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Introduction

Hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections among humans. Approximately two billion people worldwide have been exposed to HBV, and 350 million of them remain chronically infected.[1] The incidence of HBV infection and patterns of transmission vary greatly among different population subgroups throughout the world.[2] In Western countries, chronic HBV infection is relatively rare and is acquired primarily in adulthood via sexual transmission or the use of injectable drugs, whereas in Asia and most of Africa, the infection is transmitted from an infected mother to her newborn. In Japan, approximately 1.1–1.4 million people are infected with HBV.

Hepatitis B virus genomic sequences vary worldwide and have been classified into at least nine genotypes (A through J) on the basis of an intergroup divergence of ≥8% over the complete nucleotide sequence.[3,4] HBV genotypes differ in their clinical manifestations[5] and responses to therapy (e.g. interferon therapy). HBV genotypes also have distinct geographic distributions[6–8] and, in particular, genotype A is predominant in Northwestern Europe, the USA, Central Africa, and India.[9,10] The Japanese have been infected with genotypes B and C since prehistoric times.[6] However, many lines of recent evidence have revealed an increase in the prevalence of acute infection with HBV genotype A after sexual transmission among Japanese individuals.[11,12] Moreover, we previously reported that acute infection with HBV genotype A is associated with an increased risk of progression to persistent infection.[13] Therefore, we hypothesized that the distribution of genotype A chronic hepatitis B (CHB) has also increased.

In this study, we conducted a national survey of acute and chronic HBV infections to determine the geographic distribution, clinical, and virologic characteristics of genotype A HBV infection in acute hepatitis B (AHB) and CHB patients in Japan.

Patients and methods

Patients with acute hepatitis B. Between 2005 through 2010, AHB patients were recruited from 48 liver centers throughout Japan. The diagnosis of AHB was based on the rapid onset of clinical symptoms accompanied by elevated serum alanine aminotransferase (ALT) levels in addition to the detection of serum hepatitis B surface antigen (HBsAg) and a high-titer antibody to hepatitis B core antigen of the immunoglobulin M class. Patients with initial high-titer antibody to hepatitis B core antigen of the immunoglobulin G class (>10.0 S/CO) were diagnosed with an exacerbation of CHB and were excluded. Patients with acute hepatitis A, hepatitis C, or drug-induced or alcohol-induced acute hepatitis were also excluded; hepatitis D virus infection was not determined because of its extreme rarity in Japan. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions involved. Every patient provided informed consent for participation in this study.

Patients with chronic hepatitis B. We recruited 3682 patients with CHB treated at 36 liver centers spread across Japan between 2010 and 2011. In all cases, the diagnosis had been established after a follow up of at least 12 months. The patients were classified into one of the following four clinical categories:

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asymptomatic carrier state, defined by the presence of HBsAg with normal ALT levels for 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension; chronic hepatitis, defined by elevated serum ALT levels (>1.5 times the upper limit of the normal range [35 IU/L]) persisting for 6 months; cirrhosis, diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism); platelet counts <100 000/cm^3; or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

Genotyping of hepatitis B virus. The six major HBV genotypes (A through F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). The assay is based on the detection of a combination of epitopes on preS2-region products, which is specific for each genotype, by monoclonal antibodies.[14] Samples for which EIA could not determine the genotype were examined by direct sequencing of the pre-S2/S gene, followed by phylogenetic analysis as previously reported.[15]

Molecular evolutionary analysis of hepatitis B virus. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. The HBV genotype A isolates used to investigate the relationship between HBV isolates from Japanese AHB and CHB patients were the same as those used in our previous study.[11] Nucleotide sequences of HBV-DNA were aligned using the program CLUSTAL X, and genetic distance was estimated by the six-parameter method[16] in the Hepatitis Virus Database.[17] On the basis of the obtained values, phylogenetic trees were constructed using the neighbor-joining method[18] with the midpoint-rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis. Data are expressed as mean ± SD. Statistical analyses were performed using chi-squared tests and Fisher's exact tests for categorical variables. Mann–Whitney U-tests or Kruskal–Wallis one-way analyses were used for continuous variables, as appropriate. Differences were considered significant for P values <0.05. STATA Software (StataCorp LP, College Station, TX, USA) version 11.0 was used for statistical analyses.

Results

Demographic and clinical characteristics of patients with acute hepatitis B who were infected with hepatitis B virus of different genotypes. The genotype of 18 (3.2%) of the 570 patients could not be determined by EIA or phylogenetic analysis owing to low HBsAg or HBV-DNA levels, and therefore, 552 AHB patients were included in further analyses. Among the 552 AHB patients, 258 (46.7%), 65 (11.8%), and 219 (39.7%) had HBV with genotypes A, B, and C, respectively. Ten patients (1.8%) had HBV of other genotypes, including genotypes D (n = 1), E (n = 2), F (n = 2), H (n = 2), and G (n = 3). The clinical backgrounds of the 542 AHB patients infected with genotypes A, B, and C (10 patients with other genotypes were excluded, Table 1) were shown. The clinical characteristics of genotype A AHB were compared with those of non-A genotypes (genotypes B and C) statistically. AHB patients with genotype A HBV were significantly younger than those with non-A genotypes. The peak level of ALT was significantly lower for genotype A than for non-A genotypes. The duration until HBsAg was absent for genotype A was significantly longer than for non-A genotypes. In almost every case in all genotypes, sexual transmission was the primary transmission route.

Demographic and clinical characteristics of patients with chronic hepatitis B who were infected with hepatitis B virus of different genotypes. The genotype could not be determined for 63 (1.7%) of the 3682 CHB patients by EIA or phylogenetic analysis owing to low HBsAg or HBV-DNA levels. Among the 3619 CHB patients included between 2010 and 2011, 149 (4.1%), 634 (17.5%), 2810 (77.6%), 23 (0.6%), and 3 (0.08%) had the respective genotypes A, B, C, D, and F. We compared the clinical backgrounds of CHB patients infected with genotypes A, B, C, and D (Table 2). Patients with genotypes A and D were significantly younger than those with genotypes B and C. The percentage of men among patients with genotype A was significantly higher than that among patients with other genotypes. The proportion of patients with advanced-stage liver disease (cirrhosis or HCC) that was treated with nucleotide analogues or interferon was significantly higher with genotype C than with other genotypes. The platelet count was significantly lower in patients with genotype C than in those with other genotypes. The percentage of patients co-infected with human immunodeficiency virus (HIV) was significantly higher for genotype A than for the other genotypes.

Distribution of hepatitis B virus genotypes in chronic hepatitis B patients in different 10-year-age groups. The proportions of HBV genotypes in CHB patients in each generation (5-year-age groups) are shown (Fig. 1). Genotype C appeared to be prevalent in each generation group. Among younger patients (<29 years old), the percentage of genotype A was relatively high. In older patients, especially those >75 years old, the percentage of genotype B was quite high.

Distribution of asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma state among patients chronically infected with hepatitis B virus of different genotypes stratified by age. We compared the distributions of age and liver diseases in patients infected with HBV of genotypes A, B, and C (Fig. 2). For genotype A, the number of patients with liver disease was the highest in those aged 40–49 years and the lowest in those aged ≥50 years. In contrast, for genotype B, the number of patients with liver diseases was quite high in those aged ≥60 years. In genotype C, the number of patients was high for those aged ≥40 years. It appears that liver diseases have not progressed in most patients with genotype A; however, some

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Table 1 Demographic and clinical characteristics of patients with acute hepatitis who were infected with hepatitis B virus of different genotypes (*n* = 542)

	HBV genotype			
	A (*n* = 258)	B (*n* = 65)	C (*n* = 219)	*P* value
Age (years)	35.4 ± 12.5	39.2 ± 13.7	39.4 ± 13.8	0.0008
Male (%)	244 (93.8)	45 (70.8)	139 (63.5)	<0.0001
HBeAg	218/236	50/60	162/216	<0.0001
Positive rate (%)	(92.4)	(83.3)	(75.0)	
HBV-DNA (log copies/mL)	6.7 ± 1.7	5.9 ± 1.6	5.5 ± 1.4	<0.00001
ALT (IU/L)	2137 ± 1088	3078 ± 2111	2624 ± 1843	0.0052
T-bil (mg/dL)	9.2 ± 8.1	7.1 ± 6.2	7.7 ± 6.2	0.1213
PT (%)	77.4 ± 18.6	68.1 ± 27.1	69.3 ± 28.4	0.041
Duration until disappearance of HBsAg (months)	5.4 ± 7.1	2.7 ± 2.2	3.8 ± 6.2	<0.00001
Sexual transmission	187/197 (94.9)	48/50 (96.0)	140/159 (88.1)	0.059
Treatment NAs (%)	110 (42.6)	20 (30.8)	68 (31.1)	0.008
IFN (%)	7 (2.7)	0 (0)	3 (1.4)	0.108
Co-infection with HIV	24/178	1/40	3/124	<0.0001
HIV positive rate (%)	13.5	2.5	2.4	

Data are presented as *n* (%) or mean ± standard deviation.
P value: genotype A versus non-A genotypes (genotype B + C).
Maximum value during the clinical course with acute hepatitis B.
Minimum value during the clinical course with acute hepatitis B.
Transmission routes of 61 patients were unknown.
Transmission routes of 15 patients were unknown.
Transmission routes of 60 patients were unknown.
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IFN, interferon; NAs, nucleotide analogues; PT, prothrombin time; T-bil, total bilirubin.

Table 2 Demographic and clinical characteristics of patients with chronic hepatitis who were infected with hepatitis B virus of different genotypes (*n* = 3616)

	HBV genotypes				*P* value
	A (*n* = 149)	B [illegible])	C (*n* = 2810)	D (*n* = 23)	
Age (years)	42.5 ± 13.2	55.7 ± 14.4	50.0 ± 13.7	39.3 ± 10.4	<0.0001
Male (%)	105 (70.5)	372 (58.8)	1623 (57.9)	11 (47.8)	0.014
Diagnosis					
Asymptomatic carrier (%)	69 (46.6)	337 (53.2)	741 (26.4)	14 (60.9)	<0.0001
Chronic hepatitis (%)	72 (48.6)	245 (38.7)	1537 (54.7)	8 (34.8)	
Cirrhosis (%)	2 (1.4)	19 (3.0)	257 (9.1)	1 (4.3)	
HCC (%)	5 (3.4)	32 (5.1)	274 (9.8)	0 (0.0)	
Blood tests					
Platelet ($10^4/mm^3$)	19.6 ± 5.3	19.3 ± 6.3	18.1 ± 6.4	19.0 ± 5.4	0.0001
ALT (IU/L)	32.6 ± 33.4	33.0 ± 46.6	36.2 ± 44.8	45.6 ± 64.4	0.223
ALP (IU/L)	239.2 ± 131.1	243.4 ± 95.3	251.7 ± 139.2	254.1 ± 104.8	0.450
GGT (IU/L)	36.2 ± 49.3	39.3 ± 52.1	37.4 ± 55.0	24.1 ± 20.0	0.537
AFP, median (min–max)	3.3 (0.9–15 781.0)	2.9 (0.8–76 860.0)	3.0 (0.6–514 570.0)	2.5 (0.9–267.9)	0.122
HBV markers					
HBeAg (positive rate, %)	22/137 (16.1)	51/553 (9.2)	683/2634 (25.9)	3/22 (13.6)	<0.0001
HBV-DNA (log copies/mL)	4.0 ± 1.9	4.0 ± 1.7	4.3 ± 18.1	4.5 ± 2.4	0.973
Treatment					
NAs (%)	31 (20.8)	160 (25.2)	1258 (44.8)	4 (17.4)	<0.0001
IFN (%)	2 (1.3)	2 (0.3)	42 (1.5)	1 (4.3)	0.024
Co-infection with HIV (HIV positive rate, %)	11/32 (34.4)	1/115 (0.9)	3/408 (0.7)	0/2 (0.0)	<0.0001

Data are presented as *n* (%) or mean ± standard deviation.
AFP, alpha fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; IFN, interferon; NAs, nucleotide analogues.

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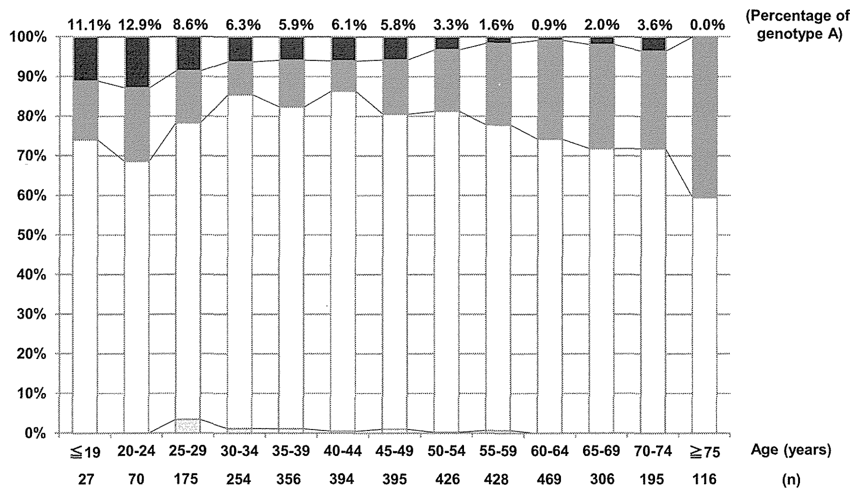

Figure 1 Distribution of hepatitis B virus genotypes in chronic hepatitis B patients in each generation (5-year-age groups) is shown. Genotype A; Genotype B; Genotype C; Genotype D.

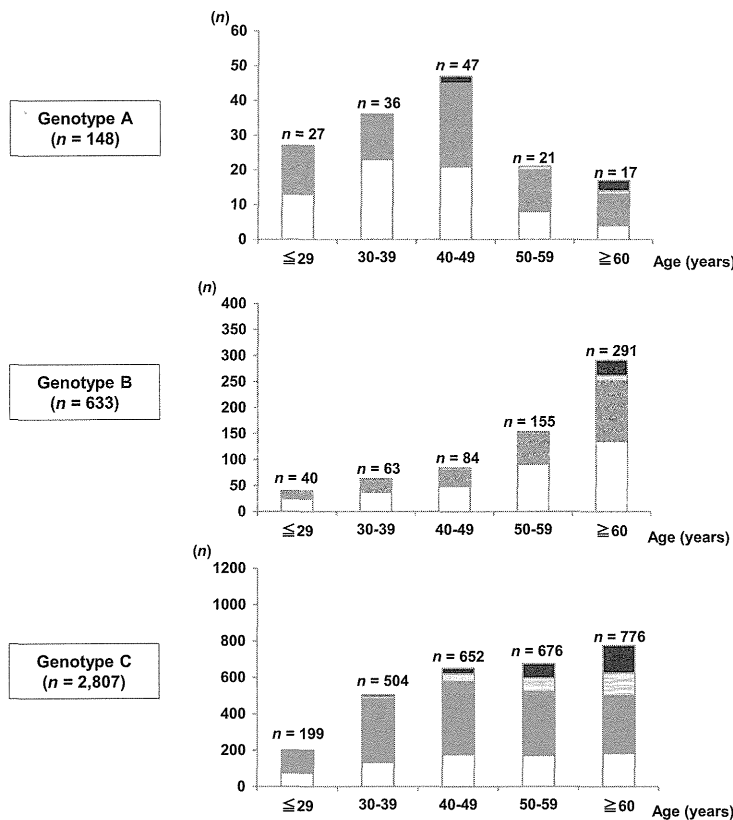

Figure 2 Distribution of hepatocellular carcinoma, cirrhosis, chronic hepatitis, and asymptomatic carrier status among the 3584 patients chronically infected with hepatitis B virus of different genotypes are stratified by age. Hepatocellular Carcinoma; Cirrhosis; Chronic Hepatittis; Asymtomatic Carrier.

patients had HCC at a relatively young age (40–49 years old). In patients infected with genotype B, the prevalence of cirrhosis and HCC was high for patients aged ≥60 years. On the other hand, liver diseases progressed at a relatively young age in patients with genotype C.

Geographic distribution of hepatitis B virus genotypes in patients with acute and chronic hepatitis B virus infection in Japan. The distribution of HBV genotypes in AHB and CHB patients in Japan differed by geographic location (Fig. 3). The number of AHB patients and percentage of

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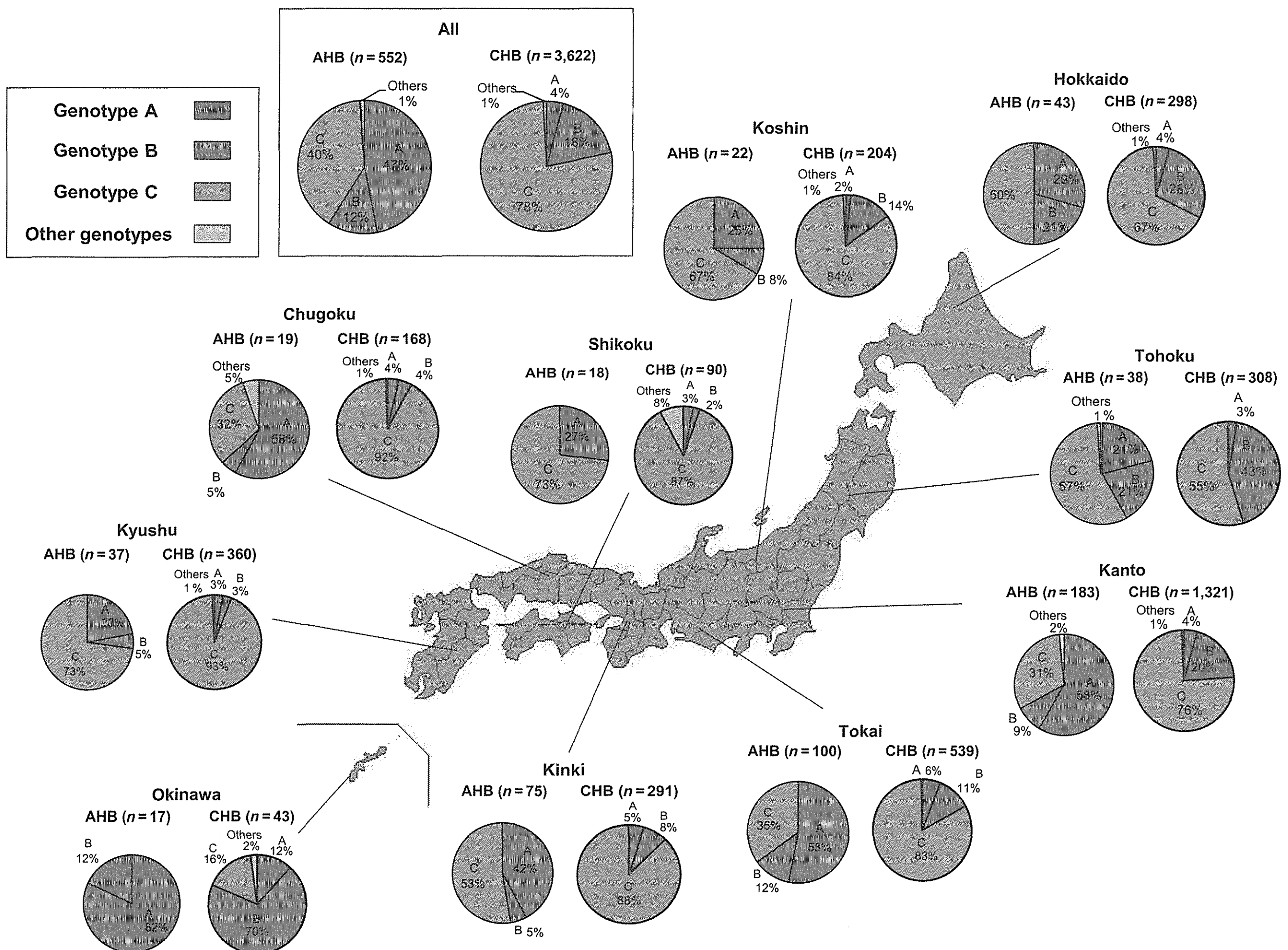

Figure 3 Geographic distribution of hepatitis B virus genotypes in patients with acute and chronic hepatitis B virus infection in Japan. AHB, acute hepatitis B; CHB, chronic hepatitis B.

genotype A were high in Kanto, Tokai, and Kinki on the Pacific side of the archipelago. Although the number of AHB patients was not as high in Chugoku and Okinawa, the percentages of genotype A were also quite high. Notably, the percentages of HBV genotype B were high in Tohoku and Okinawa as well in CHB patients as in AHB patients. In CHB patients in 2010–2011, genotype C was predominant (77.6%) in Japan. The percentage of genotype A was relatively high in Hokkaido, Kanto, Tokai, Kinki, and Okinawa areas compared with those in other areas.

Phylogenetic analyses. In 65 AHB patients and 18 CHB patients, the preS1/S gene was sequenced successfully (Fig. 4). All strains in the 65 AHB patients with genotype A in this study were classified into subtype Ae. Among the CHB patients, 4 (22.2%) and 14 (77.8%) had subtypes A1/(a) and A2/(e), respectively. These four-subtype A1 strains were present only in patients residing in Kanto and Chugoku. From the phylogenetic tree analysis, subtype A1/(a) diverged earlier than A2/(e). Almost all of the isolates in the five identical groups of subtype A2/(e) were derived from Japanese AHB patients. In each identical group, many isolates were derived from the same area (e.g. Nine strains were from Tokai, six strains from Kanto, and five strains from Okinawa in identical group 1. Four strains were from Kanto in identical group 2. Eight strains were from Kanto in identical group 3). In contrast, strains derived from CHB were not as homologous.

Discussion

This multicenter nationwide study revealed that the percentage of patients with genotype A HBV in Japanese CHB patients was 4.1% in 2010–2011. This percentage was significantly higher than that recorded in our previous nationwide study in 2000–2001 (1.7%, $P < 0.001$),[6] but it was not significantly different from that noted in our other previous study in 2005–2006 (3.5%, $P = 0.313$).[11] The finding that genotype A is spreading in young adults with CHB may support previous results regarding the recent infection pattern among adults (Fig. 1). We previously reported that the percentage of genotype A HBV among Japanese AHB patients has been increasing annually since the mid-1990s (Supporting Information Fig. S1).[19] In addition, we previously reported that the rate of progression to chronic infection after AHB caused by HBV genotype A was significantly higher than that in infections

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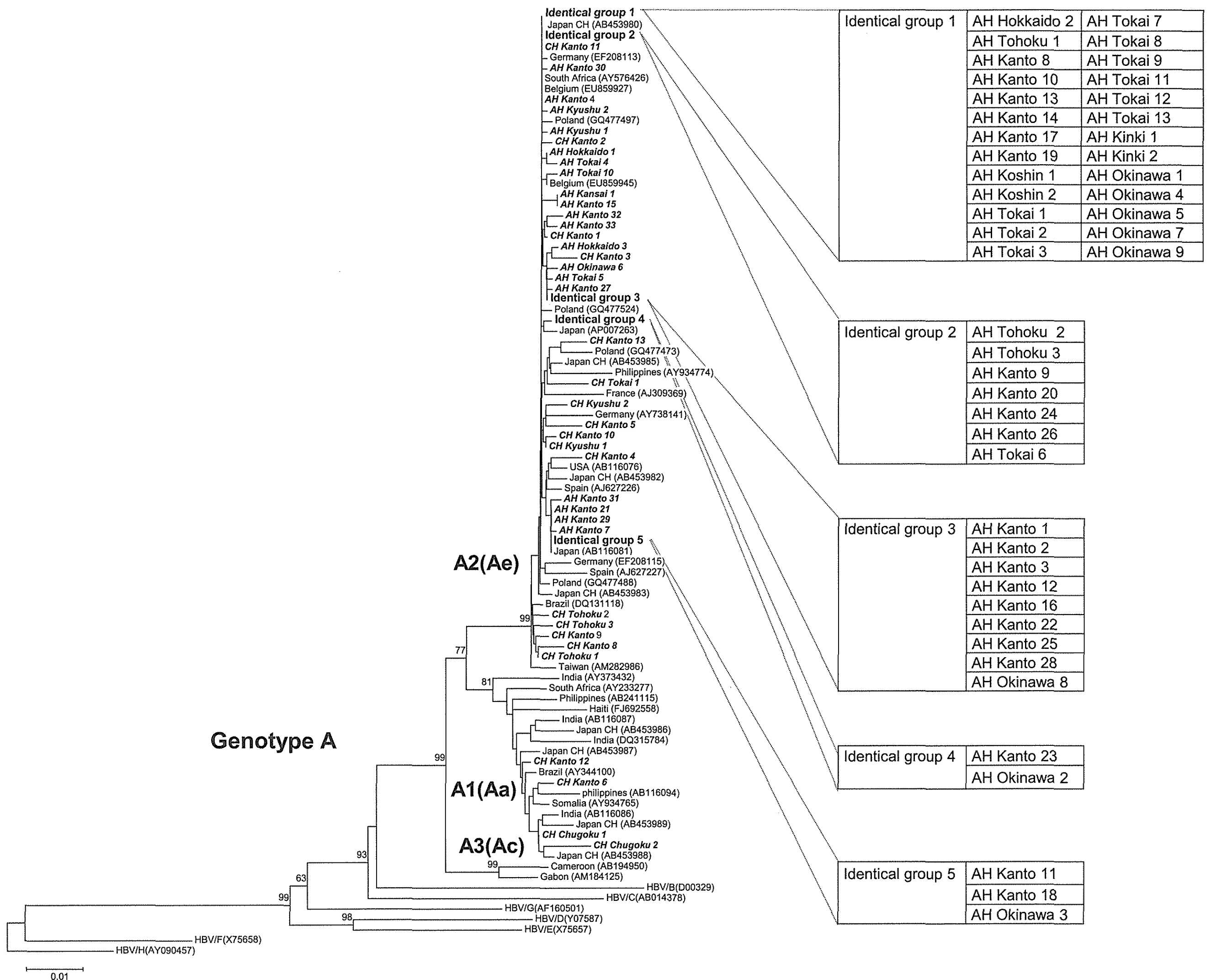

Figure 4 Phylogenetic tree was constructed based on pre-S1/S region sequences of hepatitis B virus (HBV) genotype A strains by the neighbor-joining method. Genotype A strains from 65 patients with acute infection, and 18 patients with chronic infection in this study are shown in boldface italic, and the names after AH or CH represent the areas where the patients were infected. Isolates with identical sequences are presented in brackets in "Identical groups 1 through 5" on the tree. Reference HBV isolates were obtained from our previous study and specified by their accession numbers, isolate names, and countries of origin. Bootstrap values are indicated on major phylogenetic branches. The bar at the bottom spans 0.01 nucleotide substitutions per site.

with other genotypes (7.5% vs 0.9%, respectively).[13] From these observations, we hypothesized that the percentage of genotype A in CHB patients is increasing in Japan. However, the increase of the percentage of genotype A in CHB patients was not clear from the results of this study. This trend requires verification in future nationwide surveys.

Genotype A can be classified into five subtypes (A1 [Aa], A2 [Ae], A3 [Ac], A4, and A5), although A4 and A5, which were derived from patients in Africa, were only tentatively designated as new subtypes.[20] These subtypes have distinct geographical distributions. Subtype A1 (Aa) prevails in sub-Saharan Africa and South Asia; A2 (Ae) is widely distributed in Europe and the USA, and A3 (Ac) is distributed in central Africa.[21–23] All of the 65 strains isolated from the AHB patients with genotype A in this study were classified into subtype A2 (Ae). Based on sequencing data from the USA, subtype A2(e) strains of AHB from men who have sex with men (MSM) tend to cluster phylogenetically, which indicates selective expansion of specific strains, and are significantly limited in genetic diversity.[24] Other reports from western countries have also shown sharp increases in acute infections with subtype A2(e) strains linked to high-risk practices, such as MSM activity and injection drug use.[25,26] These strains are thought to have been transmitted by foreign-born MSM and spread among subsets of the Japanese population who share specific sexual behaviors.[27–29] However, considering the results of our phylogenetic analysis, sequences derived from Japanese AHB patients were identical to or closely resembled the sequences derived from other Japanese AHB patients, rather than those obtained from

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foreigners. Therefore, subtype A2(e) may initially be spread from foreign-born MSM to the Japanese; then, it is spread from Japanese AHB patients to the Japanese general population.

Genotype A HBV is a common cause of AHB in Japan, much more common than would be expected from the relative prevalence of this genotype in patients with CHB in Japan. This may be related to the greater transmissibility of this genotype by sexual routes or to the preferential occurrence of this genotype among MSM. Because genotype A induces milder hepatitis, which is characterized by a lower peak ALT level and associated with weaker cellular immune responses, genotype A may spread unknowingly by sexual routes. The weaker immune response could be explained by slower viral replication and different expression patterns of hepatitis B e-antigen (HBeAg) by genotype A HBV, as we have previously reported.[13,30,31] Higher rates of HIV co-infection also contribute to mild acute hepatitis caused by genotype A HBV. It has previously been reported that HIV-infected adults who develop acute HBV infection are less likely to eliminate the virus because of reduced $CD4^+$ T cell counts, compared with HIV uninfected adults.[32] In this study, in both AHB and CHB patients, the percentage of patients co-infected with HIV was significantly higher with genotype A than with other genotypes, potentially because of the similarity of transmission routes, such as sexual contact among homosexual men, between genotype A HBV and HIV.

Our study has several limitations. First, the AHB and CHB patient groups were recruited over different time periods. Ideally, AHB and CHB specimens should have been recruited over the same timeframe and from the same hospitals. However, the number of new cases of AHB was smaller than that of CHB. Therefore, patients with AHB had to be included from a larger number of hospitals and for a longer time period. Second, HBV genotypes were first determined using a serological method, and DNA sequencing was used when this method failed; the proportion of use of these two techniques was different between patients with AHB and those with CHB. This happened because HBsAg is typically persistently positive, and its concentration is higher in patients with CHB, whereas in patients with AHB, HBsAg level is more often low or negative. However, because the genotypes that were determined using the serological method and those that were determined using DNA sequencing have been shown to have concordance rates exceeding 95%,[14] this difference in genotyping methods should not have influenced our results much.

In this study, the percentage distribution of advanced liver diseases (cirrhosis and HCC) in patients with genotype C was significantly higher than that in patients with other genotypes. Many lines of evidence have revealed that genotype C is associated with poor prognosis in CHB infection; one reason for this involves the high frequency of mutations at positions 1653, 1753, 1762, and 1764 in the enhancer II region.[33–35] In CHB patients, the percentage of HBeAg positivity was significantly higher for genotype C than for the other genotypes. Children infected with genotype C have been reported to achieve HBeAg seroconversion later than those with genotype B.[36] On the other hand, in CHB patients with genotype A, early stage liver diseases (asymptomatic carrier and CH) were predominant, although liver diseases progressed to cirrhosis or HCC in some patients. These differences in the clinical characteristics of each genotype among CHB patients are thought to be influenced by the difference in the time periods in which they spread (e.g. the spread of genotype A occurred more recently, compared with other genotypes in Japan) and frequency of specific mutations in each genotype.

In summary, genotype A is the predominant genotype in Japanese AHB patients. Genotype A AHB tends to result in milder hepatitis and a longer HBsAg persistence compared with other genotypes. The frequency of HIV co-infection with genotype A was higher than that with other genotypes of AHB as well as CHB. Genotype A CHB is spreading in young adults in Japan. Although early stage liver diseases are predominant in patients with genotype A CHB, liver disease progresses to cirrhosis or HCC in some patients. The distribution of HBV genotypes is considerably different between AHB and CHB in Japanese patients. Subtype A2/(e) strains with an identical or very similar sequence have spread through sexual transmission from cities on the Pacific side of the archipelago.

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Author Contributions

K. I., H. Y., H. Y., Y. K., T. S., Y. A., F. I., M. K., T. U., T. I., H. I., M. Y., Y. T., E. M., K. Y., K. M., T. M., J. T., M. K., K. M., N. M., and K. K. recruited patients and collected samples. K. I. drafted the manuscript. K. I., H. Y., M. S., and M. M. analyzed and interpreted the data. M. M. initiated, planned, and coordinated the study. All authors read and contributed to the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Annual percentage of genotype A in acute hepatitis B (1982–2010) is shown. Black bars show the percentage of genotype A, while gray bar shows non-A genotypes.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The impact of an inosine triphosphate pyrophosphatase genotype on bilirubin increase in chronic hepatitis C patients treated with simeprevir, pegylated interferon plus ribavirin

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Abstract

Background Hyperbilirubinemia, mild or moderate, is a commonly observed laboratory abnormality in chronic hepatitis C patients treated with simeprevir with pegylated interferon (Peg-IFN) plus ribavirin. In this prospective, multicenter study, we aimed to investigate the clinical features and factors associated with bilirubin increases during the therapy.

Methods A total of 192 patients with chronic hepatitis C who were treated with simeprevir with Peg-IFN plus ribavirin were analyzed.

Results The mean serum bilirubin level increased significantly during the initial 12 weeks of simeprevir administration and peaked at 2 weeks after the administration. Hyperbilirubinemia of more than 2 mg/dl developed in 18 % of the patients; in 85 % of those patients, the bilirubin levels peaked within 6 weeks and gradually decreased thereafter. A univariable analysis revealed that an increase in serum total bilirubin of 1.0 mg/dl or more from baseline was significantly associated with the sex, red blood cell count, serum hemoglobin level, serum alanine aminotransferase level, serum creatinine level and inosine triphosphate pyrophosphatase (ITPA) genotype. In the multivariable analysis, the ITPA genotype (CC odds ratio 4.990, $p = 0.011$) was found to be the only independent factor. Consistent with this result, there was a significant correlation between hyperbilirubinemia and the degree of hemolytic anemia.

Conclusions Hyperbilirubinemia develops at early time points after simeprevir administration in most cases and is dependent on the ITPA genotype. Careful attention should be paid to hyperbilirubinemia, which occurs at later time points or in patients with an ITPA non-CC genotype so that a diagnosis of liver damage with hyperbilirubinemia is not missed.

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Keywords Chronic hepatitis C · Simeprevir · Pegylated interferon plus ribavirin · Bilirubin increase · Inosine triphosphate pyrophosphatase

Abbreviations

SMV	Simeprevir
HCV	Hepatitis C virus
Peg-IFN	Pegylated interferon
RBV	Ribavirin
CH-C	Chronic hepatitis C
Plt	Platelet
WBC	White blood cell
Hb	Hemoglobin
IL-28B	Interleukin-28B
ITPA	Inosine triphosphate pyrophosphatase
ALT	Alanine aminotransferase
BMI	Body mass index
RBC	Red blood cell
OR	Odds ratio
SVR	Sustained virologic response
SOF	Sofosbuvir

Introduction

Simeprevir [SMV: once-daily second wave hepatitis C virus (HCV) NS3/4A protease inhibitor] was approved for patients with chronic HCV infections in Japan in September 2013 and by the US Food and Drug Administration in November 2013. Japanese guidelines recommend combination therapy with SMV and pegylated interferon (Peg-IFN) plus ribavirin (RBV) as a first line therapy for patients with chronic HCV genotype 1 infection [1, 2].

In patients with chronic hepatitis C (CH-C) who were treated with SMV and Peg-IFN plus RBV, mild or moderate hyperbilirubinemia was often observed as an adverse effect [3–8]. The bilirubin increase observed with this therapy is generally explained by the inhibitory effect of SMV on hepatocellular bilirubin transporters, including OATP1B1 and MRP2 [9]. Thus, a mild or moderate bilirubin increase during the treatment is not usually considered to be due to deterioration caused by liver injury; therefore, immediate drug discontinuation is not generally recommended for bilirubin increases. However, the characteristics of the bilirubin increase during treatment with SMV and Peg-IFN plus RBV have not been clarified.

In the present study, we investigated the changes in bilirubin increase and factors associated with bilirubin increase in patients with CH-C who were treated with SMV and Peg-IFN plus RBV.

Patients and methods

Patients

We performed the present study using the data from a prospective, multicenter study of SMV with Peg-IFN plus RBV combination therapy for patients with chronic HCV genotype 1 infection; that study was conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 192 consecutive patients with CH-C who began SMV with Peg-IFN plus RBV combination therapy between December 2013 and February 2014 were included in this study. All patients were Japanese.

Patients aged 20 or above with chronic HCV genotype 1 infection and a viral load of $\geq 10^5$ IU/ml at screening were eligible. The exclusion criteria were as follows: hepatitis B or human immunodeficiency virus coinfection, decompensated cirrhosis, liver failure, hepatocellular carcinoma, other forms of liver disease (e.g., alcoholic liver disease), a history of splenectomy or partial spleen embolization, chronic renal failure, depression or immunodeficiency. Informed consent was obtained from all patients before their participation in the study, which complied with the ethical guidelines of the 1975 Declaration of Helsinki (as amended in 2002) and was approved by the ethics committee of Osaka University Hospital. This study was also approved by independent or institutional review boards of all of the participating study centers (UMIN000012183).

Treatment protocol

Patients received 100 mg/day of SMV (Sovriad; Janssen Pharmaceutical K.K., Tokyo, Japan), Peg-IFN alfa-2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and RBV (Copegus; Chugai Pharmaceutical Co., Ltd.) or Peg-IFN alfa-2b (Pegintron; MSD, Tokyo, Japan) and RBV (Rebetol; MSD) for 12 weeks, followed by Peg-IFN alfa-2a or Peg-IFN alfa-2b and RBV for 12 additional weeks. SMV was administered orally once a day at a dose of 100 mg/body. In principle, Peg-IFN alfa-2a was administered subcutaneously once a week at a dose of 180 μg/body, and Peg-IFN alfa-2b was administered subcutaneously once a week at a dose of 60–150 μg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg). RBV was administered orally twice a day at an initial dose of 600–1000 mg based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg). In seven patients, the initial dose of RBV was reduced because of the assessment by the patients' doctor. We treated patients according to a standard treatment protocol for Japanese patients.

Dose modification

All dose modifications followed the manufacturers' recommendations. The dose of Peg-IFN alfa-2a was reduced to 50 % of the assigned dose if the platelet (Plt) count decreased to $<5 \times 10^4/mm^3$ or if the neutrophil count decreased to $<750/mm^3$. The dose of Peg-IFN alfa-2b was reduced to 50 % of the assigned dose if the Plt count decreased to $<8 \times 10^4/mm^3$, if the white blood cell (WBC) count decreased to $<1500/mm^3$ or if the neutrophil count decreased to $<750/mm^3$. The RBV dose was reduced from 1000 to 600 mg, from 800 to 600 mg or from 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl. Peg-IFN alfa-2a and RBV were discontinued if the Plt count decreased to $<2.5 \times 10^4/mm^3$, if the neutrophil count decreased to $<500/mm^3$ or if the Hb level decreased to <8.5 g/dl. Peg-IFN alfa-2b and RBV were discontinued if the Plt count decreased to $<5 \times 10^4/mm^3$, if the WBC count decreased to $<1000/mm^3$, if the neutrophil count decreased to $<500/mm^3$ or if the Hb level decreased to <8.5 g/dl.

Histological evaluation

Liver biopsies were performed initiating the combination therapy. The histopathological interpretation of the specimens was performed by experienced liver pathologists without biochemical, clinical or viral information for the patients in this study. The histology, activity and fibrosis were evaluated according to the METAVIR histological scoring system [10].

Safety assessment

Laboratory assessments and safety assessments were performed every week from the start of treatment to 8 weeks of treatment and then every 4 weeks from 8 to 24 weeks of treatment. Data on the adverse effects were collected, and physical examinations were performed at each visit, if clinically indicated.

Methods

Human genomic DNA was extracted from peripheral blood samples obtained from each patient. Genetic polymorphisms located near the interleukin (IL)-28B gene (rs8099917) and inosine triphosphate pyrophosphatase (ITPA) gene (rs1127354) were determined using real-time PCR. Each extracted DNA sample was subjected to PCR with primers and probes from a commercially available kit (Taqman single nucleotide polymorphism Genotyping Assays, Applied Biosystems). The genotype in the IL-28B gene (rs8099917) and genotype in the ITPA gene (rs1127354) were amplified, and the results were analyzed using real-time PCR in a thermal cycler (7900 Real-time PCR System, Applied Biosystems). Regarding the IL-28B gene (rs8099917), homozygosity for the major sequence (TT) was defined as the presence of the IL28B major genotype, whereas homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as the presence of the IL28B minor genotype. With regard to the ITPA gene (rs1127354), homozygosity for the major sequence (CC) was defined as the presence of the ITPA major genotype, whereas homozygosity (AA) or heterozygosity (CA) of the minor sequence was defined as the presence of ITPA minor genotype.

We investigated the serum bilirubin levels, changes in serum total bilirubin levels from baseline until 24 weeks after the start of treatment (Δ total bilirubin) and changes in serum Hb levels from baseline until 24 weeks after the start of treatment (Δ Hb). A significant increase in bilirubin was defined as Δ total bilirubin maximum $\geq$1.0 mg/dl.

Statistical analysis

The baseline continuous variables were expressed as the mean $\pm$ standard deviation or median and categorical variables as frequencies. Student's t-test was used for differences in the mean levels of Δ bilirubin and Δ Hb according to the ITPA genotype during the treatment period. A paired t-test was used for differences in serum bilirubin levels after the start of treatment. Correlation between the highest values of Δ Hb and Δ total bilirubin was assessed using Pearson's method. Multivariable logistic regression analyses were performed with factors associated with grade 2 hyperbilirubinemia (total bilirubin maximum >2.0 mg/dl) and factors associated with a significant increase in bilirubin (Δ total bilirubin $\geq$1.0 mg/dl). Differences were considered significant for a two-tailed *p* value <0.05. For statistical analysis we used SPSS version 19.0 J (IBM, Armonk, NY, USA).

Results

Baseline characteristics of patients with chronic HCV infection

The mean age of the patients was 61.9 years old; 92 were male and 100 were female (Table 1). Fifteen patients had compensated liver cirrhosis. The mean levels of Hb, Plt, total bilirubin and alanine aminotransferase (ALT) were 14.0 g/dl, $15.3 \times 10^4/\mu l$, 0.8 mg/dl and 63 IU/l, respectively. The patients had major genotype TT for the IL28B genotype (68.3 %) and CC for the ITPA genotype (74.7 %).

Table 1 Baseline characteristics of patients

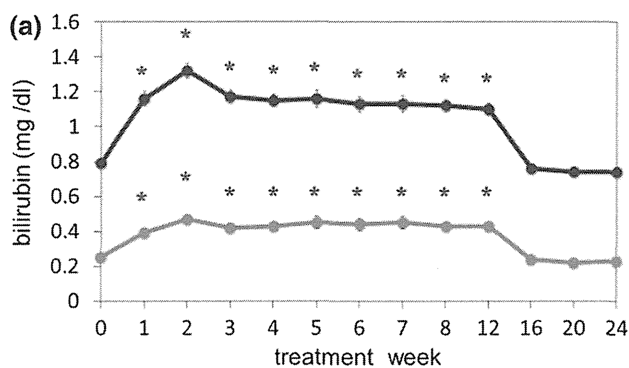
Factor	$N = 192$
Age (years old)	61.9 ± 9.8
Sex: male/female	92/100
BMI (kg/m^2)	23.3 ± 3.0
Past history of IFN: naïve/relapse/non-response	73/41/40
HCV-RNA (median, $\log_{10}$ IU/ml)	6.7
Liver histology: activity: A0/1/2/3:	1/101/62/4
fibrosis: F0/1/2/3/4	9/65/56/23/15
White blood cell (/μl)	4874 ± 1332
Neutrophils (/μl)	2524 ± 1015
Red blood cell ($\times 10^4$/μl)	440 ± 59
Hemoglobin (g/dl)	14.0 ± 1.5
Platelets ($\times 10^4$/μl)	15.3 ± 4.8
Total bilirubin (mg/dl)	0.8 ± 0.3
Direct bilirubin (mg/dl)	0.3 ± 0.2
ALT (IU/l)	63 ± 48
Creatinine (mg/dl)	0.7 ± 0.2
IL28B genotype (rs8099917): TT/TG/GG	110/49/2
ITPA genotype (rs1127354): CC/CA/AA	112/37/1
RBV dose: <10/10–12/≥12 (mg/kg)	7/123/61

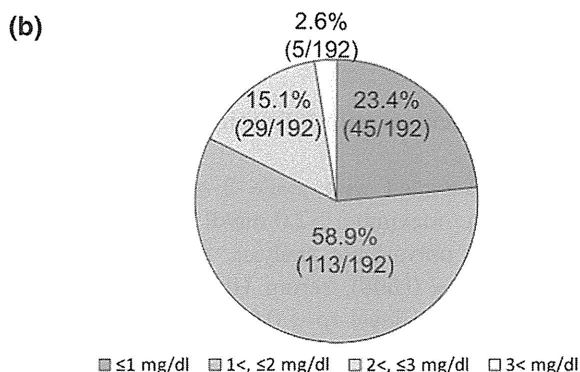
BMI body mass index, *IFN* interferon, *HCV* hepatitis C virus, *ALT* alanine aminotransferase, *IL28B* interleukin-28B, *ITPA* inosine triphosphate pyrophosphatase, *RBV* ribavirin

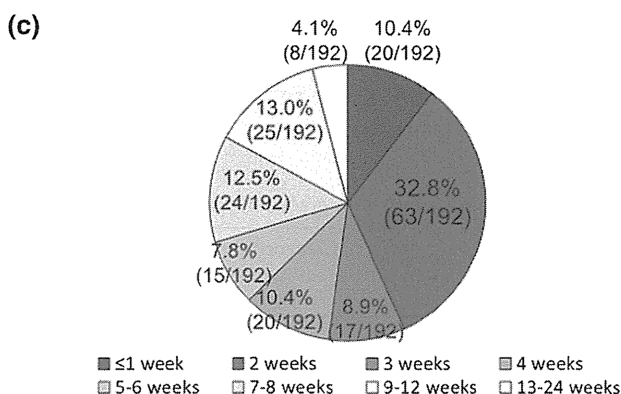
Increases in serum bilirubin levels during treatment

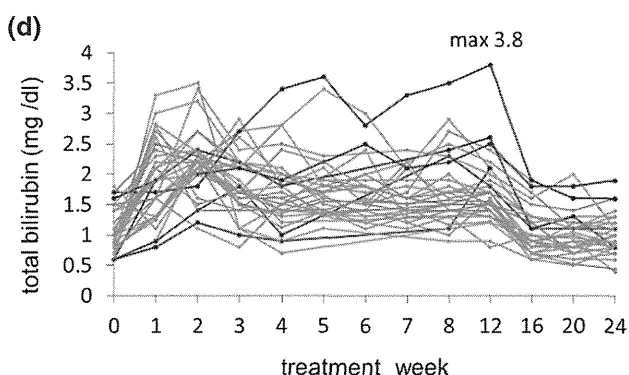
When the changes in serum bilirubin levels from the start of treatment to 24 weeks were investigated, the serum bilirubin levels peaked at 2 weeks and decreased to the baseline level after SMV administration was completed (Fig. 1a). When the distribution of the levels of the total bilirubin maximum during the treatment period were investigated, the proportions of patients with levels of total bilirubin maximum of 1 mg/dl or less, from 1 to 2 mg/dl, more than 2–3 mg/dl and more than 3 mg/dl were 23.4 % (45/192), 58.9 % (113/192), 15.1 % (29/192) and 2.6 % (5/192), respectively (Fig. 1b). According to the distribution of the timing with the total bilirubin maximum during the treatment period, the most common category was 2 weeks (32.8 %, 63/192) (Fig. 1c). There were few patients whose serum total bilirubin levels peaked after SMV administration had been completed (4.1 %, 8/192).

Hyperbilirubinemia of more than 2 mg/dl was identified as grade 2 (more than 1.5 × upper limit of normal) by the World Health Organization Toxicity Grading Scale. In the present study, there were 34 patients whose levels of total bilirubin maximum were more than 2 mg/dl (Fig. 1d). Half of these patients (17/34, 50 %) exhibited a total bilirubin increase that peaked at 2 weeks, and the total bilirubin levels peaked during the early period of SMV administration









(within 6 weeks) in most patients (85.3 %, 29/34). Hyperbilirubinemia that peaked during the later period of SMV administration (after 6 weeks) was observed in five patients

◀**Fig. 1** Increases in serum bilirubin levels during treatment. **a** Changes in serum total bilirubin and direct bilirubin levels. *Black line*, total bilirubin, *Gray line*, direct bilirubin. All data indicate the mean values and standard errors. *Asterisk*, $p < 0.05$; 0w versus each value at each time. **b** Distribution of the highest values of total bilirubin. **c** Distribution of the timing with the highest values of total bilirubin. **d** Changes in serum total bilirubin levels in patients whose levels of total bilirubin maximum were more than 2 mg/dl. *Black line*: patients whose total bilirubin levels peaked after 6 weeks (during the later period of SMV administration). *Gray line*: other patients

(14.7 %, total bilirubin maximum: 3.8); the treatment was discontinued at 12 weeks in one patient, and the total bilirubin levels decreased almost to baseline levels after SMV administration had been completed, after 12 weeks, in four patients.

The factors associated with bilirubin increase

The factors associated with grade 2 hyperbilirubinemia (total bilirubin maximum >2.0 mg/dl) are shown in Table 2. In the univariable analysis, the red blood cell (RBC) count ($p = 0.008$), serum Hb level ($p = 0.001$), serum total bilirubin level ($p < 0.001$), serum ALT level ($p = 0.004$) and ITPA genotype ($p = 0.037$) were identified as significant factors. Multivariable analysis that incorporated these five factors for grade 2 hyperbilirubinemia showed that the serum total bilirubin level [odds ratio (OR) 1.474, $p < 0.001$], serum ALT level (OR 1.016, $p = 0.011$) and ITPA genotype [OR 8.138, $p = 0.025$] were identified as significant factors.

The factors associated with significant increases in bilirubin (Δ total bilirubin $\geq$1.0 mg/dl) are shown in Table 3. In the univariable analysis, sex ($p = 0.004$), RBC count ($p = 0.001$), serum Hb level ($p = 0.001$), serum ALT level ($p = 0.030$), serum creatinine level ($p = 0.044$) and ITPA genotype ($p = 0.017$) were identified as significant factors. Multivariable analysis that incorporated these six factors concerning a significant increase in bilirubin showed that only the ITPA genotype (OR 4.990, $p = 0.011$) was identified as an independent significant factor.

Changes in Δ hemoglobin and Δ total bilirubin levels during the treatment according to ITPA genotype

The mean changes in Δ Hb and Δ total bilirubin during the treatment period according to the ITPA genotype are shown in Fig. 2. According to the presence of the ITPA genotype, decreased Δ Hb was more severe in patients with CC genotype than in patients with non-CC genotype (Fig. 2a). Regarding the changes in Δ total bilirubin, there was a significant difference between patients with the CC genotype and patients with the non-CC genotype. Patients with the CC genotype showed a clear increase in Δ total bilirubin, which peaked at 2 weeks, whereas patients with the non-CC genotype showed a mild increase in Δ total bilirubin (approximately 0.2 mg/dl increase from baseline levels on average) (Fig. 2b). To understand the association between Δ total bilirubin and Δ Hb, we examined the

Table 2 Factors associated with grade2 hyperbilirubinemia (total bilirubin maximum >2.0 mg/dl)

Factor	Category	Univariable analysis			Multivariable analysis		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age (years old)		0.979	0.944–1.015	0.241			
Sex	Male/Female	0.586	0.276–1.242	0.163			
BMI (kg/m^{2})		1.034	0.915–1.170	0.589			
Activity	0, 1/2, 3	0.690	0.291–1.633	0.398			
Fibrosis	0–2/3, 4	2.222	0.925–5.341	0.074			
White blood cell (/μl)	100/μl	0.989	0.960–1.018	0.448			
Neutrophil (/μl)	100/μl	0.999	0.963–1.037	0.964			
Red blood cell ($\times 10^4$/μl)		1.011	1.003–1.020	0.008	1.012	0.989–1.036	0.297
Hemoglobin (g/dl)		1.574	1.192–2.079	0.001	0.935	0.397–2.200	0.877
Platelets ($\times 10^4$/μl)		0.936	0.861–1.018	0.123			
Total bilirubin (mg/dl)	0.1 mg/dl	1.344	1.184–1.526	<0.001	1.474	1.230–1.765	<0.001
ALT (IU/l)		1.012	1.004–1.019	0.004	1.016	1.004–1.029	0.011
Creatinine (mg/dl)	0.1 mg/dl	0.943	0.765–1.161	0.578			
IL28B genotype	TT/non-TT	0.837	0.341– 2.053	0.698			
ITPA genotype	Non-CC/CC	4.909	1.102–21.863	0.037	8.138	1.306–50.720	0.025
RBV dose (mg/kg/day)		1.080	0.829–1.408	0.569			

BMI body mass index, *ALT* alanine aminotransferase, *IL28B* interleukin-28B, *ITPA* inosine triphosphate pyrophosphatase, *RBV* ribavirin, *OR* odds ratio

Table 3 Factors associated with significant bilirubin increases (Δ total bilirubin ≥1.0 mg/dl)

Factor	Category	Univariable analysis			Multivariable analysis		
		OR	95 % CI	*p* value	OR	95 % CI	*p* value
Age (years old)		0.990	0.960–1.022	0.550			
Sex	Male/female	0.389	0.204–0.741	0.004	0.792	0.235–2.673	0.707
BMI (kg/m$^{2)}$		1.016	0.915–1.127	0.770			
Activity	0, 1/2, 3	0.795	0.397–1.591	0.516			
Fibrosis	0–2/3, 4	1.024	0.461–2.274	0.953			
White blood cell (/μl)	100/μl	1.011	0.988–1.034	0.365			
Neutrophil (/μl)	100/μl	1.023	0.993–1.054	0.134			
Red blood cell ($\times 10^4$/μl)		1.012	1.005–1.020	0.001	1.013	0.996–1.031	0.145
Hemoglobin (g/dl)		1.508	1.192–1.908	0.001	1.141	0.599–2.170	0.689
Platelets ($\times 10^4$/μl)		0.980	0.917–1.047	0.543			
Total bilirubin (mg/dl)	0.1 mg/dl	1.076	0.973–1.189	0.155			
ALT (IU/l)		1.007	1.001–1.014	0.030	1.011	0.999–1.022	0.068
Creatinine (mg/dl)	0.1 mg/dl	1.192	1.005–1.415	0.044	1.062	0.787–1.433	0.693
IL28B genotype	TT/non-TT	1.277	0.617–2.644	0.510			
ITPA genotype	Non-CC/CC	3.864	1.273–11.728	0.017	4.990	1.456–17.106	0.011
RBV dose (mg/kg/day)		1.126	0.897–1.412	0.305			

BMI body mass index, *ALT* alanine aminotransferase, *IL28B* interleukin-28B, *ITPA* inosine triphosphate pyrophosphatase, *RBV* ribavirin, *OR* odds ratio

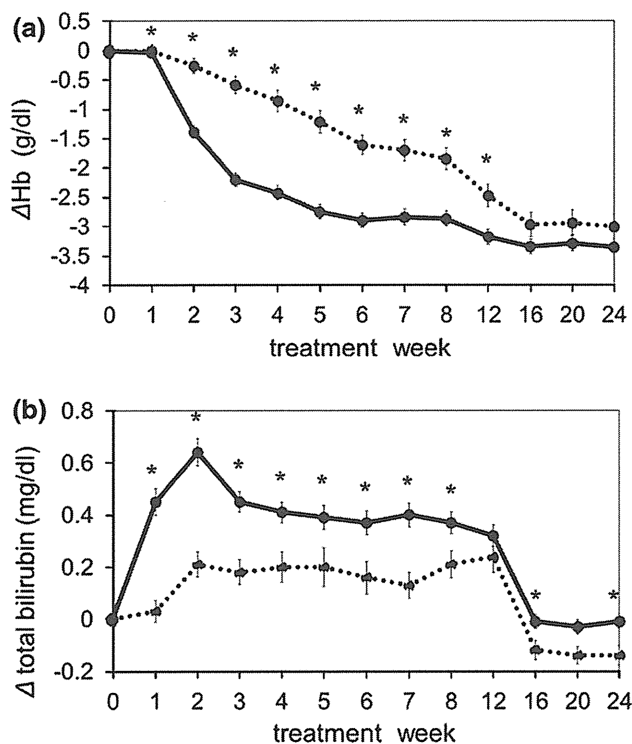


Fig. 2 Changes in serum bilirubin and hemoglobin levels during the treatment according to the ITPA genotype. **a** Δ hemoglobin levels; **b** Δ total bilirubin levels. *Solid line* ITPA CC genotype. *Dotted line* ITPA non-CC genotype. All data indicate the mean values and standard errors. *Asterisk*, $p < 0.05$; ITPA CC versus ITPA non-CC

correlation between the Δ total bilirubin maximum and Δ Hb maximum during the treatment period according to the ITPA genotype (Fig. 3). The levels of Δ total bilirubin maximum were significantly correlated with the levels of Δ Hb maximum ($r = 0.299$; $p < 0.001$).

Sustained virologic response

Sustained virologic response was 72.3 % (128/177) in the present study. The similar changes in total bilirubin levels were observed in the SVR group and non-SVR group during treatment.

Discussion

The present study was conducted to investigate the characteristics of the bilirubin increase in patients with CH-C who were treated with SMV with Peg-IFN plus RBV. Transient and mild increases in bilirubin during the treatment with SMV with Peg-IFN plus RBV were reported in Japanese phase 3 and international phase 3 trials [3–8]. The characteristics of the bilirubin increase have not been well understood in clinical practice. The present study revealed the following. Serum bilirubin levels peaked at 2 weeks and decreased to baseline levels after the SMV administration had been completed. Serum bilirubin levels were

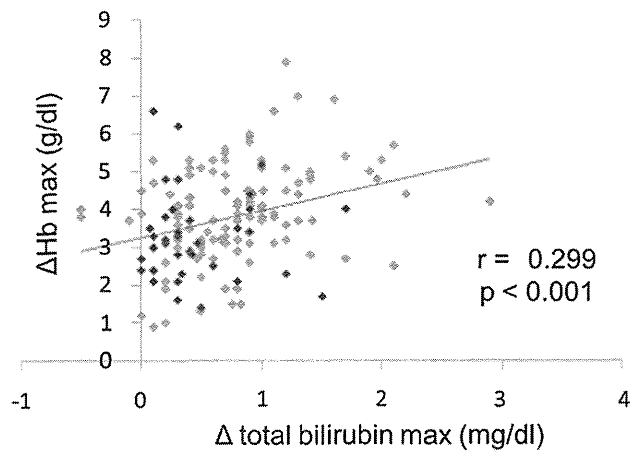


Fig. 3 Correlation between the highest values of Δ total bilirubin and Δ hemoglobin during the treatment according to ITPA genotype. *Gray point*: patients with ITPA CC genotype. *Black point*: patients with ITPA non-CC genotype

higher in patients with the ITPA CC genotype than in patients with the ITPA non-CC genotype during the treatment with SMV and Peg-IFN plus RBV.

The ITPA genotype is associated with hemolysis caused by RBV in patients with CH-C who were treated with Peg-IFN plus RBV [11–14]. ITPA genetic variants determine hemolysis caused by RBV; patients with the ITPA minor genotype showed a lower degree of hemolytic anemia than those with the ITPA major genotype. In the present study, the ITPA genotype was associated with bilirubin increases during treatment with SMV and Peg-IFN plus RBV (Tables 2, 3). When we examined the impact of the ITPA genotype on the bilirubin increase and the Hb decrease during the treatment period, significantly larger increases in serum bilirubin levels and decreases in Hb levels were observed in patients with the CC genotype than in those with the non-CC genotype (Fig. 2). The reason for the differences in the Hb decreases and bilirubin increases between patients with the CC genotype and those with the non-CC genotype may be the influence of hemolysis caused by RBV. In the COSMOS study, a total of 167 patients with chronic HCV genotype 1 infection were treated with four regimens of 150 mg SMV plus 400 mg sofosbuvir (SOF: once-daily HCV NS5B polymerase inhibitor) with or without RBV daily for 12 or 24 weeks. This study showed that in the RBV treatment groups, the Hb decrease (<10.5 g/dl of Hb) was 11.1 %, and the bilirubin increase (>2.5 × upper limit of normal) was 8.3 %, whereas in the RBV-free groups, these values were only 3.4 and 3.4 %, respectively [15, 16]. These results suggested that the Hb decrease and bilirubin increase were closely related to RBV administration during SMV plus SOF with RBV treatment. The association between the Hb decrease caused by RBV and the bilirubin increase was also reported in patients with CH-C who were treated with Peg-IFN plus RBV [17]. In the present study, a significant correlation between the Δ total bilirubin maximum and Δ Hb maximum during the treatment was observed (Fig. 3). Because patients with the non-CC genotype exhibited a small hemolytic effect, care should be taken when bilirubin increases are observed in patients with the non-CC genotype.

A transient increase in bilirubin, which peaked at 2 weeks, was observed during treatment with SMV and Peg-IFN plus RBV (Fig. 1). In patients whose levels of total bilirubin maximum were more than 2 mg/dl, serum total bilirubin levels peaked during the early period of the SMV treatment. The rapid decrease in Hb during the early period of treatment was considered to contribute to a bilirubin increase, which peaked at 2 weeks. Therefore, when bilirubin increases were observed during the later period of the treatment or after SMV administration had been completed, careful attention was required because of concerns regarding drug-induced deterioration of liver injury. In the present study, the serum bilirubin levels peaked at 2 weeks and decreased to the baseline level after SMV administration had been completed, and the bilirubin increase was higher in patients with the CC genotype than in those with the non-CC genotype. Clinicians must be careful when serum bilirubin levels peak during the later period of SMV administration, especially in patients with the non-CC genotype because factors other than hemolysis might be associated with the bilirubin increase.

In the present study, a transient increase in bilirubin, which peaked at 2 weeks, was observed, and the bilirubin increase was higher in patients with the ITPA CC genotype than in those with the ITPA non-CC genotype during treatment with SMV and Peg-IFN plus RBV. Consequently, we should pay attention to hyperbilirubinemia that peaks during the later period of SMV administration, especially in patients with the ITPA non-CC genotype.

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Compliance with ethical standards

Conflict of interest Prof. Tetsuo Takehara received a research grant from Janssen Pharmaceutical K.K., Merck Sharp & Dohme K.K. Co., Ltd. and Chugai Pharmaceutical Co., Ltd.

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SHORT REPORT Open Access

Lack of association between the *CARD10* rs6000782 polymorphism and type 1 autoimmune hepatitis in a Japanese population

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Abstract

Background: Previous genome-wide association studies have evaluated the impact of common genetic variants and identified several non-HLA risk loci associated with autoimmune liver diseases. More recent genome-wide association studies and replication analyses reported an association between variants of the *CARD10* polymorphism rs6000782 and risk of type 1 autoimmune hepatitis (AIH). In this case–control study, we genotyped 326 Japanese AIH patients and 214 control subjects.

Results: Genomic DNA from 540 individuals of Japanese origin, including 326 patients with type-1 AIH and 214 healthy controls, was analyzed for two single nucleotide polymorphisms (SNPs) in the *CARD10* gene. We selected *CARD10* rs6000782 SNPs and genotyped these using PCR–RFLP method and direct sequencing. The Chi square test revealed that the rs6000782 variant alle (c) was not associated with the susceptibility for AIH in a Japanese population [$p = 0.376$, odds ratio (OR) 1.271, 95 % confidence interval (CI) 0.747–2.161] in an allele model. Our data also showed that *CARD10* rs6000782 variants were not associated with AIH or with the clinical parameters of AIH.

Conclusions: In this study we examined an association between rs6000782 SNPs in the *CARD10* gene and type-1 AIH. Results showed no significant association of rs62000782 with type-1 AIH in a Japanese population. This study demonstrated no association between *CARD10* rs6000782 variants and AIH in a Japanese population.

Keywords: Autoimmune hepatitis, Genetic factor, Genome-wide association study, CARD10

Background

Autoimmune hepatitis (AIH) is characterized by the presence of serum antibodies, both anti-nuclear (ANA) and anti-smooth muscle antibodies (ASMA), as well as elevated immunoglobulin G levels, and interface hepatitis [1]. The genetic factors underlying the occurrence of AIH are unknown, with the exception of certain human leukocyte antigen (HLA) alleles [2]. de Boer et al. previously conducted a genome-wide association study that identified the most prominent association with AIH at rs2187668, which maps to the intronic region of *HLA-DQA1* [3]. They also showed that AIH was associated with variants of genes encoding Scr homology 2 adaptor protein 3 (*SH2B3*; rs3184504) and caspase recruitment domain-containing protein 10 (*CARD10*; rs6000782) [3]. In view of the importance of understanding the contribution of genetics to AIH, we carried out a case–control study to investigate the association between variants

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of *CARD10* rs6000782 and type 1 AIH in a Japanese population.

Methods

Study population

Consecutive type 1 AIH patients (n = 326) diagnosed according to the international diagnostic criteria for AIH [4] from the register of the Japanese National Hospital Organization (NHO) Liver Network Registry beginning in 2009 were enrolled in the present study as a multicenter cohort population [5]. Patients exhibiting primary biliary cirrhosis were excluded from the analysis. As controls, 214 healthy Japanese subjects (74 men and 140 women, with a mean age of 47.5 ± 10.8 years) with no history of liver disease were also enrolled. All patients did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. This study was conducted by adhering to the STOROBE statement (case–control studies). The study protocol was approved by the Ethics Committees of National Nagasaki Medical Center, and written informed consent was obtained from each individual.

DNA extraction and genotyping

Blood samples were taken from all study participants, and genomic DNA was isolated from peripheral blood leukocytes using a DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer's guidelines. *CARD10* rs6000782 genotypes were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. For the sequence (Fig. 1a), PCR products were treated with ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA), and then sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were analyzed using an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). Restriction fragment length polymorphism analysis was performed after the identification of single nucleotide polymorphism-specific restriction sites by NCBI Entrez SNP (http://www.ncbi.nlm.nih.gov/snp) and Takara Cut-Site Navigator (http://www.takara-bio.co.jp/enzyme). PCR restriction fragment length polymorphism genotyping to detect the 37928186A > C base pair change was performed using the following cycling profile: 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. The 365-base pair product was digested with *Xsp*I at 37 °C for 6 h and analyzed by 4 % NuSieve 3:1 agarose gel electrophoresis (Fig. 1b).

Statistical analyses

Results are expressed as mean ± SD. The statistical significance of differences between groups was calculated by either the Chi square test or Fisher's exact test for categorical data and Mann–Whitney's U test for quantitative data. Multivariate logistic regression analysis was performed with SPSS v.18 for windows (SPSS Statistics, Illinois). Deviation from Hardy–Weinberg equilibrium was assessed using the SNPAlyze software ver. 7.0 (Dynacom,

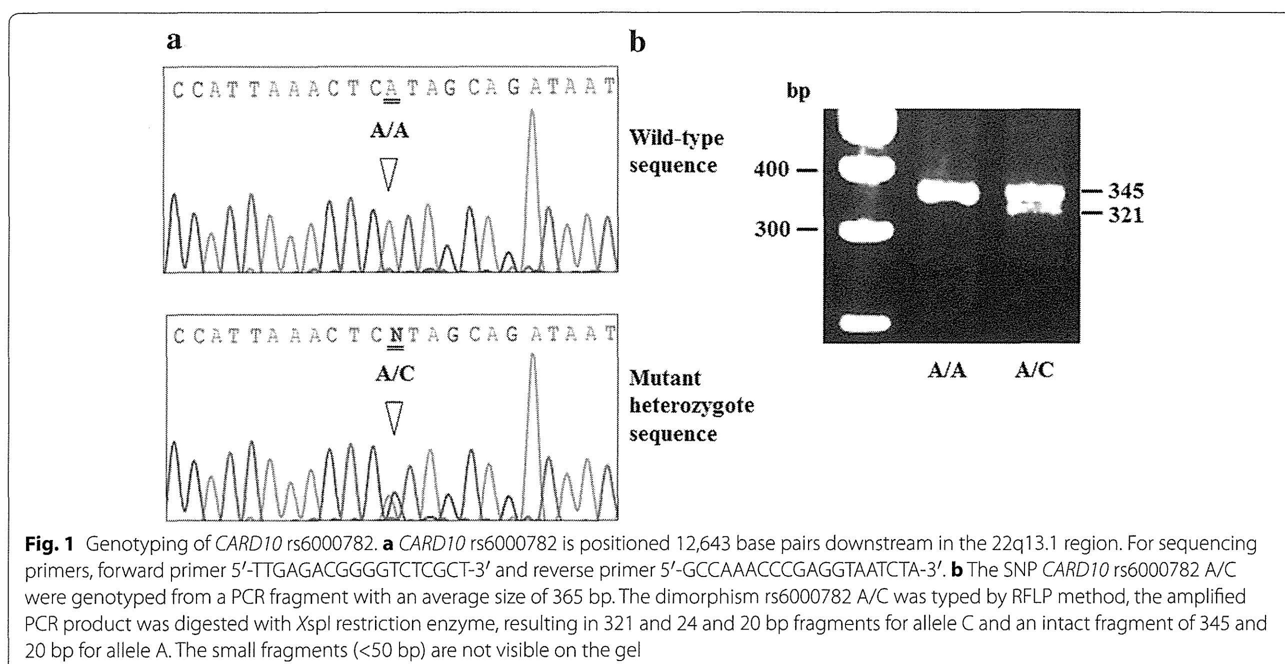

Fig. 1 Genotyping of *CARD10* rs6000782. **a** *CARD10* rs6000782 is positioned 12,643 base pairs downstream in the 22q13.1 region. For sequencing primers, forward primer 5′-TTGAGACGGGGTCTCGCT-3′ and reverse primer 5′-GCCAAACCCGAGGTAATCTA-3′. **b** The SNP *CARD10* rs6000782 A/C were genotyped from a PCR fragment with an average size of 365 bp. The dimorphism rs6000782 A/C was typed by RFLP method, the amplified PCR product was digested with XspI restriction enzyme, resulting in 321 and 24 and 20 bp fragments for allele C and an intact fragment of 345 and 20 bp for allele A. The small fragments (<50 bp) are not visible on the gel

Yokohama, Japan). A p value of <0.05 was considered significant.

Results

Demographic data of patients with AIH

Table 1 presents the demographic data of the subjected AIH patients. Among the enrolled type-1 AIH patients, 288 (88.6 %) were positive for ANA (>1:40) and 121 (38.2 %) for ASMA (>1:40). Among 326 eligible patients, 35 (10.7 %) had liver cirrhosis at the time of diagnosis, and among the remaining 291 patients without liver cirrhosis, 16 developed liver cirrhosis during the follow-up.

Table 1 Baseline characteristics of 326 Japanese AIH type 1 patients

Characteristics	N = 326 (%)
Female, n/total (%)	289/326 (88.7)
Age, years, mean ± SD	59.5 ± 13.3
Biochemistry	
AST, IU/L, median (IQR)	255.0 (92.5–723.0)
ALT, IU/L, median (IQR)	297.0 (104.5–818.0)
ALP, IU/L, median (IQR)	432.0 (316.5–584.0)
Total Bilirubin, mg/dl, median (IQR)	1.3 (0.8–4.6)
Albumin, g/dl, median (IQR)	3.9 (3.5–4.2)
IgG, mg/dl, median (IQR)	2239.0 (1809.5–2390.0)
Platelets, 10^4/μl, median (IQR)	18.5 (13.9–23.0)
Serology	
ANA ≧ 1:40, n/total (%)	288/325 (88.6)
ASMA ≧ 1:40, n/total (%)	121/317 (38.2)
Histology	
Cirrhosis, n/total (%)	51/326 (15.6)
IAIHG criteria	
Score, median (IQR)	16 (14–18)
Probable AIH, n/total (%)	126/309 (40.8)
Definite AIH, n/total (%)	183/309 (59.2)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphate, *IgG* immunoglobulin G, *ANA* anti-nuclear antibody, *ASMA* anti-smooth muscle antibody, *IQR* interquartile range, *IAIHG* International Autoimmune Hepatitis Group

Association of *CARD10* polymorphisms with type-1 AIH

Genotype distributions were in Hardy–Weinberg equilibrium in cases and controls (Table 2). Genotype frequencies and distributions, as well as odds ratios (ORs) and 95 % confidence intervals (CIs) for the association with AIH are shown in Table 3. The rs6000782 C allele was shown not to be associated with an increased risk for AIH (OR 1.271; 95 % CI 0.747–2.161; $p = 0.376$).

We also performed a detailed genotype–phenotype analysis using the clinical data. A detailed genotype–phenotype analysis using the clinical data revealed no significant association between rs6000782 and clinical findings of AIH patients (Table 4).

Discussion

AIH is characterized by an imbalanced regulation of the immune system in which innate and adaptive immune responses to hepatocyte antigens are important [6]. Genetic variation in the immune mechanisms that establish and maintain self-tolerance is likely to play a role in the development of AIH [7]. The susceptibility genes of AIH act alone or with environmental factors whose identity is mostly unknown [8]. The strongest association is with genes located within the HLA region, particularity those encoding the HLA-DRB1 alleles [2]. Up until now, evaluation of the non-HLA genetics of AIH has focused on small scale (usually non-replicated) candidate gene studies [9]. Genome-wide screening is a promising approach for the identification of the genetic determinants of complex diseases [8]. Large case–control studies with genome-wide surveys of genetic risk have been demonstrated for primary biliary cirrhosis (PBC) [10, 11]. AIH was subjected to the a similar genome-wide survey.

Table 2 Frequencies of CARD10 rs6000782 genotypes

	Locus	Genotype	Observed number (%)	Expected number[a]	p value[b]
Patients with AIH					
n = 326	rs6000782	A/A	284 (87.1)	285.3	$\chi^2 = 1.746$
		A/C	42 (12.9)	39.3	$p = 0.523$
		C/C	0	1.4	
Healthy control					
n = 214	rs6000782	A/A	193 (90.2)	192.5	$\chi^2 = 0.280$
		A/C	20 (9.3)	20.9	$p = 1.000$
		C/C	1 (0.5)	0.6	

[a] Expected genotype frequencies based on observed allele frequencies and assuming Hardy–Weinberg equilibrium

[b] p values were calculated using the Chi square test for Hardy–Weinberg equilibrium at individual loci