Journal of Infectious Diseases and Immunity Vol.3(2), pp. 30-35, February 2011
Available online at http://www.academicjournals.org/JIDI
ISSN 2141-2375 ©2011 Academic Journals

Full Length Research Paper

Tumor necrosis factor gene polymorphisms and susceptibility to rheumatoid arthritis in regional Tunisian population

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Accepted January 31, 2011

Rheumatoid arthritis (RA) is a complex multifactorial disease caused by environmental influences and an unknown number of predisposing genes. The present study was undertaken in order to investigate the possible association between RA disease and the TNFαgene promoter polymorphisms α-308A>G, α-1031 T>C and the TNFβ polymorphism+252A>G (LTA252A>G) in the Tunisian population. We compared the distribution of TNF α-308; TNF α-1031 and LTA252 polymorphisms of TNF gene between 108 patients with RA and 226 healthy controls using PCR-RFLP analysis. No deviation from Hardy-Weinberg equilibrium was detected in either set of cases or controls. The LTA252 G allele frequency was significantly increased in RA patients (p=0.01; χ^2 = 6.57 OR = 0.64, 95% CI = 0.45 to 0.91). The frequency of the LTA252GG genotype was significantly higher in RA patients than in healthy controls (p = 0.004; χ^2 = 8.15; OR = 1.99, 95% CI = 1.20 to 3.29). TNF-1031C allele frequency was significantly increased in patients with Remission RA activity (p = 0.0035; χ^2 = 8.52; OR = 6.18; 95% CI = 1.45 to 3.20). These results suggest that the LTA252A>G polymorphism of the TNF gene can be associated with the susceptibility to Rheumatoid Arthritis in our study group and the TNF-1031C allele seems to be involved in remission of RA activity. Therefore, the TNF molecule may have an important genetically and/or functionally implication in the pathogenesis of RA disease in Tunisian population.

Key words: Rheumatoid arthritis, genetic susceptibility, TNF polymorphisms, Polymerase Chain Reaction restriction fragment length-polymorphism.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common human systemic autoimmune diseases. It is characterized by chronic inflammation of the joint, which may lead to structural damage of the cartilage and bone. The worldwide prevalence of RA is about 1% and the precise etiology is still unknown (Singal et al., 1999). RA is a heterogeneous disease with a complex genetic component. Previous studies identified multiple genetic regions that might be associated with RA. Further studies have demonstrated that multiple autoimmune diseases may share common susceptibility genes. Therefore, susceptibility genes associated with other autoimmune diseases may be candidates in the study of gene susceptibility to RA.

RA is associated with cytokine network dysfunction (Choy et al., 2001) particularly TNFα which mediates a broad range of proinflammatory and immunostimulatory activeties (Beutler et al., 1989). Tumor Necrosis Factor

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Abreviations: RA, rheumatoid arthritis; **TNF,** tumor necrosis factor; **SNP,** single nucleotide polymorphism; **PCR-RFLP,** polymerase chain reaction restriction fragment length-polymorphism.

(TNF) is a multifunctional cytokine with potent proinflammatory effects, and is implicated in many inflammatory and autoimmune diseases. Cytokines such as interleukin I (IL1) and TNF are key mediators of the inflammation which induces bone and joint destruction in RA (Buchs et al., 2001; Thomson et al., 2001). The proinflammatory cytokine TNFα is a major factor involved in the RA inflammatory state (Brennan et al., 1992). The pleiotropic biological activities of TNFα are mediated by its binding to TNF receptors (TNFR) Type I and II (Hohmann et al., 1989). The TNFα gene is a member of TNF superfamily located within the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 (Carroll et al., 1987).

Several SNPs have been identified in the TNFα gene (Wilson et al., 1992; Higuchi et al., 1998; Hartl et al., 1998). Single nucleotide polymorphisms (SNPs) within the coding region of cytokine genes tend to be silent while those in promoter region have been associated with differential expression of cytokines as well as severity or susceptibility to various diseases (Bayley et al., 2004). Promoter polymorphisms and microsatellite repeats within TNF and IL-10 genes have been reported in inflammatory and infectious diseases (Bayley et al., 2004; Westendorp et al., 1997). Promoter polymorphisms at TNF have been associated with disease susceptibility, or severity of joint damage and auto antibody production in RA in different populations (Lee et al., 2007; Cvetkovic et al., 2002; Hajeer et al., 1998; Huizinga et al., 2000). However, there is little data on allelic distribution of these SNPs and its association with RA in Tunisian population.

One of the major cytokines that has been intensely investigated is TNFα a multifunctional cytokine T helper 1 (Th1) molecule (Kollias et al., 1999; Bradley et al., 2008). TNFα plays a central role in inflammation; it induces the expression of other proinflammatory molecules, chemotactic cytokines and adhesion factors (Bradley et al., 2008; Hehlgans et al., 2005). *In vivo* and *in vitro* studies have shown that high levels of TNFα lead to exacerbation of the inflammatory response. This, together with its potent immunomodulatory activities, has been suggested to be important to the pathogenesis of diseases such as asthma, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Thomas et al., 2001; Aringer et al., 2003; Brennan et al., 1998). Several TNF polymorphisms have been studied, of which we choose genotype LTA252A>G (lymphotoxin-alpha+252(A/G), TNFα-308A>G and TNFα-1031T>C polymorphisms because of their association with increased TNF secretion and their implication in susceptibility of several auto-immune disease such as systemic lupus erythematosus, insulin-dependent diabetes and inflammatory bowel disease (Lee et al., 2003) and Behçet disease (Kamoun et al., 2007).

Given the known importance of TNF in inflammatory and/or immune functions and the variation in susceptibility to immune disorders in different ethnic groups, we investigated the association of this gene with the development of rheumatoid arthritis in the Tunisian population. So the aim of this study is to investigate the possible association between LTA252A>G, TNFα-308A>G and TNFα-1031T>C polymorphisms and susceptibility to RA in Tunisian patients. Currently, there are no reports on the prevalence of the TNF SNP variant in the RA Tunisian population. To our knowledge, this is the first North African study depicting the prevalence of TNF gene polymorphisms with RA.

MATERIALS AND METHODS

Materials

Cases with RA were assessed by a rheumatologist and fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) classification criteria (Arnett et al., 1987). The healthy control subjects, unrelated Tunisian age- and sex-matched individuals had no family history of autoimmune diseases. All cases and control subjects were informed of the purpose of the study, and their consent was obtained. The ethics committee from each institute approved the study. The clinical features of the patients and controls are summarized in Table 1. A case-control study was performed to assess associations of the LTA252A>G, TNFα-308A>G and TNFα-1031T>Cpolymorphisms with RA. A total of 334 subjects of Tunisian origin, including 108 cases and 226 control subjects, were studied. The RA patients were 21 males and 87 females, recruited from Mongi Slim Hospital, between November 2007 and March 2008.

The genotype frequencies of RA patients and healthy controls conformed to the Hardy-Weinberg equilibrium (p= 0.17 for RA patients and p= 0.74 for healthy controls) for the LTA252A>G polymorphism, (p= 0.57 for RA patients and p= 0.55 for healthy controls) for the TNFα-308A>G polymorphism and (p= 0.16 for RA patients and p= 0.5 for healthy controls) for the TNFα-1031T>C polymorphism.

Genotyping

Genomic DNA was extracted from EDTA anti-coagulated whole blood using conventional salting-out procedure. The three sequences flanking TNFα-1031T>C, TNFα-308A>G and LTA252A>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. Primers (5'-GGGGAGAACAAAAGGATAAG-3') and (5'-CCCCATACTCGACTTTCATA-3') were used to amplify the 270-bp DNA fragment of the TNFα-1031T>C polymorphism. The PCR was followed by an overnight digestion with the restriction enzyme BbsI (C allele, 161 and 109 bp; T allele, 270 bp) (Noguchi et al., 2002). Digested PCR fragments were separated by a 2% agarose gel. Primers (5'-AGGCAATAGGTTTTGAGGGCCAT-3') and (5'TCCTCCCTGCTCCGATTCC G-3') were used to amplify the 107-bp DNA fragment of the TNFα-308A>G polymorphism. After

Table 1. Clinical characteristics of patients with Rheumatoid arthritis in the case-control studies.

Characterstic	RA cases	Control
Number of subjects	108	184
Age (years)	51.5 ± 30	48.3 ± 22
Age at diagnosis (years)	40.8 ± 17	-
Male	21 (19.4)	40 (21.7)
Female (%)	87 (80.5)	144 (78.2)
Menopausal (%)	41 (47.1)	-
No menopausal (%)	46 (52.8)	-
Disease duration (years)		
RA recent istallation ≤ 3years (%)	19 (17.5)	-
RA long term duration > 3years (%)	89 (82.4)	-
RA activity		
Hight DAS28>5.1 (%)	32 (29.6)	-
Moderate 3.2<DAS28<5.1 (%)	53 (49)	-
Low DAS28<3.2 (%)	9 (8.3)	-
Remission DAS28<2.6 (%)	14 (12.9)	-

amplification, the PCR products were digested with the restriction enzyme NcoI (G allele, 87 and 20 bp; A allele, 107 bp) for 6 h at 37°C, and subjected to electrophoresis using a 3.0% agarose gel (Lee et al., 2003).

Primers (5'-CCGTGCTTCGTGCTTTGGACTA-3') and (5'AGAGCTGGTGGGGACATGT CT G-3') were used to amplify the 740-bp DNA fragment to genotype the LTA252A>G polymorphism. The amplified products were digested with NcoI for TNFα-308A>G, and subjected to electrophoresis using a 1.0% agarose gel (Verity et al., 1999).

Statistical analyses

Genotype distributions were compared with those expected for samples from populations in Hardy-Weinberg equilibrium using a χ^2 test (1df). Genotype distributions were compared between the two population groups by contingency χ^2 (2 df) analysis. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated whenever applicable, to test association between genotype and RA. The significance of the OR was calculated by a 2*2 contingency 2 test (1df). Statistical significance was taken as $p<0.05$.

RESULTS

The LTA252A>G, TNFα-308A>G and TNFα-1031T>C polymorphisms were genotyped in 108 RA and 226 control subjects. The genotype frequencies of RA patients and healthy controls were in Hardy-Weinberg equilibrium. The distribution of the LTA252A>G genotype and allele frequencies differed between RA patients and controls. Results of this distribution were presented in Table 2. The LTA252A>G polymorphism is more frequent in RA than in controls groups. The frequency of subjects with the G allele was significantly higher in RA patients than in controls groups when comparing AA genotype with AG+GG genotype (GG+GA: 65.7% vs. 49.1%), p values were obtained ($p = 0.004$; $\chi^2 = 8.15$; OR = 1.99; 95% CI = 1.2 to 3.29). The LTA252A>G genotype frequencies were different between RA patients (AA: AG: GG, 34.2%: 53.7%: 12%) and controls (AA: AG: GG, 50.8%: 37.1%: 8.8%) ($p = 0.017$; $\chi^2 = 8.15$). The distribution of the G allele between RA patients (38.8%) and controls (28.9%), showed also the same results ($p = 0.01$; $\chi^2 = 6.57$; OR = 0.64, 95% CI = 0.45 to 0.91), (Table 2).

According to this study, TNFα-308A>G and TNFα-1031T>C polymorphisms were not significantly different between RA patients and healthy controls (Table 2). TNFα-308G allele frequency was (18.9%) in RA patients and 24.5% in controls ($P = 0.1$; $\chi^2 = 2.59$; OR = 1.39; 95% CI = 0.91 to 2.12). Similarly, TNF-α -1031 C allele frequency was 25.5% in RA patients and (25%) in controls ($P = 0.79$; $\chi^2 = 0.007$; OR = 0.95; 95% CI = 0.65 to 1.4) (Table 2). When analyses were carried out between LTA252A>G, TNFα-308A>G and TNF-α 1031 T>C polymorphisms and RA activity in patients, we found that the distribution of TNF-α 1031 T>C genotype frequencies differed between RA patients with Remission RA activity (DAS28<2.6), and those without this characteristics ($p=0.004$; $\chi^2 =10.71$). Similarly, the distribution of genotype frequencies obtained when comparing TT

Table 2. Comparison of TNF- α and TNF+β polymorphisms between Tunisian RA patients and healthy controls.

SNP	Genotype/Allele	RA case n=108 (%)	Control n= 226 (%)	χ^2	(P value)	OR	(95% CI)
β+252	AA	37 (34.2)	115 (50.8)				
	AG	58 (53.7)	91 (40.26)	8.15	(0.004)	1.99	(1.2-3.29)
	GG	13 (12)	20 (8.8)				
	A	132 (61.1)	321 (71)	6.57	(0.01)	0.64	(0.45-0.91)
	G	84 (38.8)	131 (28.9)				
α -308	AA	70 (64.8)	127 (56.1)				
	AG	35 (32.4)	87(38.4)	2.71	(0.25)		
	GG	3(2.7)	12(5.3)				
	A	175 (81)	341(75.4)	2.59	(0.1)	1.39	(0.91-2.12)
	G	41(18.9)	111(24.5)				
α -1031	TT	62 (57)	129 (57)				
	TC	36 (33.3)	81 (35.8)	0.58	(0.74)		
	CC	10 (9.2)	16 (7)				
	T	160 (74)	339 (75)	0.07	(0.79)	0.95	(0.65-1.40)
	C	56 (25.5)	113 (25)				

p=0.004 for genotype frequencies (obtained when comparing AA genotype and AG+GG genotype); p=0.017 and χ2=8.15 for genotype frequencies and p=0.01 for allele frequencies. HWE RA cases (p=0.17) and HWE controls (p=0.74). HWE: Hardy-Weinberg equilibrium.

comparing TT genotype and (CC+CT) genotype (Table 3) were statistically significantly different. In fact, C/C genotype frequency increases in patients with Remission RA activity compared with those without this characteristic (p = 0.0035; χ^2 = 8.52). The C allele frequency of the same polymorphism was also significantly higher in patients with Remission RA activity (42.8%) than those without this characteristic (23.4%); (p = 0.028; χ^2 = 4.8 OR = 0.41; 95% CI = 0.17 to 0.95) (Table 3).

There were no associations detected when RA patients were stratified according to sex and menopausal females.

DISCUSSION

It has been suggested that multiple genetic factors are involved in the development of RA. In this study, we have investigated the association of RA with three single nucleotide polymorphisms: LTA252A>G, TNFα-308A>G and TNF-α 1031 T>C in a Tunisian patient group by using PCR–RFLP analysis. No differences were found in the distribution of allelic or genotypic frequencies of TNFα-308A>G and TNF-α 1031 T>C polymorphisms between RA cases and healthy controls. In the meantime, a significant difference in the allele and genotype frequencies of LTA252A>G have been reported between RA patients and controls. This difference confirms the association of the LTA252 G allele with RA disease in our study group.

In this report, we confirmed the positive association of LTA252 G allele with RA in Tunisian patients. TNF-α -1031 C allele was associated with patients with Remission RA activity. However, we were unable to confirm the association of TNF-α -308G with this disease. Our results are similar to that of Panoulas et al. (2008) in UK, who found that the LT-A 252GG (LTA252 GG) genotype occurs more frequently among patients with RA than the general population. In RA, this genotype appears to associate with increased likelihood of suffering myocardial infarction (Panoulas et al., 2008). Our results agree to that of Fassmann et al. (2003) who reported that the frequency distribution of the LTA252A>G genotypes showed statistically significant differences between patients with chronic periodontitis, an inflammatory disease, and the reference group in Czech population, whereas the TNF-α -308A>G polymorphism itself showed

Table 3. Distribution of TNF- α 1031T>C polymorphism for RA patients according to the RA activity.

SNP	Genotype frequency (%)			χ^2	Allele frequency (%)		χ^2	OR
-α1031T>C	TT	TC	CC	(P value)	T	C	P value	(95%CI)
Das Remission	3 (21.4)	10(71.4)	1 (7.1)	8.52 (0.0035)	16(57.1)	12 (42.8)	4.8 (0.028)	0.41 (0.17-0.98)
Das no Remission	59(62.7)	26(27.6)	9 (9.5)		144(76.5)	44 (23.4)		

P = 0.0035 for genotype frequencies (obtained when comparing TT genotype and TC+CC genotype); p = 0.004 and χ^2 = 10.7 for genotype frequencies and p = 0.028 for allele frequencies.

no association with RA (Fassmann et al., 2003). Concerning the TNF- α -308A>G polymorphism, our results demonstrate that this polymorphism is not associated with RA susceptibility.

Our results confirms those of Lee et al. (2007) who found in a metaanalysis which included fourteen studies, 10 of Europeans, 3 of Latin Americans, and one Asian. No association between RA and the TNF α -308A allele was found in the overall population (OR 1.005, 95% CI 0.715 to 1.412, p = 0.976). However, stratification by ethnicity indicated that the TNF α -308A allele was significantly associated with RA in Latin Americans (OR 2.004, 95% CI 1.158 tob3.467, p = 0.013). Conversely, there was no association detected for the TNF- α-308A allele with RA patients from the European samples (p = 0.520; OR 0.911; 95% CI 0.684 to 1.212). This meta-analysis demonstrates that the TNF α -308 A>G polymorphism may represent a significant risk factor for RA in Latin Americans, but not in Europeans. Since Tunisia is very close to Europe, that agrees with our results.

Our results were in accordance with those reported in the French population; Mugnier et al., 2003 have found no association of TNF α -308 A with RA. This is in contrast with the study of Silvia et al. (2009) who reports that the TNF α gene is a genetic risk factor for asthma, SLE, and JRA in the pediatric Mexican population. Lack of correlation between these different ethnic groups should not necessarily be regarded as an absence of TNF polymorphisms associations with RA disease but it may reflect heterogeneity in the genetic susceptibility to this disorder. We have also identified for the first time a novel susceptible allele for RA: TNF-α -1031C allele. This allele increased with patients with Remission RA activity (p = 0.0035; χ^2 = 8.52). In Tunisia, Mariam et al. (2006) have found an association of the TNFα-1031 T>C polymorphism with susceptibility to BD (p = 0.015; χ^2 = 5.84 OR = 1.65; 95% CI = 1.08 to 2.54), but not between LTA252A>G or TNFα -308A>G polymorphism and BD disease.

In conclusion, this study suggests the possibility of a genetic element behind the increased risk of RA, in that LTA GG genotype is more frequently in patients with RA. If confirmed in prospective cohorts, this new genetic risk factor could facilitate earlier identification of RA prevention strategies. The TNF gene polymorphism may be an interesting target for novel strategies to prevent RA and early treatment in RA patients. To our knowledge, there are no reports in North Africa describing association or not association of LTA252A>G and TNF-α -1031T>C polymorphisms with RA disease. However, the present work should be regarded as a hypothesis-testing study with its limitations, and further studies using larger samples are needed to pin-point the regulatory polymorphism or haplotype and its effects on the development of certain manifestations in RA.

AKNOWLEDGMENTS

We are particularly grateful to all the patients who participated in these studies and we thank them sincerely. Many thanks for the medical staff of the Mongi Slim Hospital, particularly for A. Amari for her help. This study was supported by the grant from "Ministère de l'enseignement Supérieur et de la Recherche de la Tunisie: DGRST".

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