Human Leukocyte Antigen Polymorphisms in Italian Primary Biliary Cirrhosis: A Multicenter Study of 664 Patients and 1992 Healthy Controls

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Genetic factors are critical in determining susceptibility to primary biliary cirrhosis (PBC), but there has not been a clear association with human leukocyte antigen (HLA) genes. We performed a multicenter case-control study and analyzed HLA class II DRB1 associations using a large cohort of 664 well-defined cases of PBC and 1992 controls of Italian ancestry. Importantly, healthy controls were rigorously matched not only by age and sex, but also for the geographical origin of the proband four grandparents (Northern, Central, and Southern Italy). After correction for multiple testing, DRB1*08 [odds ratio (OR), 3.3; 95% confidence interval (CI), 2.4-4.5] and DRB1*02 (OR 0.9; 95% CI 0.8-1.2) were significantly associated with PBC, whereas alleles DRB1*11 (OR 0.4; 95% CI 0.3-0.4) and DRB1*13 (OR 0.7; 95% CI 0.6-0.9) were protective. When subjects were stratified according to their grandparental geographical origin, only the associations with DRB1*08 and DRB1*11 were common to all three areas. Associated DRB1 alleles were found only in a minority of patients, whereas an additive genetic model is supported by the gene dosage effect for DRB1*11 allele and the interaction of DRB1*11,*13, and *08. Lastly, no significant associations were detected between specific DRB1 alleles and relevant clinical features represented by the presence of cirrhosis or serum autoantibodies. In conclusion, we confirm the role for HLA to determine PBC susceptibility and suggest that the effect of HLA is limited to patient subgroups. We suggest that a large whole-genome approach is required to identify further genetic elements contributing to the loss of tolerance in this disease. (HEPATOLOGY 2008;48: 1906-1912.)

Abbreviations: AMA, anti-mitochondrial antibody; CI, confidence interval; HLA, human leukocyte antigens; OR, odds ratio; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction.

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Although the cause of primary biliary cirrhosis (PBC) remains unknown,[1] genetic susceptibility is critical in determining disease onset and severity. The role of genetic factors is supported by familial clustering, concordance rates in monozygotic twins, and multiple genetic association studies.[2,3] Nevertheless, no conclusive data on specific genes have been obtained, and proposed associations have seldom been independently replicated. An approach to the problem of PBC genetics based on linkage analysis is poorly feasible based on the rarity of the disease and the advanced age at diagnosis.

Polymorphisms of the class II human leukocyte antigens (HLA) have been extensively studied in immune-mediated diseases, and disease associations have been demonstrated in rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, and type I diabetes, among others.[4-6] Studies on these genetic factors in PBC have been performed on small populations of patients and cumulatively suggest an association with the HLA-DR beta 1 (DRB1)*8 allele,[3] but our previous study could not reproduce this finding,[7] possibly because of a geographical variability. As shown by studies of hemochromatosis,[8] the Italian population manifests a peculiar susceptibility background for complex diseases, and different areas of Italy are characterized by unique genetics.[9] The case of HLA in PBC falls within these assumptions based on a recent comparison of patients from Italy and the United Kingdom in which different HLA associations were found.[10]

To overcome the limitations of previous study and to achieve a sufficient statistical power, we initiated a national multicenter effort and collected DNA from 664 patients with PBC (the largest series ever reported in a genetic study) and 1992 healthy controls. The controls were rigorously matched for sex, age, and geographical origin of the four grandparents. These studies provide strong statistical evidence that PBC susceptibility is associated with the HLA DRB1*08 allele whereas HLA DRB1*11 and DRB1*13 confer protection from the disease. A weak association with HLA DRB1*02 was also found.

Patients and Methods

Study Population and Design. Through a multicenter case-control study that included 27 secondary and tertiary hepatology referral centers throughout Italy, we obtained whole blood samples and clinical data from 664 unrelated patients of Italian ancestry with a diagnosis of PBC. In all cases, the diagnosis was based on internationally accepted criteria, in other words, when two of the following criteria were met: elevated serum alkaline phosphatase levels for longer than 6 months, compatible or diagnostic liver histology, and positive serum anti-mitochondrial antibody (AMA).[1] Serum AMA were determined using indirect immunofluorescence, and titers 1:40 or greater were considered as positive. Eighty-five patients (13%) had undetectable serum AMA but otherwise fulfilled the diagnostic criteria and were classified as having AMA-negative PBC.[11] The geographical origin (north, center, and south of the country) of all subjects was defined on the basis of the birthplace of their grandparents for control matching purposes. Serum liver function and the levels of lipids, immunoglobulins, hepatitis B surface antigen, and antibodies to hepatitis B core antigen and hepatitis C virus were assessed by means of routine laboratory methods, and patients with signs of chronic viral infection were excluded from the study. The presence of PBC-related symptoms was defined as the occurrence of pruritus, jaundice, or major complications of cirrhosis: in other words, hepatic encephalopathy, variceal bleeding, ascites requiring diuretic therapy, or hepatocellular carcinoma. Disease duration was calculated as the time between the date of the earliest suspected evidence of liver disease and the date of blood sampling. The patients with no fibrosis on liver biopsy, that is, those with Ludwig's stage I and II,[12] were considered as having early-stage disease; those with fibrosis or cirrhosis (that is, stage III or IV) were considered as having advanced disease. Lastly, the Mayo Risk Score values were calculated at the time of enrollment.[13] Table 1 summarizes the demographic, clinical, and biochemical features of the PBC cohort included in this study at the time of enrollment. Similar to reported sex ratios, 604 of 664 (91%) PBC cases were women, and serum AMA were detected in 87% of cases. Two hundred eighty-six patients (42%) mani-

Table 1. Demographic, Clinical, and Biochemical Characteristics of Patients with PBC Enrolled in the Study

Subject Characteristics	PBC (n = 664)	Controls (n = 1992)
Female sex	604 (91%)	1812 (91%)
Age (years)	59 (21–97)	51 (22–70)
Geographical origin		
North	301 (45%)	903 (45%)
Center	171 (26%)	513 (26%)
South	192 (29%)	576 (29%)
Duration of disease (years)	10 (2–32)	–
No. of symptomatic patients	196 (30%)	–
No. with advanced stage of disease	284 (43%)	–
Total bilirubin (mg/dL) (n.v. < 1.0)	1 (0.2–40)	–
Alkaline phosphatase (IU/L) (n.v. < 279)	452 (44–3036)	–
Aspartate aminotransferase (IU/L) (n.v. < 50)	26 (12–164)	–
Serum AMA-positive	579 (87%)	–
Mayo score	5.3 (3.1–11.7)	–

Continuous variables are expressed as median (range).

fested advanced PBC, whereas 29% had symptoms at the time of enrollment.

The control population consisted of 1992 healthy subjects randomly selected from a DNA bank of over 60,000 blood and bone marrow donors. For each PBC case, three controls were enrolled after matching for sex, age (according to three age-groups: 25-45, 45-65, and older than 65 years), and geographical origin of the four grandparents. DNA samples from this population had previously been collected at the IRCCS, Ospedale Maggiore Policlinico in Milan, and stored at −80°C.

Samples from both patients and controls used in our previous work[7] and PBC samples included in a smaller association study[10] constituted a subset of the cases and controls used in the current work. The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki (Edinburgh, 2000), and all subjects enrolled in the study provided written informed consent.

DNA Extraction and HLA DRB1 Genotyping. Genomic DNA was extracted from whole blood using the standard salting out method, and samples were stored at −80°C until used. We used a reverse line blot assay for HLA-DRB1 typing (Dynal RELI SSO HLA-DRB1 Test) on polymerase chain reaction (PCR)-amplified DNA.[14] In this format, the DNA target is amplified with biotinylated primers, and the resulting PCR product is denatured and hybridized to an array of oligonucleotide probes immobilized on a nylon membrane support. After a stringent wash to remove unbound PCR sample, the presence of the PCR product bound to a specific probe is detected using a streptavidin-horseradish peroxidase enzyme that converts a soluble colorless substrate into a blue precipitate. This method allows the identification of DRB1 alleles at broad level (DRB1*01-18).

Statistical Analysis. The allele distributions in the patient and control groups, and in the groups of patients with different disease features, were compared using the Fisher's exact test. We note that DRB1 alleles were in Hardy-Weinberg equilibrium among control subjects ($P = 0.15$, exact test) when calculated as described elsewhere.[15] The relative predispositional effects method was used to seek secondary associations.[16] To compare the groups of patients defined on the basis of their HLA polymorphism status, the chi-square or Fisher's exact test were used in the analysis of categorical variables and the Mann-Whitney test in the analysis of continuous variables. All analyses were two sided, with P values of <0.05 being considered statistically significant. To control for multiple testing, the P values were corrected (Pc) by the number of comparisons according to the Bonferroni inequality method, applying a correction factor of 12, that is, the total number of the detected alleles for the DRB1 locus.

Table 2. DRB1 Allele Frequencies Observed in 664 Patients with PBC and 1992 Healthy Controls

DRB1 Allele	PBC (%)	H*	Controls (%)	H*	P†	Pc
*01	9.6	8	8.2	7		
*02	12	10	12	32	0.0034	0.041
*03	10.9	9	7.8	11		
*04	10	3	9.1	16	0.038	
*07	16.9	28	11.7	25		
*08	7.2	2	2.3		4×10^{-32}	4.8×10^{-31}
*09	0.7		0.2		0.012	
*10	1.6		1.0			
*11	13.6	16	30.0	166	1.9×10^{-24}	2.3×10^{-23}
*12	1.1		1.0	1		
*13	8.6	5	11.2	16	3.6×10^{-6}	0.000043
*14	7.8	5	5.5	5		

The prevalence (%) and number of homozygous (H) are listed for PBC and controls. P values before (P) and after correction for multiple testing (Pc) are indicated for each allele when < 0.05.

*Number of homozygotes for alleles.

†Only P values < 0.05 are shown.

These statistical comparisons were made using Stata Statistical Software (Stata Corp., College Station, TX), whereas genotypical analyses were performed using version 9.1 of the SAS programming language (SAS Institute, Cary, NC) and R (www.r-project.org/). Conditional logistic regression models were implemented to estimate odds ratios (OR) and 95% confidence intervals (CI).

Results

HLA-DRB1 Polymorphisms in Patients with PBC and Controls. The prevalence of each allele was first compared between all enrolled patients with PBC and matched controls (Table 2). After correcting for multiple testing (P_c), that is, adjusting for the compensatory decrease in PBC allele frequencies because of overrepresented alleles, HLA DRB1*08 was more frequent among patients with PBC than controls (7.2% versus 2.3%, respectively; $P_c = 4.8 \times 10^{-31}$). A second weak association with DRB1*02 was also found ($P_c = 0.041$). Negative associations were detected between PBC and DRB1*11 (13.6% in PBC versus 30% in controls, $P_c = 2.3 \times 10^{-23}$) and DRB1*13 (8.6% in PBC versus 11.2% in controls, $P_c = 0.000043$) alleles. Accordingly, carrying the DRB1*08 and DRB1*02 alleles were associated with an increased risk of having PBC (OR = 3.1; 95% CI = 1.5-4.9 and OR = 0.9; 95% CI = 0.8-1.2, respectively). Conversely, DRB1*11 (OR = 04; 95% CI = 0.3-0.4) and DRB1*13 (OR = 0.7; 95% CI = 0.6-0.9) alleles led to a reduced risk of PBC occurrence. Other DRB1 alleles were detected with similar frequencies in both populations.

Table 3. DRB1 Allele Frequencies Observed in Patients with PBC and Healthy Controls from the North, Center, and South of Italy

DRB1 allele	Patients with PBC (%)	H*	Controls (%)	H*	*P*	*Pc*
North	(n = 301)		(n = 903)			
*08	8.8	2	3.4		4×34^{-13}	5.2×10^{-12}
*11	13.6	8	26.3	50	3.9×10^{-8}	4.6×10^{-7}
*13	7.3	2	11.9	4	5.7×10^{-6}	0.000068
Center	(n = 171)		(n = 513)			
*08	7.3		2.0		1×10^{-11}	1.2×10^{-10}
*09	0.6		0.1		0.011	
*11	12.3	4	30.6	42	9.9×10^{-9}	1.2×10^{-7}
*13	9.6	1	12.6	6	0.010	
*14	10.2	1	5.6		0.013	
South	(n = 192)		(n = 576)			
*02	10.7	2	13.4	11	0.0033	0.039
*07	18	8	10.8	6	0.019	
*08	4.7		0.9		9.5×10^{-16}	1.1×10^{-14}
*09	0.8		0.1		0.000075	0.00090
*10	2.3		0.9		0.019	
*11	14.8	4	35.8	74	6.6×10^{-11}	7.9×10^{-10}

The prevalence (%) and number of homozygous (H) are listed for PBC and controls. *P* values before (P) and after correction for multiple testing (Pc) are indicated for each allele when <0.05. Only associations with uncorrected *P* values < 0.05 are illustrated.

*Number of homozygotes for alleles.

HLA-DRB1 Polymorphisms in Patients with PBC from Different Areas. When patients were subdivided according to their geographical origin determined by four grandparental information, we noted that 301 (45%) were from northern Italy, 171 (26%) from the central Italy, and 192 (29%) from the southern Italy. Table 3 illustrates the significant associations observed when HLA DRB1 allele frequencies comparisons between patients and controls were limited to each geographical region. The associations with DRB1*08 and *11 were found in all three groups, whereas the negative association between PBC, HLA DRB1*13, was found only in subjects from northern Italy, and HLA DRB1*02 was found only in subjects from southern Italy. In addition, a previously undetected association with DRB1*09 was also observed only in the southern subgroups of patients and controls.

Analysis of PBC Genotypical Associations. To further explore the DRB1 associations with PBC, the genotypes were examined for the DRB1 specificities identified in the allelic association tests. Similar to the allelic association results, DRB1*08 showed a strong positive association, *11 showed a strong protective effect and *13 a modest protective effect (Table 4). In addition, examination of the genotypes suggested a gene dosage effect because DRB1*11 homozygotes showed a lower OR than *11 heterozygotes (0.18 versus 0.33). Comparison of two copies of *11 with one copy *11 showed an OR of 0.563 (95% CI 0.327-0.967). A gene dosage effect was not observed for *13 or *08 (data not shown); however, the low frequency of these genotypes precluded a meaningful analysis.

The interaction between these DRB1 genotypes was also examined. The DRB1*11/*13 heterozygotes showed a lower OR than when only a single *11 or *13 genotype was present (Table 4). In addition, the DRB1*11/*13 heterozygote genotypes showed an effect similar to that of the *11/*11 homozygote genotypes. Thus, these results suggest a gene dosage effect of these protective alleles. When *08/*11 heterozygotes were considered, neither positive (*08) nor negative (*11) effects were observed (OR 0.94; 95% CI 0.50-1.76). Lastly, the interaction of DRB1*08, *11, and *13 genotypes was examined by analyzing the residual deviance of the chi-squared tests. An additive model was highly favored compared with a general genotypical model ($P < 0.0001$). Together these observations strongly support an additive model to explain the DRB1 association with PBC.

HLA-DRB1 Polymorphisms and PBC Clinical Features. Patients were also stratified according to disease characteristics, including disease stage, symptoms, serum AMA status, or autoimmune comorbidities. No significant changes in HLA DRB1 allele and genotypic frequencies were observed in these clinical subsets (data not shown).

Discussion

We herein report results from a large cohort of patients with PBC and demonstrate that HLA-DRB1*08 is associated with an increased risk of PBC and that DRB1*11 and 13 are associated with a protective effect. We also found a weak susceptibility association with DRB1*02.

Table 4. Genotypic Analysis of DRB1* Associations

Genotype	Analysis	Odds Ratio	95% CI	*P* Value
*08	1 versus 0*	3.32	2.44-4.51	2.0×10^{-14}
*11	1 versus 0	0.32	0.26-0.39	2.1×10^{-26}
	2 versus 0	0.17	0.10-0.29	9.1×10^{-11}
	2 versus 1	0.54	0.31-0.93	0.025
*13	1 versus 0	0.70	0.55-0.89	0.0031
*11/*13	versus 0	0.11	0.06-0.22	1.4×10^{-10}
	versus *11	0.38	0.19-0.74	0.0043
	versus *13	0.20	0.10-0.40	5.5×10^{-6}
	versus *11 or *13	0.30	0.16-0.59	0.00042
	versus *11/*11	0.77	0.34-1.76	0.54
*08/*11	versus 0	0.94	0.50-1.76	0.85
	versus *08	0.34	0.17-0.69	0.0029
	versus *11	2.81	1.48-5.34	0.0017

*The effect of one or two copies of the genotype compared with 0 or 1 copy is shown for the different genotypes. Two copies of DRB1*08 and *13 are not included since the frequency of *08/*08 and *13/*13 homozygotes were < 0.01.

The current study emphasized a close matching of ethnic background, which may decrease both type 1 error rates because of unrecognized population stratification. This is perhaps particularly important in studies of major histocompatibility complex (MHC) associations even in populations restricted to the European continent. It is known that there are substantial differences in HLA-DRB1 allele frequencies in different European populations, including regional differences within Italy. In addition, the increased population homogeneity in our regional subsets may have decreased genetic heterogeneity and resulted in increased power (lower type 2 error rate) to detect specific associations. The DRB1*08 result is contrary to our previous findings[7] and supports the need for the use of large numbers of patients and well-matched controls to provide solid evidence of genetic associations in complex diseases.

The cause of PBC autoimmunity remains enigmatic; indeed, the relative importance of genetic and environmental factors in determining disease onset remains poorly defined. Data from familial clustering, monozygotic twin concordance rates, and association studies strongly support the necessity of a permissive genetic background,[3] with the possible contribution of sex chromosome defects.[17,18] Similar to other autoimmune diseases, HLA has been widely studied in PBC susceptibility over the past decades. Data have cumulatively suggested an association with alleles and haplotypes of the HLA-DRB1 locus, particularly HLA-DRB1*08 allele.[3] However, a critical evaluation of the previously published studies on this issue manifests several potential flaws: first, the low frequency of HLA-DRB1*08 antigen; second, earlier studies did not rely on molecular analysis; third, the control matching methods often raised concerns; fourth, and more important, many studies were performed on limited numbers of patients and controls.

Several aspects constitute the strengths of the current study. First, it is important to note that all diagnoses were independently reevaluated, and only cases fulfilling international criteria were included. Second, we avoided restricting the study field to cases of advanced PBC, a flaw that limits most studies carried out at tertiary referral centers. Third, the nationwide recruitment enabled us to obtain a representative population and to match controls rigorously by geographical area, age, and sex. This is of relevance because HLA alleles have different distributions in different geographical areas within Italy,[19,20] and new population structure data are compelling.[21] The definition of the geographical origin of subjects on the basis of the birthplace of their grandparents may improve such geographical analyses, and multiple subjects would have potentially been misclassified without this information (data not shown). We also emphasize that the sample size obtained through our multicenter effort had sufficient statistical power for the chosen statistical approach and possibly overcomes the need for more complex algorithms and tests.[22]

Our data cumulatively suggest that HLA polymorphisms have distinctive characteristics in the Italian area. When data from all cases and controls were analyzed, Italian patients with PBC had a significantly lower frequency of HLA-DRB1*11 and *13, suggesting a protective role of these alleles, and a positive association with HLA-DRB1*08. These findings are in agreement with previous smaller studies from our group[7] and others.[10,23] It is of note that in these reports the population sizes were significantly smaller compared with our current study. The lack of evidence for DRB1*13 association in the southern Italian group may be secondary to the nearly null frequency of this allele in the southern population. Nevertheless, we cannot rule out the possibility that similar associations might be proven in other geographical areas when a sufficient number of patients and controls are studied. Furthermore, we report for the first time a gene dosage effect observed for the DRB1*11 allele and *11/*13 heterozygote. In addition, an additive genetic model was favored when DRB1*11, *13, and *08 genotypes were examined in all subjects. A similar effect was recently suggested for DQB1 in celiac disease,[24,25] and it is of interest that this condition shares several clinical and genetic features with PBC. Lastly, we cannot rule out the possibility that reported associations are in fact markers of different susceptibility genes in linkage disequilibrium with HLA-DRB1 alleles.

Studies from the United Kingdom and Sweden have reported contrasting data on the association of HLA-DRB1*08 with disease susceptibility and progression,[26,27] whereas data from North America suggested the association of different HLA class II polymorphisms in patients with PBC arrayed by serum AMA status.[28] We could not recapitulate the proposed endophenotypes in our population, and we hypothesize the possible role of sample size and ethnicity in determining this discrepancy.

Particular DRB1 allelic associations also suggest possible disease mechanisms. In particular, two of the alleles found to be protective for PBC have been raised previously as having a protective role for multiple infectious diseases. These studies suggest that HLA-DRB1*13 is protective for human immunodeficiency virus,[29] hepatitis B virus,[30,31] hepatitis C virus,[32] human papilloma viruses,[33] and malaria.[34] Similarly, HLA-DRB1*11 allele exerts a strong protective role against human immunodeficiency virus,[35-37] hepatitis C virus,[38,39] and human papilloma viruses.[40] DRB1*11 has also been suggested as a protective allele in autoimmune diseases,[41] particularly of the liver.[42,43] In addition, both DRB1*11 and

*13 confer resistance to several types of cancer.[44-46] Taken together, these studies suggest that both alleles might influence the penetrance of infectious agents as well as the maintenance of immune tolerance and have implications in light of the proposed infectious theory in PBC origin.[47] Furthermore, because the protective HLA alleles are associated with resistance to several infections,[48] we hypothesize that the lack of such alleles might contribute to the molecular mimicry of infectious agents leading to tolerance breakdown in PBC.[47] There have been multiple causative agents suggested as playing a role in the initiation of PBC, including viral infections, bacteria, and chemical xenobiotics.[49-51] We suggest that there will be many potential causative factors and that all will share in common a loss of tolerance to pyruvate dehydrogenase complex E2 subunit (PDC-E2), the immunodominant autoantigen of PBC. However, underlying all of these will be an element of genetic permissiveness accompanied by stochastic events. Although a large number of genetic polymorphisms have been suggested as PBC determinants,[3] only a genome-wide effort will provide solid associations, as demonstrated by recent studies in other complex autoimmune diseases.[5,52] As well illustrated by twin data, additional nongenomic factors may play a role in PBC susceptibility[2] and might thus reduce the power of a genome-wide approach.

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