TRAF1–C5 as a Risk Locus for Rheumatoid Arthritis — A Genomewide Study

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ABSTRACT

BACKGROUND
Rheumatoid arthritis has a complex mode of inheritance. Although HLA-DRB1 and PTPN22 are well-established susceptibility loci, other genes that confer a modest level of risk have been identified recently. We carried out a genomewide association analysis to identify additional genetic loci associated with an increased risk of rheumatoid arthritis.

METHODS
We genotyped 317,503 single-nucleotide polymorphisms (SNPs) in a combined case–control study of 1522 case subjects with rheumatoid arthritis and 1850 matched control subjects. The patients were seropositive for autoantibodies against cyclic citrullinated peptide (CCP). We obtained samples from two data sets, the North American Rheumatoid Arthritis Consortium (NARAC) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA). Results from NARAC and EIRA for 297,086 SNPs that passed quality-control filters were combined with the use of Cochran–Mantel–Haenszel stratified analysis. SNPs showing a significant association with disease (P<1×10−8) were genotyped in an independent set of case subjects with anti–CCP-positive rheumatoid arthritis (485 from NARAC and 512 from EIRA) and in control subjects (1282 from NARAC and 495 from EIRA).

RESULTS
We observed associations between disease and variants in the major-histocompatibility-complex locus, in PTPN22, and in a SNP (rs3761847) on chromosome 9 for all samples tested, the latter with an odds ratio of 1.32 (95% confidence interval, 1.23 to 1.42; P=4×10−4). The SNP is in linkage disequilibrium with two genes relevant to chronic inflammation: TRAF1 (encoding tumor necrosis factor receptor–associated factor 1) and C5 (encoding complement component 5).

CONCLUSIONS
A common genetic variant at the TRAF1–C5 locus on chromosome 9 is associated with an increased risk of anti–CCP-positive rheumatoid arthritis.
RHEUMATOID ARTHRITIS IS A COMMON inflammatory arthritis of unknown cause, in which both genetic and environmental risk factors have been implicated. The genetic contribution to a susceptibility to rheumatoid arthritis has been shown in studies of twins and families and in genomewide linkage scans.

Two genes have shown a strong association with susceptibility: PTPN22 and HLA-DRB1. Variants of each gene elevate the risk primarily for a subgroup of severe rheumatoid arthritis characterized by the presence of autoantibodies against cyclic citrullinated peptide (anti–CCP-positive). We have recently reported a significant association at STAT4 on chromosome 2q.

Several other promising candidate genes have been reported in the literature (e.g., CTLA4 and PADI4), but these genes have had more modest statistical evidence of association. All of the alleles associated with rheumatoid arthritis are common in healthy persons of European ancestry (allele frequency, >5%). Therefore, it seems likely that additional common genetic variants with a modest effect size (e.g., odds ratio, <1.5 per copy) remain to be discovered.

Statistical tests based on allele frequencies in case–control studies (association analyses) have more power to identify common alleles that confer a modest risk than do tests based on chromosomal segregation in families (linkage analyses). Until recently, genetic association studies were limited to small regions of the genome containing biologic candidate genes or those identified through family-based linkage studies. Recent developments in understanding patterns of human genetic variation, together with cost-effective genotyping techniques and statistical methodology, have made it possible to test, in an unbiased manner, common variants across the entire genome for the risk of disease. Current genotyping platforms are estimated to represent more than two thirds of known common genetic variation throughout the genome, encompassing more than 20,000 human genes. By comparison, fewer than 100 candidate genes have been tested for an association with the risk of rheumatoid arthritis.

Our genomewide association study involved two groups of case subjects with anti–CCP-positive rheumatoid arthritis: one group who had been treated at rheumatology clinics across North America, the North American Rheumatoid Arthritis Consortium (NARAC), and another group from a Swedish population-based study, the Epidemiological Investigation of Rheumatoid Arthritis (EIRA). In the NARAC study, case subjects were matched with control subjects according to self-reported ethnic background; in the EIRA study, case subjects were matched with control subjects according to age, sex, and geographic location.

METHODS

SUBJECTS

We refer to stage 1 as the initial genotyping of samples in the genomewide scan of single-nucleotide polymorphisms (SNPs) (called NARAC-1 and EIRA-1) and stage 2 as the replication genotyping (NARAC-2 and EIRA-2). Patients were drawn from rheumatology clinics across North America (NARAC) and Sweden (EIRA). All patients were anti–CCP-positive and met the criteria for rheumatoid arthritis adopted by the American College of Rheumatology in 1987.

The NARAC “family collection” samples were from multiplex families (primarily affected sibling pairs) in which at least one sibling had documented erosions, as seen on radiography of the hand, and at least one sibling (most often the same patient) had an onset of disease between the ages of 18 and 60 years. The other collections that make up NARAC-1 included samples from the National Data Bank for Rheumatic Diseases (mean disease duration, 10 years), the National Inception Cohort of Rheumatoid Arthritis (with patients enrolled within 6 months after clinical diagnosis), and the Study of New Onset Rheumatoid Arthritis (with patients enrolled within 12 months after clinical diagnosis).

An initial set of samples from case subjects of self-reported white ancestry was randomly drawn from all four collections (464 from NARAC, 168 from the National Data Bank for Rheumatic Diseases, 162 from the National Inception Cohort of Rheumatoid Arthritis, and 114 from the Study of New Onset Rheumatoid Arthritis) (see the Methods section in the Supplementary Appendix, available with the full text of this article at www.nejm.org). Control subjects were selected on the basis of similar self-reported ancestry from 20,000 persons who were part of the New York Cancer Project. Replication samples (NARAC-2) were randomly drawn from the same collections (except...
that no cases were drawn from the NARAC family collection and included 485 patients with anti–CCP-positive rheumatoid arthritis and 1282 control subjects from the New York Cancer Project.

Data on participation rates are not available for any of the NARAC collections of patients with rheumatoid arthritis, since recruitment of patients was performed by diverse methods, including advertising, direct mail, and physician-based enrollment. Control subjects from the New York Cancer Project were enrolled during a 2-year period by means of general advertising and point-of-service solicitation, as described previously. Written informed consent was obtained from all subjects who provided blood samples in accordance with protocols approved by the local institutional review boards.

EIRA is a population-based case–control study comprising residents of south and central Sweden who were between the ages of 18 and 70 years during the period from May 1996 to December 2005. Enrollment was limited to patients who had recently received the diagnosis of rheumatoid arthritis (within 1 year after the first onset of symptoms for 85% of patients). For each patient, a control subject was randomly selected from the study base; control subjects were matched for age, sex, and residential area. Most subjects were born in Sweden, and 97% reported having white ancestry.

We randomly selected 676 patients with anti–CCP-positive rheumatoid arthritis and 673 control subjects for genomewide genotyping (EIRA-1). Replication subjects (EIRA-2) were randomly drawn from the same study base and included 568 anti–CCP-positive case subjects and 516 control subjects. The participation rate was 96% for case subjects after recruitment from the population-based early surveillance system for rheumatoid arthritis in Sweden. Written informed consent was obtained from all subjects, and the ethics review board at the Karolinska Institutet approved the study.
Genotyping and Quality-Control Filtering

Genotyping for stage 1 was performed at the Feinstein Institute for Medical Research for the NARAC scan and at the Genome Institute of Singapore for the EIRA scan, both according to the Illumina Infinium 2 assay manual (Illumina), as previously described. The NARAC scan included 545,080 SNPs genotyped in samples from 908 case subjects and 1260 control subjects. Samples from all the NARAC case subjects were genotyped by SNP assay with Infinium HumanHap550, version 1.0 (Illumina); 601 of the controls were genotyped on the same platform, 411 on HumanHap550 (version 3.0) and 248 on Infinium HumanHap300 and HumanHap240S arrays. The EIRA scan included genotypes of 317,503 SNPs from the HumanHap300 (version 1.0) array. The data sets were filtered individually on the basis of SNP genotype call rates (>95% completeness), minor allele frequency (>0.01), and the Hardy–Weinberg equilibrium (P<1×10^{-8}). We removed subjects whose percentage of missing genotypes was more than 5%, who had non-European ancestry, who had evidence of relatedness, and who had evidence of possible DNA contamination (see the Supplementary Appendix for more details). The 297,086 SNPs that passed filters in both the NARAC and EIRA sample collections were merged into a single file for analysis.

Stage 2 genotyping of nine TRAF1–C5 haplotype SNPs was performed with the use of Sequenom iPLEX at the Broad Institute of Harvard and the Massachusetts Institute of Technology (for the NARAC-2 samples) and at the Genome Institute of Singapore (for the EIRA-2 samples), both according to the manufacturer’s specifications (see the Supplementary Appendix for additional details).

Statistical Analysis

We initially analyzed the NARAC-1 and EIRA-1 data separately and then combined the two data sets for joint analysis. Our primary analyses were performed on the combined data set from NARAC and EIRA with the use of structured association within homogeneous clusters derived through identity-by-state similarity, implemented in the PLINK tool set as a Cochran–Mantel–Haenszel stratified analysis, a method we refer to here as structured association analysis (see the Methods section in the Supplementary Appendix). A complete listing of results of the combined NARAC-1 and EIRA-1 data can also be found in the Supplementary Appendix. Additional data on NARAC-1 are available in the Database of Genotype and Phenotype (dbGaP) (accession number phs000099.v1.p1).

After we identified the TRAF1–C5 region through the genomewide scans of subjects from NARAC-1 and EIRA-1, we selected nine SNPs that lie in a 100-kb block of linkage disequilibrium for test to association with disease in NARAC-2 and EIRA-2. We selected these SNPs on the basis of linkage disequilibrium patterns within European samples from the International HapMap Project (the Centre d'Etude du Polymorphisme Humain from Utah [CEU] HapMap) with the use of the software program Tagger. We performed association analysis with the use of 2-by-2 contingency tables of allele frequencies and Fisher’s exact test. For the NARAC-2 replication samples, we performed a secondary analysis, correcting for population stratification by applying the software program EIGENSTRAT to a set of 704 SNPs informative about European ancestry and corrected along the first principal component.

Results were combined across all samples (NARAC-1, NARAC-2, EIRA-1, and EIRA-2) in three ways (see the Methods section of the Supplementary Appendix). We also carried out association analysis conditional on each SNP and haplotype with the use of combined genotype data from all four sample collections. These analyses were performed with the software program WHAP, which also provided an omnibus test for haplotype association.

To estimate power in the combined NARAC-1 and EIRA-1 scan, we considered a variety of effect sizes (as estimated by odds ratio) and allele frequencies with the use of an online genetics power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). The study had a power of about 90% to detect a disease-associated allele with a population frequency of 0.20 and an odds ratio of 1.5 (at P=5×10^{-8} under a multiplicative genetic model) but a power of only 13% to detect the same allele with an odds ratio of 1.3.

Results

Genomewide Association Analysis

We identified a set of 297,086 polymorphic SNPs genotyped in samples from 1522 case subjects with anti–CCP-positive rheumatoid arthritis and
from 1850 control subjects in the combined NARAC-1 and EIRA-1 analysis that passed our quality-control filters (see the Methods section of the Supplementary Appendix). The average call rate for these SNPs was 99.71%.

To combine results between NARAC-1 and EIRA-1 while minimizing bias caused by population stratification, we conducted a structured analysis within homogeneous clusters defined with the use of genomewide SNP data. Advantages of this approach include the ability to match case–control clusters within each collection (i.e., NARAC case and control subjects are clustered together, as are EIRA case and control subjects) and the ability to calculate odds ratios that account for population stratification. To determine whether we observed more significant results than expected by chance alone, we calculated the genomic control inflation measure, which is based on the median chi-square distribution (in which 1.0 signifies no inflation), and plotted the observed distribution, as compared with the expected distribution, of P values. After correcting for residual inflation by genomic control (1.14) and after removing SNPs from the extended major-histocompatibility-complex (MHC) region, we observed a slight excess number of SNPs in the tail of the statistical distribution (Fig. 1A). We obtained a similar result after correcting for population stratification with a principal components method (EIGENSTRAT23; genomic control, 1.08) (see Fig. 1 of the Supplementary Appendix). The inflation in the tail of the distribution could represent true positive associations (e.g., PTPN22) or could reflect the effect of an unknown source of bias in our study.

A graphical summary of the results of our genomewide association scan is shown in Figure 1B. We clearly identified SNPs in linkage disequilibrium with known susceptibility variants at HLA-DRB1 (P<1×10^-100) and PTPN22 (P=2×10^-11). Common variants within these two regions contributed the strongest statistical signal of risk for anti–CCP-positive rheumatoid arthritis in our study of patients of European ancestry.

In the combined stage 1 analysis (data not shown), a single SNP within a region on chromosome 9 in the TRAF1–C5 locus reached genomewide significance (defined here as P<5×10^-8), and several neighboring SNPs also showed a highly significant association with a diagnosis of rheumatoid arthritis. The strongest association was seen for SNP rs3761847 (P=2.8×10^-8). The minor G allele frequency was higher in case subjects (0.49) than in control subjects (0.41), providing an allelic odds ratio of 1.36 (95% confidence interval...

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**Figure 1. Results of the Genomewide Association Study.**

Panels A and B show results for 297,086 polymorphic SNPs genotyped in samples from 1522 case subjects with rheumatoid arthritis who were seropositive for autoantibodies against cyclic citrullinated peptide (anti–CCP-positive) and 1850 control subjects from the combined data sets from the North American Rheumatoid Arthritis Consortium (NARAC) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA). Panel A shows a comparison of distributions of observed versus expected P values generated by Cochran–Mantel–Haenszel stratified analysis and corrected for residual inflation by genomic control. Black data points represent the inclusion of SNPs from the major-histocompatibility-complex (MHC) locus; blue data points represent the exclusion of MHC. The most significant non-MHC SNPs are at PTPN22 and the TRAF1–C5 locus. Panel B shows SNPs plotted according to chromosomal location, with the −log_{10} P values corrected with the use of genomic control. The blue horizontal line indicates SNPs that are significant at a genomewide level (P=5×10^-8). SNPs at PTPN22 and within the MHC locus, where the HLA-DRB1 gene resides, reach genomewide significance. Multiple SNPs across the TRAF1–C5 locus on chromosome 9 (e.g., rs3761847; P=2×10^-8) have a significant association.
### Table 2: Results of Case-Control Association Analysis for Nine TRAF1–C5 Tag SNPs in all NARAC and EIRA Samples

<table>
<thead>
<tr>
<th>Position</th>
<th>SNP</th>
<th>Combined Group</th>
<th>NARAC-1</th>
<th>NARAC-2</th>
<th>EIRA-1</th>
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<td>0.12</td>
<td>0.007</td>
<td>1.27</td>
<td>0.12</td>
</tr>
</tbody>
</table>

The most significant result from the genome scan, at rs3761847, was significant in the NARAC-2 samples under the same genetic model ($P=1\times10^{-8}$), with an odds ratio of 1.37 (95% CI, 1.18 to 1.58) and showed a nonsignificant trend toward an association in the EIRA-2 replication samples, with an odds ratio of 1.11 (95% CI, 0.93 to 1.32). To provide an additional test against population stratification in the NARAC-2 replication samples, we implemented a principal components method using 704 European-derived ancestry informative markers and saw continued evidence of an association at rs3761847 ($P=0.003$).

Combining the allele counts among all 2575 samples from case subjects with anti–CCP–positive rheumatoid arthritis and 3648 samples from case subjects with anti–CCP–positive rheumatoid arthritis, including 485 anti–CCP–positive case subjects and 1282 control subjects in NARAC-2 and from 568 anti–CCP–positive case subjects and 516 control subjects in EIRA-2 to obtain additional support for our finding, as well as to perform fine mapping of the causal allele.

### Replication of TRAF1–C5 Haplotype Tag SNPs

Replication of a specific genetic model in additional samples is critical to differentiate true positive associations from statistical fluctuations. Although the combined NARAC-1 and EIRA-1 result at TRAF1–C5 was highly significant ($P=2.8\times10^{-8}$), we genotyped the most strongly associated SNPs, in addition to tag SNPs across the region, in an independent set of samples from 485 anti–CCP–positive case subjects and 1282 control subjects in NARAC-2 and from 568 anti–CCP–positive case subjects and 516 control subjects in EIRA-2 to obtain additional support for our finding, as well as to perform fine mapping of the causal allele.

A summary of the replication results for nine SNPs that capture haplotypes at TRAF1–C5 (haplotype tag SNPs) is presented in Table 2. These nine tag SNPs capture 74 of 76 (97%) of the common HapMap CEU SNPs across a 100-kb block of linkage disequilibrium that includes the TRAF1 and C5 genes, as well as another gene, PHF19 (Fig. 2).

The most significant result from the genome scan, at rs3761847, was significant in the NARAC-2 samples under the same genetic model ($P=1\times10^{-8}$), with an odds ratio of 1.37 (95% CI, 1.18 to 1.58) and showed a nonsignificant trend toward an association in the EIRA-2 replication samples, with an odds ratio of 1.11 (95% CI, 0.93 to 1.32). To provide an additional test against population stratification in the NARAC-2 replication samples, we implemented a principal components method using 704 European-derived ancestry informative markers and saw continued evidence of an association at rs3761847 ($P=0.003$).

Combining the allele counts among all 2575 samples from case subjects with anti–CCP–positive rheumatoid arthritis and 3648 samples from case subjects with rheumatoid arthritis, including $CD40 (P=3\times10^{-8})$, bradykinin receptor 1 ($BDKRI$) ($P=1\times10^{-8}$), and the 17q chemokine gene cluster containing $CCL1$, $CCL8$, and $CCL13$ ($P=4\times10^{-8}$). These SNPs require further study to determine which, if any, represent true associations with anti–CCP–positive rheumatoid arthritis. A complete list of all SNPs with $P<1\times10^{-8}$ can be found in Table 1 of the Supplementary Appendix.
control subjects and calculating the significance with a Mantel–Haenszel statistic, we observe a highly significant result at rs3761847 (P=4×10^{-14}), with an odds ratio of 1.32 (95% CI, 1.23 to 1.42) per copy of the risk allele (Fig. 2A) or P=2×10^{-16} by the omnibus haplotype test. Homozygotes for the susceptibility allele had an odds ratio of 1.87 (95% CI, 1.61 to 2.18), as compared with homozygotes for the protective allele. The attributable risk of disease conferred by the allele was 7%.

**FINE MAPPING AT TRAF1–C5**

Logistic-regression analyses that were conditional on each of the nine tag SNPs (and haplotypes defined by these SNPs) in all case–control samples showed that the most significant SNP from the genome scan (rs3761847) could explain the majority of the association signal across the locus. A neighboring SNP, rs2900180, in strong linkage disequilibrium with rs3761847, was also highly significant; the two SNPs could not be distinguished statistically in our combined sample collection. We thus considered one of these variants, or an untyped variant in strong linkage disequilibrium with rs3761847 and rs2900180, to be the causal variant.

To determine whether any putative functional variant from the public database could explain the association signal, we identified and genotyped such SNPs in the CEU HapMap and determined the extent of linkage disequilibrium between each SNP and both rs3761847 and rs2900180. (We defined putative functional motifs as those within coding exons, transcription-factor–binding sites, highly conserved regions, CpG islands, 5’ and 3’ untranslated regions, intron–exon boundaries, and microRNA binding sites.) Although no missense SNP from the database was in strong linkage disequilibrium with rs3761847, we identified a synonymous SNP in TRAF1 (rs2239657, r^2=0.97 with rs2900180) and several SNPs within highly conserved motifs for both TRAF1 and C5 (Fig. 2B).

**DISCUSSION**

This comprehensive genetic analysis of rheumatoid arthritis has led to the identification of a novel association with a 100-kb region on chromosome 9 that contains the TRAF1 and C5 genes. Our study adds to the small but growing list of validated susceptibility genes for rheumatoid arthritis that includes HLA-DRB1, PTPN22, and STAT4.

Since the most highly associated SNPs (rs376147 and rs2900180) are in linkage disequilibrium with both genes, it is not clear whether the causal alleles or group of alleles influences TRAF1 or C5 (or both) to increase susceptibility for rheumatoid arthritis. There is no known or obvious functional allele that explains these associations. In theory, the causal allele could exert its effect through a neighboring gene (e.g., PHF19), although the weight of the biologic evidence supports a role for either TRAF1 or C5. Identification of the causal allele will ultimately require the resequencing and genotyping of samples from a large number of patients with rheumatoid arthritis, together with functional studies stratified according to genotype.

The TRAF1 gene encodes an intracellular protein that mediates signal transduction through tumor necrosis factor (TNF) receptors 1 and 2 and through CD40. TNF is a critical cytokine in the pathogenesis of rheumatoid arthritis, and TNF antagonists are an effective treatment for rheumatoid arthritis. TRAF1 knockout mice have exaggerated T-cell proliferation and activation in response to TNF or when stimulated through the T-cell–receptor complex, suggesting that TRAF1 acts as a negative regulator of these signaling pathways. TRAF1 binds several intracellular proteins, including the nuclear factor-κB inhibitory protein TNFAIP3.

The clinical and biologic data for C5 are equally compelling. The complement pathway has been implicated in the pathogenesis of rheumatoid arthritis for more than 30 years. Complement activation leading to significant depletion of complement components has been shown in synovial fluid of patients with rheumatoid arthritis. C5-deficient mice are resistant to inflammatory arthritis in models with a dominant humoral component. If the causal allele acts through C5, it may do so by amplifying complement activation in joints of patients with rheumatoid arthritis.

Our initial genomewide scan (stage 1) was powered to detect moderate genetic effect sizes (odds ratio, >1.50), and only the MHC locus, PTPN22, and TRAF1–C5 achieved a P value of less
than $5 \times 10^{-8}$. Integration of data from NARAC-1 and EIRA-1 was critical in choosing SNPs for replication at TRAF1–C5, since neither study achieved a $P$ value of less than $5 \times 10^{-8}$. The recently published study by the Wellcome Trust Case Control Consortium (WTCCC) involving approximately 2000 case subjects with rheumatoid arthritis and approximately 3000 control subjects did not highlight the TRAF1–C5 region, and a SNP in near complete linkage disequilibrium with rs3761847...
(WTCCC SNP rs10118357; \(r^2=0.97\) in the CEU HapMap) was not significant at a \(P\) value of less than 0.05 in the WTCCC study. In a similar manner, our replication samples (stage 2) had limited statistical power, as made evident by a nonsignificant trend toward an association for our most significant SNP (rs3761847), in EIRA-A2 (which had a power of approximately 70% at \(P=0.05\)). Together, these findings emphasize that even rather large-scale genomewide association studies together with independent replication may have limited power to detect common risk variants of modest effect. However, we note that a recent candidate-gene study of rheumatoid arthritis supports our findings.49

There are undoubtedly additional risk variants with modest effect sizes that have yet to be discovered. For example, we integrated our data with those from the WTCCC study. Among 11 SNPs that were not within the MHC locus with moderate evidence of association (\(P<1\times10^{-5}\)) reported in the WTCCC study, we found evidence that at least 1 SNP is significant in our study (WTCCC SNP rs6902220, \(P=1.3\times10^{-5}\) in the combined NARAC-1 and EIRA-1 analysis) (Table 3 of the Supplementary Appendix). This SNP, which is located on chromosome 6q23 near the gene TNFAIP3, has been identified in a completely independent genomewide association analysis (unpublished data). Beyond the simple identification of causative alleles, it is important to recognize that genes may act at multiple different stages of disease, from early breakage of immune tolerance to the regulation of tissue destruction and the response to therapy. Therefore, continuing international collaborative studies of large cohorts of patients will be essential to understand fully the clinical significance of the wealth of genetic information that is now emerging on rheumatoid arthritis and related autoimmune disorders.

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