EXTENDED REPORT

Association of polymorphism in the transforming growth factor β1 gene with disease outcome and mortality in rheumatoid arthritis

D L Mattey, N Nixon, P T Dawes, J Kerr

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See end of article for authors' affiliations

Correspondence to: Dr D L Mattey, Staffordshire Rheumatology Centre, University Hospital of North Staffordshire, The Haywood, High Lane, Burslem, Stoke-on-Trent, Staffordshire, ST6 7AG, UK; d.l.mattey@keele.ac.uk

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Objective: To investigate whether polymorphism in the transforming growth factor β1 (TGFβ1) gene is associated with disease outcome in rheumatoid arthritis.
Methods: 208 patients with established rheumatoid arthritis were genotyped for the TGFβ1 T869C polymorphism using an amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) method. Disease severity was assessed by measuring radiographic damage by Larsen score and functional outcome by the health assessment questionnaire (HAQ). Patients were tracked on the NHS central register for notification of death, and the relation between TGFβ1 polymorphism and mortality was analysed using Cox proportional hazards regression.
Results: Patients carrying a TGFβ1 T allele had a higher mean HAQ score than those without this allele (1.60 *v* 1.22, p = 0.04). The T allele was also associated with higher five year mean area under the curve (MAUC) erythrocyte sedimentation rate (ESR), and nodular disease. Larsen score was higher in patients with the TT genotype compared with CC + CT genotypes, although this was not significant after correction for disease duration. There was a trend of increasing mortality risk with T allele dose after adjustment for age, sex, and disease duration (hazard ratio = 1.6 (95% confidence interval, 1.1 to 2.4), p = 0.01).
Conclusions: TGFβ1 T869C gene polymorphism is associated with disease outcome in rheumatoid arthritis. Carriage of the T allele (putatively associated with decreased TGFβ1 production) was associated with increased inflammatory activity and poor functional outcome, while increasing T allele dose was associated with worse survival.

The severity and long term outcome of rheumatoid arthritis have been related to various genetic factors. Although many studies have shown that polymorphism in the HLA-DRB1 gene encoding a common amino acid sequence (the shared epitope)[1] is associated with measures of disease severity, recent studies have suggested that polymorphisms at other gene loci may have an impact on the progression and severity of rheumatoid arthritis.[2–7] Several cytokine genes have been considered as likely candidates for influencing disease susceptibility or severity, and various associations with cytokine gene polymorphisms have been found.[8–12]

Recently, a study on a Japanese population of patients with rheumatoid arthritis suggested that polymorphism in the signal sequence at position +869 (T869C) of the transforming growth factor β1 (TGFβ1) gene may be associated with increased risk of rheumatoid arthritis.[13] However, a small study on a prospective cohort of white patients in New Zealand failed to find any association of the T869C polymorphism with rheumatoid arthritis prevalence or severity at two years.[14]

TGFβ has been considered an important modulator of the immune response in rheumatoid arthritis, and can have both pro- and anti-inflammatory effects. TGFβ exists as three isoforms—TGFβ1, TGFβ2, and TGFβ3—and has a broad range of biological functions including wound healing, fibrosis, immune suppression, and angiogenesis. It has chemotactic properties and may stimulate cells to produce cytokines such as interleukin (IL) 1, IL6, and tumour necrosis factor α (TNFα) at sites of inflammation.

TGFβ is also a local regulator of bone metabolism, acting downstream of oestrogen and in concert with vitamin D. Enhanced expression of TGFβ has been found in synovial effusions and synovium of patients with rheumatoid arthritis.[15–17]

The concentration of TGFβ1 in plasma has been correlated with the development of several diseases, including atherosclerosis, bone diseases, and certain forms of cancer.[18] As the level of circulating TGFβ1 appears to be under genetic control it is possible that predisposition to these diseases may be associated with particular alleles at the TGFB1 locus.[19] Various polymorphisms have been demonstrated in the TGFβ gene, and recent studies have shown associations with several diseases.[20–26] Some of these studies have reported that the T allele of the T869C polymorphism is associated with lower production of TGFβ1,[20 23 24 27] although an association with higher production has also been reported.[28 29]

As the only previous study on the association of the T869C TGFβ1 polymorphism with rheumatoid arthritis severity was carried out on patients with early disease,[14] we investigated whether this polymorphism was associated with long term outcome in a population of white patients with established disease. We also explored the association of TGFβ1 polymorphism with mortality in this group of patients.

METHODS

Study population

We studied 208 patients with long term rheumatoid arthritis (disease duration 5 to 25 years) (table 1). This was a historical cohort recruited between 1994 and 1998 in a clinic

Abbreviations: ARMS-PCR, amplification refractory mutation system–polymerase chain reaction; BMD, bone mineral density; HAQ, health assessment questionnaire; MAUC, mean area under the curve; NHSCR, National Health Service central register; ONS, Office for National Statistics; TGF, transforming growth factor

established to monitor the effects of disease modifying antirheumatic drugs. All patients were northern European white residents in north Staffordshire and satisfied the 1987 American College of Rheumatology diagnostic criteria.[30] Treatment was given as clinically indicated. Eighty nine per cent of patients were being treated with one or more disease modifying antirheumatic drugs including hydroxychloroquine, sulfasalazine, gold, or methotrexate. Fewer than 5% of patients were being treated with corticosteroids.

Ethics permission for the study was obtained from the North Staffordshire research ethics committee.

Disease outcome

Joint damage was assessed at the time of inclusion in the study by two experienced observers scoring *x* rays of the hands and feet using the standard radiographs of Larsen.[31] The majority (more than 90%) of the patients had erosions, with Larsen scores ranging between 22 and 205 (maximum possible, 210). Larsen scores were obtained on 173 of the 208 patients. Functional assessment was carried out using the health assessment questionnaire (HAQ).[32] The presence or absence of subcutaneous nodules was recorded during physical examination of each patient at the time of recruitment. Time integrated measures of disease activity were determined from five year mean area under the curve (MAUC) levels of erythrocyte sedimentation rate (ESR) and C reactive protein measured before inclusion in the study. The MAUC levels were calculated from measurements taken at approximately six monthly intervals.

Survival follow up

All patients in the study were NHS patients registered on the NHS central register (NHSCR), a computed registry of the records of all NHS patients. Access to this registry is obtained through the Office for National Statistics (ONS, General Register Office, Southport, UK). All patients in this study were tracked on the NHSCR, and notification of patient deaths was obtained from the ONS. Causes of death were coded by the ONS, using the *International Classification of Diseases*, ninth revision (ICD-9).

TGFβ1 genotyping

Genomic DNA was extracted from blood samples collected in EDTA using a DNAce Megablood kit as directed by the manufacturer (Bioline, London, UK). Laboratory personnel were blinded to the clinical characteristics of the donors and the hypothesis being investigated. Samples were genotyped for the TGFβ1 T869C polymorphism using an amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) method with the use of primers described previously by Perrey *et al*.[33] Amplification of a 241 base pair (bp) fragment was carried out using the following primer sequences: generic primer (sense): 5′-TCCGTGGGATACTGA GACAC-3′; primer C (antisense): 5′-GCAGCGGTAGCAGCAG CG-3′; primer T (antisense): 5′-AGCAGCGGTAGCAGCAG CA-3′ . The specific primer mix consisted of 10 μM generic primer and 10 μM of one of the two allele specific primers. Two internal control primers amplifying a human growth hormone sequence were used to confirm successful PCR amplification. The reaction mixtures and conditions were as described previously.[33] All amplification reactions were undertaken in a Flexigene thermal cycler (Techne (Cambridge) Limited, Cambridge, UK) using a 96 well heating block. Amplified products were visualised by electrophoresis on 2% agarose gels containing ethidium bromide (0.5 mg/ml).

Table 1 Characteristics of the study patients

Male:female (n)	93:115
Age (years)	60.0 (25 to 89)*
Age at onset (years)	49.2 (18 to 82)*
Disease duration (years)	10.0 (5 to 25)*
RF positive	66.9%
Erosions	92.8%
Nodules	19.8%

*Median (range).
RF, rheumatoid factor.

Statistical methods

Analysis of TGFβ1 T869C allele and genotype frequencies showed that they were in Hardy–Weinberg equilibrium. The association of genotypes with normally distributed outcome measures (Larsen score) was assessed using analysis of covariance (ANCOVA) with disease duration as a covariate. Association between genotypes and non-parametric data such as HAQ, MAUC, ESR, and C reactive protein was assessed using Kruskal–Wallis one way analysis of variance (ANOVA) on ranks. Where appropriate, adjustment for potential multiple testing errors was carried out, either by Bonferroni correction for normally distributed data or by the Kruskal–Wallis *z* value test for non-parametric data.

The association of TGFβ1 genotype with mortality was assessed in a Cox proportional hazard regression analysis adjusted for age, sex, and disease duration. The time intervals for those patients who were alive at the end of the study period and those who were lost to follow up were censored. The censoring date for the present analysis was 31 December 2003. Kaplan–Meier curves were constructed to illustrate the survival in patients with different TGFβ1 genotypes. The curves were compared using the log rank significance tests. All data were analysed using Number Cruncher Statistical System (NCSS) (version 5.01) and Graphpad Prism software (version 1.03).

RESULTS

TGFβ1 T869C polymorphism and disease outcome

These results are summarised in table 2. Analysis of covariance with inclusion of disease duration as a covariate showed no overall significant difference between TGFβ1 genotypes for mean Larsen score ($p = 0.09$), although there was a trend towards higher scores with increasing T allele number. Comparison of the TT genotype with the remainder (CC + CT genotypes) revealed a higher Larsen score in the TGFB1 TT genotype ($p = 0.04$), although significance was lost after correction for disease duration ($p = 0.07$). In the case of the HAQ score, patients with a CT genotype had a significantly higher score than those with a CC genotype ($p = 0.02$, after correction for multiple comparisons). However, there was essentially no difference between patients with a CT and TT genotype, and overall those with a T allele (CT or TT) had a significantly higher HAQ score than those lacking the T allele ($p = 0.02$). This remained significant ($p = 0.04$) when corrected for disease duration using ANCOVA.

Association of TGFβ1 polymorphism with time integrated disease activity

Previous studies have shown that time integrated measures of disease activity are associated with more severe radiographic and functional outcome.[34 35] We therefore examined the association of the TGFβ1 polymorphism with five year MAUC levels of ESR and C reactive protein (table 3). No significant differences were found between individual TGFβ genotypes for five year MAUC ESR or C reactive protein. However, a weakly significant difference in MAUC ESR levels was found between patients carrying a T allele and the

Table 2 Association between TGFβ1 T869C genotypes and measures of disease outcome in rheumatoid arthritis

TGFβ1 genotype	n (%)	Larsen score	n (%)	HAQ score
CC	28 (16.2)	81.1 (47.8)	30 (14.4)	1.22 (0.9)
CT	80 (46.2)	87.7 (48.0)	103 (49.5)	1.65 (0.8)
TT	65 (37.6)	101.1 (43.4)	75 (36.1)	1.56 (0.8)

Values are n (%) or mean (SD).
Differences in Larsen score between genotypes were analysed by analysis of covariance (ANCOVA) with inclusion of disease duration as a covariate. No significant difference was found between individual TGFβ1 genotypes (p = 0.09). Comparison of the TT genotype with the remainder (CC + CT) showed a higher Larsen score in the former (p = 0.04), but significance was lost after correction for disease duration (p = 0.07). The mean HAQ score was significantly higher in patients with a CT genotype than those with CC (p = 0.02, after correction for multiple comparisons). No significant difference was found between patients with a CT and TT genotype, and overall, those patients carrying a T allele had a significantly higher HAQ score than those lacking this allele (p = 0.04, after correction for disease duration).
HAQ, health assessment questionnaire.

remainder (30.6 *v* 24.3, p = 0.05). Similarly the T allele was associated with a higher MAUC C reactive protein level, although this was non-significant (p = 0.09).

Association of TGFβ1 polymorphism with nodular disease

In addition to worse radiographic and functional outcome, patients with severe rheumatoid arthritis are more likely to develop extra-articular features such as subcutaneous nodules. We examined whether the TGFB1 T allele was associated with the development of nodular disease in these patients. Comparison between individuals with and without the T allele showed a significant difference in the frequency of nodular disease. Thus 38/171 patients (22.2%) with a T allele had nodules compared with 1/26 patients (3.8%) without a T allele (odds ratio (OR) = 5.1 (95% confidence interval (CI), 1.02 to 151.8); p = 0.025).

Association of TGFβ1 polymorphism with mortality

By 31 December 2003, 58 patients (27.9%) had died (34 male, 24 female). The most common cause of death was cardiovascular disease (48.3%), followed by malignancy (24.1%). The relation of the TGFβ1 T869C polymorphism with mortality was initially examined in a multivariate Cox proportional hazards regression model which included age at entry into the study, disease duration at entry, and sex. In this model the TGFβ1 genotypes were ordered as categorical variables according to T allele number (that is, 0, 1, 2). The analysis showed a significant trend of increasing mortality risk with T allele dose (hazard ratio (HR) = 1.6 (95% CI, 1.1 to 2.4); p = 0.01) which was independent of age and male sex. In additional analyses we also included rheumatoid factor status, presence or absence of nodules, and MAUC ESR. Although MAUC C reactive protein and MAUC ESR were both associated with mortality when analysed separately, only the latter was shown to be significantly associated (p<0.0001) in a model containing both MAUC ESR and C reactive protein. The trend of increasing risk with T allele number remained significant in a model containing MAUC ESR, rheumatoid factor, and nodules, and in a stepwise model the strongest predictors of death were MAUC ESR, age, male sex, and TGFβ1 genotype (table 4). Construction of a Kaplan–Meier survival probability curve showed that poorer survival over a 10 year follow up period was particularly associated with individuals carrying the TGFβ1 TT genotype (fig 1).

Examination of the causes of death showed that the TGFβ1 TT genotype was more common in patients who had died from malignancy (12 solid tumours, two haematological) than had died from other causes, although this was not significant (64.3% *v* 33.6%, OR = 2.7 (95% CI, 0.7 to 12.0); p = 0.1). In a Cox proportional hazards regression model the TGFβ1 TT genotype was associated with an increased risk of cancer related mortality (HR = 3.5 (95% CI, 1.2 to 10.6); p = 0.02), after adjustment for age, sex, and disease duration.

DISCUSSION

Our data indicate that the T allele of the T869C polymorphism in the TGFβ1 gene is associated with certain aspects of long term disease outcome in rheumatoid arthritis. These include increased time integrated inflammatory activity, worse functional outcome, and increased frequency of rheumatoid nodules. In addition, there was a significant trend towards poorer survival with increasing T allele dose. Structural damage appeared to be greater in patients with a

Table 3 Association between TGFβ1 T869C genotypes and time-integrated measures of disease activity rheumatoid arthritis

TGFβ1 genotype	n	MAUC ESR (mm/h)	MAUC CRP (mg/l)
CC	25	24.3 (18.2)	16.2 (16.7)
CT	76	30.7 (18.1)	20.2 (18.2)
TT	57	30.8 (16.9)	23.8 (20.6)

Differences between genotypes were analysed by Kruskal–Wallis one way analysis of variance. No significant differences were found between individual TGFβ1 genotypes for five year mean area under the curve (MAUC) erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) levels. However, comparison between patients with a T allele and the remainder showed a weak significant difference in MAUC ESR levels (p = 0.05). A similar non-significant trend was seen with MAUC CRP levels (p = 0.09).

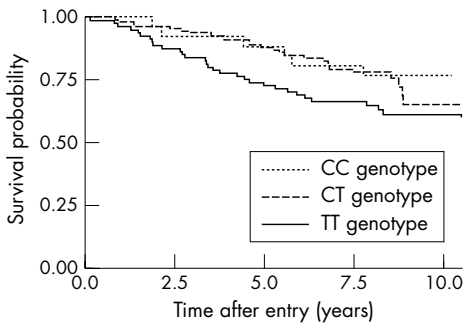

Figure 1 Kaplan–Meier survival probability curve illustrating the poorer survival of individuals with the TGFβ1 TT genotype compared with those carrying CC or CT genotypes.

Table 4 Stepwise Cox proportional hazards model showing independent predictors of mortality in patients with established rheumatoid arthritis

Step and variable	Regression coefficient	Hazard ratio (95% CI)	p Value
1. MAUC ESR	0.026	1.03/mm/h (1.01 to 1.04)	<0.0001
2. Age	0.055	1.06/year (1.02 to 1.09)	0.007
3. Male sex	0.777	2.2 (1.2 to 4.1)	0.003
4. TGFβ1 genotype*	0.463	1.6 (1.02 to 2.5)	0.04

*The TGFβ1 genotypes were ordered as categorical variables according to T allele number (that is, 0, 1, 2). The hazard ratio shows the increased risk per unit increase in T allele number.
CI, confidence interval; ESR, erythrocyte sedimentation rate; MAUC, mean area under the curve.

TT genotype, but the absence of a significant association with Larsen score after correction for disease duration did not support an association between this genotype and structural severity.

Our results are in contrast to those of a recent prospective study on a smaller number of white patients (n = 117) with early disease.[14] That study found no association with the prevalence or severity of rheumatoid arthritis. However, patients were examined only up to two years of disease duration, so it is possible that the effect of this polymorphism becomes evident at a later stage of disease.

Inconsistent findings have also been reported on the association of the T869C polymorphism with other diseases. An association with osteoporosis has been found in Japanese women[27] and in elderly white Australian women.[36] However, in the Japanese population the T allele was associated with lower bone mineral density (BMD) and increased vertebral fractures, while in the Australian patients the C allele was associated with lower BMD and an increase in prevalent fractures. In another study of German postmenopausal women the CC genotype was associated with lower BMD and greater bone loss.[28] In contrast to these reports, studies in a Chinese population, and on white women in the USA, failed to find an association.[37 38]

Reports on the association of the T869C polymorphism with TGFβ1 production have also been inconsistent. Some studies have shown that the T allele of the T869C polymorphism is associated with lower production of TGFβ1,[20 23 24 27] but the converse has also been found.[28 29] The results of our study suggest that rheumatoid patients carrying a T allele may have increased inflammatory activity over the long term. The poor outcome and earlier mortality in these patients may possibly be explained by reduced production of TGFB1 leading to worse control of inflammation. Such an effect would be cumulative so may only become evident in long term outcome studies such as this. We and others have shown previously that time integrated measures of disease activity are associated with more severe radiographic and functional outcome, and that a high level of sustained inflammation is predictive of earlier mortality in rheumatoid arthritis.[34 35 39]

It is noteworthy that T allele dose of the TGFβ1 T869C polymorphism was associated with poorer survival in this rheumatoid population. It needs to be stressed that this particular patient group already had well developed disease of five years' duration or more, so the influence of this polymorphism on the mortality of rheumatoid arthritis patients within five years of development was not investigated. However, our data indicate that in patients with established disease the carriage of a T allele increases the risk of death and that this increases with T allele dose.

In a previous Japanese study on myocardial infarction, it was suggested that the T allele was a risk factor for susceptibility to myocardial infarcts in middle aged Japanese men.[20] However, the data on TGFβ1 polymorphism and ischaemic heart disease are controversial, with the majority of studies showing no association. Although ischaemic heart disease is common in rheumatoid patients, and myocardial infarction is a frequent cause of death, we found no evidence in this study for an association of the T allele with coronary heart disease related mortality or cardiovascular mortality in general. However we did find a possible association between homozygosity for the T allele and mortality from malignancy. The TT genotype was more common in patients who had died from malignant disease, and the overall likelihood of cancer related mortality was increased in patients with this particular genotype. These data need to be treated with caution because of the relatively small number of deaths from malignancy. In other studies, carriage of one or two TGFβ1 T alleles has been associated with increased susceptibility to prostate cancer, hepatocellular carcinoma in patients with chronic hepatitis B infection, and breast cancer.[25 40–42] However, the studies on breast cancer have been inconsistent, with some showing no association.[43 44] One study on breast cancer progression showed that the CC (high producing) genotype was associated with worse survival.[45]

The evidence to date suggests that polymorphisms within the TGFβ1 gene may play a significant role in determining the development or severity of various diseases. Controversies over the association with particular diseases or disease features may arise partly from differences between ethnic groups, as well as from differences in the stage at which particular diseases have been examined. The results of our study suggest that the association of rheumatoid arthritis severity with the T869C polymorphism may be more evident in patients with well established disease where the manifestations of severe disease are more distinct than early in the disease course.

Conclusions

We have shown that polymorphism in codon 10 of the TGFβ1 gene may be associated with disease outcome and mortality in white patients with rheumatoid arthritis. Measures associated with poor outcome including increased long term inflammation, worse functional outcome, and nodular disease were associated with carriage of a T allele, which has been linked to lower production of TGFβ1 in other studies. In addition, there appears to be an association between T allele dose and earlier mortality. These findings suggest a role for TGFβ1 polymorphism in the long term outcome of rheumatoid arthritis, but this will need confirmation in studies on other rheumatoid arthritis populations.

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Authors' affiliations

D L Mattey, N Nixon, P T Dawes, Staffordshire Rheumatology Centre, University Hospital of North Staffordshire, Stoke-on-Trent, Staffordshire, UK

J Kerr, Department of Microbiology, Royal Brompton Hospital, National Heart and Lung Institute, Imperial College London, London, UK

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