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Concise report

A genome-wide association study identifies *SLC8A3* as a susceptibility locus for ACPA-positive rheumatoid arthritis

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Abstract

Objective. RA patients with serum ACPA have a strong and specific genetic background. The objective of the study was to identify new susceptibility genes for ACPA-positive RA using a genome-wide association approach.

Methods. A total of 924 ACPA-positive RA patients with joint damage in hands and/or feet, and 1524 healthy controls were genotyped in 582 591 single-nucleotide polymorphisms (SNPs) in the discovery phase. In the validation phase, the most significant SNPs in the genome-wide association study representing new candidate loci for RA were tested in an independent cohort of 863 ACPA-positive patients with joint damage and 1152 healthy controls. All individuals from the discovery and validation cohorts were Caucasian and of Southern European ancestry.

Results. In the discovery phase, 60 loci not previously associated with RA risk showed evidence for association at $P < 5 \times 10^{-4}$ and were tested for replication in the validation cohort. A total of 12 loci were replicated at the nominal level ($P < 0.05$, same direction of effect as in the discovery phase). When combining the discovery and validation cohorts, an intronic SNP in the Solute Carrier family 8 gene (*SLC8A3*) was found to be associated with ACPA-positive RA at a genome-wide level of significance RA [odds ratio (95% CI): 1.42 (1.25, 1.6), $P_{combined} = 3.19 \times 10^{-8}$].

Conclusions. *SLC8A3* was identified as a new risk locus for ACPA-positive RA. This study demonstrates the advantage of analysing relevant subsets of RA patients to identify new genetic risk variants.

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CLINICAL SCIENCE

Key words: rheumatoid arthritis, anti-citrullinated protein antibodies, joint erosions, genetic risk, genome-wide association study

Rheumatology key messages

- ACPA-positive RA has a specific genetic risk background.
- Reducing patient heterogeneity is a powerful approach to identify new risk loci for RA .
- *SLC8A3* is a new risk locus for ACPA-positive and erosive RA.

Introduction

RA is the most common inflammatory arthritis in the western world, and it develops on the background of a complex genetic susceptibility. Genome wide association studies (GWAS) have radically improved our knowledge of the genetic variability associated with the risk of developing RA. To date more than 100 different loci have been associated with RA at the genome-wide level of statistical significance [1]. However, these new risk loci collectively explain <10% of the heritability of RA. Therefore, the likelihood that additional, undiscovered loci contribute to RA risk is very high.

One major advance in the understanding of the genetic basis of RA has been the identification of a differential genetic background between ACPA-positive patients and ACPA-negative patients [2]. In ACPA-positive RA, a larger number of genes influence the risk of developing the disease and, also, they show a stronger penetrance compared with ACPA-negative patients [3]. The most compelling example of this differential genetic component is that the two loci most strongly associated with RA, *HLA-DRB1* and *PTPN22* genes, are essentially not associated with ACPA-negative RA [4]. Integrating this acquired knowledge into the study of RA genetic aetiology is proving to be a powerful strategy to identify additional risk factors [5].

Although joint destruction is the hallmark of RA and is the focus of many actual therapeutic interventions, it is not present in all patients [6]. This variability in the clinical presentation of the disease might reflect the presence of underlying genetic heterogeneity. Therefore, analysing only RA patients with radiographic joint damage represents a useful strategy to reduce heterogeneity and so increase the power to identify new genetic factors associated with the disease. In the present study we performed a case-control GWAS using ACPA-positive RA patients with radiographic joint damage in hands and/or feet. The most significant results from the GWAS that were suggestive of new risk loci for RA were subsequently corroborated using an independent validation cohort of ACPA-positive RA patients, also with radiographic joint damage. The results of this study show that analysing relevant groups of RA patients can help to identify additional genetic variation associated with disease risk.

Methods

Study subjects

In the discovery phase, a total 924 RA patients were recruited by the Immune-Mediated Inflammatory Disease Consortium [7]. All RA patients satisfied the ACR diagnostic criteria for RA, were ACPA-positive and had >2 years of follow-up since diagnosis. Importantly, all patients had erosive disease defined as ⩾1 erosions in, at least, two joint groups in hands and/or feet. All patients were Caucasian European and with all four grandparents born in Spain. Supplementary Table S1, available at *Rheumatology* Online, describes the main characteristics of the RA patient cohorts in this study.

The control cohort was also collected by the Immune-Mediated Inflammatory Disease Consortium, in collaboration with the Spanish National DNA biobank [7]. All healthy control individuals were >18 years old and without an autoimmune disease. In order to increase the power of the study, control individuals with a first or second degree relative affected with an autoimmune disease were excluded from the study. A total of 1524 healthy control individuals were recruited for analysis in the discovery phase. All controls were also Caucasian and with all four grandparents born in Spain.

The replication cohort was recruited following the same criteria as in the discovery phase. Anti-CCP-positive patients and healthy controls were all Caucasian and with all grandparents born in Spain. A total of 863 ACPA-positive patients with erosive disease and 1152 controls were recruited for the replication phase.

This study was undertaken in compliance with the Declaration of Helsinki. Informed consent was obtained from all participants, and both the protocols and study were reviewed and approved by the Vall d'Hebron University Hospital review board.

GWAS

Genome-wide genotyping was performed using Illumina Quad610 Beadchips (Illumina, San Diego, CA, USA) on 924 ACPA-positive RA patients and 1524 healthy controls. The Quad610 arrays genotype more than 550 000 single nucleotide polymorphisms (SNPs). GWAS genotyping was performed at the Centro Nacional de Genotipado (CeGen, Spain). Details on the quality control procedure used in this stage are described in 'GWAS and Replication Quality Control Procedures' in supplementary Fig. S1, available at *Rheumatology* Online.

Table 1 Association results for the new loci for ACPA-positive RA identified in our study

				GWAS		Validation		Combined	
Chr	SNP	Gene	MAF	P	OR (95%CI)	P	OR (95% CI)	P	OR (95% CI)
2	rs6435818	*SPAG16*	0.15	0.00044	1.36 (1.15, 1.62)	0.024	1.21 (1.01, 1.45)	6.76×10^{-5}	1.29 (1.14, 1.46)
3	rs2664122	*SRGAP3*	0.4	7.82×10^{-5}	1.28 (1.13, 1.44)	0.017	1.16 (1.01, 1.32)	1.91×10^{-5}	1.21 (1.11, 1.33)
3	rs807193	*CACNA1D*	0.22	1.85×10^{-5}	1.38 (1.19, 1.59)	0.043	1.15 (0.98, 1.34)	1.11×10^{-5}	1.27 (1.14, 1.41)
4	rs10517086	*LOC645481*	0.41	3.67×10^{-5}	1.29 (1.14, 1.46)	0.0044	1.19 (1.05, 1.36)	1.44×10^{-6}	1.24 (1.14, 1.36)
5	rs1991493	*EBF1*	0.21	0.00018	0.77 (0.67, 0.88)	0.013	0.84 (0.73, 0.98)	1.67×10^{-5}	0.8 (0.72, 0.89)
7	rs11496005	*AGR3*	0.35	1.43×10^{-5}	1.32 (1.17, 1.5)	0.027	1.15 (1, 1.31)	6.07×10^{-6}	1.24 (1.13, 1.36)
8	rs870615	*SGCZ*	0.28	7.41×10^{-5}	1.31 (1.15, 1.5)	0.045	1.13 (0.98, 1.31)	3.79×10^{-5}	1.23 (1.11, 1.35)
9	rs11788776	*BNC2*	0.5	7.65×10^{-5}	1.27 (1.13, 1.43)	0.048	1.11 (0.98, 1.26)	4.30×10^{-5}	1.2 (1.1, 1.3)
12	rs789331	*C12orf28*	0.38	3.41×10^{-5}	0.78 (0.69, 0.87)	0.044	0.89 (0.79, 1.01)	4.41×10^{-5}	0.83 (0.76, 0.91)
13	rs927788	*CLDN10*	0.42	6.95×10^{-5}	1.28 (1.13, 1.44)	0.039	1.12 (0.99, 1.28)	3.52×10^{-5}	1.2 (1.1, 1.31)
14	rs17175346	*SLC8A3*	0.16	8.53×10^{-5}	1.4 (1.18, 1.66)	5.97×10^{-5}	1.44 (1.2, 1.73)	3.19×10^{-8}	1.42 (1.25, 1.6)
14	rs7146876	*SERPINA13*	0.23	0.00042	1.29 (1.12, 1.49)	0.025	1.17 (1, 1.37)	8.16×10^{-5}	1.24 (1.11, 1.37)

Association results of the 12 new candidate loci for ACPA-positive RA identified in the GWAS stage and nominally replicated ($P < 0.05$) in the validation stage. GWAS: association statistics for the discovery cohort; Validation: association statistics for the validation cohort; Combined: association statistics for GWAS and validation cohorts combined; Chr: chromosome; MAF: minor allele frequency; OR (95%CI): odds ratio of SNP and 95% confidence interval; P: P-values for association; SNP: single-nucleotide polymorphism.

Replication study

Replication genotyping was performed at the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA) using the Illumina GoldenGate assay (Illumina, San Diego, CA, USA) on 863 ACPA-positive patients with joint damage and 1152 controls. Details on the quality control measures are included in 'GWAS and Replication Quality Control Procedures' in the supplementary data, available at *Rheumatology* Online.

Results

After quality-control analysis, a final number of 890 ACPA-positive RA patients and 1493 controls were used in the discovery stage. A total of 506 950 SNPs passed all quality and frequency filters and were used for association analysis.

In the discovery cohort, 25 of the established risk loci for RA were found to be significantly associated ($P < 0.05$) with ACPA-positive RA [1] (supplementary Table S2, available at *Rheumatology* Online). From these, 10 loci had not been previously associated to this specific group of RA patients. To our knowledge, *MTF1-INPP5B*, *PLCL2*, *ATG5*, *ZNF348*, *WDFY4*, *PLD4-AHNAK2* and *MED1* loci have not been previously associated with ACPA-positive RA. *RCAN1* had been analysed in a previous GWAS in ACPA-positive patients from Asian ancestry [5] but was not significantly associated.

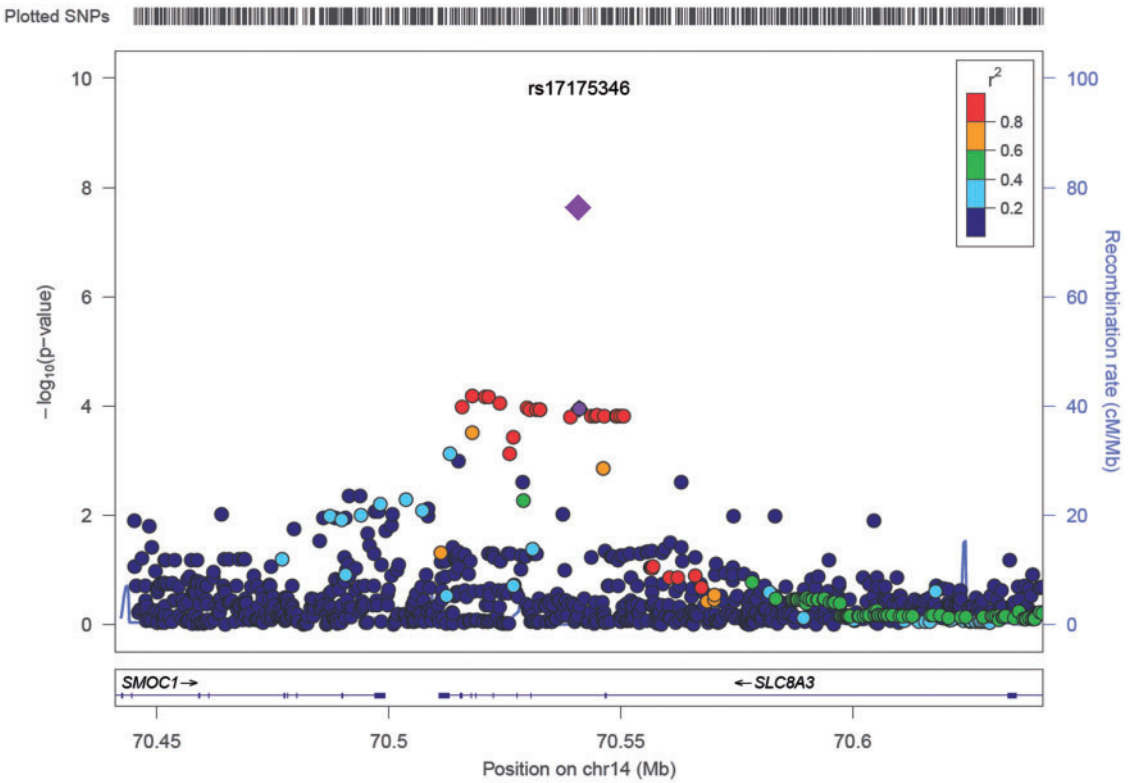
In the discovery phase, 60 genomic regions not previously associated to RA or ACPA-positive RA showed high statistical evidence of association ($P < 5 \times 10^{-4}$, supplementary Table S3, available at *Rheumatology* Online). This group of candidate risk loci was selected for validation in the independent case-control cohort. Using the validation cohort, a total of 12 loci were replicated at the nominal level loci ($P < 0.05$, same direction of effect as in the GWAS, Table 1).

When combining the data from the discovery and validation phases, SNP rs17175346, located in an intron of the solute carrier family 8 member 3 gene (*SLC8A3*) in chromosome 14q24.1, reached a genome-wide level of significance [$P = 3.19 \times 10^{-8}$, odds ratio, OR (95% CI): 1.44 (1.2, 1.73)] (Fig. 1).

In order to gain insights to the possible regulatory potential of the *SLC8A3* SNP rs17175346 associated with ACPA-positive RA we screened public functional annotation databases [8]. We found that the chromosome 14q24.1 region where rs17175346 lies has strong regulatory evidence (supplementary Fig. S2, available at *Rheumatology* Online). For example, DNAseI screening studies performed by the ENCODE project have shown that this genomic region is hypersensitive to cleavage in 112 out of 125 different human cell types, strongly supporting its role as an active genetic regulatory site. Similar evidence obtained using other genomic regulation characterization approaches like the Roadmap Epigenomics Consortium (http://www.roadmapepigenomics.org/, supplementary Fig. S3, available at *Rheumatology* Online) also support the existence of an important regulatory element in the chromosome region that harbours the SNP associated to ACPA-positive RA.

Given that the biological role of the *SLC8A3* gene is still poorly understood, we used the GeneNetwork genomic database (www.genenetwork.nl) to predict its function. This functional analysis tool uses the co-expression patterns in more than 80 000 genome-wide expression analyses in mouse and human to predict significant biological functions of genes. Using this method we found that, from all tested biological annotations, the Gene Ontology database biological processes regulation of ion transmembrane transport

Fig. 1 Association results for *SLC8A3* locus with ACPA-positive RA



Regional plot with the significance [i.e. −log10 (P-values), *y*-axis] of the SNPs in *SLC8A3* gene region in the discovery phase as a function of basepair location in chromosome 14q24.1 (*x*-axis). The validated SNP (rs17175346) is shown as a purple diamond with significance value from the combined (GWAS and validation) cohort association analysis. The remaining SNPs are shown as circles with colour coding indicating the level of LD (i.e. r^2, legend) with respect to rs17175346. The estimated recombination rates (centimorgans/megabase, right y-axis) are plotted as a continuous background line. LD: linkage disequilibrium; SNP: single-nucleotide polymorphism.

(GO:0034765) and ossification (GO:0001503) showed the most significant associations for *SLC8A3* function ($P = 1.26 \times 10^{-10}$ and $P = 1.85 \times 10^{-9}$, respectively; supplementary Table S4, available at *Rheumatology* Online).

Discussion

Using a GWAS approach, we have identified *SLC8A3* as a new risk locus for ACPA-positive RA. Analysing 890 ACPA-positive patients with joint damage and 1493 healthy controls we have identified several candidate risk loci. Using an independent cohort of 863 ACPA-positive patients with joint damage and 1152 healthy controls, we replicated the association of 12 of these new candidate risk loci for RA at the nominal level ($P < 0.05$, same direction as in GWAS). When combining the data from the discovery and validation phases, we have found a genome-wide significant association for rs17175346 (combined $P = 3.19 \times 10^{-8}$), an intronic SNP from *SLC8A3* gene located on chromosome *14q24.1*.

SLC8A3, also known as *NCX3*, encodes a highly conserved protein that mediates sodium and calcium ion exchange across the cell membrane [9]. To date, little is known about the biological processes and cell types that depend on *SLC8A3*. Recent evidence, however, indicates that it is a gene that is constitutively expressed in monocytes/macrophages [10]. Importantly, *SLC8A3* activation in cultured macrophages has been associated to an increase of TNF cytokine production [10]. TNF secretion by macrophages is clearly one of the main pathophysiological mechanisms associated with RA aetiology [11]. Therefore, genetic variants influencing the regulation of TNF secretion in this key cell type in RA could increase the risk of the disease.

In silico prediction of *SLC8A3* biological activity also suggests an association of this Na^+-dependent Ca^{2+} transporter with bone metabolism. Also, the associated SNP rs17175346 lies in a CTCF binding site, a regulatory variant that insulates from enhancer and silencer signals, and it has been characterized in bone forming cells (osteoblasts) (supplementary Table S5, available at *Rheumatology* Online). In RA, the disequilibrium between enhanced osteoclast differentiation and the inhibition of osteoblast-mediated bone repair contributes to bone

erosion, which is the hallmark of the disease. *SLC8A3* has been shown to be expressed in osteoblasts during their differentiation and following bone formation [12]. Furthermore, there is increasing evidence that SLC8A3 is the main cellular translocator of Ca^{2+} from osteoblasts into the bone extracellular matrix [13]. Our results therefore suggest that genetic variation in the biological pathways affecting the target tissue in RA can also increase the risk of developing the disease.

Joint destruction is the most important severity feature of RA. In the present study we recruited ACPA-positive patients with radiographic joint damage, thus increasing the homogeneity of the patient cohort. To our knowledge, this is the first GWAS for RA where all patients both in the discovery and in the replication cohort are positive for ACPA and joint destruction in hands and/or feet. Using this approach we have increased the homogeneity of the patient cohort and we have therefore significantly increased the power to identify new genetic variants relevant for this predominant group of patients. A recent meta-analysis with Caucasian European and Asian RA cohorts increased to 101 the number of genetic variants associated to RA [1]. Despite the large sample size of this study, *SLC8A3* SNP rs17175346 did not show evidence of statistical association ($P > 0.05$, supplementary Table S6, available at *Rheumatology* Online). In this meta-analysis, however, patients were selected neither for positivity to ACPA nor for the presence of erosions. ACPA-negative patients and patients without erosions can represent up to 30% of individuals diagnosed with RA [6]. As suggested previously, including different patient subsets in the genetic analysis can clearly undermine the statistical power to identify new risk variants in RA [14, 15]. Of relevance, one of the nominally replicated genes in this study, *SPAG16*, has been recently found to be associated with the radiological progression rate in RA at the genome-wide level of significance [16]. Despite being a GWAS for a RA phenotype (and therefore a case-only study), it shares several features with the present GWAS for disease risk. Like in our study, in this recent GWAS only ACPA-positive RA patients were analysed. Also, similar to our study, the associated *SPAG16* variant does not show a significant association in the global RA meta-analysis study ($P > 0.05$, data not shown). Together, these results highlight the importance of patient selection criteria in the identification of additional relevant genetic variants in RA.

In the validation phase we replicated the association of 11 additional risk loci with ACPA-positive RA at the nominal level ($P < 0.05$). Although none of these additional loci reached a genome-wide level of statistical significance after combining both cohorts (i.e. $P < 5 \times 10^{-8}$), there is a clear enrichment of nominally significant genes ($P = 0.00017$, binomial test). This result clearly supports that within this group of replicated genes there are additional true risk factors for ACPA-positive RA. Apart from *SPAG16*, another highly suggestive candidate for ACPA-positive RA risk based on its biological function is early B cell factor (*EBF1*) gene. *EBF1* activity has shown to be crucial for B cell lineage commitment to mature antibody-secreting cells [17]. Variation at this gene has been recently associated with the risk of SS [18]. If validated in an independent dataset, this gene would add to the group of B cell pathway genes that have been previously associated with RA susceptibility [1].

In this study, we performed a GWAS in ACPA-positive RA with joint damage. We have identified *SLC8A3* as a new risk locus for ACPA-positive RA and we have also identified several additional loci with suggestive evidence of association with this prevalent disease group. These findings underline the importance of patient selection to characterize the missing heritability of RA.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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