Genetic variants associated with rheumatoid arthritis patients and serotypes in European populations

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Abstract

Objective

To replicate the association of rheumatoid arthritis (RA) susceptibility loci in an independent European sample and to assess their specificity with anti-citrullinated protein antibodies (ACPA) status.

Methods

A selection of 64 SNP previously associated with RA have been typed in a cohort of 267 RA patients (169 ACPA-positive and 98 ACPA-negative) and 152 controls from the Rheumatology Units of the University Hospital of Pisa (Italy) and the University of Pécs Medical Center (Hungary). Regression analyses were performed first considering overall RA patients and secondly, taking both serotype subgroups as different disease entities. The results have been adjusted for age, gender and origin of individuals.

Results

The well-known CD2, REL, TNFAIP3, IRF5, PTPRC, and CCR6 have been confirmed as RA disease associated loci together with recently discovered BACH2, RASGRP1, and IKZF3 loci, taking all RA patients as a unique phenotype. Results from both serological subgroups separately reflect the specificity of these susceptibility loci and show additional ACPA-positive specific associations for variants at IL6R, IL2RA, BLK, DDX6, IL6, and TLE3 genes.

Conclusion

The results from GAPAID project are consistent with previously established RA disease associations for CD2, PTPRC, REL, CCR6, TNFAIP3, IRF5, BLK, IL2RA, and DDX6 loci. In addition, IL6R, BACH2, RASGRP1, TLE3, and IKZF3 are replicated for the first time in an independent European population and IL6 appears to be a suggestive new RA associated locus. The stratified analysis based on ACPA status provides further support for distinct genetic aetiologies of RA subsets, which might have therapeutic implications.

Key words

rheumatoid arthritis, ACPA, SNP, genetic association studies

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Introduction

Rheumatoid arthritis (RA) is one of the most common autoimmune disorders affecting approximately 0.5-1.0% of the population worldwide (1), mainly females. The disease is characterised by chronic, systemic inflammation that may affect many tissues leading to joint destruction, functional disability and decreased life expectancy (2, 3). Most RA patients present different autoantibodies in the serum, which can be useful in establishing the diagnosis and classifing it into clinically different subtypes (4, 5). Rheumatoid factor (RF) has been considered the most important autoantibody in RA for many years, with a range of sensitivity of 70–80%, but it is also present in other autoimmune and non autoimmune conditions, as well as in healthy – mostly elderly – individuals (7-9). In contrast, anti-citrullinated protein antibodies (ACPA) measured as anti-cyclic citrullinated peptide (anti-CCP) (6) have a higher specificity (88–98%) and a similar sensitivity as compared with RF (70–80%) (7, 10, 11). Nevertheless, not all RA patient produce present ACPA autoantibodies and both positive and negative serotypes are considered two different clinical entities of the same disease (12). In fact, ACPA are actually considered a well-established diagnostic and prognostic marker for RA; positivity to these autoantibodies predicts a more aggressive and destructive condition of the disease than ACPA-negative RA (13).

Genetic studies of RA in European ancestry populations, including Genome Wide Association Studies (GWAS) and meta-analysis-based works, have identified several dozens of RA risk *loci* (14-17). Most of them have been identified and validated in RA patients seropositive for ACPA autoantibodies and little is known about their contribution in seronegative RA disease. Also, most recently discovered RA *loci* have not yet been validated in external cohorts of patients. Independent validations in sets of patients from different origin are essential in order to include new markers in routine clinical diagnostics. The GAPAID (Genes And Proteins for AutoImmunity Diagnostics) consortium was created within the European Union's Seventh Framework Programme for Research and Technological Development (FP7), with the aim of validating gene and protein biomarkers for autoimmune diseases, such as RA, and to develop a novel platform for diagnosis and prognosis of the disease. In this context, the aim of the present study is to replicate the association with RA of 64 selected *loci* in a multicentre European population and to assess their specificity with ACPA status in a stratified analysis.

Materials and methods

Ethic statement

This study was approved by the Ethics Committee of the University Hospital of Pisa (reference number: 45066/2012) and the Hungarian Scientific and Research Ethics Board (ref. number: 24973-1/2012 EKU). The procedures followed were in accordance with the Helsinki Declaration of 1975. All the patients gave written informed consent.

Sample

A cohort of 267 RA patients (cases) and 152 healthy blood donors (controls) was recruited from two centres, the Clinical Immunology Unit of the University of Pisa (Italy) and the Department of Rheumatology and Immunology of the University of Pécs (Hungary) (Table I). The recruitment period was between August 2012 and October 2013 and all the RA patients fulfilled the American College of Rheumatology (ACR) classification criteria for RA (6).

Clinical and serological data of RA patients were evaluated retrospectively (Table I). Sera samples of all healthy controls and Italian RA patients were processed to measure RA disease-specific autoantibodies against ACPAs by using the AESKULISA CCP commercial ELISA kit from AESKU Diagnostics (Germany) according to the manufacturer's instructions. ACPA antibody status of RA patients from Pécs was collected from patients' history. These anti-CCP assessments were carried using commercial ELISA kits – (Cogent Diagnostics; United Kingdom) before 2006 and CCPlus® Immunoscan (Euro Diagnostica; Sweden) after 2006

Table I. Clinical data of individuals included in the study.

Variable	Italian cases	Hungarian cases	Total cases	Italian controls	Hungarian controls	Total controls
Individuals (n)	131	136	267	100	52	152
Age at inclusion, SD (years)	60.5±13.0	58.4±11.9	59.4±12.5	39.5±11.3	39.7±9.9	39.5±10.8
Sex, female (%)	73.3	83.1	78.3	28	80.8	46.1
Rheumatoid Factor positivity (%)	54.9	58.1	56.5	n.d.	n.d.	n.d.
Anti-CCP positivity (%)	61.8	64.7	63.3	n.d.	n.d.	n.d.
Medium ACPA titer, <60 units (%)	12	13.9	13	n.d.	n.d.	n.d.
High ACPA titer, >60 units (%)	88	86.1	87	n.d.	n.d.	n.d.

– according to the manufacturer's instruction. The sensitivity and specificity of these different commercial kits are very similar, which makes results from one population and from the other comparable between them.

SNP selection and genotyping

A total of 64 single nucleotide polymorphisms (SNP) were compiled for RA study in GAPAID project (Table II). The list includes genetic markers from those well-known RA susceptibility *loci* reported in the most relevant GWAS and meta-analyses in European or European ancestry populations along with recently identified ones (14-16, 18). Genetic markers from genes related to different clinical features have been also included for GAPAID project objectives and tested in the present study in the context of susceptibility to the disease (19-24).

DNA from buffy coat samples was purified using NucleoSpin® 96 Blood Core Kit (Macherey-Nagel). DNA quantity (ng) and quality (260/280 and 260/230 absorbances) were checked with Qubit® fluorometer (Life Technologies) and NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific) respectively, before the genotyping process. Genotyping of selected SNP was performed by TaqMan® OpenArray® technology (Life Technologies) based on the 5´–3´ exonuclease activity of polymerase. For each array, 2 negative controls and 46 samples were included. TaqMan® Genotyper Software v. 1.3 (Life Technologies) was used for allele assignment.

Before statistical analyses three quality criteria were checked with *PLINK* v.2.050 software (25): SNP call rate (min. 95%), sample call rate (min. 95%) and conformity of genotype proportions to Hardy-Weinberg equilibrium (HWE) in the overall population. SNPs that did not fulfil any of these criteria were eliminated from the analysis.

Statistical analyses

Logistic regression analyses were performed with the above mentioned *PLINK* v.2.050 software with the aim of identifying RA susceptibility *loci* or ACPA-positive and/or ACPA-negative specific genetic markers. All the analyses were carried out under additive, dominant and recessive genetic models. Age, gender, and the origin data of individuals were included as covariates in all the analyses in order to control their effect. Odds ratio (OR) values have been also estimated with a confidence interval of 95%. Adjusted *p*-values lower than 0.05 were considered to be statistically significant.

Results

After the genotyping process, SNP rs3807306 (*IRF5*), rs2275806 (*GATA3*) and rs3025058 (*MMP3*), and 58 indi-

Table II. Selected SNP. The gene or nearest gene from the analysed SNP is indicated.

Gene	Location	SNP ID	Gene	Location	SNP ID
CD2	1p13.1	rs798000	*TAGAP*	6q25.3	rs629326
FCGR2A	1q23	rs10494360	*TNFAIP3*	6q23	rs6920220
		rs1801274			rs10499194
FCGR2B	1q23	rs1050501	*ELMO1*	7p14.1	rs75351767
FCGR3A	1q23	rs396991	*IL6*	7p21	rs1800795
IL10	1q31-q32	rs1800896	*IRF5*	7q32	rs10488631
IL6R	1q21	rs2228145			rs3807306
MMEL1	1p36	rs2843401	*BLK*	8p23-p22	rs4840565
MTHFR	1p36.3	rs1801133	*CCL21*	9p13	rs2812378
PADI4	1p36.13	rs2240336	*TRAF1*	9q33-q34	rs10739580
POU3F1	1p34.1	rs883220			rs3761847
PTPN22	1p13.2	rs2476601	*ARID5B*	10q21.2	rs12764378
PTPRC	1q31-q32	rs2014863	*GATA3*	10p15	rs2275806
AFF3	2q11.2-q12	rs10209110	*IL2RA*	10p15-p14	rs10795791
CD28	2q33	rs1980422	*PRKCQ*	10p15	rs947474
CTLA4	2q33	rs11571302	*CD5*	11q13	rs595158
		rs3087243	*DDX6*	11q23.3	rs4938573
REL	2p13-p12	rs13031237	*MMP3*	11q22.3	rs3025058
		rs34695944	*SCGB1A1*	11q12.3	rs3741240
SPRED2	2p14	rs6546146	*TRAF6*	11p12	rs570676
STAT4	2q32.2-q32.3	rs13426947	*KIF5A*	12q13.13	rs10683701
		rs7574865	*RASGRP1*	15q14	rs8043085
DNASE1L3	3p14.3	rs35677470	*TLE3*	15q22	rs8026898
IL2-IL21	4q27	rs78560100	*IRF8*	16q24.1	rs13330176
RBPJ	4p15.2	rs932036	*IKZF3*	17q21	rs12936409
ANKRD55	5q11.2	rs71624119	*TYK2*	19p13.2	rs34536443
GIN1	5q21.1	rs39984	*CD40*	20q12-q13.2	rs4810485
IL4	5q31.1	rs2070874			rs6032662
SLC22A4	5q31.1	rs1050152	*RCAN1*	21q22.12	rs2834512
BACH2	6q15	rs72928038	*RUNX1*	21q22.3	rs9979383
CCR6	6q27	rs3093024	*IL2RB*	22q13.1	rs3218251
PRDM1	6q21	rs6911690	*TMEM187*	Xq28	rs13397

Table III. Results from regression analyses on the overall RA population and on both ACPA-positive and ACPA-negative serotypes separately. *P*-values have been adjusted for age, gender and origin of individuals.

	SNP ID	Gene	Minor allele (Tested allele)	Genetic Model*	OR (95% CI)	*p*-value
RA *vs*. Controls	rs798000	*CD2*	G	ADD	1.77 (1.04-3.02)	0.035
(222 *vs*. 139)	rs2014863	*PTPRC*	C	REC	3.89 (1.28-11.84)	0.017
	rs34695944	*REL*	C	ADD	1.65 (1.05-2.60)	0.030
	rs13031237	*REL*	T	ADD	1.68 (1.07-2.64)	0.025
	rs72928038	*BACH2*	A	REC	9.57 (1.02-89.75)	0.048
	rs3093024	*CCR6*	A	REC	2.09 (1.04-4.18)	0.037
	rs6920220	*TNFAIP3*	A	ADD	2.35 (1.32-4.17)	0.003
	rs10499194	*TNFAIP3*	T	DOM	0.52 (0.29-0.95)	0.035
	rs10488631	*IRF5*	C	ADD	2.46 (1.27-4.76)	0.007
	rs8043085	*RASGRP1*	T	REC	7.71 (1.41-42.02)	0.018
	rs12936409	*IKZF3*	T	REC	3.47 (1.46-8.25)	0.004
ACPA+ *vs*. Controls	rs2228145	*IL6R*	C	DOM	0.40 (0.19-0.87)	0.020
(142 vs. 139)	rs13031237	*REL*	T	ADD	1.73 (1.00-2.98)	0.049
	rs3093024	*CCR6*	A	REC	3.17 (1.35-7.53)	0.008
	rs6920220	*TNFAIP3*	A	ADD	3.19 (1.56-6.53)	0.001
	rs1800795	*IL6*	C	REC	2.79 (1.06-7.33)	0.038
	rs4840565	*BLK*	C	DOM	2.17 (1.06-4.53)	0.040
	rs10795791	*IL2RA*	G	ADD	1.80 (1.07-3.05)	0.028
	rs4938573	*DDX6*	C	DOM	2.34 (1.04-5.28)	0.040
	rs8043085	*RASGRP1*	T	REC	12.34 (1.42-107.50)	0.023
	rs8026898	*TLE3*	A	REC	6.54 (1.28-33.49)	0.024
	rs12936409	*IKZF3*	T	REC	3.97 (1.41-11.20)	0.009
ACPA- *vs*. Controls	rs2014863	*PTPRC*	C	REC	7.39 (2.23-24.55)	0.001
(80 *vs*. 139)	rs10499194	*TNFAIP3*	T	DOM	0.47 (0.23-0.98)	0.045
	rs10488631	*IRF5*	C	ADD	3.04 (1.42-6.51)	0.004

*ADD: additive; DOM: dominant; REC: recessive.

viduals (45 cases and 13 controls) were removed for statistical analyses due to their low call rate. All remaining SNP fit HWE in the overall population. Thus, a total of 61 SNP and 361 individuals (222 cases and 139 controls) were downstream analysed.

Results from the logistic regression analyses are shown in Table III. Several well-established RA susceptibility *loci* have been confirmed in our population when RA patients were considered as a unique entity: *CD2*, *PTPRC, REL*, *CCR6, TNFAIP3*, and *IRF5*. In all these cases the minor allele conferred a higher disease risk (OR range: 1.6–3.8), with the exception of the protective effect of T allele from rs10499194 located in *TNFAIP3 locus* with an OR of 0.52. In addition, *BACH2*, *RASGRP1*, and *IKZF3* genes also emerged as associated with disease susceptibility in this analysis.

When ACPA positive and ACPA negative patients were separately analysed, *REL*, *CCR6, RASGRP1*, and *IKZF3* conferred predisposition to ACPA-positive RA, and *PTPRC* and *IRF5* to the ACPA-negative disease. In the case of *TNFAIP3 locus*, the minor allele from SNP rs6920220 is associated with ACPA-positive RA, and SNP rs10499194 to the seronegative one. In addition, the analysis of ACPA-positive patients shows other six genes specific for this serotype, *loci* not detected when all RA patients are considered as a unique entity: *IL6R*, *IL6, BLK*, *IL2RA*, *DDX6*, and *TLE3*.

Discussion

Rheumatoid arthritis is a complex multifactorial autoimmune disease caused by the interplay of genetic and environmental factors. In the present study, a total of 15 RA associated *loci* have been detected. Among them, several previously reported associations have been confirmed, such as those for *CD2*, *PTPRC, REL*, *CCR6, TNFAIP3*, *IRF5*, *BLK, IL2RA*, and *DDX6* genes. The validation of these RA susceptibility *loci*, even with the limited sample size of the study, gives reliability to the association detected for those genes replicated and suggested for the first time here (*IL6R, BACH2*, *IL6, RASGRP1*, *TLE3,* and *IKZF3*).

On the whole, our results confirm the genetic diversity of ACPA positive and ACPA negative RA. In fact, so far most RA risk alleles have been described as conferring predisposition to ACPA positive RA (14-16). That is the case for the well established *CD2*, *PTPRC, REL*, *CCR6*, *TNFAIP3*, *BLK, IL2RA*, and *DDX6* RA susceptibility *loci*. In the present study all of them, except *CD2* and *PTPRC*, were confirmed to be associated with ACPA positivity. Interestingly, *TNFAIP3 locus* is also related to negative serotype for the first time showing some overlapping of risk factors for the two RA subgroups. Although shared risk alleles have been previously reported for both ACPA serotypes this genetic overlap seems to be partial or incomplete (26, 27). Furthermore, this association, as well as the new protective effect of *PTPRC* against ACPA-RA disease, needs to be confirmed in an independent patient cohort. Even so, the results confirm the contribution of *IRF5* to RA risk in ACPA- patients previously described (28).

As mentioned above, other six genes have been detected linked to RA:

IL6R, BACH2, *IL6, RASGRP1*, *TLE3,* and *IKZF3*. This is the first study suggesting the association for *IL6* gene, namely with ACPA+ serotype, and replicating the associations for *IL6R, BACH2, RASGRP1*, *TLE3,* and *IKZF3 loci* reported by Eyre *et al*. (16) in an independent cohort of European origin and with an allelic effect in the same direction as previously described.

IL-6 is one of the key cytokines involved in RA development being the IL6R/IL6 pathway a major therapeutic target for the disease. Tocilizumab, an anti-interleukin-6 receptor antibody, is an effective biologic drug for RA (29) and the functional SNP rs2228145 in *IL6R* has been described as predictor of response to therapy (30). Recently, a DNA methylation-based analysis has also reported *IL6R* as a target gene for RA (31). Up to now, polymorphisms in IL6, such as rs1800795, have been related to the biological response to rituximab in several autoimmune diseases (23) but they have not been tested in a RA susceptibility context. In the present study, both rs2228145 (*IL6R*) and rs1800795 (*IL6*) are specifically associated with the disease in the ACPA positive subgroup, suggesting a potential role of these *loci* in the variable response to biological therapy in different subsets of RA patients.

SNP rs72928038 from *BACH2* gene was described as a suggestive RA susceptibility *locus* by Eyre *et al*. (16), and confirmed more recently in a validation study (32). In the present study this relation has also been replicated. BACH2 encodes a B cell–specific transcription factor that has been shown to regulate the *PRDM1* gene, a well-known RA susceptibility *locus* in mice (33). The intronic SNP rs72928038 has already been described associated with other autoimmune diseases such as type1 diabetes (34), Crohn's disease (35), and celiac disease (36).

RASGRP1 and *TLE3 loci* have been detected associated with risk to ACPA+ RA disease as previously described (16). *RASGRP1* encodes a guanine nucleotide exchange factor required for the activation of Ras/mitogen-activated protein kinase pathways (37) that critically mediates the development and function of both T and B lymphocytes (38). Polymorphisms in this gene have been previously described associated with type 1 and 2 diabetes (39, 40). Regarding *TLE3*, the encoding protein is a transcriptional co-repressor of nuclear factor-kB and lead to anti-inflammatory activity (41).

Finally, Stahl *et al*. (14) suggest a RA risk allele in the 17q12 *locus* in European ancestry populations. This association was confirmed in a meta-analysis of a multiethnic population (42), where authors proposed the *IKZF3-ORMDL3-GSDMB* region as the most likely RA associated *locus*. Concretely, *IKZF3* has been later described as a RA susceptibility gene, specific for ACPA+ serotype, combining Immunochip and GWAS data (16). The present study replicates for the first time this association in an independent European population. IKZF3 is a member of the IKAROS transcription factors family implicated in the regulation of B cell lymphocyte proliferation and differentiation (43). Polymorphisms in this gene or surrounding have also been associated with other diseases such as systemic lupus erythematosus, type 2 diabetes or Crohn's disease (34, 44, 45).

In the past few decades, the development of several new effective biologic DMARDs (disease-modifying anti-rheumatic drugs), together with early diagnosis, early aggressive therapy, treat to target approach, and tight disease activity control, have improved the management of RA (46). However, DMARDS – both synthetic and biologic – can have serious adverse effects and not all the patients respond well to a certain therapy. Pharmacogenomics is a relatively new field of research aiming at the identification of subgroups of RA patients with a distinct genetic background, who respond better or worse than other RA patients to a certain drug, thus allowing a personalised therapeutic approach in the management of RA (47, 48). In that sense, the results of this study contribute to a better understanding of the different genetic subtypes in RA, establishing the basis for further research in pharmacogenomics.

Conclusions

In the present study 9 well-established RA susceptibility *loci* have been confirmed (*CD2*, *PTPRC, REL*, *CCR6, TNFAIP3*, *IRF5*, *BLK*, *IL2RA*, and *DDX6*), together with *IL6R, BACH2, RASGRP1*, *TLE3,* and *IKZF3* replicated for the first time in an independent European population and *IL6* reported as a suggestive new RA associated gene. The stratified analysis based on ACPA status provides further support for distinct genetic aetiologies of RA subsets emphasising the need to consider them separately in genetic as well as functional studies of this disease.

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