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

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

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Key words: rheumatoid arthritis, TGF beta, polymorphism, disease outcome, mortality
Short running title: TGFβ1 polymorphism and RA
Abstract

Objective: To investigate whether polymorphism in the transforming growth factor β1 (TGFβ1) gene is associated with disease outcome in rheumatoid arthritis (RA).

Methods: Two hundred and eight patients with established RA 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 National Health Service Central Register for notification of death, and the relationship 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 patients without this allele (1.60 v 1.22, p = 0.04). The T allele was also associated with higher 5-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 significant trend of increasing mortality risk with T allele dose after adjustment for age, sex and disease duration (hazard ratio 1.6, 95% CI 1.1 – 2.4, p = 0.01).

Conclusion: The TGFβ1 T869C gene polymorphism is associated with disease outcome in RA. 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.
Introduction
The severity and long term outcome of rheumatoid arthritis (RA) have been associated with a number of 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 RA [2-7]. A number of cytokine genes have been considered as likely candidates for influencing disease susceptibility and/or severity, and several associations with cytokine gene polymorphisms have been found [8-12].

Recently, a study on a Japanese population of RA patients suggested that polymorphism in the signal sequence at position +869 (T869C) of the transforming growth factor beta 1 (TGFβ1) gene may be associated with increased risk of RA [13]. However, a small study on a prospective cohort of Caucasian patients in New Zealand failed to find any association of the T869C polymorphism with RA prevalence or severity at two years [14].

TGFβ has been considered an important modulator of the immune response in RA, and can demonstrate both pro-and anti-inflammatory effects. TGFβ exists as 3 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 IL-1, IL-6 and TNFα at sites of inflammation. TGFβ is also a local regulator of bone metabolism, acting downstream of estrogen and in concert with vitamin D. Enhanced expression of TGFβ has been found in synovial effusions and synovium of patients with RA [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]. Since 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 TGFβ1 locus [19]. A number of polymorphisms have been demonstrated in the TGFβ gene and recent studies have demonstrated associations with various diseases [20-26]. Several 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].

Since the only previous study on the association of the T869C TGFβ1 polymorphism with RA severity was carried out on patients with early disease [14] we decided to investigate whether this polymorphism was associated with long term outcome in a population of Caucasian patients with established disease. We have also investigated whether this TGFβ1 polymorphism is associated with mortality in this group of patients.
Patients and Methods

Study population.
Two hundred and eight patients with long-term RA (disease duration 5 - 25 years) were studied (Table 1). This was an historical cohort recruited between 1994 and 1998 in a clinic established to monitor the effects of disease modifying anti-rheumatic drugs. All patients were northern European Caucasians resident in north Staffordshire and satisfied the 1987 ACR diagnostic criteria [30]. Therapy was administered as clinically indicated. Eighty nine per cent of patients were being treated with one or more DMARDs including hydroxychloroquine, sulphasalazine, 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 (>90%) of patients had erosions, with Larsen scores ranging between 22-205 (maximum possible=210). Larsen scores were obtained on 173/208 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 (CRP) measured prior to inclusion in the study. The MAUC levels were calculated from measurements taken at approximately 6 monthly intervals.

Survival follow-up
All patients in this study were National Health Service (NHS) patients registered on the NHS Central Register (NHSCR), a computerised registry of the records of all NHS patients. Access to this registry is obtained via 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, Humbar Road, London, England). 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 bp fragment was performed using the following primer sequences: Generic primer (sense): 5’ - TCCGTGGGATCTGAGACAC - 3’; Primer C (antisense): 5’ - GCAGCGGTAGCAGCGAGC - 3’; Primer T (antisense): 5’ – AGCAGCGGTAGCAGCAGCA GCA – 3’. The specific primer mix consisted of 10 uM generic primer and 10 uM 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 performed in a Flexigene Thermal Cycler (Techne (Cambridge) Limited, Cambridge, England) using a
96 well heating block. Amplified products were visualised by electrophoresis on 2% agarose gels containing ethidium bromide (0.5 mg/ml).

**Statistical Methods**

Analysis of TGFβ1 T869C allele and genotype frequencies demonstrated 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 CRP levels was assessed using Kruskal-Wallis one way analysis of variance (ANOVA) on ranks. Where appropriate, adjustment for potential multiple testing errors was performed, either by Bonferroni correction for normally distributed data or by 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 December 31, 2003. Kaplan-Meier curves were constructed in order 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

Analysis of covariance (ANCOVA) with inclusion of disease duration as a covariate revealed 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 TGFβ1 TT genotype (p = 0.04), although significance was lost after correction for disease duration (p = 0.07). In the case of the HAQ score those 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 5-year MAUC levels of ESR and CRP (Table 3). No significant differences were found between individual TGFβ genotypes for 5-year MAUC ESR or CRP. However, a weak significant difference in MAUC ESR levels was found between patients carrying a T allele and the remainder (30.6 v 24.3, p = 0.05). Similarly the T allele was associated with a higher MAUC CRP 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 RA are more likely to develop extraarticular features such as subcutaneous nodules. We examined whether the TGFβ1 T allele was associated with the development of nodular disease in these patients. Comparison between individuals with and without the T allele revealed a significant difference in the frequency of nodular disease. Thus, 38/171 (22.2%) of patients with a T allele had nodules compared with 1/26 (3.8%) patients without a T allele (OR 5.1, 95% CI 1.02 - 151.8, p = 0.025).

Association of TGFβ1 polymorphism with mortality

By 31 December 2003, 58/208 (27.9%) patients had died (34 male, 24 female). The most common cause of death was cardiovascular disease (48.3%), followed by malignancy (24.1%). The relationship 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 (i.e. 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 – 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 CRP 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 CRP. The trend of increasing risk with T allele number remained significant in a model containing MAUC ESR, RF 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 illustrated 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 frequent in patients that had died from malignancy (12 solid tumours, 2 haematological) than had died from other causes, although this was not significant (64.3 v 33.6 %, OR 2.7 95% CI 0.7 – 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 – 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 RA. 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 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 (n = 117) of Caucasian patients with early disease [14]. This study found no association with the prevalence or severity of RA. 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 Australian white 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 fracture. 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 demonstrated 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 suggested [28, 29]. The results of our study suggest that RA 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 TGFβ1 leading to poorer 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 RA [34, 35, 39].

It is noteworthy that T allele dose of the TGFβ1 T869C polymorphism was associated with poorer survival in this RA population. It needs to be stressed that this particular patient group already had well developed disease of 5 years or more, so the influence of this polymorphism on the mortality of RA patients within 5 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 (MI), the T allele was suggested to be a risk factor for susceptibility to MI in middle-aged Japanese men [20]. However, the data on TGFβ1 polymorphism and ischemic heart disease (IHD) are controversial, with the majority of studies showing no association. Although IHD is common in RA patients, and MI is a
frequent cause of death, we found no evidence in this study for an association of the T allele with MI related or cardiovascular mortality. However we did find a possible association between homozygosity for the T allele and mortality due to malignancy. The TT genotype was more frequent in patients that 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 low number of deaths due to 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 studies showing no association [43, 44]. One study on breast cancer progression showed that the CC (high producing) genotype was associated with poorer survival [45].

The evidence to date suggests that polymorphisms within the TGFβ1 gene may play a significant role in determining the development and/or severity of a number of 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 RA 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.

In summary, we have shown that polymorphism in codon 10 of the TGFβ1 gene may be associated with disease outcome and mortality in Caucasian patients with RA. 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 RA, but this will need confirmation in studies on other RA populations.

**Competing interests**
None declared

**Acknowledgements**
We wish to thank Mrs June Fisher and Mrs Sheila Clarke (metrologists) for their help with data collection, and Professor Peter Jones (Department of Mathematics, Keele University) for statistical advice. Work supported by the Haywood Rheumatism Research and Development Foundation.

**Ethics approval**
Ethics permission for the study was obtained from the North Staffordshire Research Ethics Committee, Stoke-on-Trent.

**Figure legend**
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.
References


Table 1. Characteristics of rheumatoid arthritis (RA) patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>male:female</td>
<td>93:115</td>
</tr>
<tr>
<td>Age (median + range), yrs</td>
<td>60.0 (25 – 89)</td>
</tr>
<tr>
<td>Age at onset (median + range), yrs</td>
<td>49.2 (18-82)</td>
</tr>
<tr>
<td>Disease duration (median + range), yrs</td>
<td>10.0 (5 – 25)</td>
</tr>
<tr>
<td>Rheumatoid factor positive (%)</td>
<td>66.9%</td>
</tr>
<tr>
<td>Erosions (%)</td>
<td>92.8%</td>
</tr>
<tr>
<td>Nodules</td>
<td>19.8%</td>
</tr>
</tbody>
</table>
Table 2. Association between TGFβ1 T869C genotypes and measures of disease outcome in RA.

<table>
<thead>
<tr>
<th>TGFβ1 genotype</th>
<th>n (%)</th>
<th>Larsen score (SD)</th>
<th>n (%)</th>
<th>HAQ score (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>28 (16.2)</td>
<td>81.1 (47.8)</td>
<td>30 (14.4)</td>
<td>1.22 (0.9)</td>
</tr>
<tr>
<td>CT</td>
<td>80 (46.2)</td>
<td>87.7 (48.0)</td>
<td>103 (49.5)</td>
<td>1.65 (0.8)</td>
</tr>
<tr>
<td>TT</td>
<td>65 (37.6)</td>
<td>101.1 (43.4)</td>
<td>75 (36.1)</td>
<td>1.56 (0.8)</td>
</tr>
</tbody>
</table>

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) demonstrated 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).
Table 3. Association between TGFβ1 T869C genotypes and time-integrated measures of disease activity RA.

<table>
<thead>
<tr>
<th>TGFβ1 genotype</th>
<th>n</th>
<th>MAUC ESR (SD) mm/hr</th>
<th>MAUC CRP (SD) mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>25</td>
<td>24.3 (18.2)</td>
<td>16.2 (16.7)</td>
</tr>
<tr>
<td>CT</td>
<td>76</td>
<td>30.7 (18.1)</td>
<td>20.2 (18.2)</td>
</tr>
<tr>
<td>TT</td>
<td>57</td>
<td>30.8 (16.9)</td>
<td>23.8 (20.6)</td>
</tr>
</tbody>
</table>

Differences between genotypes were analysed by Kruskal-Wallis one-way analysis of variance. No significant differences were found between individual TGFβ1 genotypes for 5-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).
Table 4. Stepwise Cox proportional hazards model showing independent predictors of mortality in patients with established RA

<table>
<thead>
<tr>
<th>Step and variable</th>
<th>Regression coefficient</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MAUC ESR</td>
<td>0.026</td>
<td>1.03/mm/hr (1.01 – 1.04)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2. Age</td>
<td>0.055</td>
<td>1.06/yr (1.02 – 1.09)</td>
<td>0.007</td>
</tr>
<tr>
<td>3. Male sex</td>
<td>0.777</td>
<td>2.2 (1.2 – 4.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>4. TGFβ1 genotype*</td>
<td>0.463</td>
<td>1.6 (1.02 – 2.5)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*The TGFβ1 genotypes were ordered as categorical variables according to T allele number (i.e. 0, 1, 2). The hazard ratio shows the increased risk per unit increase in T allele number.
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