TNF+489 polymorphism does not contribute to susceptibility to rheumatoid arthritis

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ABSTRACT

Objectives. *To determine if a tumour necrosis factor (TNF +489) polymorphism is associated with susceptibility to rheumatoid arthritis (RA).*

Methods. *Two European populations were studied: 217 controls and 238 patients from the north of England and 145 controls and 179 patients from Spain. HLA-DRB1 and TNF+489 markers were typed using polymerase chain reaction based methods.*

Results. *Strong associations were demonstrated with shared epitope (SE) encoding HLA-DRB1 alleles in the English (OR= 2.9 [2.2-3.9]) and Spanish (OR= 2.3 [1.6-3.3]) populations, however no association was found with TNF+489 alleles. Furthermore carriage of TNF+489A was not associated with the presence of radiological erosions, rheumatoid nodules or rheumatoid factor.*

Conclusion. *The role of the TNF locus in the genetic background of RA is unclear, however, our data does not support the previous reported association of the TNF+489A allele with RA susceptibility or severity.*

Introduction

Susceptibility to rheumatoid arthritis (RA) is multifactorial with both environmental and genetic factors being important with the latter contributing around 30% of total susceptibility (1). The identification of susceptibility determinants in a genetically complex disease such as RA is difficult, however it is clear that a major genetic contribution, perhaps as much as one third of the total, lies within the Major Histocompatibility Complex (MHC). Alleles of HLA-DRB1 which encode a similar sequence have been most convincingly implicated (2). This sequence, termed the shared epitope (SE), encodes amino acids 69-73 of the mature peptide and is important in determining the repertoire of peptides which are bound by the class II molecules and presented to T cells (3). The linked DQ locus has also been implicated but it is not clear if this association is primary or secondary to linkage disequilibrium with SE alleles (4).

The class III region of the MHC lies telomeric of the DR and DQ loci. This region is gene dense with approximately 1 gene per 15 kilobases (kb) of DNA and encodes many immunologically important proteins including several complement proteins, the 70 kd heat shock protein, and at the telomeric end, approximately 900 kb telomeric of DRB1, the tumour necrosis factor (TNF) cluster containing the genes for TNF, lymphotoxin α and β (5). In view of the gene location and biological activities of TNF there has been intense effort to determine if polymorphism within the TNF gene contributes to the genetic background of RA.

A number of single nucleotide polymorphisms (SNPs) have been identified within the TNF promoter region at -1031, -862, -856, -574, -376, -308 and -238. Some of these have been associated with altered gene function by some, but not all groups (6,7). The TNF -308 variant does not appear to be associated with susceptibility to rheumatoid arthritis (8), however, carriage of the TNF-238A allele has been associated with protection from severe forms of RA (9). Two SNPs have also been identified within intron 1 of TNF: +489 (G to A Transition) and +691 (G deletion) and disease association have recently emerged (10, 11). Carriage of TNF +489A has been associated with both decreased risk of developing RA and a 3.9-fold reduced risk of developing erosive RA in a Dutch study. Furthermore, this association was independent of SE alleles (11). We have recently reported the association of TNF+ 489A with DRB1*11 (12). We therefore undertook to re-examine the role of the TNF+489 polymorphism in rheumatoid arthritis by performing case control studies in two European populations.

Materials and methods

Patients and controls

Populations from two regions of Europe were used; from Galacia, Spain (Controls, n = 145; patients, n = 179) and Sheffield, England (controls, n = 217; patients, n = 238). All the Spanish RA patients were recruited from the Division of Rheumatology of the Hospital Xeral-Calde (Lugo, Spain). This

hospital is the only referral center for a mixed rural (60%) and urban population of almost 250,000 people living in the area surrounding Lugo city, in the middle of the province of Lugo, in Galicia, Northwestern Spain. This long time neglected area of the inner of Galicia has important peculiarities previously reported elsewhere (13) (14). Of note, it is not a coastal area and it has been relatively isolated from the rest of Galicia for many centuries due to geographical problems. This population seems to be Caucasoid of Celtic origin (15), is relatively static, and no major migration has occurred in the area during the last two decades.

The English cohorts consisted of random blood donor and patients attending the Rheumatology outpatient clinic in the Royal Hallamshire Hospital, Sheffield. All patients satisfied the 1987 revised American College of Rheumatology criteria for the diagnosis of RA. Controls were ethnically matched, healthy individuals. Measures of clinical phenotype including the presence of radiological erosions, rheumatoid nodules and rheumatoid factor were available in the Spanish population and have been reported previously (16).

HLA typing

DNA was extracted from EDTA anticoagulated blood by standard methods. HLA-DRB1 was typed in the Spanish population using a semi-automated commercially available reverse dot blot method, INNO-LIPA (Abbott laboratories, England) and, in the English population, by polymerase chain reaction sequence specific primer reactions (PCR-SSP) amplification followed by agarose gel electrophoresis (17).

TNF microsatellites

TNF microsatellites were typed in the Spanish population and the results have been reported previously (16).

TNF +489 typing

Typing was performed by 5'-nuclease PCR using the PE Biosystems Taqman allelic discrimination system: 20 ng of genomic DNA was used as template for amplification using oligonucleotides and final concentrations (nM) as follows: Forward: 5'-ATA CAC ACT TAG TGA GCA CCT TCC AT-3' (300), Reverse: 5'- GGT GAA AGA TGT GCG CTG ATA G-3' (300), Allele 1 Probe (FAM label) 5'-CGT CTT TCT CCA CGT TTT TTT CTC TCC AT-3' (50) and Allele 2 Probe (TET label) 5'- CGT CTT TCT CCA TGT TTT TTT CTC TCC ATC-3' (100) in a total volume of 25 µl containing 1x TaqMan Universal Master Mix™ and made up with sterile water. The cycling conditions were as follows: an initial cycle of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 62°C for 1 minute, with a final holding cycle of 15°C. Genotype was ascertained using an ABI Prism® 7200 Sequence Detection System machine.

Statistical analysis

The strength of association between RA and SE or TNF +489 alleles was assessed using odds ratio (OR) and 95% confidence intervals (CI). The associations between TNF+489 and TNF microsatellites were calculated using Fishers Exact Test. The SE alleles were defined as DRB1* 01, *0401, *0404, *0405, *0408, *10.

Results

Strong associations with SE alleles of HLA-DRB1 were seen in both populations; England OR = 2.9 (2.2-3.9), Spain OR = 2.3 (1.6-3.3). Carriage of TNF+489 alleles did not differ between controls and patients in either population (Table I). The frequency of the TNF+489A allele was identical (0.08) in the English controls and patients compared with 0.13 and 0.09 in the Spanish controls and patients respectively.

Carriage of allele 3 of the TNFβ microsatellite had previously been reported to be an independent risk factor for RA in this Spanish population (16). We therefore examined the association of TNF+489 alleles with TNF microsatellite alleles. Significant associations were found in the Spanish control population between TNF+489A and TNFa10 ($\chi 2 = 28$, $p < 0.0001$) and TNFd4 ($\chi 2 = 4.8$, $p = 0.03$). No association was found between TNF+489A and TNFb3 ($\chi 2 = 2.2$, $p = 0.14$) in the Spanish patient cohort.

Finally we examined if this TNF marker was associated with clinical features of RA. Although the numbers of patients in some groups were relatively small, we did not find any evidence of a genetic association of this marker with the presence of radiological erosions, subcutaneous nodules or rheumatoid factor in the Spanish population (Table II).

Discussion

Association studies are a powerful method of detecting weak genetic effects in polygenic diseases however a major problem inherent to this type of study is population stratification, it is therefore essential that genetic associations are confirmed in separation populations. In this study we were not able to detect an association of TNF+489A with the development of RA or with the presence of clinical markers of severe disease. The original study from the Netherlands had reported a reduction in carriage of TNF+489A from 12% in controls to 6.8% and 7.1% in the patient populations. In addition carriage of TNF+489A was associated

Table I. Carriage rates of TNF+489 alleles in two European control and RA populations.

	TNF+489 G,G	TNF+489 G,A or A,A	Odds Ratio
England			
Controls	181	35	
RA	201	37	1.1 (0.6-1.7)
Spain			
Controls	76	26	
RA	112	24	1.6 (0.85-3.0)

Table II. Carriage of the TNF+489A allele is not associated with the presence of radiological erosions, rheumatoid nodules or rheumatoid factor in Spanish patients.

	Erosions		Nodules		Rheumatoid factor	
TNF+489	-	+	-	+	-	+
G,G	30	69	99	13	28	84
A,G	6	15*	21	3†	5	19¶

*OR = 0.9 (0.3-2.6); †OR = 1.1 (0.3-4.2); ¶ OR = 1.3 (0.4-3.7)

with a 3.9-fold decrease in risk of developing erosive disease compared with that of patients who were homozygous for the common allele (TNF+489G). These associations were found to be independent of SE alleles suggesting that this marker might be an independent risk factor for the development of rheumatoid arthritis.

However our study, involving two large populations from different regions within Europe, did not find evidence implicating carriage of TNF+489 in the genetic background of RA. With the combined data set of 692 observations we should be able to detect a difference in frequency of the TNF+489GA or +489AA genotypes of 0.07 compared with the observed value of 0.18 (these values chosen to match the 40% reduction reported previously (11), with 80% power at the 5% level of significance. No association between TNF +489 and SE +ve alleles was found. We have previously reported genetic association of TNF +489 A with HLA-DRB1*11 (12). The frequency of the TNF +489 allele seems to vary across Europe from 0.12 in Italy, Spain and the Netherlands to 0.08 in England. The Spanish RA cohort studied here has been previously reported to show independent associations with DRB1 and TNF alleles. This may be due to linkage disequilibrium with a nearby variant, however, our results are consistent with the TNF+489 variant being neutral in RA.

Despite these results, there is increasing evidence that a second susceptibility locus for RA lies in the distal class III or class I regions of the MHC. Two family based studies using the transmission disequilibrium test have examined if TNF microsatellite alleles are more frequently transmitted to affected offspring than expected. A study of 52 Spanish RA families found preferential transmission of TNF haplotypes to affected offspring which was independent from transmission of SE alleles (18), these results were supported by a study of 50 multiplex Irish RA families (19). Recently several case control studies, involving typing of a large number of polymorphic markers spanning the MHC, have also shown evidence for a second susceptibility locus in the region telomeric of DRB1 (20, 21) with some evidence implicating a 70 kb region between the TNF and HLA-B loci (22). This region is known to contain four expressed genes; NFKBIL1 (IκBL), ATP6G, BAT1 and MICB, which therefore represent attractive candidate genes. However, it must be stressed that not all studies have confirmed these associations to be independent from DRB1 (23).

Further evidence for the role of a second genetic susceptibility locus within the MHC has also been reported in other autoimmune diseases. Two reports have described associations of carriage of TNF-308A with increased risk of developing systemic lupus erythematosus, independent from class II and complement cluster alleles (24, 25). Recently other autoimmune diseases including scleroderma (26), giant cell arteritis (27), coeliac disease (28) and myasthenia gravis (29) have also been reported to show secondary independent genetic associations with the telomeric class III/centromeric class I regions. Susceptibility genes for autoimmune diseases have been shown to cluster non-randomly across the genome, suggesting that distinct autoimmune diseases may be controlled by common sets of susceptibility genes (30). The above reports are consistent with the idea that a second MHC susceptibility locus for a range of autoimmune diseases may lie telomeric of the class II region.

Although we were not able to replicate the findings reported by van Krugten *et al*. implicating the TNF+489A allele with RA susceptibility, there is an increasing body of evidence implicating the telomeric region of the MHC in the genetic background of RA. The identification of this locus should be greatly facilitated by our increasing knowledge of the gene content of the MHC and the generation of a high density genetic map of this region.

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