

# A tumour necrosis factor $\alpha$ polymorphism is not associated with rheumatoid arthritis

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## Abstract

**Objective**—To determine whether a polymorphism within the tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) gene is associated with susceptibility to, or severity of, rheumatoid arthritis (RA).

**Methods**—Consecutive patients with recent onset RA were enrolled in a prospective trial. DNA was collected, disease activity was measured at presentation, and radiographic progression at three years was assessed. Typing of TNF $\alpha$  was by polymerase chain reaction and single stranded conformation polymorphism analysis.

**Results**—No association of TNF $\alpha$  alleles and susceptibility to, or severity of, RA was demonstrated.

**Conclusions**—These results indicate that this TNF $\alpha$  polymorphism does not play a part in the genetic background of RA.

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The aetiology of rheumatoid arthritis (RA) is believed to be both genetic and environmental, with genetic factors contributing 15-30% of the overall risk.<sup>1</sup> A large part of the inherited component is associated with the major histocompatibility complex (MHC), which is a 4 megabase (Mb) stretch of DNA on the short arm of chromosome 6.<sup>2</sup> Several alleles of the HLA-DRB1 locus, including the serotypes HLA-DR1, -DR4 and -DR10, have been associated with RA. These susceptibility alleles share a common peptide sequence in the region of the protein that binds antigen and presents them to T cells;<sup>3</sup> this sequence is commonly referred to as the shared epitope. These shared epitope encoding alleles are not only involved in susceptibility, but are also associated with disease severity.<sup>4</sup> Furthermore, homozygosity for the susceptibility allele DRB\*0401 is associated with major organ involvement.<sup>5</sup>

The gene for tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) lies within the class III region of the MHC, approximately 1 Mb telomeric of the DR locus. The importance of TNF $\alpha$  as an inflammatory mediator in RA has been demonstrated by the presence of high levels in rheumatoid synovial fluid,<sup>6</sup> the development (in transgenic mice overexpressing TNF $\alpha$ ) of a symmetrical polyarthritis similar to RA,<sup>7</sup> and the protective effects of TNF $\alpha$  antibodies in RA.<sup>8</sup> In view of the chromosomal localisation, the biological effects, and its implication in chronic inflammation, there has been

speculation that polymorphism in this gene may be implicated in the pathogenesis or clinical manifestations of RA.

Increased production of TNF $\alpha$  has been demonstrated in DR3 and DR4 positive individuals compared with DR2 positive individuals,<sup>9</sup> suggesting that production is under genetic control. Transcription of the TNF $\alpha$  gene is regulated by the promoter region, which consists of an 1100 base pair (bp) stretch of DNA lying between the TNF $\alpha$  and lymphotoxin- $\alpha$  genes. We have recently described the first known polymorphism within the human TNF $\alpha$  gene.<sup>10</sup> This lies within the promoter region and involves the substitution of guanine by adenosine in the uncommon (TNF2) allele. In this study, we tested the hypothesis that this polymorphism influences the susceptibility to, or progression of, recent onset rheumatoid arthritis.

## Patients and methods

### PATIENTS AND CONTROLS

Patients were seen in the department of Rheumatology of the University Hospital, Nijmegen, Netherlands. One hundred and forty seven unrelated adult white patients who met the American College of Rheumatology criteria for RA with a disease duration less than one year at entry, were included.<sup>11</sup> At entry, the median age of the RA patients was 55 years, 62% were women, and 80% were IgM rheumatoid factor positive (enzyme linked immunosorbent assay:  $\geq 10$  IU/ml). The control group consisted of 135 healthy unrelated white blood donors from the same geographical region.

Disease progression was followed prospectively and in 115 patients, radiographs were available at entry and after a three year follow up. In this group median age was 55 years, 64% were women and 75% were IgM rheumatoid factor positive. The median number of swollen joints was 18 and of painful joints, 16. Disease activity score (DAS) (a sensitive index for disease activity<sup>12</sup>) was calculated using the Ritchie articular index, erythrocyte sedimentation rate (ESR), number of swollen joints and general health (visual analogue scale of 10 cm: 0 = best possible, 10 = worst possible).

### RADIOGRAPHIC SCORING

Radiographs were scored blind by one observer (NdV) as described previously.<sup>13</sup> Briefly, at each selected joint interface the erosions were

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Table 1 Comparison of TNF $\alpha$  genotypes in all patients, shared epitope (SE) positive and negative subgroups, and controls

	TNF2-ve	TNF2+ve	OR
All			
Controls	90	45	
Patients	95	52	1.1 (0.7-1.8)
SE positive			
Controls	43	17	
Patients	74	27	0.9 (0.5-1.9)
SE negative			
Controls	47	28	
Patients	21	25	2 (0.95-4.2)

OR = Odds ratio.

graded 0-5 in the hands and 0-10 in the feet, and joint space narrowing was graded 0-4 (1 = focal; 2 = less than 50%; 3 = more than 50% or subluxation; 4 = ankylosis). Erosion and narrowing progression scores were calculated by subtracting the initial score from the score after three years follow up.

#### DNA PREPARATION

DNA was isolated from anticoagulated peripheral blood mononuclear cells using standard methods.

#### TNF $\alpha$ GENOTYPING

A 107 bp stretch of the TNF $\alpha$  promoter was amplified (sense primer 5'-AGGCAATAGG-TTTTGAGGGCCAT-3'; antisense primer 5'-TCCTCCCTGCTCCGATTCCG-3') by polymerase chain reaction (PCR) using 1.25 U *Taq* DNA polymerase, 0.2  $\mu$ mol/l of each deoxyribonucleotide, 2.5 mmol/l MgCl<sub>2</sub>, 50 mmol/l KCl, 10 mmol/l Tris/HCl pH 9.0, 1% Triton X-100 and 100-500 ng DNA template in 50  $\mu$ l reaction. Cycles were: 1  $\times$  94°C for three minutes; 60°C for one minute; 72°C for one minute, 35  $\times$  94°C for one minute; 60°C for one minute; 72°C for one minute, and a final cycle with an elongation period of five minutes. The genotype was determined using single stranded conformational polymorphism analysis as previously described.<sup>14</sup> PCR product (50  $\mu$ l) was denatured with 2.5  $\mu$ l 1 mol/l NaOH, heated to 42°C for five minutes, and 6  $\mu$ l 100% formamide added. The samples were loaded on a 9% polyacrylamide gel and electrophoresed for 16 hours at 4 V/cm and 4°C. The DNA was stained with ethidium bromide and visualised under ultraviolet light.

Table 2 Clinical and radiological characteristics in TNF2 positive and negative patients in the prospective study group

Characteristic*	TNF2-ve (n = 80)	TNF2+ve (n = 35)	Mean diff	95% CI
At entry				
Swollen joints (n)	17.63 (7.95)	16.60 (7.21)	1.03	-2.07 to 4.13
Painful joints (n)	16.44 (10.11)	14.51 (10.06)	1.93	-2.12 to 5.98
DAS	4.36 (1.10)	4.38 (1.18)	-0.02	-0.47 to 0.43
Erosion score	1.30 (1.07)	1.06 (1.25)	0.24	-0.21 to 0.69
Joint narrowing score	0.93 (1.10)	0.69 (1.05)	0.24	-0.20 to 0.68
Radiographic progression after three years				
Erosion score	3.15 (1.92)	3.81 (2.25)	-0.66	-1.47 to 0.15
Joint space narrowing	3.18 (2.26)	3.82 (2.59)	-0.64	-1.59 to 0.31

Values are mean (SD) or absolute numbers. \*For the purpose of statistical testing, radiographic scores were transformed by taking the square root to approximate a normal distribution. DAS = Disease activity score, a composite index of disease activity (see Patients and Methods). No significant differences.

#### HLA-DRB1 TYPING

For HLA-DRB1 typing a polymerase chain reaction amplification of exon 2 was performed. The resulting products were screened with sequence specific oligonucleotide (SSO) probes. In every screening procedure, SSO probes were end labelled using T4 polynucleotide kinase, hybridised to the dotblotted PCR product, washed, and detected by autoradiography. PCR amplified DNA of homozygous typing cells were processed together with the samples to ascertain specific hybridisation.

Shared epitope positive alleles comprised the following: DRB1\*0101, \*0102, \*0401, \*0404, \*0405, \*0408, \*0409, \*0410, \*1402, \*1406, \*1409, and \*1001.

#### STATISTICAL METHODS

Odds ratios (OR) with 95% confidence limits are shown. The significance of association between clinical parameters and genotypes was assessed using a *t* test. The association of the TNF $\alpha$  polymorphism with radiographic progression was also tested in an analysis of covariance, correcting for the effects of the following prognostic variables: age (55 and younger *v* 56 and older), gender, presence of the shared epitope, rheumatoid factor positivity, number of swollen joints, ESR, and radiographic score at entry. In these analyses, ESR and radiographic scores were transformed to approximate a normal distribution by taking the square root.

We enrolled in the study a sufficient number of patients to be able to detect a doubling of allele frequency (odds ratio = 2.5) at  $\alpha = 0.05$  (type I error) and  $\beta \leq 0.1$  (type II error,  $\geq 90\%$  power).

#### Results

There was no significant difference in TNF $\alpha$  genotypes between controls and patients (table 1). Thus our data do not support the hypothesis that the polymorphism studied here is responsible for the MHC association with susceptibility to RA. Carriage of the shared epitope was strongly associated with disease susceptibility: OR = 2.75 (1.5-4.1).

To test if the TNF $\alpha$  polymorphism studied might be an additional risk factor for RA, supplementing that of the shared epitope, a 2  $\times$  2 table analysis was performed. Patients and controls were divided into groups based on the presence or absence of the allele TNF2 and the shared epitope. In this analysis no significant effect of the TNF $\alpha$  polymorphism could be demonstrated (table 1).

To establish if there was an association with disease severity, rather than with susceptibility, we examined the relationship of genotypes with clinical parameters at presentation. There was no significant association with any of these (table 2). In addition, there was no association with radiographic progression after three years, as assessed by joint erosion and joint space narrowing, even after correction for the prognostic variables.

## Discussion

Our results show that there is no significant association of TNF2 with RA. The possibility that this polymorphism may be a marker for a secondary, though weaker, disease association locus is not supported by our finding of no difference in the carriage rates of TNF2 in shared epitope negative patients and controls. In addition, although TNF2 homozygosity was increased in shared epitope negative patients compared with controls, this was not significant because of the small numbers (four of 46 and three of 75, respectively).

TNF $\alpha$  is a potent immunomodulator and has been shown to stimulate cartilage and bony resorption. Because DR3 has been shown to be associated with high production and is in strong linkage disequilibrium with TNF2, we speculated that there may be an association of this allele with severity of disease. Again, we found no evidence that this was the case, as assessed by several clinical parameters at presentation or by radiographic progression at three years.

The polymorphic site is 308 bases upstream of the start of the first exon of TNF $\alpha$ , within an 1100 bp stretch of DNA between the TNF $\alpha$  and lymphotoxin- $\alpha$  genes. This region has been shown to control the rate of transcription of TNF $\alpha$ .<sup>15</sup> The polymorphism is contained within a consensus sequence for the transcription factor AP-2. We have evidence from reporter gene constructs, containing the two allelic promoters, which have been into human B cells, that the TNF2 allele is a more powerful inducer of transcription compared with TNF1 (A G Wilson, unpublished observation).

These results indicate that this polymorphism in the TNF $\alpha$  promoter region is not important in susceptibility to, or severity of, RA. One intriguing aspect is the reported association of the RA susceptibility/severity marker, HLA-DR4, with increased TNF $\alpha$  production. This association cannot be explained by presence of the TNF2 allele, as it is not in linkage disequilibrium with HLA-DR4.<sup>14</sup> Perhaps as yet unidentified poly-

morphisms lie in other regions of the TNF $\alpha$  gene, which may explain these observations.

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