


Genome-wide Association Studies of Specific Antinuclear Autoantibody Subphenotypes in Primary Biliary Cholangitis

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Anti-nuclear antibodies to speckled 100 kDa (sp100) and glycoprotein 210 (gp210) are specific serologic markers of primary biliary cholangitis (PBC) of uncertain/controversial clinical or prognostic significance. To study the genetic determinants associated with sp100 and gp210 autoantibody subphenotypes, we performed a genome-wide association analysis of 930 PBC cases based on their autoantibody status, followed by a replication study in 1,252 PBC cases. We confirmed single-nucleotide polymorphisms rs492899 ($P = 3.27 \times 10^{-22}$; odds ratio [OR], 2.90; 95% confidence interval [CI], 2.34-3.66) and rs1794280 ($P = 5.78 \times 10^{-28}$; OR, 3.89; 95% CI, 3.05-4.96) in the human major histocompatibility complex (MHC) region associated with the sp100 autoantibody. However, no genetic variant was identified as being associated with the gp210 autoantibody. To further define specific classical human leukocyte antigen (HLA) alleles or amino acids associated with the sp100 autoantibody, we imputed 922 PBC cases (211 anti-sp100-positive versus 711 negative cases) using a Han Chinese MHC reference database. Conditional analysis identified that HLA-DR β 1-Asn77/Arg74, DR β 1-Ser37, and DP β 1-Lys65 were major determinants for sp100 production. For the classical HLA alleles, the strongest association was with *DRB1*03:01* ($P = 1.51 \times 10^{-9}$; OR, 2.97; 95% CI, 2.06-4.29). Regression analysis with classical HLA alleles identified *DRB1*03:01*, *DRB1*15:01*, *DRB1*01*, and *DPB1*03:01* alleles can explain most of the HLA association with sp100 autoantibody. **Conclusion:** This study indicated significant genetic predisposition to the sp100 autoantibody, but not the gp210 autoantibody, subphenotype in PBC patients. Additional studies will be necessary to determine if these findings have clinical significance to PBC pathogenesis and/or therapeutics. (HEPATOLOGY 2019;70:294-307).

Primarily biliary cholangitis (PBC) is an autoimmune disease characterized by chronic inflammation and destruction of intrahepatic bile ducts. PBC is mainly observed in middle-aged women. Patients with PBC generally present with abnormal liver tests including elevation of alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT), and combinations of symptoms, including

Abbreviations: AA, amino acid; AMA, anti-mitochondrial antibody; ANA, anti-nuclear antibody; CI, confidence interval; gp210, glycoprotein 210; GWA, genome-wide association; HLA, human leukocyte antigen; IL12, interleukin 12; IL12R, IL12 receptor; LD, linkage disequilibrium; MAF, minor allele frequency; MHC, major histocompatibility complex; OD, optical density; OR, odds ratio; PBC, primary biliary cholangitis; PBS, phosphate-buffered saline; SNP, single-nucleotide polymorphism; sp100, speckled 100 kDa.

Received October 29, 2018; accepted March 3, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30604/supinfo.

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Supported in part by grants from the National Natural Science Foundation of China (81870397 to X.D.L.; 81620108002, 81771732, 81830016, to X.M.; 81570469 to R.Q.T.) and from the Jiangsu Provincial Research Fund (BE2017713, to X.D.L.; BL2018657, to Y.T.).

pruritus, fatigue, and portal hypertension.⁽¹⁾ Anti-mitochondrial antibodies (AMA) are the serological hallmark for PBC and are found in nearly 95% of patients.⁽²⁾ Several large-scale genome-wide association (GWA) studies in European, Japanese, and Han Chinese PBC cohorts have pointed to a strong genetic

predisposition to PBC.⁽³⁻⁷⁾ Specific human leukocyte antigen (HLA) alleles and common polymorphisms in interleukin 12 (*IL12*), IL12 receptor (*IL12R*), tumor necrosis factor superfamily member 15 (*TNFSF15*), *IL21*, *IL21R*, and other immune-associated genes have been implicated in PBC pathogenesis.^(3,5,6) Although

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DOI 10.1002/hep.30604

Potential conflict of interest: Nothing to report.

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these studies provided many insights in our understanding of PBC pathogenesis, no further studies have examined the genetics of subphenotypes in PBC.

Other than AMA, autoantibodies to glycoprotein 210 (gp210) and speckled 100 kDa (sp100), the two subtypes of anti-nuclear antibodies (ANA), are also highly specific to PBC and can be detected in 10%–40% of PBC patients.^(8–10) Anti-gp210 and sp100-positive serum samples present typical rim and multinuclear dot staining patterns, respectively. Anti-gp210 antibody recognizes a single epitope containing 25 amino acids (AAs) in the C-terminal tail of nuclear pore gp210 protein.⁽¹¹⁾ Several studies have indicated that autoantibody to gp210 can serve as an indicator of poor prognosis. Muratori et al. found that anti-gp210 positivity was associated with higher serum cholestatic parameters including ALP, GGT, and bilirubin.⁽⁸⁾ Unfavorable clinical outcome, more rapid disease progression, and significantly higher hepatic failure were found in anti-gp210-positive patients.^(11–13) Recently, Yang et al. found that anti-gp210 positivity is associated with more severe forms of PBC in Chinese patients with prognostic value.^(14,15) Sp100 is a component of promyelocytic leukemia protein (PML) and sp100-containing nuclear bodies. Anti-sp100 antibodies in PBC patients recognize multiple epitopes in sp100.⁽¹⁶⁾ Studies on the clinical significance of anti-sp100 antibody have thus far generated mixed results.^(17–20) A few early studies indicated that the anti-sp100-positive patient group tended to have earlier disease onset and more frequently had cirrhosis compared to anti-sp100-negative patients. Studies by Nakamura et al.⁽¹²⁾ and Yang et al.⁽¹⁴⁾ found no correlation of anti-sp100 positivity with disease prognosis, but neither study investigated the subtypes of autoantibodies. Two semiquantitative analyses of anti-sp100 antibody titers during the course of the treatment found a correlation of anti-sp100 titers with Mayo risk score, an indicator of disease severity.^(17,19) Because sp100 autoantibodies recognized multiple epitopes on sp100, an earlier study found a change of some anti-sp100 epitopes during the course of disease progression and ursodesoxycholic acid (UDCA) treatment in some PBC patients, but the overall anti-sp100 status remained unchanged.⁽²¹⁾ Unfortunately, no subsequent follow-up or replication studies were reported by other researchers. Therefore, it remains undetermined whether any anti-sp100 immunoglobulin subtypes or titers can serve as indicators of disease progression.

We recently performed a large-scale GWA analysis in a Han Chinese PBC cohort and identified multiple common variants in major histocompatibility complex (MHC) region, *TNFSF15*, *IL21*, *IL21R*, chemokine (C-X-C motif) receptor 5, cluster of differentiation 58, signal transducer and activator of transcription 4, and other immune-related genes associated with PBC.⁽⁶⁾ To further define the role of genetic factors associated with different PBC subphenotypes (anti-gp210/anti-sp100) at the whole-genome level, we undertook serological analysis of anti-gp210/anti-sp100 status in our PBC cases. Combining the serological results and genome-wide single-nucleotide polymorphism (SNP) data, we systematically performed the association analysis to pinpoint the genetic variants associated with gp210 and sp100 autoantibody subphenotypes in PBC. The results presented in this report indicate a strong genetic predisposition to the sp100 autoantibody subphenotype in PBC patients.

Patients and Methods

PATIENT INFORMATION AND GENOME-WIDE GENOTYPING

The PBC patients recruited in this study have been reported previously.⁽⁶⁾ The PBC patients were recruited with the approval of the research ethics boards of Southeast University and Jiaotong University and in accordance with the guidelines of the Declaration of Helsinki (2008). Informed consent was obtained from all subjects recruited. In brief, all patients self-reported Han Chinese origin and were diagnosed based on the criteria recommended by the American Association for the Study of Liver Diseases.⁽²²⁾ SNP genotyping was performed using Han Chinese population-specific HumanOmniZhongHua-8 BeadChip (Illumina, San Diego, CA). The quality control of genotyping results was detailed in our GWA report.⁽⁶⁾ We removed SNPs with a call rate of <90%, a minor allele frequency (MAF) < 1%, and a Hardy-Weinberg equilibrium P value < 1×10^{-4} . Potential genetic relatedness based on pairwise identity by descent for all of the successfully genotyped individuals was examined using PLINK 1.07 software.⁽²³⁾ If first-degree or second-degree relative pairs were identified (PI-HAT value > 0.25), we removed the samples with the lower genotyping call rates of the related individuals. SNPs located on the

X, Y, or mitochondrial chromosomes and all copy number variation probes were excluded.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR ANTI-sp100 AND ANTI-gp210 ANTIBODIES

Serum samples were stored at -80°C in aliquot before use. Enzyme-linked immunosorbent assay (ELISA) plates (96-well, 100 ng/well) were coated overnight at 4°C with purified sp100 antigen (AA position 296-386) or gp210 antigen (AA position 1863-1887). The plate was further incubated with 1% bovine serum albumin (BSA) for 2 hours at room temperature and washed 3 times with phosphate-buffered saline (PBS). Serum samples were diluted (1:200) with PBS buffer containing 1% BSA. After incubation for 1 hour at 37°C , the plate was washed 4 times with PBS and incubated with 1:40,000 diluted peroxidase-conjugated anti-human immunoglobulin G antibody (Genscript, China) at room temperature for 1 hour. Next, after washing 4 times with PBS, the plate was incubated with 100 μL of chromogenic substrate (3,3',5,5'-tetramethylbenzidine) for 20 minutes, with addition of 2 M sulfuric acid (50 μL /well) as the stop solution. Optical density (OD) was read by an ELISA plate reader with absorbance at 450 nm. The results with an OD ratio higher than 3 times over negative controls were defined as antibody-positive. Samples with an OD ratio between 2 and 3 times were retested and excluded if ambiguous on multiple testing with different serum dilutions.

SNP GENOTYPING

SNP genotyping in the replication study was performed by TaqMan assay for rs492899 and rs9380343 and by Sanger DNA sequencing for rs1794280. The primer and probe sequences are listed in Supporting Table S1. The TaqMan assay was performed using a Bio-Rad CFX Manager Real-Time PCR system in 10 μL of reaction mixtures containing $1 \times$ PCR buffer, 2 mM deoxyribonucleotide triphosphate mix, 2 μM of each primer, 3 μM of labeled probe, 0.25 U Taq HS polymerase (Takara, Dalian), 5 ng of genomic DNA, and 6.15 μL deionized water. PCR conditions were as follows: 95°C for 10 minutes, followed by 40

cycles of 95°C for 30 seconds and 60°C or 62°C for 30 seconds.

IMPUTATION OF HLA ALLELES, AAs, AND SNPs IN MHC REGION AND ASSOCIATION ANALYSIS

The genotyped 7,889 SNPs in the MHC region (chromosome 6 region at 29-34 Mb, University of California Santa Cruz hg19 assembly) included on the HumanOmniZhongHua-8 BeadChip were extracted for analysis. Imputation was performed using a Han MHC reference panel built by deep sequencing that contains 29,948 variants for 10,689 healthy Han Chinese subjects.⁽²⁴⁾ SNP2HLA and Beagle software was used to impute SNPs, two-digit and four-digit HLA alleles, and HLA AAs.^(25,26) We applied post-imputation quality control criteria of $\text{MAF} > 0.01$, imputation quality ($r^2 > 0.3$). Finally, we excluded subjects and SNPs with $< 90\%$ genotyping completion and/or Hardy-Weinberg equilibrium $P < 1 \times 10^{-4}$ or $\text{MAF} < 0.01$. A total of 23,137 variants were imputed. Among them, there were 22,317 SNP or insertion/deletions (including 7,889 input SNPs from GWA), 657 AA variants from HLA class I and II antigens, and 163 classical HLA alleles (66 two-digit alleles and 97 four-digit alleles). Linkage disequilibrium (LD) analysis for selected SNPs was performed using the Haploview program.⁽²⁷⁾

STATISTICAL ANALYSIS

Variant associations were tested using logistic regression models with autoantibody status (positive/negative) as the outcome variable. The statistical results were presented without genomic control correction as there is negligible evidence of population stratification in our previous study.⁽⁶⁾ Stepwise conditional analysis were used to identify independent variants of the HLA allele or AA polymorphisms for two subphenotypes of PBC by selecting the most significant associated variant as a covariate until no variant satisfied the threshold ($P < 2.11 \times 10^{-6}$). Meta-analysis based on a fixed-effect model for the two independent PBC cohorts was performed using Stata12.0 software (Stata Corporation, College Station, TX).

Results

DETERMINATION OF ANTI-sp100 AND ANTI-gp210 STATUS IN PBC GWA AND REPLICATION COHORTS

Anti-gp210 was detected in 350/912 (38.38%) of the cases, while anti-sp100 was found in 211/922 (22.89%) subjects. Of the 904 subjects successfully tested for both gp210 and sp100 antibodies, 87 (9.62%) were positive for both autoantibodies and 432 (47.79%) were negative for both antibodies. In the replication cohort, 1,252 subjects were analyzed. Anti-gp210 was detected in 444 (35.46%) of the cases, while anti-sp100 was found in 234 (18.69%) subjects. Among them, 122 (9.74%) were positive for both autoantibodies, and 696 (55.59%) were negative for both antibodies (Table 1).

GWA ANALYSIS AND REPLICATION STUDY FOR ANTI-gp210 SUBPHENOTYPE

In total 794,694 autosomal SNPs were analyzed between 350 anti-gp210-positive and 562 negative subjects. No SNP achieved genome-wide significance ($P < 5 \times 10^{-8}$) (Fig. 1 and Table 2), and the strongest association was the HLA class II SNP rs1794280 ($P = 1.86 \times 10^{-6}$; odds ratio [OR], 0.40; 95% confidence interval [CI], 0.27-0.59). There were 15 SNPs in the MHC region with $P < 1.0 \times 10^{-5}$. We performed LD analysis using these SNPs and

identified two separate LD blocks (Supporting Fig. S1). To further test for possible HLA associations, we selected one SNP from each of two separate LD blocks (rs1794280 and rs9380343) for replication in the second PBC cohort with 444 anti-gp210-positive and 808 anti-gp210-negative subjects. We found no significant association with rs1794289 ($P = 0.11$; OR, 0.75; 95% CI, 0.53-1.07) and rs9280343 ($P = 0.08$; OR, 1.16; 95% CI, 0.98-1.37) in the replication cohort. Meta-analysis, including 794 anti-gp210-positive and 1,370 negative subjects showed decreased association compared to the GWA result (Table 3). Our results did not confirm the association of rs1794280 and rs9380343 with anti-gp210 phenotype.

GWA ANALYSIS AND REPLICATION STUDY FOR ANTI-sp100 SUBPHENOTYPE

In total, 794,531 autosomal SNPs were analyzed between 211 anti-sp100-positive and 711 negative subjects. We observed a strong association in the HLA class II gene region, with six SNPs reaching genome-wide significance (Fig. 1). The strongest association was also with rs1794280 ($P = 4.83 \times 10^{-10}$; OR, 2.79; 95% CI, 2.00-3.90) but with the minor allele as the predisposing variant (Table 2). For the top nine SNPs with $P < 1 \times 10^{-7}$, we performed LD analysis and identified two independent LD groups (Supporting Fig. S2). The top SNPs from each group (rs1794280 and rs492899) were selected for replication using the additional 234 anti-sp100-positive

TABLE 1. Sample Information for the Discovery and Replication Study for Anti-sp100 and Anti-gp210 Among PBC Patients

	Number	Anti-gp210			Anti-sp100			Anti-gp210/ Anti-sp100	Anti-gp210/ Anti-sp100
		Positive	Negative	Missing	Positive	Negative	Missing	Both Positive	Both Negative
Total cases	2,182	794	1,370	18	445	1,729	8	209	1,128
GWAS cases	930	350	562	18	211	711	8	87	432
Male	3	1	2		0	3		0	2
Female	927	349	562		211	708		87	430
Age (mean \pm SD)	54.20 \pm 6.36	54.04 \pm 11.31	54.47 \pm 6.36		54.91 \pm 2.83	53.97 \pm 0.71		54.66 \pm 7.79	56.51 \pm 2.12
Replication cases	1,252	444	808	0	234	1,018	0	122	696
Male	185	82	103		43	142		29	89
Female	1,067	362	705		191	876		93	607
Age (mean \pm SD)	55.93 \pm 2.83	56.75 \pm 6.36	55.48 \pm 7.79		57.16 \pm 13.44	55.65 \pm 2.83		57.85 \pm 16.26	55.31 \pm 7.78

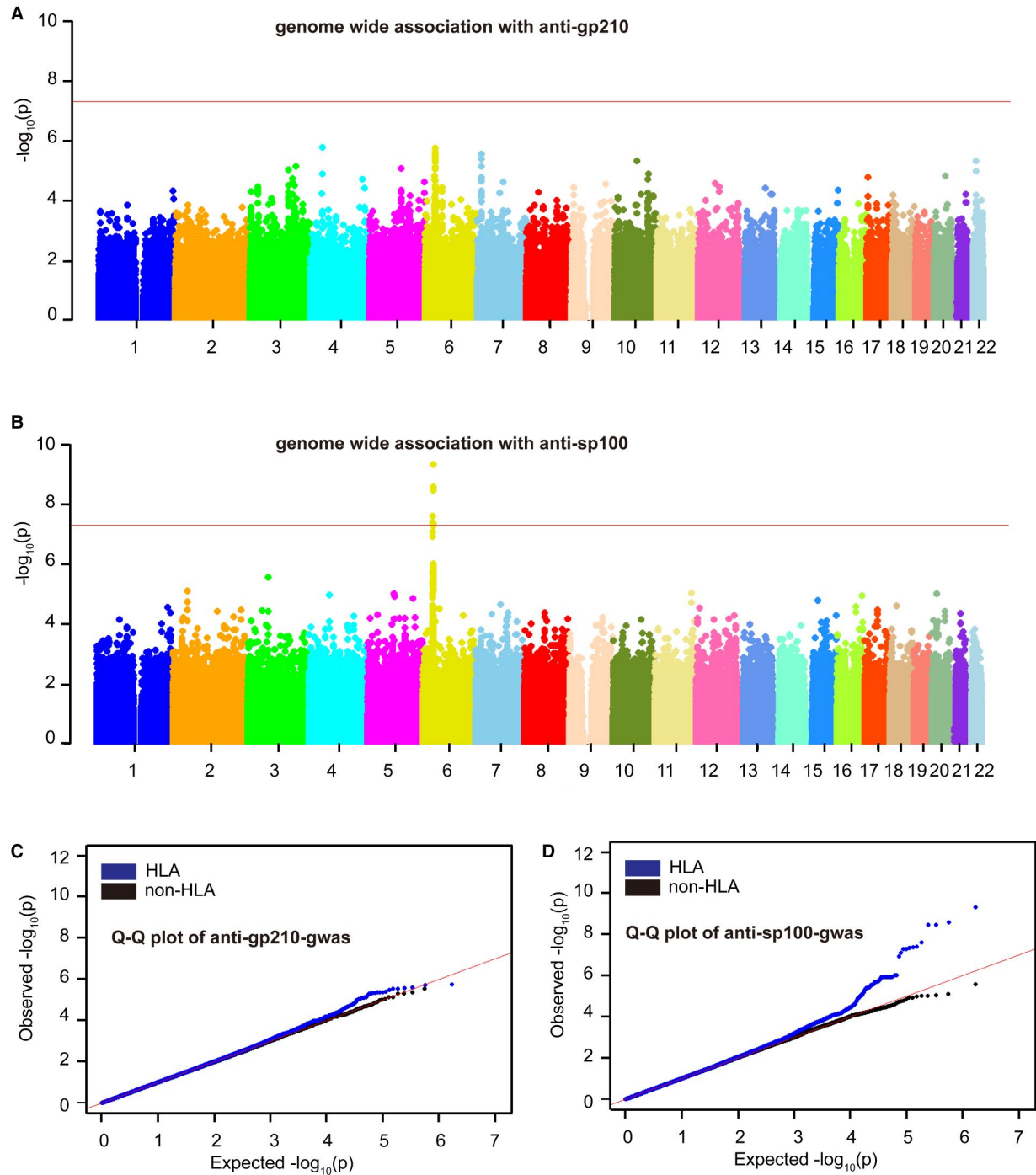


FIG. 1. Manhattan and Q-Q plots of anti-gp210 and anti-sp100 GWA results. (A) Manhattan plot of anti-gp210-GWA results; (B) Manhattan plot of anti-sp100 GWA results; (C) Q-Q plot of anti-gp210-GWA results; (D) Q-Q plot of anti-sp100 GWA results. (A,B) The horizontal axis shows genomic position, and the vertical axis shows the $-\log P$ values for association. The horizontal red line ($P = 5 \times 10^{-8}$) was set as the GWA significance threshold. (C,D) The red line is for null expectation, and the plot in blue is for P values including HLA SNPs, whereas the plot in black is for the P value after excluding SNPs in the MHC region.

and 1,018 negative subjects. In the replication study, both rs1794280 and rs492899 also showed significant associations with $P = 4.25 \times 10^{-21}$ (OR, 4.85;

95% CI, 3.49-6.73) and 1.14×10^{-13} (OR, 2.82; 95% CI, 2.13-3.75), respectively. Meta-analysis using both data sets (445 anti-sp100-positive and

TABLE 2. Genetic Association Analysis of Anti-gp210 or Anti-sp100 With GWAS Data in Han Chinese PBC

Association	SNP	Position (hg19)	A1/A2	Antibody-Positive	Antibody-Negative	<i>P</i>	OR (95% CI)	Location
Anti-gp210*	rs1794280	32667171	T/A	0.041	0.098	1.86×10^{-6}	0.40 (0.27-0.59)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQA2</i>
	kgp1505269	33053609	A/G	0.492	0.387	2.50×10^{-6}	1.53 (1.28-1.83)	Missense variant of <i>HLA-DPB1</i>
	rs9380343	33079166	A/G	0.498	0.394	2.64×10^{-6}	1.53 (1.28-1.82)	Upstream of <i>HLA-DPB2</i>
	rs9348906	33080360	C/A	0.496	0.393	2.92×10^{-6}	1.53 (1.28-1.82)	Missense variant of <i>HLA-DPB2</i>
	rs10484569	33058952	A/G	0.485	0.383	3.29×10^{-6}	1.52 (1.27-1.81)	Downstream of <i>HLA-DPB1</i>
	rs2068204	33058718	A/G	0.485	0.384	3.91×10^{-6}	1.52 (1.27-1.81)	Downstream of <i>HLA-DPB1</i>
	rs4282438	33072172	C/A	0.484	0.383	4.27×10^{-6}	1.51 (1.27-1.81)	Upstream of <i>HLA-DPB2</i>
	rs3097649	33056962	A/G	0.484	0.383	4.38×10^{-6}	1.51 (1.27-1.81)	3'UTR of <i>HLA-DPB1</i>
	rs9296081	33076661	C/G	0.485	0.385	4.64×10^{-6}	1.51 (1.27-1.80)	Upstream of <i>HLA-DPB2</i>
	rs6457713	33077776	A/G	0.485	0.385	4.64×10^{-6}	1.51 (1.27-1.80)	Upstream of <i>HLA-DPB2</i>
	rs9296080	33076539	A/G	0.485	0.385	5.51×10^{-6}	1.51 (1.27-1.80)	Upstream of <i>HLA-DPB2</i>
	rs3134977	32651641	A/C	0.041	0.093	7.97×10^{-6}	0.42 (0.28-0.62)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQB2</i>
	rs3129716	32657436	G/A	0.041	0.093	7.97×10^{-6}	0.42 (0.28-0.62)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQB2</i>
	rs7770370	33048921	A/G	0.368	0.466	8.60×10^{-6}	0.67 (0.56-0.80)	Intron variant of <i>HLA-DPB1</i>
	rs9357156	33041073	A/C	0.275	0.368	9.06×10^{-6}	0.65 (0.54-0.79)	Intron variant of <i>HLA-DPA1</i>
Anti-sp100 [†]	rs1794280	32667171	T/A	0.140	0.055	4.83×10^{-10}	2.79 (2.00-3.90)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQA2</i>
	rs3134977	32651641	A/C	0.132	0.053	3.52×10^{-9}	2.69 (1.92-3.78)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQB2</i>
	rs3129716	32657436	G/A	0.132	0.053	3.52×10^{-9}	2.69 (1.92-3.78)	Intergenic between <i>HLA-DQB1</i> and <i>HLA-DQB2</i>
	rs492899	31933518	G/A	0.169	0.082	2.49×10^{-8}	2.28 (1.70-3.06)	Intron variant of <i>SKIV2L</i>
	rs9268577	32395942	A/G	0.126	0.053	4.01×10^{-8}	2.55 (1.81-3.59)	Between <i>BTNL2</i> and <i>HLA-DRA</i>
	rs9986640	32764970	A/G	0.101	0.038	4.50×10^{-8}	2.84 (1.93-4.18)	Intergenic between <i>HLA-DQB2</i> and <i>HLA-DOB</i>
	rs9501212	32760006	G/A	0.101	0.038	5.18×10^{-8}	2.83 (1.92-4.16)	Intergenic between <i>HLA-DQB2</i> and <i>HLA-DOB</i>
	rs6925976	32755092	C/A	0.101	0.038	5.18×10^{-8}	2.83 (1.92-4.16)	Intergenic between <i>HLA-DQB2</i> and <i>HLA-DOB</i>
	rs474534	31938107	G/A	0.185	0.096	8.19×10^{-8}	2.13 (1.61-2.82)	Intron variant of <i>DOMSZ</i>

**P* values are calculated based on a data set of 350 anti-gp210-positive and 562 anti-gp210-negative PBC cases. All SNPs in the chromosome 6 MHC region with *P* values $< 1 \times 10^{-5}$ are shown.

[†]*P* values are calculated based on a data set of 211 anti-sp100-positive and 711 anti-sp100-negative PBC cases. All SNPs in the chromosome 6 MHC region with *P* values $< 1 \times 10^{-7}$ are shown.

Abbreviations: A1, minor allele; A2, major allele; UTR, untranslated region.

1,729 negative subjects in total) for rs1794280 and rs492899 showed $P = 5.78 \times 10^{-28}$ (OR, 3.89; 95% CI, 3.05-4.96) and 3.27×10^{-22} (OR, 2.90; 95% CI, 2.34-3.66), respectively (Table 3). These results confirmed a strong association of HLA class II gene polymorphisms with the generation of sp100 autoantibody in PBC patients.

IMPUTATION ANALYSIS OF HLA SNP, HLA ANTIGEN VARIANTS, AND CLASSICAL HLA ALLELES WITH sp100 AUTOANTIBODY

To further define the genetic basis of sp100 autoantibody production in the MHC region, we conducted

TABLE 3. Summary of ANA (Anti-sp100 and Anti-gp210) Association for Genotyped Variants in PBC Cohorts at Two-Stage Study

	Anti-sp100				Anti-gp210			
	rs1794280		rs492899		rs1794280		rs9380343	
Position (hg19)	32667171		31933518		32667171		33079166	
A1/A2	T/A		G/A		T/A		T/C	
Subgroups	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
GWAS								
Number	211	711	211	711	350	562	350	562
Genotypes (AA/AB/BB/missing)	3/53/152/3	4/65/638/4	5/62/144/0	5/98/608/0	0/26/322/3	7/94/456/4	92/159/98/1	102/233/226/1
MAF	0.140	0.055	0.169	0.081	0.041	0.098	0.498	0.394
OR (95% CI)	2.79 (2.00-3.90)		2.28 (1.70-3.06)		0.40 (0.27-0.59)		1.53 (1.28-1.82)	
P	4.83 × 10 ⁻¹⁰		2.49 × 10 ⁻⁸		1.86 × 10 ⁻⁶		2.64 × 10 ⁻⁶	
Replication								
Number	218	951	230	996	414	755	436	782
Genotypes (AA/AB/BB)	5/69/144	5/73/873	7/75/148	13/130/853	5/38/371	5/104/646	91/216/129	154/354/274
MAF	0.181	0.044	0.193	0.078	0.058	0.075	0.456	0.423
OR (95% CI)	4.85 (3.49-6.73)		2.82 (2.13-3.75)		0.75 (0.53-1.07)		1.16 (0.98-1.37)	
P	4.25 × 10 ⁻²¹		1.14 × 10 ⁻¹³		0.11		0.08	
Meta-analysis								
OR (95% CI)	3.89 (3.05-4.96)		2.90 (2.34-3.66)		0.55 (0.42-0.72)		1.29 (1.15-1.47)	
P	5.78 × 10 ⁻²⁸		3.27 × 10 ⁻²²		1.71 × 10 ⁻⁵		3.96 × 10 ⁻⁵	

Abbreviations: A1, minor allele; A2, major allele.

imputation analysis using a Han-MHC reference panel built by sequencing 10,689 healthy control Han Chinese subjects.⁽²⁴⁾ Analysis using 211 anti-sp100-positive versus 711 negative subjects identified 46 SNPs, five AA variants, and four HLA alleles (one of two digits and three of four digits), all in the HLA class II gene region, reaching genome-wide significance (Table 4; Supporting Table S3). Among the most significant SNPs, three (rs77689370, rs9269941, and rs67476479) were coding variants for the *DRB1* gene.

Strong associations with specific AAs corresponding to the key residues that distinguish the specific classical alleles were observed, including DRβ1-AAs 77, 74, 71, and 70, located within the third hypervariable region (AAs 67-74) on the alpha helix of the DRβ1 chain, where they directly interact with antigens presented to helper T cells (Fig. 2). The most associated AA was in DRβ1 at position 74 with the AA arginine and at position 77 with asparagine (N), having predisposing effects ($P = 1.51 \times 10^{-9}$; OR, 2.97; 95% CI, 2.06-4.29). The strong association in DRβ1 was also found at position 71 with a lysine residue ($P = 1.87 \times 10^{-8}$; OR, 2.73; 95% CI, 1.90-3.92) and at position 70, with a glutamine residue ($P = 1.75 \times 10^{-7}$; OR, 1.82; 95%

CI, 1.45-2.88). The strong predisposing effect was also observed in DQα1 at position 207 with the presence of the AA methionine (M) or absence of valine (V).

Among 163 classical HLA alleles imputed, the strongest association was with *DRB1*0301* ($P = 1.51 \times 10^{-9}$; OR, 2.97; 95% CI, 2.06-4.29). *DQB1*0201* is in complete LD with *DRB1*0301* and DRβ1-Arg74 and showed the identical signal. A strong association was also observed for *DQA1*0102* ($P = 8.10 \times 10^{-9}$; OR, 2.04; 95% CI, 1.60-2.61), which is in complete LD with DQα1-Met207. A smaller signal (not in LD with *DRB1*0301*) was observed with *DPB1*0301* ($P = 1.32 \times 10^{-6}$). A weak association was observed in the *HLA-B* and *HLA-C* alleles ($P > 1 \times 10^{-4}$). There was no association ($P < 1 \times 10^{-2}$) observed for the *HLA-A* and *HLA-DPA1* alleles.

DRβ1-Asn77/Arg74, DRβ1-Ser37, AND DPβ1-Lys65 WERE MAJOR DETERMINANTS FOR sp100 AUTOANTIBODY

To further define the association of specific AAs within different HLA antigens and identify

TABLE 4. Summary of the Classic HLA Alleles and AA Polymorphisms Associated With Anti-sp100

Variants	Position (hg19)	A1/A2	Reference of A1		P	OR (95% CI)	Region
			Anti-sp100+	Anti-sp100–			
SNP							
rs1794280	32667171	T/A	0.140	0.051	5.98×10^{-10}	3.00 (2.09-4.31)	Between <i>DQB1</i> and <i>DQA2</i>
6-32723737	32723737	–CC/A	0.111	0.036	1.26×10^{-9}	3.37 (2.23-5.09)	<i>DQB2</i>
rs113547322	32206619	T/G	0.313	0.177	1.36×10^{-9}	2.12 (1.66–2.72)	Close to <i>NOTCH4</i>
rs9269173	32447188	A/T	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	Between <i>DRA</i> and <i>DRB5</i>
rs77689370	32549563	A/G	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
rs9269941	32551939	T/G	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
rs67476479	32551948	P/A	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
HLA alleles							
<i>HLA_DRB1_03</i>	32552064	P/A	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
<i>HLA_DRB1_0301</i>	32552064	P/A	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
<i>HLA_DQA1_0102</i>	32608306	P/A	0.313	0.182	8.10×10^{-9}	2.04 (1.60–2.61)	<i>DQA1</i> noncoding
<i>HLA_DQB1_0201</i>	32631061	P/A	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DQB1</i> noncoding
<i>HLA_DPBI_03</i>	33049368	P/A	0.100	0.039	1.32×10^{-6}	2.70 (1.78-4.09)	<i>DPBI</i> noncoding
<i>HLA_DPBI_0301</i>	33049368	P/A	0.100	0.039	1.32×10^{-6}	2.70 (1.78-4.09)	<i>DPBI</i> noncoding
AA polymorphisms							
AA_DRβ1_77_32551939	32551939	N/T	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
AA_DRβ1_74_32551948_R	32551948	P/A	0.135	0.050	1.51×10^{-9}	2.97 (2.06-4.29)	<i>DRB1</i> coding
AA_DQα1_207_32610462_M	32610462	P/A	0.313	0.182	8.10×10^{-9}	2.04 (1.60–2.61)	<i>DQA1</i> coding
AA_DQα1_207_32610462_V	32610462	A/P	0.313	0.182	8.10×10^{-9}	2.04 (1.60–2.61)	<i>DQA1</i> coding
AA_DRβ1_71_32551957_K	32551957	P/A	0.135	0.054	1.87×10^{-8}	2.73 (1.90-3.92)	<i>DRB1</i> coding
AA_DRβ1_70_32551960_Q	32551960	P/A	0.410	0.276	1.75×10^{-7}	1.82 (1.45–2.28)	<i>DRB1</i> coding

Abbreviations: A1, effective allele; A2, alternative allele; A, absent; P, present.

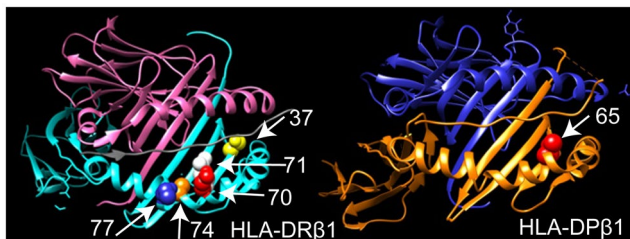


FIG. 2. Three-dimensional ribbon models for the HLA-DR and HLA-DP proteins associated with the production of anti-sp100 autoantibodies. HLA-DR and HLA-DP protein structures were based on Protein Data Bank entries 3pdo and 3lqz with a direct view of the peptide-binding groove, respectively, prepared using the program UCSF Chimera. AA positions identified by the association analysis are shown as spheres.

putative functional/causal variants, we first tested each AA within HLA-DRβ1 using logistic regression analysis. Due to complete LD between DRβ1

Arg74 and Asn77 ($r^2 = 1$), we selected Asn77 for conditional analysis because the only polymorphic AA residues were Asn and Thr in the Han Chinese cohort. Conditioning on DRβ1-AA77 eliminated the effects of DRβ1-AAs 74, 71, and 70. The remaining association within DRβ1 was observed at AA position 37 with a serine residue ($P = 6.87 \times 10^{-4}$), located in the P9 peptide-binding pockets. Further conditional analysis with both DRβ1-Asn77 and Ser37 eliminated the effect within DRβ1 (Fig. 3A-C; Supporting Figs. S3 and S4). Thus, DRβ1-Asn77 (and/or Arg74) and Ser37 account for all of the observed associations within HLA-DRβ1.

After conditioning on DRβ1-Asn77 and Ser37 in the MHC region, we only observed an association ($P < 1 \times 10^{-4}$) within the *HLA-DPB1* region, with the strongest association coming from rs3128928 ($P = 5.04 \times 10^{-7}$) and DPβ1-Lys65 ($P = 4.21 \times 10^{-6}$).

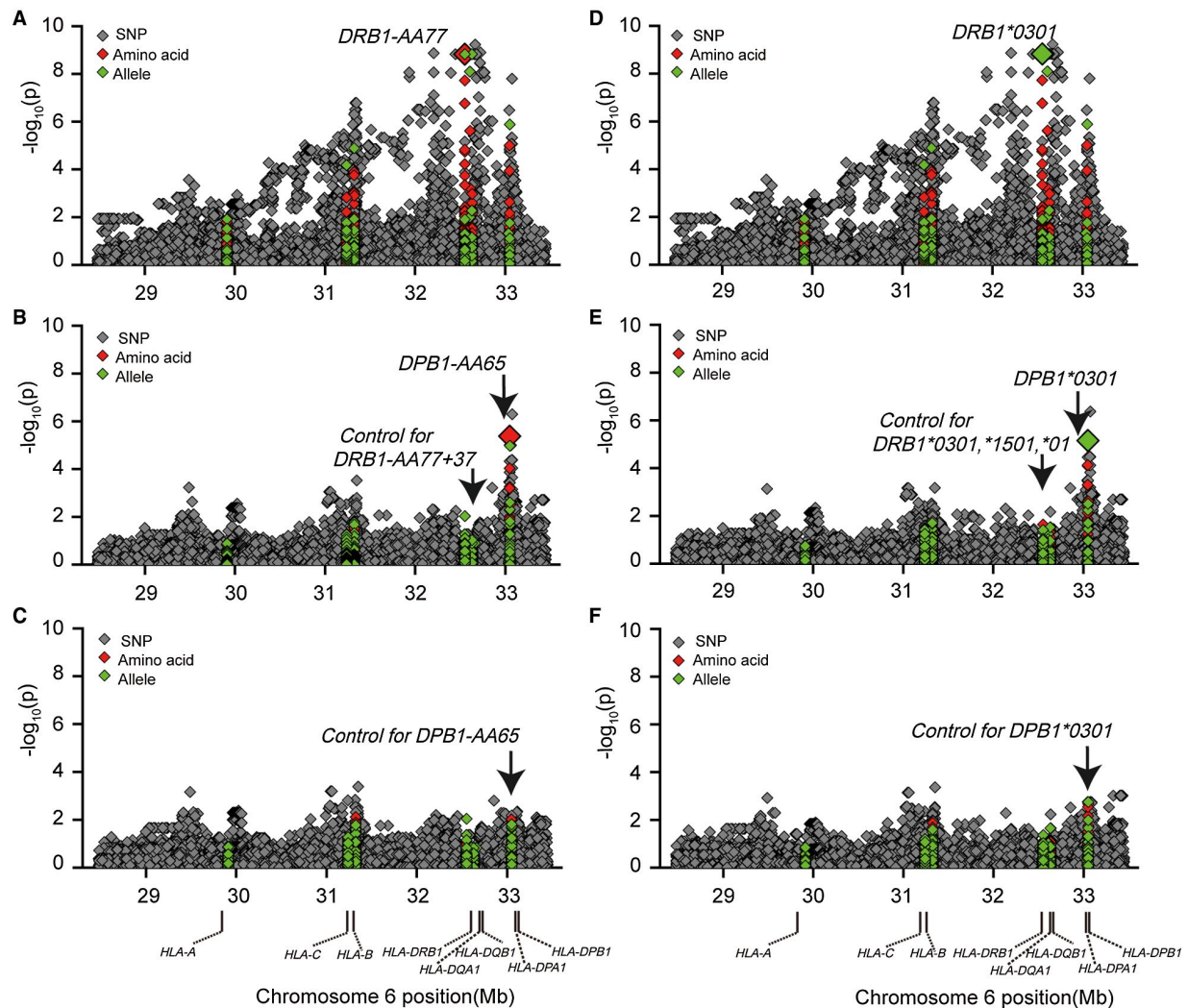


FIG. 3. Conditional analysis of genotyped and imputed HLA SNPs, AAs, and classical alleles with anti-sp100 subphenotype in the PBC GWA cohort. (A) Association results before conditional analysis, marked with AA77 in HLA-DR β 1. (B) Conditional analyses controlling for HLA-DR β 1 AA77 and AA37. (C) Conditional analysis by further controlling for HLA-DP β 1 AA65. (D) Association results before conditional analysis, marked with DRB1*0301 in the DRB1 locus. (E) Conditional analysis by further controlling for DRB1*0301, DRB1*1501, and DRB1*01. (F) Conditional analysis by further controlling for DPB1*0301.

Further conditioning on DP β 1-Lys65 or rs3128928 eliminated all of the effects within the HLA-DP β 1 region (Fig. 3A-C; Supporting Fig. S3). These results indicate that DR β 1-Asn77/Arg74, DR β 1-Ser37, and DP β 1-Lys65 were likely the major determinants for sp100 production. We note that there was a small residual signal at rs2442753 ($P = 4.1 \times 10^{-4}$) (Supporting Table S3) approximately 20 kb proximal to MHC class I-related chain A and within the intron of a putative noncoding RNA (LOC105379664).

CLASSICAL HLA-DRB1*03:01, HLA-DRB1*15:01, AND HLA-DRB1*01 ALLELES AND DPB1*03:01 ALLELE CAN EXPLAIN THE DIFFERENTIAL PRODUCTION OF ANTI-sp100 ANTIBODY IN PBC COHORTS

We also tried to further characterize the association of sp100 autoantibody with classical HLA alleles. Because the most significant associations

were with HLA-DR β 1 AAs (77, 74, 71, and 70) and alleles (*DRB1*03:01*), we first performed stepwise regression conditioning on different *DRB1* alleles as covariates within the *DRB1* region. When conditioning on *DRB1*03:01* ($P = 1.51 \times 10^{-9}$), the next most significant allele is *DRB1*15:01* ($P = 3.41 \times 10^{-3}$; OR = 1.72; 95% CI, 1.20-2.47). When conditioning on *DRB1*03:01* and *DRB1*15:01*, the remaining most associated allele is *DRB1*01* ($P = 3.38 \times 10^{-3}$, OR = 3.52, 95% CI, 1.52-8.16). Afterward, there is no significant association observed within the *DRB1* region (Fig. 3D-F; Supporting Table S3). These results indicated that there are three independent alleles for the sp100 autoantibody within the *DRB1* region. After conditioning on these three alleles (*DRB1*03:01*, *DRB1*15:01*, and *DRB1*01*), the remaining signals ($P < 1 \times 10^{-4}$) within the MHC region only resided in the *HLA-DPB1* region, peaked with SNP rs3128928 ($P = 4.30 \times 10^{-7}$; OR, 2.60; 95% CI, 1.80-3.77) or the classical allele *DPB1*03:01* ($P = 7.01 \times 10^{-6}$; OR, 2.79; 95% CI, 1.78-4.36). Conditioning on a combination of *DRB1* (**03:01*, **15:01* and **01*) and *DPB1*03:01* alleles, we observed no significant residue signal remaining in the *HLA-DPB1* region (Fig. 3F; Supporting Table S3). Similar to the AA results presented above, there was a small residual signal ($P = 4.1 \times 10^{-4}$) with rs2442753. These results clearly indicate that production of sp100 autoantibody can be mostly explained by a combination of risk alleles of *DRB1* (**03:01*, **15:01*, and **01*) and *DPB1*03:01*, with *DRB1*03:01* possessing the major effect.

Discussion

A large-scale GWA analysis conducted by our group indicated a strong genetic predisposition to PBC in Han Chinese.⁽⁶⁾ Here, we report a genetic study of PBC subphenotypes based on PBC-specific ANAs against gp210 and sp100. A common feature in autoimmune diseases is the generation of high-titer ANAs. Among the many ANAs identified so far, some are not disease-specific, existing in many different autoimmune diseases and even some cancers, lung diseases, and infectious diseases; but there are also disease-specific ANAs. ANAs to gp210 and sp100 are two autoantibodies highly specific to PBC.⁽¹⁰⁾ Several GWA studies in

different autoimmune diseases have identified significant associations of different ANAs with specific polymorphisms and classical HLA alleles.⁽²⁸⁻³⁰⁾ Previously, two studies investigated the association of specific HLA class I and II alleles with ANAs in PBC, but these studies have been confined to small sample sets and limited HLA alleles.^(31,32) The results presented in this report also have merits over previous reported imputations in other HLA fine-mapping studies. The first lies in the population-specific gene chips used in GWA with 7,889 markers in the MHC region included on the Illumina HumanOmniZhongHua-8 BeadChip. The second is the Han Chinese-specific MHC panel generated by deep sequencing of 10,689 Han Chinese individuals.⁽²⁴⁾ Therefore, we were able to perform the association analysis during the GWA stage using a large number of SNPs (including population-specific SNPs) in the MHC region and apply these SNPs in accurate imputation of the remaining SNPs, AA variation in HLA antigens, and classical HLA alleles.

In this study, we observed striking differences in the association of genetic predisposition of two PBC-specific ANAs to gp210 and sp100. A highly significant association was only observed with sp100 autoantibody but not with gp210. Anti-gp210 antibody in PBC patients recognizes a single epitope within a 15-AA stretch in the carboxyl terminus of gp210, a member of the nuclear pore complex that regulates the flow of macromolecules between the nucleus and the cytoplasm.⁽³³⁻³⁵⁾ The mechanism of generation of anti-gp210 antibody is still a matter of debate and could arise by molecular mimicry of the *Escherichia coli* mutY gene product containing a similar antigenic determinant or by abnormal expression of gp210 in patients with PBC.⁽³³⁾ A lack of association in HLA polymorphism with gp210 autoantibody may not support the notion of a molecular mimic of external bacterial protein epitopes in the generation of the gp210 autoantibody because this requires extended processing of bacterial protein fragments and presentation by antigen-presenting cells and T cells. The autoimmune target of anti-sp100 is the sp100 nuclear antigen, a major component of PML-sp100 nuclear bodies. Different from anti-gp210, sp100 autoantibody in PBC patients recognized multiple epitopes, including linear and conformational

epitopes.⁽³⁶⁾ Two major linear epitope sequences recognized by anti-sp100 share sequence similarities with several viral proteins found in the Epstein-Barr virus protein sequences.⁽³⁷⁾ In our analysis, we found HLA-DRβ1-Asn77/Arg74, DRβ1-Ser37, and HLA-DPβ1-Lys65 associated with the sp100 autoantibody. All of these AAs locate within the peptide-binding grooves, which imply a functional impact of sp100 epitope presentation in autoantibody production. Therefore, our results may suggest significantly different mechanisms in the pathogenesis of two subphenotypes associated with ANAs to sp100 and gp210. The HLA association for anti-sp100 autoantibody indicates that anti-sp100 production is an adoptive immune response and T cell-dependent. The lack of specific HLA association for anti-gp210 may suggest a T cell-independent response.

Our data show that although the sp100 autoantibody is highly specific to PBC, genotypes in HLA genes that are most strongly associated with sp100 autoantibody are not the same as those associated with PBC disease *per se* (Supporting Table S2). Indeed, this is also true for HLA antigens and AAs; *HLA-DRB1*08:03*, *HLA-DRB1*07:01*, and *DPB1*17:01* are the major predisposing alleles for PBC (manuscript in preparation). One speculative explanation for these surprising discrepancies is that *HLA-DRB1*08:03*, *HLA-DRB1*07:01*, and *DPB1*17:01* may present specific mitochondrial antigens that are critical for disease susceptibility, whereas these HLA antigens do not preferentially present either sp100 or gp210. AMA-M2 autoantibodies (pathognomonic for PBC) recognize multiple different proteins, mainly the E2 subunits of the 2-oxo-acid dehydrogenase complex (ODGC) including pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex, and branched-chain 2-oxoacid dehydrogenase complex, with, to a less extent, the E1 and E3 subunits of ODGC. Therefore, *HLA-DRB1*08:03*, *HLA-DRB1*07:01*, and *DPB1*17:01* may highly predispose for PBC but have very little effect on positivity for gp210 and sp100.

A potential limitation in our study lies in the measurement of both antibodies only at a single time point at or after diagnosis for more than half of our PBC cases. There were reports that change of anti-sp100 titers has been found during the course of the treatment, but conversion of anti-sp100

positivity was rarely found.⁽¹⁹⁾ But conversion of anti-gp210 positivity was reported in some of the patients treated with UDCA in Nakamura et al.'s study of Japanese PBC cohort, although this report was not confirmed by other studies.⁽³⁸⁾ This conversion during the treatment may increase the false-negative rate in the study cohort, which may reduce the power of our study to detect a genetic association for anti-gp210. However, this may not be a critical factor in the lack of association observed in our gp210 groups because both of our GWA and replication cohorts exhibit significantly higher anti-gp210-positive rates compared with the study by Nakamura et al. (38.4% and 35.5% versus 26.1%). At the same time, the sample size (930 GWA and 1,252 replication samples) in the current study should provide sufficient power to detect genetic associations. We also note that the current study is confined to PBC patients in the Han Chinese population. Additional studies will be necessary to ascertain whether the association of sp100 to particular HLA antigens and amino acids found in our study is also present in other populations.

As reviewed at the beginning of this article, the clinical significance of anti-sp100 and anti-gp210 is uncertain.^(8,11,12,17-20) Further studies will be needed to determine if PBC disease progression, endophenotypes, and, importantly, responses to different therapeutic regimens are associated with the presence or titers of these autoantibodies. We also note that in future PBC studies it may be useful not only to stratify patient groups based on variants that predispose to PBC itself but also to examine whether stratification based on the HLA associations of sp100 are associated with particular endophenotypes or therapeutic responses.

In summary, the current study addressed genetic determinants associated with sp100 and gp210 autoantibody subphenotypes in PBC and identified specific SNPs, HLA alleles, and AAs associated with the sp100 autoantibody but not with the gp210 autoantibody. The striking difference in genetic predisposition observed between the sp100 and gp210 subphenotypes may indicate distinct roles of these two autoantibodies in the pathogenesis of PBC.

Acknowledgment: We thank the patients for their participation in this study. Patient samples and clinical information were provided by the Jiangsu Provincial PBC Collaboration Group, Shanghai Renji Hospital, and Shuguang Hospital.

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