Aguilar-Medina M, Estrada-Parra S, González-y-Merchand J, Favila-Castillo L, Monroy-Ostria A, Estrada-Garcia ICE (2003) Quantification of cytokine gene expression using an economical real-time polymerase chain reaction method based on SYBR® Green I. Scand J Immunol 57(5):439–445

20. Myasoedova E, Kremers HM, Fitz-Gibbon P, Crowson C, Therneau T, Gabriel S (2009) Lipid profile improves with the onset of rheumatoid arthritis. Ann Rheum Dis 68 (Suppl3):78

21. Toms TE, Symmons DP, Kitas GD (2010) Dyslipidaemia in rheumatoid arthritis: the role of inflammation, drugs, lifestyle and genetic factors. Curr Vasc Pharmacol 8(3):301–326

22. Matuszewska A, Madej M, Wiland P (2015) Immunological markers of rheumatoid arthritis. Postepy Higi Med Dosw (Online) 70:251–257

23. Dörner T, Egerer K, Feist E, Burmester GR (2004) Rheumatoid factor revisited. Curr Opin Rheumatol 16(3):246–253

24. Aletaha D, Alasti F, Smolen JS (2015) Rheumatoid factor, not antibodies against citrullinated proteins, is associated with baseline disease activity in rheumatoid arthritis clinical trials. Arthritis Res Ther 17(1):229

25. Arnab B, Biswadip G, Arindam P, Shyamash M, Anirban G, Rajan P (2013) Anti-CCP antibody in patients with established rheumatoid arthritis: does it predict adverse cardiovascular profile? J Cardiovasc Dis Res 4(2):102–106

26. Shafiaa S, Shaha Z, Sofib F, Rasoola R, Gulla A (2016) Anti-CCP is associated with greater disease burden in Kashmiri population with rheumatoid arthritis. Rheumatology (Sunnyvale) 6(190): 2161–1149.1000190

27. Mjaavatten MD, van der Heijde D, Uhlig T, Haugen AJ, Nygaard H, Sidenvall G, Helgetveit K, Kvien TK (2010) The likelihood of persistent arthritis increases with the level of anti-citrullinated peptide antibody and immunoglobulin M rheumatoid factor: a longitudinal study of 376 patients with very early undifferentiated arthritis. Arthritis Res Ther 12(3):R76. doi:10.1186/ar2995

28. Singh HV, Shrivastava AK, Raizada A, Singh SK, Pandey A, Singh N, Yadav D, Sharma H (2013) Atherogenic lipid profile and high sensitive C-reactive protein in patients with rheumatoid arthritis. Clin Biochem 46(12):1007–1012. doi:10.1016/j.clinbiochem.2013.03.023

29. Ghosh B (2016) Correlation of serum uric acid with disease activity and C-reactive protein in patients suffering from rheumatoid arthritis. Open J Rheumatol Autoimmun Dis 6(03):79

30. Ajay K, Pranshi M, Neelima S (2012) Study of non-enzymatic antioxidant and lipid peroxide levels in rheumatoid arthritis. UJMD 1:10–13

31. Danve A, Sehra S, Jaykumar D, Kulkarni S (2016) Tumor necrosis factor in-hibitors may improve glycemic control in patients rheumatoid arthritis and concomitant diabetes mellitus. Osteol Rheumatol Open J 1(1):17–19

32. Schett G (2012) Pathophysiology of subchondral bone erosions in rheumatoid arthritis. Arthritis Res Ther 14:1–8

33. Oregon-Romero E, Vazquez-Del Mercado M, Ruiz-Quezada SL, Navarro-Hernandez RE, Rangel-Villalobos H, Martinez-Bonilla G, Bernard-Medina AG, Armendariz-Borunda J, Garcia-Banuelos J, Munoz-Valle JF (2008) Tumor necrosis factor alpha-308 and -238 polymorphisms in rheumatoid arthritis. Association with messenger RNA expression and sTNF-alpha. J Investig Med 56(7): 937–943.

34. Aranda F, Perés Wingeyer SD, Schneeberger E, Valerio M, Saint Martin E, Dal Pra F, Correa MÁ, Citera G, Martínez L, Mannucci P (2016) The− 308 G/A polymorphism in the tumor necrosis factor-α gene is not associated with development and progression of rheumatoid arthritis in Argentinean patients. Int J Rheum Dis 19(5): 476–481

35. Gambhir D, Lawrence A, Aggarwal A, Misra R, Mandal SK, Naik S (2010) Association of tumor necrosis factor alpha and IL-10 promoter polymorphisms with rheumatoid arthritis in North Indian population. Rheumatol Int 30(9):1211–1217. doi:10.1007/s00296-009-1131-0

36. Priyanka R, Kawaljit M, Ajay K, Priyanka S, RubinaS AB (2014) Association of tumor necrosis factor-alpha -308 G>A polymorphism with rheumatoid arthritis in two north Indian cohorts. Arch Rheumatol 29:241–249

37. Lagha A, Zidi S, Stayoussef M, Gazouani E, Kochkar R, Kochbati S, Almawi W, Yacoubi-Loueslati B (2015) Interleukin-1β, interleukin1-Ra, interleukin-10, and tumor necrosis factor-α polymorphisms in Tunisian patients with rheumatoid arthritis. Pathol Biol 63(4):179–184

38. Li F, Xie X, Chen J, Gao J, Lu F (2015) Association of TNF-α gene polymorphisms with the risk of rheumatoid arthritis in Han Chinese population from Hunan. Zhong Nan Da Xue Xue Bao Yi Xue Ban = J Cent S Univ Med Sci 40(9):945