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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Genetic polymorphisms in CTLA4 and SLC4A2 are differentially associated with the pathogenesis of primary biliary cirrhosis in Japanese patients

Yoshihiro Aiba · Minoru Nakamura · Satoru Joshita · Tatsuo Inamine · Atsumasa Komori · Kaname Yoshizawa · Takeji Umemura · Hitomi Horie · Kiyoshi Migita · Hiroshi Yatsuhashi · Makoto Nakamuta · Nobuyoshi Fukushima · Takeo Saoshiro · Shigeki Hayashi · Hiroshi Kouno · Hajime Ota · Toyokichi Muro · Yukio Watanabe · Yoko Nakamura · Toshiki Komeda · Masaaki Shimada · Naohiko Masaki · Tatsuji Komatsu · Michiyasu Yagura · Kazuhiro Sugi · Michiaki Koga · Kazuhiro Tsukamoto · Eiji Tanaka · Hiromi Ishibashi · The PBC Study Group in NHOSLJ

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Abstract

Background Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis (PBC). In order to dissect the genetic basis for the production of these autoantibodies, as well as the development and progression of PBC in Japanese patients, we examined single nucleotide polymorphisms (SNPs) in cytotoxic T-lymphocyte antigen 4 (CTLA4) and solute carrier family 4 anion exchanger, member 2 (SLC4A2), which have been associated with the pathogenesis of PBC in Caucasian patients.

Methods Four SNPs for both CTLA4 and SLC4A2 were genotyped, using the polymerase chain reaction–restriction fragment length polymorphism method and TaqMan assay, in 450 Japanese PBC patients and 371 sex-matched healthy controls.

Results The CTLA4 rs231775, rs3087243, and rs231725 SNPs were significantly associated with PBC susceptibility. The CTLA4 rs231725 SNP was significantly associated with progression to late-stage disease. The CTLA-4 haplotype 1 (rs231775 G, rs231777 C, rs3087243 G, rs231725 A; GCGA) was a risk factor for PBC susceptibility but a protective factor for PBC progression. Conversely, the CTLA-4 haplotype 2 (ACAG) was a protective and risk factor, respectively, for PBC susceptibility and progression. In addition, the CTLA4 rs231777 SNP and haplotype 3 (ATGG) was significantly associated with anti-gp210

NHOSLJ: National Hospital Organization Study Group for Liver Disease in Japan.

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Y. Aiba · M. Nakamura (✉) · A. Komori · H. Horie · K. Migita · H. Yatsuhashi · H. Ishibashi
Clinical Research Center, National Hospital Organization Nagasaki Medical Center, 2-1001-1 Kubara, Omura, Nagasaki 856-8562, Japan
e-mail: nakamuram@nmc.hosp.go.jp

M. Nakamura · A. Komori · K. Migita · H. Yatsuhashi · H. Ishibashi
Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Omura, Japan

M. Nakamura · M. Nakamuta · N. Fukushima · T. Saoshiro · S. Hayashi · H. Kouno · H. Ota · T. Muro · Y. Watanabe · Y. Nakamura · T. Komeda · M. Shimada · N. Masaki · T. Komatsu · M. Yagura · K. Sugi · M. Koga
Headquarters of PBC Study Group in National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ), Omura, Japan

S. Joshita · K. Yoshizawa · T. Umemura · E. Tanaka
Division of Gastroenterology and Hepatology, Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

T. Inamine · K. Tsukamoto
Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

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antibody production, while SLC4A2 haplotype 4 (rs2069443 A, rs2303933 G, rs2303937 A, rs2303941 T; AGAT) and haplotype 3 (AAGC) were significantly associated with PBC susceptibility and anti-centromere antibody production, respectively.

Conclusions CTLA4 and SLC4A2 genetic polymorphisms are differentially associated with PBC development and progression, as well as anti-gp210 or anti-centromere antibody production, in Japanese PBC patients.

Keywords PBC · SNPs · CTLA4 · SLC4A2 · Autoantibody

Introduction

Primary biliary cirrhosis (PBC) is a chronic liver disease that leads to the destruction of intrahepatic bile ducts, progressive cholestasis, and eventually liver cirrhosis. Although the pathogenesis of PBC is still unknown, frequent familial clusters and a high concordance rate in monozygotic twins suggest that genetic backgrounds strongly influence PBC pathogenesis [1].

Many candidate gene association studies have been performed and several single nucleotide polymorphisms (SNPs) have been associated with PBC, including human leukocyte antigen (HLA), cytotoxic T-lymphocyte antigen 4 (CTLA4), solute carrier family 4 anion exchanger, member 2 (SLC4A2), tumor necrosis factor, and programmed cell death 1 and multidrug resistance protein 3 [2–13]. In addition to these genes, recent genome-wide association studies have revealed that IL12A, IL12RB2, IRF5-TNPO3, 17q12-21, and MMEL1 loci were associated with PBC susceptibility in Caucasians [14–16].

CTLA4 is predominantly expressed on activated and regulatory T cells, where it functions as a co-inhibitory molecule that interacts with B7.1 (CD80) and B7.2 (CD86) that are expressed on antigen-presenting cells. Many studies have shown that CTLA4 genetic polymorphisms are associated with disease susceptibility and severity in various autoimmune diseases, such as type I diabetes, rheumatoid arthritis, and Grave's disease [17, 18]. However, studies on the association between PBC and CTLA4 genetic polymorphisms have yielded conflicting results among different ethnic groups, and only a limited number of studies have assessed autoantibody production [6, 8, 19–21].

SLC4A2, which is occasionally referred to as anion exchanger 2, is distributed in various tissues and functions as an Na^+-independent Cl^-/HCO_3^- exchanger that regulates intracellular and transepithelial acid–base transport, including bicarbonate secretion into bile in cholangiocytes [22, 23]. SLC4A2 expression in liver biopsy sections, as well as in peripheral blood mononuclear cells, was significantly reduced in PBC patients compared to healthy subjects and patients with non-PBC liver diseases [24, 25]. In addition, the in vitro activity of SLC4A2 in cholangiocytes was lower in PBC patients as compared to healthy subjects or patients with non-PBC liver diseases [26]. These reports indicated that SLC4A2 might be involved in the pathogenesis of PBC. Indeed, SLC4A2 genetic polymorphisms were associated with anti-mitochondrial antibody (AMA) production and PBC progression during ursodeoxycholic acid (UDCA) treatment in Caucasian patients [11, 20].

We previously reported that anti-gp210 and anti-centromere antibodies were differentially associated with PBC progression; the presence of anti-gp210 antibodies is a strong risk factor for progression to hepatic failure, while a positive anti-centromere antibody status is a significant risk factor for progression to portal hypertension [27]. These results may imply that several different genetic polymorphisms are potentially involved in the production of these autoantibodies as well as PBC progression. However, aside from HLA studies, there are no data on genetic polymorphisms that are associated with the production of these autoantibodies [3].

In the present study, we dissected the genetic basis for the production of these autoantibodies, as well as PBC development and progression, by examining CTLA4 and SLC4A2 SNPs, which have been reportedly associated with disease susceptibility and progression, as well as AMA production, in Caucasian PBC patients [8, 9, 11–13, 20, 21].

Methods

Subjects

The study subjects included 450 PBC patients and 371 healthy controls, who were enrolled in two different PBC cohort studies that included the National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ) (cohort 1) and the Shinshu PBC Study Group in Japan (cohort 2). Patients were diagnosed with PBC based on the following three clinical criteria: (1) the presence of detectable AMA in the serum; (2) elevated liver enzymes, including alkaline phosphatase, at the initial diagnosis; and (3) compatible histological features according to Scheuer's classification. Patients who had acute hepatitis, a chronic hepatitis virus B or C infection, alcoholic liver disease, and other chronic liver diseases were excluded from this study. The demographics and clinical data of the patients at the end of the observation period are shown in Table 1. The sex distribution and age of the healthy controls were as

follows: female 310 (83.6%), median age 44 years, range 25–84 years. The study protocol was approved by the Committee for Ethical Issues dealing with the Human Genome and Gene Analysis at the Clinical Research Center in the National Hospital Organization (NHO) Nagasaki Medical Center and Shinshu University School of Medicine, and written informed consent was obtained from each subject.

Classification of clinical stages of PBC

PBC patients were classified into the following three different clinical stages based on the liver biopsy and/or clinical manifestations: clinical stage I, Scheuer's stage 1 or 2 in the liver biopsy or unknown histological stage without any signs indicating portal hypertension or liver cirrhosis; clinical stage II, Scheuer's stage 3 or 4 in the liver biopsy or any histological stage with signs of portal hypertension or liver cirrhosis but without persistent jaundice (total bilirubin <2 mg/dL); and clinical stage III, any Scheuer's stage with persistent or progressive jaundice (total bilirubin ≥2 mg/dL).

Clinical stage I was defined as early-stage disease, whereas clinical stages II and III were defined as late-stage disease. Clinical stages I and II were also defined as a non-jaundice stage, whereas clinical stage III was defined as a jaundice stage. The observation period was defined as the time frame from the initial diagnosis until death, liver transplantation, death from non-liver associated diseases, or the end of the follow-up period, whichever came first. The demographics of the early- and late-stage patients at the end of the observation period are shown in Table 2.

Detection of autoantibodies

AMA and anti-centromere antibodies were detected using commercially available enzyme-linked immunosorbent assay kits (MBL, Nagoya, Japan). Anti-gp210 antibodies were detected as previously described [28]. These autoantibodies were assayed at least twice during the

Table 1 Demographics and clinical data of PBC patients

Characteristics	Cohort 1 PBC ($n = 331$)	Cohort 2 PBC ($n = 119$)	Combined PBC ($n = 450$)
Female (%)	86.4	87.4	86.4
Age (years)[a]	64 (31–87)	58 (34–85)	63 (31–87)
Observation period (years)[b]	69.1 ± 63.2	70.6 ± 50.9	69.4 ± 60.1
Clinical stage I/II/III (%)	71.3/23.0/5.7	75.6/24.4/0	72.4/23.3/4.2
AMA^+ (%)	89.4	86.6	88.7
$gp210^+$ (%)	35.4	31.1	34.0
$Cenp^+$ (%)	25.0	34.4	27.3

PBC primary biliary cirrhosis, *AMA^+* anti-mitochondrial antibody-positive, *$gp210^+$* anti-gp210 antibody-positive, *$Cenp^+$* anti-centromere antibody-positive

[a] Median (range)

[b] Mean ± standard deviation

Table 2 Characteristics of PBC patients in early and late stages

Characteristics	Cohort 1			Cohort 2			Combined		
	Early ($n = 236$)	Late ($n = 95$)	*P*	Early ($n = 87$)	Late ($n = 32$)	*P*	Early ($n = 323$)	Late ($n = 127$)	*P*
Female (%)	88.1	82.1	0.16	87.4	87.5	1	87.9	82.1	0.22
Age (years)[a]	64	68	0.001	58	59	0.72	62	66	0.003
Observation period (years)[b]	61.7 ± 58.9	87.3 ± 69.8	0.002	71.0 ± 55.9	69.8 ± 34.3	0.89	64.2 ± 58.1	82.9 ± 63.1	0.003

Early early stage (clinical stage I), *Late* late stage (clinical stage II + III)

[a] Median (range)

[b] Mean ± standard deviation

observation period, using serum samples obtained at different time points.

DNA preparation and genotyping

Genomic DNA was extracted from the peripheral whole blood of subjects using a NucleoSpin Blood Quick Pure (Macherey-Nagel, Düeren, Germany) according to the manufacturer's protocol.

CTLA4 and SLC4A2 SNPs were analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and TaqMan SNP Genotyping assays as previously described [6, 10]. All primers and probes for the TaqMan SNP Genotyping assays were purchased from Applied Biosystems (Foster City, CA, USA). The PCR primers and restriction enzymes for PCR–RFLP are shown in Supplementary Table 1 and were purchased from Sigma-Aldrich (St. Louis, MO, USA) and New England BioLabs (Ipswich, MA, USA), respectively.

Selection of SNPs and haplotypes

Only one tag SNP, CTLA4 rs231777 SNP, was selected based on the linkage disequilibrium and haplotype blocks of all the SNPs (>5% minor allele frequency) in CTLA4 chromosomal region 2q 33 containing the 2-kb up-stream region on the HapMap Japanese in Tokyo (JPT) data (http://www.hapmap.org) using the default setting of the iHap website. Other CTLA4 SNPs (>5% minor allele frequency), rs231775, rs3087243, and rs231725, which were reportedly associated with CTLA4 function, were also chosen. Four SLC4A2 tag SNPs (rs2069443, rs2303933, rs2303937, and rs2303941) were selected based on linkage disequilibrium and haplotype blocks from all SNPs (>5% minor allele frequency) in SLC4A2 chromosomal region 7q 35–36 containing the 5-kb up- and down-stream regions on the HapMap JPT data using the default setting of the Haploview 4.1 software program (Broad Institute, Cambridge, MA, USA).

Statistical analysis

The clinical characteristics were compared between the early and late stage using an unpaired Student's t test, Mann–Whitney U test, and Fisher's exact test. Hardy–Weinberg equilibrium was evaluated based on the χ^2 test for goodness of fit. The associations between SNPs and disease susceptibility and progression, as well as autoantibody production, were examined using the χ^2 test. Multiple testing in the allele and haplotype analyses was corrected using Bonferroni's correction and permutation test (1000 simulations), respectively. These analyses were conducted with Statcel2 and SNPAlyze ver.7.0 software (Dynacom, Yokohama, Japan). Statistical significance was defined as $P < 0.05$.

Results

CTLA4 SNPs were significantly associated with PBC susceptibility in Japanese patients

First, we performed Hardy–Weinberg equilibrium tests to determine whether the CTLA4 and SLC4A2 SNPs genotyped in this study were associated with homogeneous genetic backgrounds. All CTLA4 and SLC4A2 SNPs were in Hardy–Weinberg equilibrium (Supplementary Table 2).

Subsequently, we analyzed the association between CTLA4 and SLC4A2 SNPs and PBC susceptibility. The frequencies of major alleles G, G, and A at CTLA4 rs231775, rs3087243, and rs231725 SNPs, respectively, were significantly increased in PBC patients compared to controls in cohort 1, cohort 2, and the combined cohort (Table 3). The frequency of the G allele at the SLC4A2 rs2303937 SNP was significantly decreased in PBC patients compared to controls in cohort 1, but not in cohort 2 or the combined cohort (Table 3).

CTLA4 SNPs were significantly associated with progression to late-stage disease

To determine the associations between the CTLA4 and SLC4A2 SNPs and PBC progression, we compared the frequencies of these SNPs between the early and late stages.

Although gender was not significantly different between early- and late-stage disease, PBC patients in the early stage tended to be significantly younger and have a shorter observation period than late-stage patients (Table 2). These results imply that a few patients in the early stage might progress to late-stage disease in the future.

The frequency of the A allele at the CTLA4 rs231725 SNP was significantly decreased in PBC patients with late-stage disease compared to those with early-stage disease in both cohort 1 and cohort 2 (Table 4). The significance of this SNP for progression to late-stage disease was markedly increased when cohort 1 and cohort 2 were combined, even after Bonferroni's correction. The frequencies of G alleles at the CTLA4 rs231775 and rs3087243 SNPs were significantly decreased in late-stage patients compared to early-stage patients in the combined cohort; the significance disappeared after Bonferroni's correction (Table 4).

On the other hand, there were no significant differences in the frequencies of all alleles at the SLC4A2 SNPs between early- and late-stage disease (Table 4). Furthermore, there were no significant differences in the

Table 3 The associations of *CLTA4* and *SLC4A2* SNPs with susceptibility in PBC patients

	Allele	Cohort 1		Cohort 2		Combined		
		Controls/PBC (%)	*P*	Controls/PBC (%)	*P*	Controls/PBC (%)	OR (95% CI)	*P* (Pc)
CTLA4 SNP rs ID								
rs231775	G	58.3/64.0	0.04	56.3/67.2	0.02	57.8/65.1	1.35 (1.10–1.65)	0.003 (0.024)
	A	41.7/36.0		43.7/32.8		42.2/34.9		
rs231777	C	86.5/87.5	0.64	89.9/93.2	0.23	87.4/89.0	1.12 (0.86–1.57)	0.33 (–)
	T	13.5/12.5		10.1/6.8		12.6/11.0		
rs3087243	G	70.0/77.0	0.01	66.1/75.2	0.04	69.4/76.6	1.47 (1.18–1.83)	0.001 (0.008)
	A	30.0/23.0		33.9/24.8		30.6/23.4		
rs231725	A	54.4/60.9	0.02	50.0/61.3	0.02	53.2/61.0	1.37 (1.13–1.67)	0.002 (0.016)
	G	45.6/39.1		50.0/38.7		46.8/39.0		
SLC4A2 SNP rs ID								
rs2069443	A	70.0/71.3	0.62	68.1/73.7	0.26	69.5/71.8	1.12 (0.90–1.39)	0.32 (–)
	C	30.0/28.7		31.9/26.3		30.5/28.2		
rs2303933	G	72.5/72.1	0.85	78.7/73.7	0.27	74.0/72.4	0.92 (0.74–1.15)	0.49 (–)
	A	27.5/27.9		21.3/26.3		26.0/27.6		
rs2303937	G	58.9/52.7	0.03	77.1/70.3	0.84	57.2/52.7	0.84 (0.69–1.02)	0.09 (–)
	A	41.1/47.3		22.9/29.7		42.8/47.3		
rs2303941	T	74.4/76.4	0.40	63.4/52.7	0.12	76.4/76.3	0.99 (0.79–1.25)	0.95 (–)
	C	25.6/23.6		36.6/47.3		23.6/23.7		

PBC primary biliary cirrhosis, *SNP* single nucleotide polymorphism, *OR* odds ratio, *CI* confidence interval, *Pc P* value corrected by Bonferroni

Table 4 The associations of *CLTA4* and *SLC4A2* SNPs with progression in PBC patients

	Allele	Cohort 1		Cohort 2		Combined		
		Early/late (%)	*P*	Early/late (%)	*P*	Early/late (%)	OR (95% CI)	*P* (Pc)
CTLA4 SNP rs ID								
rs231775	G	65.7/60.0	0.17	72.4/53.1	0.005	67.5/58.3	0.67 (0.50–0.91)	0.009 (0.072)
	A	34.3/40.0		27.5/46.9		32.5/41.7		
rs231777	C	87.5/87.4	0.96	94.2/90.3	0.30	89.3/88.1	0.86 (0.55–1.34)	0.61 (–)
	T	12.5/12.6		5.8/9.7		10.7/11.9		
rs3087243	G	78.8/72.6	0.10	79.3/64.1	0.02	78.9/70.5	0.64 (0.46–0.88)	0.007 (0.056)
	A	21.2/27.4		20.7/35.9		21.1/29.5		
rs231725	A	63.6/54.2	0.03	66.1/51.6	0.01	64.4/52.8	0.62 (0.46–0.83)	0.001 (0.008)
	G	36.4/45.8		33.9/48.4		35.6/47.2		
SLC4A2 SNP rs ID								
rs2069443	A	71.6/70.5	0.78	70.7/81.5	0.13	71.4/73.0	1.08 (0.77–1.51)	0.67 (–)
	C	28.4/29.5		29.3/18.5		28.627.0		
rs2303933	G	71.2/74.2	0.43	75.0/70.4	0.51	72.0/73.4	0.94 (0.70–1.31)	0.73 (–)
	A	28.8/25.8		25.0/29.6		28.0/26.6		
rs2303937	G	53.4/51.1	0.59	52.9/55.6	0.87	53.3/52.0	0.95 (0.71–1.28)	0.76 (–)
	A	46.6/48.9		47.1/44.4		46.7/48.0		
rs2303941	T	76.1/77.4	0.72	76.5/74.1	0.12	76.2/76.6	0.97 (0.69–1.38)	0.93 (–)
	C	23.9/22.6		23.5/25.9		23.8/23.4		

PBC primary biliary cirrhosis, *SNP* single nucleotide polymorphism, *Early* early stage (clinical stage I), *late* late stage (clinical stage II + III), *OR* odds ratio, *CI* confidence interval, *Pc P* value corrected by Bonferroni

Table 5 The associations of *CLTA4* and *SLC4A2* SNPs with anti-gp210 antibody production in PBC patients

	Allele	Cohort 1		Cohort 2		Combined		
		$gp210^{-/+}$ (%)	*P*	$gp210^{-/+}$ (%)	*P*	$gp210^{-/+}$ (%)	OR (95% CI)	*P* (Pc)
CTLA4 SNP rs ID								
rs231775	G	66.0/60.8	0.17	70.7/59.5	0.09	67.3/60.5	0.74 (0.56–0.99)	0.04 (0.32)
	A	34.0/39.2		29.3/40.5		32.7/39.5		
rs231777	C	89.6/83.2	0.02	94.4/90.3	0.24	94.4/84.9	0.56 (0.37–0.85)	0.006 (0.048)
	T	10.4/16.8		5.6/9.7		5.6/15.1		
rs3087243	G	77.1/76.7	0.95	77.1/70.3	0.24	77.2/75.2	0.89 (0.65–1.23)	0.49 (–)
	A	22.9/23.3		22.9/29.7		22.8/24.8		
rs231725	A	63.4/56.0	0.06	63.4/52.7	0.07	63.9/55.2	0.70 (0.53–0.92)	0.01 (0.08)
	G	36.6/44.0		36.6/47.3		36.1/47.8		
SLC4A2 SNP rs ID								
rs2069443	A	69.8/73.3	0.35	71.6/78.3	0.38	70.3/74.3	1.23 (0.89–1.69)	0.23 (–)
	C	30.2/26.7		28.4/21.7		29.7/25.7		
rs2303933	G	73.3/70.7	0.47	77.7/65.0	0.08	76.0/69.5	0.79 (0.57–1.08)	0.14 (–)
	A	26.7/29.3		22.3/35.0		24.0/30.5		
rs2303937	G	52.8/52.2	0.87	53.1/55.0	0.88	52.9/52.7	0.99 (0.74–1.32)	1.00 (–)
	A	47.2/47.8		46.9/45.0		47.1/47.3		
rs2303941	T	77.1/75.9	0.72	75.4/76.7	1.00	76.7/76.0	0.96 (0.69–1.34)	0.86 (–)
	C	22.9/24.1		24.6/23.3		23.3/24.0		

PBC primary biliary cirrhosis, *SNP* single nucleotide polymorphism, $gp210^{-/+}$ anti-gp210 antibody-negative/positive, *OR* odds ratio, *CI* confidence interval, *Pc P* value corrected by Bonferroni

Table 6 The associations of *CLTA4* and *SLC4A2* SNPs with anti-centromere antibody production in PBC patients

	Allele	Cohort 1		Cohort 2		Combined		
		$Cenp^{-/+}$ (%)	*P*	$Cenp^{-/+}$ (%)	*P*	$Cenp^{-/+}$ (%)	OR (95% CI)	*P*
CTLA4 SNP rs ID								
rs231775	G	63.4/66.5	0.48	71.2/59.8	0.08	65.3/64.2	0.96 (0.70–1.30)	0.81
	A	36.6/33.5		28.8/40.2		34.7/36.8		
rs231777	C	86.8/89.0	0.46	92.8/93.9	1.00	88.2/90.7	1.30 (0.79–2.12)	0.34
	T	13.2/11.0		7.2/6.1		11.8/9.3		
rs3087243	G	76.8/77.4	0.87	79.5/67.1	0.04	77.5/74.0	0.83 (0.59–1.16)	0.29
	A	23.2/22.6		20.5/32.9		22.5/26.0		
rs231725	A	63.4/62.2	0.68	64.7/54.9	0.16	61.4/59.8	0.93 (0.69–1.26)	0.64
	G	36.6/37.8		35.3/45.1		38.6/40.2		
SLC4A2 SNP rs ID								
rs2069443	A	70.1/73.8	0.37	72.7/75.8	0.73	70.6/74.3	1.20 (0.85–1.70)	0.30
	C	29.9/26.2		27.3/24.2		29.4/25.7		
rs2303933	G	73.8/68.3	0.17	71.8/77.3	0.49	73.4/70.9	0.88 (0.63–1.24)	0.49
	A	26.2/31.7		28.2/22.7		26.6/29.1		
rs2303937	G	50.6/58.5	0.08	50.0/60.6	0.17	50.5/59.1	1.42 (1.04–1.93)	0.03[a]
	A	49.4/41.5		50.0/39.4		49.5/40.9		
rs2303941	T	78.9/70.1	0.03	75.8/75.8	1.00	78.2/71.7	0.71 (0.50–1.00)	0.06
	C	21.1/29.9		24.2/24.2		21.8/28.3		

PBC primary biliary cirrhosis, *SNP* single nucleotide polymorphism, $Cenp^{-/+}$ anti-centromere antibody-negative/positive, *OR* odds ratio, *CI* confidence interval

[a] The significant association disappeared after Bonferroni's correction

frequencies of all tested CTLA4 and SLC4A2 alleles between patients in the non-jaundice stage and those in the jaundice stage (Supplementary Table 3).

CTLA4 and SLC4A2 SNPs were associated with anti-gp210 and anti-centromere antibody production, respectively

We next analyzed the associations between CTLA4 and SLC4A2 SNPs and the production of AMA and anti-gp210 and anti-centromere antibodies (Tables 5 and 6 and Supplementary Table 4). The frequency of the C allele at the CTLA4 rs231777 SNP was significantly decreased in anti-gp210 antibody-positive patients compared to anti-gp210 antibody-negative patients in cohort 1. Although this SNP was not significantly different between the two groups in cohort 2, this SNP was significantly different in the combined cohort, even after Bonferroni's correction. Although the frequencies of the major alleles, the G allele at CTLA4 rs231775 SNP and the A allele at CTLA4 rs231725 SNP, were significantly decreased in anti-gp210 antibody-positive patients compared to anti-gp210 antibody-negative patients in the combined cohort, the significance disappeared after Bonferroni's correction (Table 5).

The frequency of the G allele at the SLC4A2 rs2303937 SNP was significantly increased in anti-centromere antibody-positive patients compared to anti-centromere antibody-negative patients in the combined cohort, but the statistical significance disappeared after Bonferroni's correction (Table 6).

On the other hand, there were no CTLA4 or SLC4A2 SNPs that were significantly associated with AMA production (Supplementary Table 4).

CTLA4 and SLC4A2 haplotypes were differentially associated with PBC development and progression to late-stage disease and anti-gp210 or anti-centromere antibody production

Haplotype analysis revealed that CTLA4 haplotype 1 (rs231775 G, rs231777 C, rs3087243 G, rs231725 A; GCGA) was significantly increased in PBC patients compared to controls, while CTLA4 haplotype 2 (ACAG) was significantly decreased in PBC patients compared to controls. Conversely, CTLA4 haplotype 1 and haplotype 2 were significantly decreased and increased, respectively, in PBC patients with late-stage disease compared to those with early-stage disease. In addition, CTLA4 haplotype 3 (ATGG) and haplotype 1 were significantly increased and decreased, respectively, in anti-gp210 antibody-positive patients compared to anti-gp210 antibody-negative patients (Table 7).

SLC4A2 haplotype 4 (rs2069443 A, rs2303933 G, rs2303937 A, rs2303941 T; AGAT) and haplotype 1 (AGGT) were significantly increased and decreased, respectively, in PBC patients compared to controls. Furthermore, SLC4A2 haplotype 3 (AAGC) was significantly increased in anti-centromere antibody-positive patients compared to anti-centromere antibody-negative patients (Table 8).

Table 7 The associations of *CTLA4* haplotypes with susceptibility, progression, and anti-gp210 antibody production in PBC patients

CTLA4 haplotype	Controls/PBC (%)	OR (95% CI)	*P**	Early/late (%)	OR (95% CI)	*P**	$gp210^{-/+}$ (%)	OR (95% CI)	*P**
1. GCGA	50.2/60.4	1.52 (1.24–1.85)	<0.001	60.5/55.9	0.65 (0.48–0.87)	0.006	63.1/55.2	0.72 (0.54–0.96)	0.03
2. ACAG	28.9/22.9	0.73 (0.58–0.91)	0.001	25.1/28.2	1.49 (1.07–2.08)	0.02	22.3/24.0	1.10 (0.79–1.52)	0.56
3. ATGG	10.3/10.0	0.94 (0.69–1.29)	0.68	10.3/10.0	1.18 (0.75–1.87)	0.47	8.5/15.1	1.91 (1.25–2.93)	0.001
4. GCGG	3.5/4.3	0.67 (0.42–1.06)	0.13	3.1/4.7	1.18 (0.57–2.44)	0.66	3.6/4.6	1.30 (0.60–2.59)	0.39

CTLA4 haplotype rs231775–rs231777–rs3087243–rs231725, *PBC* primary biliary cirrhosis, *Early* early stage (clinical stage I), *late* late stage (clinical stage II + III), $gp210^{-/+}$ anti-gp210 antibody-negative/positive, *OR* odds ratio, *CI* confidence interval, *P** permutation *P* value

Table 8 The associations of *SLC4A2* haplotypes with susceptibility, progression, and anti-centromere antibody production in PBC patients

SLC4A2 haplotype	Controls/PBC (%)	OR (95% CI)	*P**	Early/late (%)	OR (95% CI)	*P**	$Cenp^{-/+}$ (%)	OR (95% CI)	*P**
1. AGGT	30.1/25.6	0.80 (0.64–1.00)	0.05	25.4/26.1	1.05 (0.75–1.47)	0.82	24.6/28.5	1.21 (0.86–1.71)	0.25
2. CGAT	25.5/28.8	0.85 (0.68–1.06)	0.15	25.7/25.0	0.97 (0.69–1.36)	0.81	26.5/23.4	0.85 (0.60–1.21)	0.36
3. AAGC	18.8/20.4	0.90 (0.70–1.15)	0.44	19.4/17.9	0.91 (0.62–1.32)	0.63	17.3/23.2	1.44 (1.00–2.09)	0.05
4. AGAT	11.4/15.7	1.45 (1.08–1.95)	0.02	15.2/17.0	1.13 (0.76–1.69)	0.54	16.7/13.4	0.78 (0.50–1.20)	0.25

SLC4A2 haplotype rs2069443–rs2303933–rs2303937–rs2303941, *PBC* primary biliary cirrhosis, *Early* early stage (clinical stage I), *late* late stage (clinical stage II + III), $Cenp^{-/+}$ anti-centromere antibody-negative/positive, *OR* odds ratio, *CI* confidence interval, *P** permutation *P* value

Discussion

A significant association between the CTLA4 rs231725 SNP and PBC susceptibility was first reported in the United States and was replicated in Canada by Juran et al. [7, 21]. A modest association between the CTLA4 rs231725 SNP and PBC susceptibility was also recently found in the combined Shinshu and Nagasaki cohort in the Japanese population [6]. The present study increased the number of controls and PBC patients in the Nagasaki cohort, and further confirmed that the CTLA4 rs231725 SNP is significantly associated with PBC susceptibility in the Japanese population. In addition, the CTLA4 rs3087243 and rs231775 SNPs were associated with PBC susceptibility in the present two different Japanese cohorts. These results are consistent with previous reports from the United Kingdom, China, Italy, France, and North America [4, 9, 11–13].

In the present haplotype analysis, CTLA4 haplotype 1 (rs231775 G, rs231777 C, rs3087243 G, rs231725 A; GCGA) was significantly associated with PBC susceptibility in the Japanese population, whereas CTLA4 haplotype 2 (ACAG) was associated with PBC resistance in Japanese patients. These results are consistent with the risk and protective haplotypes for PBC susceptibility previously reported from North America [12, 21]. A functional analysis of CTLA4 SNPs showed that the G allele at the CTLA4 rs231775 SNP resulted in a Thr-to-Ala amino acid substitution, leading to diminished CTLA4 expression on the cell surface [29]. Soluble CTLA4 mRNA is expressed at lower levels in healthy controls who have the G allele compared to those who have the A allele of the CTLA4 rs3087243 SNP [30]. CTLA4 SNP rs231725 might be associated with the expression of CTLA4 via the stability of its mRNA, since the CTLA4 rs231725 SNP is located at the 3′-flanking region of the CTLA4 gene. Thus, the functional alterations associated with these CTLA4 SNPs might similarly operate in PBC development in Japanese patients.

As for PBC progression, the CTLA4 rs231725 SNP was significantly associated with progression to late-stage disease. In addition, haplotype analyses revealed that CTLA4 haplotype 1 (rs231775 G, rs231777 C, rs3087243 G, rs231725 A; GCGA) was a risk factor for PBC susceptibility but was a protective factor for PBC progression. Conversely, CTLA4 haplotype 2 (ACAG) was a protective and risk factor, respectively, for PBC susceptibility and progression. Thus, the haplotype that confers PBC susceptibility confers resistance to PBC progression, and vice versa. These results might imply that PBC development and progression have different components of pathogenesis in terms of CTLA4 genetic polymorphisms. A previous report showed that a CTLA4 haplotype containing rs231775 G, rs231777 C, rs3087243 G, and rs231725 A was a risk factor for both susceptibility and progression to orthotopic liver transplantation (OLT) in Caucasian PBC patients [21]. However, there was no significant association between CTLA4 haplotypes and progression to hepatic failure and/or OLT in Japanese patients in the present study. Since the number of patients who progressed to hepatic failure and/or OLT was only 10% of the patients with late-stage disease in our cohort, further study is needed to dissect the association between CTLA4 SNPs and severe PBC progression.

SLC4A2-deficient mice exhibit PBC-like features characterized by portal inflammation with $CD4^+$ and $CD8^+$ T-lymphocyte infiltration, elevated alkaline phosphatase and alanine aminotransferase levels, and prevalence of AMA, indicating that functional alterations in SLC4A2 are involved in PBC pathogenesis in humans [31]. Indeed, genetic SLC4A2 polymorphisms were associated with AMA production and PBC progression in Caucasian PBC patients [11, 20]. In the present study, although SLC4A2 SNPs and haplotypes were not significantly associated with PBC progression, SLC4A2 haplotype 1 (rs2069443 A, rs2303933 G, rs2303937 G, rs2303941 T; AGGT) and haplotype 4 (AGAT) were significantly associated with PBC susceptibility. Further studies are required to confirm the associations between SLC4A2 SNPs and haplotypes and PBC susceptibility and progression among various ethnicities.

In cohort studies in the United States, the CTLA4 rs231775 G/G genotype and rs231725 A/A genotype tended to be more prevalent in AMA-positive PBC patients compared to AMA-negative PBC patients [8, 21]. Furthermore, a CTLA4 SNP haplotype was associated with AMA production [21]. In addition, the SLC4A2 rs2303929 and rs3793336 SNPs were associated with AMA production in Caucasian PBC patients [20]. These results indicate that genetic polymorphisms in both CTLA4 and SLCA42 are involved in autoantibody production in PBC patients. Although the CTLA4 and SLC4A2 SNPs were not significantly associated with AMA production in the present study in Japanese PBC patients, we found, for the first time, that the CTLA4 rs231777 and SLC4A2 rs2303937 SNPs were associated with anti-gp210 and anti-centromere antibody production, respectively. There were also significant associations between haplotypes carrying these risk alleles and anti-gp210 or anti-centromere antibody production. Moreover, the association of these SNPs with autoantibody production was still significant, even after a multivariate analysis, by taking into account the HLA-DRB1 polymorphisms in which HLA-DRB1*0405 and *0803 were significantly associated with anti-gp210 and anti-centromere antibody production, respectively (data not shown) [3].

Aberrant self-antigen expression and presentation during apoptosis are thought to be one mechanism that contributes to the breakdown of immunological tolerance. In fact, abnormal expression of pyruvate dehydrogenase E2 (PDC-E2), which is a major target antigen for AMA, was observed in the apical region of the biliary epithelium in PBC patients [32]. Apoptotic blebs containing intact PDC-E2 were found in biliary epithelial cells during apoptosis [33]. Aberrant gp210 expression was observed in biliary epithelial cells in the small bile ducts of PBC patients, and the degree of gp210 expression was positively correlated with PBC disease activity [34]. Therefore, it is possible that CTLA4 SNPs play an important role in the breakdown of immunological tolerance to the gp210 antigen that is aberrantly presented, although the localization of the gp210 antigen during apoptosis remains to be determined. On the other hand, SLC4A2 participates in lymphocyte activation by regulating intracellular pH homeostasis [35]. The characteristic features of PBC, including AMA production, were observed in SLC4A2-deficient mice. The expression of genes related to antigen presentation was also increased in cholangiocytes from SLC4A2-deficient mice [31]. Thus, alterations in these SLC4A2 functions, which are possibly associated with genetic polymorphisms in SLC4A2, might be involved in anti-centromere antibody production.

In conclusion, we confirmed that CTLA4 SNPs are associated with PBC susceptibility and progression in the Japanese population. Furthermore, we showed, for the first time, that CTLA4 and SLC4A2 SNPs are associated with anti-gp210 and anti-centromere antibody production, respectively. Since anti-gp210 and anti-centromere antibodies are different risk factors for the hepatic failure-type and portal hypertension-type of PBC progression, the results in the present study may imply that genetic CTLA4 and SLC4A2 polymorphisms are differentially involved in multiple steps of PBC pathogenesis.

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Conflict of interest None.

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Human Leukocyte Antigen Class II Molecules Confer Both Susceptibility and Progression in Japanese Patients With Primary Biliary Cirrhosis

Takeji Umemura,[1] Satoru Joshita,[1] Tetsuya Ichijo,[1] Kaname Yoshizawa,[1,4] Yoshihiko Katsuyama,[2] Eiji Tanaka,[1] and Masao Ota[3]; The Shinshu PBC Study Group

Along with twin and family studies, recent genome-wide association studies suggest that genetic factors contribute to the susceptibility and severity of primary biliary cirrhosis (PBC). Although several reports have demonstrated that the human leukocyte antigen (HLA) *DRB1*08:03* allele is associated with disease susceptibility in Japan, the precise analysis of HLA haplotypes and the role of amino acid alignment have not been fully clarified. We investigated HLA class I A, B, and C and HLA class II DRB1 and DQB1 alleles and haplotypes in 229 Japanese patients with PBC and compared them with the published data of 523 healthy subjects. Significant associations were found with PBC susceptibility for the *DRB1*08:03-DQB1*06:01* (13% versus 6%; $P = 0.000025$; odds ratio [OR] = 2.22) and *DRB1*04:05-DQB1*04:01* haplotypes (17% versus 13%; $P = 0.044$; OR = 1.38). Conversely, there were significant protective associations with the *DRB1*13:02-DQB1*06:04* (2% versus 5%; $P = 0.00093$; OR = 0.27) and *DRB1*11:01-DQB1*03:01* haplotypes (1% versus 4%; $P = 0.03$; OR = 0.37). The frequency of the *DRB1*09:01-DQB1*03:03* haplotype was significantly higher in patients who had received orthotopic liver transplantation (33% versus 11%; $P = 0.0012$; OR = 3.96). Furthermore, the frequency of serine at position 57 ($P = 0.0000015$; OR = 1.83) of the DRβchain differed the most in patients with PBC, compared with healthy subjects. *Conclusion*: This study established the role of HLA haplotypes in determining PBC susceptibility and progression in the Japanese population. Further resequencing of the HLA region is required to more precisely identify the genetic components of PBC. (HEPATOLOGY 2012;55:506-511)

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often leads to cirrhosis and liver failure.[1] Although twin and family studies suggest that genetic factors contribute to disease susceptibility and severity,[2,3] the cause of PBC remains poorly understood.[4] Significant associations of genetic factors, including HLA alleles, tumor necrosis factor alpha,[5–8] and cytotoxic T-lymphocyte antigen 4,[8–14] with PBC have been reported. Among these, only HLA has consistently been associated with PBC susceptibility.[15] The HLA-*DRB1*08* family of alleles has been the most frequently described determinant for susceptibility to PBC[16–21]; HLA-*DRB1*08:03* has been associated with PBC in the Japanese.[22–26] However, HLA DQB1 alleles and haplotypes have not been fully investigated, and large cohort studies have indicated that HLA-*DRB1*11* and *DRB1*13* alleles are, in fact, protective against PBC.[20,21,26] Because recent genome-wide studies of PBC have shown the strongest association signals in the HLA region,[27–30] we sought to determine whether particular HLA alleles or haplotypes or DRB1 allele amino acid alignments were associated with susceptibility to PBC or disease progression in the Japanese population.

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; OLT, orthotopic liver transplantation; OR, odds ratio; PBC, primary biliary cirrhosis.

From the [1]Department of Medicine, Division of Hepatology and Gastroenterology, Shinshu University School of Medicine, Matsumoto, Japan; [2]Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan; [3]Department of Legal Medicine, Shinshu University School of Medicine, Matsumoto, Japan; and [4]Department of Gastroenterology, NHO Shinshu Ueda Medical Center, Ueda, Japan.

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The Shinshu PBC Study Group includes the following members: Yuki Ichikawa-Yamada, Takefumi Kimura, Susumu Morita, Atsushi Kamijo, Michiharu Komatsu, Akihiro Matsumoto (Shinshu University School of Medicine), and Nobuyoshi Yamamura (Department of Gastroenterology, Suwa Red Cross Hospital).

Table 1. Demographic and Clinical Characteristics of Patients With PBC

Characteristics	PBC (n = 229)	
Median age, years (range)	57	(27-86)
Female, n (%)	204	(89)
Late stage of disease, n (%)	50	(22)
Cirrhosis, n (%)	42	(18)
OLT, n (%)	15	(7)
Serum AMA positive, n (%)	209	(91)

Abbreviations: PBC, primary biliary cirrhosis; OLT, orthotopic liver transplantation; AMA, antimitochondrial antibody.

Patients and Methods

Subjects. Clinical and biochemical features of 229 PBC patients who were enrolled for this study between January 2005 and September 2010 are shown in Table 1. The racial background of all patients was Japanese. HLA class I and II allelic genotypes from 523 healthy subjects obtained in a previous study were available as controls.[31] In addition, HLA class II allelic genotypes from 130 patients with chronic hepatitis C virus infection were adopted from another study as comparison cases having another liver disease.[32] The diagnosis of PBC was based on criteria from the American Association for the Study of Liver Diseases.[33] Serum antimitochondrial antibody was determined using indirect immunofluorescence, and a titer of $\geq$1:40 was considered to be positive.[34] Our serological protocol did not include testing for particular antinuclear antibodies, such as ani-gp210 antibody reactivity, or antimitochondrial antibody titration. All patients were negative for hepatitis B surface antigen, antibody to hepatitis B core antigen, antibody to hepatitis C virus, and antibody to human immunodeficiency virus. Patients were classified into two stages of PBC, based on their most recent follow-up: Early-stage patients were histologically Scheuer stage I or II[35] or of unknown histological stage without liver cirrhosis, and late-stage patients were histologically Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure.[14] Liver cirrhosis was diagnosed by histological examination and/or characteristic clinical signs of advanced liver disease.[36] All subjects provided written informed consent for this study, which was approved by the institutional ethics committee.

HLA Class I and II Typing. Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate–lyzed and proteinase K–treated cells, as described previously.[37] HLA typing was carried out using a Luminex multianalyzer profiling system (Luminex, Austin, TX) with a LAB type SSO One Lambda typing kit (One Lambda, Inc., Canoga Park, CA), which is based on polymerase chain reaction sequence-specific oligonucleotide probes. HLA genotypes were determined by sequence-based typing. Peptide sequences of all HLA-DRB1 alleles in the IMGT/HLA database release 3.4.0 (April 2011) were aligned.

Statistical Analysis. Phenotype frequencies were estimated by direct counting for each HLA allele. The significance of an association was evaluated by determining the standard P values after chi-squared analysis or Fisher's exact test. A P value of less than 0.05 was considered statistically significant. Association strength was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI).

Results

Distribution of HLA A, B, and C Alleles. Among HLA class I alleles, the frequencies of *A*02:01* and *C*03:03* were significantly increased in patients with PBC, compared with healthy subjects (16% versus 11%, $P = 0.0029$, and 18% versus 13%, $P = 0.012$, respectively) (Table 2). In contrast, patients had significantly lower frequencies of *A*02:06* (6% versus 9%; $P = 0.038$), *A*33:03* (4% versus 8%; $P = 0.0025$), *B*44:03* (2% versus 7%; $P = 0.0011$), *C*08:01* (5% versus 10%; $P \doteq 0.005$), *C*14:03* (3% versus 7%; $P = 0.0018$), and *C*15:02* (2% versus 4%; $P = 0.03$) alleles, compared with controls (Table 2). No other HLA A, B, or C alleles differed significantly between the groups.

Distribution of HLA-DRB1 and DQB1 Alleles. Among DRB1 alleles, *DRB1*04:05* and *DRB1*08:03* were significantly associated with PBC, compared with healthy subjects (17% versus 13%, $P = 0.044$, and 13% versus 6%, $P = 0.000025$, respectively) (Table 2). Patients with PBC had a significantly lower frequency of *DRB1*11:01* (1% versus 4%; $P = 0.02$) and *DRB1*13:02* (3% versus 6%; $P = 0.029$) allele carriage, compared with controls (Table 2). Among DQB1 alleles, the *DQB1*04:01* and *DQB1*06:01* alleles were significantly associated with an increased risk of PBC (18% versus 13%, $P = 0.02$, and 23% versus

Address reprint requests to: Takeji Umemura, M.D., Ph.D., Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. E-mail: tumemura@shinshu-u.ac.jp; fax: +81-263-32-9412 Masao Ota, Ph.D., Department of Legal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. E-mail: otamasao@shinshu-u.ac.jp; fax: +81-263-37-3084.

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Additional Supporting Information may be found in the online version of this article.

Table 2. Statistical Analysis of Representative HLA Antigens Among Patients With PBC and Healthy Subjects

HLA	Frequency (%) Patients With PBC (2n = 458)	Healthy Subjects (2n = 1,032-1,046)	P Value	OR (95% CI)
*A*02:01*	16	11	0.0029	1.63 (1.19-2.24)
*A*02:06*	6	9	0.038	0.61 (0.39-0.95)
*A*33:03*	4	8	0.0025	0.43 (0.25-0.74)
*B*44:03*	2	7	0.0011	0.34 (0.18-0.65)
*C*03:03*	18	13	0.012	1.48 (1.10-1.99)
*C*08:01*	5	10	0.005	0.51 (0.32-0.81)
*C*14:03*	3	7	0.0018	0.38 (0.21-0.70)
*C*15:02*	2	4	0.03	0.44 (0.21-0.90)
*DRB1*04:05*	17	13	0.044	1.38 (1.02-1.87)
*DRB1*08:03*	13	6	0.000025	2.22 (1.53-3.20)
*DRB1*11:01*	1	4	0.02	0.35 (0.15-0.83)
*DRB1*13:02*	3	6	0.029	0.49 (0.27-0.91)
*DQB1*03:01*	6	12	0.00027	0.44 (0.29-0.69)
*DQB1*04:01*	18	13	0.02	1.45 (1.07-1.95)
*DQB1*06:01*	23	15	0.000091	1.75 (1.32-2.30)
*DQB1*06:02*	7	12	0.019	0.61 (0.41-0.91)
*DQB1*06:04*	2	5	0.0041	0.35 (0.17-0.72)

Abbreviations: HLA, human leukocyte antigen; PBC, primary biliary cirrhosis; OR, odds ratio; CI, confidence interval.

15%, $P = 0.000091$, respectively) (Table 2). Conversely, *DQB1*03:01* (6% versus 12%; $P = 0.00027$), *DQB1*06:02* (7% versus 12%; $P = 0.019$), and *DQB1*06:04* (2% versus 5%; $P = 0.0041$) all conferred a reduced risk of PBC occurrence (Table 2). No other HLA DRB1 or DQB1 alleles were significantly associated with PBC, compared with healthy subjects. We also examined the influence of DRB1 and DQB1 allele homozygosity with PBC susceptibility and protection, but found no significant associations. However, the *DRB1*08:03* and *DQB1*06:01* alleles were significantly associated with PBC, compared to comparison cases with chronic hepatitis C (13% versus 5%, $P = 0.0017$, and 23% versus 16%, $P = 0.02$, respectively) (Supporting Table 1). Conversely, *DQB1*03:01* and *DQB1*06:04* had significantly lower frequencies in patients with PBC than in chronic hepatitis C controls (6% versus 12%, $P = 0.0076$, and 2% versus 5%, $P = 0.041$) (Supporting Table 1).

Distribution of Haplotypes Among PBC Patients and Controls. The frequency of the *DRB1*08:03-DQB1*06:01* haplotype in patients with PBC was 13% and significantly higher than the 6% observed in healthy subjects ($P = 0.000025$; OR = 2.22) (Table 3). However, there was no significant difference between the groups regarding the *DRB1*15:02-DQB1*06:01* haplotype (10% versus 9%; $P = 0.47$). There was also a modest relationship between carriage of the *DRB1*04:05-DQB1*04:01* haplotype and disease susceptibility (17% versus 13%; $P = 0.044$; OR = 1.38). In contrast, protective effects were seen for the *DRB1*13:02-DQB1*06:04* haplotype (2% versus 5%; $P = 0.00093$; OR = 0.27) and *DRB1*11:01-DQB1*03:01* haplotype (1% versus 4%; $P = 0.03$; OR = 0.37) in our cohort.

Association Between HLA and Clinical Findings. PBC patients were stratified according to history of orthotopic liver transplantation (OLT) and disease progression. The HLA-*DRB1*09:01* and *DQB1*03:03* alleles (33% versus 11%, $P = 0.0012$, and 33% versus 12%, $P = 0.0022$, respectively) and the *DRB1*09:01-DQB1*03:03* haplotype (33% versus 11%; $P = 0.0012$; OR = 3.96; 95% CI: 1.75-8.95) were all significantly associated with OLT (Table 4). Homozygosity for the *DRB1*09:01* and *DQB1*03:03* alleles (43% versus 4%, $P = 0.0012$, and 43% versus 4%, $P = 0.00076$, respectively) and the *DRB1*09:01-DQB1*03:03* haplotype (43% versus 4%; $P = 0.0012$; OR = 16.50; 95% CI: 2.10-129.63) was significantly correlated with OLT. When PBC patients with cirrhosis (n = 42) were compared to those without (n = 187), similar significant genetic associations of the *DRB1*09:01* and *DQB1*03:03* alleles (23% versus 10%, $P = 0.0043$, and 23% versus 11%, $P = 0.0094$, respectively) and the *DRB1*09:01-DQB1*03:03* haplotype (23% versus 10%; $P = 0.0043$; OR = 2.51; 95% CI: 1.37-4.62) with disease progression were found (Table 4). Homozygosity for the *DRB1*09:01* and *DQB1*03:03* alleles (27% versus 3%, $P = 0.007$, and 27% versus 2%, $P = 0.0049$, respectively) and the *DRB1*09:01-DQB1*03:03* haplotype (27% versus

Table 3. Haplotype Distribution in PBC Patients and Healthy Subjects

Allele at Each Locus		Patients With PBC (%)	Healthy Subjects (%)		
DRB1	*DQB1*	2n = 458	2n = 1,032	P value	OR (95% CI)
**08:03*	**06:01*	60 (13)	66 (6)	0.000025	2.22 (1.53-3.20)
**04:05*	**04:01*	79 (17)	136 (13)	0.044	1.38 (1.02-1.87)
**13:02*	**06:04*	7 (2)	56 (5)	0.00093	0.27 (0.12-0.60)
**11:01*	**03:01*	6 (1)	36 (4)	0.03	0.37 (0.15-0.88)
**15:02*	**06:01*	47 (10)	92 (9)	0.47	
**09:01*	**03:03*	58 (13)	138 (13)	0.77	

Abbreviations: PBC, primary biliary cirrhosis; OR, odds ratio; CI, confidence interval.

Table 4. HLA Allele and Haplotype Distribution for OLT Status and Clinical Disease Progression

Allele at Each Locus		OLT (%)	Non-OLT (%)	P Value	Cirrhosis (%)	Noncirrhosis (%)	
DRB1	DQB1	2n = 30	2n = 428		2n = 84	2n = 372	P Value
*09:01		10 (33)	48 (11)	0.0012	19 (23)	39 (10)	0.0043
	*03:03	10 (33)	51 (12)	0.0022	19 (23)	42 (11)	0.0094
*09:01	*03:03	10 (33)	48 (11)	0.0012	19 (23)	39 (10)	0.0043
*08:03	*06:01	6 (20)	54 (13)	0.38	8 (10)	52 (14)	0.37
*04:05	*04:01	5 (17)	76 (18)	0.92	9 (11)	72 (19)	0.09

Abbreviations: HLA, human leukocyte antigen; OLT, orthotopic liver transplantation.

3%; $P = 0.007$; OR = 13.45; 95% CI: 1.36-133.18) was significantly correlated with cirrhosis, as well. No other HLA class I or II alleles or haplotypes were significantly associated with disease progression.

Distribution of DRB1 Amino Acid Residues. The amino acid sequence encoded by the second exon of *HLA-DRB1* was determined for each subject. The prevalence of glycine at position 13 ($P = 0.0013$; OR = 1.60), tyrosine at positions 16 ($P = 0.0013$; OR = 1.60) and 47 ($P = 0.00017$; OR = 1.62), serine at position 57 ($P = 0.0000015$; OR = 1.83), and leucine at position 74 ($P = 0.0000069$; OR = 2.01) was significantly higher in patients with PBC, compared with healthy subjects (Table 5). In contrast, serine at position 13 ($P = 0.000037$; OR = 0.51), histidine at position 16 ($P = 0.0029$; OR = 0.66), and phenylalanine at position 47 ($P = 0.000096$; OR = 0.61) conferred protection against the disease.

Analysis of the amino acid residues encoded by *DRB1*09:01* revealed six unique differences from those encoded by other *DRB1* alleles: lysine at position 9, aspartic acid at position 11, tyrosine at position 26, histidine at position 28, glycine at position 30, and valine at position 78 (Table 6).

Discussion

The present study examined HLA class I and II alleles and haplotypes and amino acid residues in patients with PBC in the Japanese population. Our key findings were as follows: (1) The HLA *DRB1*08:03-DQB1*06:01* haplotype was significantly associated with disease pathogenesis, which was in agreement with several Japanese studies linking *DRB1*08:03* with PBC; (2) Japanese PBC patients had significantly lower frequencies of HLA *DRB1*13:02-DQB1*06:04* and *DRB1*11:01-DQB1*03:01* haplotypes, suggesting protection by these haplotypes to the disease, as indicated by recent reports in Europe; (3) the existence of a relationship between HLA haplotype and OLT and disease progression; and (4) PBC-associated alleles have specific antigen presentation profiles.

The HLA- *DRB1*08:03* ($P = 0.000025$) and *DQB1*06:01* ($P = 0.000091$) alleles were strongly associated with PBC susceptibility. Although a relationship between *DRB1*08:03* and PBC has already been reported in the Japanese population, an association with the *DQB1*06:01* allele has not been investigated in a large cohort like ours. *DQB1:06:01* is known to be in linkage disequilibrium with *DRB1*08:03* or *DRB1*15:02* in the Japanese population. Our data clearly show that the *DRB1*08:03-DQB1*06:01* haplotype was significantly associated with PBC ($P = 0.000025$), but the *DRB1*15:02-DQB1*06:01* haplotype was not. This suggests that the *DRB1*08:03* allele and/or the *DRB1*08:03-DQB1*06:01* haplotype might play a crucial role in PBC development in Japan. However, because *DRB1*08:03* was found in only 13% of PBC patients in this study, other candidate genes and environmental factors require further study. The *DRB1*04:05-DQB1*04:01* haplotype was also found to be weakly associated with susceptibility to PBC. Because our previous reports showed that this haplotype was strongly associated with autoimmune hepatitis and autoimmune pancreatitis in the Japanese population,[38,39]

Table 5. Frequency of Different Amino Acids at HLA DRB1

Residue	Amino Acid	PBC (%) 2n = 458	Healthy Subjects (%) 2n = 1,032	P Value	OR (95% CI)
13	Glycine	98 (21)	150 (15)	0.0013	1.60 (1.21-2.12)
	Serine	55 (12)	214 (21)	0.000072	0.52 (0.38-0.72)
16	Tyrosine	98 (21)	150 (15)	0.0013	1.60 (1.21-2.12)
	Histidine	346 (76)	850 (82)	0.0029	0.66 (0.51-0.86)
47	Tyrosine	344 (75)	672 (65)	0.00017	1.62 (0.26-2.07)
	Phenylalanine	114 (25)	364 (35)	0.00017	0.62 (0.48-0.79)
57	Serine	157 (34)	224 (22)	0.0000004	1.88 (1.48-2.40)
74	Leucine	90 (20)	112 (11)	0.0000069	2.01 (1.48-2.72)

Abbreviations: OR, odds ratio; CI, confidence interval.

Table 6. Amino Acid Differences Between the *DRB1*09:01* Allele and Other HLA *DRB1* Alleles

	Position					
Allele	9	11	26	28	30	78
*DRB1*09:01*	Lysine	Aspartic acid	Tyrosine	Histidine	Glycine	Valine
Other	Tryptophan	Leucine	Leucine	Glutamic acid	Cysteine	Tyrosine
	Glutamic acid	Valine	Phenylalanine	Aspartic acid	Tyrosine	
		Glycine			Lysine	
		Serine			Arginine	
		Proline			Histidine	

Abbreviation: HLA, human leukocyte antigen.

deeper evaluation of *DRB1*04:05-DQB1*04:01* with regard to autoimmune diseases and PBC may uncover key relationships of clinical value. Recently, genome-wide association studies showed that HLA and other non-HLA genes were associated with susceptibility to PBC in Europe and North America.[27–30] Accordingly, similar studies are now being performed to clarify the genes responsible for PBC in Japan.

This study shows, for the first time, that the *DRB1*13:02-DQB1*06:04* and *DRB1*11:01-DQB1*03:01* haplotypes played protective roles against PBC in the Japanese population. Our data support the recent consensus that *DRB1*11* and **13* confer resistance in Europe and Japan,[20,21,26] although we cannot exclude the possibility that these associations are only linkage markers for a yet undefined gene for PBC. Multiple lines of evidence show that *DRB1*11* and *DRB1*13* alleles are also protective against several infectious diseases. Because bacterial infections have been reported as possible causes of PBC,[40,41] HLA alleles or haplotypes that are resistant to such agents might influence protection against PBC development.[15]

Interestingly, this study revealed a novel association between the *DRB1*09:01-DQB1*03:03* haplotype and PBC progression. Although Nakamura et al.[26] reported that *DRB1*09:01* was associated with disease progression of non-jaundice-type PBC, there have been no reports of a connection between HLA haplotypes and OLT or cirrhosis in Japan. Several studies from the United Kingdom and Sweden[19,42] have reported that *DRB1*08:01* is associated with both susceptibility and progression to the disease, but a study from Italy could not confirm this.[21] Homozygosity of the *DRB1*09:01-DQB1*03:03* haplotype was also associated with disease progression in our cohort. The reasons for this observation are unknown; however, the association of this particular HLA haplotype and disease progression is striking. Because only 15 (7%) and 42 (18%) of our 229 patients had OLT and cirrhosis, respectively, further longitudinal follow-up studies in larger cohorts from different ethnicities are required. A recent study uncovered that anti-gp210 and anticentromere antibodies may be risk factors for the progression of PBC.[43] It would be of interest to assess associations between these autoantibodies and HLA haplotypes in the future.

Last, the present study determined and analyzed the amino acid sequence encoded by the *DRB1* allele in relation to disease susceptibility. The incidence of glycine-13, tyrosine-16, and leucine-74 encoded by *DRB1*08:03* was higher and that of serine-13, histidine-16, and phenylalanine-47 encoded by *DRB1*11* and *DRB1*13* was lower in PBC patients. These data are consistent with a report by Donaldson et al.[20] Serine-57 had the highest frequency among patients in our cohort ($P = 0.0000004$), likely because it is encoded by *DRB1*04:05* and *DRB1*08:03*, which are both significantly associated with PBC susceptibility in the Japanese population. Serine-57 relevance was not found in a European study,[20] probably because frequencies of the *DRB1*04* and *DRB1*08* alleles therein were found in 10% and 7%, respectively, of patients.[21] The amino acid residue at position 57 influences the binding of antigen side chains associated with the 9th pocket of the expressed DR molecule, which might factor predominantly in susceptibility to PBC in Japanese cases. Interestingly, amino acid residues lysine-9, aspartic acid-11, tyrosine-26, histidine-28, glycine-30, and valine-78 were encoded by *DRB1*09:01* only, suggesting that some or all of these may contribute to disease progression in Japanese patients.

In conclusion, the *DRB1*08:03-DQB1*06:01*, *DRB1*13:02-DQB1*06:04*, and *DRB1*11:01-DQB1*03:01* haplotypes are associated with either PBC susceptibility or protection in the Japanese population. *DRB1*09:01-DQB1*03:03* is a novel haplotype associated with the progression of PBC that has several uniquely expressed amino acids. Other specific amino acid residues in the DRβchain appear to contribute to susceptibility or resistance to PBC. Genome-wide analysis and resequencing of the entire HLA region will be necessary to provide more precise genetic information on susceptibility to PBC in Japan.

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REVIEW ARTICLE

Characteristics of autoimmune hepatitis in the Asia-Pacific Region: historical review

Mikio Zeniya · Hiroki Takahashi

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Abstract
Background Autoimmune hepatitis (AIH) has been considered a relatively rare disease in Asia, including Japan, where there is a high frequency of infection with hepatitis viruses.
Method We reviewd the ethnic differences of clinical features of autoimmume hepatitis by reported paper.
Result Immunogenetic predisposition, especially differences in human leukocyte antigens (HLAs), has been pointed out as one of the factors for the occurrence of AIH. In other words, HLA-DR3, which is the first disease-susceptibility gene for AIH discovered in UK and the USA, is extremely rare in Asia including Japan. Moreover, HLA-DR4, which is more frequent than HLA-DR3 in Asia, is associated with a favorable response to treatment and improved prognosis. This also explains why diagnosis and treatment of AIH have not drawn as much attention as viral hepatitis. However, as a survey conducted in Japan shows, the number of patients diagnosed with AIH is increasing. However, the number of elderly patients positive for HLA-DR4 is also increasing in UK and the USA.
Conclusion In the era of genome-wide association studies, further progress of studies on AIH, a typical disease model for immunological liver cell damage, is expected.

Keywords Autoimmune hepatitis · HLA-DR · Ethnic difference

M. Zeniya (✉) · H. Takahashi
The Jikei University Graduate School of Medicine
Gastroenterology, Tokyo, Japan
e-mail: zeniya@jikei.ac.jp

Introduction

In 1951, Kunkel et al. [1] reported the case of a young woman positive for lupus erythematosus (LE) cells with active chronic hepatitis associated with hyper γ-globulinemia without any apparent known causes such as infection with hepatitis viruses. Because she was positive for LE cells, this disease was designated as lupoid hepatitis at first [2]. Later, Mackay et al. [3] proposed to designate this unknown hepatic disease state as autoimmune hepatitis (AIH) on the basis of immunological characteristics, such as hyper-γ-globulinemia and the marked presence of plasma cells among invasive cells in the portal region; such is the designation that has been used to the present day. Later, it was revealed that antinuclear antibody (ANA) and antismooth muscle antibody, which are autoantibodies, are frequently detected in AIH, and they became important clinical indicators. When the concept of AIH was proposed, the presence of hepatitis B virus and its diagnostic method had been established; however, hepatitis C virus had not been identified yet. Consequently, AIH was classified into the category of non-A, non-B hepatitis. It was a fact that, in case of hyper γ-globulinemia or positivity of autoantibodies such as ANA, AIH was diagnosed by excluding other factors, especially drug-induced liver damage.

In 1989, the establishment of a method to identify hepatitis C-related antibodies and the subsequent discovery of the hepatitis C virus genome [4] facilitated the diagnosis of hepatitis C. As a result, the disease-independence of AIH became apparent, and the pathology of AIH was confirmed as a disease due to autoimmune phenomena [5]. Thus, in many studies on AIH done before the discovery of hepatitis C virus, it was naturally impossible to avoid the inclusion of patients with hepatitis C. Therefore, it is difficult to assess the results obtained before the establishment of methods for the diagnosis of hepatitis C virus infection.

In Asia, the rate of infection with hepatitis virus is high for both Type B and Type C. The pathology of non-viral hepatitis has rarely attracted much clinical attention except for regionally observed hepatic schistosomiasis infection. In 1980, after the designation of AIH was proposed, a sectional meeting on AIH was organized by the Specific Disease Investigation Research Team of the Japanese Ministry of Health and Welfare (at that time), and a nationwide study of case series was started [6]. Thereafter, nationwide surveys have been continuously conducted up to the present date (Table 1) [7–9]. It is historically interesting that the early investigation was conducted to determine whether infection with hepatitis B virus was associated with the pathogenesis of AIH by inducing autoimmune phenomena. This investigation showed that AIH was not associated with hepatitis B virus in Japan. Although the early study of AIH case series was conducted nationwide, it involved an extremely small number of cases. However, the number of reported cases has apparently been increasing since the establishment of diagnostic methods for hepatitis C virus infection. Moreover, in 1998, when the methods for the diagnosis of hepatitis C were established, the International Autoimmune Hepatitis Group (IAIHG) presented the criteria for the diagnosis of AIH [10], resulting in further accuracy of the diagnosis. In this review, we summarize the differences of clinical features of AIH between Asia-Pacific region and UK/USA based on the results of studies of nationwide survey done in Japan as well as studies reported from Asian Pacific countries. We also refer to present issues concerning AIH.

Human leukocyte antigens and ethnicity

In UK and the USA, human leukocytes antigen (HLA)-DR3 is reported as a disease-susceptibility gene for AIH [11]. Interestingly, the proportion of HLA-DR3 carriers is extremely low in Asia, especially in Japan (Table 2) [12]. Thus, AIH due to this disease-susceptibility gene is extremely rare in Japan. In UK and the USA, as suggested by the fact that AIH was also known as juvenile cirrhosis, rapid progression of the disease in young patients was also considered as a characteristic of AIH. Such cases of young patients are rare in Japan [13].

Figure 1 shows the age of AIH onset in UK, the USA, and Japan [14–16]. As evident from this figure, the number of young patients is extremely small in Japan. Yet, Seki et al. [17] reported that AIH occurred even in Japan, despite the extremely small number of HLA-DR3 carriers, and that the disease-susceptibility gene for the disease was HLA-DR4 in this country. Of note is that the peak age of AIH onset among patient positive for HLA-DR4 is in the middle aged, whereas AIH commonly occurs in young people positive for HLA-DR3. In Japan, which has the extremely small number of HLA-DR3 carriers, the age of AIH onset is in the middle or older aged; therefore, there is naturally a single peak of disease onset centered in the middle aged. A subsequent study revealed that AIH occurred even in those negative for HLA-DR3 in UK and the USA, and that the disease-susceptibility gene in those subjects was HLA-DR4 [18]. Thus, there would be two disease-susceptibility genes in UK and the USA. HLA-DR3 and HLA-DR4 act as disease-susceptibility genes for young people and those in the middle or older aged, respectively. The larger number of disease-susceptibility genes eventually leads to differences in the number of cases of AIH, and there are more cases in UK and the USA than in Asian countries, which share common genetic factors with Japan. Figure 2 shows the frequencies of chronic liver disease by factor in several countries [6]. Although the significant difference in the rate of infection

Table 1 Summary of the Japanese nationwide survey on AIH

Year	No. of patients (M:F)	Event
1975–1979	170 + α	Anti-LSP, ADCC
1979–1982	69 + α	
1983–1984	52 + α	
1975–1984 total	314 (1:10)	ASGP-R
1986–1987	167 (1:20)	
1988–1989	200 (1:7)	HLA-B8-DR3 followed DR4
		HCV discovered
1990–1994	496 (1:7.1)	IAIH group criteria
1995–1997	413 (1:6.3)	
1998–2000	330 (1:6.6)	Modified scoring
2009	1,056 (1:6)	Simplified criteria

Table 2 Gene frequencies of serologically typed HLA-DR, DQ loci in Japanese

	%		%
DR1	5.5	DQ1	45.6
DR3	0.2	DQ2	0.6
DR4	22.8	DQ3	18.8
DR8	13.3	DQ4	14.9
DR9	13.0	DQ7	15.2
DR10	0.6	DQBL	4.8
DR12	7.0	DR11	2.6
DR14	5.5	DR13	7.8
DR15	17.4	$n = 898$	
DR16	0.8		
DRJ25	1.9		
DR2LU	0.0		
DRBL	1.0		

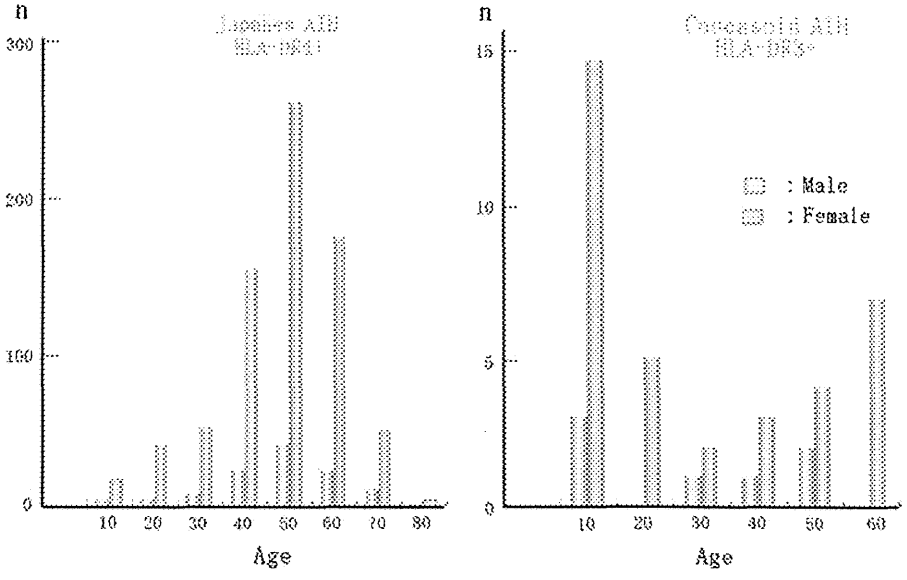

Fig. 1 Prevalence of AIH is greatest among white groups who have a high frequency of HLA-DR3 and HLA-DR. AIH has at least two different genetic predispositions that may affect clinical features. HLA-DR4 positive AIH patients tend to be older and female dominant. However, these features have been changing, recently the number of an older age group of patients with AIH increased in Western countries. Referred and modified from Refs. [15, 16]

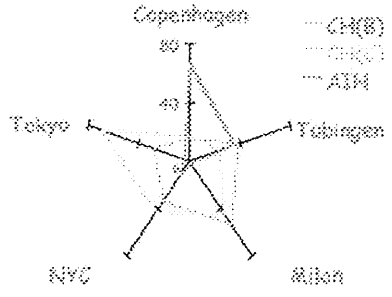

Fig. 2 Differences in frequency of major types of chronic hepatitis (percent distribution by reported five regions), exemplified by reported data (referred and modified from [6]). Hepatitis virus infection rate might relate to the frequency of AIH [6]

Table 3 Incidence of patients with autoimmune liver diseases cases/100,000/year [13]

	Europe			Asia
	England	France	Austria	Japan
AIH typel	0.1–0.2	0.12	1.2	0.08–0.015
AIH type2	Rare	0.03	Rare	Rare
PBC	0.07–0.15	0.67	20	0.3–0.6
PSC	−0.08	Rare	−5	Rare

Incidence of AH is different, but in PBC this different is not observed

with hepatitis virus among the countries is an important factor, different frequencies strongly suggest the presence of more disease-susceptibility genes. In fact, the reported incidence is clearly higher in UK and the USA than in Japan (Table 3) [6]. However, as described later, it should be further noted that the number of people diagnosed with AIH is on the increase in Japan and that middle age or older patients positive for HLA-DR4 are also increasing in UK and the USA. Moreover, the number of male patients also gradually tends to increase in Japan. Nevertheless, although there is a marked difference in the frequency of AIH among UK, the USA, and Asia, such difference is not observed in primary biliary cirrhosis (PBC), which is another distinctive autoimmune liver disease. The existence of disease susceptibility conferred by HLA-DR in the region is of great importance for the progression of the disease due to autoimmune reactions. In this regard, a molecular study confirmed that the so-called anchor peptides to which antigens and probably autoantigens are attached, are common in both HLA-DR3 and DR4 carriers in UK and the USA (Table 4) [19]. Unfortunately, despite these studies, identification of AIH autoantigens has not been successful. Involvement of the epitope spreading observed during disease progression is also suggested as a factor for the occurrence of AIH, and it will become an important issue in the future.

In South America, HLA-DR13 that is different from the previously mentioned disease-susceptibility genes has been reported in pediatric AIH [20]. In addition, the anchor peptides for this HLA clearly differ from those for HLA-DR3 and -DR4. This indicates the existence of additional AIH-susceptibility genes. However, many cases reported in South America occurred after the discovery of hepatitis A. Thus, they may be caused by autoimmune phenomena based on viral infection, and it is appropriate to consider those cases as different from AIH due to unknown causes. In regard to a relationship between viral infection and autoimmune reactions, AIH may be associated with unknown viruses, retroviruses, etc.; thus, further analyses will be necessary in the future. Moreover, analyses of