TNF microsatellites polymorphism is associated with rheumatoid arthritis. Confirming evidence in north-western Colombians

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Abstract

Objective

To examine the contribution of tumor necrosis factor alpha (TNF) microsatellite (a to e) polymorphism to the genetic risk of developing rheumatoid arthritis (RA) in a northwestern Colombian population.

Methods

This was an association study in which 108 RA patients and 222 matched individuals were enrolled. HLA-DRB1 and DQB1 polymorphisms were evaluated to examine for linkage disequilibrium between these loci and TNF microsatellites. Genotyping was performed using denaturing polyacrylamide gels and polymerase chain reaction-sequence techniques.

Results

By unconditional logistic regression analysis, the TNFa6 allele (OR= 2.37, 95%CI 1.07-5.24) and the TNFb4 allele (OR= 3.01, 95%CI 1.07-9.00) were observed to be associated with disease. These associations were independent of HLA-DR and HLA-DQ since linkage disequilibrium between HLA class II and TNF microsatellites was not observed. In addition, patients with the TNFa8 allele had a five times greater risk of developing extra-articular manifestations as compared to patients without this allele (OR = 5.07, 95%CI 1.14 – 22.52), regardless of age and the duration of disease. Haplotype analysis disclosed a protective effect for TNFa7/b7/c1/d4/e3/-308G/-238G.

Conclusion

These results confirm that the TNF locus exerts a primary influence on both susceptibility to and the severity of RA.

Key words

TNF, rheumatoid arthritis, genetic, microsatellites, HLA, Latin America.

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Introduction

Rheumatoid arthritis (RA) is a systemic chronic, inflammatory disease that affects approximately 0.4% of the Latin American population (1, 2). It is characterized by the infiltration of the joints by leucocytes leading to the destruction of cartilage and bones, but other systemic features contribute significantly to morbidity. The clinical presentation and course of RA (i.e., the phenotype) varies among populations. This might be attributed to the effects of genotype (i.e., polymorphic genes) on phenotype as a result of environmental or stochastic effects (1, 3). From a genetic point of view RA is a complex disease, meaning that its inheritance does not follow a single-gene dominant or single-gene recessive Mendelian law, and hence it is polygenic (1, 3).

Among the genetic factors in RA, HLA is a well-known susceptibility locus and accounts for about 30% of the genetic component of disease (1, 3). Associations of some HLA-DR alleles with the susceptibility to (4) and the severity of disease have been documented (5). Refinements of those early studies have led to the now prevailing "shared epitope" hypothesis (6), which states that a sequence encompassing the amino acid residues 67 to 74 in the third hypervariable region of DRβ1 confers susceptibility to RA, rather than a specific allele being uniquely associated with RA (6). This association suggests that *HLA-DRB1* is one of the primary genes involved in the development of disease. Nevertheless, other major histocompatibility complex (MHC) genes may also contribute to the risk of RA.

Analysis of this hypothesis is not an easy endeavour as linkage disequilibrium is extremely strong in the MHC region and certain combinations of alleles are often preferentially present together on the same haplotype. One candidate gene in this region is the one that codes for tumor necrosis factor alpha (*TNF*), which is a pro-inflammatory cytokine that is an important pathogenic mediator in RA (7), and its neutralization has been developed as an effective treatment for disease (8). *TNF* is located within the class III region of the MHC, at 6p21.31 and is highly polymorphic (9). Five microsatellites and numerous single nucleotide polymorphisms (SNPs) in its promoter have been described (10).

Fine mapping studies in which both *TNF* microsatellites and SNPs have been examined are scarce. Martinez *et al.* (11) examined 52 families in a Spanish cohort in which *HLA-DRB1, TNF* SNPs promoter, and *TNF*a and *TNF*b microsatellites were typed. Their data suggested that *TNF*a6 and *TNF*b5 were independent markers of RA susceptibility in their population, pointing to a genetic role of the *TNF* region in the pathogenesis of RA (11).

Taking into account firstly that microsatellite polymorphism may vary across populations, and secondly that replication studies are important to confirm previous associations, we examined the influence of *TNF* microsatellite polymorphism on the risk of developing RA in a northwestern Colombian population.

Patients and methods

Patients and controls

The study included 108 patients with RA (97 women and 11 men). Their main characteristics are shown in Table I. All patients met the American College of Rheumatology classification criteria (12). Demographic and clinical data [including extra-articular manifestations (EAM)] were recorded for the patients, including rheumatoid nodules, lung and heart involvement, Sjögren's syndrome, scleritis and/or episcleritis,

Table I. Clinical characteristics of the RA patients studied.

Characteristic	Case group N = 108
Mean age (yrs.)	48.5 ± 14.2
Mean age at onset (yrs.)	30 ± 10
Mean disease duration (yrs.)	6.5 ± 6
Extraarticular manifestations*	45.4%
Rheumatoid factor positive**	80%
Anti-CCP2 antibody positive***	96%

*Corresponds to at least one of the following: lung or heart involvement, Sjögren's syndrome, vasculitis, nodules, episcleritis, Felty's syndrome, or Raynaud's phenomenon.
**by turbidimetry [(+) > 40 U].
***by ELISA [(+) > 30 IU].

vasculitis, Felty's syndrome, and Raynaud's phenomenon (1).

The control group was composed of 222 individuals who were not related to the patients and who had no history of chronic inflammatory autoimmune or chronic infectious diseases; they were matched to the patients by gender, ethnicity, and socioeconomic status. Their mean age was 47 ± 9 years, and 90% were women.

This research was conducted in compliance with Resolution 008430 of 1993 of the Ministry of Health of Colombia, and was classified as research with minimal risk. The local Ethics Committee approved the study.

Historical and genetic evidence suggests that the population of Antioquia is suitable for studies involving the genetic mapping of complex traits. The state of Antioquia (capital, Medellín) is located in the northwestern part of Colombia between the central and western branches of the Andean Mountains and is inhabited by the Paisa community, a description of which has already been published (13, 14). Anthropological and historical studies describe this population as the most clearly defined in Colombia. Its ethno-historical origin stems most probably from the Spaniards, Jews (Christianized Sephardim or Marranos), and Basques. The admixture between Paisa and African or Amerindian populations has been historically documented as low (15). Several lines of genetic evidence suggest that the Paisa community exhibits the features of a genetically isolated community (16). Firstly, the identity coefficient method has estimated the ancestral ethnic components as being 85% Caucasian and 15% Amerindian (13). The African contribution to the Paisa community was estimated as being not significantly different from 0 (14). Secondly, strong admixture distortions in the gender vectors of racial blending in this community were found, with more than 96% of the chromosomes being of Caucasian origin and most of the mitochondrial component being of Amerindian origin (17).

TNF *microsatellite typing*

Genomic DNA was extracted from 10 ml of an EDTA-anticoagulated blood sample using the standard salting-out technique. Primer sequence and reaction conditions were carried out as previously described (18, 19). Following amplification, *TNFa*, *TNFb*, *TNFc*, *TNFd* and *TNFe* microsatellite alleles were resolved on denaturing polyacrylamide gels composed of 6% acrylamide (19:1 with bis-acrylamide, both from Bio-Rad, Hertfordshire, UK) and 7 M urea (Sigma, Pool, UK). We included positive controls of known genotype and ladder marker (Promega, Bogotá, Colombia) in each reaction.

To confirm the genotype obtained by the denaturing polyacrylamide gels, 10% of the samples were sequenced on an automated DNA sequencer (Abi-Prism 3100 Genetic Analyzer, Applied Biosystems). The primers used for sequencing were the same as those used for the genotyping.

HLA typing

We have previously reported that *HLA-DRB1*0404* is associated with RA in our population (20). In this study, however, *HLA-DRB1* and *DQB1* genotyping were performed in the patient group in order to examine for linkage disequilibrium between these loci and *TNF* microsatellites. HLA class II (DRB1 and DQB1) allele typing was performed by polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) reverse dot blot hybridization using according to IMGT/HLA version 2.6, July 2004 (Amplicor, Hoffmann La Roche, Basel, Switzerland).

Table II. Linkage disequilibrium analysis in RA patients for *TNF* microsatellites, SNPs, *HLA-DRB1,* and *HLA-DQB1*.

	TNFa	TNFb	TNFc	TNFd	TNFe	TNF-308	TNF-238	HLA-DRB1
TNFb	0.00009							
TNFc	0.0041	0.0028						
TNFd	0.0009	0.0001	0.00001					
TNFe	0.0028	0.00009	0.00001	0.00001				
TNF-308	0.8756	0.2535	0.4626	0.0108	0.4250			
TNF-238	0.9998	0.9999	0.9999	0.9999	0.9999	0.9999		
HLA-DRB1	0.8961	0.2259	0.6615	0.8867	0.3348	0.8096	0.9999	
HLA-DQB1	0.1368	0.0391	0.3126	0.9213	0.0887	0.6343	0.9999	0.4814

Results correspond to *p*-values, and were obtained by the likelihood-ratio test whose empirical distribution is achieved by a permutation procedure (45). Significant differences indicate linkage disequilibrium.

Statistical and genetic analysis

Data were analyzed using the Statistical Analysis Systems software, version 9 (SAS Institute Inc., Cary, NC). Allele frequencies were estimated by the gene-counting method, and exact tests were performed to identify any departure from the Hardy-Weinberg proportions and any differences in allele frequencies between cases and controls. Haplotypic frequencies were estimated by maximum-likelihood using PROC HAPLOTYPE from the SAS/Genetics program. Population heterogeneity among the case and control sets was examined by means of Wright's F statistics according to the non-biased method of Weir and Cockerham (21).

Linkage disequilibrium analysis was carried out with the Arlequin software program, version 2.000. Unconditional logistic regression was performed to estimate the odd ratios (ORs) and 95% confidence intervals of the different alleles among patients and controls, using the most frequent allele as the reference group. All logistic models were adjusted by age and gender. A nominal p-value < 0.05 was considered statistically significant.

Results

Genetic structure analysis

Hardy-Weinberg equilibrium was seen in all samples and strong linkage disequilibrium among all microsatellites was observed in both the cases and controls (Table II). Population stratification was not seen in our sample since the Fst subdivision coefficient was not

significantly different from 0. Therefore, patients and controls came from a similar genetic background.

TNF *microsatellite polymorphism and disease*

Fourteen *TNFa, 7 TNFb, 2 TNFc, 7 TNFd* and *3 TNFe* alleles were observed in our population. Determination of allele frequencies showed that the most frequent at each microsatellite were *TNF* a7 (23%), b7 (41.4%), c1 (76%), d4 (51.1%) and e3 (85.4%).

By unconditional logistic regression we observed statistical differences for the *TNF*a6 allele between cases and controls (9.7% *vs* 5.5%, OR = 2.37, 95%CI 1.07–5.24 $p = 0.03$) and for *TNF*b4 (5.1% *vs* 2.3%, OR = 3.10, 95%CI 1.07–9.00 $p = 0.04$) (Table III). After adjustment for age and disease duration, intra-group analysis showed that the *TNF*a8 microsatellite allele was more frequent in patients with EAM (13.8%) (recorded in dichotomous form as present or absent, and corresponding to at least one of the EAM defined in the Patients and methods section) compared to those without EAM (5.3%) (OR = 5.07, 95%CI 1.14–22.52). Analysis of haplotypic frequencies showed that the haplotype *TNF*a7/b7/c1/d4/e3/-308G/-238G was less frequent in cases compared to controls (8.9% *vs* 4.4, $p = 0.04$) (Table IV).

Linkage disequilibrium analysis

To test for independent associations, *TNF* microsatellites and *HLA-DRB1* status were examined in all patients. In this analysis we also included the previously reported results of *TNF*-238 and -308 SNPs (22). There was no linkage disequilibrium between the *HLA-DRB1* locus and any of the *TNF*microsatellites. Nor there was any linkage disequilibrium between *TNF*microsatellites and SNPs (Table II).

Table III. Frequencies, odds ratios and *p*-values for the different TNF polymorphisms.

Polymorphism		Cases (%)	Controls (%)	Odds ratio (95% CI)	*p*-value
TNFa					
	7	19.4	22.9	1.00 (reference)	
	2	8.8	5.5	2.10 (0.95, 4.68)	0.07
	3	10.6	16.3	0.83 (0.40, 1.72)	0.62
	5	4.2	5.0	1.04 (0.37, 2.91)	0.93
	6	**9.7**	**5.5**	**2.37 (1.07, 5.24)**	**0.03**
	8	8.8	11.9	0.77 (0.36, 1.63)	0.49
	11	15.3	18.1	0.88 (0.46, 1.69)	0.70
	12	9.7	9.2	1.05 (0.50, 2.24)	0.89
TNFb					
	7	34.7	41.4	1.00 (reference)	
	3	8.3	10.7	0.99 (0.47, 2.11)	0.98
	4	**5.1**	**2.3**	**3.10 (1.07, 9.00)**	**0.04**
	5	8.3	5.1	1.43 (0.61, 3.32)	0.41
	6	42.6	39.8	1.14 (0.73, 1.79)	0.55
TNFc					
	1	78.7	75.9	1.00 (reference)	
	2	21.3	24.1	1.03 (0.63, 1.69)	0.89
TNFd					
	4	51.4	51.1	1.00 (reference)	
	2	6.9	3.2	2.01 (0.76, 5.31)	0.16
	5	19	25.3	0.82 (0.49, 1.40)	0.47
	6	20.4	16.9	1.44 (0.86, 2.39)	0.16
TNFe					
	3	83.2	85.4	1.00 (reference)	
	1	11.2	11.7	1.19 (0.64, 2.24)	0.59
	2	5.6	2.9	2.74 (0.98, 7.61)	0.06

Allele most frequent registered in controls was used as the reference allele. Odds ratio were adjusted by gender and age. Only alleles with a frequency at least 5% were analyzed (by logistic regression).

Discussion

The results of this study indicate that *TNF*a6 and b4 are associated with RA in our population, and that this association is *HLA-DRB1* and *–DQB1* independent since linkage disequilibrium between the TNF and HLA loci was not observed. These data are in agreement with previous reports showing an HLA-independent association between *TNF*a6 and RA both in Europeans (i.e., English and Spaniards) (11, 23) and Latin-Americans (i.e., Peruvians) (24). Other reports have shown associations with the *TNF*a2 and *TNF*a11 alleles (25-28). Although clinical and genetic heterogeneity might explain these diverse results, there is agreement for a genetic role of the *TNF* locus on susceptibility to RA.

Logistic analysis showed that patients with the *TNF*a8 allele had a five times higher risk of developing EAM compared to patients without this allele. In a previous report, Mattey *et al*. (29) also showed that the *TNF*a8 allele was associated with EAM in patients from northwest Spain (29). Our population exhibited an increased frequency of EAM compared to the Spanish cohort (45.4% *vs* 19.3%; OR = 3.5, 95%CI 2.04–5.87 $p = 0.0001$). When we compared age at onset (30 ± 10 *vs* 49.6 ± 11.8 years, $p = 0.0001$) and disease duration (6.5 ± 6 *vs* 13.2 ± 7.9 years, $p = 0.0001$) between Colombian and Spanish patients, we also found statistically significant differences. These results indicate that our RA cohort was characterized by a more aggressive disease pattern than patients from northwest Spain (29). Besides age at onset and duration of disease, other factors such as access to medical care could also account for the differences in the course of RA observed between Colombians and Spaniards. In fact, only 12% of our RA patients attended a rheumatology clinic within the first year of disease (1). Despite this, the *TNF*a8 allele consistently influenced the presence of EAM in both populations.

Family studies and segregation analysis have shown that proband sex is an important risk factor for developing RA (30, 31). Most of our patients were females, in whom the *TNF*a6 allele has been reported to be more common than in males (32), but the low number of males included in our study precludes an accurate analysis in this respect. Others have reported that the *TNF*a6

Table IV. Haplotypic frequencies in the cases and controls.

TNFa	TNFb	TNFc	TNFd	TNFe	TNF-308	TNF-238	Controls (2N = 332)	Cases (2N = 202)	*p*-value
11	6	1	4	3	G	G	0.160	0.134	0.41
12	6	1	4	3	G	G	0.055	0.084	0.20
7	7	1	4	3	G	G	0.089	0.044	0.04
7	7	1	6	3	G	G	0.097	0.061	0.14
8	6	1	4	3	G	G	0.068	0.049	0.38

Global *p*-value = 0.99. Only haplotypes with frequencies ≥ 5% in either controls or cases are shown.

allele belongs to an extended *HLA-DRB1*0401/HLA-B44* haplotype, predominantly found in female but not in male patients (32). Thus, the influence of gender could also explain the association of *TNF*a6 with RA. However, the frequency of the *HLA-DRB1*0401-TNF*a6 haplotype in our population was low (2.8%). Concerning *HLA-DRB1*, the **0404* allele was associated with RA in our population, as it has been reported to be in most Latin American populations (3). The *HLA-DRB1*0401* allele occurred at a very low frequency among our RA patients and was not observed in the healthy population (20).

Studies have shown that the *TNF* –308A allele is associated with RA, both as a susceptibility and as a severity factor (22, 33-37). In our population, we previously reported that this allele is a predisposing factor for RA (22). When data from our study on *TNF* -308 and -238 SNPs were included in the present analysis, no linkage disequilibrium between microsatellites and these SNPs was found. As the gametic phase was unknown, linkage disequilibrium between pairs of loci was tested by the likelihood-ratio test whose empirical distribution was obtained by a permutation procedure (38). Positive selection among these different and nearly genetic factors for a common disease may explain the lack of expected linkage disequilibrium (39). The HLA-independent association between the *TNF*a6 and -308A alleles and RA might also be a consequence of molecular evolution. Evidence exists to suggest that some particular genes belonging to the MHC class III region are more ancestral than MHC class II and I due to the physiological role that their proteins play in host defence. In fact, MHC class III region products (i.e. TNF-α) form a part of innate immunity whereas gene products belonging to class II form a part of acquired immunity. This is supported by findings showing that MHC classes I and II were translocated recently (40).

Results from association studies may be influenced by several factors, including population stratification. Our control population was matched to the patients by age, sex, origin, ethnicity and socioeconomic status, with environmental conditions that were similar to those of the patients. The Fst was not different from 0, indicating that our sample was not stratified and that both the cases and controls had a similar genetic background (21).

Replication of promising initial results is a necessary step in order to assess the contribution of genes to human disease. With this goal in mind we conducted the present study, which confirms earlier results (11, 24). It is noteworthy that even though our p-value was 0.03 by logistic regression, when combined with the p-value (0.004) from the earlier study by Martinez *et al*. (11) a total p-value of 0.00012 was obtained, which suggests reproducibility (41). In other words, when the results of the present study are interpreted in the context of earlier results, we have suggestive evidence that the association between *TNF* microsatellites and RA is true.

The *TNF*b4 allele was also found to be associated with RA in our population. This allele, as part of the *TNF*a11b4 haplotype, has been associated with RA in a Japanese cohort (28) and with high sensitivity to infliximab therapy (42). This haplotype was scarce (2%) in our population. *TNF*b4 could have a *TNF*a11-independent effect on RA. The *TNF*b6 allele frequency in our population was unexpectedly higher than in Europeans (18), but similar to that reported in Taiwanese (43). We also found interesting similarities in allele frequencies between Asians and Colombians in other polymorphic genes such as *PTPN22* (44).

Finally, haplotype *TNF*a7/b7/c1/d4/e3/-308G/-238G was observed to be a protective factor for RA in Colombians. Its relative frequency in the cases was about half its relative frequency in controls.

In conclusion, our results confirm the significant influence that the *TNF* locus exerts on both the susceptibility to and the severity of RA.

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