EXTENDED REPORT

Polymorphisms in the tumour necrosis factor gene are not associated with severity of inflammatory polyarthritis

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Background: Tumour necrosis factor alpha (TNFα) is a powerful inflammatory mediator in rheumatoid and other types of inflammatory arthritis. Polymorphisms within the TNFα gene have previously been investigated to determine their role in the aetiopathogenesis of rheumatoid arthritis (RA), but it is unclear whether reported associations are with susceptibility to, or severity of, disease.
Objective: To examine the association between both individual TNFα single nucleotide polymorphisms (SNPs) and haplotypes with the development and severity of erosions by 5 years in patients with inflammatory polyarthritis (IP).
Methods: 438 patients from the Norfolk Arthritis Register observational inception cohort of patients with IP were *x* rayed 5 years after disease onset. They were genotyped for nine SNPs mapping to the TNFα gene, using a SNaPshot primer extension assay. Haplotypes were constructed in patients with IP, who were compared for the presence and extent of erosions at 5 years.
Results: No association between individual TNFα SNPs or haplotypes in the patients who developed erosions at 5 years compared with those who remained non-erosive was found. Restricting analysis to patients who satisfied ACR criteria for RA by 5 years did not affect the conclusions.
Conclusion: The TNFα gene does not seem to be associated with severity as assessed by erosive outcome at 5 years in patients with IP.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder with a prevalence of 0.8%.[1] Based on twin studies performed in the UK population, a strong genetic component to disease susceptibility has been suggested with a heritability approaching 60%.[2] Linkage and association with HLA has been widely replicated, but HLA is estimated to account for only ~30% of the total genetic component to susceptibility.[3] Furthermore, it is unclear whether the HLA region harbours more than one susceptibility gene. Three recent studies using high density microsatellite mapping across the classical HLA region have suggested that a second region, close to the class III region, may contribute to disease susceptibility.[4–6] The tumour necrosis factor α (TNFα) gene lies within this region and is a major candidate.

Although a number of proinflammatory cytokines are important in inflammatory polyarthritis (IP), there is now a wealth of experimental evidence to support a pivotal role of TNFα as the prime mediator of inflammation. This is based on observations that TNFα is up regulated in RA joints and its blood concentration correlates with disease activity. TNFα induces destruction of bone, and cartilage induces synoviocyte proliferation and promotes angiogenesis (reviewed by Feldmann *et al*[7]). Furthermore, insertion of a human TNFα transgene into a variety of mouse strains leads to the spontaneous development of a chronic, erosive arthritis.[8] The central role of TNFα as an inflammatory mediator in RA was illustrated when it was demonstrated that antibodies directed against epitopes of TNFα have a powerful anti-inflammatory action when given to patients with resistant RA and other inflammatory arthritides.[9] Polymorphism within the gene, leading to higher levels of circulating TNFα, might predispose to the development of chronic inflammation in susceptible people. TNFα is, therefore, a prime candidate RA severity gene.

The TNFα locus has been screened for polymorphisms, and at least 12 single nucleotide polymorphisms (SNPs) mapping to promoter, exonic, intronic, or 3′ regulatory regions of the gene have been identified, to date. In the UK population, however, only nine appear to be polymorphic.[10] A number of previous studies have investigated the association of TNFα SNPs mapping to regulatory and exonic regions with RA, but none have investigated the association with RA of haplotypes of these SNPs. The data are also inconsistent in that the −857, −376, −308, and −238 promoter polymorphisms as well as the +489 exonic SNP have been associated with susceptibility to, or severity of, RA in some studies[11 12] but not in others.[13–19] Most of these studies have investigated association using prevalent RA cases of varying severity recruited from hospital, and the separation of susceptibility from severity effects for the TNFα locus is hampered using this study design. To examine the question of whether TNFα SNPs are associated more with severity than susceptibility, ideally association should be investigated in an inception cohort of patients, all followed up prospectively to quantify disease outcome. The Norfolk Arthritis Register (NOAR) provides such a resource. It aims to collect information on all incident cases of IP (defined as swelling of two or more joints lasting for four or more weeks after exclusion of other causes) presenting to a primary care physician in a geographically defined region and is a true community based inception cohort.[20] Data collection is not restricted to patients fulfilling American College of Rheumatology (ACR) criteria for RA as these criteria perform poorly in early disease, and it is in early disease that the identification of genes that

Abbreviations: ACR, American College of Rheumatology; CI, confidence interval; DMARD, disease modifying antirheumatic drug; IP, inflammatory polyarthritis; NOAR, Norfolk Arthritis Register; OR, odds ratio; RA, rheumatoid arthritis; RF, rheumatoid factor; SNP, single nucleotide polymorphism; TNFα, tumour necrosis factor α

determine prognosis is likely to have greatest impact by allowing better targeting of aggressive treatments.[21] The development of erosions is accepted as an objective and reliable outcome measure and the presence of erosions is used in many studies as a surrogate marker for quantifying disease severity.[22]

This study, therefore, aimed at examining whether TNFα SNPs or haplotypes were associated with severe outcome as measured by presence and extent of radiographic erosions by 5 years.

PATIENTS AND METHODS

Study design

A prospective cohort study was undertaken to investigate the association of nine TNFα SNPs with radiographic severity of IP in UK patients. Each of the nine individual TNFα SNP alleles together with the most commonly observed derived haplotypes were assessed as to their role in predicting the presence and severity of erosions by 5 years. Similar analyses were also undertaken to assess the role of TNFα in determining the presence of seropositivity for rheumatoid factor (RF) and in distinguishing between subjects with IP whose disease had evolved into RA compared with those who remained ''undifferentiated''.

Patients

Details of the case ascertainment procedure have been described in more detail previously.[20] Briefly, all cases newly presenting to primary care with IP within the region formerly known as the Norwich Health Authority are referred to, and assessed by, a research nurse using a standard questionnaire and examination. Baseline clinical data are recorded and blood taken for rheumatoid factor (RF) and C reactive protein analysis and for DNA extraction. Patients are reviewed annually. This includes documenting any disease modifying treatment started in the previous year. At each assessment, subjects are also scored as to whether 1987 ACR classification criteria for RA[23] are satisfied, using either a point or a cumulative evaluation, as described previously.[24] Patients with an alternative diagnosis, other than psoriatic arthritis, RA, or postviral arthritis are excluded from the analysis.

All patients with IP had radiographs of the hands and feet 5 years after registration. Details of the *x* ray scoring are described elsewhere.[25] Briefly, *x* ray findings of the hands and feet were scored by two observers using the Larsen method.[26] A third observer arbitrated in cases of disagreement. For the purposes of this analysis the first 438 consecutive subjects who were successfully followed up for 5 years and had provided a blood sample for DNA were studied.

Genotyping

Genotyping was performed using a SNaPshot primer extension assay as described previously.[10] Polymerase chain reaction primers, probes, and reaction conditions have been described previously.[10]

Statistical analysis

Patients were defined as erosive if they had a Larsen score $\geqslant 2$ in any joint and both the presence of any erosion, as well as the actual Larsen score, at 5 years were used as outcome measures to assess the role of the TNFα polymorphisms investigated. Allele and genotype frequencies of each SNP were compared between patients with erosions at 5 years and those without using the χ^2 test. Odds ratios (ORs) and 99% confidence interval (CI) were calculated. For analysis of the Larsen score the subjects were divided into three groups: those with no erosions (n = 244), those with erosions and Larsen score less than or equal to the median (n = 101), and those with erosions and a score greater than the median (n = 93). The OR and 99% CI for carriage of one allele for each SNP were calculated for each group of Larsen scores, with the lowest (Larsen score of zero) acting as the referent group. Subgroup analysis was also undertaken, restricting analysis to those (*a*) who satisfied ACR criteria for RA by 5 years or (*b*) were RF positive by 5 years. Haplotypes were constructed using the EM algorithm implemented in HelixTree (Golden Helix Inc, Montana, USA). The resulting haplotype frequencies were compared between patients who developed erosions and those who did not using the software programme CLUMP.[27] As treatment with disease modifying antirheumatic drugs (DMARDs) may mask genetic associations, all analyses were repeated after adjusting for DMARD and/or steroid use.

Associations with nine SNPs were investigated in this study and, in addition, the data were stratified as described above to fully explore whether the SNPs are associated with susceptibility to, or severity of, IP. However, as the SNPs are tightly linked, applying a Bonferroni correction would be overly conservative as the test assumes independence of loci. The strategy adopted in this study was to calculate 99% CIs rather than 95% CIs—that is, to assess association at the more stringent $p<0.01$ significance level. The study had 80% power to detect the effect of an SNP with a 10% minor allele frequency conferring an OR of 2.2 at the 1% significance level, assuming a dominant mode of inheritance.

RESULTS

Patients

Table 1 shows the clinical characteristics of the IP cohort. Sixty eight per cent of patients were female. By five years, 194 were erosive, with Larsen scores ranging from 2 to 138 and 78.3% satisfied ACR criteria for RA (table 1). The remainder had undifferentiated IP.

Association with severity

For eight of the nine TNF SNPs analysed (SNPs −1031, −863, −857, −376, −238, +489, +851, and +1304) there were no differences in either allele or genotype frequencies between the patients with IP developing erosions and those remaining non-erosive at 5 years (table 2). The −308G TNF allele was associated with erosive outcome at 5 years, but the 99% confidence interval included unity. Furthermore, no trend of increasing allele frequencies with extent of radiographic erosions (indicating a dose-response effect) was noted either for the −308 or for any of the other SNPs studied (table 2). Similarly, restricting the analysis to those patients with IP who by five years were either (*a*) RF positive or (*b*)

Table 1 Characteristics of the cohort with IP (n = 438)

Patient characteristics	
Age (years), mean (SD)	55.4 (14.4)
Sex (female)	298 (68.0)
RF positive	
At baseline	113 (25.8)
At 5 years	166 (37.9)
RA criteria positive	
At baseline	197 (45.0)
At 5 years	343 (78.3)
Copies of shared epitope	
0	176 (40.5)
1	199 (45.7)
2	60 (13.8)
Erosive at 5 years	194 (44.3)
Median Larsen score at 5 years in those with erosions (IQR)	25 (4–30)

Results are given as No (%) unless otherwise stated.
IQR, interquartile range.

Table 2 Comparison of allele frequencies in patients with IP who develop erosions by 5 years and those who remain non-erosive and comparison of allele frequencies by tertile of Larsen score compared with patients who remain non-erosive

				Tertile of Larsen score at 5 years by carriage of allele OR (99%CI)		
SNP allele	Erosive (%) (n = 194)	Non-erosive (%) (n = 244)	OR (99%CI)	Lowest (reference) (n = 244)	Middle (n = 101)	Highest (n = 93)
−1031						
T	297 (77.3)	401 (82.2)				
C	87 (22.7)	87 (17.8)	1.35 (0.86 to 2.12)	1.0	1.19 (0.69 to 2.04)	1.54 (0.90 to 2.62)
−863						
C	302 (80.3)	406 (86.0)				
A	74 (19.7)	66 (14.0)	1.51 (0.92 to 2.48)	1.0	1.34 (0.74 to 2.41)	1.70 (0.95 to 3.03)
−857						
C	342 (90.5)	424 (89.1)				
T	36 (9.5)	52 (10.9)	0.86 (0.48 to 1.54)	1.0	1.03 (0.51 to 2.07)	0.68 (0.30 to 1.53)
−376						
G	376 (96.9)	481 (98.6)				
A	12 (4.1)	7 (1.4)	2.19 (0.59 to 9.42)	1.0	2.10 (0.49 to 8.96)	2.29 (0.54 to 9.78)
−308						
G	329 (86.1)	391 (80.8)	1.48 (0.90 to 2.45)	1.0	1.46 (0.80 to 2.67)	1.49 (0.80 to 2.80)
A	53 (13.9)	93 (19.2)				
−238						
G	345 (93.2)	423 (94.4)				
A	25 (6.8)	25 (5.6)	1.23 (0.55 to 2.73)	1.0	0.95 (0.35 to 2.56)	1.52 (0.63 to 3.64)
+489						
G	349 (90.0)	438 (90.1)				
A	39 (10.0)	48 (9.9)	1.02 (0.55 to 1.87)	1.0	1.22 (0.62 to 2.43)	0.80 (0.36 to 1.77)
+851						
A	363 (94.0)	457 (94.8)				
G	23 (6.0)	25 (5.2)	1.16 (0.51 to 2.61)	1.0	1.26 (0.51 to 3.12)	1.05 (0.39 to 2.83)
+1304						
A	351 (91.4)	436 (90.8)	1.07 (0.56 to 2.08)	1.0	1.03 (0.48 to 2.20)	1.12 (0.50 to 2.51)
G	33 (8.6)	44 (9.2)				

satisfied ACR criteria for RA did not affect the results (table 3). As both these latter two indicators (presence of RF and satisfaction of ACR criteria) may be surrogate markers of severity, allele frequencies for the nine SNPs were also compared between seropositive and seronegative patients and between patients with RA and IP. However, no differences were detected (table 4).

HelixTree software (Golden Helix Inc, Montana, USA) was used to estimate haplotypes. Among the patients as a whole, six out of a possible 512 TNF haplotypes accounted for 92% of those observed, indicating strong linkage disequilibrium across the gene. No differences in the frequencies of these TNF haplotypes were seen between those patients who developed erosive changes at 5 years and those who remained non-erosive ($p = 0.65$) (table 5).

DISCUSSION

In the most extensive analysis to date of the role of polymorphism within the TNFα gene and IP, we found that none of the SNPs tested were associated with the presence or extent of radiographic erosions. Similarly, haplotypes of TNFα SNPs showed no association with the development of erosions by 5 years.

Previous studies of the TNFα gene have primarily focused on investigation of a few SNPs with susceptibility to RA and, as far as we know, this is the first report attempting to systematically examine all of the polymorphisms and haplotypes within this gene to determine whether they might usefully predict outcome.

A number of methodological issues need to be considered in interpreting these data. We have chosen to investigate

Table 3 Comparison by TNF SNP allele in subgroups of patients with IP who develop erosions by 5 years and those who remain non-erosive

	Erosive *v* non-erosive if RF positive by 5 years (n = 166)		Erosive *v* non-erosive if RA defined by ACR criteria by 5 years (n = 343)	
TNF SNP	OR (99% CI)‡	p Value‡	OR (99% CI)‡	p Value‡
TNF−1031*C/T	1.00 (0.44 to 2.26)	1.00	1.44 (0.85 to 2.45)	0.08
TNF−863*C/A	1.17 (0.52 to 2.66)	0.62	1.55 (0.88 to 2.74)	0.05
TNF−857*C/T	1.18 (0.39 to 3.57)	0.71	1.02 (0.51 to 2.03)	0.95
TNF−376*G/A	0.19 (0.01 to 3.43)	0.14	1.95 (0.48 to 7.89)	0.22
TNF−308*G/A†	1.38 (0.53 to 3.62)	0.39	1.49 (0.83 to 2.66)	0.08
TNF−238*G/A	0.59 (0.11 to 3.21)	0.42	1.36 (0.49 to 3.76)	0.44
TNF+489*G/A	0.89 (0.28 to 2.88)	0.80	1.19 (0.60 to 2.35)	0.51
TNF+851*A/G	0.46 (0.05 to 4.11)	0.36	1.45 (0.55 to 3.78)	0.32
TNF+1304*A/G	1.84 (0.47 to 7.20)	0.25	1.12 (0.52 to 2.42)	0.70

The two subgroups shown are patients with IP who are RF positive by 5 years and patients who satisfy ACR classification criteria for RA by 5 years
†OR of having an erosive outcome in the presence of the rare allele except for TNF-308*G/A where the odds ratio relates to presence of the common TNF-308*G allele; ‡analysis has been adjusted to account for possible confounding by treatment.

Table 4 Comparison by TNF SNP allele of RF positive IP (by 5 years) compared with RF negative patients with IP and patients with RA defined by ACR criteria (by 5 years) with patients who do not satisfy criteria by 5 years (uIP)

	RF positive *v* RF negative by 5 years		RA defined by ACR criteria *v* uIP by 5 years	
TNF SNP	OR (99% CI)‡	p Value‡	OR (99% CI)‡	p Value‡
TNF−1031*C/T	1.32 (0.82 to 2.13)	0.13	0.99 (0.60 to 1.63)	0.94
TNF−863*C/A	1.63 (0.98 to 2.71)	0.01	0.84 (0.48 to 1.49)	0.44
TNF−857*C/T	0.92 (0.48 to 1.76)	0.75	0.73 (0.36 to 1.47)	0.25
TNF−376*G/A	1.44 (0.41 to 4.99)	0.45	0.34 (0.05 to 2.36)	0.15
TNF−308*G/A†	0.72 (0.43 to 1.23)	0.12	0.90 (0.54 to 1.51)	0.61
TNF−238*G/A	0.87 (0.36 to 2.07)	0.68	1.65 (0.74 to 3.67)	0.11
TNF+489*G/A	0.89 (0.46 to 1.71)	0.65	0.63 (0.31 to 1.30)	0.10
TNF+851*A/G	0.50 (0.19 to 1.33)	0.07	1.10 (0.47 to 2.56)	0.77
TNF+1304*A/G	0.50 (0.23 to 1.05)	0.02	1.45 (0.76 to 2.79)	0.14

†OR of having an erosive outcome in the presence of the rare allele except for TNF-308*G/A where the odds ratio relates to presence of the common TNF-308*G allele; ‡analysis has been adjusted to account for possible confounding by treatment.

association with outcome after the onset of IP rather than restrict the study to those with RA. Classification of a subject with new onset IP as RA is hazardous given the changing nature of the disease as it evolves.[28 29] However, restricting analysis to the subgroup of subjects (n = 343 (78.3%)) who were RA positive by five years did not affect the findings.

Studies of the prediction of outcome are subject to the possible negative confounding effect that subjects with potentially more severe disease might be more likely to be more aggressively treated, hence masking an apparent risk. Indeed, we have shown within the NOAR cohort that subjects with more severe disease at baseline were more likely to be treated early with DMARDs.[30] In an ideal cohort, treatment would be standardised, but this is not possible in an observational study. We attempted to assess the possible influence of treatment effects by adjusting for the ever use of any DMARD (including steroids) but even after including this adjustment, none of the SNPs were associated with disease severity. This adjustment was rather crude and, possibly, subsequent differences in treatment between those with and without a particular TNFα allele might have masked an effect.

We classified patients' erosive status and severity based on radiograph data for the hands and feet. This takes no account of the erosive status in other joints, but previous studies have suggested that, in RA at least, there is reasonable correlation between radiological status in small and large joints.[31]

The most extensively investigated polymorphism is the −308 TNF promoter polymorphism. This has been investigated primarily in relation to susceptibility, but results have been conflicting, with RA susceptibility being associated with the rare A allele in some series,[12 32] with the common G allele in others,[11 13 33] and with neither in yet others.[14 17] One previous study reported an association of the −308A allele with higher cumulative disease scores and lower functional class but, confusingly, association of the −308G allele with overall susceptibility.[34] In our larger study using an inception cohort of patients with IP, we found that the −308G allele showed a trend towards worse radiological outcome, as measured by the presence/absence of erosions (associated at the 5% but not the 1% significance level). The frequency of the associated allele did not increase with increasing Larsen score, indicating no allele dose-response effect. Similarly, when the analysis was restricted to patients satisfying ACR classification criteria for RA by 5 years, the −308 TNF SNP was not associated with the presence of erosions by 5 years. Taken together, the results suggest that the −308 TNF SNP is unlikely to have a significant role in determining development of erosive outcome at 5 years in patients with IP. Functional studies of the −308 TNF SNP have produced conflicting results, with some investigators reporting higher transcription of the gene in the presence of the A allele,[35–37] whereas others have not.[38–41]

Two studies have suggested that the −238GA genotype is associated with a reduction in the extent of erosive change.[42 43] This has not been confirmed in the current, larger study despite very similar allele frequencies to those found previously. The current results are also supported by previous transfection studies, which failed to demonstrate a functional role for this SNP.[33 44]

Transcription of other cytokines is determined by combinations of polymorphisms acting in concert with each other.[45] Similarly, it may be postulated that combinations of coding SNP alleles may contribute to the structure and function of the TNF molecule and, in turn, these different combinations may have different effects on severity of disease. This will only be detected if haplotype analysis is undertaken, but no previous studies have undertaken full TNFα haplotype analysis. No differences in haplotype frequencies were observed between patients who developed erosions and those

Table 5 Haplotype frequencies for nine TNF SNPs in patients with IP with erosions at 5 years compared with non-erosive patients

	SNP									Haplotype frequency, No (%)	
Haplotype*	−1031	−863	−857	−376	−308	−238	+489	+851	+1304	Erosive	Non-erosive
A	T	C	C	G	G	G	G	A	A	95 (49.0)	119 (48.7)
B	C	A	C	G	G	G	G	A	A	32 (16.3)	30 (12.1)
C	T	C	C	G	A	G	G	A	A	26 (13.2)	44 (18.1)
D	T	C	T	G	G	G	A	A	A	13 (6.5)	19 (7.7)
E	C	C	C	G	G	A	G	G	G	6 (3.2)	6 (2.5)
F	T	C	C	G	G	G	G	A	G	5 (2.6)	8 (3.4)

*These six haplotypes account for 92% of the haplotypes in patients with IP.

that did not, suggesting that the TNFα gene does not play a part in determining erosive outcome. However, these polymorphisms may be important in determining treatment responses. Establishing whether these polymorphisms play a part in predicting which patients respond to new biological treatments that interfere with the TNFα pathway will be important as not all patients respond to these expensive treatments.

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