Prevalence of functional haplotypes of the peptidylarginine deiminase citrullinating enzyme gene in patients with rheumatoid arthritis: no influence of the presence of anti-citrullinated peptide antibodies

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Abstract

Objective

*Citrullinated peptides produced by enzymatic deimination of arginine residues in proteins by peptidylarginine deiminases are of particular interest in the pathogenesis of rheumatoid arthritis (RA). One type of citrullinated protein – the cyclic citrullinated peptide – is the target of the anti-cyclic citrullinated peptide antibody, the most sensitive and specific autoantibody in RA. The peptidylarginine deiminase type 4 (*PADI4*) gene, which codes one of the PADI enzyme isotypes, has genetic variants that confer susceptibility to RA in Asian, but not in European populations.*

Methods

Genetic associations were examined in 214 Hungarian RA patients characterized for the presence of anti-CCP and rheumatoid factor. The patients were characterized for the existing haplotypes of the PADI4 gene (defined by the combinations of 4 exonic padi4_89: 163G/A, padi4_90: 245T/C, padi4_92: 335C/G, padi4_104: 349T/C and 2 intronic padi4_94: 17535226C/T and padi4_102: 17546809C/T variants) by the PCR-RFLP method.

Results

None of the PADI4 *haplotypes was accumulated in RA patients. One new finding was that we also did not detect the accumulation of any haplotypes either in the anti-CCP or in the RF-positive subgroups of patients.*

Conclusion

The data presented here show that none of the naturally occurring haplotypes of the PADI4 *gene conferred susceptibility to RA in an average group of Hungarian patients; this is in agreement with findings for other European populations. In addition, none of the functional* PADI4 *haplotypes were associated with the pathologic immune response, which was evidenced by the absence of accumulation of anti-CCP-positive subjects in the specific* PADI4 *haplotypes.*

Key words

PADI4, rheumatoid arthritis, haplotype, Caucasian, RF, anti-CCP.

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This work was supported by a grant from the Hungarian National Science Foundation OTKA T049589 and by the Ministry of Health, ETT497/2006.

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Received on June 21, 2006; accepted in revised form on January 18, 2007.

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Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting approximately 0.5%-1% of the population worldwide (1), including Hungary (2). The disease is considered to be one of the most common autoimmune disorders and is caused by a combination of environmental and genetic factors (3). Various genes are involved in the development of RA, including allelic variants of *HLA, TNFR2, SLC22A4, RUNX1, PTPN22* and *PADI4* (4-15); a major susceptibility factor is the HLA-DRB1 gene. Various alleles of the HLA-DRB1 gene code for a common amino acid sequence called the "shared epitope" on the DRβ1-chain of the protein, an epitope which seems to be associated with RA (14).

The peptidylarginine deiminase citrullinating enzymes (PADIs; E.C. 3.5.3.15.) are involved in the post-translational deimination of arginine residues to citrulline in proteins (10, 16-18). Citrullination partially unfolds the proteins via loss of the positive charge in the arginine moiety, which can affect the antigenicity of the protein chains. PADIs play a specific role in the pathogenesis of rheumatoid arthritis (13, 18-20), as citrullinated proteins are the targets of anti-citrullinated peptide antibodies (ACPAs), including that against the cyclic citrullinated peptide (anti-CCP), which is the most sensitive RA-specific autoantibody (4, 21). The antibodies are usually generated at an early stage of the disease (6, 17, 22). Amongst the naturally occurring variants of the *PADI4* gene, some have been found to confer susceptibility to RA in Asian populations (10, 13, 23). Recent studies on Europeans, including British, French and Spanish Caucasian populations, could not confirm these findings (6, 22, 24). Only one German case-control study showed an association of a functional haplotype with the disease (25).

Rheumatoid factor (RF) is a special autoantibody in RA. IgG-RF molecules agglutinate due to their self-binding capacity and the complexes thus formed can further activate the immune system (26). RF is present in approximately 75% of RA patients although, like anti-CCP, it can also be found in other inflammatory and infectious diseases and even in a small proportion (3-5%) of the healthy population. A strong correlation has been observed between anti-CCP- and RF-positivity: 58-72% of RF-positive patients proved to be positive for anti-CCP as well. In comparison to positivity for just one of the two factors, this combined seropositivity is associated with more progressive and erosive RA (27).

Variations in the amino acid sequence of the PADIs can influence their immunological features; these variants can have different immune responses and ultimately the coded characters can thereby affect the production of ACPAs. The aim of this present study was to: (i) define the haplotypes and their frequencies in a Hungarian population of RA patients; (ii) test if any of the haplotypes can confer susceptibility for RA in the average population; and (iii) study the haplotype distribution in serologically characterized subgroups and to test thereby the possible associations of the haplogroups with anti-CCP or RF positivity, alone or in combination. One interesting aspect of Hungary is that its original inhabitants were of Asian origin whereas now its population is mixed, and a special feature of this study is that it focused on a specific target population – Caucasian Hungarians (28).

Materials and methods

Subjects

We examined 214 patients with the typical symptoms and a diagnosis of RA (41 males, 173 females, mean age 57.1 ± 14.5 years). 194 carefully selected, clinically healthy subjects (108 males, 86 females, mean age 36.5 ± 10.5 years) served as controls. The controls did not have any evidence or history of major metabolic disease; special care was taken to exclude patients with a history of immunological diseases. All control subjects were from the same geographic area. All of our patients and controls were unrelated Caucasians. All RA patients fulfilled the diagnostic criteria of the American College of Rheumatology (29). During the entire study period the guidelines and regulations approved by the local Ethical Committee and the Helsinki Declaration in 1975 were followed.

Table I. Age-group specific distribution of rheumatoid factor (RF)- and anti-cyclic citrullinated peptide (anti-CCP)-seropositivity in the patients with rheumatoid arthritis.

Age groups		RF		Anti-CCP		RF and anti-CCP	
		Positive	Negative	Positive	Negative	Positive	Negative
Total	(n = 214)	156 (72.9%)	58 27.1%)	139 (65.0%)	75 (35.0%)	120 (56.1%)	43 (20.1%)
< 30	(n = 15)	9 (60.0%)*	6 (40.0%)*	5 (33.3%)*	10 (66.7%)*	4 (1.86%)	6 (2.80%)
31-50	(n = 45)	33 (73.3%)	12 (26.7%)	29 (64.4%)	16 (35.6%)	28 (13.1%)	7 (3.27%)
51-70	(n = 115)	84 (73.0%)	31 (27.0%)	78 (67.8%)	37 (32.2%)	65 (30.4%)	23 (10.7%)
> 70	(n = 39)	30 (76.9%)	9 (23.1%)	27 (69.2%)	12 (30.8%)	23 (10.7%)	7 (3.27%)

*$p < 0.05$ or less versus all other age groups.

Serological testing

For each RA patient, sera from non-hemolyzed blood was tested for the presence of RF using the Rheumatoid Factor Screen ORG522S test by ORGENTEC Diagnostika GmbH (Mainz, Germany). Anti-CCP antibodies were detected using an enzyme immunoassay (Euro-Diagnostica, Malmö, Sweden) following the manufacturer's instructions.

Genotyping methods

Genomic DNA was extracted from peripheral blood leukocytes using a routine desalting method. We examined four exonic PADI4 SNPs: padi4_89 (163G/A, GenBank rs11203366), and padi4_90 (245T/C, GenBank rs11203367) in exon 2, padi4_92 (335C/G, GenBank rs874881) in exon 3 and padi4_104 (349C/T, GenBank rs1748033) in exon 4 and two intronic SNPs of the same gene, padi4_94 (17535226C/T on chromosome 1, GenBank rs2240340) and padi4_102 (17546809C/T on chromosome 1, GenBank rs2240337). The nomenclature followed is that of Suzuki *et al*. (13). The naturally occurring haplotypes are listed in Table II. Each of the exonic genetic variants are associated with an amino acid change in the protein products: a Gly55Ser, Val82Ala, Gly112Ala, and Leu117Leu modification, respectively.

The following primers were designed and used to amplify the examined sequences: for padi4_89*G/A forward 5'-CTCCTCACTGCATCCTCTGCT-3', reverse 5'-CTTTCATCGTCAGGGTCACCTCTA-3'; for padi4_90*T/C forward 5'-CAAAGTCCCACGATCTGCAAG-3', reverse 5'-AGGACACTATGG CTGGAAGAAGC-3'; for padi4_92*G/C forward 5'-AGCTTTTTGCTTTCCCTCCATT-3' and reverse 5'-GTCTGACTGGCTAGAAACCATGC-3'; for padi4_94*C/T forward 5'-CTCACCAACCTCTCCTGGTAC-3' and reverse 5'-TCACCAATTGTGGGTTCAGA-3'; for padi4_102*C/T forward 5'-CTGGCCCAGGCACCACCAG-3' and reverse 5'-AGGGTTTCGGCAGCTGTGCC -3', and for padi4_104*C/T forward 5'-CATCACAGTTGTGGCCCCG-3' and reverse 5'-GCGGGTGATGTCTGCGCCC-3'. The mismatch bases are underlined.

The PCR amplifications were performed on MJ Research PTC 200 thermal cyclers according to the following protocol: initial denaturation at 95ºC for 2 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec (at 60ºC in the case of padi4_102 and padi4_104, and at 57ºC in the case of padi4_94) and extension at 72ºC for 30 sec. Final extension was carried out at 72ºC for 5 min. Each polymerase chain reaction contained 200 μM of each dNTP, 1 unit of Taq polymerase, 5 μl of reaction buffer (100 mM Tris HCl, pH = 9.0; containing 500 mM KCl, 15 mM $MgCl_2$), 0.2 μM of each primer and 1 μl DNA to be amplified) in a final volume of 50 μl.

The amplicons were digested with allele-specific restriction endonucleases, HaeIII for padi4_89, *MlsI* for padi4_90 *HpaII* for padi4_92, *KpnI* for padi4_94, *RsaI* for padi4_102 and *PasI* for padi4_104. In all amplicons there was an obligatory cleaving site to enable us to control the efficacy of the digestion.

Statistical analysis

Statistical analysis was carried out using Excel and SPSS 11.5. for Windows. Association of RA and the examined serologic features with each of the *PADI4* haplotyes was tested using the chi-square test.

Results

We divided our RA patients into four groups by age (Table I). Significantly fewer seropositive subjects were found in the youngest group (< 30 years of age) than in the other three (for RF: $\chi^2 = 3.79$, $p = 0.051$; for anti-CCP: $\chi^2 = 19.24$, $p < 0.001$). The frequency of RF positivity in patients under 30 years of age was 61%, whereas in the other groups of patients it increased to 73%. In the case of anti-CCP the difference was much more striking: 33% of the patients under 30 were seropositive, while 64% of the older patients had anti-CCP positivity.

DNA was available from 203 RA patients and 194 control subjects. The allele frequencies for all the single nucleotide variants of the six examined loci were in Hardy-Weinberg equilibrium both in the RA and the control subjects. None of the variants showed significant accumulation in RA patients compared to controls (data not shown). For padi4_102, the prevalence rate of the T allele was significantly higher in the control group than in the patients with RA (15.2% *vs* 24.4%; $\chi^2 = 5.05$; $p = 0.025$; OR = 0.54; 95%CI: 0.32-0.93).

The haplotype frequencies are shown in Table II. Haplotype 1 (characterized by padi4_89*A, padi4_90*C, padi_92*C, padi4_94*C, padi4_102*C and padi4_104*C) and haplotype 2 (padi4_89*G, padi4_90*T, padi4_92*G, padi4_94*T, padi4_102*C and

Table II. Haplotype structure and frequency in the PADI4 gene.

Haplotype ID	SNP ID (padi4_x)						Haplotype frequency				Regression analysis			
	89	90	92	94	102	104	Case (n = 334)		Control (n = 260)		χ^2	*p*	OR	(95%CI)
Haplotype 1	A	C	C	C	C	C	170	(50.9)	125	(48.1)	0.47	0.495	1.12	(0.81-1.55)
Haplotype 2	G	T	G	T	C	T	92	(27.5)	60	(23.1)	1.53	0.216	1.27	(0.87-1.84)
Haplotype 3	G	T	G	T	T	T	20	(5.98)	16	(6.15)	0.01	0.933	0.97	(0.49-1.91)
Haplotype 4	G	T	G	T	C	C	28	(8.38)	24	(9.23)	0.13	0.717	0.90	(0.51-1.59)
Other	NA	NA	NA	NA	NA	NA	29	(8.68)	34	(13.1)	NA	NA		NA

Values represent the numbers of cases, with the relative frequencies (expressed as a percentage) between parentheses; in the last column the ranges of odds ratios are given between parentheses. Chi-squares and ORs have been calculated for the haplotype frequencies in cases vs. controls. NA: not applicable.

Table III. Haplotype distribution in male and female RA patients and controls.

	RA patients				Controls			
	Male (n = 64)		Female (n = 270)		Male (n = 142)		Female (n = 118)	
Haplotype 1	35	(54.7)	135	(50.0)	70	(49.3)	55	(46.6)
Haplotype 2	13	(20.3)	79	(29.3)	35	(24.6)	25	(21.2)
Haplotype 3	3	(4.68)	17	(6.30)	6	(4.23)	10	(8.47)
Haplotype 4	7	(10.9)	21	(7.78)	9	(6.34)	15	(12.7)
Other	6	(9.38)	18	(6.67)	22	(15.5)	8	(6.78)

Values represent the numbers of cases, with the relative frequencies (expressed as percentages) given between parentheses.

padi4_104*T) were the most frequent. We also detected haplotype 1B (classification by Hoppe *et al.*; see ref. 25), which is formed from three different haplotypes determined by the six SNPs studied. Coexistence of the padi4_89*A, padi4_90*C and padi4_92*G alleles was common in these. Frequency of the 1B haplotype did not differ significantly between cases and controls. There was no accumulation of any haplotypes in the RA patients compared with the controls. No association was found between the haplotype and the gender of the subjects (Table III). None of the haplotypes showed an increased frequency in the RF- and anti-CCP- positive RA subjects (Table IV). Moreover, no difference was observed in the distribution of the *PADI4*-haplotypes in patients with combined seropositivity (RF plus anti-CCP), nor in patients with combined seronegativity.

Discussion

Amongst the naturally occurring variants of the *PADI4* gene, some have been reported to confer susceptibility to RA in Asian populations (10, 13, 23), whereas examinations of European groups, including British, French and Spanish Caucasian populations, have failed to confirm these associations (6, 22, 24), except for a German cohort studied by Hoppe *et al.* (25). One explanation for these differences could be the differing genetic structure of the populations examined. In this context, the Hungarians are unique in the Carpathian basin because a large proportion of the earliest inhabitants came from the east, beyond the Urals. Ancient tribes settled in Hungary 1100 years ago and mixed with the indigenous population, and over the course of history several other ethnic groups mingled with them (28). The relative incidence rates of haplotypes fundamentally different from those of the European lineages is still not known, however.

Another explanation for the discrepancies between studies could be linked to immunological considerations. PADIs are involved in the post-translational deimination of arginine in proteins; the resulting citrullination partially unfolds proteins via loss of the positive charge of the arginine moiety (10, 16-18). PADIs play a specific role in the pathogenesis of rheumatoid arthritis, as the citrullinated proteins generated by them are the immuno-targets of ACPAs, including anti-CCP. Functional studies on the naturally occurring variants of the *PADI4* gene products have revealed that haplotype (genotype) can affect the stability of the mRNA transcripts, which in turn can have an effect on the biochemistry of the PADI4 enzyme. The biochemical properties of the PADI4 enzyme variants could theoretically influence the immune response against cyclic-citrullinated peptides. Therefore, in the present study we did not confine ourselves to an examination of the distribution of *PADI4* gene haplotypes in Hungarians, but also tested for possible correlations between haplotypes and seropositivity.

The rate of RF and anti-CCP seropositivity was similar to that reported for other Caucasian European populations (4). Both markers were present in 56% of all RA patients and this range was also consistent with other European data. Seropositivity for both factors had already developed in 4% of the patients before 30 years of age, although peak accumulation was found in the age range of 51-70 years (data not shown). This is also commonly observed in other populations.

Our findings on allele and haplotype frequencies are similar to those previously described in other European studies (6, 22, 24). The same haplotypes also exist in the Japanese population, although haplotype 1B has only been identified in the German, British and our Hungarian populations (24,

Table IV. Haplotype distribution and frequencies of PADI4 SNPs in RA patients positive for rheumatoid factor (RF), anti-CCP or both.

	RA patients					
	RF		Anti-CCP		RF and anti-CCP	
	Positive (n = 242)	Negative (n = 92)	Positive (n = 214)	Negative (n = 120)	Positive-positive (n = 200)	Negative-negative (n = 78)
Haplotype 1	125 (51.7)	45 (48.9)	108 (50.5)	62 (51.7)	102 (51.0)	39 (50.0)
Haplotype 2	68 (28.1)	24 (26.1)	63 (29.4)	29 (24.2)	59 (29.5)	20 (25.6)
Haplotype 3	13 (5.37)	7 (7.61)	12 (5.61)	8 (6.67)	10 (5.00)	5 (6.41)
Haplotype 4	22 (9.09)	6 (6.52)	19 (8.88)	9 (7.50)	18 (9.00)	5 (6.41)
Other	14 (5.79)	10 (10.9)	12 (5.61)	12 (10.0)	11 (5.50)	9 (11.5)

Values represent the numbers of cases, with the relative frequencies (expressed as a percentage) between parentheses.

25). The majority of RA patients and controls had haplotypes 1 and 2. This means that the Hungarian population under study here did not differ from other Europeans in this respect. No accumulation of any specific haplotype was observed in the patients with RA, which means that none of the major haplotypes confer susceptibility to the disease.

Further analysis in patient groups characterized for the presence of anti-CCP and/or RF antibodies also did not reveal the accumulation of any haplotypes in the seropositive RA patients. This means that none of the haplotypes created a predisposition for the abnormal immune response characterized by the production of either anti-CCP, RF, or a combination of them. This observation does on the other hand suggest that the nature of the examined variants has no significant effect on the sequence of events responsible for the abnormal immune response.

Acknowledgements

We are grateful to Edit Papp, Judit Oksai, Jánosné Zentai and Ibolya Farkas for their excellent technical assistance.

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