HLA-DRB1*0404 is strongly associated with high titers of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis

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Abstract

Objective

*To test whether the presence of RA associated HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*0404 alleles individually influences anti-cyclic citrullinated peptide antibodies (anti-CCP) production.*

Methods

The frequency of anti-CCP antibodies was calculated in the sera of 260 RA patients expressing either two (double dose genotypes SE+/SE+), one (single dose genotypes SE+/SE-) or no RA associated HLA-DR alleles (SE-/SE-). Anti-CCP antibodies titers were also determined.

Results

*RA associated HLA-DR alleles are not mandatory for production of anti-CCP. We found that 68% of SE-/SE- patients were anti-CCP positive. There was no significant difference in anti-CCP between SE negative patient (SE-/SE-) and patients expressing at least one SE (SE+/SE+ and SE+/SE-) (p=0.140). We observed no statistical difference in anti-CCP between RA patients expressing one or two SE (82% vs. 77%, p=0.577). Among SE+/SE-patients, HLA-DRB1*0404 was associated with anti-CCP with a statistically significant difference compared with SE negative patients (90% anti-CCP positive, p=0.02). HLA-DRB1*0404 was also associated with high titers of anti CCP with a statistically significant difference compared with HLA-DRB1*0401 and HLA-DRB1*0101 patients (p=0.025).*

Conclusions

*The RA-associated HLA-DRB1*0404 allele was the most strongly associated with the presence of anti-CCP in RA sera. Moreover, HLA-DRB1*0404 patients had higher titers of anti CCP than patients with other RA associated HLA-DR alleles.*

Key words

Rheumatoid arthritis, HLA-DRB1*0404, anti-CCP.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease with a prevalence of 0.5% worldwide. The etiology of RA is unknown, however a genetic predisposition to RA is well established (1). HLA-DR genes are the strongest genetic component in RA (2). HLA-DR alleles whose B1 chain contains the "shared epitope" (SE), a conserved 5 amino acid motif, carry susceptibility to develop RA. This motif is (according to the one letter amino acid code) QKRAA/ QRRAA/ RRRAA. RA associated HLA-DR alleles include HLA-DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0408, and HLA-DR1 (3). These alleles are not equally associated with risk to develop RA. Indeed, HLA-DR genotypes containing two susceptibility alleles (SE+/SE+) confer a higher risk than genotypes containing only one susceptibility allele (SE+/SE-) which confer a higher risk than DR genotypes containing no susceptibility allele (SE-/SE-) (4). The maximal risk to develop RA is observed in individuals expressing both HLA-DRB1*0401 and HLA-DRB1*0404 (5). However, how these HLA-DRB1 alleles influence the development of RA is unknown.

In this study, we focused on HLA-DRB1*0401, HLA-DRB1*0404 and HLA-DRB1*0101. Because these alleles are associated with different risks to develop RA, we supposed that they act by different mechanisms, helping autoantigen processing or autoantibody production. Among the various autoantibodies known in RA, autoantibodies to citrullinated proteins (ACPA) are highly specific (6). ACPA recognize citrullin (a posttranslationally modified form of arginin) on different proteins like filaggrin, vimentin or fibrin (7, 8). ACPAs are detected by a commercial enzyme-linked immunoabsorbent assay containing a synthetic cyclic citrullinated peptide (anti-CCP) (9). Recent studies have described the association of SE positive alleles with anti-CCP production in different populations of patients with RA (10- 14). Among RA associated alleles, the strongest association was observed with HLA-DR4 and the lowest was with HLA-DR1 (15, 16).

To evaluate the influence of HLA-DRB1*0401, HLA-DRB1*0404 and HLA-DRB1*0101 on anti-CCP production, we quantified anti-CCP in RA patients from Southern France expressing either two, one or no RA associated HLA-DR alleles. Our most striking finding is that HLA-DRB1*0404 is strongly associated with anti-CCP in RA.

Methods

Patients and controls

We studied 260 patients with RA from the rheumatology unit at La Conception Hospital in Marseille (mean age 55.1 years, mean disease duration 11.5 years, 78% female, 91% receiving disease modifying anti rheumatic drugs (DMARDs) and 64% anti-TNF-α). All RA patients fulfilled the American College of Rheumatology 1987 revised criteria (17). The control groups were composed of 126 patients with other type of arthritides: 53 patients with spondylarthropathy (AS) from the rheumatology unit at La Conception Hospital in Marseille, 73 patients with systemic sclerosis (SSc) (from a national cohort elaborated in collaboration with Hospital Cochin, Saint Antoine, Saint Louis, Paris, Hospital Claude Huriez, Lille and Hospital La Conception, Marseille) and 56 healthy controls. Healthy controls were recruited among laboratory staff volunteers and volunteer bone marrow donors after health evaluation to discard autoimmune pathologies. All participants had given informed consent.

HLA-DRB1 typing

HLA-DR typing was performed by PCR/sequence specific oligonucleotide analysis (18). The following alleles were classified as SE positive: HLA-DRB1*0101, HLA-DRB1*0102, HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0405, HLA-DRB1*0408 and HLA-DRB1*1001. In our cohort, 25% of RA patients expressed two SE (SE+/SE+), 54% carried one SE (SE+/SE-) and 20% were SE negative (SE-/SE-).

Anti-CCP2 antibodies

Anti-cyclic citrullinated peptide IgG antibodies were detected by a second-generation ELISA (Immunoscan RA Mark 2, Eurodiagnostica, Sweden). All

Table I. Distribution of shared epitope and anti-CCP positivity in RA patients and controls*.

	RA patients Number (%)	Spondylarthropathy Number (%)	Systemic sclerosis Number (%)	Healthy controls Number (%)
SE+/SE+	54/66 (82)	1/3 (33)	0/0	0/0
SE+/SE-	109/141 (77)	0/12	1/15 (7)	0/18
SE-/SE-	36/53 (68)	1/38 (3)	3/58 (5)	0/38
Total	199/260 (77)	2/53 (4)	4/73 (5)	0/56

*anti-CCP: anti-cyclic citrullinated peptide; RA: rheumatoid arthritis; SE: shared epitope.

Table II. Frequency of RA patients whose sera were positive for anti-CCP by number of SE*.

	Number of patients	Anti-CCP>25 U/ml Number (%)	*p* versus SE-/SE- group
SE+/SE+	66	54 (82)	0.124
HLA-DRB1*0401/ HLA-DRB1*0404	22	19 (86)	0.175
HLA-DRB1*0101/ HLA-DRB1*0401	13	10 (77)	0.767
HLA-DRB1*0101/ HLA-DRB1*0101	8	6 (75)	0.995
HLA-DRB1*0404/ HLA-DRB1*0404	4	3 (75)	0.792
HLA-DRB1*0401/ HLA-DRB1*0401	5	3 (60)	0.891
HLA-DRB1*0101/ HLA-DRB1*0404	3	3 (100)	-
other SE+/SE+	11	10 (91)	0.240
SE+/SE-	141	109 (77)	0.248
HLA-DRB1*0404/SE-	41	37 (90)	0.02§
HLA-DRB1*0401/SE-	50	36 (72)	0.814
HLA-DRB1*0101/SE-	50	36 (72)	0.814
SE-/SE-	53	36 (68)	
Total	260	199 (77)	

*anti-CCP: anti-cyclic citrullinated peptide; RA: rheumatoid arthritis; SE: shared epitope.
§ p=0.056 versus HLA-DRB1*0401/SE- group and HLA-DRB1*0101/SE- group.

Table III. Frequency of RA patients whose sera were positive for high titer of anti-CCP*.

	Number of patients	Anti-CCP>200 U/ml Number (%)	p versus SE-/SE- group
SE+/SE+	66	36 (55)	0.999
HLA-DRB1*0401/ HLA-DRB1*0404	22	13 (59)	0.809
HLA-DRB1*0101/ HLA-DRB1*0401	13	8 (62)	0.799
HLA-DRB1*0101/ HLA-DRB1*0101	8	3 (38)	0.668
HLA-DRB1*0404/ HLA-DRB1*0404	4	2 (50)	0.635
HLA-DRB1*0401/ HLA-DRB1*0401	5	3 (60)	0.872
HLA-DRB1*0101/ HLA-DRB1*0404	3	1 (33)	0.949
other SE+/SE+	11	6 (54)	0.819
SE+/SE-	141	67 (48)	0.618
HLA-DRB1*0404/SE-	41	27 (66)	0.289 §
HLA-DRB1*0401/SE-	50	20 (40)	0.268
HLA-DRB1*0101/SE-	50	20 (40)	0.268
SE-/SE-	53	28 (53)	
Total	260	131 (50)	

*anti-CCP: anti-cyclic citrullinated peptide; RA: rheumatoid arthritis; SE: shared epitope.
§ p=0.025 versus HLA-DRB1*0401/SE- group and HLA-DRB1*0101/SE- group.

the assays were performed in duplicate. A test result above 25 U/ml was considered positive. A test result above 200 U/ml was considered as a high titer of anti-CCP (10).

Statistical analysis

Chi-square test, Fisher exact test were used to compare anti-CCP status between genotypic groups. Kruskal-Wallis test was used to compare the medians of anti-CCP titers from the different groups of patients. $p<0.05$ was considered significant.

Results

Sensitivity and specificity of anti-CCP in RA patients

The sera of 260 patients with rheumatoid arthritis (RA), 73 patients with systemic sclerosis (SSc), 53 patients with spondylarthropathy (AS) and 56 healthy controls were tested for the presence of anti-CCP2 antibodies. A test result above 25 U/ml was considered positive (Table I). The percentage of patients whose sera were positive for anti-CCP was calculated in groups of patients expressing either two SE (SE+/SE+), one SE (SE+/SE-) or no RA associated allele (SE-/SE-).

We found that 77% of RA patients were positive for anti-CCP. Among control groups, 5% of SSc patients, 4% of AS patients and 0% of healthy controls were positive. Thus, in our RA population, anti-CCP have a sensitivity of 77% and specificity of 97%.

*HLA-DRB1*0404 is associated with anti-CCP antibodies*

To evaluate the influence of RA associated HLA-DR alleles on anti-CCP production, we compared the frequency of anti-CCP positive sera among groups of patients expressing either two (SE+/SE+), one (SE+/SE-) or no RA associated HLA-DR alleles (SE-/SE-) (Table II).

We found that RA associated HLA-DR alleles are not mandatory for production of anti-CCP. Indeed 68% of SE-/SE- patients had anti-CCP. There was no significant difference in anti-CCP between SE negative patient (SE-/SE-) and patients expressing at least one SE (SE+/SE+ and SE+/SE-) (p=0.140 by Chi square test). We observed no statis-

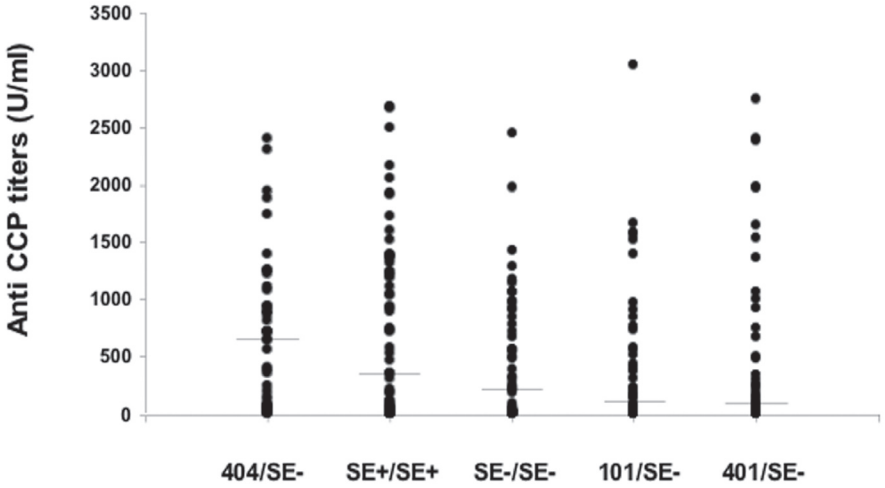

Fig. 1. Anti-CCP titers in 260 RA patients. Serum samples with a test result above 25 U/ml were considered positive. Horizontal bars indicate median titers of anti CCP antibodies for each group: 660, 362, 227, 115 and 87 U/ml respectively. HLA-DRB1*0404 is associated with a significantly higher median of anti CCP using a Kruskal Wallis test (p=0.017).

tical difference between RA patients expressing one or two SE (82% *vs*. 77%, p=0.577 by Chi square test). Among SE+/SE+ patients, there was only a trend towards association between anti-CCP and the HLA-DRB1*0401/HLA-DRB1*0404 genotype (86% anti-CCP positive, p=0.175 by Chi square test). Among SE+/SE- patients, we found that HLA-DRB1*0404 was associated with anti-CCP with a statistically significant difference compared with SE-/SE- patients (90% anti-CCP positive, p=0.02 by Chi square test).

*HLA-DRB1*0404 is associated with higher titers of anti-CCP*

To assess whether anti-CCP titers correlated with presence of particular SE alleles, we made a quantitative analysis of anti-CCP. A test result above 200 U/ml was considered as a high titer of anti-CCP (Table III).

We observed that 55% of SE+/SE+ patients, 48% of SE+/SE- patients and 53% of SE-/SE- patients were high anti-CCP positive. Among SE+/SE- patients, we found that HLA-DRB1*0404 was associated with high titers of anti-CCP with a statistically significant difference compared with HLA-DRB1*0401 and HLA-DRB1*0101 patients (p=0.025 by Chi square test). HLA-DRB1*0404 was also associated with the highest median titers of anti-CCP and this result is significant using a Kruskal Wallis test (p=0.017) (Fig. 1).

Discussion

Most patients with RA express particular HLA-DR alleles whose DRB1 chains share a highly conserved amino acid motif called shared epitope (SE). HLA-DRB1*0404 and HLA-DRB1*0401 confer high risk while others like HLA-DRB1*0101 carry lower risks of RA (19). Moreover, a dose effect has been observed in SE positive HLA-DRB1 genotypes. In particular, individuals expressing both HLA-DRB1*0401 and HLA-DRB1*0404 are exposed to maximal risk to develop RA. This synergic effect is still unexplained and respective contribution of each allele to the pathogenesis of RA is not understood. In addition, RA associated alleles are not associated with the same degree of disease severity. Several studies have shown an association between HLA-DR4 alleles and extra-articular disease manifestations (20). In particular, HLA-DRB1*0401 predisposes to more severe disease (21). These data suggest that RA associated HLA-DR alleles can contribute to the development of RA by different mechanisms.

RA sera contain a family of autoantibodies directed against various citrullinated proteins. They can be detected by anti-CCP assay. Anti-CCP are highly specific for RA and are reported to be a good predictor for the development of RA. In this work, we studied the association between anti-CCP production and HLA-DRB1*0101, 0401 and 0404 in single or double dose genotypes. To that, we quantified anti-CCP in sera of RA patients from Southern France expressing different HLA-DRB1 genotypes.

We found that the presence of RA associated HLA-DR alleles is not mandatory for production of anti-CCP. In a previous study, we observed a similar result when we compared the frequency of sera positive for anti-citrullinated fibrinogen (AFIBA) among the groups of patients expressing two, one or no RA associated HLA-DR alleles. Fifty five percent of the SE negative patients had antibodies to citrullinated fibrinogen (22).

We could find a higher percentage of anti-CCP in patients expressing at least one SE allele compared to SE negative patients, but the difference was not statistically significant (79%, p=0.140). This is different from the Leiden early arthritis cohort in which anti-CCP were positive in 61% of patients expressing at least one SE allele compared to 36% of SE negative patients (10). In the North American RA consortium, SE alleles are also strongly associated with anti-CCP (11). The relatively small sample available could explain this discrepancy.

In addition, we expected higher anti-CCP positivity in patients expressing two SE alleles compared to patients expressing one SE allele. The association of two SE alleles with anti-CCP positivity has been described in the Leiden early arthritis cohort (by comparing with the healthy control group without SE as the referent) (13). In our cohort, a similar percent of RA patients expressing either two or one SE were anti-CCP positive. Moreover, there was no difference in anti-CCP titer between patients expressing two or one SE alleles. The same result was observed in the Leiden early arthritis cohort. Patients carrying two SE alleles did not have a significantly higher anti-CCP titer compared with patients carrying one SE allele (10).

Among single dose SE patients, we tested whether anti-CCP antibodies were associated with a particular HLA-DR allele. We observed that HLA-

DRB1*0404 was strongly associated with anti-CCP production in RA. Moreover, HLA-DRB1*0404 was also associated with high titers of anti-CCP (the highest median anti-CCP titer and the most important percentage of sera with anti-CCP above 200 U/ml). These data are consistent with studies which revealed a highly significant association between anti-CCP and HLA-DR4 (15). HLA-DRB1*0401 is associated with anti-CCP in Northern European and Portuguese populations (12, 15, 16, 23). This is the first description of association between HLA-DRB1*0404 and anti-CCP production in RA.

How HLA-DRB1*0404 can contribute to the production of anti-CCP antibodies remains to be explained. We recently observed that HLA-DRB1*0404 is also associated with anti-citrullinated fibrinogen in RA sera. Indeed, 83% of RA patients expressing HLA-DRB1*0404 have autoantibodies to citrullinated fibrinogen (22). Moreover, by screening synovial proteins with sera of RA patients homozygous for HLA-DR alleles, we observed that sera of RA patients homozygous for HLA-DRB1*0404 recognized a 100 kD synovial protein identified as calpastatin, the natural inhibitor of calpains (proteases involved in cartilage destruction). Indeed, 50% of RA patients expressing HLA-DRB1*0404 have autoantibodies to synovial calpastatin (24).

Our data support the idea that HLA-DRB1*0404 carries an original function allowing its association with many autoantibody responses in RA.

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