alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was 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. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: logit(p) = −3.905+0.083*age+(−0.929)*sex+(−0.684)**DPB1*02:01*+1.814*c1+(−0.478)*c2+0.782*c3. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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CASE STUDY Open Access

Efficacy and tolerability of Entecavir for hepatitis B virus infection after hematopoietic stem cell transplantation

Jun Aoki[1], Kiminori Kimura[2*], Kazuhiko Kakihana[1], Kazuteru Ohashi[1] and Hisashi Sakamaki[1]

Abstract

Introduction: Hepatitis B virus (HBV) flare is a serious problem following hematopoietic stem cell transplantation (HSCT), and the mortality rate is high if severe hepatitis occurs.

Case description: Although Entecavir (ETV) is a standard antiviral drug for HBV infection, the efficacy and safety of ETV therapy in HSCT are still unclear.

Discussion and Evaluation: To examine the efficacy and tolerability of ETV treatment in HSCT, we retrospectively identified 5 patients who received ETV for treatment of HBsAg carrier among patients undergoing HSCT in our institute. We reviewed their clinical information such as clinical course of serum HBV DNA levels, administration period and dose of ETV, and adverse events. There were no episodes of HBV flare or reactivation after HSCT in all patients during the observation period, as a 10-fold rise in HBV DNA levels or positive conversion of HBsAg were not observed.

Conclusion: ETV monotherapy is effective and safe for HBsAg carrier patients following HSCT.

Keywords: Hepatitis B virus; Hematopoietic stem cell transplantation; Entecavir

Introduction

Hepatitis B virus (HBV) flare and reactivation after hematopoietic stem cell transplantation (HSCT) is a life-threatening complication in patients with HBV infection (Liang et al. 1999). HBV related hepatitis is generally observed in hepatitis B surface antigen (HBsAg)-positive and/or HBV DNA-positive patients (Hui et al. 2005). Recently, however, HBV reactivation was reported in patients with even though resolved HBV infection that was indicated negative HBsAg and positive anti-hepatitis B core antibody (HBcAb) and/or HBsAb) at a lower rate (Knoll et al. 2004). It is well established that the frequency of HBV reactivation is higher in HSCT patients (Hammond et al. 2009). The underlying mechanism of HBV flare and reactivation following HSCT is likely to be related to impaired cellular immunity caused by prior chemotherapy and conditioning regimens. Furthermore, administration of immunosuppressive agents, including calcineurin inhibitors and steroids for graft-versus-host disease (GVHD), may exacerbate HBV replication (Xunrong et al. 2001).

HBV-infected patients at high risk of HBV flare and reactivation are recommended to receive preemptive antiviral therapy following HSCT. Lamivudine (LAM), an analogue of cytidine, is available for antiviral therapy in HSCT, (Tomblyn et al. 2009) and inhibits HBV reverse transcriptase, resulting in suppression of HBV replication. Several studies have reported the efficacy of LAM treatments in HSCT patients (Hsiao et al. 2006; Giaccone et al. 2010).

However, LAM treatment has the potential to induce development of drug-resistant mutations owing to the low genetic barrier. Since a small number of mutations are required for LAM resistance, the incidence of LAM-resistant HBV has recently been increasing, and LAM resistance is associated with a rebound in viral load and hepatitis. In fact, LAM treatments showed high resistance and recurrence rates in patients with chronic

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hepatitis B infection (CHB) undergoing liver transplantation (Perrillo et al. 2001; Mutimer et al. 2000; Lo et al. 2001). Similarly, the appearance of drug-resistant mutants and HBV DNA breakthrough within LAM treatments has been reported in HSCT patients (Hsiao et al. 2006). Thus, more effective agents with lower resistance rates are required in both liver transplantation and HSCT.

Entecavir (ETV), a cyclopentyl guanosine nucleoside analogue, has been approved for treatment of patients with CHB. ETV inhibits reverse transcriptase, DNA replication and transcription. Compared with LAM, ETV has greater antiviral potency and a higher genetic barrier to resistance (Lai et al. 2002). Several studies have shown the superiority of ETV treatments in liver transplantation (Xi et al. 2009; Fung et al. 2011), however, ETV treatment for patients undergoing HSCT has not been reported, and its role in HSCT remains unclear. Here, we describe HBsAg carrier patients administered ETV for treatment following HSCT, and consider its efficacy and tolerability.

Patients and methods

Patients

This study was approved by the local medical ethics committee of Tokyo Metropolitan Komagome Hospital. We retrospectively identified HBsAg carrier patients (serum HBsAg-positive and HBV-DNA positive) who received ETV for prophylaxis of HBV flare among patients undergoing HSCT between September 2006 and August 2011. We Laboratory data and clinical information were obtained from our institution's electronic medical records.

Hematopoietic stem cell transplantation methods

In our institution, myeloablative conditioning regimens were administered to allogeneic recipients aged <60 years, while elderly patients received fludarabine-based reduced-intensity regimens. GVHD prophylaxis usually comprised short-course methotrexate and cyclosporine A (CsA) or tacrolimus (FK506). For acute GVHD treatment, methylprednisolone (mPSL) 2 mg/kg i.v. in divided dose daily was administered. Antibacterial prophylaxis was provided by tosufloxacin. Steroid-resistant GVHD was treated with a steroid pulse (mPSL 1000 mg i.v. in divided dose daily for 3 days) or mycophenolate mofetil (MMF) 1000 mg twice daily p.o.. Antifungal prophylaxis consisted of fluconazole or itraconazole. Acyclovir or valacyclovir was administered for herpes simplex virus prophylaxis and ganciclovir or foscarnet was administered against cytomegalovirus reactivation.

Entecavir therapy

ETV 0.5 mg once daily p.o. was administered to patients with HBsAg-positive as primary treatments. The ETV dose was adjusted according to the kidney function. None of the patients received LAM or hepatitis B immunoglobulin at the time of transplantation or during the post-transplant period.

Hepatitis B virus assay

The levels of HBsAg, anti-HBc and anti-HBs were determined using commercially available chemiluminescence enzyme immunoassay kits (LUMIPULSE Presto HBsAg, LUMIPULSE Presto HBsAb-N, LUMIPULSE Presto HBcAb-III; Fujirebio, Tokyo, Japan). The cut-off index (COI) of the assay of HBsAg, anti-HBs and anti-HBc was 1.0, 10 mIU/mL and 1.0, respectively. The serum HBV DNA concentrations were quantified using the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics, Basel, Switzerland). The four major HBV genotypes (A–D) were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed against distinct epitopes on the preS2-region products using commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co. Ltd., Tokyo, Japan) (Orito et al. 2001). HBV DNA sequences bearing the core promoter and precore or core regions were amplified by PCR with heminested primers. The PCR products were directly sequenced by the dideoxy chain termination method using a Big Dye Terminator (Applied Biosystems, Foster city, CA) and an ABI PRISM 3100-avant analyzer (Aritomi et al. 1998).

Case description

Patient profiles

Among the 376 patients who received HSCT in our hospital between September 2006 and August 2011, six HBsAg-positive and HBV-DNA positive patients were identified (Figure 1). Of those patients, five patients received ETV for prevention of HBV flare (Table 1). Four patients were inactive chronic hepatitis B (ICHB) (#1, #2, #4 and #5) and other patients were CHB (#3), respectively. Patient #5 has developed HBV reactivation during pre-transplantation therapy at previous treated hospital. Four patients underwent allogeneic HSCT, while one patient underwent autologous HSCT (#1). Within 4 allogeneic recipients, patient #4 received a graft from a matched related donor, while the other three patients received grafts from matched unrelated donors. All of donors were serum HBsAg negative. Myeloablative conditioning regimens were administered to three allogeneic recipients and a fludarabine-based non-myeloablative conditioning regimen was administered to the remaining allogeneic recipient. For GVHD prophylaxis, one patient (#2) received CsA and methotrexate and three patients received FK506 and methotrexate. At the time of transplantation, two patients had undetectable serum HBV DNA levels. Three patients were HBV genotype C and one patient was genotype B. In addition,

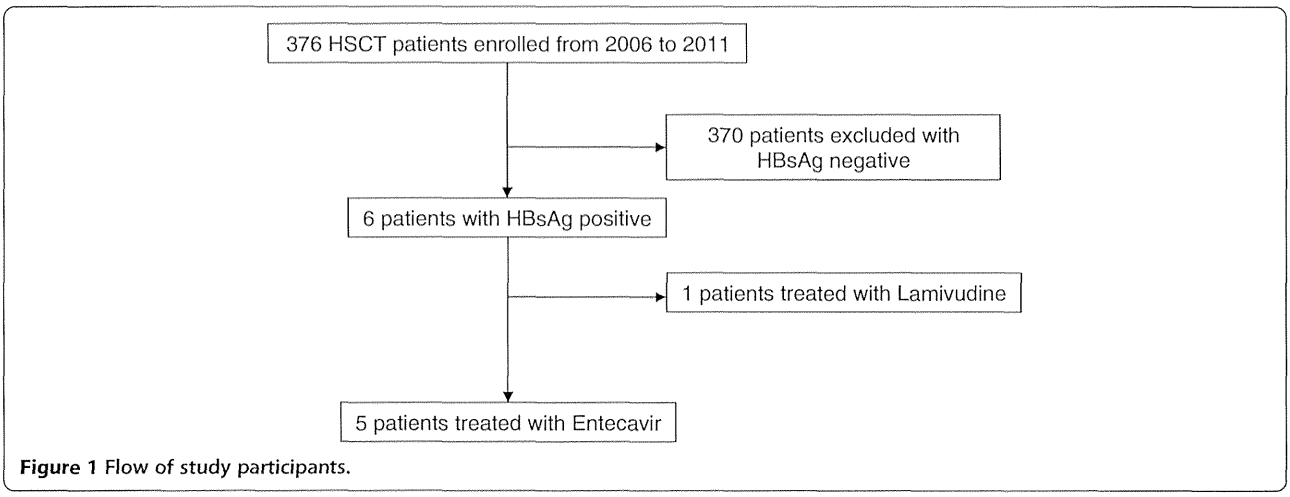

Figure 1 Flow of study participants.

HBV core and precore promotor mutations were not detected in all patients (Table 2).

ETV administration can prevent HBV flare and reactivation following HSCT.

The median observation period was 12.5 months (range, 2–50 months). ETV 0.5 mg once daily p.o. was administered as primary treatments for HBV infection. The ETV dose was increased after HSCT with patient #4 due to elevation of HBV viral load. Nevertheless all allogeneic-HSCT recipients suffered from mucous membrane disorder, no patient discontinue ETV oral administration. #4 and #5 patients developed renal disorder due to toxoplasmosis and adverse event of FK506 respectively. The ETV doses were reduced according to the kidney function in these patients. ETV was well tolerated in all cases and no patients discontinued ETV during the follow-up period for severe adverse events. There was no adverse drug interaction between ETV and other drugs. Median neutrophil engraftment time was 20 days (range 16–32 days) in allogeneic HSCT patients (#2-5). No cytopenia due to ETV treatment was observed in all patients. At the last follow-up, three patients (#1, #2 and #4) had died and the causes of mortality were aspergillus pneumonia, aggravation of underlying disease and toxoplasmosis, respectively.

In respect to immunosuppressive agent, two patients (#4 and #5) received mPSL 2 mg/kg i.v. in divided dose daily for acute GVHD in a short period. Patients #2 and #3 suffered chronic GVHD and continued to receive systemic steroid (mPSL 0.5 mg/kg and PSL 0.5 mg/kg i.v. in divided dose daily, respectively) and FK506 for the observation period. Patient #2 received a steroid pulse (mPSL 1000 mg i.v. in divided dose daily for 3 days) against exacerbation of chronic GVHD. Nevertheless, steroid treatment continued during GVHD in these patients and HBV DNA was not detected, indicating that ETV effectively protected against HBV flare and reactivation. In patients #3 and #4, MMF was also added for steroid-refractory GVHD.

There were no episodes of HBV flare or reactivation after HSCT in all patients during the observation period, as a 10-fold rise in HBV DNA levels or positive conversion of HBsAg were not observed. As shown in Figure 2, although patient #3 showed a significantly high HBV DNA level during the HSCT period, HBV flare was not observed. Serum HBsAg was not detected in patient #3

Table 1 Characteristics of 5 HSCT recipients treated with Entecavir

Case	Age (y)	Sex	Disease	Transplantation	GVHD prophylaxis	Steroid administration	cGVHD	Conditioning regimen	Observation period	Sstatus	Cause of death
#1	64	F	MM	Autologous	(–)	No	-	Mel	2 M	Dead	MM
#2	55	F	MDS-RAEB	Allogeneic	FK + MTX	Yes	Yes	BU + CY	9 M	Dead	Aspergillus Pneumonia
#3	24	F	MDS-RCMD	Allogeneic	FK + MTX	Yes	Yes	BU + CY	50 M	Alive	-
#4	58	F	AML	Allogeneic	CsA + MTX	Yes	No	BU + CY	4 M	Dead	Toxoplasmosis
#5	63	F	T-LBL	Allogeneic	FK + MTX	Yes	No	Flu + Mel + TBI(4Gy)	16 M	Alive	-

Abbreviation: MM multiple myeloma, *MDS* myelodysplastic syndrome, *RAEB* refractory anemia with excessive blast, *RCMD* refractory cytopenia with multilineage dysplasia, *AML* acute myeloid leukemia, *T-LBL* T lymphoblastic lymphoma, *FK* tacrolimus, *CsA* ciclosporin A, *Mel* melfalan, *BU* busulfan, *CY* cyclophosphamide, *TBI* total body irradiation, *Flu* fludarabine.

Table 2 HBV markers of 5 HSCT recipients treated with Entecavir

Case	Genotype	HBsAg	HBsAb	HbcAb	BCP/Precore	Initial serum HBV DNA (xLog Copies/ml)	Flare after HSCT
#1	ND	(+)	(−)	(+)	ND	4	No
#2	C	(+)	(−)	(+)	ND	Undetectable	No
#3	B	(+)	(−)	(+)	Wild type	>7.6	No
#4	C	(+)	(−)	(+)	Wild type	Undetectable	No
#5	C	(+)	(−)	(+)	Wild type	7.4	No

Abbreviation: BPC basal core promoter, *ND* not determined.

at 18 months after the start of ETV. Subsequently, patient #3 showed reduction of HBV DNA to an undetectable level at the last follow-up.

Discussion and evaluation

A phase III trial reported that ETV has superior virological, histological and biochemical efficacy as an antiviral drug for HBV infection compared with LAM (Chang et al. 2006). Furthermore, ETV showed a low resistance rate compared with LAM (Tenney et al. 2009). Recent case reports have described successful treatment of HBV reactivation after HSCT with ETV (Christopeit et al. 2010; Milazzo et al. 2011). Therefore, ETV is a strong candidate as a substitute for LAM for HBV prophylaxis in HSCT.

The present study has shown the safety and efficacy of ETV treatment for protection against HBV flare after HSCT. Similar to previous reports for liver transplantation, (Fung et al. 2011) all the patients continued ETV through to the last follow-up without any intolerable adverse events. Intense immunosuppression during HSCT owing to the conditioning regimen and GVHD prophylaxis is a serious risk for HBV flare and reactivation. However, no HBV flare after HSCT was documented during our observation period with ETV treatment. Although blood count after HSCT is not stable, no cytopenia due to ETV was observed. Furthermore, no drug

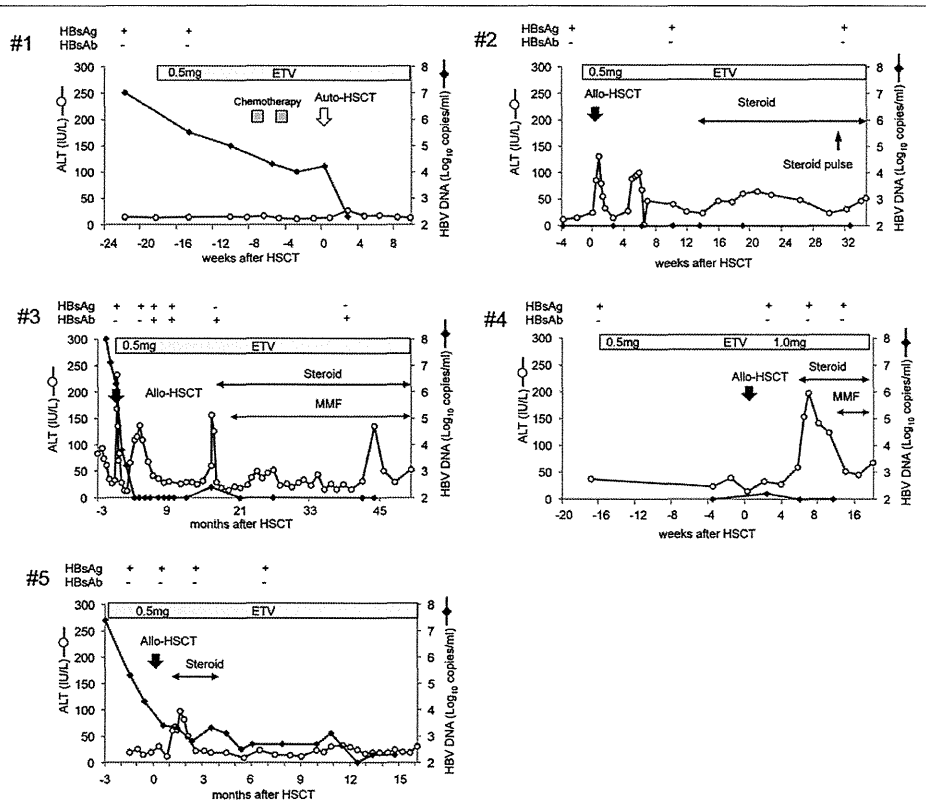

Figure 2 Clinical courses of HSCT patients treated with ETV. The X-axis shows the time course, and the Y-axes show the ALT levels and HBV DNA copy numbers, respectively. Transitions of HBsAg and HBsAb are shown at the top. No HBV DNA elevation was observed except for patient #3. HBV DNA of patient #3 became detectable level transiently. ALT elevation after HSCT was observed in all allogeneic HSCT recipients (#2-5). Causes of ALT elevation were considered GVHD or VOD.

interaction with ETV was documented after HSCT. Our study has demonstrated that ETV treatment is a promising candidate for HBV flare and reactivation prophylaxis in HSCT, similar to the case for liver transplantation.

Seroclearance of HBsAg and HBV DNA was observed with patient #3, despite a high HBV DNA level during the HSCT period. The timing of the serum HBsAg clearance was consistent with that in a liver transplantation study (Fung et al. 2011). Of the 5 patients tested, HBsAb was detectable with 1 patient (#3). The time of detection of HBsAb was 6 weeks after HSCT. HBsAb with patient #3 was detectable during the follow-up period. This positive conversion of HBsAb might be associated with HBsAg clearance (Fung et al. 2011).

All allogeneic HSCT patients showed serum ALT elevation after HSCT. Almost all cases showed ALT elevation without HBV DNA elevation or positive conversion of HBsAg, and we considered that these liver injuries were caused by GVHD or drug-induced hepatotoxicity. Serum ALT elevation and positive conversion of HBV DNA were observed at the same time with patient #3 at 17 months after the HSCT period. However, chronic GVHD symptoms, such as skin keratinization and oral dryness, were exacerbated around the same time and ALT decreased after systemic steroid and MMF administration. Moreover, the positive reaction for HBV DNA was transient and the presence of HBsAg conversely became negative. Collectively, based on these findings, we suggest that the ALT elevation might have been caused by chronic GVHD.

There are some limitations in this study, since it was retrospective and the number of patients was small. Half of the patients died within 1 year after HSCT and their observation periods were insufficient. In future, a large prospective study of ETV prophylaxis in HSCT is required.

Conclusions

In conclusion, our study showed that ETV monotherapy may be effective and safe after HSCT for patients with HBV. Our study will contribute to future clinical trials.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

K.K and H.S designed this study. J.A and K.K wrote the manuscript. J.A collected clinical information. K.K and K.O contributed the collection of clinical information. All authors read and approved the final manuscript.

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Hepatology Research

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Editorial

Should we try antiviral therapy for hepatitis C virus infection with pyoderma gangrenosum-like lesions?

See article in *Hepatology Research* **44**: 238–245
Eradication of hepatitis C virus could improve immunological status and pyoderma gangrenosum-like lesions
Yasuteru Kondo, Tomoaki Iwata, Takahiro Haga, Osamu Kimura, Masashi Ninomiya, Eiji Kakazu, Takayuki Kogure, Tatsuki Morosawa, Setsuya Aiba and Tooru Shimosegawa

Pyoderma gangrenosum (PG) is a rare, ulcerative and painful neutrophilic dermatosis without any infectious signs. PG usually begins with pustules, red papules, plaques or nodules that rapidly grow to ulcerations with undetermined purple borders.[1] Five clinical variants have been characterized: classic, bullous, pustular, vegetative and peristomal types. Although PG is idiopathic in 25–50% of cases, approximately 50% of clinical cases are associated with underlying systemic diseases such as inflammatory bowel disease, rheumatological disease, paraproteinemia, hematological malignancies, and HIV and hepatitis virus infection.[2] PG may be due to neutrophilic dysfunction, autoinflammation, genetic factors and/or inflammatory cytokines.[3] PG is diagnosed by excluding other cutaneous ulcerative diseases by biopsy and identification of clinical features, because PG has no specific histopathological features. There is no gold standard for treatment of PG, therefore, therapy should focus on the associated underlying systemic disease. In addition to local wound management and pain control, topical or systemic agents have been considered. For mild and superficial lesions, topical agents (e.g. corticosteroids, tacrolimus and cyclosporin) are administrated. When more effective treatment is required, corticosteroids and cyclosporin are considered as first-line systemic agents. Recently, the use of tumor necrosis factor (TNF)-α inhibitors for PG has emerged, and infliximab is the only efficacious systemic agent.[4,5]

Hepatitis C virus (HCV) infection may present with various cutaneous manifestations such as urticaria, pruritus, cryoglobulinemia, erythema multiforme, granuloma annulare, porphyria cutanea tarda, vasculitis, lichen planus, vitiligo, erythema nodosum, pityriasis rubra pilaris and perniosis-like lesions.[6] PG is thought to be an extrahepatic cutaneous manifestation of HCV infection, although its frequency is low.[7] Only few cases of PG associated with chronic hepatitis C have been reported. Smith *et al.* reported the first case of chronic hepatitis C and PG.[8] Several skin biopsies were performed, but leukocytoclastic vasculitis in cutaneous ulcerative lesions on the lower legs, with typical clinical manifestations of PG, was not observed. Therefore, it was suggested that the PG-like lesion was not associated with mixed cryoglobulinemia (MC) because of the absence of histological findings. Treatment with interferon (IFN)-α-2a was started and the lesion improved within 5 weeks. Keane *et al.* reported another case, in which biopsies of ulcerative lesions in the lateral aspect of the calf showed a dense mixed lymphocytic infiltration in the dermis and vasculitis in the erythematous ulcer rim. Treatment with topical clobetasol and systemic minocycline resulted in improvement within several weeks, and there was no recurrence during 4 months of follow-up.[9] Currently, no specific treatment regimen for PG associated with chronic hepatitis C has been established, because there are few case reports and the pathogenic mechanism is still unknown.

In general, PG is also associated with the administration of drugs such as granulocyte colony-stimulating factor,[10] antipsychotic agents and IFN-α.[11] Indeed, IFN-α treatment has some well-known cutaneous side-effects such as dry skin, hair loss and vitiligo.[12,13] Furthermore, cutaneous ulcerations, indurated erythema and leukocytoclastic vasculitis may occur at IFN-α injection sites. The pathogenesis of local cutaneous reactions against IFN-α is still unknown. Several case reports have suggested that PG results from IFN-α treatment during the course of hematological malignancies. One case was improved by treatment with cyclosporin A and prednisone.[14] In addition, serum levels of TNF-α, interleukin

(IL)-6 and soluble IL-2 receptor increased when the cutaneous lesions appeared and returned to normal levels when the lesion healed. This indicated that the onset of PG was required for elevated inflammatory cytokine levels.[14] Furthermore, Yurci *et al.* reported the first PG case resulting from pegylated (PEG)-IFN-α-2a during the treatment of chronic hepatitis C.[15] Four weeks after starting PEG-IFN-α-2a treatment, cutaneous ulcerative lesions were clinically suspected as PG appeared, and treatment was discontinued after 8 weeks. After withdrawal of PEG-IFN-α-2a, PG-like lesions improved. Thus, PEG-IFN-α-2a may trigger cutaneous side-effects because pegylation allows stable serum levels of drugs.

Thus, the pathogenesis of PG with chronic hepatitis C is still unclear, suggesting that PG related to chronic hepatitis C is not associated with MC, but with other immunological mechanisms. In a study reported in this issue, Kondo *et al.* examined whether peripheral blood mononuclear cells (PBMC), including activated B cells, T-helper (Th)1 cells, Th2 cells, Th17 cells and $CD4^+CD25^+IL7R^-$ regulatory T cells (Treg) were involved in PG disease activity.[16] They demonstrated that Th17 cell numbers were markedly higher in patients with chronic hepatitis C and PG compared with those without extrahepatic immunological complications. In addition, after clearance of HCV following IFN therapy, the abnormal immunological status returned to normal and the PG-like lesions recovered without immunosuppressive therapy. These findings suggest that Th17 cells have an important role in the onset of PG, although it is still unknown whether these cells are HCV specific. As reported in this issue, HCV-infected CD4 T cells are required for disease progression, suggesting that HCV-specific Th17 cells are responsible for development of PG.

Th17 immunity has been identified during HCV infection, and PBMCs from HCV antibody positive patients secrete IL-17, IFN-γ, IL-10, and transforming growth factor (TGF)-β in response to stimulation with HCV non-structural protein (NS)4. The authors suggested that both HCV-specific Th1 and Th17 cells were suppressed by NS4-induced production of the innate anti-inflammatory cytokines, IL-10 and TGF-β.[17] This suggests that HCV infection suppresses antigen-specific Th17 cell functions and is responsible for induction of persistent chronic infection.

Although it has not been demonstrated that PG-like lesions are not associated with cryoglobulinemia, Kondo *et al.* have suggested an alternative hypothesis in which the pathogenesis of PG-like lesions with chronic hepatitis C is associated with Th17-related immunity. To the best of our knowledge, this report is the first evidence that the onset of PG is related to the number and cytokine production of Th17 cells.

According to the study reported in this issue and other recent reports, PG may be induced by IFN treatment as well as HCV infection. Kondo *et al.* have demonstrated that IFN treatment is more effective than immunosuppressants for the treatment of PG in the case of HCV infection. Thus, when a case presents with HCV and PG it may be useful to consider IFN treatment. The validity of IFN for PG should be verified by accumulating a greater number of clinical cases.

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Original Article

Risk factors for hepatitis B virus recurrence after living donor liver transplantation: A 17-year experience at a single center

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Aim: The incidence of hepatitis B virus (HBV) recurrence after liver transplantation (LT) has been reduced by prophylaxis with hepatitis B immunoglobulin (HBIG) and nucleoside analogs, but the factors associated with HBV recurrence are unclear. The aim of this study was to determine the risk factors associated with HBV recurrence after living donor LT (LDLT).

Methods: A retrospective review was performed for 45 patients (28 male and 17 female; median age, 54 years) who underwent LDLT for HBV-related liver disease and were followed up for at least 6 months between October 1996 and June 2013. The virological data, tumor burden, antiviral therapy and immunosuppressive therapy were evaluated and compared between the HBV recurrence ad non-recurrence groups.

Results: Seven of the 45 patients (15.6%) developed post-LT HBV recurrence. The median interval between LDLT and HBV recurrence was 23.7 months (range, 0.8–35.9). Three of the seven patients (42.9%) developed recurrence after cessation of HBIG, and three (42.9%) were cases with hepatocellular carcinoma (HCC) recurrence after LDLT. The remaining case underwent transplantation from a donor with positive hepatitis B surface antigen. Based on the univariate and multivariate analyses, HBIG cessation (hazard ratio [HR], 20.17; 95% confidence interval [95% CI], 2.091–194.593; $P=0.009$) and HCC recurrence (HR, 30.835; 95% CI, 3.132–303.593; $P=0.003$) were independent risk factors for HBV recurrence after LDLT.

Conclusion: In LDLT patients, cessation of HBIG and HCC recurrence were risk factors associated with HBV recurrence, so careful monitoring for serological HBV markers is needed in patients with these factors.

Key words: hepatitis B immunoglobulin, hepatitis B virus recurrence, hepatocellular carcinoma, living donor liver transplantation

INTRODUCTION

HEPATITIS B IS a leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide. Before the advent of an effective means for preventing the virtually universal re-infection of the graft, the outcome of liver transplantation (LT) for hepatitis B virus (HBV)-related liver diseases was dismal, and this often led to HBV recurrence rates greater than 80% and mortality rates of 50% at 2 years.[1] Prophylaxis with hepatitis B immunoglobulin (HBIG) and nucleoside analogs, such as lamivudine, has markedly decreased the recurrence rate of HBV through their synergistic effects.[2] However, approximately 10% of transplanted patients still develop HBV recurrence.[3,4] In previous studies, the factors associated with HBV recurrence were reported to be a high pre-LT HBV DNA level,[5,6] hepatitis B e-antigen (HBeAg) positivity,[7] non-fulminant hepatitis B,[8] immunosuppression from steroids and systemic chemotherapy,[9] and pre-LT HCC and post-LT HCC recurrence.[10–12]

In this study, we noted that a group of patients still developed HBV recurrence after living donor LT (LDLT). We analyzed a retrospective series of 45 patients who underwent LDLT for HBV-related liver disease and were followed for at least 6 months, and we evaluated their virological and biochemical data, tumor burden, antiviral therapy and immunosuppressive therapy, as well as the eventual development of HCC recurrence. The aim of this

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study was to determine the risk factors associated with HBV recurrence after LDLT.

METHODS

Immunoprophylaxis

ALL PATIENTS WERE treated with a combination HBIG (Hebsbulin-IH; Japan Blood Products Organization, Tokyo, Japan) and at least one nucleoside agent (lamivudine, adefovir, entecavir or a combination thereof) for HBV prophylaxis after transplantation.

Nucleoside analog therapy was initiated when the patients were referred to the hospital and indicated for LDLT, if they had not yet been treated with this agent. For fulminant hepatitis B patients, lamivudine or entecavir was initiated when the etiology was verified to be HBV. HBIG at 10 000 U was administrated i.v. during the anhepatic phase during the operation, followed by 5000 U/day for 1 week after the LDLT. Thereafter, 3000–5000 U of HBIG was administrated every 2–3 months. The targeted level of hepatitis B surface antibody (HBsAb) was more than 500 IU/L for the cases before March 2004, and was more than 200 IU/L for the first year and more than 100 IU/L thereafter for the cases after April 2004.[13]

Immunosuppression

A calcineurin inhibitor, such as cyclosporin or tacrolimus, with or without mycophenolate mofetil, was used as the immunosuppressive therapy after LDLT. The immunosuppressive dosing was adjusted according to the therapeutic drug levels and renal function. A gram of methylprednisolone was given after reperfusion, and the dose was tapered from 200 mg to 20 mg daily in a week, then switched to oral prednisolone, and finally tapered off in 6 months.

Serological monitoring

The recurrence of the HBV was defined as the appearance of the hepatitis B surface antigen (HBsAg) in the serum after LDLT.[8,10] Standard biochemical tests of liver function were performed at each follow-up visit. The serum HBsAg, HBsAb and HBV DNA were tested monthly. From 1996 until March 2004, the HBV DNA levels were quantified with a transcription-mediated amplification assay (Mitsubishi Chemical Medience, Tokyo, Japan), which has a detection range of 3.7–8.7 log genome equivalents (LGE)/mL. Thereafter, all HBV DNA levels were tested with a polymerase chain reaction (PCR) assay (SRL, Tokyo, Japan), which has a detection range of 2.6–7.6 log copies/mL. The YMDD mutant was detected using a PCR enzyme-linked minisequence assay (SRL).

Surveillance for HCC recurrence

After LDLT, patients with known HCC were followed regularly in our outpatient hepatology clinic. Surveillance with computed tomography was performed every 3–4 months. If there were concerns about HCC recurrence, whole-body computed tomography or magnetic resonance imaging was ordered at the discretion of the patient's physician.

Statistical analysis

Continuous variables were compared by the Mann–Whitney *U*-test. Categorical variables were compared by the χ^2-test and Fisher's exact tests. A Cox regression analysis was used to determine the predictors of the time to HBV recurrence. The variables reaching statistical significance by the univariate analysis were then included in the multivariate analysis. The cumulative incidence of patient survival and HBV recurrence after LDLT were calculated using the Kaplan–Meier method, and the difference was evaluated by the log-rank test. A value of $P < 0.05$ was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS, Chicago, IL, USA).

RESULTS

Demographics

A RETROSPECTIVE REVIEW of the medical record database was performed for 45 patients (28 male and 17 female; median age, 54 years) who underwent LDLT for HBV-related liver disease and were followed up at least 6 months between October 1996 and June 2013 at Kyushu University. Table 1 summarizes the patients' data at the time of LDLT. The median follow-up time after LDLT was 66 months (range, 9–174). Hepatitis C virus co-infection was present in three patients (6.7%). HCC was present in 28 patients (62.2%). Twenty-five patients with HCC (89.3%) were diagnosed by preoperative computed tomography or magnetic resonance imaging, whereas three patients (10.7%) were diagnosed incidentally by a pathological examination of the explant. Six patients (21.4%) had evidence of vascular invasion. HCC beyond the Milan criteria was present in eight patients (28.8%) by preoperative imaging, and in 15 patients (53.8%) by explant pathology, respectively.

Pre-LT HCC therapy was administrated to 17 of the 28 patients with HCC (60.7%). Among them, two patients (7.1%) received pre-LT systemic chemotherapy, which consisted of a combination of an antimetabolite (5-fluorouracil), platinum-based agent (cisplatin) and anthracycline (epirubicin). Fourteen patients (50%) received a combination of local ablative therapy in the

Table 1 Characteristics of the 45 patients with hepatitis B virus-related liver disease

	HBV-related transplantation ($n=45$)
Age (years)	54 (31–67)
Sex	
Male	28 (62.2%)
Female	17 (37.8%)
HCV co-infection	3 (6.7%)
Primary disease	
Acute liver failure	12 (26.7%)
Liver cirrhosis	33 (73.3%)
HBsAg positivity	41 (91.1%)
HBeAg positivity	10 (22.2%)
HBV DNA (log copies/mL)	
Unknown	5 (11.1%)
<2.6	20 (44.4%)
2.6–5	8 (17.8%)
>5	12 (26.7%)
HCC	28 (62.2%)
By preoperative imaging	25 (89.3%)
Incidental on explant	3 (10.7%)
Vascular invasion	6 (21.4%)
Beyond Milan criteria by:	
preoperative imaging	8 (28.8%)
explant pathology	15 (53.8%)
Pre-LT systemic chemotherapy	2 (7.1%)
Post-LT systemic chemotherapy	3 (10.7%)
Pre-LT antiviral therapy	
None	4 (8.9%)
LAM	22 (48.9%)
LAM + ADV	6 (13.3%)
ETV	13 (28.9%)
Corticosteroid therapy >6 months	12 (26.7%)
Median follow-up period (months)	66 (9–174)

Qualitative variables are expressed as the numbers of patients, with percentages in parentheses, and quantitative variables are expressed by the medians, with ranges in parentheses.
ADV, adefovir; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LAM, lamivudine; LDLT, living donor liver transplantation.

form of arterial chemoembolization, radiofrequency ablation and/or an ethanol injection prior to LDLT. One patient (3.6%) underwent surgical resection of the tumor prior to LDLT.

Of the 28 patients with HCC, three patients (10.7%) received post-LT systemic chemotherapy. Among them, one patient received post-LT chemotherapy for the treatment of combined hepatocellular and cholangiocellular carcinoma. The post-LT chemotherapy regimens included a combination of antimetabolites (5-fluorouracil, gemcitabine), a platinum-based agent (cisplatin), anthracycline (epirubicin) and multikinase inhibitor (sorafenib). Prior to LDLT, 41 patients (91.1%) were treated with antiviral therapy consisting of lamivudine, adefovir, entecavir or a combination thereof.

Overall HBV recurrence

Seven of the 45 patients (15.6%) developed post-LT HBV recurrence. The median interval between LDLT and the development of HBV recurrence was 23.7 months (range, 0.8–35.9). The overall actuarial rates of HBsAg recurrence after LDLT at 1, 3 and 5 years were 6.7%, 17.9% and 17.9%, respectively (Fig. 1). Table 2 shows the results of the univariate analysis of risk factors associated with HBV recurrence after LDLT. The factors significantly associated with HBV recurrence were cessation of HBIG ($P=0.039$) and HCC recurrence ($P=0.021$). According to the multivariate analysis, the same factors were found to be independently associated with a higher risk of HBV recurrence after LDLT: cessation of HBIG (hazard ratio [HR], 20.17; 95% confidence interval [95% CI], 2.091–194.593; $P=0.009$) and HCC recurrence (HR, 30.835; 95% CI, 3.132–303.593; $P=0.003$) (Table 3).

HBIG cessation

Three of the seven patients (42.9%) with HBV recurrence were cases in whom HBIG was suspended during combined prophylaxis after LDLT (Table 4, cases 1–3). In case 1, the HBIG was suspended while the patient received HBV

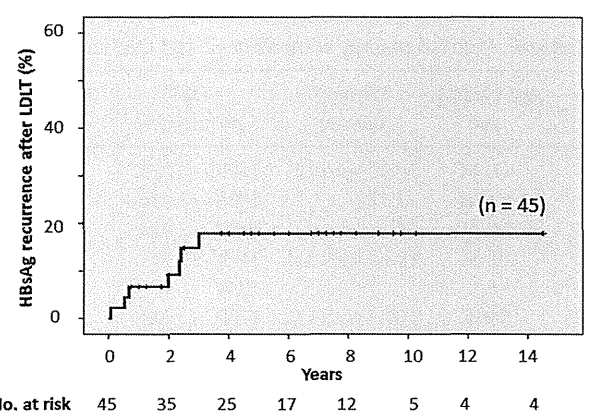

Figure 1 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence in the 45 patients. LDLT, living donor liver transplantation.

Table 2 Results of the univariate analysis of factors associated with HBV recurrence after LDLT

	HBsAg recurrence ($n = 7$)	HBsAg non-recurrence ($n = 38$)	*P*
Age (years)	53 (46–64)	53.5 (31–67)	0.778
Male sex	4 (57.1%)	24 (63.2%)	0.538
Acute liver failure	1 (14.3%)	11 (43.2%)	0.387
Pre-LT HBeAg positivity	1 (14.3%)	9 (23.7%)	0.506
HBV DNA level at LT >5 log copies/mL ($n = 40$)	2 (33.3%; $n = 6$)	10 (29.4%; $n = 34$)	0.595
HBIG cessation	3 (42.9%)	3 (7.9%)	0.039
Pre-LT HCC	4 (57.1%)	24 (63.2%)	0.468
Beyond Milan criteria by			
preoperative imaging	2 (28.6%)	6 (15.8%)	0.363
explant pathology	4 (57.1%)	11 (28.9%)	0.154
HCC recurrence	3 (42.9%)	2 (5.3%)	0.021
Post-LT systemic chemotherapy	2 (28.6%)	1 (2.6%)	0.059
Duration of corticosteroids (months)	3.7 (1.4–8.4)	2.9 (0.2–23.9)	0.309
Post-LT antiviral therapy	4/2/1	19/5/14	0.402
LAM/LAM + ADV/ETV	(57.1%/28.6%/14.3%)	(50%/13.2%/36.8%)	

Qualitative variables are expressed as the numbers of patients, with percentages in parentheses, and quantitative variables are expressed by the medians, with ranges in parentheses.
ADV, adefovir; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LDLT, living donor liver transplantation.

Table 3 Results of the multivariate analysis of factors associated with HBV recurrence after LDLT

	Hazard ratio	95% CI	*P*
HCC recurrence	30.835	3.132–303.593	0.003
HBIG cessation	20.170	2.091–194.593	0.009
Age (>55)	–	–	0.732
Sex, male	–	–	0.529
Post-LT systemic chemotherapy	–	–	0.863

CI, confidence interval; HBIG, hepatitis B immunoglobulin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living donor liver transplantation.

vaccination. However, HBsAg and HBV DNA reemerged 2 months after the cessation of HBIG, and the vaccination had failed. Adefovir was subsequently added to his treatment regimen, and the HBV DNA immediately became undetectable. In cases 2 and 3, HBIG was suspended for financial reasons, and HBsAg reemerged 4 and 6 months after the suspension of HBIG, respectively. After HBIG was reintroduced, the HBsAg disappeared and HBsAb reappeared immediately. HBIG was also suspended in three of the 38 patients (7.9%) who did not have HBV recurrence, and these were the cases in whom HBV vaccination was successfully performed. The indication of HBV vaccination was patients who have a normal or near

Table 4 Antiviral therapy administrated and the outcomes of patients with HBV recurrence after LDLT

	Age (years), sex	Primary disease	HBV prophylaxis	Cessation of HBIG	Time to HBV recurrence (months)	Time to HCC recurrence (months)	Outcome
1	53, M	HCC with LC	HBIG + LAM	+	24	–	Alive
2	47, M	ACLF	HBIG + LAM	+	28	–	Alive
3	54, M	LC	HBIG + LAM	+	6	–	Alive
4	46, M	HCC with LC	HBIG + LAM, ADV	–	8	5	Died
5	59, F	HCC with LC	HBIG + LAM, ADV	–	36	15	Died
6	47, F	HCC with LC	HBIG + ETV	–	29	30	Alive
7	64, F	LC	HBIG + LAM	–	1	–	Alive

ACLF, acute-on-chronic liver failure; ADV, adefovir; ETV, entecavir; HBcAb, hepatitis B core antibody; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LC, liver cirrhosis; LDLT, living donor liver transplantation.

normal liver function tests with a low level of immunosuppression, and the follow-up period after LDLT was at least a year. HBIG was suspended 1–4 weeks before starting HBV vaccination.[14]

As shown in Figure 2, the cumulative HBsAg recurrence rates were significantly higher in patients with HBIG cessation than in those receiving combined prophylaxis ($P = 0.013$).

Pretransplant HCC and HCC recurrence

Hepatocellular carcinoma was present in 28 patients (62.2%), and the cumulative HBsAg recurrence rates after LDLT were not significantly higher in patients with HCC than in those without HCC ($P = 0.711$) (Fig. 3a). Between the patients with HCC beyond and within the Milan criteria based on the preoperative imaging, there was no statistically significant difference in the cumulative HBsAg recurrence rates ($P = 0.370$) (Fig. 3b). Meanwhile, in patients with HCC beyond and within the Milan criteria diagnosed by the explant pathology, the cumulative HBsAg recurrence rates were higher (with marginal significance) in the patients with HCC beyond the Milan criteria than in those with HCC within the Milan criteria ($P = 0.068$) (Fig. 3C).

Among these 28 patients with HCC, five patients (17.9%) developed HCC recurrence after LDLT, and HBV recurrence occurred in three of these five patients (60%) (Table 4, cases 4–6). In case 4, the tumors were beyond the Milan criteria at LDLT, and HCC recurred 5 months after transplantation. Despite the use of chemotherapy and radiation, the HCC had grown and the HBsAg reappeared 8 months after transplantation during systemic chemotherapy. In case 5, there were multiple HCC tumors, and the disease was beyond the Milan criteria at LDLT. At 15 months after transplantation, HCC recurred as lung metastasis, and HBsAg recurrence was observed at 36 months after LDLT during systemic chemotherapy. In both cases 4 and 5, the HBV DNA increased in spite of combined therapy with entecavir, adefovir and HBIG.[15] Both patients finally died of recurrent HCC, at 12 and 43 months after transplantation, respectively.

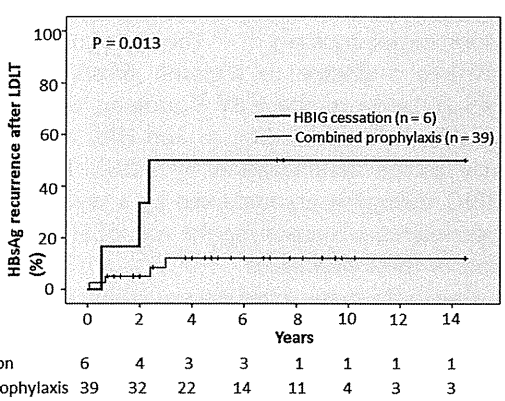

Figure 2 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence in patients with cessation of hepatitis B immunoglobulin (HBIG) and in those receiving combined prophylaxis (HBIG + antiviral agent). LDLT, living donor liver transplantation.

In case 6, the HCC was within Milan criteria at LDLT, and HBsAg reemerged 29 months after LDLT, which was 1 month prior to the detection of HCC recurrence. The patient underwent several operations for the treatment of metastasis and, thereafter, the HCC has been under control. She has not received systemic chemotherapy or radiation therapy, and the HBV DNA levels have been undetectable by combined prophylaxis with entecavir and HBIG. In patients with HCC recurrence, the cumulative HBsAg recurrence rate after LDLT was significantly higher than that in patients without HCC recurrence ($P < 0.001$) (Fig. 3d).

The remaining patient with HBV recurrence (Table 4, case 7) was the only case of transplantation from a living donor with positive HBsAg.[16] This living donor was the patient's son whose blood type was identical. He had no history of liver dysfunction, and was referred to as a "healthy carrier". Because no other living donors were available and brain-dead donors are rarely available in Japan, we decided to proceed to LDLT with this donor. To date, the patient has been doing well at 12 years after transplantation and the donor has also been doing well.

Overall survival

The overall survival after LDLT was not significantly reduced for patients with HBV recurrence, with probabilities at 1, 3 and 5 years of 100%, 91.5% and 91.5%, respectively, for patients without HBV recurrence versus 100%, 85.7% and 71.4%, respectively, for patients with HBV recurrence ($P = 0.250$) (Fig. 4a). However, if the six cases of HBIG cessation were excluded ($n = 39$), the cumulative survival rate was significantly reduced for patients with HBV recurrence, with probabilities at 1, 3 and 5 years of 100%, 90.7% and 90.7%, respectively, for patients without HBV recurrence versus 100%, 75% and 50%, respectively, for patients with HBV recurrence ($P = 0.037$) (Fig. 4b).

DISCUSSION

IN OUR STUDY, the demographic, virological, tumor burden, antiviral therapy and immunosuppressive therapy of patients with and without HBC recurrence after LDLT were analyzed to identify the risk factors for HBV

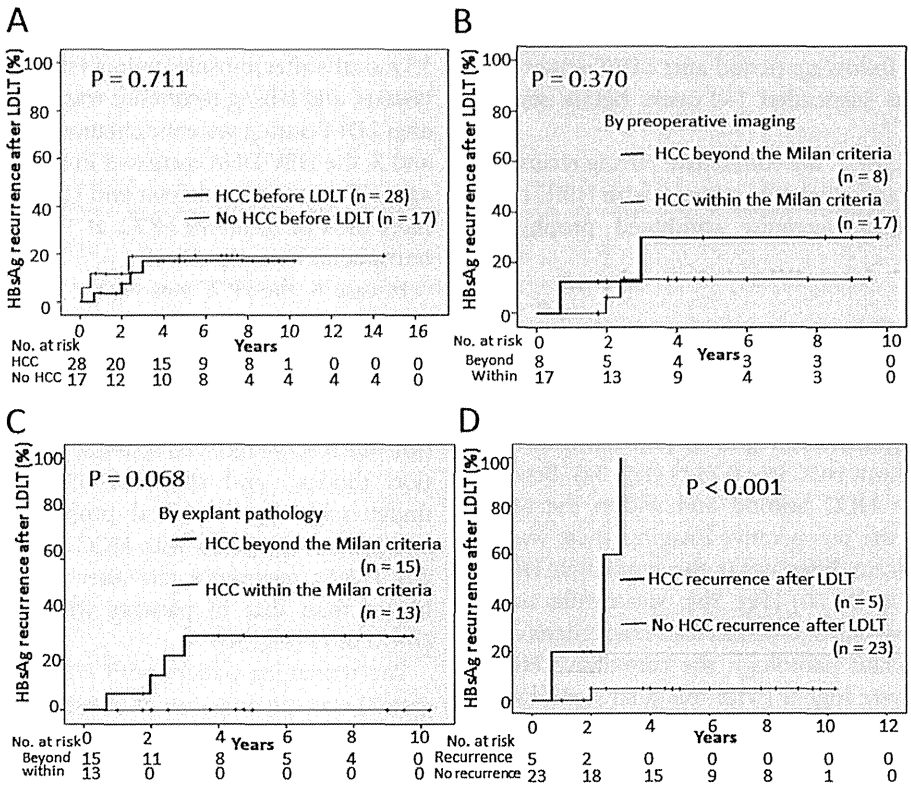

Figure 3 Cumulative rates of hepatitis B surface antigen (HBsAg) recurrence: (a) in patients with and without hepatocellular carcinoma (HCC) before living donor liver transplantation (LDLT); (b) beyond and within the Milan criteria by preoperative imaging; (c) beyond and within the Milan criteria by explant pathology; (d) with and without the recurrence of HCC. LDLT, living donor liver transplantation.

recurrence. We showed that both cessation of HBIG and HCC recurrence were independent risk factors for HBV recurrence after transplantation. Previous studies have demonstrated that pre-LT HBeAg positivity, non-fulminant hepatitis B, immunosuppression from steroids and systemic chemotherapy after LDLT are risk factors for HBV recurrence,[7–9] however, we could not find a statistically significant correlation with those factors and HBV recurrence in the present study. Although a high pre-LT HBV DNA level was also reported to be an independent risk factor for HBV recurrence,[5,6] we also found no statistically significant correlation with this factor. A limitation of our retrospective study is that not all of the HBV DNA levels were known at the time of LDLT. Therefore, the effects of antiviral therapy on the viral load were not available for all patients. However, all patients had undetectable HBsAg and HBV DNA levels after LDLT.

The mechanisms by which HBIG protects the transplanted liver against HBV reinfection are not fully understood. One hypothesis is that HBIG protects naive hepatocytes against the HBV released from extrahepatic sites by blocking a putative HBV receptor.[17,18] Previous studies reported that recurrent hepatitis B during the first 6 months post-LT is usually related to inadequate HBIG doses in patients with a high viral load pre-LT, whereas late recurrence is caused mainly by the selection of immune escape mutants.[19–21] The most common mutation involves a glycine to arginine substitution at codon 145 (G145R) of the HBV S protein. This mutation results in reduced binding to anti-HBs, and such viruses may escape neutralization by HBIG. The cessation of HBIG therapy is accompanied by a reversion to a wild-type sequence, supporting the role of HBIG in the selection of these mutations.

Due to the many drawbacks of HBIG, including its high cost, several trials have attempted to minimize the dose of HBIG in selected patients.[22,23] Recently, Fung *et al.* reported that a HBIG-free regimen of entecavir monotherapy was effective for suppressing HBV after LT.[24] Yi *et al.* also reported the efficacy of sequential entecavir monotherapy after 1-year combination therapy.[25] Compared with lamivudine, entecavir has greater antiviral potency and a