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Identification of AF4/FMR2 family, member 3 (*AFF3*) as a novel rheumatoid arthritis susceptibility locus and confirmation of two further pan-autoimmune susceptibility genes

Anne Barton[1,*†], Steve Eyre[1,†], Xiayi Ke[1], Anne Hinks[1], John Bowes[1], Edward Flynn[1], Paul Martin[1], YEAR Consortium[‡], BIRAC Consortium[‡], Anthony G. Wilson[2], Ann W. Morgan[3], Paul Emery[3], Sophia Steer[4], Lynne J. Hocking[5], David M. Reid[5], Pille Harrison[6], Paul Wordsworth[6], Wendy Thomson[1] and Jane Worthington[1]

[1]arc-Epidemiology Unit, Stopford Building, The University of Manchester, Manchester, UK, [2]School of Medicine & Biomedical Sciences, Sheffield University, Sheffield S10 2JF, UK, [3]Section of Musculoskeletal Disease, Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK, [4]Clinical and Academic Rheumatology, Kings College Hospital NHS Foundation Trust, Denmark Hill SE5 9RS, London, UK, [5]Musculoskeletal and Genetics Section, Division of Applied Medicine, University of Aberdeen, Aberdeen AB25 2ZD, UK, and [6]Botnar Research Centre, Institute of Musculoskeletal Sciences, University of Oxford, Oxford OX3 7LD, UK

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The concept of susceptibility genes common to different autoimmune diseases is now firmly established with previous studies demonstrating overlap of loci conferring susceptibility to type 1 diabetes (T1D) with both Coeliac disease and multiple sclerosis. Rheumatoid arthritis (RA) is an archetypal autoimmune disease and we, therefore, targeted putative T1D susceptibility loci for genotyping in UK RA cases and unrelated controls. A novel RA susceptibility locus at *AFF3* was identified with convincing evidence for association in a combined sample cohort of 6819 RA cases and 12 650 controls [OR 1.12 95% confidence intervals (CI) 1.07–1.17, $P = 2.8 \times 10^{-7}$]. Association of two previously described loci (*CTLA-4* and 4q27) with RA was also replicated (OR 0.87, 95% CI 0.82–0.94, $P = 1.1 \times 10^{-4}$ and OR 0.86, 95% CI 0.79–0.94, $P = 5.4 \times 10^{-4}$, respectively). These findings take the number of established RA susceptibility loci to 13, only one of which has not been associated with other autoimmune diseases.

INTRODUCTION

Rheumatoid arthritis (RA) is a disease in which uncontrolled inflammation of synovial joints may cause progressive joint damage and consequent long-term disability. It is a typical autoimmune disease, being characterized by the elaboration of auto-antibodies to immunoglobulins (rheumatoid factor) and cyclic citrullinated peptides (anti-CCP antibodies) and by genetic associations with the class II region of the major histocompatibility complex. Genome wide association (GWA) and subsequent validation studies have successfully identified a number of novel RA susceptibility loci but it is already clear that simply following up the top tiers of significantly associated markers from such studies is unlikely to identify all the relevant disease-associated loci (1–6). Clustering of autoimmune diseases, including type 1 diabetes (T1D) and RA, is apparent in families and it has already been established that RA and T1D share common susceptibility loci (7). For

*To whom correspondence should be addressed. Tel: +44 1612755037; Fax: +44 1612755043; Email: anne.barton@manchester.ac.uk
†These two authors contributed equally to the work.
‡Supplementary note online.

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example, a non-synonymous single nucleotide polymorphism (SNP) in the *PTPN22* gene (*PTPN22*R620W*) has been associated with susceptibility to both diseases as has polymorphism at the interleukin 2 receptor alpha (*IL2RA*) locus (1,8). Given that T1D is an autoimmune disease for which a large number of susceptibility variants have already been identified, we sought to explore the hypothesis that putative T1D susceptibility genes/loci are also associated with RA.

RESULTS

T1D putative genetic susceptibility loci were selected for investigation from information available from a previous study (8), excluding markers mapping to the *INS* (not a strong candidate gene for RA), *CLEC2D2* (associated by multi-locus imputation analysis only) and *PTPN22* (RA data published previously) (2) genes (8,9). Eighteen single nucleotide polymorphisms (SNPs) mapping to 14 distinct loci were genotyped in 3962 UK RA cases and 3531 unrelated UK controls. A Bonferroni correction of 14 was applied to correct for the number of loci studied, resulting in a *P*-value threshold of 0.004 for claims of significance. The sample size had >80% power to identify association at this threshold assuming the same effect sizes as reported previously for T1D for most of the loci investigated (Supplementary Material, Table S1) (8,9).

Of the 18 SNPs tested, four variants mapping to three loci (*AFF3, CTLA4* and *IL2/21*) showed evidence for association with RA (Table 1). These included two SNPs [rs10865035 OR 1.13 (1.06–1.21), $P = 4.0 \times 10^{-4}$ and rs1160542 OR 1.12 (1.05–1.20), $P = 0.001$] mapping to the *AFF3* locus, which are highly correlated ($r^2 = 0.97$) with each other. Conditional logistic regression analysis suggested that the primary effect is from rs10865035. A perfect proxy for this variant was genotyped as part of the Wellcome Trust Case Control Consortium (WTCCC) study (rs9653442) and also showed evidence for association in that cohort of 1860 RA cases and 2920 controls (trend $P = 4.8 \times 10^{-4}$) (1). For the rs10865035 SNP, data was available from an additional cohort of 997 RA cases, recruited as part of an inception cohort study of RA outcome and 6199 controls with published genotype frequencies available (8). In total, therefore, data was available for 6819 RA cases and 12 650 controls. Allele frequencies were similar across the three control cohorts with no evidence of heterogeneity ($P = 0.39$). Hence, a combined analysis across all samples was undertaken and confirmed strong evidence for association of this locus with RA susceptibility (OR 1.12 95% CI 1.07–1.17, $P = 2.8 \times 10^{-7}$).

A variant mapping to the *CTLA-4* gene showed the strongest evidence for association with RA in the current cohort (rs3087243 OR 0.87, 95% CI 0.82–0.94, $P = 1.1 \times 10^{-4}$). There have been previous reports of association of this variant with RA but findings have been inconsistent, probably reflecting the modest sample sizes used in many of the previous investigations (10,11). Combining the results from two previous well-powered studies with the current data, the SNP is confirmed as being associated with RA susceptibility [OR 0.88 (0.83–0.93), $P = 2.6 \times 10^{-6}$] (Fig. 1) (11).

A SNP mapping within the *IL2-IL21* locus on chromosome 4q27, previously reported to be associated with T1D, Coeliac

Table 1. T1D-associated SNPs tested for association with RA

Marker	Chr	Locus/Gene	HWE control	MAF case	MAF control	Genotype frequency cases (%)			Genotype frequency controls (%)			Trend *P*-value	Allelic OR (95% CI)
rs1160542	2	*AFF3*	0.32	0.47	0.45	833 (22.4)	1860 (49.9)	1033 (27.7)	574 (19.3)	1493 (50.3)	900 (30.3)	0.001	1.12 (1.05–1.20)
rs10865035	2	*AFF3*	0.26	0.49	0.46	882 (24.2)	1786 (49.0)	976 (26.8)	596 (20.3)	1488 (50.6)	854 (29.1)	4.0×10^{-4}	1.13 (1.06–1.21)
rs1990760	2	*IFIH1*	0.77	0.39	0.39	594 (15.5)	1803 (47.1)	1427 (37.3)	525 (15.3)	1627 (47.3)	1286 (37.4)	0.839	1.01 (0.94–1.08)
rs3087243	2	*CTLA4*	0.74	0.42	0.46	677 (18.5)	1760 (48.0)	1232 (33.5)	634 (20.8)	1523 (50.0)	892 (29.2)	1.1×10^{-4}	0.87 (0.82–0.94)
rs17388568	4	*IL2*	0.77	0.28	0.28	308 (7.9)	1530 (39.3)	2057 (52.8)	271 (7.8)	1415 (40.6)	1797 (51.6)	0.46	0.97 (0.91–1.05)
rs6822844	4	*IL2_IL21*	0.19	0.16	0.18	95 (2.4)	1052 (27.1)	2739 (70.5)	125 (3.6)	1003 (29.0)	2326 (67.4)	5.4×10^{-4}	0.86 (0.79–0.94)
rs6897932	5	*IL7R*	0.06	0.27	0.29	270 (6.8)	1573 (39.9)	2100 (53.3)	267 (7.6)	1482 (42.3)	1756 (50.1)	0.007	0.91 (0.84–0.97)
rs3194051	5	*IL7R*	0.45	0.27	0.26	296 (7.5)	1551 (39.4)	2088 (53.1)	239 (6.8)	1318 (37.7)	1942 (55.5)	0.03	1.08 (1.00–1.17)
rs7722135	5	*CCNH*	0.19	0.22	0.22	198 (5.5)	1211 (33.4)	2217 (61.1)	146 (4.3)	1185 (34.6)	2094 (61.1)	0.39	1.04 (0.96–1.12)
rs213950	7	*CFTR*	0.19	0.41	0.40	654 (16.6)	1890 (48.0)	1397 (35.4)	570 (16.3)	1639 (46.8)	1294 (36.9)	0.26	1.04 (0.97–1.11)
rs2666236	10	*NRP1*	0.89	0.42	0.40	673 (17.2)	1921 (49.1)	1316 (33.7)	569 (16.3)	1689 (48.3)	1240 (35.4)	0.09	1.06 (0.99–1.13)
rs2292239	12	*ERBB3*	0.39	0.35	0.35	518 (13.2)	1692 (42.9)	1730 (43.9)	432 (12.4)	1558 (44.7)	1498 (42.9)	0.90	1.00 (0.93–1.07)
rs17696736	12	*C12ORF30*	0.66	0.45	0.44	825 (20.9)	1899 (48.2)	1217 (30.9)	671 (19.2)	1707 (48.8)	1119 (32.0)	0.08	1.06 (0.99–1.13)
rs12708716	16	*KIAA0350*	0.63	0.36	0.36	507 (12.9)	1791 (45.4)	1643 (41.7)	453 (12.9)	1596 (45.6)	1455 (41.5)	0.88	1.00 (0.93–1.06)
rs2542151	18	*PTPN2*	0.13	0.17	0.17	123 (3.1)	1133 (28.7)	2696 (68.2)	111 (3.2)	954 (27.2)	2442 (69.6)	0.27	1.05 (0.96–1.14)
rs763361	18	*CD226*	0.84	0.48	0.46	942 (23.9)	1902 (48.2)	1100 (27.9)	745 (21.2)	1750 (49.9)	1012 (28.9)	0.03	1.08 (1.01–1.15)
rs6017667	20	*C20ORF168*	0.14	0.35	0.36	474 (12.2)	1799 (46.3)	1615 (41.5)	483 (13.9)	1571 (45.2)	1422 (40.9)	0.14	0.95 (0.89–1.02)
rs380421	20	*C20ORF168*	0.10	0.35	0.36	469 (12.0)	1821 (46.4)	1631 (41.6)	479 (13.8)	1560 (44.9)	1436 (41.3)	0.19	0.96 (0.89–1.02)

The gene name refers to the nearest gene in the region although SNPs are not necessarily intra-genic.
Chr, chromosome; HWE, *P*-value statistic for Hardy–Weinberg equilibrium test; MAF, minor allele frequency; OR, odds ratio (95% confidence intervals).

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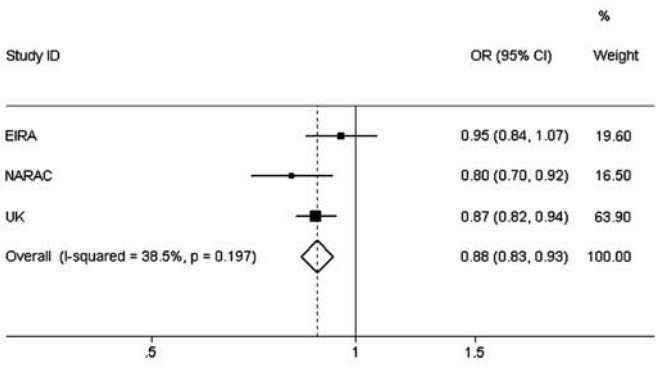

Figure 1. Meta-analysis of current data for the rs3087243 SNP in the *CTLA-4* gene with data from a previous study in two well-powered cohorts of RA patients and controls.

disease and RA, was also associated with RA susceptibility in the current series (9). As reported previously, it was the common allele that predisposed to disease [rs6822844 OR 0.86, (0.79–0.94), $P = 5.4 \times 10^{-4}$].

The three loci show evidence for association with T1D and RA and are strong candidates for pan-autoimmunity susceptibility genes. If these loci predispose to autoimmunity in general, it might be expected that associations would be stronger in subsets of patients with auto-antibodies. However, following stratification by presence of auto-antibodies, the strength of associations was similar between auto-antibody positive and negative individuals at all three SNPs indicated by the overlapping confidence intervals (Table 2).

Finally, although the common allele of a non-synonymous SNP (rs6897932) mapping to the *IL7R* gene previously associated with both T1D and multiple sclerosis was somewhat over-represented in RA cases (OR 0.90, 95% CI 0.84–0.97; trend $P = 0.007$), this did not achieve statistical significance at the corrected *P*-value threshold (8,12,13).

DISCUSSION

The concept of pan-autoimmunity susceptibility genes is now firmly established with recent reports of overlap between T1D and Coeliac disease as well as T1D and multiple sclerosis (14,15). By targeting variants with suggestive evidence for association with T1D, we have identified a novel association of the *AFF3* locus with RA susceptibility. In addition, association of two other loci, the *CTLA4* and *IL2-IL21* regions, has also been confirmed.

The association of variants in the *AFF3* locus with susceptibility to RA has not previously been described. The variant was selected for genotyping because there was suggestive evidence for association with T1D in one large case–control series ($P = 5.0 \times 10^{-6}$), although this was not subsequently replicated in a smaller family-based study (8). However, we have found evidence for association of the variant in three independent case–control series including UK patients with RA and controls with combined analysis reaching a threshold that some regard as achieving genome-wide significance ($P < 5 \times 10^{-7}$). The associated variants map to the 5′ and promoter region of the *AFF3* gene, which is strongly conserved in

Table 2. Results for SNPs with evidence for association with T1D and RA after stratifying by auto-antibody status in RA patients

SNP	Locus	Control genotype counts: *n* (%) 1/1 1/2 2/2			CCP neg+RF neg genotype counts: *n* (%) 1/1 1/2 2/2			OR (95% CI)[a]	CCP pos +/or RF pos genotype counts: *n* (%) 1/1 1/2 2/2			OR (95% CI)[a]
rs10865035	*AFF3*	854 (29.1)	1488 (50.6)	596 (20.3)	104 (27.6)	177 (46.9)	96 (25.5)	1.14 (0.98–1.33)	661 (24.1)	1352 (49.3)	728 (26.6)	1.14 (1.06–1.22)
rs3087243	*CTLA4*	892 (29.2)	1523 (50.0)	634 (20.8)	112 (29.8)	195 (51.9)	69 (18.4)	0.94 (0.81–1.1)	518 (35.4)	1303 (45.9)	938 (18.6)	0.87 (0.81–0.94)
rs6822844	*IL2_21*	2326 (67.3)	1003 (29.0)	125 (3.6)	277 (72.1)	97 (25.3)	10 (2.6)	0.81 (0.66–1.0)	2061 (70.9)	775 (26.7)	71 (2.4)	0.85 (0.77–0.93)

RF, rheumatoid factor; CCP, anti-cyclic citrullinated peptide antibody; Pos, positive; Neg, negative.
Number of patients for whom auto-antibody data available: RF pos=2470; RF neg=934; Anti-CCP pos=1446; Anti-CCP neg=674; CCP neg+RF neg (auto-antibody negative)=400; CCP pos +/or RF pos (auto-antibody positive)=2958.
[a]OR (95% CI)=odds ratio and 95% confidence intervals.

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evolution and is preferentially expressed in lymphoid cells (16). It encodes a nuclear factor that can interact with DNA and contains transcriptional activation domains, making it a strong candidate autoimmunity gene.

Our findings also support association of the *CTLA4* gene with RA susceptibility. CTLA4 is a co-stimulatory receptor expressed on activated T cells which down-regulates the T cell response. The rs3087243 SNP maps within the 3′ region of the *CTLA-4* gene and previous functional studies have identified a correlation between the ratio of soluble to full length CTLA4 mRNA levels with genotype at this variant (17). Interestingly, the fusion protein, Abatacept (Orencia), a biologic drug with proven efficacy in RA, is a CTLA-4 analogue, highlighting the fact that relatively modest genetic effects may identify targets for treatment which are highly effective in large numbers of patients (18).

A region on chromosome 4q27 also showed evidence for association with RA susceptibility in our UK series. The region exhibits substantial linkage disequilibrium encompassing both the *IL2* and *IL21* genes. Since both are good candidates as autoimmunity susceptibility genes, identifying the causal polymorphism to determine exactly how it predisposes to autoimmune diseases will be challenging.

It is interesting to note that the *IL7R* gene showed a trend towards association with RA. The rs6897932 SNP is located in exon 6 and is associated with increased exon skipping resulting in an alteration in the ratio of the membrane-bound to soluble receptor. The IL7 pathway is important in T cell growth and plays a role in regulating cytokine production and, interestingly, expression of IL7R has been correlated with disease activity in peripheral blood mononuclear cells from patients with RA (19). However, association in other RA data sets is required before this locus can be confidently confirmed as an RA susceptibility gene.

In summary, by targeting loci with prior evidence for association with T1D, we have identified a novel association of *AFF3* with RA susceptibility. We have also confirmed association of RA with two other loci (*CTLA4* and *IL2-21*/4q27) so that a total of 13 loci have now been confirmed as associated with RA susceptibility (*HLA DRB1, PTPN22, STAT4, TRAF1/C5*, 6q23*, IL2RB*, 12q13, 10p15*, CD40, MMEL1, AFF3*, 4q27 and *CTLA-4*). Interestingly, for all but one of these loci (*TRAF1/C5*), association with other autoimmune diseases, including systemic lupus erythematosus, Graves disease and coeliac disease, has been described, firmly establishing the concept of common susceptibility loci for autoimmunity.

MATERIALS AND METHODS

Sample collections

Patients with RA ($n = 3{,}962$) were recruited from six centres (Manchester, Sheffield, Leeds, Aberdeen, Oxford and London) across the UK as described previously (2). All cases were Caucasian of Northern European descent and all fulfilled the 1987 American College of Rheumatology classification criteria modified for genetic studies (20,21). Clinical and demographic characteristics of the cohort are detailed in Supplementary Material, Table S2. Briefly, 72% subjects were female, 72% were rheumatoid factor positive and 68% carried ACPA antibodies as recognized by the anti-CCP antibody test. Therefore, this cohort is representative of a hospital-based series of RA subjects. Healthy Caucasian controls ($n = 3531$) were recruited from five of the six centres (cases only recruited from London). All participants were recruited after providing informed consent and the study was approved by the North West Research Ethics Committee (MREC 99/8/84). Additional patient samples ($n = 997$) from subjects satisfying ACR criteria for RA (20,21) were obtained from an inception cohort of patients with inflammatory polyarthritis recruited from the Norfolk Arthritis Register (NOAR). Sixty-eight percent of patients were female, 39% were RF positive at baseline and 52% had developed erosions by 5 years. Local Ethics approval was obtained and all patients provided informed consent (LREC 2003-075).

Immunogenetics

Serum RF and anti-CCP antibody titre were measured using commercially available kits [RF-PAIA Immunoturbidimetric Assay for rheumatoid factor, Diastat^{TM} Anti-CCP Kit (Axis-Shield Diagnostics Limited, UK)]. Patients with titres $\geq$40 units/ml and $\geq$5 units/ml were defined as positive for RF and anti-CCP antibodies, respectively. Anti-CCP status was available only for a subset of the total validation case cohort ($n = 2370$).

Genotyping

Genotyping was performed using the Sequenom iPlex platform (www.sequenom.com) according to the manufacturer's instructions. All genotyping was performed at the **arc** Epidemiology Unit, Manchester. Details of the primer and probe sequences used for the SNPs are available on request. Only samples and SNPs exceeding a 90% success rate were used in the analysis.

Analysis

Genotype counts in cases and controls were analysed using PLINK software using the χ^2 test for trend (22). A corrected significance threshold of $P < 0.004$ was selected based on a Bonferroni correction for 14 independent loci tested as part of this study. Power calculations were performed using the CaTS power calculator tool (http://www.sph.umich.edu/csg/abecasis/cats/). Stratification analysis was performed by subgrouping the cases into anti-CCP antibody positive and negative subgroups for comparison with the controls. Stepwise logistic regression was used to determine whether the association observed at the *AFF3* locus was primarily due to linkage disequilibrium between the associated SNPs or whether independent effects existed. The possibility of population stratification was not examined because genome-wide data was only available for the WTCCC data set. However, in that cohort, no evidence for global population stratification in the UK population tested was identified ($\lambda_{GC} = 1.03$ for RA samples versus controls) and allele frequencies in the two other UK control cohorts examined as part of the current study did not differ from the control cohort tested as part of the WTCCC study (1).

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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