EXTENDED REPORT

Investigation of association between the *TRAF* family genes and RA susceptibility

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Objective: The tumour necrosis factor (TNF) receptor-associated factor (TRAF) family is important in activating multiple inflammatory and immune related processes induced by cytokines such as TNFα and interleukin-1. These genes therefore represent strong candidate susceptibility factors for rheumatoid arthritis (RA). A study was undertaken to investigate the association between single nucleotide polymorphisms (SNPs) spanning six *TRAF* genes and RA in a British population.

Methods: Twenty-three haplotype tagging (ht) SNPs and 26 random SNPs spanning the six *TRAF* genes were initially tested for association in a cohort of 351 unrelated patients with RA and 368 controls. Any SNPs demonstrating an association were genotyped in further samples. Sequenom MassARRAY technology was preferentially used for genotyping. Both single point and haplotypic analyses were performed.

Results: Forty-four SNPs were successfully genotyped and conformed to Hardy-Weinberg expectation. A single SNP, rs7514863, mapping upstream of the *TRAF5* gene and affecting a putative transcription factor binding site, demonstrated a significant association across the entire cohort of 1273 cases with RA compared with 2463 healthy controls (OR for minor T allele 1.2 (95% CI 1.06 to 1.36), p = 0.005). The association was stronger in the subgroup carrying at least one copy of the shared epitope alleles (OR 1.43 (95% CI 1.18 to 1.73), p = 0.0003).

Conclusion: These findings provide evidence for the association of an SNP upstream of a strong candidate RA susceptibility gene, *TRAF5*, in a large cohort of patients and controls. Further association and functional studies are required to investigate the role of this variant, or one in linkage disequilibrium with it, in RA disease causation.

Rheumatoid arthritis (RA) is a multifactorial disease in which both genetic and environmental factors are thought to interact to result in the persistent synovial inflammation characteristic of this condition. Both clinical and laboratory based research have shown that variation in levels of the pro-inflammatory cytokine tumour necrosis factor α (TNFα) plays a prominent part in the pathology of this disease. Numerous studies have therefore been performed to identify genetic variants in the gene encoding TNFα (the *TNFα* gene), which may influence the expression and/or function of this protein and contribute towards the pathology of RA. However, these studies have so far proved inconclusive. For example, one promoter polymorphism, *TNF-308G/A*, has been associated with RA susceptibility in some studies[1 2] or with RA severity.[3 4] Other studies have found no such association.[5 6] Furthermore, there are as many reports confirming a direct functional effect of this promoter polymorphism on TNFα protein levels[7–10] as there are opposing it.[11–13] Thus, as genetic and functional analyses have failed to confidently identify variants in the *TNFα* gene region that account unequivocally for varying levels of the protein, alternative genetic regulatory factors may be important. In turn, these may also contribute to RA susceptibility.

The TNFα cytokine exerts its function by interacting with membrane bound receptors and activating intracellular signalling cascades. These pathways comprise a complex network of proteins, each of which contributes to the final TNFα induced response. Potentially, any factor involved in regulating and/or mediating TNFα activity may contribute towards the pathology of chronic inflammatory diseases by perturbing pathways of TNFα signalling. Good examples of this are the chronic auto-inflammatory TNF receptor associated periodic fever syndromes which result from germline mutations in the gene region encoding the extracellular domain of type 1 (55 kDa) TNFα receptor (TNF-R1; TNFRSF1A). The TNF receptors play a key role in both regulating and mediating TNFα activity. Consequently, the mechanism by which several of the *TNFRSF1A* mutations contribute to the pathology of these syndromes has been ascribed to defects in cell surface shedding with a subsequent increase in TNFα inflammatory signalling or impaired TNFα apoptotic signalling (reviewed by Stojanov and McDermott[14]).

The TNF receptor-associated factor (TRAF) family is an important group of intracellular adapter proteins for a wide variety of receptors including the TNFα and interleukin (IL)-1 receptor superfamilies. These molecules are responsible for transducing extracellular cytokine signals from the corresponding cell surface receptors and activating intracellular signalling cascades. Collectively, the TRAF family are responsible for regulating nuclear factor kappa B and Jun N-terminal kinase signalling pathways, which play crucial roles in cell proliferation and differentiation, apoptosis, bone remodelling and, importantly, further activation/inhibition of cytokines such as TNFα.

The six major TRAF proteins are encoded by individual genes mapping to different regions of the genome. Interestingly, evidence of linkage to the regions harbouring some of these *TRAF* genes has previously been detected in whole genome scans of families with RA. For example, the *TRAF5* gene maps to chromosome 1q31–32 and evidence for linkage to this region has repeatedly been demonstrated in European RA linkage studies.[15 16] In addition, a recent single nucleotide polymorph-

Abbreviations: IL, interleukin; MAF, minor allele frequency; RA, rheumatoid arthritis; RF, rheumatoid factor; SE, shared epitope; SNP, single nucleotide polymorphism; TNFα, tumour necrosis factor α; TRAF, TNF receptor-associated factor

ism (SNP) based genome-wide linkage scan has identified evidence for linkage to chromosome 11p12 where the *TRAF6* gene maps.[17] Finally, peaks of linkage have also been demonstrated close to the *TRAF1* and *2* genes on chromosome 9q34, the *TRAF3* gene on chromosome 14q32 and the *TRAF4* gene on chromosome 17q11, although not all have been replicated.[15 16 18 19]

Together, these data support the hypothesis that TRAF proteins may play a role in the pathology of RA. We have therefore investigated the contribution of genetic variants spanning the six *TRAF* encoding genes to the susceptibility to RA.

METHODS

Patients

A multistage case-control association study was used to investigate the role of the *TRAF* family of genes in RA susceptibility. Patients with RA were selected from two main sources: (1) the Arthritis Research Campaign (ARC)'s National Repository of patients and families with RA and (2) local general practices in the Norfolk and Norwich area (www.arc.man.ac.uk/). All cases satisfied the 1987 ACR criteria modified for genetic studies.[20 21] Unrelated controls with no history of inflammatory joint disease were recruited from healthy blood donors and general practice registers. In addition, a subset of 2024 subjects from the 1958 birth cohort were also included.[22] This cohort comprises approximately 17 000 randomly selected individuals from across England, Scotland and Wales who were all born during one week in March 1958 and followed prospectively. In total, DNA was available for 1469 cases with RA and 2760 controls. All subjects were of UK Caucasian ethnic origin. The available demographic data for these samples are shown in table 1.

SNP selection

The CEPH (Utah residents with ancestry from northern and western Europe, CEU) population phase 1 dataset from the Hapmap project was used to identify 23 haplotype tagging (ht) and 9 random SNPs across the six gene regions (www.hapmap.org). Haplotypes and htSNPs were defined using the confidence intervals method within Haploview.[23 24] One non-synonymous SNP (rs1131877), mapping to exon 3 of the *TRAF3* gene, was identified and substituted as a htSNP. In addition, 17 SNPs were selected from other public databases to increase the coverage across the genes (www.ncbi.nlm.nih.gov/). Only SNPs with a minor allele frequency (MAF) $\geq$5% were included in this selection process. Details of the SNPs selected across each gene are shown in table 2 and in supplementary fig 1 available online at http://ard.bmj.com/supplemental.

Table 1 Demographic data for cases with rheumatoid arthritis and controls

Characteristic	Cases [n]	Controls [n]
Median (IQR) age of disease onset* (years)	44 (32, 57) [1418]	48 (48, 48) [2440]
Proportion of females (%)	67 [977/1455]	52 [1360/2636]
Proportion seropositive for RF (%)	79 [1101/1394]	–
Proportion with erosive disease (%)	68 [767/1135]	–
Proportion positive for SE (1 and/or 2 copies) (%)	80 [862/1082]	46 [798/1722]
Total subjects available	1469	2760

IQR, interquartile range; RF, rheumatoid factor; SE, shared epitope.
*For non-1958 birth cohort controls, age when bled was used.

Genotyping

Forty-eight SNPs were genotyped by either MassARRAY Homogenous MassEXTEND (hME) or iPLEX assays followed by matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (MS) (Sequenom, Cambridge, UK). Multiplex assays were designed using the MassARRAY Assay Design software and accompanying online bioinformatic tools ProxSNP, PreXTEND, and PleXTEND (www.realsnp.com). All assays were performed as recommended by the manufacturer (www.sequenom.com). MassARRAY Typer software was used for automated genotype calling. One htSNP (rs6540679) was genotyped using a Taqman 5′ allelic discrimination assay (Applied Biosystems, Warrington, UK) as described elsewhere.[25] Primer and probe details for these assays are presented in supplementary tables 1–3 available online at http://ard.bmj.com/supplemental.

Genotyping was performed in three stages. First, all SNPs were genotyped in a small subset of samples, specifically 351 unrelated cases with a family history of RA and 368 controls. Second, any SNPs showing a trend towards an association ($p<0.05$) at a single point or haplotypic analysis were replexed and genotyped in an additional 594 unrelated cases and 368 controls. Finally, any SNPs still showing an association ($p<0.05$) in the combined group were genotyped in the remaining 524 unrelated cases and 2024 controls from the 1958 birth cohort.

Power

Based on the average previously reported MAF across the 49 SNPs (25%), the initial cohort of 351 cases and 368 controls had 80% power to detect loci conferring an odds ratio (OR) of 1.6 at the 5% significance level, assuming a dominant model. However, as these cases were selected because of the presence of a family history, this power may be underestimated.

Statistical analysis

SNPs that were consistently problematic (genotyped successfully in <70% of individuals) or deviated from Hardy-Weinberg equilibrium in controls ($p<0.01$) were excluded from further analyses. If any htSNPs were rejected at this point, alternatives were identified from the Hapmap CEU dataset and genotyped as above. Furthermore, the Tagger option within Haploview software, which implements a pairwise r^2 method for selecting tagging SNPs, was used to ensure all common variation described by Hapmap was captured by the successfully genotyped SNPs.[24 26]

Both allelic and genotypic associations between single SNPs and RA susceptibility were analysed using χ^2 tests. Logistic regression and/or stratification analyses were performed to investigate the impact of sex, the presence of erosive disease, rheumatoid factor (RF) and shared epitope (SE) status on any SNPs showing an association across the entire cohort ($p<0.05$). As there were no available data regarding RF status in the controls, this factor could not be included in any logistic regression model. Logistic regression was performed to investigate interactions between associated SNPs ($p<0.05$) and SE status. These analyses were limited to SNPs with evidence for an association in order to limit the number of tests performed. All statistical analyses were performed using STATA software (StataCorp, Texas, USA). Finally, both Haploview and Helixtree software were used to determine haplotype frequencies using a standard expectation-maximisation algorithm and to investigate haplotypic associations (Golden Helix Inc, Montana, USA).[24]

RESULTS

Stage 1: Genotyping of 49 SNPs in the first subset of 351 cases and 368 controls

Forty-nine SNPs spanning the six *TRAF* genes were selected and genotyped in the first subset of 351 cases with RA and 368

Table 2 Details of *TRAF* genes and SNPs selected for genotyping

Gene	Location	Size (kb)	SNPs
TRAF1	9q33–34	24.5	2 ht and 1 random
TRAF2	9q34	40	6 ht and 3 random
TRAF3	14q32.32	130	6 ht and 14 random
TRAF4	17q11–12	6	2 ht
TRAF5	1q32	48	5 ht and 5 random
TRAF6	11p12	21	2 ht and 3 random

ht, haplotype tagging; SNP, single nucleotide polymorphism.

healthy controls. Of these SNPs, 44 were successfully genotyped and conformed to Hardy-Weinberg expectation (see supplementary table 4 available online at http://ard.bmj.com/supplemental). Four random SNPs mapping to the *TRAF 2*, *3* and *6* genes failed at the genotyping stage. The remaining SNP—an htSNP (rs12569232) from *TRAF5*—did not meet Hardy-Weinberg expectation in controls. Re-analysis of the *TRAF5* haplotype blocks indicated that this SNP was only tagging itself and the remaining variation across the second linkage disequilibrium block was defined by the remaining successfully genotyped htSNP. As the other 22 htSNPs were successfully genotyped, most of the common variation (MAF ⩾5%) identified in the Hapmap dataset was captured in this study. Furthermore, the Tagger program in Haploview also confirmed that the previously described variation was captured across all six *TRAF* genes.

Of the 44 SNPs analysed, 36 did not have an association with RA susceptibility, either singly or by haplotype (see supplementary tables 4 and 5 available online at http://ard.bmj.com/supplemental). This excluded the *TRAF 1*, *3*, *4* and *6* genes from further analysis. A three-marker haplotype (rs7048473, rs2811761 and rs10781522) defining the promoter and 5′ region of *TRAF2* showed a significant association with RA (global haplotype $p = 0.013$). In particular, the only haplotype containing the minor G allele for rs2811761 showed the greatest significance, which coincided with the single point association demonstrated to this SNP (single point $p = 0.02$). An additional association was observed with the random SNP rs4880075 mapping adjacent to this haplotype block (single point $p = 0.05$). Thus, these four *TRAF2* SNPs were carried forward to the second stage of genotyping.

An effect was also found with a three-marker haplotype (rs7514863, rs6540679 and 10863888) in the promoter and 5′ region of the *TRAF5* gene (global haplotype $p = 0.02$). However, two of these SNPs showed stronger single point associations (rs7514863: minor T allele OR 1.49 (95% CI 1.12 to 1.97), $p = 0.004$; rs10863888: minor A allele OR 1.39 (95% CI 1.11 to 1.75), $p = 0.003$). A random SNP (rs12123230) mapping adjacent to this haplotype block also demonstrated a genotypic association ($p = 0.017$). These four *TRAF5* SNPs were carried forward to the second stage of genotyping.

In order to investigate potential interactions between the genes encoding *TRAF2* and *TRAF5* on the susceptibility to RA, interaction analyses using logistic regression tests were performed between each of the four *TRAF2* SNPs and each of the four *TRAF5* SNPs. No significant interaction effect was found (data not shown). Furthermore, none of these eight SNPs showed an interaction effect with SE status (data not shown).

Stage 2: Genotyping of 8 SNPs in the larger subset of 945 cases and 736 controls

Following genotyping in an additional 594 cases and 368 controls, eight SNPs were analysed in the combined cohort of 945 cases and 736 controls. No association was found, using either single point or haplotype analysis, with the four *TRAF2* SNPs (see supplementary tables 6 and 7 available online at http://ard.bmj.com/supplemental). Only one SNP, rs7514863, upstream of *TRAF5* remained associated (T allele OR 1.36 (95% CI 1.12 to 1.65), $p = 0.002$). Although a haplotype association was seen, this was less significant than the SNP alone (global haplotype $p = 0.02$; see supplementary tables 6 and 7 at http://ard.bmj.com/supplemental). Thus, only rs7514863 was genotyped in the remaining samples.

Stage 3: Genotyping of the rs7514863 *TRAF5* SNP in the entire cohort

The rs7514863 SNP was genotyped in the remaining cases and controls. Combined analysis showed a significant association across the entire cohort of 1463 cases with RA and 2763 healthy controls (T allele OR 1.2 (95% CI 1.06 to 1.36), $p = 0.005$, table 3). Following stratification analysis, this association appeared restricted to SE positive patients (table 4). Although not always statistically significant due to limited numbers of male patients and RF negative patients, the ORs associated with this SNP were similar across all other subgroups (table 4). Logistic regression adjusting for SE and sex confirmed these findings (adjustment for SE: OR 1.3 (95% CI 1.1 to 1.5), $p = 0.002$; adjustment for sex: OR 1.2 (95% CI 1.1 to 1.4), $p = 0.005$). However, no statistical interaction was found between SE status and rs7514863 following logistic regression ($p = 0.19$).

DISCUSSION

Despite multiple lines of evidence implicating the TNFα cytokine in the pathology of RA, the role of variation in the *TNFα* gene remains undetermined. In this study we have investigated the hypothesis that other factors regulating and/or

Table 3 Frequencies and allelic association analysis of rs7514863 in the complete dataset

Allele and genotypes	Cases (n = 1273)	Controls (n = 2463)	OR (95% CI)
T	467 (0.18)	778 (0.16)	1.2 (1.06 to 1.36) p = 0.005
AA	848 (0.67)	1756 (0.71)	
AT	383 (0.30)	636 (0.26)	
TT	42 (0.03)	71 (0.03)	

OR, odds ratio; CI, confidence intervals.

Table 4 Stratification analysis of rs7514863 in the complete dataset

Stratification	Proportion of the risk T allele [n]		Allelic association	
	Cases	Controls	OR (95% CI)	p Value
Female	0.19 [853]	0.16 [1211]	1.23 (1.04 to 1.44)	0.013
Male	0.18 [408]	0.16 [1149]	1.17 (0.94 to 1.44)	0.16
RF positive*	0.19 [912]	0.16 [2463]	1.20 (1.05 to 1.40)	0.0072
RF negative*	0.19 [250]	0.16 [2463]	1.22 (0.96 to 1.55)	0.1
Erosive disease*	0.18 [438]	0.16 [2463]	1.20 (0.99 to 1.44)	0.06
One copy of SE	0.20 [470]	0.15 [578]	1.39 (1.11 to 1.75)	0.0043
Two copies of SE	0.20 [285]	0.14 [147]	1.53 (1.05 to 2.25)	0.029
At least one copy of SE	0.20 [755]	0.15 [725]	1.43 (1.18 to 1.73)	0.0003
Negative for SE	0.17 [190]	0.16 [827]	1.03 (0.77 to 1.39)	0.84

RF, rheumatoid factor; SE, shared epitope; OR, odds ratio; CI, confidence intervals.
Values in square brackets show number of individuals conforming to the corresponding characteristic.
*All controls were used in the analysis.

mediating TNFα signalling may instead contribute to RA susceptibility. The TRAF family are strong candidates because of their essential role in transducing cytokine signals, such as TNFα, from their cell surface receptors and activating downstream intracellular signalling cascades. An association between RA susceptibility and six of the TRAF family genes was therefore investigated. A seventh member of this family has recently been identified and may also warrant investigation.

A significant association was observed between a single SNP, rs7514863, located upstream of the *TRAF5* gene and a large panel of 1273 cases with RA compared with 2463 healthy controls (OR for minor T allele 1.2 (95% CI 1.06 to 1.36), $p = 0.005$). Although this effect appeared to be restricted to individuals positive for SE, there was no evidence of a statistical interaction in that there was no evidence for a multiplicative effect in RA susceptibility. Although anti-cyclic citrullinated peptide (anti-CCP) antibody status was not available for the present cohort, a strong correlation between anti-CCP antibodies and SE alleles has previously been demonstrated. It is therefore possible to speculate that the *TRAF5* association may simply be restricted to a subset of patients with RA characterised by these antibodies.[27]

A relatively small sample size was tested in the first stage of genotyping, limiting the power to detect weak genetic effects. For instance, there was just 45% power to detect an OR of 1.5 at the 5% significance level, assuming a dominant model at the SNP with the lowest MAF of 7%. This calculation assumes that the causal variant was genotyped or was in perfect correlation with the genotyped marker. The use of tagging SNPs therefore further reduces power. The selection of patients with a family history of RA for the first stage of genotyping will have partially compensated for this but, nonetheless, the power was insufficient to detect weak effects in the initial phase of this study. The possibility that the lack of association of the other *TRAF* genes with RA susceptibility represents a false negative finding cannot therefore be excluded.

Conversely, it is possible that the association found between the rs7514863 polymorphism and RA represents a false positive finding, particularly as multiple SNPs were tested without any correction to the test statistics. The use of a multi-staged study design does not solve this problem as data from previous stages were carried forward. A Bonferroni correction would have been overly stringent because, although htSNPs were selected to reduce redundant genotyping, there was some degree of linkage disequilibrium between these and the random SNPs genotyped. For this study we used permutation testing (10 000 permutations) at each stage of the analysis, which suggested that the rs7514863 association was more likely to represent a true finding than to result from chance effects ($p<0.05$). However, replication in other populations is required before the association can be confidently confirmed.

If the present observation is a true finding, there are several possible explanations for this positive association: either the rs7514863 SNP is the functional polymorphism or it is acting as a marker for an alternative causal variant. In order to capture potential promoter and regulatory sequences, any SNPs located up to 20 kb upstream of the *TRAF* genes were included in the selection process. Consequently, the rs7514863 SNP, which maps ~15 kb upstream of the *TRAF5* gene and within the 10th intron of the preceding *Rest co-repressor 3* gene (NCBI gene id: 55758), was identified as a htSNP. Several bioinformatics databases such as TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and Alibaba2 (http://www.gene-regulation.com) were used to investigate the potential functional effects of this associated SNP. Interestingly, this region was consistently identified as a potential binding site for the immune and inflammatory response related CCAAT/enhancer binding protein which may be disrupted by the minor T allele of the associated variant. It may therefore be possible that this predicted transcription factor binding site is involved in the regulation of the downstream immune-related *TRAF5* gene. Numerous examples of long range gene regulation can be found in the literature[28] in addition to transcription factor binding site mapping within adjacent gene regions.[29] Furthermore, there are examples in which mutations in both these factors contribute towards human disease (reviewed by Lettice and Hill[30] and Dermitzakis *et al*[31]). Based on current information regarding the function of the Rest co-repressor 3 protein, it does not appear to be a strong candidate for RA susceptibility. However, we cannot exclude it from having a potential role in the pathology of RA without first performing functional analyses.

Alternatively, it is possible that the associated rs7514863 SNP is acting as a marker for a second variant within the *TRAF5* gene region. For instance, this SNP demonstrated complete linkage disequilibrium with the rs11582143 SNP in the Hapmap dataset (D' and $r^2 = 1$; see supplementary fig 1 available online at http://ard.bmj.com/supplemental) which we confirmed in a subgroup of 368 controls from the present cohort ($D' = 1$, $r^2 = 0.98$; data not shown). This rs11582143 variant maps to the 5′ UTR region of the *TRAF5* gene. However, neither of the above bioinformatics websites predicted any potential functional effect for this SNP. Furthermore, it is also possible that the associated SNP is in linkage disequilibrium with another form of genetic variant not considered here—for example, a copy number polymorphism—which may contribute to RA susceptibility.

Although the TRAF5 adapter protein is not the primary member involved in TNFα signalling, there are several reasons why it may be important. First, the TRAF family proteins

operate as components of multi-subunit signalling complexes in which individual proteins may inhibit, supplement or act as surrogates for each other. In particular, there is redundancy between TRAF2- and TRAF5-mediated nuclear factor kappa B activation.[32] Indeed, TRAF5 demonstrates binding specificities for TNFα receptor 2 (TNFR2; TNFRSF1B) and lymphotoxin β and, thus, has the potential to mediate these receptor signals and contribute to key immuno-inflammatory pathways.[33] Second, in vitro experiments and gene knockout mouse models have implicated TRAF5 in several T cell related functions. For instance, in an asthma mouse model, mice deficient in TRAF5 developed more pronounced lung inflammation with higher levels of Th2 cytokine production.[34] In vitro studies have also confirmed that TRAF5 is important for Th2 cell differentiation and cytokine production[34] as well as T cell activation and cell survival.[35] Furthermore, this latter study implicated TRAF5 in T cell mediated autoimmune responses. Finally, although the *TRAF5* gene is ubiquitously expressed, it shows particularly high levels in the spleen, lung and thymus. Thus, abnormal TRAF5 activity could play an important role in the pathology of autoimmune and inflammatory diseases.

In summary, we have found evidence for an association of an SNP upstream of a strong candidate RA susceptibility gene, *TRAF5*, in a large cohort of patients and controls from a UK population. Replication of these findings in similarly sized sample series from other populations is required to confirm the association with RA. Further association and functional studies are necessary to investigate the role of this variant, or one in linkage disequilibrium with it, in the causation of RA as well as other autoimmune and inflammatory conditions.

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Further details are given in the tables and figure in the online supplement available at http://ard.bmj.com/supplemental.

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