Data description

The ALD patients were categorized according to their etiologies including viral hepatitis [hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), or hepatitis E virus (HEV)], alcohol-induced hepatitis, drug-induced hepatitis, and autoimmune hepatitis (AIH). The patients' age, sex, length of stay, in-hospital mortality, and total costs for hospitalization were summarized for each subgroup.

The DPC database includes the records of clinical practices performed on each patient. The clinical procedures examined during hospitalization were use of prostaglandin E1, corticosteroid injection, albumin preparation, platelet transfusion, fresh-frozen plasma transfusion, continuous hemodiafiltration (CHDF), plasma apheresis, plasma exchange, and liver transplantation.

Estimation of incidence of hospitalization for ALD

We estimated the incidence of hospitalization for ALD based on the number of beds in all acute care hospitals in Japan, and hospitals that had joined the DPC database. We assumed that there was no seasonality in the hospitalizations for ALD. To adjust for the influence of bed volume imbalance, we stratified the hospitals based on bed volume categories. The estimated annual number of ALD cases (Y_i) and the 95 % confidence interval (CI) were calculated with the following equation using Wald confidence intervals for the population proportion [11]:

$$Y_i/N_i = p_i \pm Z\sqrt{p_i(1-p_i)/(n_i \times 2)},$$

where N_i is the total number of beds in all acute care hospitals in Japan, n_i is the number of beds in the DPC hospitals, $p_i = X_i/(n_i \times 2)$ (X_i is the observed number of ALD cases in DPC hospitals between July and December, 2007–2010), and $Z = 1.96$.

Estimation of incidence of hospitalization for acute or acute-on-chronic liver failure

In the present study, we included the patients with alcoholic hepatitis which were usually excluded from the disease entity of ALF [6, 7, 12]. We defined the fatal form of ALD in the present study as acute or acute-on-chronic liver failure (A/AoCLF). A/AoCLF (or ALF) is not covered by a distinctive ICD code, and the clinical data that define the presence of A/AoCLF (or ALF) were not accessible. Therefore, we assumed that those who underwent plasma exchange were A/AoCLF cases for estimation of the mortality from A/AoCLF in the DPC database. This can be an acceptable approximation of the number of A/AoCLF cases with a minimal possibility of underreporting [13], because more than 90 % of ALF patients in Japan undergo plasma exchange [4]. We estimated the incidence of hospitalization for A/AoCLF in the same way used for ALD.

Statistical analysis

The examined variables were expressed as the median with the 1st and 3rd percentiles (continuous variables) and frequencies (categorical variables). The significance of differences among groups was assessed by the Chi square test. The threshold for significance was a value of $P < 0.05$. All statistical analyses were conducted using IBM SPSS version 19.0 (IBM SPSS, Armonk, NY, USA).

Results

Etiologies and clinical characteristics of ALD in Japan

A total of 10509 ALD cases were identified between 1 July and 31 December in 2007–2010. Overall, 54.7 % of cases (5748) were male, and the median age was 53 years. The peak age for male patients was in their 60s, while the peak age for female patients was in their 50s (Fig. 1). The most frequent cause of ALD was indeterminate (35.1 %), followed by drugs (16.1 %) and alcohol (15.6 %) (Fig. 2a). When restricted to A/AoCLF cases, HBV-induced A/AoCLF accounted for 20.3 % of all cases of A/AoCLF

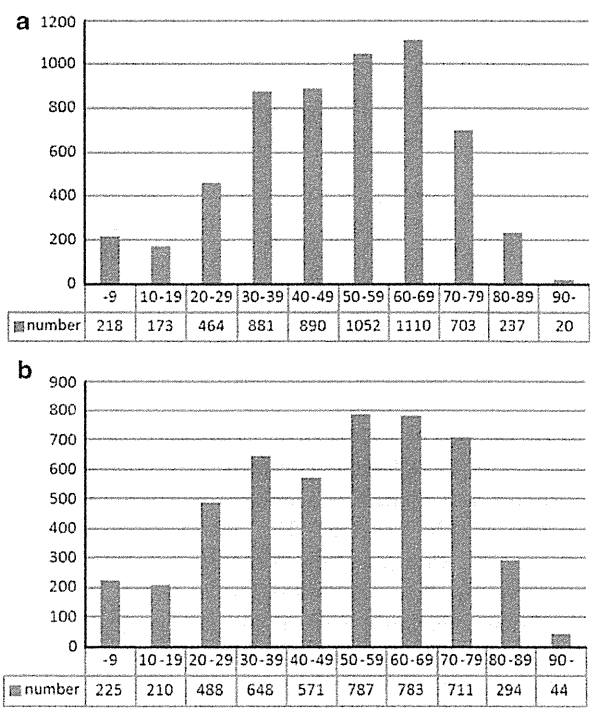

Fig. 1 Age distributions of the 10509 patients with ALD

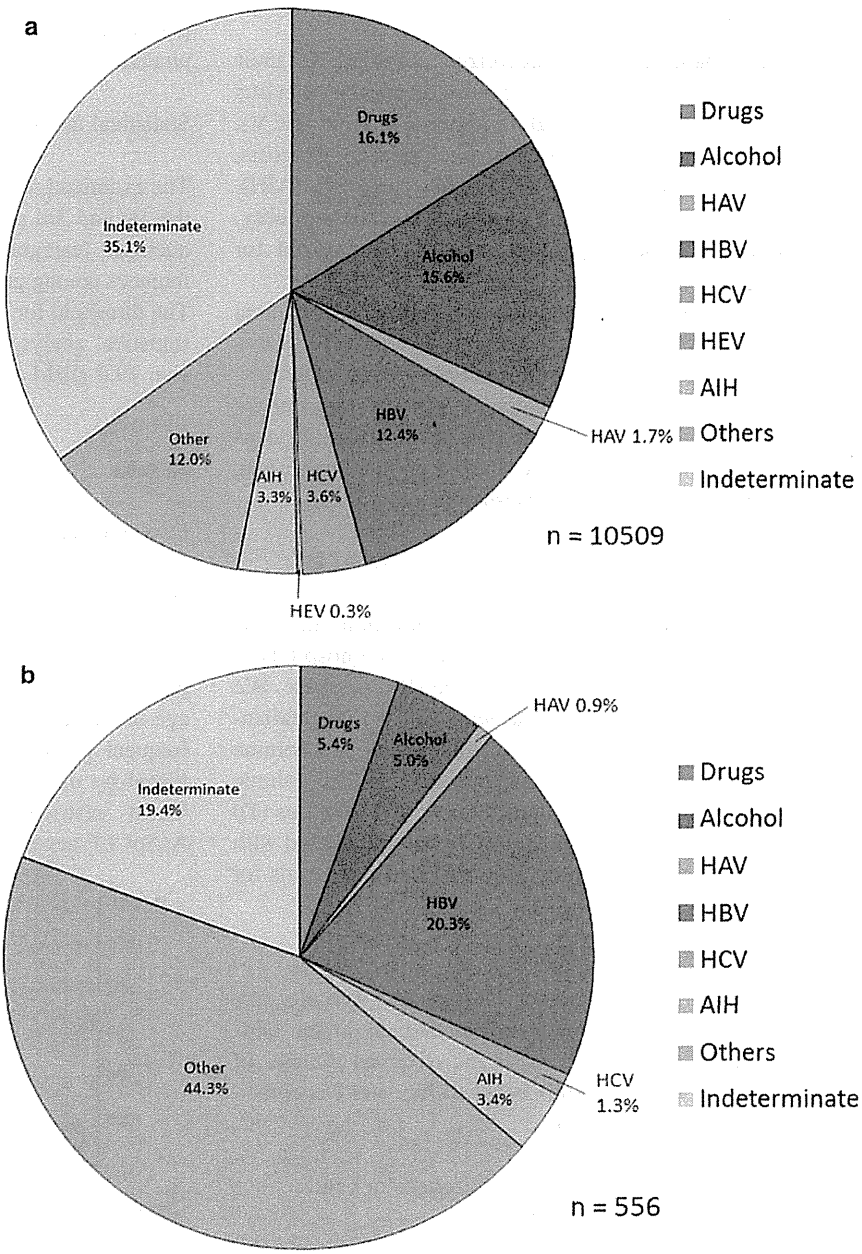

Fig. 2 **a** Etiologies of ALD in the total of 10509 patients in the DPC database between 2007 and 2010. **b** Etiologies of A/AoCLF in the total of 556 patients in the DPC database between 2007 and 2010

(Fig. 2b). Table 1 shows the etiologies and clinical characteristics of ALD cases in Japan. The proportion of male patients was highest in alcoholic hepatitis (83.7 %) and lowest in AIH (21.6 %). The overall mortality rate was 5.9 % (622 cases), and the etiologies of ALD affected the clinical outcomes. ALD induced by HAV or HCV had favorable outcomes with in-hospital mortality rates of approximately 2 %, compared with ALD induced by HBV, AIH, or HEV that had in-hospital mortality rates of 6.1–7.1 %. The hospitalization related to AIH was associated with the longest hospital stay and highest cost (median: 30 days and US$17183, respectively), followed by HEV-induced hepatitis (median: 27 days and US$15822, respectively).

Clinical practices

Table 2 summarizes the clinical procedures performed for the ALD cases. Overall, only 55 patients (0.5 %) underwent liver transplantation. Various treatments other than liver transplantation were provided to the ALD patients. Plasma exchange and CHDF were performed in 556

Table 1 Etiologies and clinical characteristics of 10509 cases of ALD

Features	Drugs ($n = 1690$)	Alcohol ($n = 1642$)	HAV ($n = 177$)	HBV ($n = 1303$)	HCV ($n = 383$)	HEV ($n = 28$)	AIH ($n = 342$)	Others[c] ($n = 1260$)	Indeterminate ($n = 3684$)
Age (years)[a]	63 (49–74)	55 (45–65)	47 (32–60)	41 (31–56)	57 (42–67)	57 (48–65)	60 (49–62)	32 (18–54)	53 (34–68)
Male sex. n (%)	679 (40.2)	1374 (83.7)	101 (57.1)	917 (70.4)	209 (54.6)	22 (78.6)	74 (21.6)	615 (48.8)	1757 (47.7)
Length of stay (days)[a]	16 (10–25)	14 (9–23)	16 (11–25)	18 (11–26)	13 (8–21)	27 (19–51)	30 (18–47)	13 (8–22)	14 (9–23)
In-hospital mortality. n (%)	48 (2.8)	57 (3.5)	3 (1.7)	80 (6.1)	9 (2.3)	2 (7.1)	23 (6.7)	144 (11.4)	256 (6.9)
Hospitalization costs (US$)[a, b]	9227 (5856–14322)	8278 (5225–13029)	9211 (6764–15075)	10801 (6862–16905)	7609 (3962–12572)	15822 (11720–25552)	17183 (11146–27143)	8393 (5312–20354)	8629 (5515–13888)

[a] Median (1st quartile–3rd quartile)

[b] The exchange rate was assumed to be 80 Japanese yen for US$1

[c] Epstein–Barr virus infection. 479 (38.1 %) cases; cytomegalovirus infection. 178 (14.1 %) cases; echinococcus infection. 31 (2.5 %) cases; herpes simplex virus infection. 7 (0.6 %) cases; Wilson disease. 6 (0.5 %) cases; leptospiral infection. 1 (0.1 %) case; adenovirus infection. 1 (0.1 %) case; other or unspecified viruses or parasites. 554 (44.0 %) cases

(5.3 %) and 360 (3.4 %) cases of all ALD cases, respectively. Plasma exchange was performed in 133, 141, 122, and 160 cases of all ALD cases in 2007, 2008, 2009, and 2010, respectively. Fresh-frozen plasma, platelet, and albumin preparations were used in 961 (9.1 %), 350 (3.3 %), and 748 (7.1 %) cases, respectively. Corticosteroids injection was most commonly used in AIH. The overall mortality in patients who underwent plasma exchange was 44.2 % (246/556 cases).

ALD in children

Of all the ALD cases, infant (0–3 years) and child (4–18 years) cases accounted for 261 (2.5 %) and 494 (4.7 %) cases, respectively. The etiologies of pediatric ALD were HBV in 5.8 % of cases, HAV in 1.6 %, AIH in 0.9 %, HCV in 0.8 %, and other causes in 45.1 %. The remaining 41.6 % of cases were considered indeterminate. The distribution of the etiologies differed from that in adults (Fig. 3). Overall, the in-hospital mortality rates of infant, child, and adult cases were 2.7 % (7/261), 1.0 % (5/494), and 6.3 % (610/9754), respectively. The mortality was significantly lower in infants and children than in adults (Chi square test; $P = 0.03$ and $P < 0.001$, respectively).

Estimated incidences of hospital admission for ALD and A/AoCLF in Japan

The estimated annual incidence of ALD in Japan, calculated by the equation using Wald confidence intervals, was 16645 cases (95 % CI: 15877–17413) (Table 3). According to the Population Census Data, the population of Japan in 2008 was approximately 127 million, indicating that the estimated annual incidence of hospitalization for ALD was 131.1 cases/1 million people. The annual numbers of A/AoCLF cases were estimated to be 598, 662, 643, and 698 cases in 2007, 2008, 2009, and 2010, respectively.

Discussion

In the present study, we used a large nationwide administrative claims database to evaluate the incidence of hospitalization related to ALD in Japan, which was estimated to be 131.1 cases/1 million people/year. Although Japan has a regulation requiring reports for several infectious diseases, our previous study analyzing the incidence of acute hepatitis B [13] using the DPC database revealed underreporting of acute hepatitis B in the National Epidemiological Surveillance for Infectious Disease, which is based on the Infectious Control Law [14]. Non-mandatory reporting systems will inevitably result in underestimation

Table 2 Clinical practices

Treatments	Drugs (n = 1690)	Alcohol (n = 1642)	HAV (n = 177)	HBV (n = 1303)	HCV (n = 383)	HEV (n = 28)	AIH (n = 342)	Others (n = 1260)	Indeterminate (n = 3684)
Transplantation	0 (0)	0 (0)	0 (0)	10 (0.8)	1 (0.3)	0 (0)	3 (0.9)	34 (2.7)	7 (0.2)
Plasma exchange	30 (1.8)	28 (1.7)	5 (2.8)	113 (8.7)	7 (1.8)	0 (0)	19 (5.6)	246 (19.5)	108 (2.9)
CHDF	11 (0.7)	19 (1.2)	2 (1.1)	61 (4.7)	3 (0.8)	0 (0)	13 (3.8)	160 (12.7)	91 (2.5)
Plasmapheresis	0 (0)	5 (0.3)	0 (0)	0 (0)	0 (0)	1 (3.6)	0 (0)	2 (0.2)	3 (0.1)
Prostaglandin E1	23 (1.4)	25 (1.5)	2 (1.1)	15 (1.2)	3 (0.8)	0 (0)	3 (0.9)	38 (3.0)	34 (0.9)
Cyclosporin A	5 (0.3)	0 (0)	0 (0)	15 (1.2)	3 (0.8)	0 (0)	3 (0.9)	11 (0.9)	12 (0.3)
Corticosteroids injection	215 (12.7)	103 (6.3)	25 (14.1)	167 (12.8)	9 (2.3)	3 (10.7)	105 (30.7)	329 (26.1)	499 (13.5)
Fresh-frozen plasma	51 (3.0)	74 (4.5)	20 (11.3)	158 (12.1)	9 (2.3)	8 (28.6)	45 (13.2)	327 (26.0)	269 (7.3)
Platelet transfusion	17 (1.0)	16 (1.0)	4 (2.3)	43 (3.3)	9 (2.3)	2 (7.1)	15 (4.4)	150 (11.9)	94 (2.6)
Albumin preparation	47 (2.8)	107 (6.5)	8 (4.5)	80 (6.1)	11 (2.9)	3 (10.7)	46 (13.5)	212 (16.8)	234 (6.4)

Data are shown as n (%)

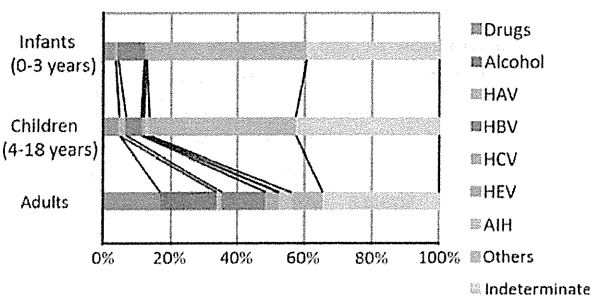

Fig. 3 Etiology of acute hepatitis in each generation

of the occurrence of disease, and thus impair health policy evaluation and decision making. In the DPC database, the diagnosis upon hospitalization is a required item, and is thought to be completely free from recall bias.

The frequency distribution of etiologies differs geographically worldwide [15, 16]. In a previous ALF study from the United States, drugs (including acetaminophen)-induced ALF were shown to be responsible for more than 50 % of ALF cases [17]. In the present study, drug-induced A/AoCLF accounted for only 5.4 % of all cases of A/AoCLF, which is far lower than the value reported in the previous ALF study from the United States. Consistent with the present study, drug-induced ALF was reported to account for about 5–15 % of cases in previous nationwide analyses in Japan [4, 6, 18]. On the other hand, HBV-induced ALF accounted for the largest proportion (about 30–40 %) of ALF cases in Japan [4, 6, 18], which is much higher than the value reported in the United States [17]. In the present study, HBV-induced A/AoCLF constituted 20.3 % of A/AoCLF cases, which is lower than the values in previous reports in Japan. ICD-10 code-based diagnosis of chronic hepatitis may have been attached to more than a few inactive HBV carriers. We excluded such patients from the present study, although acute exacerbation of hepatitis in asymptomatic HBV carriers is considered as acute hepatitis in Japan. This may explain the discrepancy.

The DPC database includes the records of clinical practices performed on each patient. Thus, we can track the use of medications and procedures including plasma exchange and liver transplantation. Artificial liver support with plasma exchange plays a central role in the treatment of ALF in Japan. The results showed that the annual estimated numbers of A/AoCLF cases were 598–698 from 2007 to 2010. These figures may be acceptable in light of an epidemiological survey of nationally-designated intractable diseases, which recently estimated the number of ALF patients in Japan to be 429 cases/year [19]. The overall fatality of A/AoCLF was estimated to be 44.2 % in the present study. In Japanese nationwide studies, the survival probabilities of patients with ALF were reported to be 47.8 % in the survey from 1998 to 2003 [4], and 47.4 % in the survey from 2004 to 2009 [18]. Similarly, the probability of spontaneous survival was reported to be approximately 45 % in the United States [17].

In the present study, ALD induced by HAV or HCV had favorable outcomes with regard to mortality, compared with that induced by HBV, HEV, or AIH, which is compatible with previous studies that reported favorable outcomes of ALF induced by HAV and unfavorable outcomes of ALF induced by HBV or AIH [17]. Moreover, the mortality of infants and children hospitalized for ALD was significantly lower than that of adults. We cannot know the severity of ALD from the DPC database, which may leave room for the possibility that children with ALD were more prone to be admitted to hospitals with less severe conditions. Favorable outcomes of ALF in children were also reported [20].

Table 3 Estimated number of annual ALD patients in Japan

Bed volume	Number of acute care beds in Japan (N_i)	Number of acute care Beds in DPC hospitals (n_i)	Number of ALD patients in DPC hospitals for 2 years (X_i)[a]	Estimated number of all ALD patients in Japan (Y_i) (95 % confidence interval)
≤399	566658	119853	4843	11449 (10997–11900)
400–599	175715	89627	3008	2949 (2801–3096)
600–799	88870	49740	1477	1319 (1225–1414)
≥800	78995	50245	1181	928 (854–1003)
Total	910238	309465	10509	16645 (15877–17413)

[a] Data were collected from 6 months (July–December) of each 4 years (2007–2010)

Table 4 Estimated number of annual ALD patients and annual incidence (per 1 million people) of acute hepatitis in Japan

Features	Drugs	Alcohol	HAV	HBV	HCV	HEV	AIH	Others	Indeterminate
Estimated number	2788 (2476–3100)	3021 (2709–3334)	267 (169–366)	1815 (1552–2076)	574 (428–720)	40 (1–79)	474 (340–609)	1918 (1653–2183)	5748 (5293–6203)
Annual incidence/1 million people	22.0 (19.5–24.4)	23.8 (21.3–26.3)	2.1 (1.3–2.9)	14.3 (12.2–16.4)	4.5 (3.4–5.7)	0.32 (0.01–0.62)	3.7 (2.7–4.8)	15.1 (13.0–17.2)	45.3 (41.7–48.9)

Data are shown as n (95 % confidence interval)

In the present study, ALD induced by indeterminate etiology accounted for the greatest proportion (35.1 %) of all cases of ALD. A previous study showed the possibility that patients with ALF induced by HBV, AIH, or drugs may be included in ALF with indeterminate etiology, using a data-mining approach [21]. However, data on a total of 104 items, including information inaccessible in the DPC database such as past history or laboratory data, are required to categorize patients by this approach. Future improvements to the DPC database are encouraged to enable access to more information, which will allow us to undertake further useful approaches.

Alcoholic hepatitis often develops in patients with chronic liver disease caused by habitual alcohol consumption. Thus, in the recently determined criteria for ALF (as well as the previous criteria for FH) in Japan, patients with alcoholic hepatitis are usually excluded from the disease entity of ALF (or FH) [4, 6, 7, 12]. However, alcoholic hepatitis may develop in patients with minimal liver injury, and is still included as an etiological factor for the disease entity of ALF in Europe and the United States [5]. In addition, alcoholic hepatitis is associated with a high fatality rate [22]. Therefore, alcohol-induced ALD is thought to constitute a major health burden. For these reasons, in the present study, we did not exclude patients with alcoholic hepatitis unless accompanied by another diagnosis such as chronic hepatitis or cirrhosis.

This study has several limitations. First, the sample collection in the DPC database is not based on a random sampling method, and thus the hospital distribution tends to be biased. Although the DPC database represents approximately 40 % of all admissions to secondary and tertiary care hospitals in Japan, participating hospitals tend to be medium-to-large-sized institutions with beds for more severe ALD patients. The mortality could,, therefore be overestimated by excluding less severe patients in small-sized hospitals. However, ALD is relatively common and hospitalization related to ALD in small-to-medium-sized hospitals is thought to account for a considerable portion. Indeed, 4843 (46.1 %) cases were derived from hospitals with less than 400 beds, as shown in Table 3. Hence, this limitation may be not too serious. Second, the DPC database leaves room for the possibility of inaccurate reporting of diagnoses. Although we excluded cases with diagnoses of chronic liver diseases, some patients with acute exacerbation of chronic liver disease might have been registered as ALD, resulting in an overestimation of the ALD incidence in Japan. Third, as noted above, important clinical data such as prothrombin time or degree of encephalopathy were unavailable in the DPC database. Consequently, we could not learn from the database the etiology or clinical characteristics of ALF cases, which should be characterized by the presence of encephalopathy and prothrombin international normalized ratio of >1.5. Fourth, the DPC survey is only conducted between July and December each year, and therefore data between January and June were not available. The sample may therefore be biased, especially in ALDs related to seasonal causes such as HAV or HEV.

Fifth, patients may have been referred from the first hospital to another hospital for specialized treatment, such as liver transplantation. In this case, the two admissions would be recorded separately in the DPC database, leaving the possibility that such patients were enrolled in the analyses in a duplicated manner. Finally, the DPC database only includes inpatient data, and; therefore, we cannot know the incidence of ALD cases treated in outpatient settings from the database. However, the most severe cases are likely to be included in the inpatient database (Table 4).

In conclusion, the present study has demonstrated the incidence of ALD and the clinical practices performed on ALD patients in Japan using the nationwide DPC database. The overall in-hospital mortality of ALD in Japan was 5.9 % in the DPC database, which was affected by the etiologies as well as the patients' background characteristics. Since the DPC database does not cover the whole admission in Japan and also does not cover patients in outpatient settings, the overall burden of the disease may still remain to be evaluated. Improvement of public health surveillance systems is necessary for population-based patient monitoring.

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Conflict of interest None of the authors have any conflicts of interest.

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MAJOR ARTICLE

High Levels of Hepatitis B Virus After the Onset of Disease Lead to Chronic Infection in Patients With Acute Hepatitis B

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Background. Some patients with acute hepatitis B virus (HBV) infection develop chronic infection. However, the method for identifying these patients has not been established.

Methods. We followed 215 Japanese patients with acute HBV infection until the clearance of hepatitis B surface antigen (HBsAg) or the development of chronic infection. Levels of HBsAg and HBV DNA were serially monitored from the onset.

Results. Of the 215 patients, 113 (52.5%) possessed HBV genotype A, 26 (12.0%) genotype B, and 73 (34.0%) genotype C. Twenty-one of the 215 (9.8%) developed chronic infection, with the persistence of HBsAg for >6 months. The rate of chronicity of genotype A, B, and C was 12.4%, 3.8%, and 8.2%. Of the 21 patients, only 6 (2.8%) patients, including 5 with genotype A, failed to clear HBsAg within 12 months. Levels of HBsAg at 12 weeks and HBV DNA at 4 weeks were useful for distinguishing the patients who became chronic from those who did not ($P < .001$ and $P < .001$, respectively). Likewise, the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks were useful for discriminating between the patients who lost HBsAg within 12 months and those who did not ($P < .01$ and $P < .05$, respectively).

Conclusions. In acute HBV infection, clearance of HBV may happen between 6 and 12 months from the onset. Only those who fail to clear HBV within 12 months from the onset may develop chronic infection.

Keywords. hepatitis B virus antigen; hepatitis B virus; genotype.

The clinical outcome of acute hepatitis B is self-limited in the majority of immunocompetent adults. However, some patients run a prolonged or even chronic course, or are complicated by acute liver failure. Several factors are implicated in different clinical courses.

Hepatitis B virus (HBV) genotypes and subtypes are known to influence the clinical outcome of acute hepatitis B. For instance, HBV subgenotype B1 is associated with fulminant hepatic failure in acute hepatitis B [1]. On the other hand, genotype A is associated with chronic sequelae [2–5]. Furthermore, patients with subgenotype C2 are more likely to develop chronic infection than those with subgenotype B2 [6]. These characteristics may reflect viral kinetics in acute HBV infection that would differ among HBV infections with distinct genotypes/subgenotypes, but little is known about them.

Quantitation of hepatitis B surface antigen (HBsAg), in addition to HBV DNA, has been introduced to analysis of viral kinetics in patients with chronic hepatitis B in recent years. HBsAg levels are also useful for estimating

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viral loads and predicting the response to antiviral treatments [7–9], and for determining the natural history of chronic hepatitis B [10, 11]. Therefore, HBsAg and HBV DNA would be instrumental in foretelling the outcome of acute hepatitis B. However, the clinical utility of these markers in patients with acute hepatitis B is largely unknown.

Therefore, the aim of the present study was to examine differences in viral kinetics among patients with acute hepatitis B, who were infected with HBV of different genotypes, and evaluate the usefulness of quantifying HBsAg and HBV DNA for predicting the clinical outcome.

PATIENTS AND METHODS

Patients

This was a retrospective study of patients who were diagnosed with acute hepatitis B in our institutions during 1994 through 2010. Criteria for the diagnosis of acute hepatitis B were (1) acute onset of liver injury without a previous history of liver dysfunction; (2) detection of HBsAg in the serum; (3) immunoglobulin M (IgM) antibody to HBV core (anti-HBc) in high titers (detectable in serum samples diluted 10-fold) [3]; (4) absence of a past or family history of chronic HBV infection; and (5) exclusion of coinfection with hepatitis A virus, hepatitis C virus, or other hepatotropic viruses by serologic testing. Among the 232 patients who met these criteria, 215 patients (159 men and 56 women with a mean age of 31.8 ± 10.0 years) whose serum samples were available for virologic analyses were included in the study. No patient developed liver failure.

No patient received antiviral treatment. Of the 215 patients, 159 (74.0%) patients could be regularly followed up until the confirmation of clinical outcomes. Based on the duration of HBsAg (defined as the interval between the onset [defined by the first visit] and the last visit with detectable HBsAg), we classified the 159 patients into the following 4 groups (the duration of HBsAg is indicated in parentheses): group 1 (<3 months); group 2 (3–6 months); group 3 (>6–12 months); and group 4 (>12 months). Changes in virologic parameters were analyzed in relation with clinical characteristics. The study was approved by the ethics committees of our institutions, and written informed consent was obtained from each patient.

Quantification of Serologic Markers for HBV Infection and HBV DNA

HBsAg had been measured quantitatively by chemiluminescent enzyme-linked immunosorbent assay (ELISA; Sysmex JAPAN Co, Ltd, Kobe, Japan) every 2–4 weeks, until the clinical outcome was known. It has a dynamic range of 0.03–2, 500 IU/mL. Serum samples scaling out from this range were diluted so as to contain them within it. Antibody to hepatitis B s antigen (anti-HBs), hepatitis B e antigen (HBeAg), and IgM anti- HBc were determined by ELISA (Abbott JAPAN Co, Ltd, Tokyo, Japan). Levels of HBV DNA were determined using the COBAS TaqMan HBV v.2.0 kit (Roche Diagnostics, Basel, Switzerland), which has a dynamic range over 2.1–9.0 log copies/mL.

HBV Genotyping

The HBV genotype was determined by a genotype-specific probe assay (Smitest HBV genotyping Kit, Genome Science, Fukushima, Japan) as previously reported [12].

Molecular Evolutionary Analyses

HBV genotype A started to prevail in Japan merely several years ago, suggesting that it was imported to Japan only recently [3, 13]. Therefore, genomic sequences of HBV genotype A (HBV/A), recovered from sera of patients with acute HBV infection, would be closely related to one another and with those reported from abroad. To evaluate this possibility, 20 HBV/A samples were selected randomly and sequenced by the method reported previously [14].

The number of nucleotide substitutions per site was estimated by the 6-parameter method [15], and a phylogenetic tree was constructed by the neighbor-joining method [16] based on the numbers of substitutions. To confirm the credibility of phylogenetic analyses, bootstrap resampling tests were carried out 1000 times [17].

Statistical Analyses

Categorical variables were compared by χ^2 test or Fisher exact test, and continuous variables by the Mann-Whitney U test. $P < .05$ was considered statistically significant. Receiver operating characteristic (ROC) analysis was performed to compute the area under the ROC curves for viral markers to determine cutoff points for predicting the outcome.

RESULTS

Distribution of HBV Genotypes in Patients With Acute Hepatitis B

HBV genotypes were determined in 215 of the 232 (93%) patients with acute hepatitis B. Of the 215 patients, genotype A was detected in 113 (52%), B in 26 (12%), C in 73 (33%), D in 1 (1%), E in 1 (1%), and F in 1 (1%). The distribution of genotypes was compared among 4 periods during 1994 through 2010 (Table 1). The proportion of patients with genotype A gradually increased to 65.9% in 2007–2010; it was higher than those in the earlier periods (34.4% in 1994–1998 [$P = .002$], 36.8% in 1999–2002 [$P = .002$], and 51.9% in 2003–2006 [$P = .093$]).

Phylogenetic Relationship Among HBV Strains of Genotype A

We randomly selected 11 HBV/A strains sampled in 2007–2010 and 9 of those in 2001–2006, and constructed a molecular evolutionary tree (Figure 1). All 20 samples had similar nucleotide sequences with a concordance of 99%. They were close to previously

Table 1. Prevalence of Hepatitis B Virus Genotypes in Patients With Acute Hepatitis B During 4 Chronologic Periods

Period	Genotype A	Genotype B	Genotype C	Others
1994–1998 (n = 32)	11[a] (34.4%)	3 (9.3%)	18 (56.3%)	0
1994–1998 (n = 38)	14[b] (36.8%)	4 (10.5%)	20 (52.7%)	0
1994–1998 (n = 54)	28[c] (51.9%)	6 (11.1%)	19 (35.1%)	1 (1.9%)
1994–1998 (n = 91)	60[a,b,c] (65.9%)	13 (14.3%)	16 (17.6%)	2 (2.2%)
Total (N = 215)	113 (52.5%)	26 (12.0%)	73 (34.0%)	3 (1.5%)

[a] $P = .0032$.

[b] $P = .0014$.

[c] $P = .02$.

reported genotype A2 sequences from Western countries. The results support the possibility that HBV/A was imported to Japan only recently and has been spreading throughout the country.

Clinical Features Among Patients Infected With HBV of Different Genotypes

Clinical features of patients with acute hepatitis B of different genotypes are compared in Table 2. The mean age was no different among patients infected with HBV of different genotypes. The proportion of men was higher in genotype A or B than C infection (93.8% or 80.7% vs 39.7% [A vs C, $P < .001$; B vs C, $P < .001$]).

The maximum alanine aminotransferase (ALT) level was lower in patients with genotype A than in those with genotype C (2126 ± 938 vs 2857 ± 1668 IU/L, $P = .002$). The maximum bilirubin level was higher in patients with genotype A (7.1 ± 6.4 mg/dL) or C (9.0 ± 7.5 mg/dL) than in those with genotype B (4.8 ± 3.3 mg/dL) (A vs B, $P = .003$; B vs C, $P < .001$). Regarding viral markers, the peak HBV DNA level was higher in patients with genotype A than in those with genotype C (6.3 ± 1.7 vs 4.9 ± 1.5 log copies/mL, $P < .001$). HBeAg was detected in 95 of the 121 (77.3%) patients with genotype A, 24 of the 28 (88.5%) with genotype B, and 37 of the 58 (65.5%) with genotype C (A vs C, $P = .036$). Men who have sex with men were more frequently represented among patients with genotype A than B or C (31.4% vs 4.8% or 11.3% [A vs B, $P = .017$; A vs C, $P = .002$]). Antibody to human immunodeficiency virus (anti-HIV) was examined in 72 of the 113 (63.7%) patients with genotype A, 7 of the 26 (26.9%) with genotype B, 58 of the 73 (79.5%) with genotype C, and 1 with genotype E. Anti-HIV was detected in 7 of the 72 (9.7%) patients with genotype A, and the other 96 patients tested for anti-HIV showed negative results. All of the 7 patients with anti-HIV cleared HBsAg from the serum within 6 months without antiviral treatment.

Among the 215 patients whose HBV genotypes were determined, 159 could be followed until the confirmation of clinical outcomes. The distribution of HBsAg-positive period is compared among patients with different genotypes. Group 1 (HBsAg persisting for <3 months) comprised 84 patients; group 2 (3–6 months) comprised 54 patients; group 3 (>6–12 months) comprised 15 patients; and group 4 (>12 months) comprised 6 patients. HBsAg remained >6 months in 21 of the 215 (9.8%) patients, including 14 of the 113 (12.4%) with genotype A, 1 of the 26 (3.8%) with genotype B, and 6 of the 73 (8.2%) with genotype C. Among the 21 patients, 15 (71.4%) cleared HBsAg within 12 months from the onset, and were classified into group 3. The remaining 6 (5 with genotype A and 1 with genotype B) who failed to clear HBsAg within 12 months were classified into group 4. All of the 6 were negative for anti-HIV. The proportion of group 4 was 6.0% in the patients with genotype A, 4.0% in those with genotype B, and 0% in those with genotype C.

The mean duration of HBsAg was 13.9 ± 8.7 weeks in patients with genotype A, 7.1 ± 5.3 weeks in those with genotype B, and 9.6 ± 7.6 weeks in those with genotype C, presuming the duration of HBsAg in group 4 at 12 months. The duration was longer in patients with genotype A than in those with B or C (A vs B, $P < .001$; A vs C, $P = .04$).

Prediction of the Outcome by the Duration of HBsAg

Table 2 shows that the duration of HBsAg among patients with genotype A varied to a higher extent than that among those with other genotypes. Therefore, we determined HBsAg and HBV DNA levels serially, and evaluated them for the ability to predict the outcome of acute hepatitis B in patients with genotype A.

Serial changes in HBsAg levels are shown in Supplementary Figure 1*A*. HBsAg levels declined more slowly in group 2 than group 1, as well as in group 3 than group 2. In group 4, HBsAg reelevated at 12 weeks after the onset. Figure 2 compares HBsAg levels among groups 1–4 at different intervals from the onset. HBsAg at 8 weeks from the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBsAg at 12 weeks from the onset was helpful for discriminating among groups 2, 3, and 4.

Prediction of the Outcome by HBV DNA

We also studied serial changes of HBV DNA in patients with genotype A, and examined if they also were useful for predicting the clinical outcome of acute hepatitis B. Supplementary Figure 1*B* shows serial changes in HBV DNA levels in patients in 4 groups. Although the reelevation of HBV DNA was not observed, the decline of HBV DNA was quite slow in group 4. Figure 3 compares HBV DNA levels among groups 1–4 at different intervals from the onset. HBV DNA at 4 weeks from

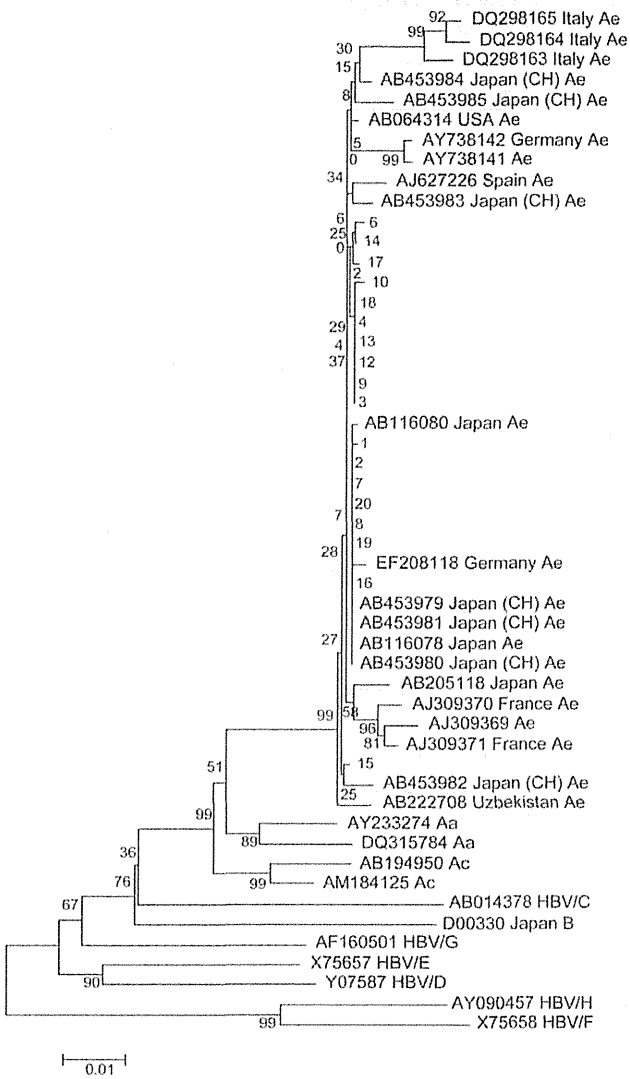

Figure 1. Evolutionary relationships of 86 hepatitis B virus genotype A taxa, including 20 from the present cases. The evolutionary history, inferred using the neighbor-joining method, shows that all 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBV DNA levels at 8 weeks from the onset were useful for discriminating between group 4 and group 3, as well as for distinguishing group 3 or 4 from group 1 or 2.

Levels of HBsAg and HBV DNA for Predicting Persistent Infection

As the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks from the onset were useful for distinguishing group 4 from the other groups, we evaluated the appropriate levels for predicting persistent infection in patients with genotype A. When we set the cutoff value of HBsAg at 1000 IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (98.1%). Likewise, when we set the cutoff value of HBV DNA at 10^6 log IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (96.4%). Therefore, HBsAg at 12 weeks >1000 IU/mL or HBV DNA at 8 weeks >10^6 log copies/mL is useful for predicting persistent infection.

DISCUSSION

In Japan, as shown in Table 1, the dominant HBV in acute hepatitis has been shifting from genotype C to A [3, 5, 14, 18]. The fact that nucleotide sequences of HBV/A isolates from patients

Table 2. Baseline Characteristics and the Duration of Hepatitis B Surface Antigen in Patients With Acute Hepatitis B Virus With Different Hepatitis B Virus Genotypes

	HBV Genotypes					
Features	A (n = 113)	B (n = 26)	C (n = 73)	D (n = 1)	E (n = 1)	F (n = 1)
Age, y	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a,b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c,d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT, IU/L	2126 ± 938[e,*]	2394 ± 820	2857 ± 1668[e]	4180	1175	1533
Bilirubin, mg/dL	7.1 ± 6.4[f,*]	4.8 ± 3.3[f,g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA, log copies/mL	6.3 ± 1.7[h,*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i,*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg*						
Group (mo)						
1 (<3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
2 (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
3 (>6–12)	9 (10.8%)	0	6 (12.5%)	0	0	0
4 (>12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; anti-HIV, antibody to human immunodeficiency virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

[a] $P < .001$.

[b] $P < .001$.

[c] $P = .017$.

[d] $P = .002$.

[e] $P = .002$.

[f] $P = .003$.

[g] $P < .001$.

[h] $P < .001$.

[i] $P = .036$.

* Data from anti-HIV-positive patients are excluded.

with acute hepatitis B in this study were very close to one another suggests that most HBV/A strains were imported recently and have spread rapidly, which may be attributed to the features of HBV/A in transmission routes and viral kinetics. We have reported that patients with genotype A tend to have multiple sexual partners [5]. Consequently, chances of secondary transmission of HBV/A would be higher than those of other genotypes, which may increase the number of patients who contract HBV/A infections. On the other hand, HBsAg persisted longer in patients with genotype A than B or C, which is consistent with the in vivo experiment using chimera mice carrying human hepatocytes showing that proliferation of HBV starts later and lasts longer in genotype A than in B or C infection [19].

Our results have shown that 6% of the patients with genotype A develop persistent infection. Because liver cirrhosis or hepatocellular carcinoma can develop in a substantial population of HBV carriers [20, 21], it is important to distinguish the patients in whom HBV infection becomes chronic, and follow them carefully. Although polymorphisms in host genes may be useful for identifying patients who are prone to develop chronic HBV infection [22], simple surrogate markers for the outcome have not been reported. Our data indicate that it would be difficult to predict the clinical outcome based on serum levels of viral markers at the first visit alone. This is understandable, because the dose of infecting virus, as well as the interval between infection and the first visit, can vary widely. Hence, we set out to analyze changes in serum levels of viral markers.

As seen in Figure 2, HBsAg levels at 12 weeks from the onset were most useful for discriminating among groups 2, 3, and 4 in the genotype A infection. Therefore, the outcome of acute hepatitis B may be predictable at this time point. Of note is the reelevation of HBsAg observed in group IV (Supplementary Figure 1*A*). Reelevation of viral markers suggests prolonged viral proliferation in the liver, and may be useful to identify the patients who may develop chronic infection.

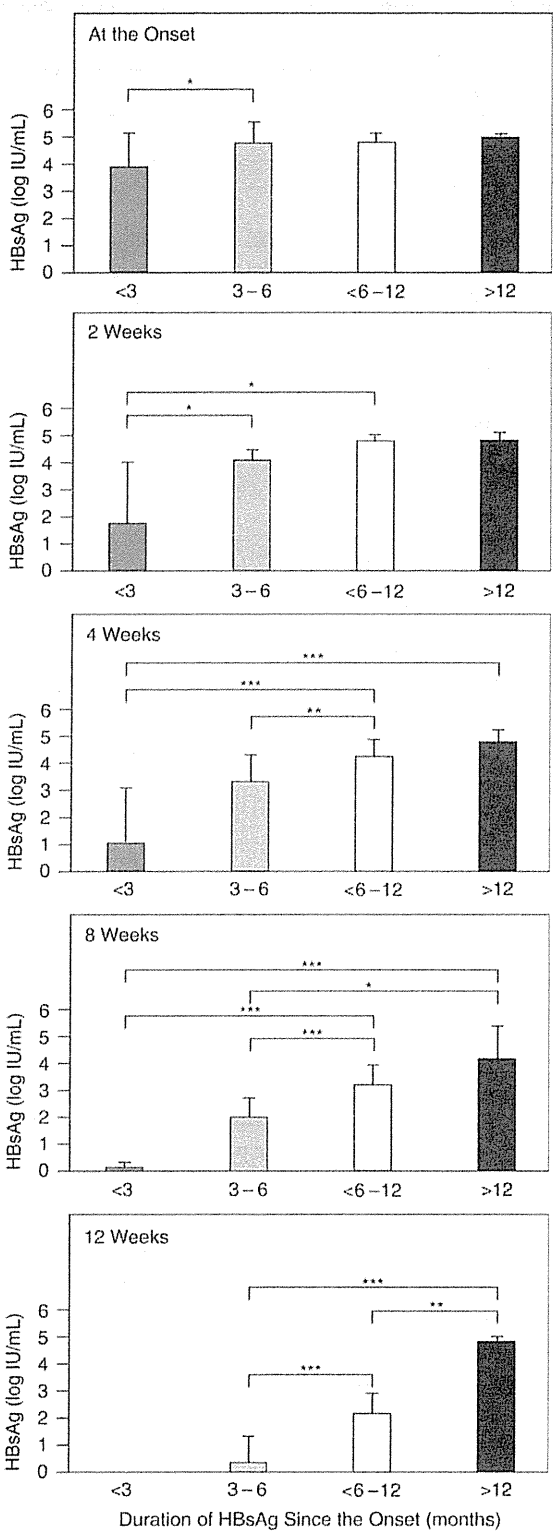

Figure 2. Levels of hepatitis B surface antigen in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A *P< .05; **P< .01; ***P< .001. Abbreviation: HBsAg, hepatitis B surface antigen.

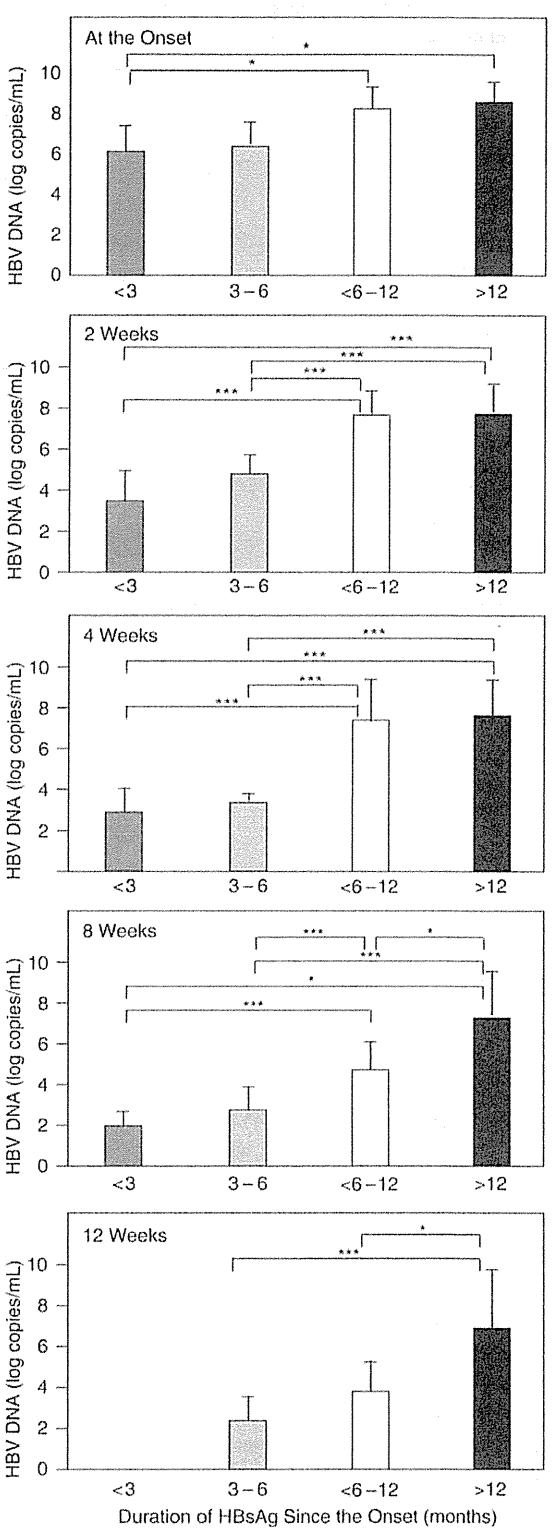

Figure 3. Levels of hepatitis B virus DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A. *P< .05; **P< .01; ***P< .001. Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

As shown in Figure 3, HBV DNA levels at 4 weeks from the onset can discriminate groups 1/2 from groups 3/4. Furthermore, HBV DNA levels at 8 weeks from the onset can distinguish group 4 from group 1, 2, or 3. Therefore, the combination of HBV DNA levels at weeks 4 and 8 would be useful for predicting the outcome. For the prediction of a chronic outcome, HBV DNA level at 8 weeks from the onset is a useful surrogate marker of the outcome as well as HBsAg level at 12 weeks. There were differences in viral kinetics among groups 1, 2, 3, and 4.

Our present study showed that 15 of the 215 patients (7.0%) cleared HBsAg from >6 to 12 months after the onset. Sixty percent of the 15 patients had HBV/A. Although these patients met the criteria of chronic infection, they finally cleared HBsAg from the sera. Therefore, we would like to propose that transition to chronic infection in acute hepatitis B be judged at 12 months from onset in patients with genotype A; further studies in larger cohorts are necessary. One reason for our proposal is the indication of antiviral treatment. Antiviral treatment in patients with acute hepatitis B is not indicated because previous studies failed to show the efficacy of antiviral treatments in the patients with acute hepatitis B [23, 24]. However, if patients who actually develop chronic infection can be identified and treated by antiviral treatment, the number of those who develop secondary infection may be markedly reduced. Evaluation of the efficacy of antiviral treatments by prospective studies, based on surrogate markers for the outcome, should be conducted as the next step. HBeAg, which was reported to be useful as a surrogate marker for chronicity, should also be assessed as a surrogate marker [25, 26].

Our study has some limitations. First, the lack of data in early stages made it difficult to study viral kinetics precisely. Second, viral kinetics in the infection with each HBV genotype were obtained from a restricted number of patients, not large enough to establish the usefulness of changes in viral markers in earlier stages of HBV infection. Third, anti-HIV was not checked in all patients due to the lack of informed consent. Fourth, HBsAg and HBV DNA were not determined 24 weeks after onset when discrimination between groups 3 and 4 may be possible more easily. Fifth, the maximum levels of ALT and bilirubin may be affected by the time of blood test. Validation studies in larger cohorts are necessary to evaluate the feasibility of our hypotheses.

In conclusion, we have shown that viral kinetics and the clinical outcome are different among patients with acute hepatitis B who are infected with HBV of distinct genotypes. HBsAg levels at 12 weeks and HBV DNA at 8 weeks after the onset would be useful to predict the clinical outcome of patients with acute hepatitis B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

IL28B minor allele is associated with a younger age of onset of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

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Abstract
Background IL28B polymorphisms were shown to be associated with a response to peg-interferon-based treatment in chronic hepatitis C (CHC) and spontaneous clearance. However, little is known about how this polymorphism affects the course of CHC, including the development of hepatocellular carcinoma (HCC). We evaluated the influence of IL28B polymorphisms on hepatocarcinogenesis in CHC patients.
Methods We genotyped the rs8099917 single-nucleotide polymorphism in 351 hepatitis C-associated HCC patients without history of IFN-based treatment, and correlated the age at onset of HCC in patients with each genotype.
Results Frequencies of TT, TG, and GG genotypes were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The mean ages at onset of HCC for TT, TG, and GG genotypes were 69.9, 67.5 and 66.8, respectively. In multivariate analysis, IL28B minor allele (TG and GG genotypes) was an independent risk factor for younger age at onset of HCC ($P = 0.02$) in males ($P < 0.001$) with higher body mass index (BMI; $P = 0.009$). The IL28B minor allele was also associated with a lower probability of having aspartate aminotransferase-to-platelet ratio index (APRI) >1.5 (minor vs. major, 46.7 vs. 58.6 %; $P = 0.01$), lower AST (69.1 vs. 77.7 IU/L, $P = 0.02$), lower ALT (67.8 vs. 80.9 IU/L, $P = 0.002$), higher platelet count (12.8 vs. 11.2 × 10^4/μL, $P = 0.002$), and higher prothrombin time (79.3 vs. 75.4 %, $P = 0.002$).
Conclusions The IL28B minor allele was associated with lower inflammatory activity and less progressed fibrosis of the liver; however, it constituted a risk factor for younger-age onset of HCC in CHC patients.

Keywords rs8099917 · Hepatocarcinogenesis · Interferon-λ · Risk allele · Fibrosis

Abbreviations

AFP	α-Fetoprotein
APRI	Aminotransferase platelet ratio index
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IL28B	Interleukin 28B
PCR	Polymerase chain reaction
peg-IFN	peg-Interferon
RIG- I	Retinoic acid-inducible gene-I
SNP	Single-nucleotide polymorphism
SVR	Sustained viral response
TLR3	Toll-like receptor 3

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Currently, patients with chronic

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hepatitis C (CHC) are treated with a combination of peg-interferon (peg-IFN) and ribavirin [2, 3]. Recently, HCV nonstructural 3/4A serine protease inhibitors combined with PEG-IFN and RBV were reported to achieve higher sustained viral response (SVR) rates in genotype 1 patients compared to conventional PEG-IFN/RBV. These triple therapies are considered to be the next standard of care for patients with CHC virus infection [4, 5].

Genetic variations near the interleukin 28B (IL28B) gene, encoding the type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [6–8] and also spontaneous clearance of HCV [9]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via the retinoic acid-inducible gene-I (RIG-I) and toll like receptor 3 (TLR3) and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [10, 11].

Genetic polymorphisms of genes involved in innate immunities are likely to influence the strength and nature of this defense system [12]. Besides its antiviral properties, IFN-λ exhibits antitumor activity; in fact, several experimental studies in cell lines and in animal models demonstrated that the activation of type III IFN induces apoptosis [13] and antitumor activities [14–16]. Thus, this genetic factor is thought to influence the natural course of HCV infection, including the development of HCC. However, little is known about the influence of IL28B polymorphisms on hepatocarcinogenesis in patients with CHC.

In the present study, we examined the association between the rs8099917 single-nucleotide polymorphism (SNP) at the IL28B locus with the age at onset of HCC and other clinical findings in patients with CHC who had no history of receiving IFN-based treatment.

Materials and methods

Patients

The patients analyzed in the present study were derived from an HCV study cohort of the University of Tokyo Hospital. In this cohort, we enrolled the patients who visited the liver clinic at our institute between August 1997 and April 2009, and agreed to provide blood samples for human genome studies along with written informed consent according with the Declaration of Helsinki. All patients underwent laboratory blood tests at the time of enrollment in our cohort. The result of the blood tests were recorded with the information on alcohol consumption and BMI of each patient. The patients who were positive for hepatitis B surface antigen and had a history of biliary disease were excluded. All subjects in our cohort were Japanese, and this research project was approved by the ethics committees of the University of Tokyo (No. 400).

From this cohort, we examined the patients who had developed new-onset HCC and received initial therapy in our institute by January 31, 2010, and with available sample for genotyping. We excluded the patients with a history of receiving IFN-based treatment. Finally, 351 patients were enrolled for this study, and the association between the age at onset of HCC and the IL28B genotype was analyzed. Patient follow-up and Diagnosis of HCC was performed as previously described [17, 18].

IL28B genotyping

Human genomic DNA was extracted from the whole blood of each patient. Genotyping for the IL28B rs8099917 T/G polymorphism was performed by polymerase chain reaction (PCR) using the TaqMan predesigned SNP Genotyping Assay (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Allele-specific primers were labeled with fluorescent dye (FAM or HEX) and used in the PCR reaction. Aliquots of the PCR products were genotyped using an allele-specific probe of the SNP on a real-time PCR thermocycler (MX3000P, Stratagene, La Jolla, CA). Samples were subjected to 50 cycles of denaturation for 15 s at 92 °C, annealing of primers for 30 s at 60 °C, and elongation for 30 s at 60 °C.

Study endpoint

We analyzed the relationship between the age at onset of HCC (the primary endpoint of this study) and host factors, including the IL28B genotypes, sex, BMI, alcoholic consumption, and HCV genotype. We also examined the relationship between IL28B genotypes and the clinical findings at the time of enrollment in our cohort (the secondary endpoint), such as the biochemical markers and presence of liver fibrosis. Liver biopsies were only available in a small number of patients (48); liver fibrosis was assessed using the aspartate aminotransferase platelet ratio index (APRI), and an APRI of >1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4) [19].

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD) while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the Chi square test, and stepwise logistic regression analyses were used to adjust the influence of IL28B genotype by other covariates such as sex, BMI (<25

or not), and alcoholic consumption (<50 g/day or not). For continuous data, the univariate associations were evaluated using the Student's t test or nonparametric Wilcoxon rank-sum test as appropriate. Since the age at onset of HCC (the primary endpoint of this study) satisfied the assumption of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$), we used stepwise regression analysis to adjust the influence of IL28B genotype by sex, BMI (<25 or not), and alcoholic consumption (<50 g/day or not). All statistical analyses were two-sided, and the threshold of the reported P values for significance was accepted as <0.05. All statistical analyses were performed using R 2.13.1 software (http://www.r-project.org).

Results

Patient characteristics

Patient characteristics are shown in Table 1. Frequencies of the rs8099917 TT, TG, and GG genotype were 74.3 % (261/351), 24.8 % (87/351), and 0.9 % (3/351), respectively. The SNP genotype distribution was in Hardy–Weinberg equilibrium (P value was not significant). We defined the IL28 major genotype as homozygous for the major sequence (TT) and the IL28B minor genotype as homozygous (GG) or heterozygous (TG) for the minor sequence. The mean age at onset of the HCC patients was 69.3 years, and approximately 60 % were male. The mean age at the time of enrollment was 67.2 years and the follow-up period was 27.9 months in average.

Table 1 Clinical characteristics and genotype distributions in the study cohort ($n = 351$)

Parameter	Values
Mean age at onset of HCC, in years	69.26 ± 8.07
Mean age at the time of enrollment, in years	67.16 ± 8.32
Male sex	200 (57.0 %)
BMI >25	70 (20.0 %)
Alcohol consumption (>50 g/day)	75 (21.4 %)
IL28B genotype	
TT	261 (74.3 %)
TG	87 (24.8 %)
GG	3 (0.9 %)
T allele frequency	0.87
HCV genotype	
Genotype 1	240 (68.4 %)
Genotype 2	91 (25.9 %)
Not tested	20 (5.7 %)

Continuous variables were represented as the mean ± standard deviation (SD) and categorical variables were as number and frequencies (%)

Primary endpoint

Table 2 shows the age at onset of patients with HCC and the associations among IL28B genotypes, sex, BMI, alcohol consumption, and HCV genotype. The mean age at onset in patients with HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). In multivariate analysis, the age at onset of HCC was significantly younger in patients with the IL28B minor genotype ($P = 0.02$, Fig. 1), independently of male sex ($P < 0.001$) and higher BMI ($P = 0.009$). The characters of HCC, such as sizes (2.56 vs. 2.40 cm, $P = 0.41$) or the numbers (1.94 vs. 2.23, $P = 0.54$) at diagnosis were not significantly different between IL28B major and minor genotypes. We also analyzed the interval between blood transfusion and the onset of HCC in 161 patients who have histories of blood transfusion which had been the major cause of HCV infection in Japan [20]. The mean interval between blood transfusion and the onset of HCC for the IL28B major and minor genotypes were 39.09 ± 9.99 and 38.86 ± 9.27 years, respectively ($P = 0.9$; data not shown).

Secondary endpoint

Table 3 shows the clinical findings and associations between the IL28B genotypes at the time of enrollment in our cohort. The IL28B major genotype was significantly associated with a higher probability of having an APRI >1.5 (58.62 vs. 46.67 %, $P = 0.01$; Fig. 2), a lower platelet count (11.15 vs. 12.80 × 10^4/μL, $P = 0.002$), a higher AST level (77.69 vs. 69.12 IU/L, $P = 0.02$), a higher ALT level (80.92 vs. 67.79 IU/L, $P = 0.002$), and a lower prothrombin time (75.40 vs. 79.27 %, $P = 0.002$) compared to the IL28B minor genotype after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment of our cohort. A lower γ-GTP level was significantly associated with the IL28B major genotype in univariate analysis, and alcoholic consumption, sex, and age were stronger factors associated with the γ-GTP level. Thus, after adjustment for these factors, the IL28B genotype was not extracted as a significant factor associated with the γ-GTP level. Histological assessments of liver fibrosis were performed in 248 patients at the time of initial therapy. The prevalence of histologically proved liver cirrhosis (F4) was 65.6 % (118/180) in patients with major genotype and 51.5 % (35/68) in those with minor genotype. The prevalence of liver cirrhosis was significantly higher in patients with major genotype after adjustment for sex, BMI, alcoholic consumption, and the age at the time of initial therapy for HCC ($P = 0.045$, data not shown).

Table 2 Factors associated with the age at onset of HCC

Variable	Mean	Standard deviation (SD)	*P* value	
			Univariate	Multivariate[a]
IL28B genotype			0.02	0.02
Major (TT)	69.88	7.97		
Minor (TG/GG)	67.48	8.17		
Sex			<0.001	<0.001
Male	67.94	8.48		
Female	71.02	7.16		
BMI			0.01	0.009
>25	66.87	9.11		
≤25	69.86	7.70		
Alcohol consumption			0.11	–
>50 (g/day)	67.78	9.37		
≤50 (g/day)	69.67	7.65		
HCV genotype			0.29	–
Genotype 1	69.65	7.59		
Genotype 2	68.22	8.79		

[a] Stepwise regression analysis for the age at onset of HCC (the dependent variable) using IL28B genotype, sex, BMI, alcohol consumption, and HCV genotype as independent variables

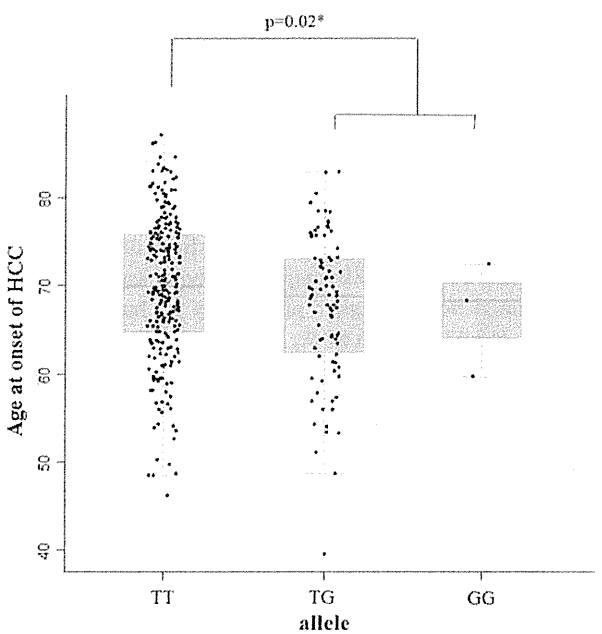

Fig. 1 *Box and whisker and dot plot* distributions of the age at onset of HCC in each genotype. The mean age at onset of HCC for the IL28B major and minor genotypes were 69.88 ± 7.97 and 67.48 ± 8.17, respectively, and was significantly higher in patients with the IL28B major genotype than in those with the minor genotype ($P = 0.02$). *P values after adjustment for sex, BMI, and alcoholic consumption

Discussion

In the present study, we evaluated the association between the IL28B polymorphism and the age at onset of HCC in patients with CHC. The IL28B minor genotype was significantly associated with younger age at onset of HCC with well known risk factors for the development of HCC such as male gender and higher BMI [21] without prior IFN-based treatment. Our previous study analyzing a susceptibility locus for HCV-induced HCC using a genome-wide association study (GWAS) could not detect the significant association between IL28B genotypes and the development of HCC in a cross-sectional distribution analysis between patients with and without HCC in more than 3,000 samples [22]. Also, IL28B alleles were not identified as a susceptibility locus for HCV-induced HCC in another GWAS study [23]. The cross-sectional distribution analyses may have underestimated the susceptibility to HCC because it could not take into consideration the future development of HCC and the duration after the past onset of HCC. Moreover, although GWAS would provide an effective and unbiased approach for revealing risk alleles for genetically complex non-Mendelian disorders, the risk of multiple comparisons made in a GWAS have resulted in reports of false positive results (Type 1 errors), and if the correction is overly conservative or the power is inadequate, false negative results (Type 2 errors) [24–26]. The relation between IL28B polymorphism and the susceptibility to HCC is still controversial. A previous study from Japan reported that the rs8099917 TT genotype was associated with a lower incidence of HCC even in non-responders to IFN based treatment [27] that was in agreement with the present study. Another study from Italy evaluating the association between genome frequency and the presence of cirrhosis due to hepatitis C, hepatitis B, alcohol use, and other factors also showed a higher prevalence of the IL28B minor allele in patients with HCC

Table 3 Associations between the IL28B genotype and clinical findings at the time of enrollment in our cohort

Variable	Mean/proportion (standard deviation; SD)		*P* values	
	Major (TT)	Minor (TG/GG)	*P* value	Adjusted *P* value[¶]
APRI >1.5[a]	58.62 % (52.38–64.66)	46.67 % (36.07–57.69)	0.07	0.01[‖]
Platelet count ($\times 10^4/\mu L$)	11.15 (5.00)	12.80 (5.43)	0.01	0.002**
AST (IU/L)	77.69 (45.14)	69.12 (38.16)	0.12	0.02**
ALT (IU/L)	80.92 (60.45)	67.79 (41.78)	0.17	0.002**
T.B (mg/dL)	0.90 (0.40)	0.83 (0.39)	0.02	–
Alb (g/dL)	3.69 (0.46)	3.71 (0.46)	0.9	–
ALP (IU/L)[b]	236.4 (81.75)	216.4 (58.96)	0.08	0.11**
γGTP (IU/L)[c]	76.83 (65.34)	87.23 (42.92)	0.005	–
PT (%)[d]	75.40 (13.36)	79.27 (13.13)	0.02	0.002**

[¶] Adjusted for sex, BMI, alcoholic consumption, and the age at enrollment (independent variables). The dependent variables of each *P* values are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, platelet count, AST, ALT and so on)

[‖] *P* value by stepwise logistic regression analysis

** *P* value by stepwise regression analysis

[a] Odds ratio (95 % CI) for major allele was 1.88 (1.13–3.11), and 95 % confidence interval (CI) of each proportion is parenthesized for this outcome

[b] Missing in 115 patients

[c] Missing in 112 patients

[d] Missing in 4 patients

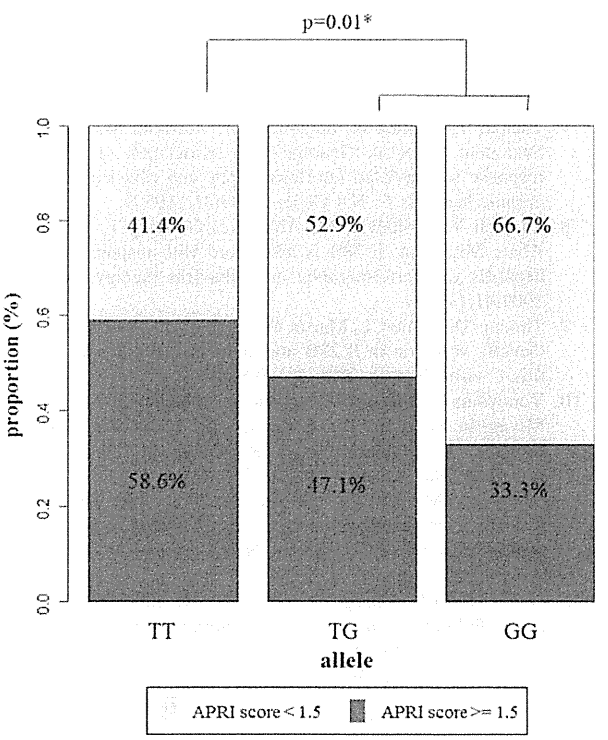

Fig. 2 *Bar plot* the proportion of having an AST-to-platelet ratio (APRI) score >1.5 in each allele. **P* values after adjustment for sex, BMI, alcoholic consumption, and the age at enrollment

compared to those without HCC [28]. However, other studies showed no relation between IL28B polymorphism and the susceptibility to HCC [29–32]. Some studies have reported the HCV genotype 1 as a risk factor associated with HCC in patients who had CHC [33–35]; however, we could not find a significant association between the HCV genotype and hepatocarcinogenesis in the present study. Our data showed no relationship between the duration of HCV infection in the patients with a history of blood transfusion. The mean age of blood transfusion was not significantly different between patients with major and minor genotypes (28.99 in major genotype vs. 27.60 in minor genotype, $P = 0.18$). Moreover, older age at HCV infection was reported to be associated with more rapid disease progression [36]. Thus, the difference in the duration of HCV infection may have little effect on the result of the present study. The IL28B genotype may have a critical role in the onset of HCC. Moreover, only about 45 % of all patients in the present study have the history of blood transfusion; hence, further analysis with larger samples may be indicated.

Previous studies evaluating patients with chronic HCV infection showed severer histological inflammatory activity and fibrosis, as well as higher ALT levels and APRI scores in patients homozygous for the IL28B major alleles [29, 32, 37, 38]. Similarly, in the present study, the IL28B

major genotype was significantly associated with a higher probability of having an APRI >1.5 and a higher ALT level; and the prevalence of histologically proved liver cirrhosis (F4) was significantly higher in patients with major genotype at the age at the time of initial therapy for HCC. Given the association between the IL28B major allele and the severe inflammatory activity or progressed fibrosis, the IL28B allele is thought to be associated with the susceptibility to HCC via a mechanism that is independent of controlling an activity of HCV infection.

Recent experimental studies have suggested that IFN-λ has an antitumor activity. In esophageal cancer cell lines expressing IFN-λ receptor complexes, IFN-λ1 suppressed growth via the induction of the G1 phase arrest or apoptosis [39]. An antitumor activity of IFN-λ was also shown in the B16 melanoma, BNL hepatoma, Colon 26, and neuroendocrine BON1 tumor cells [40–43]. One probable explanation for the paradoxical result of the present study is that the more aggressive inflammatory activity of patients with IL28B major genotype may reflect a stronger immune response to the virus, which may also have anti-tumor effects. However, the innate immune responses and antitumor activity via IFN-λ, as well as the mechanism underlying the association of the IL28B genotype, have not been elucidated. Further studies are needed to determine the functional role of the IL28B gene in relation to the course of chronic HCV infection, including hepatocarcinogenesis.

Because of the retrospective design, this study is limited by the absence of some important clinical details such as information about the histological findings of fibrosis and inflammation. Although the APRI is a useful index for the prediction of fibrosis, the limitation of this score has been reported in previous studies [44, 45]. Prospectively designed studies are needed to confirm our findings. However, observing chronic HCV-infected patients without antiviral treatment would be nearly impossible in the future. In this regard, the present study may have important implications.

In conclusion, the IL28B minor genotype was associated with a younger age of onset of HCC in patients with CHC, and this association was completely independent of the response to IFN-based treatment. Hepatocarcinogenesis appeared to be suppressed in patients who had CHC with the IL28B major genotype, despite higher inflammatory activity and progressed fibrosis of liver. The current findings may provide a clinically important information in the follow-up or HCC screening of cirrhotic patients.

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Conflict of interest None of the authors have any conflicts of interest.

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