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Associations between genetic factors, tobacco smoking and autoantibodies in familial and sporadic rheumatoid arthritis

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Abstract

Objectives. The objective of this study was to investigate the association between genes (*HLA-DRB1* and *PTPN22*) and tobacco smoking, separately as well as combined, and serological markers of rheumatoid arthritis (RA) in a French population of RA.

Methods. 274 RA patients with half of them belonging to RA multicase families, were genotyped for *HLA-DRB1* allele and for *PTPN22-1858* polymorphism. IgM rheumatoid factor and anti-CCP antibodies were determined by ELISA method. The search for association relied on Chi-square test and odds ratio with 95% confidence interval calculation. The interaction study relied on the departure-from-additivity-based method.

Results. The presence of at least one *SE* allele was associated with anti-CCP antibodies presence (82.5% versus 68.4%, $P=0.02$), particularly with *HLA-DRB1*0401* allele (28.0% versus 16.4%, $P=0.01$). Tobacco exposure was associated with anti-CCP antibodies, but only in presence of *SE*. A tendency toward an interaction was found between tobacco, the presence of at least one *HLA-DRB1*0401* allele and anti-CCP antibodies (attributable proportion due to interaction= +0.24 [-0.21 +0.76]). The cumulative dose of cigarettes smoking was correlated with anti-CCP antibodies titers ($r=0.19$, $P=0.04$). The presence of both *SE* and *1858T* alleles was associated with a higher, but not significantly different, risk for anti-CCP antibodies presence than for each separately. No association was found between *PTPN22-1858T* allele and tobacco smoking for autoantibodies positivity.

Conclusion. Our findings suggest an association between *SE* alleles and tobacco smoking for anti-CCP positivity and a tendency toward an interaction between the *HLA-DRB1*0401* allele and smoking for anti-CCP positivity in this sample of RA.

Key-Words: Rheumatoid Arthritis, Shared Epitope, *PTPN22*, anti-CCP antibodies, tobacco smoking.

Rheumatoid arthritis (RA) pathogenesis is multifactorial, involving both genetic and environmental factors. Although the association of some *HLA-DRB1* alleles with RA has been reported nearly three decades ago, the underlying biological mechanism of this association remains unknown. The presence of the RAA sequence at positions 72 to 74 of the HLA-DR beta chain molecule, for all *HLA-DRB1* alleles known to be associated with RA, led to the shared epitope (SE) hypothesis [1]. The modelisation of the SE component in RA has recently been successful [2, 3].

Rheumatoid factor (RF) production in RA is generally associated with a more severe phenotype of the disease. RF production has been associated with carriage of SE alleles. Furthermore, several publications showed that the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies together with SE alleles carriage was associated with a very high relative risk for future development of RA [4, 5]. Those reports finally lead to the hypothesis that SE alleles contribute to the RA only by the development of anti-CCP antibodies and do not independently constitute a risk factor of RA [6-8]. Moreover, the combination of the *PTPN22 1858T* variant, another genetic factor RF positive RA-associated and -linked, and anti-CCP antibodies was recently reported to give a much higher relative risk for developing RA than the combination of the *PTPN22 1858T* variant and HLA-SE [9].

Numerous environmental risk factors have been studied in RA, but tobacco smoking is to date the only well-established environmental risk factor [10]. A gene-environment interaction between tobacco smoking and SE alleles was reported to increase the risk of RF positive RA [11, 12]. More recently, a model has been proposed in which smoking, in the context of SE alleles may trigger specific immune reactions to citrullinated proteins [13]. However, smoking seems to be a risk factor for anti-CCP antibodies production restricted to RA patients who carry SE alleles [14]. No association was reported between *PTPN22 1858T* variant and tobacco exposure in RA.

Here, we tested the hypothesis of an association between genes (*HLA-DRB1* and *PTPN22*) and tobacco smoking, separately as well as combined, and serological markers of rheumatoid arthritis (RA) in a French population of familial and sporadic RA.

PATIENTS AND METHODS

Patients. We studied 274 RA patients of French Caucasian origin as defined for each of the 4 grand-parents, 196 patients being index cases of trio families *ie* one RA patient and both parents and 78 patients being index cases of affected sibling pair families. RA diagnosis fulfilled the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria [15]. All individuals provided informed written consent and the study was approved by the hospital Bicêtre ethics committee (Kremlin-Bicêtre, Assistance Publique-Hôpitaux de Paris).

In this RA patients sample, 89% of the RA patients were females, 47% belonged to RA multicase families, and at the time of serum collection, the mean age of RA onset was 34 ± 11 years and the mean disease duration was 12 ± 9 years. Erosions were present in 82% of the patients and rheumatoid nodules in 19%.

Genotyping. Blood samples were collected for DNA extraction and genotyping. *HLA-DRB1* typing was performed with the polymerase chain reaction – sequence specific primer method using the Dynal Classic SSP DR low resolution and the Dynal Classic high resolution SSP for subtyping of *HLA-DRB1**01, *04, *11, *13 and *15 alleles (Dynal Biotech, Lake Success, NY). SE alleles were *HLA-DRB1**0101, *0102, *0401, *0404, *0405, *0408 and *1001. Alleles were then classified in three groups, S_2 , S_{3P} and L , according to their 70-74 amino acid sequence, as previously reported [2, 3]. Genotyping of the *PTPN22-1858 T/C* variant was performed by PCR-restriction fragment length polymorphism, as previously reported [16].

This T variant eliminated a restriction site for *RsaI* enzyme, and genotypes were secondly checked by a PCR-restriction fragment length polymorphism using the *XcmI* enzyme for which a restriction site was created when the T allele was present.

Autoantibodies status. IgM rheumatoid factor was provided by ELISA method (QUANTA, Lite™ RF IgM, INOVA diagnostics, San Diego, USA), and the anti-CCP status was provided by an anti-CCP antibody ELISA (Immunoscan RA, Euro-Diagnostic, Malmö, Sweden). Both ELISA tests were performed on the same serum sample according to the manufacturer's instructions.

Tobacco exposure. Information about tobacco exposure was collected by using a questionnaire sent to each RA patient [11]. Following questions about smoking were asked: (1) Do you smoke? (2) If not, did you ever smoke? (3) In which year did you start smoking? (4) In which year did you stop smoking? (5) What sort of tobacco did you smoked: cigarettes, cigars, pipes? (6) Average number of cigarettes smoked per day: 1 to 5, 6 to 9, 10 to 19, ≥ 20 cigarettes? (7) Duration in years of smoking exposure: < 10 , 10 to 19, ≥ 20 years? Unanswered questionnaires were completed by telephone. RA patients who were smoker at the year of the serum collection were considered as current smokers, those who reported that they were smokers and stopped smoking before the year of serum collection were defined as ex-smokers. Current smokers and ex-smokers were defined as ever smokers, and RA patients who reported they had never smoked were defined as never smokers. The cumulative dose of cigarettes smoked was then expressed as pack-year, one pack-year being the equivalent of 20 cigarettes smoked per day for one year.

Statistical analysis. Statistical analysis to search for association between exposure to genetic factors (*SE* alleles and/or *PTPN22 1858T* allele) and/or tobacco smoking with RF or anti-CCP positivity, relied on chi-square or Fisher's exact test when appropriate, odds ratio (OR) and 95% confidence interval (95% CI) with 2x2 or 2x3 tables. In order to search for biological interaction, we used the departure-from-additivity-based method. Attributable proportion (AP) due to interaction was determined by using the following formula,

$AP = (R_{11} - R_{10} - R_{01} + R_{00}) / R_{11}$, AP and 95% CI were calculated with the SYSTAT program [17, 18]. The correlation study between the cumulative dose of cigarettes smoking expressed in pack-years and the anti-CCP antibodies titers relied on a correlation test. Finally, search for association between exposure to genetic factors (*SE* alleles and/or *PTPN22 1858T* allele) and/or tobacco smoking with RF or anti-CCP positivity was performed with the same statistical tests than for the global sample, in the subgroup of familial RA and in the subgroup of sporadic RA.

RESULTS

RA sample characteristics. 218 (79.6%) RA patients carried at least one *SE* allele within 88 patients homozygous for *SE* alleles, whereas 89 (32.6%) RA patients carried at least one T allele of *PTPN22-1858* variant within 9 patients homozygous for the *PTPN22-1858* T allele. 190 (69%) RA patients had a positive RF and 217 (79%) patients had anti-CCP antibodies, and 172 (63%) were both RF and anti-CCP antibodies positive. Among the 243 (89%) RA patients for whom the information about tobacco exposure was known, 122 (50%) were ever smokers, 43 (35%) being current smokers and 79 (65%) being ex-smokers. There were significantly more men in the subgroup of patients exposed to tobacco (18.0% versus 1.6%, $P = 2 \times 10^{-5}$).

Association between autoantibodies status and *SE* alleles

RF and *SE* alleles

The presence of at least one *SE* allele in the genotype was not associated with RF positive RA (81.0% RF positive versus 76.0% RF negative, OR=1.3 [0.7-2.5]). The hierarchy of the genotypes risk in the RF positive subgroup was different from that previously reported in the

literature, S_2/S_2 being the most at risk genotype followed by S_2/S_{3P} and S_{3P}/S_{3P} genotypes. The distribution of *HLA-DRB1* alleles was similar in RF positive and negative RA patients.

Anti-CCP antibodies and *SE* alleles

The presence of at least one *SE* allele in the genotype was significantly associated with anti-CCP antibodies positive (82.5% in anti-CCP positive versus 68.4% in anti-CCP negative, OR=2.2 [1.2-4.2]), particularly for the subgroup of patients homozygous for *SE* (OR=5.2 [2.0-13.6]) (Table 1a). The hierarchy of the genotypes risk in the anti-CCP positive subgroup was similar to that previously reported in the literature, although 95% CI largely overlapped (Table 1b).

Table 1. Association between anti-cyclic citrullinated peptide (anti-CCP) antibodies and shared epitope (*SE*) alleles.

a)	Anti-CCP +	Anti-CCP -	OR	95% CI
<i>SE/SE</i>	81	7	5.2	2.0-13.6
<i>SE/X</i>	98	32	1.1	0.7-2.9
<i>X/X</i>	38	18	1	-

b)	Anti-CCP +	Anti-CCP -	OR	95% CI
S_2/S_{3P}	54	4	5.8	1.8-18.6
S_2/S_2	14	2	2.8	0.6-13.6
S_{3P}/S_{3P}	13	1	4.3	0.5-35.6
S_2/L	46	11	1.9	0.8-4.6
S_{3P}/L	52	21	1.2	0.5-2.5
L/L	38	18	1	-

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies. OR= odds ratio; 95% CI= 95% confidence interval. S_2 = *HLA-DRB1**0401, *1303; S_{3P} = *0101, *0102, *0404, *0408, *1001; *L*= all other *HLA-DRB1* alleles.

The distribution of *HLA-DRB1* alleles was similar in anti-CCP positive and negative RA patients, except for *HLA-DRB1**0401 allele (28.0% of *HLA-DRB1**0401 alleles in anti-CCP positive versus 16.4% in anti-CCP negative, OR=1.9 [1.1-3.3]).

Association between autoantibodies status, *SE* alleles and cigarettes smoking

No effect of tobacco exposure on RF status was observed.

Tobacco exposure was significantly associated with anti-CCP antibodies occurrence, but only in presence of *SE* alleles (OR=2.9 [1.2-7.4]) (Table 2a). We found a negative, but not statistically significant interaction between those factors, AP= -0.83 [-1.75 +0.09]. We identified an association between tobacco exposure and *HLA-DRB1**0401 allele (*0401) for the presence of anti-CCP antibodies (Table 2b), and a tendency toward a positive but not statistically significant interaction between those factors, AP= +0.24 [-0.21 +0.76].

Table 2. Odds ratio (OR) and 95% confidence interval (95% CI) for developing anti-cyclic citrullinated peptide (anti-CCP) antibodies in the presence of tobacco exposure (TE) and shared epitope (SE) alleles.

a)	TE	SE	Anti-CCP+	Anti-CCP-	OR	95% CI
	-	-	15	11	1	-
	+	-	21	5	2.9	0.8-10.1
	-	+	78	17	3.3	1.3-8.5
	+	+	77	19	2.9	1.2-7.4

b)	TE	*0401	Anti-CCP+	Anti-CCP-	OR	95% CI
	-	-	40	18	1	-
	+	-	51	18	1.3	0.6-2.7
	-	+	52	10	2.3	0.9-5.5
	+	+	48	6	3.4	1.2-9.4

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies.

Association between anti-CCP antibodies status and cigarettes smoking

Tobacco exposure was not associated with RF positive nor with anti-CCP positivity. However, the risk for anti-CCP positivity increased with the number of years of smoking (Table 3) and was statistically significant at ≥ 20 years of tobacco exposure (OR=3.7 [1.1-12.8]).

Table 3. Odds ratio (OR) with 95% confidence interval (95% CI) of developing anti-cyclic citrullinated peptide (anti-CCP) antibodies rheumatoid arthritis for ever-smokers compared with never-smokers by duration of smoking.

Number of years of smoking	CCP+	CCP-	OR	95% CI
< 10	27	11	0.7	0.3-1.6
10 to 19	29	10	0.8	0.4-2.0
≥ 20	42	3	3.7	1.1-12.8
Never smokers	93	28	1	-

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies.

The risk for anti-CCP antibodies also increased with the number of cigarettes smoked per day, rising from less than 1 for <10 cigarettes per day to 3.3 when ≥ 20 cigarettes were smoked (Table 4).

Table 4. Odds ratio (OR) with 95% confidence interval (95% CI) of developing anti-cyclic citrullinated peptide (anti-CCP) antibodies rheumatoid arthritis for ever-smokers compared with never-smokers by intensity of smoking.

Number of cigarettes smoked a day	CCP+	CCP-	OR	95% CI
1 to 5	32	10	0.9	0.4-2.1
6 to 9	17	6	0.8	0.3-2.3
10 to 19	33	7	1.4	0.5-3.4
≥ 20	16	1	3.3	0.4-26.4
Never smokers	93	28	1	-

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies.

Finally, the risk for anti-CCP antibodies increased up to 4.2 for a cumulative dose of cigarettes smoked ≥ 20 pack-years (Table 5).

Table 5. Odds ratio (OR) with 95% confidence interval (95% CI) of developing anti-cyclic citrullinated peptide (anti-CCP) antibodies rheumatoid arthritis for ever-smokers compared with never-smokers by cumulative dose of smoking.

Number of pack-years	CCP+	CCP-	OR	95% CI
< 10	52	19	0.8	0.4-1.6
10 to 19	26	4	1.8	0.6-5.6
≥ 20	20	1	4.2	0.5-32.4
Never smokers	93	28	1	-

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies.

Indeed, we observed a correlation between the cumulative dose of smoked cigarettes expressed in pack-years, and anti-CCP antibodies titers ($r=0.19$, $P=0.04$).

Association between autoantibodies, tobacco exposure, *SE* and *PTPN22-1858 C/T* genotype. We did not find any association between *PTPN22-1858T* allele and tobacco exposure neither for RF nor for anti-CCP antibodies positivity. We observed that the presence of both genetic factors, *i.e.*, *SE* and *PTPN22-1858T* alleles, was associated with a higher, but not statistically significant, risk to develop anti-CCP antibodies (OR= 2.9 [1.2-7.1]) than for each genetic factor separately (Table 6a). A negative but not statistically significant interaction between those factors was observed, AP= -0.27 [-1.08 +0.54]. Considering only the *HLA-DRB1*0401* allele among the *SE* alleles, the risk for anti-CCP antibodies was similar in the presence of both genetic factors (*HLA-DRB1*0401* and *PTPN22-1858T* alleles) or in the presence of *HLA-DRB1*0401* allele alone, as 95% CI largely overlapped (Table 6b), and a negative but not statistically significant interaction was observed, AP= -0.15 [-0.91 +0.60].

Table 6. Odds ratio (OR) and 95% confidence interval (95% CI) for developing anti-cyclic citrullinated peptide (anti-CCP) antibodies in the presence of shared epitope (*SE*) alleles and/or *1858T* allele of *PTPN22* gene.

a)	<i>SE</i>	<i>1858T</i>	Anti-CCP+	Anti-CCP-	OR	95% CI
	-	-	26	15	1	-
	+	-	116	27	2.5	1.2-5.3
	-	+	12	3	2.1	0.5-8.6
	+	+	62	12	2.9	1.2-7.1

b)	* <i>0401</i>	<i>1858T</i>	Anti-CCP+	Anti-CCP-	OR	95% CI
	-	-	73	31	1	-
	+	-	69	11	2.6	1.2-5.5
	-	+	34	9	1.5	0.7-3.6
	+	+	40	6	2.7	1.0-6.9

Anti-CCP+= presence of anti-CCP antibodies; anti-CCP-=absence of anti-CCP antibodies.

The search for association between genes, tobacco smoking and autoantibodies positivity remained not significantly different in the subgroup of familial RA (78 affected sibling pairs families and 51 trio families with at least one first or second degree relative affected by RA). In the 145 trio families without any family history of RA, the presence of at least one *SE* allele in the genotype was significantly associated with anti-CCP positivity (OR=2.7 [1.1-6.2]), particularly for the subgroup of patients homozygous for *SE* (OR=14.5 [1.7-120.2]), and the presence of both genetic factors, ie *SE* and *PTPN22-1858T* alleles, was associated with a higher, but not statistically significant, risk to develop anti-CCP antibodies (OR= 4.8 [1.4-16.2]) than for each genetic factor separately.

DISCUSSION

In this study, we aimed at evaluating the association between the two RA genetic factors (*SE* and *PTPN22-1858T* alleles), and/or tobacco exposure and autoantibodies (RF and anti-CCP antibodies) positivity in a French familial and sporadic RA sample. We failed to identify any association between RF and *SE*, nor between RF and tobacco smoking. We observed that the presence of at least one *SE* allele was associated with anti-CCP antibodies presence (82.5% versus 68.4%, $P=0.02$), particularly with *HLA-DRB1*0401* allele (28.0% versus 16.4%, $P=0.01$). Tobacco exposure was significantly associated with anti-CCP antibodies, but only in presence of *SE*. The hierarchy of *HLA-DRB1* genotypes risk was respected in the anti-CCP positive subgroup, probably because of the high prevalence of the *HLA-DRB1*0401* allele in this population. A tendency toward a positive but not statistically significant interaction was observed between tobacco, the presence of at least one *HLA-DRB1*0401* allele and anti-CCP antibodies ($AP= +0.24 [-0.21 +0.76]$). The risk for anti-CCP positivity in RA index cases increased as the number of cigarettes smoked per day increased and as the number of years of smoking increased. The cumulative dose of cigarettes smoking was correlated with anti-CCP antibodies titers ($r=0.19$, $P=0.04$). The presence of both *SE* and *PTPN22-1858T* alleles was associated with a higher, but not significantly different, risk to develop anti-CCP antibodies than for each genetic factor separately. But this increased risk disappeared when considering

only the *HLA-DRB1*0401* allele within the *SE*. No association was found between *PTPN22-1858T* allele and tobacco smoking for autoantibodies positivity.

We found here an association between the anti-CCP positivity, tobacco exposure and *SE* alleles, particularly with the *HLA-DRB1*0401* allele. Moreover the anti-CCP antibodies titers were correlated with the intensity of the tobacco exposure, suggesting a strong effect of this environmental factor on autoantibodies production, through a gene-environment association. This gene-environment association was not observed for *PTPN22-1858 T* allele. However *PTPN22* gene encodes a protein which is not involved in the antigene recognition and this gene has a minor effect on RA susceptibility in comparison with *SE*; a larger sample size should be required to observe such a gene-environment association. Surprisingly, we failed to identify any association between RF and anti-CCP antibodies presence and the *PTPN22-1858 T* allele. Indeed, Dieudé et al reported linkage to and association with this allele and the RF positivity in trio families [16]. This difference may be explained by the fact that, in this study, we pooled these trio families and the affected sibling pairs families in an exposed-not exposed study. Furthermore RF status was determined by an Elisa test for IgM on a serum sample collected at the inclusion in the genetic study whereas in the previous study, RF was considered as positive when at least one RF positive result (determined by latex fixation, or Waaler Rose assay or by laser nephelometry) was observed during the disease course.

Although the sample size was limited in this study, our findings of association between *SE*, anti-CCP antibodies and tobacco exposure were similar to those already reported in the literature [11]. Recently, gene-gene and gene-environment interactions in RA were compared in three large case-control studies [19]. This article reported an interaction between *SE* and *PTPN22-1858T* alleles for developing anti-CCP positive RA, and the absence of an interaction between smoking and the *PTPN22-1858T* allele. The association between tobacco exposure, anti-CCP antibodies and *HLA-DRB1*0401* allele is interesting as the citrullination of peptides such as vimentin selectively increased their binding to HLA-DR molecules containing the *SE* motif [20]. Indeed, Hill et al. reported that *HLA-DRB1*0401* transgenic mice had stronger immune response to citrullinated peptides than to native arginine-containing peptides [21].

In this study, we did not find any association between tobacco exposure, *SE* alleles and rheumatoid factor positivity although this association was previously reported in the literature. This observation could be due to the long RA duration in this sample and the possible disappearance of the rheumatoid factor during the evolution of the disease, and maybe to the fact that we chose to test only IgM RF and not IgA RF. Moreover the sample size is rather small to study the interaction between genetic factors and environmental factors, such as tobacco smoking. Replication studies with larger sample size of unselected RA patients, should be required before clinical application.

Finally, it should be of great interest to go further by performing immunological studies to investigate the functional interaction between tobacco exposure, anti-CCP antibodies and *HLA-DRB1*0401* allele, and to determine which component of the tobacco smoke should be responsible of such an autoimmune reaction.

In conclusion, our findings suggest an association between *SE* alleles and tobacco smoking for anti-CCP positivity and a tendency toward an interaction between the *HLA-DRB1*0401* allele and smoking in the development of anti-CCP positivity in this sample of French familial and sporadic RA.

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