Tumour necrosis factor gene polymorphisms in Egyptian patients with rheumatoid arthritis and their relation to disease activity and severity

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Abstract

Aim of the study: The present case control study was conducted to assess the association of LTA 252 A>G, TNF-α 308 G>A, and TNF-α 1031 T>C gene polymorphisms with rheumatoid arthritis (RA), and their involvement in disease activity and severity.

Material and methods: A total of 70 Egyptians, including 35 RA patients and 35 healthy control individuals, were included in the study. The RA patients comprised 34 females and one male. Cases with RA were diagnosed by a rheumatologist and fulfilled the 2010 ACR/EULAR criteria. Modified disease activity score (DAS28) was used to assess disease activity. Van Der Heijde-modified Sharp score (vdHSS) was used to assess radiological changes for assessment of disease severity. PCR-RFLP was used to detect the association of LTA 252 A>G, TNF-α 308 G>A, and TNF-α 1031 T>C gene polymorphisms with RA.

Results: TNF-α 308 G allele and TNF-α 308 GG genotype were significantly higher in RA patients compared to healthy control subjects (p = 0.04 and p = 0.001, respectively). TNF-α 308 G allele and GG genotype were significantly higher in the RA non-remission group compared to the remission group (p = 0.008, p < 0.001). Patients with the TNF-α 308 AG genotype had higher mean of Sharp score compared to the patients with the GG and AA genotypes (p = 0.007). There was no significant association between LTA 252 A>G and TNF-α 1031 T>C gene polymorphisms with RA.

Conclusions: Our results suggest that TNF-α 308 G/A gene polymorphism is genetically associated with RA and involved in disease activity and severity in Egyptian patients.

Key words: rheumatoid arthritis, genetic susceptibility, TNF polymorphisms, polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP).

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects 1% of the population worldwide [1]. It is a systemic disease that involves the joints, organs, and other areas of the body such as the skin, eyes, heart, lungs, kidney, and spleen [2, 3].

RA displays complex inheritance resulting from an intricate interplay between an individual’s environmental and genetic background [4]. It has a multifactorial aetiology, including a wide spectrum of clinical manifestations, variability in disease, progression, severity, and response to therapies [5].

Increased expression of pro- and anti-inflammatory cytokines detected in the affected tissues and serum of RA patients clearly illustrates the role of cytokines in the aetiology of RA [6]. Tumour necrosis factor (TNF), a proinflammatory cytokine, has been shown to play a significant role in the pathogenesis of multiple autoimmune diseases including RA [6-8].

TNF-α and lymphotoxin alpha (LTA) are closely related cytokines that share 30% of amino acid residues and have the same cell surface receptor [9]. Nedwin et al. [10] reported that the TNF-α and LTA genes are positioned in tandem on chromosome 6 between the class I and class II cluster of the major histocompatibility complex (chromosome 6p21.1-6p21.3). The proinflammatory cytokine TNF-α is one of the cardinal factors involved in RA inflammatory state [7]. TNF-α pleiotropic biological activities are mediated binding to TNF receptors (TNFR) type I and II [11].

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TNF-α plays a pivotal role in inflammation by inducing the expression of other proinflammatory molecules, chemotactic cytokines, and adhesion factors [12-14]. In vivo and in vitro studies have illustrated that high levels of TNF-α lead to exacerbation of the inflammatory response. This, together with its strong immunomodulatory activities, has been suggested to be important in the pathogenesis of various diseases such as asthma, systemic lupus erythematosus (SLE), and RA [7, 15, 16].

Amongst the five polymorphisms (at positions +691, -238, -308, -851, and -857) of TNF-α 308 gene, which were detected by PCR-single strand conformation polymorphism (PCR-SSCP) analysis, TNF-α 308 polymorphism has been reported to be associated with autoimmune diseases including RA [14, 17]. The genetic variation on position -308 resulted in two allelic forms in which the existence of guanine (G) defines the common variant, and the existence of adenine (A) determines the less common one. LTA G/A polymorphism is linked closely to TNF-α, has also been shown to contribute to the susceptibility of multiple autoimmune diseases [18, 19]. A polymorphism has been detected at position +252 within the first intron of the TNF-β gene, consisting of a G (LTA +252 G) on one allele and an A (LTA +252 A) on the alternate allele. The presence of G at this position determined the mutant allele known as LTA*1 (allele-1), which is the less frequent allele in white subjects and is associated with increased TNF-α and LTA production [20, 21].

A study of a Japanese population has indicated that LTA (+252) polymorphism together with HLADRB1*0405 may have an influence on the predisposition to RA [22]. To the best of our knowledge there are only two cohorts elucidating the role of TNF-α 308 G/A polymorphism in RA patients in Egypt. On the other hand, there are no reports investigating the association of other polymorphisms among Egyptian RA patients. Given the known importance of TNF gene in inflammatory and/or immune functions and the variation in susceptibility to immune disorders in different ethnic groups, we investigated the possible association between LTA 252 A>G, TNF-α 308 G>A, and TNF-α 1031 T>C polymorphisms and susceptibility to RA in Egyptian patients. In addition, we investigated the association of these polymorphisms with disease activity and severity.

Material and methods

Patients

A total of 70 Egyptian persons, including 35 cases and 35 control subjects, were included in the study. The RA patients comprised 34 females and one male. The RA patients were recruited from the outpatient clinic of the Rheumatology and Rehabilitation Department of Beni Suef University Hospital, Egypt, between March 2014 and January 2015. Cases with RA were diagnosed by a rheumatologist and fulfilled the 2010 ACR/EULAR criteria for diagnosis of RA [23]. The healthy control subjects were unrelated Egyptian age- and sex-matched individuals who had no family history of autoimmune diseases. The control group lived in the same geographical area and had the same ethnic origin as the patients. All cases and control subjects were informed of the purpose of the study, and their consent was obtained. The local Ethics Committee approved the study.

Clinical and laboratory assessment

Blood samples were obtained from all patients for determination of erythrocyte sedimentation rate (ESR; Westergreen), C-reactive protein (CRP), and rheumatoid factor (RF) by semi-quantitative latex (AVITEX® CRP and AVITEX® RF, Omega Diagnostics). CRP was considered positive when > 6 mg/l while RF was considered positive at ≥ 8 IU/ml. Anti-cyclic citrullinated peptide (anti-CCP) was determined using enzyme-linked immunosorbent technique (ELISA) using QUANTA Lite® CCP3 IgG ELISA, INOVA Diagnostics. According to the manufacturer’s protocol, serum was considered positive when the reading was ≥ 20 units.

The modified disease activity score DAS28 was calculated for all patients [24]. DAS28 score of higher than 5.1 is indicative of high disease activity, whereas a DAS28 below 3.2 indicates low disease activity. A patient is considered in remission if the DAS28 score is lower than 2.6.

In all patients, plain radiographs of both hands and feet in the posteroanterior projection were obtained. Van Der Heijde-modified Sharp score (vdHSS) was used to assess radiological changes [25].

Genotyping

Genomic DNA was extracted from EDTA anti-coagulated whole blood using QIAamp DNA Mini Kit (Cat. no. 51104, QIAGEN) according to the manufacturer’s protocol. The three sequences flanking TNF-α 1031 T>C, TNF-α 308G>A, and LTA 252 A>G single nucleotide polymorphisms were amplified by polymerase chain reaction (PCR). Genotyping of these polymorphisms was determined by a restriction fragment length polymorphism (RFLP) assay [26].

The 270-bp region of the TNF-α 1031 gene, encompassing the 1031T/C polymorphism site, was amplified via polymerase chain reaction (PCR) using the sense (5′-GGGGAGAACAAAAGGATAAG) and antisense (5′-CCCCATCTCGACTTTCATA) primer pair [27]. The total reaction volume was 25 µl: 5 µl DNA, 12.5 µl Dream Taq green PCR master mix (Fermentas), 1 µl of each primer and 5.5 µl nuclease-free water. Initially, the PCR reaction was subjected to denaturation for 5 min at 95°C, followed by 30 cycles of amplification (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C). A final elongation step...
(5 min at 72°C) was applied at the end of the 30 cycles [28]. The PCR is followed by digestion with the restriction enzyme BbsI (Thermo Scientific Cat. no. FD0574) according to the manufacturer’s protocol (C allele, 159 and 111 bp; T allele, 270 bp) (Fig. 1) [28]. Digested PCR fragments were separated by 4% agarose gel electrophoresis stained with ethidium bromide followed by ultraviolet visualisation.

The primers (5’-AGGCAATAGGTTTTGAGGCCCATT-3’) and (5’TCCCTCCCTGCTCCGATTCC G-3’) were used to amplify the 107-bp DNA fragment of the TNF-α 308 G>A polymorphism. The total PCR reaction mixture was 25 µl: 5 µl DNA, 12.5 µl Dream Taq green PCR master mix (Fermentas), 1 µl of each primer and 5.5 µl nuclease-free water. The PCR Cycling conditions for TNF-α 308 G>A were performed according to the protocol described by Bonyadi et al.: 5 min for initial denaturation at 95°C; 35 cycles at 95°C for 1 min for denaturation, 30 s at 65°C for annealing and 30 s at 72°C for extension, followed by 5 min at 72°C for final extension [28]. After amplification, PCR products were digested (at 37°C) by NcoI (Thermo Scientific Cat. no. FD0574) according to the manufacturer’s protocol (G allele, 87 and 20 bp; A allele, 107 bp) (Fig. 2) [28]. Digested PCR products were electrophoresed in 4% agarose gel stained with ethidium bromide and followed by ultraviolet visualisation. Primers (5’-CCTGTTGGGTCCGCCTTTGAC-3’) and (5’AGGCTGTGGGGGACATGTA CTG-3’) were used to amplify the 740-bp DNA fragment to genotype the LTA 252 A>G polymorphism. The total PCR reaction mixture was 25 µl: 5 µl DNA, 12.5 µl Dream Taq green PCR master mix (Fermentas), 1 µl of each primer and 5.5 µl nuclease-free water. The PCR cycling conditions were performed according to the protocol described by Cabrara et al. [29] with a slight modification: initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification: 94°C for 45 sec., 62.5°C for 45 sec, and a final extension at 72°C for 1.5 min. PCR products were digested (at 37°C) by NcoI (Thermo Scientific Cat. no. FD0574) according to the manufacturer’s protocol. Digested PCR products were electrophoresed in 4% agarose gel stained with ethidium bromide and followed by ultraviolet visualisation. The digested products generated 185 bp and 555 bp bands for the G allele and a 107 bp band for the A allele (Fig. 3) [29].

**Statistical analysis**

The collected data review, coding, and statistical analysis was done using SPSS software (Statistical Package for Social Science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows. Mean, median, range, and standard deviation were calculated to measure central tendency and dispersion of quantitative data, while the frequency of occurrence was calculated to measure qualitative data. Student’s t-test was used to determine the significance of the difference between two means. χ² was carried out for comparison of qualitative data, and Fisher’s exact test was used when the cell count was less than 5. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated whenever applicable, to test the association between genotype and RA. Analysis of variance (ANOVA) test was used to determine the difference between more than two means. The significance of the OR was calculated using a 2 × 2 contingency table. Genotype distributions were compared with those expected for samples from populations in
Hardy-Weinberg equilibrium using an $\chi^2$ test (1 df). The level of significance was taken at a $p$-value of $< 0.05$.

Results

The demographic, clinical, and laboratory data of RA patients and control group are shown in Table 1.

Concerning $LTA$ $252$ $A>G$ gene polymorphism, the genotype frequencies of RA patients and healthy controls conformed to the Hardy-Weinberg equilibrium ($p = 0.2531$ and $p = 0.224$, respectively). Similarly, for the $TNF-\alpha$ $1031$ $T>C$ gene polymorphism, the genotype frequencies of RA patients and healthy controls conformed to the Hardy-Weinberg equilibrium ($p = 0.97$; $p = 0.13$, respectively). As regards the $TNF-\alpha$ $308A>G$ gene polymorphism, the genotype frequencies for cases showed deviation from HWE ($p = 0.0019$), while the genotype frequencies of controls conformed to HWE ($p = 0.08$) (Table 2).

Analysis of the distribution of $TNF-\alpha$ $308$ $G/A$ revealed that the frequency of the G allele was higher than the A allele in the non-remission group. On the other hand, only two patients carrying the A allele were in remission, and there were no patients in remission carrying the A allele. The difference between the two groups was statistically significant ($p = 0.008$). On comparing the distribution $TNF-\alpha$ $308$ genotypes in both remission and non-remission group patients, the GG genotype was more frequently represented than the AG genotype, and the difference was highly statistically significant ($p < 0.001$) (Table 3).

On comparing the frequency of distribution of $TNF-\alpha$ $308$ genotypes with disease severity, we detected that the patients with heterozygous mutant type (AG) had higher Sharp score than the patients with the wild type (GG) genotype and the homozygous mutant (AA) genotype, respectively, and the difference was statistically significant ($p = 0.007$) (Table 4).

On the other hand, analysis of the frequency of distribution of genotypes in $TNF-\alpha$ $1031$ and $LTA$ $+252$ polymorphism according to disease severity was statistically non-significant ($p = 0.105$ and $p = 0.691$, respectively) (Table 4).

We did not detect any association between the distribution of $TNF-\alpha$ $308$, $TNF-\alpha$ $1031$, and $LTA$ $252$ genotypes and age/sex of patients disease duration, CRP, RF, and anti-CCP positivity (data not shown).

Discussion

RA is a complex, multifactorial, inflammatory disease of unknown aetiology with considerable variability. Both
Table 2. Comparison of TNF-α and TNF-β polymorphisms between Egyptian rheumatoid arthritis patients and healthy controls

|                          | Cases (n = 35) | Controls (n = 35) | Odds ratio and 95% confidence interval | \(\chi^2\) | p-value     |
|--------------------------|---------------|------------------|---------------------------------------|-----------------|-------------|
| **TNF-α 308, n (%)**     |               |                  |                                       |                |             |
| GG                       | 30 (85.7)     | 19 (54.3)        | Reference                             | < 0.001\*      |             |
| AG                       | 3 (8.6)       | 16 (45.7)        | 8.42 (1.9-42.3)                       | 11.32           | 0.266       |
| AA                       | 2 (5.7)       | 0 (0.0)          | 0.0 (0.0-7.18)                        | 1.24            |             |
| A                        | 7 (10)        | 16 (45.7)        | Reference                             | 4.21            | 0.040\*     |
| G                        | 63 (90)       | 54 (77.14)       | 2.67 (0.94-7.791)                     |                 |             |
| **TNF-α 1031, n (%)**    |               |                  |                                       |                |             |
| TT                       | 24 (68.5)     | 21 (60.0)        | Reference                             |                 |             |
| TC                       | 10 (28.6)     | 14 (40.0)        | 0.63 (0.20-1.90)                      | 0.85            | 0.355       |
| CC                       | 1 (2.9)       | 0 (0.0)          | NA                                    | 0.86            | 0.354       |
| T                        | 58 (82.86)    | 56 (80.0)        | Reference                             | 0.189           | 0.664       |
| C                        | 12 (17.14)    | 14 (20.0)        | 1.21 (0.47-3.084)                     |                 |             |
| **LTA 252, n (%)**       |               |                  |                                       |                |             |
| AA                       | 15 (42.9)     | 18 (51.4)        | Reference                             |                 |             |
| AG                       | 18 (51.4)     | 16 (45.7)        | 1.35 (0.46-3.95)                      | 0.38            | 0.540       |
| GG                       | 2 (5.7)       | 1 (2.9)          | 2.40 (0.15-74.6)                      | 0.50            | 0.481       |
| A                        | 48 (68.57)    | 52 (74.29)       | Reference                             | 0.56            | 0.454       |
| G                        | 22 (31.43)    | 18 (25.71)       | 0.76 (0.33-1.67)                      |                 |             |

\*Significant difference (p-value < 0.05), allele frequency was calculated according to Hardy-Weinberg equation (HWE), NA – non-applicable

Environmental and genetic factors have been related to susceptibility to disease initiation as well as outcome of disease course [30]. Fifty per cent of risk of developing RA is attributable to genetic factors [31]. Huge progress has been made in the detection of genetic regions that are characterised by structural variation (single nucleotide polymorphisms); more than 30 genetic regions are associated with the incidence of the occurrence of RA [32].

Single nucleotide polymorphisms are considerable and spread throughout the genome. Such disparities are associated with diversity in the population, susceptibility to diseases, and differential response to medical treatment [33].

Polymorphisms situated within the promoter region of TNF-α and the intron 1 polymorphism of LTA, in particular, have been linked with altered levels of circulating TNF-α [34].

In the present study, on investigating the genetic association of TNF-α 308 A/G and RA, the G allele and the wild type (GG) genotype were more frequently represented among RA patients (\(p = 0.040, \ p < 0.001\); respectively). In accordance with our results, Mosaad et al. reported that the G allele and the GG genotype were more frequently represented among RA patients compared to the healthy control group [35]. Moreover, several studies conducted in other parts of the world are in agreement with our results [36, 37].

However, in contrast to the results of the current study, Hussein et al. [33] reported that TNF 308 AA genotype was more frequently prevalent among the patients and associated with RA susceptibility. Similarly, in contrast to our findings, numerous studies showed significantly higher frequency of allele A or genotype AA in RA patients compared to the control group, suggesting that TNF-α 308 A allele is a predisposing factor of RA [38-40]. On the other hand, non-significant association between TNF-α 308 polymorphism and RA susceptibility was observed in other case control studies [41, 42].

Our results regarding TNF-α 308 G/A polymorphism could be explained by the fact that these differences in findings may be attributed to the ethnicity-related genetic constitution in different populations, which is evident from the significant variability in genotype data of TNF-α 308 polymorphism among the healthy subjects of various ethnicities [37]. Hence, the joint gene-environment synergy might be responsible for the considerable differences in the results of polymorphism association studies on RA patients from diversities in ethnicities and/or geographical locations [42]. The difference in results between patient groups in different
In accordance, Al Rayes et al. found no significant difference in the frequency distribution were similar between patients and healthy controls. In accordance, Al Rayes et al. found no significant difference in the frequency distribution were similar between patients and healthy controls [37]. Moreover, Al-Rayes et al. reported that both G allele and GG genotype were similarly distributed in cases and controls [43].

In contrast, Santos et al. [44] reported an association between A allele and RA. These studies showed that TNF-β+252 polymorphism together with HLA-DRB1*0405 has an influence on susceptibility to RA. Similarly, Al-Rayas et al. reported that GG genotype of TNF-β (+252) polymorphism was more frequent in RA patients as compared to the control group [37].

In the current study, analysis of the genetic influence of TNF-α 308 allele and on disease activity represented by TNF-α 308 allele and on disease activity represented by Sharp score was highest among patients carrying the AG genotype compared to patients carrying the AA and GG genotypes (p = 0.007), the heterozygous mutant (AG). The effect of polymorphism on disease severity might result in modification of RA disease activity [43].

In accordance with the results of the current study, Petra et al. and Hussein et al. indicated a positive association between GG genotype and disease activity [33, 45]. On the other hand, Nemec et al. did not detect an association between TNF-α 308 G/A promoter SNP and RA disease activity represented by disease activity score DAS28 scoring system [38].

In the current study, analysis of disease severity represented by Sharp score revealed that regarding TNF-α 308 G/A SNP Sharp score was highest among patients carrying the AG genotype compared to patients carrying the AA and GG genotypes (p = 0.007), the heterozygous mutant (AG). The effect of polymorphism on disease severity...

| Table 3. Comparison of TNF-α and TNF-β polymorphisms distribution according to DAS28 scoring system |
|-------------|----------------|----------------|
| DAS28       | Remission (n = 1) | Non-remission (n = 34) |
| TNF-α 308, n (%) | GG 0 (0.0) | 30 (88.2) |
|             | AG 3 (8.9)   | 2 (5.9)   |
|             | AA 1 (2.9)   | 1 (3.0)   |
|             | A 2 (100) | 5 (7.4) |
|             | G 63 (92.6) | 0 (0.0) |
| TNF-α, n (%) | CC 10 (29.4) | 3 (9.1) |
|             | TT 23 (67.7) | 7 (20.6) |
|             | C 12 (34.4) | 3 (9.1) |
|             | T 56 (82.4) | 4 (12.1) |
| LTA 252, n (%) | GG 0 (0.0) | 2 (5.9) |
|             | AG 17 (50.0) | 13 (38.2) |
|             | AA 15 (44.1) | 7 (20.6) |
|             | G 30 (90.0) | 17 (50.0) |
|             | A 71 (89.0) | 32 (94.1) |

Significant difference (p-value < 0.05), remission group – DAS28 < 2.6, nonremission group includes: low-activity patients (DAS28 ≥ 2.6 to < 3.2), moderate-activity patients (DAS28 ≥ 3.2 to < 5.2), and high-activity group (DAS28 ≥ 5.1), allele frequency was calculated according to HWE.

| Table 4. Comparison between disease severity (represented by Sharp score) and gene polymorphisms in RA patients |
|-------------|----------------|----------------|
| Genotypes | Sharp score (mean ±SD) | ANOVA test (F) | p-value |
| TNF-α 308 | GG 34.13 ±24.24 | 5.79 | 0.007* |
|             | AG 80.00 ±10.00 | 2.42 | 0.105 |
|             | AA 21.00 ±12.72 | 0.691 | 0.37 |
| LTA 252 | GG 37.50 ±24.74 | 32.87 ±24.75 | 0.007* |
|             | GA 41.00 ±28.67 | 41.00 ±28.67 | 0.007* |
|             | AA 32.87 ±24.75 | 32.87 ±24.75 | 0.007* |

Significant difference (p < 0.05), the total number of cases analysed for each gene is 35.
could be explained by differences the rate of TNF-α synthesis. Thus, the production of TNF-α may be associated with TNF-α promoter SNP. In fact, the role of linkage disequilibrium is intense in this area, and it may be difficult to study the role of SNPs separately [38]. Moreover, circu-
luating TNF-α levels might be under a complex regulatory process. Circulating TNF-α level is regulated at different stages: gene transcription, post transcription control of mRNA stability, cleavage of the membrane form to release the soluble form, and the expression of receptors [46].

Our results regarding radiological joint damage were in accordance with Rezaieyazdi et al. [47], who documented the association between heterozygous mutant (AG) genotype with a worse course of the disease. However, in contrast, Nemeč et al. [38] reported an association between severe course of RA and TNF-α 308 GG genotype. This was also reported by Barton et al. [48], who reported that the G allele showed a tendency towards worse radiological outcome at five years, as measured by the presence or absence of erosions, in patients with inflammatory arthritis. On the other hand, Mosaad et al. reported that RA patients with A allele tend to have an increased number of erosions [38]. By contrast, there was no significant association between erosive disease and TNF-α, in Turkish patients and Polish patients [49].

In the current study, no statistically significant association was found between TNF-α 1031 T/C polymorphism and RA disease activity and severity. In agreement with the results of the current study, Barton et al. reported the absence of a significant association between TNF-α 1031 and disease severity in RA patients [48]. Contrary to our findings, Karray et al., pointed out that the C allele and CC genotype were significantly higher in patients in remission of RA activity than in those from the non-remission group [27]. There was no statistically significant association detected as regards disease activity and severity in the Egyptian patients.

Conclusions

The results of the current study suggest that TNF-α 308 G/A SNP can be genetically associated with the susceptibility to RA in our study group and might be involved in both disease activity and severity. Therefore, the TNF molecule might have major genetic and/or functional involvement in the pathogenesis of RA and might also be implicated in disease activity and severity in the Egyptian patients.

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