The role of eight polymorphisms in three candidate genes in determining the susceptibility, phenotype, and response to anti-TNF therapy in patients with rheumatoid arthritis

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ABSTRACT

Objective. *Several single nucleotide polymorphisms (SNPs) have been associated with rheumatoid arthritis (RA) such as peptidylarginine deiminase-4* (PADI4)*, osteopontin* (OPN)*, and perforin* (PRF1) *genes. Thus, we aimed at analysing the influence of eight SNPs in these candidate genes on RA susceptibility and their association with laboratory and clinical features in terms of response to anti-TNF therapy.*

Methods. *We performed a case-control study on 377 Caucasian RA patients and 391 healthy, ethnicity-matched, population-based controls. All subjects were genotyped for PADI4_89/94, PADI4_92, PADI4_104, PADI4_100 in* PADI4*; -156G/GG and +1239A/C in* OPN *and A91V and N252S in* PRF1 *genes. The patients were stratified for shared epitope (SE) HLA-DRB1. rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA) were analysed. The patients started anti-TNF treatment and they were evaluated at baseline and after 12 weeks. Disease activity was evaluated with DAS28 and response to treatment with EULAR criteria.*

Results. *A statistically significant association between RA and OPN -156G/GG was found (p=0.023). SE was firmly confirmed to be associated with RA (OR=3.68; $p<10^{-10}$). No other statistically significant association with clinical and laboratory features were observed.*

Conclusion. *For the first time, in an Italian cohort, we report the association between -156G/GG in* OPN *gene and RA susceptibility. Short-term response to anti-TNF therapy was not influenced by the genetic variants studied.*

Introduction

Rheumatoid arthritis (RA, OMIM no. 180300) is a chronic, systemic, inflammatory disease affecting primarily the joints and resembling the features of autoimmunity. The multifactorial etiology of RA suggests that both environmental and genetic factors are responsible for disease susceptibility and phenotype (1). In addition to the known association with Shared Epitope (SE), recently, several single nucleotide polymorphisms (SNPs), identified by means of genome wide analysis (GWA), raised to attention not only in association with disease susceptibility but also in determining disease phenotype and response to therapy (1-4).

Indeed, early T cell activation gene 1 (also called osteopontin (*OPN*)), peptidylarginine deiminase-4 (*PADI4*), and perforin 1 (*PRF1*) seem to be involved in different stages of the pathogenesis of autoimmune diseases such as enhancement of Th1 response, autoantibody production and cytotoxicity (5-7). The role of SNPs in these codifying genes has been investigated, showing a possible role in influencing the susceptibility and phenotype of a number of autoimmune diseases including RA (5-7).

To the best of our knowledge, there are no data concerning the role of these SNPs in influencing the response to anti-TNF therapy in RA. In the present study, we investigated the role of 8 SNPs in three candidate genes, *OPN, PADI4,* and *PRF1,* in determining RA susceptibility and phenotype in a cohort of Italian patients, with regard to response to anti-TNF therapy.

Materials and methods

Three-hundred and seventy-seven Caucasian patients (70 (18.57%) males, and 307 (81.43%) females) affected with RA, diagnosed according to the American College of Rheumatology (ACR) criteria 1987 (8), were enrolled in this case-control study in five Italian Rheumatology units (Sapienza University of Rome; University of Pavia; University of Siena; San Camillo Hospital, Rome; University of Padova). Three hundred and ninety-one healthy Caucasian, ethnicity matched, population-based subjects (181 (46.29%) males, 210 (53.71%) females) served as controls.

Blood samples were obtained from all subjects before anti-TNF therapy and at 12 weeks follow-up. Genomic DNA and sera were collected using standard protocols and stored at -70°C until use. Rheumatoid factor (RF, Behring; Germany) and anti-citrullinated protein/peptide antibodies (ACPA, Axis Shield; Dundee, Scotland) were detected using ELISA following the manufacturer's instructions; erythrocyte sedimentation rate (ESR) was evaluated using Westergren's method.

Genotyping

All the subjects were genotyped for 4 SNPs in *PADI4*: PADI4_89/94 (rs11203366), PADI4_92 (rs874881) PADI4_104 (rs1748033), PADI4_100 (rs2240335); for 2 SNPs in *OPN*: -156G/GG (rs11439060) and +1239A/C (rs9138); and for 2 SNPs in *PRF1*: A91V (rs35947132) and N252S (rs28933375). The primers and methods used for the genotyping of the studied SNPs and of HLA-DRB1 SE were previously described (5, 9-11).

Clinical evaluation

Three-hundred and seventy-seven patients (mean age 51.4 years (range 18–82), mean disease duration 152.7 months (range 6–528)) started anti-TNF therapy with either adalimumab 40 mg every other week, subcutaneously, Humira, Abbott Immunology, USA, or etanercept 50 mg once a week, subcutaneously, Enbrel, Wyeth, USA, because refractory to conventional immunosuppressive therapy. DMARDs and glucocorticoids were maintained stable during the follow-up. Patients were evaluated at baseline (before starting TNF antagonist), and at 12 weeks follow-up. Data were collected into a standardised form including: demographic characteristics, date of diagnosis, co-morbidities, past and present treatments, anti-TNF agent prescribed with date of beginning, and concomitant medications. Disease activity was measured with the Disease Activity Score in 28 joints (DAS28), and response to therapy was evaluated according to the European League Against Rheumatism (EULAR) response criteria (12).

Statistical analysis

All comparisons of gene and genotype frequencies between the groups were performed by using contingency table and Pearson χ^2. Corrections were made where necessary for the sample size (Fisher's exact test) and for the number of comparisons (P*c* with Bonferroni post-test) All the *p*-values were two-tailed, $p<0.05$ were considered to be significant. The association between polymorphisms and susceptibility to the disease was measured with odds ratio (OR) and 95% confidence intervals (CI). Linear association was calculated by means of Mantel-Haenszel test. For each polymorphism, the power of study was at least more than 80% considering the respective OR and frequency of the SNP in the healthy population. For this purpose we used the Power calculator for genetic study programme. The other statistical analyses were performed by means of SPSS software 13.0 (Statistical Package for Social Sciences). ELISA comparisons were performed with the non-parametric Mann-Whitney U-test. Additionally, DAS28 changes for genotypes were analysed by ANOVA according to the genotypes at baseline, and after 12 weeks. Finally, we performed a generalised linear models multivariable analysis nested by patient and visit using DAS28 as the dependent variable and age, gender, RF, ACPA, disease duration, *OPN, PADI4, PRF1* and *SE* genotypes alone and in combination as independent variables.

The study was performed according to the Helsinki criteria, was approved by local ethics committees, and patients' written informed consent was provided.

Table I. Clinical and serological parameters of the RA patients at baseline and at 12-week follow-up.

Data	Baseline	At 12 weeks follow-up	*p*-value (baseline *vs.* 12 weeks)
ESR (mm/h) mean ±SD	30.5 ± 21.5	21.7 ± 16	<0.0001
Tender joint count (mean ±SD)	14.5 ± 7.5	6.9 ± 6	<0.0001
Swollen joint count (mean ±SD)	6.9 ± 5.6	2.4 ± 3.4	<0.0001
Disease activity VAS for patient (mean ±SD)	62.8 ± 20	41.8 ± 20.4	<0.0001
Disease activity VAS for physician (mean ±SD)	54.1 ± 18.5	38.4 ± 17.2	<0.0001
HAQ (mean ±SD)	2.3 ± 1.2	1.06 ± 0.7	<0.0001
DAS28 (mean ±SD)	5.5 ± 1.2	4.2 ± 1.3	<0.0001
EULAR response			
None (n/%)	–	113/30.0	
Moderate (n/%)	–	211/56.0	
Good (n/%)	–	53/14.0	
ACPA	289.9 ± 342.8	372.2 ± 324.5	N.S.

SD: standard deviation; ESR: erythrocyte sedimentaion rate; VAS: visual analogue scale; HAQ: health assessment questionnaire; DAS28: disease activity score on 28 joints; EULAR: European League Againts Rheumatism; ACPA: Anti-citrullinated protein antibodies. N.S.: Non significant.

Table II. Association of OPN-156G/GG genotype frequencies with RA susceptibility.

	OPN-156G/GG			
	G/G n. (%)	G/GG n. (%)	GG/GG n. (%)	
Patients (n=362)	139 (38.4)	182 (50.3)	41 (11.3)	*p*-value=0.023
Controls (n=380)	182 (48.0)	155 (41.0)	43 (11.0)	Chi square=7.54 OR=1.47

RA: rheumatoid arthritis.

Table III. Stratification of OPN -156 G/GG according with the presence of SE.

	SE carriers (n=250)			
	OPN -156 G/G	OPN -156 G/GG	OPN -156 GG/GG	
Case (n=187)	61 (33%)	96 (52%)	30 (15%)	*p*=0.04
Controls (n=63)	33 (52%)	25 (40%)	5 (8%)	
	SE negative (n=338)			
Case (n=151)	68 (45%)	68 (45%)	15 (10%)	*p*=NS
Controls (n=187)	91 (49%)	75 (40%)	21 (11%)	

SE: shared epitope.

Results

Table I shows clinical and demographic data of the RA patients. Response to anti-TNF therapy, measured with EULAR criteria, was overall good at 12 weeks follow-up. No evidence of deviations from Hardy-Weinberg equilibrium for any of the markers was found.

At first, we performed a case-control analysis to compare the haplotypic frequencies between 377 consecutively recruited RA patients and 391 ethnically matched controls. SE was firmly confirmed to be strongly associated with RA (OR=3.68; CI 2.57–5.25; *p*-value<10^{-10}; χ^2=139.02 *pc*<0.0001). *OPN* -156G/GG in the *OPN* gene was found the only other SNP associated with RA susceptibility (χ^2=7.54; *p*=0.023; OR 1.47, Table II; considering only alleles OR=1.47; CI 1.09–1.97; *p*-value=0.009; χ^2=6.65 *pc*>0.05).

When we stratified this association for the presence/absence of the HLA-DRB1 SE, the OPN -156G/GG was associated with disease susceptibility only in the SE positive population (*p*=0.04, Table III). No significant differences in gene and genotype frequencies were observed between patients and controls for the other SNPs studied. This lack of association with disease susceptibility was reproduced when the groups were stratified for the presence of the HLA-DRB1 SE.

We then performed multiple regression analysis for the studied SNPs, showing that PADI4 89/94 was the only associated polymorphism with RA susceptibility (*p*<0.0001, χ^2=16.054, supplementary Table I).

SE positive patients were more frequent in ACPA positive patients compared with ACPA negative or controls (respectively 53%, 33%, 27% in ACPA positive, ACPA negative and controls, *p*>0.05). OPN showed similar frequencies in ACPA ± populations. PADI4_89/94 and PADI4_92, two SNPs showing a high linkage disequilibrium (D'=1.0, r^2=0.98), were associated with the presence of ACPA at 12 weeks (*p*=0.04 and *p*=0.02, respectively).

Response to anti-TNF therapy was assessed according to genotype frequencies. Patients with different *OPN*, *PRF1* or *PADI4* genotypes showed similar percentages of EULAR responders versus non responders. Sex and TNF inhibitor agent used did not modify the percentages of EULAR response within the genotyping subgroups. Furthermore, changes in the individual components of the DAS28 (*i.e.* mean ESR, swollen joint count, tender joint count, and patient's self-estimated general health) did not differ between patients with different genotypes. The multivariable analysis using DAS28 as the dependent variable, and age, gender, RF, ACPA, disease duration, *OPN, PADI4, PRF1* and *SE* genotypes alone and in combination as independent variables failed to find any significant association between response to anti-TNF therapy and any of the genotypes.

Supplementary Table I.

	Chi-square	*p*-value
PRF1 N252S	0.343	0.558
PRF1 A91V	3.297	0.192
PADI4_89/94	16.054	0.000
PADI4_92	2.187	0.335
PADI4_104	4.056	0.132
PADI4_100	3.206	0.201
OPN -156	3.721	0.156
OPN +1239	1.683	0.431

Supplementary Table II. a) Association of PADI4_89 genotype frequencies with ACPA; **b)** Association of PADI4_92 genotype frequencies with ACPA.

a PADI4_89	GG n. (%)	GA n. (%)	AA n. (%)	
ACPA- (n=53)	7 (13.2)	34 (64.2)	12 (22.6)	*p*-value=0.04
ACPA+ (n=134)	17 (12.7)	61 (45.5)	56 (41.8)	Chi square=6.432
b PADI4_92	CC (n/%)	CG (n/%)	GG (n/%)	
ACPA- (n=56)	8 (14.3)	35 (62.5)	13 (23.2)	*p*-value=0.02
ACPA+ (n=137)	19 (13.9)	57 (41.6)	61 (44.5)	Chi square=8.3542

Discussion

The results of our study showed for the first time an association between *OPN* -156G/GG (rs9138) polymorphism and RA susceptibility, and confirmed that SE and PADI4 are associated with disease susceptibility. These results require further confirmation in larger populations. Nevertheless, our observation is promising at the light of functional and genetic data previously reported for *OPN* and *PADI4*. Concerning OPN, this is one of the major non-collagenous bone matrix proteins produced by osteoblasts and osteoclasts (5). It acts like a Th1 cytokine, by stimulating T cell proliferation, by enhancing the production of IFN-γ, by improving the expression of cytokines such as TNF leading into the recruitment of inflammatory cells (5). The gene polymorphism *OPN -156G/GG (*rs9138) is located in the 5' flanking region, and seems to be implicated in the regulation of gene expression (5). This increased amount may be responsible of an excessive function of the protein resulting in amplification and perpetuation of inflammation. Indeed, OPN mRNA levels are increased in $CD4^+$ synovial T cells from RA patients, and these levels correlate with higher OPN levels in synovial fluids (13).

We further confirmed in an Italian cohort that SE and PADI4 are associated with disease susceptibility. SE was observed more frequently in ACPA positive patients. Citrullination can be influenced by the presence of specific HLA system genotypes, and it appears that these events occur more frequently in patients showing early erosions and early disease onset. This evidence was already found and confirmed in recent studies, although the mechanism and the real significance of HLA-DRB1 SE and ACPA binomial are yet to be determined (9). According to data reported in the literature (14), PADI4 has been found associated with susceptibility to RA, especially in the ACPA positive population. Recent evidences suggest that RA can be distinguished in dif-

ferent subsets depending on ACPA status, thus the role exerted by SE and PADI4 variants might prove useful in predicting distinct disease course and pathogenesis. Both PADI4_89/94 and PADI4_92 (rs11203366 and rs874881) seem responsible of ACPA production (9). The mechanism by which this phenomenon takes place remains unclear, even if several replication studies reported similar data. We found no association between the studied variants and response to therapy measured with EULAR criteria. Nonetheless, an overall good EULAR response was achieved in our patients, supported by changes in the continuous variable DAS28 during the study. Finally, the polymorphisms in PRF1 gene were not found associated with disease susceptibility nor with phenotype. We expected to find moderate to large effects on disease susceptibility when studying PRF1 SNPs, and the study was powered accordingly. However the effects found were small and statistically not significant, with OR close to 1 (no effect).

In conclusion, RA causes direct and indirect costs and the biologic treatment for RA is not always satisfactory, yet expensive, yet not risk-free. The capability to distinguish not only patients who will develop RA, but also to identify sub-sets depending on clinical features and response to therapy by means of individual genetic pattern, can be extremely useful as for the patients as for the whole health system. Further replication studies are needed to better clarify these issues.

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