Rheumatology 2008;47:1761–1767
Advance Access publication 3 October 2008
doi:10.1093/rheumatology/ken376

Fine mapping of the MHC Class III region demonstrates association of *AIF1* and rheumatoid arthritis

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Objectives. The heritability of RA has been estimated to be ~55%, of which the MHC contributes about one-third. *HLA-DRB1* alleles are strongly associated with RA, but it is likely that significant non-DRB1 MHC genetic susceptibility factors are involved. Previously, we identified two three-marker haplotypes in a 106-kb region in the MHC class III region immediately centromeric to TNF, which are strongly associated with RA on *HLA-DRB1*0404* haplotypes. In the present study, we aimed to refine these associations further using a combination of genotyping and gene expression studies.

Methods. Thirty-nine nucleotide polymorphisms (SNPs) were genotyped in 95 DRB1**0404* carrying unrelated RA cases, 125 DRB1**0404*-carrying healthy controls and 87 parent-case trio RA families in which the affected child carried *HLA-DRB1*04*. Quantitative RT–PCR was used to assess the expression of the positional candidate MHC class III genes *APOM, BAT2, BAT3, BAT4, BAT5, AIF1, C6orf47, CSNK2β* and *LY6G5C*, and the housekeeper genes, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and β_2-microglobulin (*B2M*) in 31 RA cases and 21 ethnically, age- and sex-matched healthy controls. Synovial membrane specimens from RA, PsA and OA cases were stained by an indirect immunoperoxidase technique using a mouse–anti-human AIF1 monoclonal antibody.

Results. Association was observed between RA and single markers or two marker haplotypes involving *AIF1, BAT3* and *CSNK*. *AIF1* was also significantly overexpressed in RA mononuclear cells (1.5- to 1.9-fold difference, $P = 0.02$ *vs HPRT*, $P = 0.002$ *vs B2M*). AIF1 protein was clearly expressed by synovial macrophages in all the inflammatory synovial samples in contrast to the non-inflammatory OA samples.

Conclusions. The results of the genotyping and expression studies presented here suggest a role for *AIF1* in both the aetiology and pathogenesis of RA.

Key words: Rheumatoid arthritis, Immunogenetics, Major histocompatibility complex.

Introduction

RA is a common complex disease affecting ~1% of most populations worldwide. Twin studies have estimated the heritability of RA to be ~55%, and that about one-third is contributed by the MHC region [1]. The *HLA-DRB1* locus in the class II region of the MHC is consistently associated with RA susceptibility in diverse ethnic groups. Susceptibility to RA is conferred by specific *HLA-DRB1* alleles that encode a conserved linear sequence of amino acids in the DRβ1 chain, at positions 67–74, known as the shared epitope (SE) [2]. However, the SE does not explain all of the MHC associations in RA. RA is associated with non-SE *HLA-DRB1* alleles in some populations [3, 4], different SE alleles have different strengths of disease association [5] and some MHC haplotypes carrying the same DRB1 alleles are differentially associated with RA [6, 7]. Recently, Amos and colleagues [8] have demonstrated significant linkage of RA to the MHC in families where the affected relative pairs do not carry SE alleles [logarithm of the odds ratio (LOD) = 3.9]. Consequently, it appears that other genes within the MHC) are involved in disease susceptibility. Although many studies have investigated the possibility of non-*HLA-DRB1* MHC susceptibility genes, most of these studies have taken inadequate account of the extensive linkage disequilibrium in the MHC, resulting in failure to distinguish true associations from those due to linkage disequilibrium with *HLA-DRB1* alleles [9]. Very few studies have been performed that control for the DRB1 associations of RA at allelic level, and can thus accurately study association as opposed to linkage disequilibrium with HLA-DRB1 [6, 10, 11].

We have previously identified a single nucleotide polymorphism (SNP) haplotype, which includes the lymphotoxin alpha (LTA) and TNF loci in the MHC class III region of chromosome 6 that shows differential association with RA on *HLA-DRB1*0404* and *DRB1*0401* haplotypes, suggesting the presence of additional non-*HLA-DRB1* RA susceptibility genes on these haplotypes [7]. These findings are supported by Jawaheer *et al.* [6], who demonstrated association of RA with different MHC haplotypes carrying either *HLA-DRB1*0404* or *DRB1*0401*. Using microsatellite markers we refined the regions identified in our own studies, identifying two three-marker haplotypes in a region of 122 kb in the MHC class III region immediately centromeric to TNF which are strongly associated with RA on *HLA-DRB1*0404*, but not on *DRB1*0401*, haplotypes [10]. The most strongly associated haplotype, d6s2671/3-MN6s1997/5-d6s273/5, spans 106 kb and encompasses 12 genes. The genes in this interval include allograft inflammatory factor 1 (*AIF1*), HLA-B associated transcripts (*BAT2*, *BAT3*, *BAT4*, *BAT5*), Apolipoprotein M (*APOM*), Ly6 Superfamily members *LY6G5B, LY6G5C*, *LY6G6C* and *LY6G6D*, Casein Kinase 2subunit beta (*CSNK2B*) and *C6orf47*.

In the present study, we aimed to refine these associations further using a combination of genotyping and gene expression studies. Over 600 SNPs were identified in the candidate region by a search of electronic databases including dbSNP. Of these, 39 SNPs, either exonic or lying close to exon–intron boundaries, and with published heterozygosity data, were genotyped in *HLA-DRB1*0404* unrelated cases and controls and in parent-case trio families with children carrying *HLA-DRB1*0404*. Nine of these positional candidate genes showed nominal association with RA ($P < 0.05$) and we investigated the expression of these using

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Submitted 22 January 2008; revised version accepted 19 August 2008.

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real-time quantitative PCR in peripheral blood mononuclear cells (PBMCs) from RA patients and matched healthy controls. We also compared the expression of AIF1 in the synovium from inflammatory and non-inflammatory disorders.

Materials and methods

Study population

Cases and controls in this study were British Caucasians. All subjects gave informed written consent and approval had been granted from the local ethics committee. For the genotyping studies, 95 unrelated *HLA-DRB1*04*-positive RA cases, 125 *DRB1*04*-positive unrelated controls and 87 parent-case trios were available. All cases satisfied the 1987 ACR criteria for RA [12], and all unrelated cases and controls carried *DRB1*0404*, as the specific aim of this project was to map the HLA-class III associations we had previously observed on the background of *HLA-DRB1*0404*-bearing haplotypes. In total, there were 306 *DRB1*0401*, 290 *DRB1*0404* and 366 non-*DRB1*0401/0404* haplotypes available for study. Of the cases, 103 *DRB1*0401*, 128 *DRB1*0404* and 127 non-*DRB1*0401/0404* haplotypes were available for study.

For the RTq–PCR study, we tested 31 Caucasian RA patients fulfilling the 1987 ACR criteria for RA [12]. These patients were taking standard DMARDs and were compared with 21 ethnically, age- and sex-matched healthy controls.

Genotyping studies

SNP selection and genotyping. Cases, controls and families were genotyped for 39 SNPs in this region. Eleven SNPs (rs2736182, rs9688644, rs1062968, rs6921213, rs1065503, rs3130049, rs2242655, rs2295664, rs2254083, rs10298 and rs2242654) proved to be monomorphic and were excluded from further analysis. The average spacing of the remaining SNPs was 3.9 kb. Details of the polymorphic SNPs, including their locations are summarized in Table 1. All SNPs were genotyped by KBiosciences (Hoddesdon, Herts, UK) using a mixture of competitive allele PCR (KASPar) and TaqMan® genotyping assays (Applied Biosystems, CA, USA). Details of SNP genotyping protocols are available on request. *HLA-DRB1*04* subtyping was performed by PCR/amplification refractory mutation system (ARMS) as previously described [7].

RTq–PCR study

RNA extraction. Peripheral blood was collected into pre-cooled EDTA tubes and all samples were processed within 60 min. Mononuclear cells were isolated from 15 ml of peripheral whole blood by Ficoll density gradient centrifugation. RNA extraction was performed using the Qiagen RNAeasy kit according to the manufacturer's instructions (Qiagen, Sussex, UK). The RNA samples were DNase treated. The quality and concentration of the RNA samples were assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA).

cDNA synthesis. The same reverse transcriptase and protocol was used for all cDNA synthesis reactions. Each reaction contained 2 μg of total RNA, and 2 μl of oligodT primer (100 ng/μl) in a 20-μl reaction volume, which was denatured at 75°C for 10 min then cooled on ice for 2 min. Samples were then treated with DNase by the addition of 8 μl of 5× first strand buffer, 4 μl of 0.1 M dithiothreitol (DTT), 2 μl of dNTPs (10 mM each), 1 μl of DNase (100 U/μl), 0.5 μl of RNase inhibitor (40 U/μl) and 2.5 μl of water. The mixture was then incubated 37°C for 30 min. The DNase was inactivated at 75°C for 5 min, following which the mixture was immediately placed on ice for 2 min. One microlitre each of Moloney Murine Leukemia Virus (M-MLV) (200 U/μl) and RNase inhibitor were added and the sample incubated at 42°C for 90 min then heat inactivated at 94°C for 5 min. The final cDNA concentration was diluted 1:3 with sterile RNAse free water, and stored at −20°C.

Primer and probes for RTq–PCR. In our real-time study, mRNA expression of the nine MHC class III genes that showed evidence of genetic association with RA in any analysis: *APOM, BAT2, BAT3, BAT4, BAT5, AIF1, C6orf47, CSNK2B* and *LY6G5C*, and of two housekeeper genes, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and β_2-microglobulin (*B2M*), was measured. The primers and probes for *HPRT* were designed using Primer Express 1.5 (Applied Biosystems, sequences available on request). The primers and probes for the 11 genes studied were from 'Assay on Demand' kits (Applied Biosystems).

RTq–PCR protocol. RTq–PCR was performed using a Rotorgene 3000 (Corbett Research, Sydney, Australia), and a primer/probe-based system. RTq–PCRs contained 5 μl of universal qPCR mastermix (Eurogentec, Hampshire, UK), 0.5 μl of 'Assay on Demand' (Applied Biosystems), 3.5 μl of RNAse free water and 1 μl of cDNA template. Two housekeeping genes, *HPRT* and *B2M*, were studied. After an initial single cycle of 50°C for 2 min and 95°C for 10 min, the PCR programme was 45 cycles of 95°C for 15 s and 60°C for 60 s. All RTq–PCRs included a standard curve (SC) using pooled cDNA diluted over a representative range of concentrations, and a common reference calibrator to check for intra-run variability. All standards were run in triplicate; all samples were run in duplicate.

The effect of SNPs on expression of the genes listed above was investigated using publicly available data from lymphoblastoid cell lines from 206 families of British descent ($n = 830$ individuals). In these samples, expression has been assessed using Affymetrix gene-expression chips (U133 Plus 2.0 chips, including 54 675 distinct probe targets), and tested for association with genomewide SNP genotype data from Illumina HumHap300 and Sentrix-1 chips (covering in total 408 273 SNPs) [13]. Heritability of expression levels and the contribution of individual SNPs to that heritability was also assessed within the studied families.

Immunohistochemistry

Fresh specimens of SM were obtained from patients undergoing hip or knee arthroplasty for OA ($n = 6$), RA ($n = 4$) or PsA ($n = 2$). SM was fixed in formalin and embedded in paraffin wax; 5 μm sections were stained by an indirect immunoperoxidase technique using mouse–anti-human AIF1 mAb (ab15690, Abcam, Cambridge, UK) at a 1:100 dilution.

Serial sections were similarly stained with the mAbs, F7.2.38, L26 and KP1 (Dakopats, UK) directed against CD3 (T cells), CD20 (B cells) and the CD68 (macrophages), respectively. Antigens were detected by incubation with labelled polymer and diaminobenzidine (Envision + kit; Dakopats). The sections were counterstained with haematoxylin. Sections treated with an isotype control IgG2b antibody, (ab9403-1, Abcam) in primary antibody diluent alone were negative controls.

Statistical analysis

Genotyping studies. Genotypes were checked for Hardy–Weinberg equilibrium by chi-squared test (1df) compared with expected values. Mendelian inheritance of markers was verified in single-case families.

Coverage of genetic variation by genotyped SNPs in genes targeted was assessed using HapMap data from Caucasian families (HapMap data release 23a, www.hapmap.org), using Haploview 4.1.

Association analysis was performed using the programme 'Whap' (http://pngu.mgh.harvard.edu/purcell//whap/). Whap uses the expectation-maximisation (EM) algorithm to determine the posterior probability of possible haplotypes, then uses all

TABLE 1. Details of 28 polymorphic SNPs typed in this study

Gene name	SNP (rs number)	SNP Alleles	SNP location in gene and detail	Position
AIF1	rs2259571	A/C	Uncertain. Either UTR, intron 2 or intron 3.	31691806
AIF1	rs2269475	G/A	Uncertain. Either exon 3, non-synonymous coding SNP, Tryptophan to Arginine, or intron 4.	31691910
BAT2	rs2857693	A/C	UTR	31696363
BAT2	rs1046080	A/C	Exon 13, non-synonymous coding SNP, Lysine to Threonine change.	31703787
BAT2	rs2242660	C/T	Intron 15	31705732
BAT2	rs2736158	C/G	Exon 17, non-synoymous coding SNP, Alanine to Glycine change.	31708283
BAT2	rs1046089	G/A	Exon 23, non-synonymous coding SNP, Histidine to Arginine change.	31710946
BAT2	rs11538264	C/T	Exon 24, non-synonymous coding SNP, Methionine to Valine.	31711168
BAT2	rs9267522	A/G	Exon 25, synonymous coding SNP, Glutamine	31711749
BAT2	rs3132453	A/C	Exon 27, non-synonymous coding SNP, Leucine to Valine change.	31712023
BAT3	rs2178899	A/T	UTR	31714735
BAT3	rs1052486	C/T	Exon 14, non-synonymous coding SNP, Proline to Serine change.	31718665
BAT3	rs805299	A/T	UTR	31727631
BAT3	rs813115	C/T	UTR	31727999
APOM	rs805264	C/T	Intron 1	31731852
APOM	rs707921	G/T	Intron 5	31733520
C6orf47	rs3130617	C/T	Exon 1, non-synonymous coding SNP, Arginine to Glycine change.	31735502
BAT4	rs7029	A/G	Exon 3, synonymous coding SNP, Arginine.	31737932
BAT4	rs7992	A/G	Exon 3, synonymous coding SNP, Arginine.	31738220
CSNK2B	rs14365	A/G	Exon 3, synonymous coding SNP, Tyrosine.	31743689
CSNK2B	rs5872	A/T	UTR	31745713
LY6G5B	rs9267532	T/C	Exon 3, non-synonymous coding SNP, Cystine to Arginine.	31747958
LY6G5C	rs13295	A/G	UTR	31752619
LY6G5C	rs2280800	G/T	Intron 2	31754377
BAT5	rs10573	G/A	Exon 18, synonymous coding SNP, Proline.	31763417
BAT5	rs1475865	A/G	Exon 12, synonymous coding SNP, Leucine.	31765392
LY6G6D	rs405722	T/G	UTR	31793924
LY6G6C	rs805294	C/T	Intron 2	31796196

Position is given in base pairs from the p-telomere of chromosome 6, as per HapMap Data Release July 2006. UTR: untranslated region. *AIF1* translation/transcription patterns are uncertain in public databases.

possible haplotypes in the analysis, weighted by their likelihood. To allow both case/control and parent-case trio data to be combined in the analysis, the parent-case trio contribution was restricted to the within-family component of variation. The population prevalence of RA was set at 1%. Single-marker analysis was performed, either controlling for the effect of linkage disequilibrium with *HLA-DRB1* or not, as specified in the results, using logistic regression implemented within Whap. Where single-marker findings achieved $P < 0.1$, two-marker haplotypes with neighbouring SNPs were also studied. Only haplotypes making up >5% of the total available for study were analysed. Results are presented as uncorrected *P*-values. *P*-values corrected for the number of markers and haplotypes studied were calculated by permutation testing within Whap. To correct for multiple testing, the number of independent markers genotyped was assessed using spectral decomposition as implemented by the programme SNPSpD [14], which takes into account linkage disequilibrium between marker pairs. Note that because the analysis involves within-family association, conventional measures of effect sizes such as odds ratios are not available.

RTq–PCR analysis

RTq–PCR measures the increase in fluorescent signal (δRn) generated by the accumulation of product. The threshold cycle (C_T) is the cycle at which a statistically significant increase in δRn is first detected and is related to the amount of target cDNA. All measurements were performed in duplicate. Analysis was done using the two SC method. Patient and control findings were compared by analysis of variance.

Results

Genotyping studies

All 28 SNP analysed in these studies were in Hardy–Weinberg equilibrium. Spectral decomposition analysis indicates that an uncorrected *P*-value of 0.0035 (corrected for 14.4 independent observations) was equivalent to a corrected *P*-value of 0.05.

Of the 28 SNPs genotyped, 26 had been typed by the International HapMap project (rs813115 in *BAT3*, and rs5872 in *CSNK2B* had not been). These 26 SNPs captured 78/96 SNPs (81%) in the 12 positional candidate genes with $r^2 > 0.8$ (mean $r^2 = 0.989$).

Single- and two-marker haplotype results are summarized in Table 2. Genotype counts from unrelated cases and controls (excluding parent-case trios) are provided in Supplementary Table 1 (see supplementary data available at *Rheumatology* Online). Three regions demonstrate nominally significant association with RA ($P < 0.05$ uncorrected), involving SNPs in *AIF1*, *BAT3* and *CSNK2B*. At *AIF1*, association with the two-marker combination *AIF1.1-AIF1.2* showed global association with a *P*-value of 0.0067 ($P_{corrected} = 0.1$), and of the three-marker combination *AIF1.1-AIF1.2-BAT2.1* ($P = 0.003$, $P_{corrected} = 0.043$). The associations at *BAT3* and *CSNK2B* were

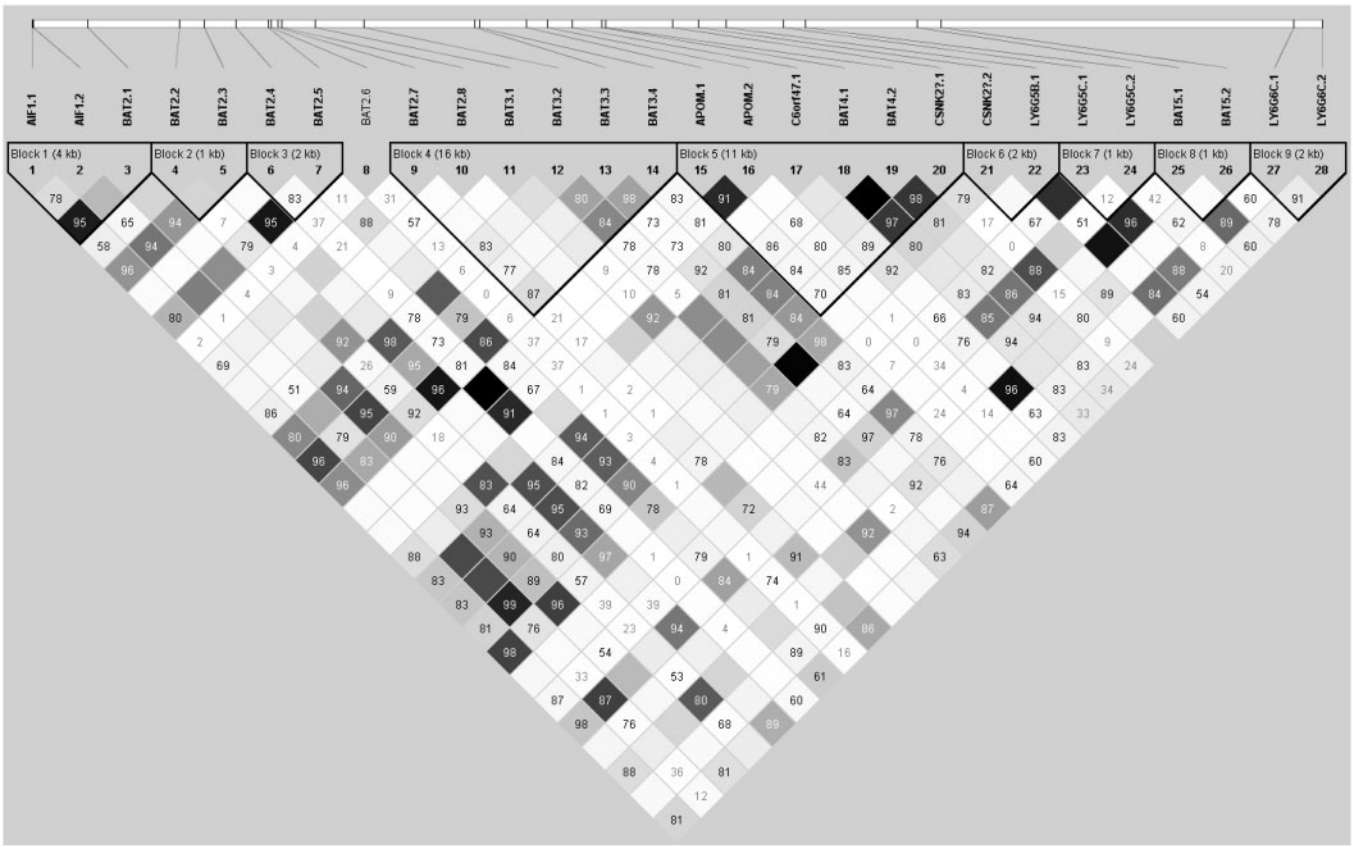

FIG. 1. Pairwise linkage disequilibrium (r^2) across study region. Haplotype blocks define by 'solid spine of LD' option in Haploview 4.1. The significantly associated markers AIF1.1, AIF1.2 and BAT2.1 form one haplotype block. The block shades represent strength of linkage disequilibrium ranging from $r^2 = 1$ in black to $r^2 = 0$ in white.

not significant ($P_{\text{corrected}} > 0.3$) and were therefore not studied further.

Linkage disequilibrium (LD) in the region was assessed and r^2-values are shown in Fig. 1. A high degree of LD was observed between markers, which did not decay with distance. There was no significant correlation between LD and intermarker distance ($r = -0.06$, $P = 0.34$). Nine blocks of LD were identified, including one block containing markers AIF1.1, AIF1.2 and BAT2.1, in which significant association with disease was observed.

RTq–PCR study

Reproducibility and accuracy. Two housekeeping genes were run for each positional candidate gene to improve accuracy of results. Both of the housekeeping genes, *HPRT* and *B2M*, were stably expressed. In addition, the common reference sample did not vary between reactions. Lastly, the SC produced nearly identical results in reactions performed on separate occasions. These quality control measures demonstrate that these RTq–PCR findings are highly reproducible.

RTq–PCR findings. A summary of the RTq–PCR results is shown in Table 3. *AIF1* was significantly over expressed in RA case mononuclear cells (1.5- to 1.86-fold difference, $P = 0.02$ *vs HPRT*, $P = 0.002$ *vs B2M*). *C6orf47* was significantly under-expressed in RA mononuclear cells (4-fold, $P = 0.03$ *vs HPRT*, $P = 0.02$ *vs B2M*). We were unable to detect expression of *LY6G5C*; it may not be expressed in mononuclear cells, or, its expression may be too low to be detected by this method. There was no significant difference in mRNA expression in RA case mononuclear cells compared with controls for the following six genes: *APOM, BAT2, BAT3, BAT4, BAT5* and *CSNK2B*.

TABLE 2. Single-marker and two-marker haplotype results, uncontrolled and controlled for the effect of linkage disequilibrium between SNPs and *HLA-DRB1*, and the association of DRB1 with RA

SNP		Uncontrolled		Controlled	
Name	Number	Single marker	Two marker	Single marker	Two marker
AIF1.1	rs2259571	NS	–	0.095	0.0067
AIF1.2	rs2269475	NS	–	NS	–
BAT2.1	rs2857693	NS	0.016	NS	–
BAT2.2	rs1046080	0.0072	0.03	NS	–
BAT2.3	rs2242660	NS	0.031	NS	–
BAT2.4	rs2736158	0.01	NS	x	–
BAT2.5	rs1046089	NS	–	NS	–
BAT2.6	rs11538264	NS	–	NS	–
BAT2.7	rs9267522	NS	0.056	NS	–
BAT2.8	rs3132453	0.024	0.041	x	–
BAT3.1	rs2178899	NS	–	NS	0.045
BAT3.2	rs1052486	NS	–	0.024	NS
BAT3.3	rs805299	NS	–	0.023	NS
BAT3.4	rs813115	NS	NS	NS	NS
APOM.1	rs805264	0.067	0.04	x	x
APOM.2	rs707921	0.0097	0.0074	x	–
C6orf47.1	rs3130617	0.044	NS	NS	–
BAT4.1	rs7029	NS	–	NS	–
BAT4.2	rs7992	NS	–	NS	–
CSNK2β.1	rs14365	NS	–	0.084	0.016
CSNK2β.2	rs5872	NS	–	0.07	0.029
LY6G5B.1	rs9267532	NS	–	x	x
LY6G5C.1	rs13295	NS	–	x	–
LY6G5C.2	rs2280800	NS	–	NS	–
BAT5.1	rs10573	NS	0.041	X	–
BAT5.2	rs1475865	0.024	NS	NS	–
LY6G6C.1	rs405722	NS	NS	x	–
LY6G6C.2	rs805294	NS	–	NS	–

SNP names are the name of the gene, followed by the number assigned to each SNP within the relevant gene. NS = $P > 0.1$. Analysis was restricted to DRB1*0404 haplotypes where the major SNP-DRB1 haplotype contributed <0.95 of all the observed haplotypes. Where this was not the case, the result is denoted by 'x'. Results are uncorrected for multiple comparisons.

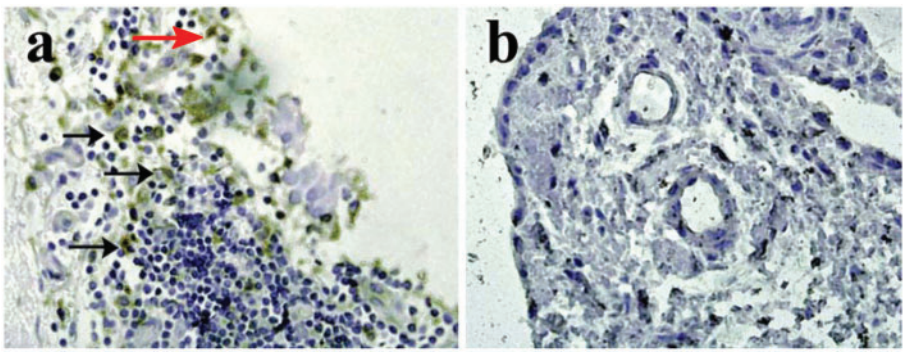

FIG. 2. Representative histological sections of (**a**) RA synovium showing AIF1 expression in intimal (red arrow) and subintimal macrophages (black arrows). In contrast, (**b**) OA synovium shows no staining for AIF1 (immunoperoxidase, original magnification 300×).

However, *BAT3* showed a non-significant, 2-fold decrease in expression in cases compared with controls.

For the case data, disease activity measures were recorded. Expression levels did not significantly correlate with CRP, ESR or anti-cyclic citrullinated peptide (CCP) antibody levels. Notably, all of the RA cases had moderate disease activity (mean ESR = 47 mm/h, mean CRP = 51 mg/l, mean anti-CCP antibody level = 59 U/ml). All patients were receiving disease modifying treatment at the time of study (16 were on anti-TNF biologics, six on MTX and seven on corticosteroids).

Data from lymphoblastoid cell lines indicate that AIF1 expression is highly heritable (heritability = 53%), and strongly influenced by variants close to or within *AIF1*. The SNP rs2269475 genotyped in our study was strongly associated with AIF1 expression in lymphoblastoid cell lines ($P = 4.4 \times 10^{-6}$, explaining 12% of the heritability of AIF1 expression). The SNP most strongly associated with AIF1 expression, rs2857694 ($P = 2.3 \times 10^{-10}$, explaining 21% of heritability of AIF1 expression), is in strong LD with marker rs2259571 which was associated with RA in the current study ($r^2 = 0.84$, $D' = 1$). Apart from markers in and around *AIF1*, no other marker genomewide achieved experiment-wise association with AIF1 expression in this data set. No SNP was associated with expression levels of the other positional candidate genes studied here at experiment-wise significance levels.

Immunohistochemistry

AIF1-expressing cells were found in the subintima of all RA and PsA synovial specimens (Fig. 2a). In serial sections, it was evident that AIF1+ cells were CD68+ subintimal macrophages and not CD3 or CD20 lymphocytes. AIF1 expression was also noted in scattered synovial lining cells in the thickened intima of some RA and PsA cases. The OA synovial specimens which did not contain significant inflammatory infiltrate showed little or no staining for AIF1 (Fig. 2b). In contrast, increased AIF1 staining of subintimal macrophages was seen in the one case of 'inflammatory OA', in which the synovium contained numerous scattered chronic inflammatory cells including lymphocytes, plasma cells and macrophages.

Discussion

In this study, we have further refined the 106-kb interval in the MHC class III region that we have previously shown to be involved in RA independently of *HLA-DRB1* [10]. Using high density SNP typing and a case–control association study design, we identified one region (*AIF1-BAT2*) with significant association with RA. We were not able to determine whether *AIF1.1* and *AIF1.2*, or *BAT2.1*, were associated with RA independently of each other.

TABLE 3. Findings of RTq–PCR study in RA cases and unrelated gender, age- and ethnicity-matched controls

Gene of interest	House-keeper	Expression ratio	*P*-value
AIF1	HPRT	1.6	0.02
	β_2-Microglobulin	1.8	0.002
BAT2	HPRT	0.9	0.7
	β_2-Microglobulin	1.1	0.8
BAT3	HPRT	0.6	0.09
	β_2-Microglobulin	0.5	0.06
APOM	HPRT	1.1	0.4
	β_2-Microglobulin	1.1	0.5
C6orf47	HPRT	0.3	0.03
	β_2-Microglobulin	0.2	0.02
BAT4	HPRT	0.9	0.07
	β_2-Microglobulin	0.8	0.07
CSNK2B	HPRT	0.9	0.4
	β_2-Microglobulin	0.9	0.1
BAT5	HPRT	1.0	0.8
	β_2-Microglobulin	1.0	0.9

Although many associations were apparent in the analyses involving *HLA-DRB1*04* matched cases and controls most did not persist after fully controlling for linkage disequilibrium with *HLA-DRB1* (Table 2). This is consistent with our previous demonstration of marked differences in MHC haplotypes even between closely related *HLA-DRB1*04* subtypes [7, 10]. This highlights the necessity of controlling for linkage disequilibrium with *HLA-DRB1* at the allelic level in such studies; simple matching for possession of HLA-DR4 or shared-epitope carriage is insufficient to distinguish true association from linkage disequilibrium effects.

No other published study to date has studied this region in this depth, controlling completely for linkage disequilibrium with *HLA-DRB1*. In a study of 54 MHC microsatellite markers in 469 multicase RA families, with appropriate control for the effects of HLA-DRB1, Jawaheer and colleagues [6] reported association with one microsatellite haplotype on the ancestral A1-B8-DRB1*03 haplotype, a non-SE encoding haplotype. The region of association covered an ~500 kb segment of the central MHC, which did not include *HLA-DRB1*. There was also evidence of an additional susceptibility element in the class I region of the MHC against the background of DRB1*0404. These findings are consistent with our results showing different disease-modifying elements on the background of DRB1*0401 and DRB1*0404 [7, 10]. Whether the genetic effects observed in the current study are also seen in association with non-*DRB1*0404* MHC haplotypes will require further studies matching for each specific MHC-DRB1 haplotype studied. Kilding and colleagues [11] studied 13 SNPs across the MHC class III region controlling for *HLA-DRB1* in 164 British Caucasian families, and reported association with SNPs in *LST1*, *BAT1* and *PG8*, but none of the associations was significant after correction for the number of markers studied. Association of RA with alleles of the D6S273

microsatellite, lying between *HSP70* and *BAT2*, has previously been reported [15]. Zanelli and colleagues [16] also reported association of the region immediately telomeric to *TNF* in RA, consistent with our own findings. However, neither of these studies fully controlled for linkage disequilibrium with *HLA-DRB1* at allelic levels.

To assist in clarifying which specific genes were involved in RA, we investigated the expression of the positional candidate genes by RTq–PCR. Significant differential expression of two genes (*AIF1* and *C6orf47)* in the original 106-kb region of interest was demonstrated by PBMCs from RA patients. C6orf47 is a novel protein, which interacts with fibroblast growth factor receptor 3 (FGFR3), an important mediator of cartilage development in the growth plate; *FGFR3* mutations are involved in achondroplasia and thanatophoric dysplasia and it seems an unlikely candidate for RA [17]. Further, it was not associated with RA in the current genetic association study. Conversely, *AIF1*, which encodes a 17 kDa IFN-γ inducible Ca^{++}-binding EF hand protein, is a good candidate gene for involvement in RA, a Th1 cytokine-mediated disease. Historical data from lymphoblastoid cell lines indicate that AIF1 expression is strongly influenced by *cis*-acting genetic variants, in contrast to each of the other genes in the region studied. This would support the hypothesis that the association of *AIF1* with RA operates through effects on AIF1 transcription. Our own findings, and the range of conditions in which AIF1 has been implicated, suggest that the gene has a role in a diverse range of inflammatory conditions rather than being specific for RA. This is further supported by the finding that AIF1 overexpression is found not only in synovium from RA patients, but also in PsA, and not in non-inflammatory OA controls.

Our findings support recent observations made by Kimura and colleagues [18], who not only demonstrated AIF1 expression in RA (but not OA) synovium but also that this was strongly correlated to IL-6 levels. They also noted that exposure to AIF1 stimulated synovial proliferation and IL-6 production. Overexpression of AIF1 has also been identified in a microarray study of RA synovium; eight other MHC genes were differentially expressed in RA synovium but none of these lie within 300 kb of *AIF1* [19]. This makes it unlikely that other genes in linkage disequilibrium with *AIF1* but not studied in our RTq–PCR study are also differentially expressed in RA synovium. Our findings extend these observations by demonstrating that *AIF1* polymorphisms are directly associated with RA, and thus suggest that AIF1 is not only involved in the pathogenesis of RA but also in its genetic aetiology.

Although only moderately powered, this is one of the largest studies to date of non-*HLA-DRB1* MHC genes in RA [9], and one of few that can differentiate true from spurious association due to the known differential associations of *HLA-DRB1* SE alleles with RA and the effects of linkage disequilibrium in the MHC. Nonetheless, studies involving larger sample sizes and more dense genotyping will still be required to replicate these findings and provide a comprehensive analysis of the region. The level of statistical association we observed is also amongst the strongest reported for non-DRB1 MHC associations in RA, particularly having corrected as we have for the number of SNPs genotyped. As is the case with all genetics studies, replication of these findings is required. The combination of our genetic, gene-expression and immunohistochemistry data, along with the observations of Kimura and colleagues strengthens the likelihood that these are true positive findings.

AIF1 originally came to attention because it is highly expressed in response to balloon-induced vascular injury and allograft rejection [20]. Its expression is induced by a variety of pro-inflammatory cytokines, including IFN-γ, IL-1β and TNF-α. Transient transfection of macrophage cell lines with AIF1 constructs reduce macrophage apoptosis, promote iNOS production and increases macrophage migration, whereas down-regulation of AIF1 using siRNA has the opposite effect [21]. AIF1 overexpressing transgenic mice are partially resistant to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, an IL-1-dependent model of IBD, raising the possibility that AIF1 is involved in the control of Th-1 responses [22]. The effect of loss-of-function of AIF1 has not yet been reported in *in vivo* models. The expression of AIF1 in different forms of arthritis, its correlation with disease severity, and the effect of genetic variation in *AIF1* on its expression and function await further studies.

In summary, we have shown a significant association between RA and an MHC haplotype containing *AIF1-BAT2*, independent of *HLA-DRB1*. Using RTq–PCR, we have demonstrated increased expression of AIF1, but not BAT2, in PMBCs of RA patients compared with healthy controls. Finally, we have shown expression of AIF1 in synovial macrophages in the lining layer of all the inflamed SMs (including RA, PsA and inflammatory OA) compared with non-inflamed OA controls. These findings combined with the recent data from Kimura and colleagues [18], strongly suggest that AIF1 has a significant role in the aetiopathogenesis of RA.

Rheumatology key messages

- The MHC is strongly associated with RA, largely but not entirely due to association of alleles of HLA-DRB1 with the disease.
- This study confirms that the gene *AIF1*, located within the MHC class III region, is overexpressed in peripheral blood mononuclear cells and synovial biopsies from RA patients.
- Polymorphisms of *AIF1* are associated with RA independent of the nearby HLA-DRB1, suggesting that *AIF1* is directly involved in RA pathogenesis.

Acknowledgements

The researchers would like to thank the individuals who provided samples for this study, and are grateful for the financial support of the Arthritis Research Campaign (UK), and Arthritis Australia.

Disclosure statement: I.E.A. is an employee of Schering-Plough Corp., Inc and receives salary/stock from them. L.R.C., following completion of the work detailed in the paper, accepted an appointment at GlaxoSmithKline. All other authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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