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BRIEF REPORT

Association Between HLA Haplotypes and Increased Serum Levels of IgG4 in Patients With Primary Sclerosing Cholangitis

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Increased serum levels of IgG4 have been reported in 9%–15% of patients with primary sclerosing cholangitis (PSC); it is not clear whether this increase contributes to pathogenesis. We performed genetic analyses of the HLA complex in patients with PSC from Norway, Sweden, and from the United States. We found an association between levels of IgG4 above the upper reference limit and specific HLA haplotypes. These patients had a significantly lower frequency of the strongest PSC risk factor, HLA-B*08, than patients without increased IgG4, and significantly higher frequencies of HLA-B*07 and HLA-DRB1*15. HLA genotype therefore might affect the serum concentration of IgG4, and increased IgG4 might be a marker of a distinct phenotype of PSC.

Keywords: Genetic Association Study; Immunogenetics; IgG4-Associated Cholangitis; Immune Response.

The clinical phenotype of primary sclerosing cholangitis (PSC) is heterogeneous. Several comorbidities are found only in subgroups of the patients, for example, inflammatory bowel disease (IBD) (up to 80%), other autoimmune disorders (approximately 25%), and features of autoimmune hepatitis (approximately 10%).[1] There also is reason to believe that the nature of the bile duct affection varies (eg, small-duct affection only is found in approximately 10% of the patients).[1] The finding of increased serum concentrations of IgG4 in a proportion of PSC patients first was reported in 2006.[2] Later studies have corroborated this observation, yielding frequencies of 9%–15%.[2–4] Increased IgG4 in PSC seems to be a marker of a more severe disease course.[2] How or whether it may relate to IgG4-associated cholangitis (IAC), occurring in the context of systemic inflammatory IgG4-related disease,[5] is obscure. In contrast to PSC, IAC responds to immunosuppression, but to what extent that also pertains to PSC patients with increased IgG4 is undetermined.[6]

Recently, it was shown that the IgG4-producing B cells in IAC show a large degree of clonality,[7] suggesting the presence of specific antigenic triggers. There also is considerable evidence to support an autoimmune component to the pathogenesis in PSC,[1] but how this relates to high IgG4 concentrations observed in a fraction of patients is unknown. The strongest genetic risk factors in PSC are encoded within the HLA complex on chromosome 6p21.[8] Because of genetic properties of the HLA complex (strong linkage disequilibrium) and the presence of multiple, independent association signals, it has proven exceedingly difficult to determine the biologically relevant gene variants.[8,9] We hypothesized that increased IgG4 concentrations serve as a marker for a pathogenetically distinct group of PSC patients, and therefore aimed to explore the clinical features and HLA background of this group.

We determined the IgG4 level in 263 Norwegian PSC patients (Supplementary Table 1 and Supplementary Materials and Methods section). Several IgG4 assays with different upper reference levels (URLs) exist. In this study, an increased serum IgG4 concentration was defined as greater than either of the following: 1.35 g/L (suggested threshold for IAC[4] and similar to the 1.4 g/L URL used to determine increased IgG4 level in PSC by Mendes et al[2]), or 2.01 g/L (URL of the assay used in the present study). According to these thresholds, an increased IgG4 level was found in 47 (18%) and 23 (9%) patients, respectively (Supplementary Table 1).

Abbreviations used in this paper: **IAC, IgG4-associated cholangitis; IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis; URL, upper reference limit.**

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Table 1. HLA Associations in Norwegian PSC Patients Stratified According to IgG4 Concentrations Using the Upper Reference Limit (IgG4 > 2.01) as a Cut-Off Value

	Allele frequency, n (%)			High IgG4 vs low IgG4		Low IgG4 vs control		High IgG4 vs control	
HLA allele[a]	Control (2n[b] = 736)	Low IgG4 (2n[b] = 480)	High IgG4 (2n[b] = 46)	OR (95% CI)	P[c]	OR (95% CI)	P	OR (95% CI)	P[c]
B*07	118 (16)	63 (13)	11 (24)	**2.1 (1.1–4.3)**	**.04**	0.8 (0.6–1.1)	.15	1.7 (0.8–3.3)	.17
B*08	94 (13)	196 (41)	12 (26)	**0.5 (0.3–1.0)**	**.05**	4.7 (3.5–6.2)	<.001	2.4 (1.2–4.8)	.01
DRB1*03	106 (14)	191 (40)	16 (35)	0.8 (0.4–1.5)	.48	3.9 (3.0–5.2)	<.001	3.2 (1.7–6.0)	<.001
DRB1*04	157 (21)	36 (8)	4 (9)	1.3 (0.5–3.4)	.77	0.3 (0.2–0.4)	<.001	0.4 (0.2–1.0)	.04
DRB1*07	55 (7)	17 (4)	2 (4)	1.5 (0.4–5.2)	.68	0.5 (0.3–0.8)	.005	0.7 (0.2–2.3)	.57
DRB1*11	43 (6)	10 (2)	2 (4)	2.5 (0.7–9.3)	.29	0.4 (0.2–0.7)	.002	0.9 (0.3–3.0)	.67
DRB1*13:01	47 (6)	65 (14)	6 (13)	1.0 (0.4–2.3)	.91	2.3 (1.6–3.4)	<.001	2.3 (1.0–5.4)	.12
DRB1*15	112 (15)	69 (14)	12 (26)	**2.1 (1.1–4.2)**	**.04**	0.9 (0.7–1.3)	.70	**2.0 (1.0–3.9)**	**.05**

CI, confidence interval; OR, odds ratio.
[a]Only previously PSC-associated HLA alleles are shown; for a full listing see Supplementary Tables 2 and 3.
[b]2n, number of individuals in the group × 2 alleles per individual.
[c]Bold entries are alleles that are significantly different between high and low IgG4, as well as alleles that are associated with high but not low IgG4 levels when compared with healthy controls.

In line with previous observations,[2] increased IgG4, irrespective of the cut-off level, was associated with more advanced disease stage, as evaluated by liver biochemistry and revised Mayo risk score (Supplementary Table 1). Considering increased IgG4 as a phenotype, there was a significant association between increased IgG4 (cut-off value, 1.35) and reduced transplant-free survival from the time of the PSC diagnosis ($P = .05$) (Supplementary Figure 1).

To test the hypothesis of a genetic basis for increased IgG4 level in patients with PSC, we compared PSC patients with high and low IgG4 levels, and both of these groups with healthy controls (n = 368) for *HLA-B* and *HLA-DRB1*, focusing on previously identified PSC-associated alleles (Table 1 and Supplementary Tables 2 and 3). By using an IgG4 level higher than 1.35 as the cut-off value, the strongest genetic risk factor in PSC,[8] the HLA-B*08 allele, was less prevalent in patients with high than with low IgG4 levels (29% vs 42%; $P = .02$) (Supplementary Table 2). When considering URL (IgG4 > 2.01) as the cut-off value, a significantly reduced HLA-B*08 frequency still was observed in the high IgG4 group, with the additional observations that HLA-B*07 and DRB1*15 were significantly more prevalent in PSC patients with high than with low IgG4 levels (Table 1).

To validate these findings, we included PSC patients from Sweden (n = 68) and the United States (n = 90), focusing on high IgG4 levels using the cut-off IgG4 value greater than the URL, as a number of different IgG4 assays were applied (Supplementary Materials and Methods section). By using imputed HLA data,[8] the significantly lower frequency of HLA-B*08 and the higher frequencies of HLA-B*07 and DRB1*15 in PSC patients with high IgG4 levels were confirmed in the combined Swedish-US panel (Table 2). A meta-analysis of all patients yielded *P* values of .004, .005, and .002 for the differences observed for HLA-B*07, B*08, and DRB1*15, respectively (Table 2). When comparing PSC patients with healthy controls in the Norwegian panel, HLA-DRB1*15 was associated only with PSC patients with IgG4 levels greater than 2.01 (odds ratio, 2.0; 95% confidence interval, 1.0–3.9; $P = .05$) (Table 1). This observation also was replicated in the combined Swedish-US panel (odds ratio, 3.1; 95% confidence interval, 1.5–6.6; $P = .003$) (Supplementary Table 4).

Studies on the genetics of systemic IgG4-related disease have been very limited. An association with the HLA-DRB1*0405-DQB1*0801 haplotype was observed in a Japanese population of IgG4-associated autoimmune pancreatitis.[10] This haplotype rarely is observed in the Norwegian population, which means that results cannot be compared. HLA-B*08 is part of the HLA-B*08-DRB1*03 haplotype, which has been associated with multiple autoimmune diseases.[11] The increased frequency of HLA-B*08 in the low IgG4 level group parallels our observation of higher HLA-B*08 frequency in large- than small-duct PSC,[12] suggesting that exclusion of patients showing extreme phenotypes leaves a genetically more homogeneous PSC population.

The increased frequencies of both HLA-B*07 and DRB1*15 (serotype DR2) observed in patients with IgG4 level greater than the URL may be explained by the typical co-occurrence of these 2 alleles on the same conserved haplotype (AH7.1). An association of HLA-DR2 with PSC first was described in 1991, in a study in which clinical differences also suggested that HLA-DR2 and DR3 could represent different etiologic subsets of PSC.[13] Association studies for HLA-DR2/DRB1*15 in PSC have yielded different results. Except for one study in Italian patients with PSC,[14] significant associations have been detected only when patients carrying HLA-DRB1*03 (serotype DR3) and DRB1*13:01 (serotype DR6) were excluded from the analysis.[9,15] Our study explains the presence of this secondary association with DR2 in PSC by stratification of the patient population according to serum IgG4 concentrations.

Table 2. HLA Allele Frequencies and Replication Association Analyses in PSC Patients From Sweden and the United States Comparing Individuals With High and Low IgG4 Levels

	Sweden			United States			Sweden + United States		Norway + Sweden + United States[b]	
	Allele frequency, n (%)			Allele frequency, n (%)						
HLA allele[a]	Low IgG4 ($2n^d = 124$)	High IgG4[c] ($2n^d = 12$)	OR	Low IgG4 ($2n^d = 162$)	High IgG4[c] ($2n^d = 18$)	OR	OR_{CMH}	P_{CMH}[e]	OR_{CMH}	P_{CMH}[e]
B*07	18 (15)	4 (33)	3.0 (0.9–10.1)	26 (16)	5 (28)	2.1 (0.7–5.9)	2.3 (1.0–5.5)	.05	2.2 (1.3–2.8)	.004
B*08	45 (36)	2 (17)	0.4 (0.1–1.6)	48 (29)	2 (11)	0.4 (0.1–1.3)	0.3 (0.1–0.9)	.04	0.4 (0.2–0.8)	.005
DRB1*15	28 (23)	4 (33)	1.8 (0.6–5.8)	27 (16)	7 (39)	3.2 (1.2–8.6)	2.4 (1.1–5.4)	.03	2.2 (1.3–2.8)	.002

CMH, Cochran–Mantel–Haenszel test; 95% CI, low IgG4 level defined as control population; OR, odds ratio.
[a]Only alleles significantly different between high and low IgG4 PSC (upper reference limit as the cut-off level) were analyzed in the replication panel.
[b]Meta-analysis across both the Sweden + US replication panels and the original Norwegian panel (Table 1).
[c]High IgG4 level was defined as above the upper reference limit.
[d]2n, number of individuals in the group × 2 alleles per individual.
[e]No significant heterogeneity of the odds ratios (Breslow–Day test, $P > .05$ for all 3 alleles).

Although the power of this study was limited because the groups with high IgG4 levels were small, the independent replication of the main findings represents a strength of the study. The study specifically was assessing differences in established PSC HLA risk factors between patients with high and low IgG4 levels. Because of multiple testing concerns and low effect size of non-HLA associations in PSC,[8] associations outside the HLA complex warrant further investigations in accordingly sized study panels. Furthermore, because most of the patients in the present study were included before the awareness of the IAC entity, we lack available data to evaluate the presence of pancreatic disease and systemic IgG4-related disease because relevant investigations were not performed during this period. In the present study, the similar prevalence of high IgG4 levels (9%–10%) and HLA associations in patient panels analyzed with different assays but using the URL as a cut-off value, suggest that categorizing patients according to the URL rather than an absolute IgG4 threshold value is beneficial. However, in the Norwegian panel, a more advanced disease stage and a different HLA association was observed also in patients with IgG4 above 1.35, which is lower than the URL of 2.01, meaning that IgG4 concentrations less than the URL could be clinically relevant.

In conclusion, we report that PSC patients with increased serum IgG4 concentrations show distinct HLA associations, suggesting that the IgG4 concentrations in part are determined genetically, and that phenotypic heterogeneity may contribute to the complexity of the HLA associations observed in PSC. The possibility that an increased IgG4 level is a marker for a distinct PSC entity should be explored in studies of pathophysiology or therapeutic trials targeting this subpopulation.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.01.041.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Study Population

Norwegian PSC patients and controls. We recruited the PSC patients and collected sera at the time of admittance to Oslo University Hospital Rikshospitalet (Oslo, Norway) during the time period 1992–2010 (Supplementary Table 1). All unrelated PSC patients with available stored pretransplant serum and DNA were included in the study (n = 263). The diagnosis of PSC was based on accepted criteria, with typical large-duct affection on cholangiography.[1] We selected ethnically matched and sex-matched healthy controls (n = 368; 70% male) randomly from the Norwegian Bone Marrow Donor Registry and obtained written informed consent from all study participants. The study was approved by the Regional Committee for Medical and Health Research Ethics in Southeastern Norway.

Regarding comorbidities, IBD was diagnosed and classified according to commonly accepted clinical, endoscopic, radiologic, and histologic criteria.[2] Biliary cancer was defined as the accumulated frequency of cholangiocarcinoma and cancer of the gallbladder.

Swedish and US PSC patients and controls. PSC patients from Sweden were included if both IgG4 data and imputed HLA data from previous studies were available (Supplementary Table 5).[3,4] For HLA association analyses, all Swedish controls analyzed in the same study (n = 2464) were included.[4]

PSC patients from the United States were recruited from the Mayo Clinic and included if IgG4 data from the research database or medical records were available in addition to imputed HLA data from a previous study (Supplementary Table 1).[4] For HLA association analyses, all US controls (n = 648) recruited from the same center were included.

Laboratory Tests and Genotyping of the Norwegian Panel

The median time from the diagnostic cholangiography to sampling and biobanking of the serum included for analysis was 0.6 years (range, -0.6 to 29.0 y). Serum samples were stored at -80°C and subjected to up to 2 freeze/thaw cycles before IgG4 analysis, which was performed in all samples at the same time point. The distribution of IgG, sodium, potassium, and albumin, which were measured in the same serum and at the same time as IgG4, seemed stable over time.

We measured serum IgG4 concentrations by particle-enhanced immunonephelometry on a BN ProSpec nephelometer with the N-LATEX IgG4 kit (Siemens Healthcare Diagnostics, Munich, Germany). This method has a coefficient of variation of 4.5%–4.9% and performs close to method mean on external quality control (United Kingdom National External Quality Assessment Service). The method's reference interval is 0.030–2.010 g/L (age, ≥19 y). Because there is no definitive cut-off level for clinical evaluation of increased serum IgG4 concentration, we divided the patients into groups of low and high IgG4 levels, applying a cut-off value of 1.35 g/L, as recently suggested as a threshold for diagnosing IgG4-associated cholangitis,[5] and similar to the 1.4 g/L threshold applied for PSC patients by Mendes et al.[6] We also performed a re-analysis of the data applying a higher cut-off value of 2.01 g/L, which is the URL of the assay used in the present study.

Regarding the concentration of IgG4 in relation to disease duration and stability over time, there was no correlation between observation time from the time of diagnosis to the time of serum sampling and IgG4 levels or the prevalence of increased IgG4. In a set of 14 individuals with 2 sets of blood samples with a median of 8.0 years (range, 3.4–16.7 y) of observation time between the samples, we found a significant correlation between the measurements (Spearman rank correlation coefficient, 0.78; $P = .002$).

Information with regard to leukocyte and thrombocyte count, international normalized ratio, prothrombin time (replaced by the international normalized ratio in Norway in 1998, meaning that usually either the international normalized ratio or prothrombin time was available, but not both) and serum concentrations of bilirubin, albumin, creatinine, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were available from databases of clinical biochemistry. Biochemical data were retrieved from a date as close to the day the blood used for the IgG4 concentration measurement was drawn (ie, the same day or up to 1 week earlier).

Information with regard to presence of perinuclear antineutrophil cytoplasmic antibodies (analyzed by indirect immunofluorescence in stored pretransplant sera at 1:20 dilution) was available for 259 (98%) patients.

Sequencing-based *HLA-B* and *HLA-DRB1* genotyping was performed as described previously.[7] The genotypes were resolved to a consistent 2-digit resolution, except for HLA-DRB1*13, which was resolved to a 4-digit level to take into account the known association with HLA-DRB1*13:01.[8] For *HLA-B* it was necessary to resolve to serologic level in the Norwegian panel to ensure comparability with the data available from the controls.

Laboratory Tests and Genotyping of the Replication Panel From Sweden and the United States

Briefly, as part of a previous study, the Swedish PSC patients were invited in 2008 to provide sera for an IgG4 study of patients with PSC.[3] IgG4 was analyzed using a nephelometric method with 1.4 g/L as the URL (Minineph, The Binding-Site, Oxford, UK).[3] For the US patients, IgG4 concentration measurements were collected from the research database, most often retrospectively. Several different assays were used. For both the Swedish and US patients, only the URL was used as cut-off value to make the data comparable.

For the patients and controls from Sweden and the United States, data on the HLA alleles B*07, B*08, and DRB1*15 were available from a previous study.[4] By using a

dense set of single-nucleotide polymorphisms genotyped in the HLA complex, imputation of *HLA-B* and *DRB1* alleles was performed using HLA*IMPv2.[9,10] In a benchmarking experiment performed in conjunction with the original study,[4] the imputation accuracy (per allele) for HLA-B*07 was 99% and for HLA-B*08 was 98%. For HLA-DRB1*15 the imputation accuracy was 100%. In total, 464 samples were included in the benchmarking analysis for *HLA-B* and 387 samples were included for *HLA-DRB1*.

Statistical Analysis

Statistical analysis was performed using the chi-square test for comparison of categoric variables, except for the Fisher exact test where appropriate. The Mann–Whitney *U* test was applied for continuous variables.

The odds ratios and confidence intervals in the HLA association tests were calculated using the Woolf formula with the Haldane correction,[11] except for meta-analyses, in which Cochran–Mantel–Haenszel statistics were applied. Uncorrected *P* values less than .05 were considered statistically significant for alleles shown to be associated with PSC in previous studies.[7,12]

We analyzed survival by applying Kaplan–Meier curves for liver transplantation–free survival from the time of the diagnostic cholangiogram. The log-rank test was applied to compare the groups with low and high IgG4 levels.

The time-dependent Cox regression model was applied to predict the prognosis. We calculated the prognostic index according to the multiple time–dependent prognostic model defined by Boberg et al,[13] as follows: prognostic index = 1.04 (ln [bilirubin] - 3.31) – 0.12 (albumin – 37.27) + 0.013 (age at diagnosis – 36.04). The higher revised Mayo risk score was calculated according to the following formula[14]: Mayo risk score = (0.0295 * [age in years]) + (0.5373 * LN [total bilirubin level in mg/dL]) - (0.8389 * [serum albumin level in g/dL]) + (0.5380 * LN [aspartate aminotransferase level in IU/L]) + (1.2426 * [points for variceal bleeding]).

The PASW Statistics 18 software (IBM, Armonk, NY), Prism 6.0 software (GraphPad Software, La Jolla, CA), and MS Excel software (Microsoft, Redmond, WA) were applied to perform all the statistical analyses.

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