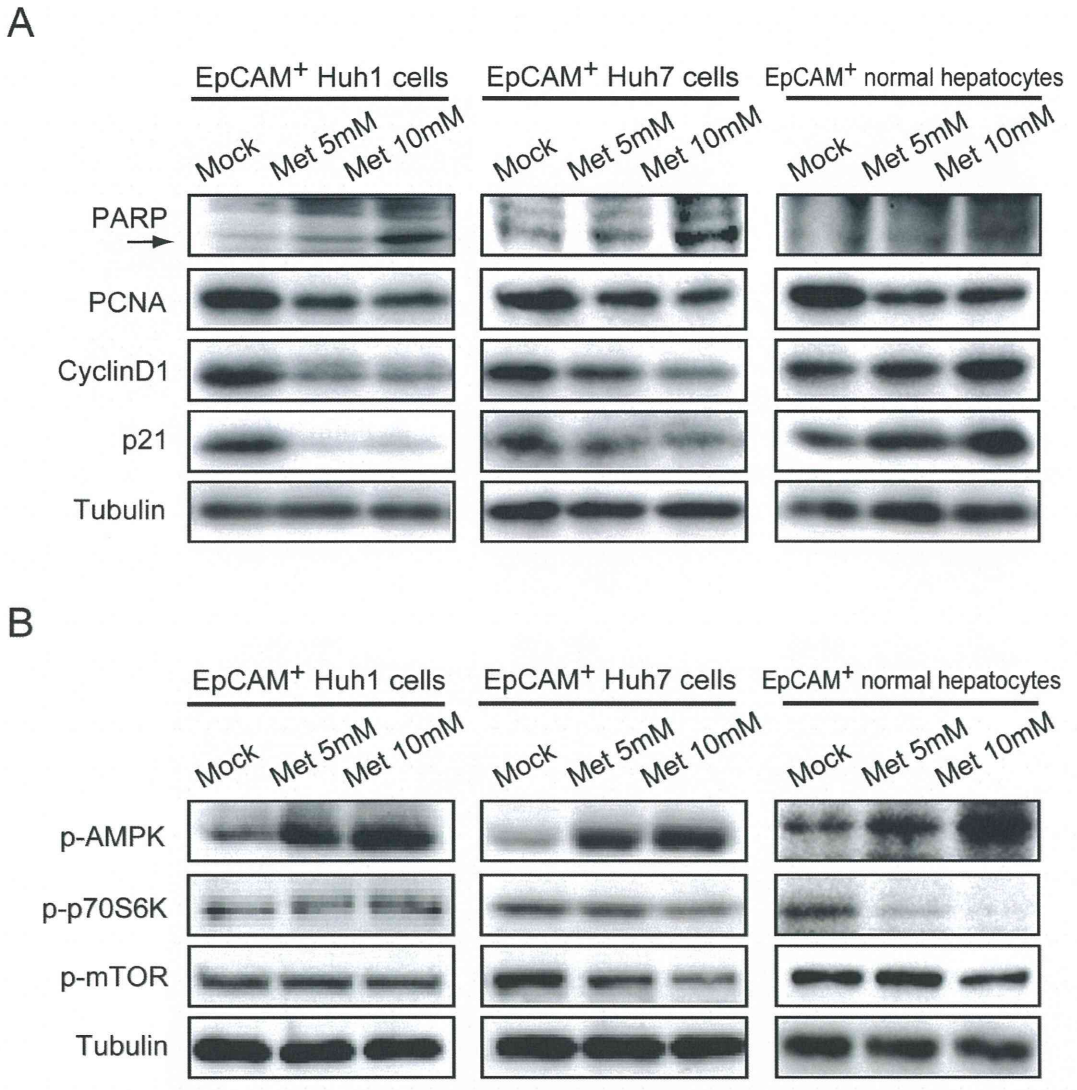

Figure 6. Cell growth inhibition, induction of apoptosis, and inhibition of the mTOR pathway after metformin treatment in HCC cells and normal hepatocytes. (A) EpCAM$^+$ cells were subjected to Western blot analysis using anti-PARP, PCNA, cyclin D1, p21, and tubulin (loading control) antibodies. The arrow indicates the cleaved forms of PARP. (B) EpCAM$^+$ cells were subjected to Western blotting using anti-phospho-AMPK, phospho-p70S6K, phospho-mTOR, and tubulin (loading control) antibodies.
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(Invitrogen). One thousand cells were plated onto ultra-low attachment six-well plates (Corning, Corning, NY) for the sphere formation assay. The number of spheres (>100 μm in diameter) was counted on day 14 of culture. A single cell suspension derived from original spheres was obtained for the secondary sphere formation using a Neurocult chemical dissociation kit (StemCell Technologies, Vancouver, BC, Canada). Paraffin-embedded sections of spheres were subjected to hematoxylin & eosin (H&E) staining and immunostaining with anti-EpCAM (Cell Signaling Technology, Danvers, MA) and anti-AFP (Dako Cytomation, Carpinteria, CA) antibodies for the pathological analysis.

Growth Curves

The proliferation of HCC cells treated with metformin was examined using trypan blue staining after 48 and 96 hours of culture.

Detection of Apoptotic Cells

To detect apoptosis, cells were stained with an anti-CASP3 antibody (Chemicon, Temecula, CA), followed by Alexa-555-conjugated goat anti-rabbit IgG (Molecular Probes). Apoptotic cells were also evaluated by staining with Annexin V-allophycocyanin (APC) (BD Biosciences, San Jose, CA) and PI using FACSCanto (BD Biosciences).

Cell Sorting and Analysis

Single-cell suspensions were stained with an APC-conjugated anti-EpCAM antibody (Biolegend, San Diego, CA) or APC-conjugated anti-CD133/1 antibody (Miltenyi Biotec, Auburn, CA). After incubation, 1 μg/ml of PI was added to eliminate dead cells. Flow cytometric cell sorting and analysis were performed using FACSAria or FACSCanto (BD Biosciences).

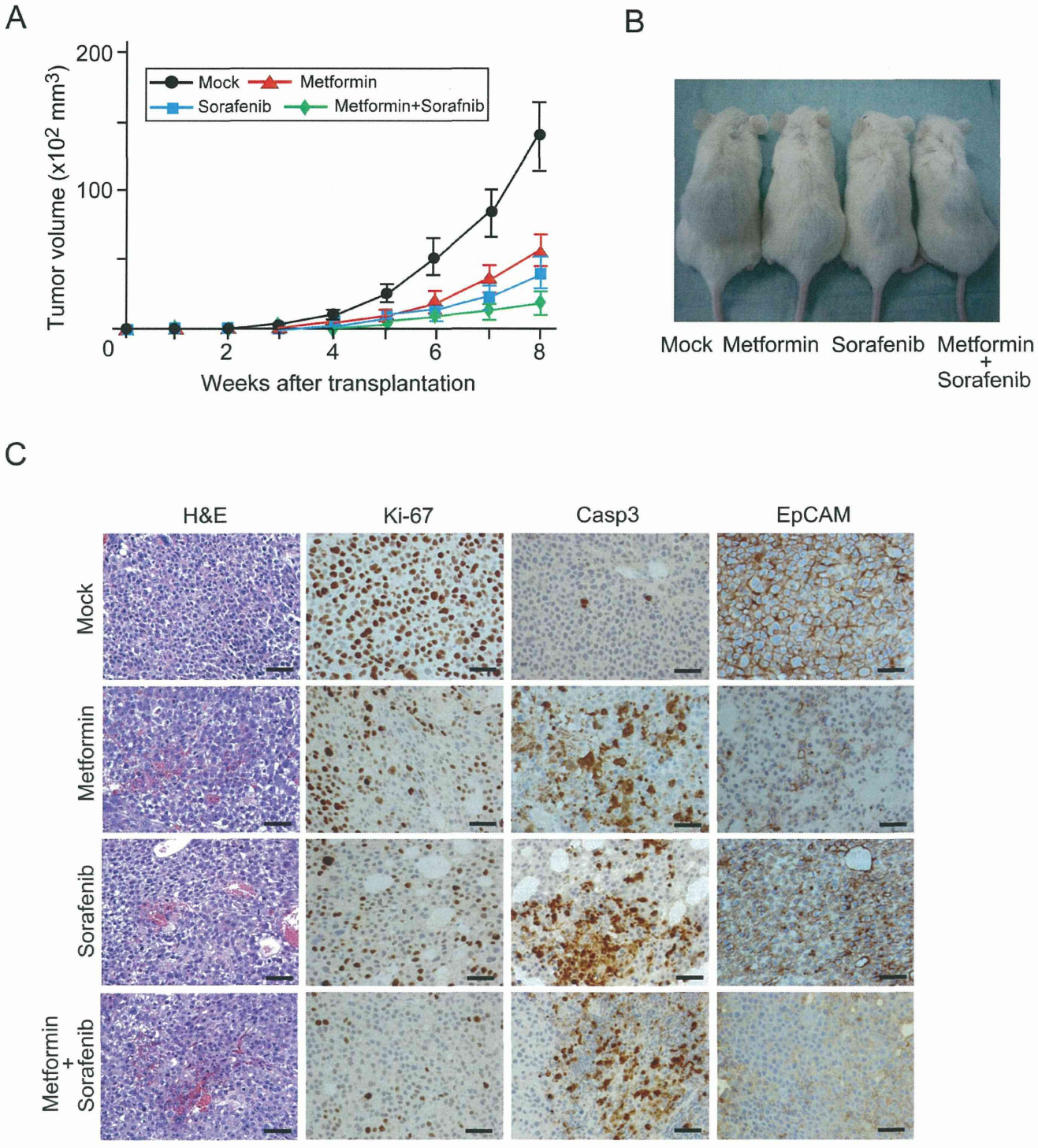

Figure 7. Inhibition of xenograft tumor growth by the administration of metformin and/or sorafenib. (A) A total of 2×10^6 Huh7 cells were transplanted into NOD/SCID mice. Tumor volume was monitored weekly after the cell transplantation. *Statistically significant ($p<0.05$). (B) Representative images of recipient mice treated with metformin and/or sorafenib 6 weeks after the transplantation. (C) Hematoxylin and eosin (H&E) staining and immunohistochemical analysis of subcutaneous tumors.
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Western Blotting

Sorted HCC cells were subjected to Western blot analysis using anti-EpCAM (Abcam, Canbridge, UK) and anti-tubulin (Oncogene Science, Cambridge, MA) antibodies. Metformin-treated cells were also subjected to Western blotting using anti-PARP (Cell Signaling Technology), anti-PCNA (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-cyclin D1 (BD Biosciences), anti-p21 (Cell Signaling Technology), anti-phospho-AMPK (Cell Signaling Technology), anti-phospho-mTOR (Ser2448, Cell Signaling

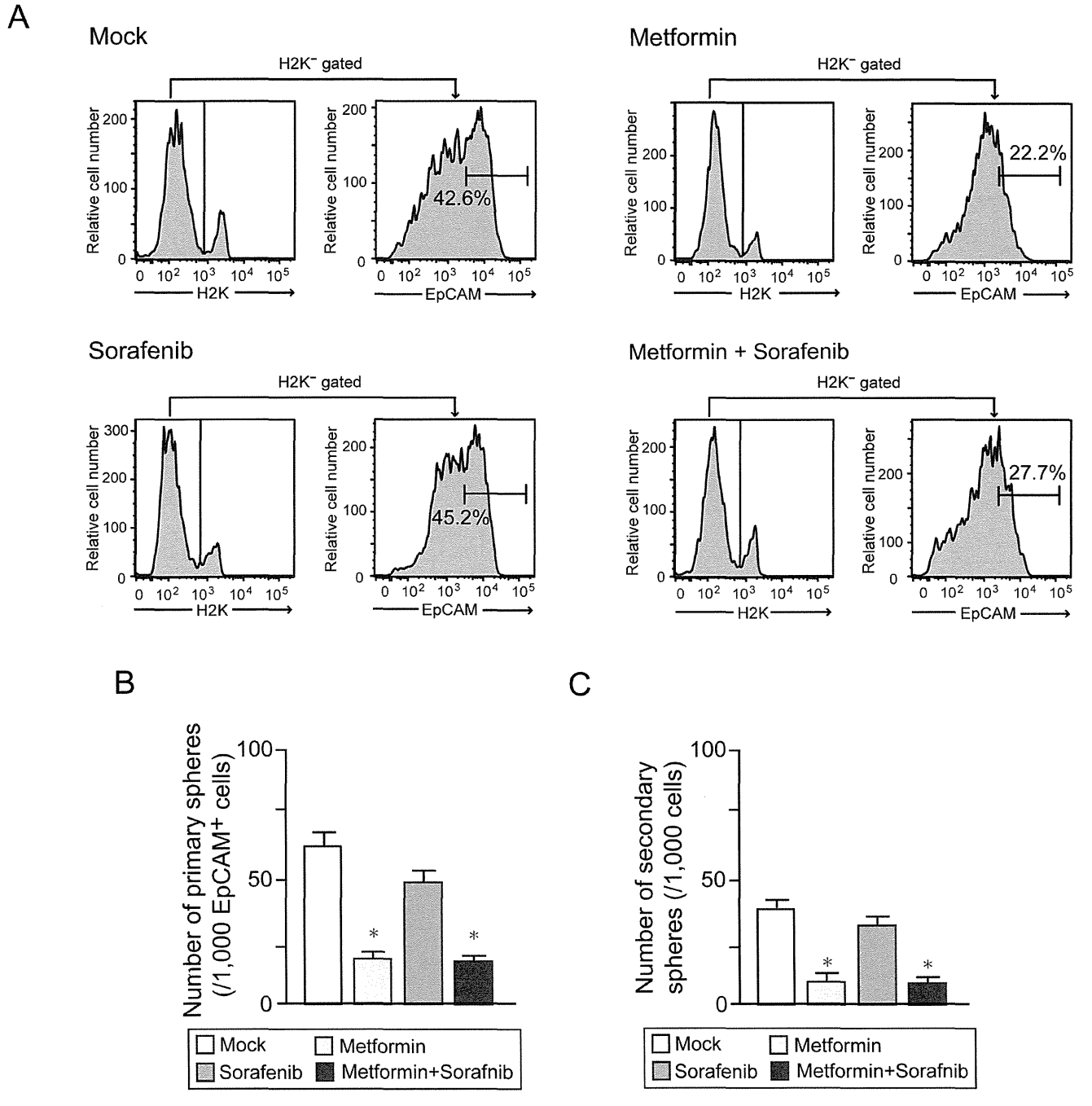

Figure 8. Re-analysis of xenograft tumors. (A) Flow cytometric analysis of subcutaneous tumors. The percentages of positive fractions for the indicated markers are shown as the mean values for three independent analyses. (B) Number of large spheres generated from 1,000 EpCAM$^+$ HCC cells treated with metformin. *Statistically significant ($p<0.05$). (C) Number of secondary spheres 14 days after replating. *Statistically significant ($p<0.05$).
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Technology), anti-phospho-p70 S6 Kinase (Thr389, Cell Signaling Technology), and anti-tubulin antibodies.

Xenograft Transplantation Using NOD/SCID Mice

In the metformin and/or sorafenib treatment model, a total of 2×10^6 Huh7 cells were transplanted into the subcutaneous space of the backs of NOD/SCID mice. Metformin (250 mg/Kg, by intraperitoneal injection) and sorafenib (10 mg/Kg, by gavage) were administered daily. Tumor formation and growth were observed weekly. To analyze subcutaneous tumors, small pieces of tumors were put in DMEM containing 5 mg/ml collagenase type II (Roche) and digested. The cell suspension was centrifuged on Ficoll (IBL, Gunma, Japan) to remove dead cells and debris. Harvested cells were subjected to flow cytometric analyses and sphere formation assays. Subcutaneous tumors were also subjected to H&E staining and immunohistochemical staining with an anti-EpCAM antibody (Cell Signaling Technology), anti-CASP3 antibody (Chemicon), and anti-Ki67 antibody (DAKO, Carpin-

teria, CA). These experiments were performed in accordance with the institutional guidelines for the use of laboratory animals.

Statistical Analysis

Data are presented as the mean ± SEM. Significant differences between 2 groups were analyzed using the Mann-Whitney U test. P values less than 0.05 were considered significant.

Author Contributions

Conceived and designed the experiments: TS TC AI OY. Performed the experiments: TS TC KY FK YZ MO SK. Analyzed the data: TM SO ES YO AT MT YT. Contributed reagents/materials/analysis tools: TS TC AI. Wrote the paper: TS TC AI.

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PLOS ONE

New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

* Resolved individuals were HBsAg negative and HBcAb positive.

** 419 of 467 healthy controls were de-identified, without information on age.

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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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検査値の読み方　連載

ALT正常HBe抗体陽性症例の経過

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はじめに

B型肝炎ウイルス(HBV)の自然経過において，HBe抗原の陰性化，HBe抗体の陽転(seroconversion；SC)はウイルスの活動性低下を意味し，肝炎も鎮静化すると考えられていたが，SC後にウイルス学的，生化学的に不安定な経過を示す例も少なくないことが明らかにされている．

「キャリア」という言葉は，臨床的にはいろいろな場面で用いられているが，「キャリア＝病的問題がない」と短絡的に判断される場面も見受けられ，慎重な評価や対応が必要である．

本稿では，ALT正常HBe抗体陽性者の背景や経過につき，当科の成績を示し，経過観察に関し考察する．

Ⅰ．ALT正常HBe抗体陽性者 ― 当科の解析

1．検討対象

1）対　象

当科に通院中で，2年以上，無治療のまま血清ALT値が持続的に30 IU/l以下の期間を有するHBV陽性例を91例認めた．そのなかで，ALT値が30 IU/l以下の時点で，HBs抗原とHBコア関連抗原(HBcr抗原)の同時期測定が可能であった87例を対象とした．

2）検討項目，測定方法

HBs抗原，および，HBcr抗原の測定時期を起点としたHBVマーカーとALT値変動と，経過中発癌の有無を検討した．

HBs抗原，HBe抗原，HBe抗体はCLIA(chemiluminescent immunoassay)法，HBcr抗原はCLEIA (chemiluminescent enzyme immunoassay)法，HBV DNA量はreal time PCR (polymerase chain reaction)法で測定した．また，HBV genotype判定は，EIA (enzyme im-

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munoassay)法，あるいは，PCR-invader 法，HBV precore 変異は ELMA (enzyme linked mini-sequence assay)法，HBV core promoter 変異は ELSPA (enzyme linked specific probe assay)法により測定した．

3）病期の判定

組織学的検査，画像検査，治療歴や経過所見から，慢性肝炎，あるいは，肝硬変の判定を実施した．これらの所見で明らかな慢性肝病変の所見を認めない症例は「慢性所見なし」とし，無症候性に対応するものと判定した．

4）HBs 抗原/HBcr 抗原による HBV レベルの群別

厚生労働省「B 型肝炎の核酸アナログ薬治療における治療中止基準の作成と治療中止を目指したインターフェロン治療の有用性に関する研究」班では，「核酸アナログ薬中止に伴うリスク回避のための指針 2012」を策定した[1]．その基準では，HBs 抗原と HBcr 抗原の量で，それぞれ 3 段階に分別して 0/1/2 点のスコアを配し，二つの合計点数を判定指標とした．本稿では，このスコアをウイルスレベル評価に用いた．

5）対象症例の背景

87 例の内訳は，男性 32 例，女性 55 例，年齢は中央値 53 歳(23～77 歳)，病期別には，慢性所見なし 22 例，慢性肝炎 52 例，肝硬変 13 例で，経過観察中の肝癌発生は 14 例(16.1%)に認めた．経過中の HBs 抗原消失は 2 例．HBV genotype は，A 型 2 例，B 型 26 例，C 型 58 例であった(判定保留 1 例)．ALT 値(IU/*l*)の中央値は 18(7～30)，HBs 抗原(log IU/m*l*)は 2.57 (−1.00～4.64)，HBcr 抗原(log U/m*l*)は 3.0 未満(<3.0～6.1)，HBV DNA 量(log copies/m*l*)は 3.4(検出せず～6.7)であった．観察期間は，平均 7.1 年(中央値 7.4 年)であった．

2．ウイルス学的・生化学的評価

1）HBV レベルの分布

前述したように，2 年以上 ALT 値が 30 IU/*l* 以下の時点で，HBs 抗原と HB コア関連抗原(HBcr 抗原)，HBV DNA 量を測定した 87 例の HBs 抗原，HBcr 抗原，HBV DNA 量の分布を示す(図 1)．

対象症例の HBs 抗原(log IU/m*l*)は，1.9 未満が 36.8%，1.9～2.9 が 23.0%，2.9%以上が 40.2%であった．HBcr 抗原(log U/m*l*)では，70%程度が 3.0 未満と低値で，4.0 以上の症例は約 10%であった．HBV DNA 量(log copies/m*l*)は，3.0 未満は 36.9%，4.0 未満だと 65.6%であるが，一部には ALT 正常でも 6.0 以上の症例も認めた．

上記マーカーと年齢との関連では，いずれも，年齢上昇とともに低下する傾向にある．HBs 抗原は，50 代以降から低値域に分布が広がる傾向を示し，HBcr 抗原の定量測定可能例は，50 代以降は少ない．HBV DNA 量も 50 代以降から，低値に収束する傾向を認めた．

2）HBV DNA 量と ALT 値の関連性

対象症例の ALT 値と HBV DNA 量との相関性は低かった．ALT 値を正常範囲内で二分した場合には，正常上限の 50%である 15 IU/*l* 以下(low normal ALT)の症例は 28 例，16 IU/*l* 以上(high normal ALT)の症例は 59 例であった．前者の HBV DNA 量(log copies/m*l*)は，中央値 3.14，最大 4.17，4.0 以上の症例は 5 例(17.9%)であったが，後者では，それぞれ，3.72，6.65，25 例(43.1%)と，ALT 正常範囲のなかでも HBV レベルに差がある傾向を認めた．

HBV DNA 量と ALT 値の関連を年代別に見ると，HBV DNA 量の分布は，若年代では低値から高値まで広い分布を示し，高齢化とともに低値域に収束する傾向を認めた．HBV DNA 量と ALT 値に相関性を認めたのは 30 代と 40 代

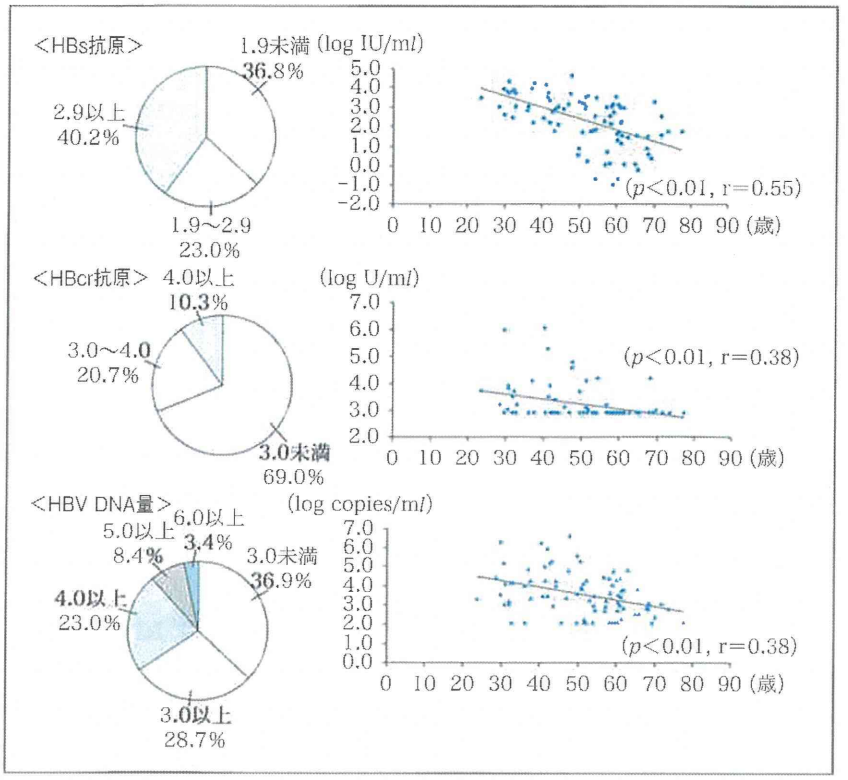

図1 HBs 抗原，HBcr 抗原，HBV DNA 量のレベル別分布と年齢別推移

の症例で，相関係数は，それぞれ，0.56，0.54であった．20代では，HBV DNA 量が高値でもALT 値との相関は低く，50 代以降は HBV DNA 量が全体に低値となり，ALT 値との相関性は低下していた(図 2)．

3）生化学的，ウイルス学的安定例

今回の対象 87 例中，HBs 抗原，HBcr 抗原測定時点で，「血清 ALT 値が 30 (IU/l) 以下，HBV DNA 量が 4.0 (log copies/ml) 未満」を満たす症例は 57 例であったが，前後の各 1 年間もこの条件を維持していた例は 35 例であった．この安定化維持例は，それ以外の 52 例と比較した場合，基準時の ALT 値，HBV DNA 量は低値の傾向を示したが，そのほかに，高齢，HBs 抗原量低値，HBcr 抗原量の低値，の傾向を認めた(表 1)．

4）HBs 抗原/HBcr 抗原の総合スコア解析

HBs 抗原と HBcr 抗原をスコア化し，総合スコアを 0/1/2/3/4 の 5 段階に分けた場合，各群の平均年齢は 61.0/49.6/50.5/40.8/44.1(歳)，HBV DNA 量の平均値は 2.90/3.94/3.19/3.97/4.93 (log copies/ml) であった．スコア低値例で高齢，HBV DNA 量低値の傾向を認めた．

3．肝発癌との関連

1）HBs 抗原/HBcr 抗原の総合スコアと肝発癌例

経過中に肝癌発生を認めた症例は 14 例であったが，HBs 抗原/HBcr 抗原の総合スコア別の発癌例は，総合スコア 0/1/2 で，それぞれ，5 例 (17.2%)/5 例 (29.4%)/4 例 (23.5%) であった．スコアが 3 および 4 の症例からの発癌は認めず，スコア低値例のほうが発癌の頻度が高かった．

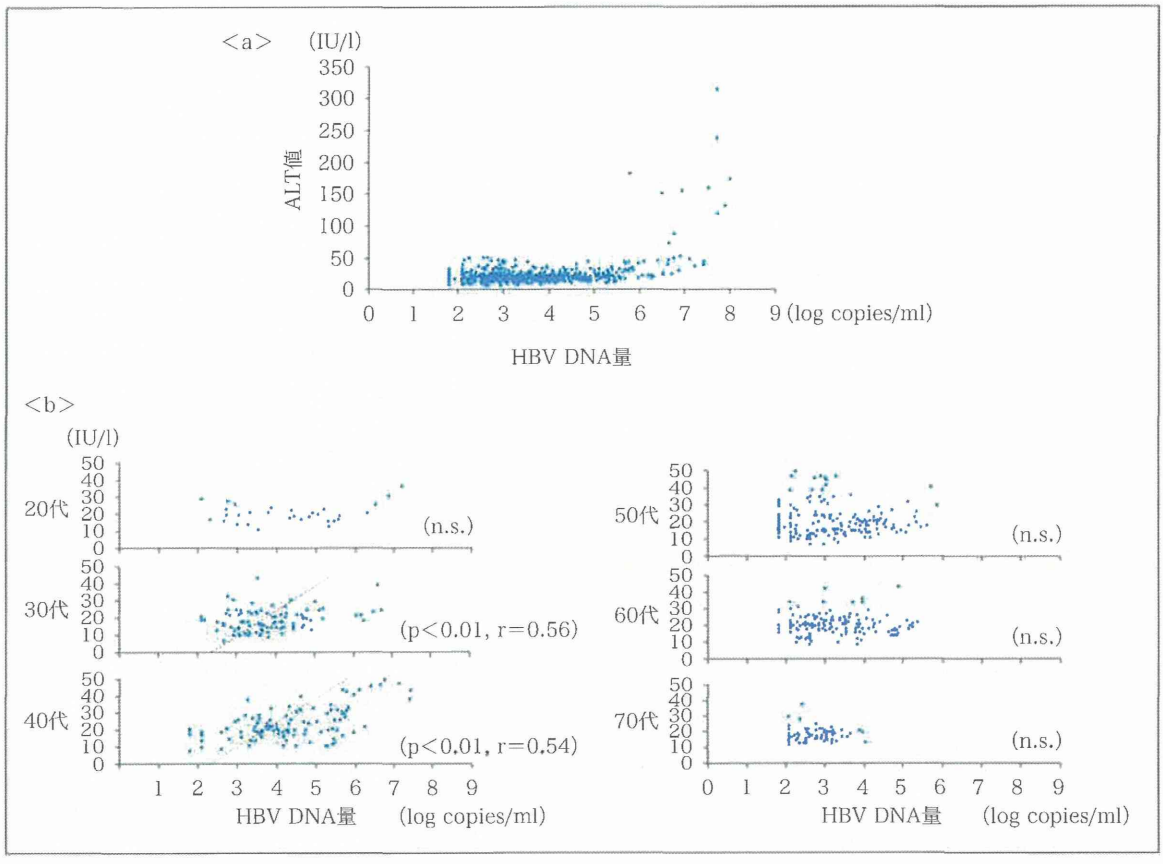

図2 年代別の HBV DNA 量と ALT 値の関連性
a：HBV DNA 量と ALT 値の関連
b：年代別の関連(ALT 値は 50 IU/*l* までの範囲を表示)

表1 安定化維持の有無と背景

	安定化維持例	非安定例	*p*
症例数	35	52	
男/女 比	14/21	18/34	n. s.
年齢(歳)	55. 9±12. 0	48. 4±12. 1	<0. 01
病期(ASC/CH/LC)	8/20/7	14/32/6	n. s.
HCC 合併	8 例(22. 9%)	6 例(11. 5%)	n. s.
HBV genotype(A/B/C)※	1/8/25	1/18/33	n. s.
ALT(IU/*l*)	16. 6±5. 2	19. 1±4. 5	<0. 01
Plt(×10^4/μl)	16. 5±5. 7	17. 9±4. 4	n. s.
AFP(ng/m*l*)	4. 0±5. 2	3. 6±2. 6	n. s.
HBsAg(log IU/m*l*)	3. 58±0. 53	3. 94±0. 93	<0. 01
HBcrAg(<3. 0/3≤)(log U/m*l*)	29/6	31/21	0. 02
HBV DNA(log copies/m*l*)	2. 72±0. 54	4. 08±1. 08	<0. 01
Precore(wild/mutant)※	3/16	9/31	n. s.
CP 変異(wild/mutant/感度以下)※	2/10/7	14/22/4	n. s.

※：判定保留・未測定例を除く

表2　肝発癌の有無と背景因子

	肝発癌あり	発癌なし	p
症例数	14	73	
男/女 比	11/3	21/52	0.01
年齢(歳)	61.3±9.3	49.6±12.3	<0.01
病期(ASC or CH/LC)	6/8	68/5	0.01
HBV genotype(A or B/C)※	0/14	28/44	<0.01
ALT(IU/l)	16.5±3.4	18.4±5.1	n.s.
Plt($\times 10^4/\mu l$)	13.0±5.3	18.2±4.5	<0.01
AFP(ng/ml)	4.0±3.8	3.7±4.0	n.s.
HBsAg(log IU/ml)	1.48±1.37	2.49±1.25	0.01
HBcrAg(<3.0/3≤)(log U/ml)	12/2	48/25	n.s.
HBV DNA(log copies/ml)	2.88±0.70	3.66±1.15	0.02
Precore(wild/mutant)※	0/6	12/41	n.s.
CP 変異(wild/mutant/感度以下)※	0/6/0	16/29/8	n.s.

※：判定保留・未測定例を除く

2）肝癌発生例の背景因子

経過中に肝癌の発生した14例を非発癌例と比較すると，男性，高齢，病期進展，HBV genotype C，血小板低値，HBs抗原低値，HBV DNA量低値，などの傾向を認めた(表2)．

臨床的に肝硬変と診断されている13例からは8例(61.5%)の発癌を認め，慢性肝炎の所見や既往が明らかな例では52例中6例(11.5%)，「慢性所見なし」群の22例からは発癌を認めなかった．血小板数(/μl)別の経過中発癌例は，15万未満，15万～20万，20万以上では，それぞれ，30例中9例(30.0%)，31例中5例(16.1%)，26例中0例であった．

Ⅱ．考　察

1．ALT正常HBe抗体陽性者と「キャリア」

慢性HBV感染者の自然経過について，当初はHBe抗原が活動性判定の指標と考えられ，HBV持続感染者で，HBe抗原/抗体のSCにより，ウイルスの活動性は低下し肝炎も鎮静化すると考えられていた．しかし，SC後にウイルス学的，生化学的に不安定な経過を示す例も少なくないことが明らかになっている．

Yimら[2]は，B型肝炎の経過を，immune tolerant期，immune clearance期(HBe抗原陽性慢性肝炎期)，inactive carrier期，reactivation(HBe抗原陰性慢性肝炎期)に分けているが，個人差が大きく，すべての患者が同様の経過をとるわけではない．

一方，「キャリア」という言葉は，HBVキャリア，無症候性キャリア(asymptomatic carrier)，非活動性キャリア(inactive carrier)，健康キャリア(healthy carrier)など，臨床的にはいろいろな表現に用いられる．

一般的には，「無症候性キャリア」とは，HBV持続感染者のなかで，肝機能検査所見が正常な者を指し，画像検査，肝生検などで明らかな肝障害を確認しえた者は除外，とすることが多い．しかし，血液生化学検査のみで評価する場合には進展例も含まれる．今回の検討でも，「ALT正常HBe抗体陽性者」として囲い込みをした場合，肝硬変例が15%程度含まれている．したがって，病期や病態の判断には，血液生化学検査に加え，画像あるいは組織検査の情報を併せ

て行う必要がある.

また，ワンポイントの検査でALT値が正常であっても，経過中にALT値の変動を認める例も多く，ウイルス学的マーカーも含めた安定化予測には，年齢やHBVマーカーのレベル，正常範囲のなかでのALTレベル(low normal ALT，high normal ALT)などを参考に経過を観察し，肝炎再燃の可能性を推測する必要がある.

2．非活動性キャリアの定義

平成25年4月，日本肝臓学会から「B型肝炎治療ガイドライン(第1版)」が公表された．このガイドラインでは，「治療適応のないHBe抗原セロコンバージョン後の非活動性キャリア」は，「抗ウイルス治療がなされていないdrug freeの状態で，1年以上の観察期間のうち3回以上の血液検査で①HBe抗原が持続陰性，かつ②ALT値が持続正常(30 IU/l以下)，かつ③HBV DNAが4.0 log copies/ml未満，のすべてを満たす症例」と定義された.

ただし，前述のように，血液検査のみでは病期進行例の除外が不十分となるため，画像所見や血小板数などの所見も合わせて判断を行うべきである.

3．ALT正常HBe抗体陽性例からの肝発癌

今回の対象で経過中肝発癌を14例(16.1%)に認めた．これらは，高齢，男性，HBV genotype C，血小板低値の傾向を示したが，HBVマーカーのレベルは低値の傾向を示し，HBs抗原/HBcr抗原によるHBVレベル別には，むしろ，総合スコア低値例で発癌が多い傾向を認めた．発癌が高齢者に多い結果である可能性も考慮すべきであるが，肝癌好発例の拾い上げに際しては，HBVレベルの低い例に注意が必要であった.

HBV持続感染者からの発癌に関して，Yangら[3]は，性別，年齢，ALT値，HBV要因(HBe抗原，HBV DNA量，genotype)に加えて，肝癌家族歴，飲酒歴を含めてスコア化したノモグラムがリスク予測に有用であることを報告しており，参考になるものと思われる.

ま と め

ALT値が持続正常，HBe抗体陽性のキャリアは，画像あるいは組織所見を含めて判断をする必要がある．長期的なALT値の正常化維持に関しては，病期，年齢，HBVマーカーのレベル，ALT値などを参考に予測し，経過観察を継続する．一方，肝発癌例ではHBVレベルは低い症例が多い傾向を示しており，HBe抗体陽性のALT正常者の肝炎活動性，発癌に関しては，別視点でのfollowが必要である.

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B型肝炎の自然予後（無治療住民検診での長期予後）

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索引用語：B型肝炎，自然予後，住民コホート，HBs抗原自然消失，肝癌

1 はじめに

B型肝炎ウイルス(HBV)感染症は，非感染例に比べ明らかに肝発癌リスクが高く[1]，わが国では肝癌リスクの第2位，アジア全体では第1位を占めている．日本赤十字社の初回献血者から推定した，本邦におけるHBV感染者数は，Tanakaらの報告によれば15～65歳において967,753名(95％ CI：806,760～1,128,745)，内訳は男性571,210名(95％ CI：479,267～663,152)，女性396,543名(95％ CI：327,494～465,593)と見積もられている[2]．しかしながらHBVによる肝病態進展や肝発癌機序についてはいまだ不明な点が多く，その解明にはまず，本邦におけるB型肝炎の自然予後がどのようなものかを質の高いコホートで解析・検証する必要がある．これまで，キャリアにおける肝発癌関連因子としていくつかのウイルス側，宿主側因子などが報告されているものの，これらはcross-sectional studyあるいは病院患者コホートなどの限られた集団で検討されたものがほとんどである．

HCV感染と異なり，無症候性キャリア(ASC)が多く潜在するHBV感染は，無治療で経過を追った場合の肝発癌の頻度やHBs抗原自然消失，非感染者との生命予後に差異があるかどうかなど，感染に伴う自然予後の把握が難しい．

そこで本稿では，主に住民を対象としたコホート研究でこれまで明らかにされているB型肝炎の自然予後について，国内外の論文をもとに紹介したい．

2 アジアにおけるB型肝炎自然予後

Kusakabe，Mizokamiらは住民コホートを用いて，本邦におけるHBV単独感染者のHCC発生と関連するリスク因子を前向きコホートで解析している[3]．このコホートは，多目的コホート研究(JPHC研究[4,5])の一環で，全国6保健所管内に住む40～69歳の男女19,393人を平成17年まで(平均観察12.7年)に追跡した調査結果に基づくものであり，期間中110例の肝発癌を認めた．このうち13例がHBV単独感染例(図1)，78例がHCV単

Hisayoshi WATANABE et al：Long-term clinical outcomes in patients with hepatitis B: natural history population-based cohort studies
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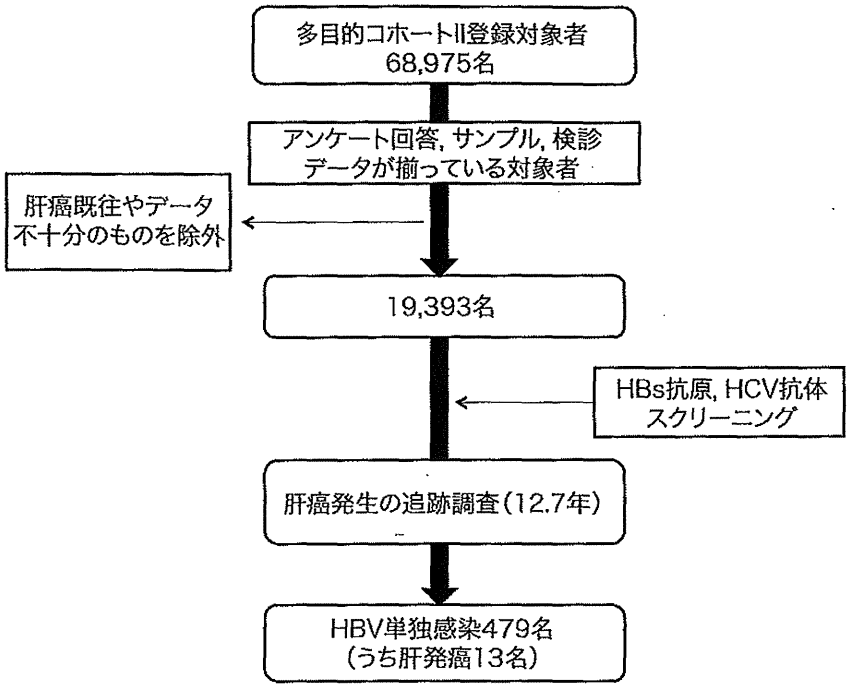

図1　コホート概要(文献3より引用改変)

表1　発癌例と非発癌例の比較(文献3より引用改変)

	HCC (n = 13)	Non-HCC (n = 466)	P
年齢	58.8 ± 6.3	55.1 ± 8.5	NS
男性	11 (85%)	209 (45%)	<0.005
BMI	22.3 ± 2.9	23.4 ± 3.0	NS
アルコール多量摂取	0	36 (8%) 92 (20%)	NS
喫煙	7 (54%)		<0.005
ALT (IU/L)	44.7 ± 30.0	23.8 ± 20.4	<0.005
γ-GTP (IU/L)	31.7 ± 16.2	23.2 ± 25.2	NS
HBe抗原陽性	3 (23%)	14 (3%)	<0.005
HBcr抗原 (kU/mL)	39,276 ± 121,639	6,486 ± 47,987	<0.005
HBcr抗原陽性	7 (54%)	99 (21%)	<0.005
HBV DNA (log copies/mL)	6.1	4.1	<0.005
HBV DNA ≧ 5 log copies	6 (46%)	39 (8%)	<0.005
Genotype B	4 (31%)	264 (57%)	NS
Genotype C	9 (69%)	202 (43%)	NS
C1653T	6 (46%)	116/421 (28%)	NS
T1753V	6 (46%)	78/421 (19%)	<0.005
A1762T/G1764A	11 (87%)	142/421 (34%)	<0.005
G1896A	11 (87%)	348/421 (83%)	NS

独感染例，2例がHBV・HCV共感染例，17例が非B非C例であった．HBV単独感染者における肝癌(HCC)例，非肝癌(Non-HCC)例の背景を比較すると，男性，喫煙者の割合，ALT値，HBe抗原陽性，HBcr抗原陽性，HBV DNA値がHCC群において有意に高く，

表2　HBV，HCV感染状況による肝発癌ハザード比(文献8より一部改変)

感染状態	対象	人年	HCC例	ハザード比(95% CI)
HBs抗原 (-) / HCV抗体 (-)	5,744	53.504	16	1.0 (reference)
HBs抗原 (+) / HCV抗体 (-)	335	2,981	15	17.1 (8.4～34.8)
HBs抗原 (-) / HCV抗体 (+)	360	3,731	12	10.4 (4.9～22.1)
HBs抗原 (+) / HCV抗体 (+)	14	133	3	115.0 (32.5～407.3)

表3　HBs抗原陰性化例の臨床背景の比較(文献9より一部改変)

	HBs抗原陰性化あり (n=20)	HBs抗原陰性化なし (n=81)	P
年齢(歳)	56	50	0.038
男性	8 (40%)	36 (44%)	>0.2
肝硬変合併	4 (20%)	15 (18%)	1.0
ALT (IU/L)	26	35	0.057
HBV genotype (A:B:C:不明)	0: 2: 18: 0	3: 7: 69: 2	>0.2
HBe抗原	3 (15%)	35 (43%)	0.02
HBs抗原 (Log IU/mL)	1.7	3.3	<0.001
HBcr抗原 (Log U/mL)	3.0	4.7	<0.001
HBV DNA (log copies/mL)	3.0	5.7	<0.001

core promotor二重変異(A1762T/G1764A)の頻度も高かった(表1).

さらにCox比例ハザードモデルにて肝発癌リスク因子を解析すると，core promotor二重変異のみが独立したHCCリスク因子であった(ハザード比7.05，95% CI 1.03-48.12，$P=0.046$)．患者コホートを用いたCore promotor二重変異とHCC発生リスクに関してはこれまでいくつか報告されてきたが[6,7]，この研究は本邦における住民ベースのHBV感染者のHCC発生リスクを調べた初めての大規模前向きコホート研究である．住民コホートという性格上，ほとんどの対象者はHBV健常キャリアと推定されるが，サンプル数をさらに増やした前向き研究が期待される．

韓国ではHBV高浸淫地域におけるHBV/HCV共感染例のHCCリスクについて報告がある[8]．6,694人を平均9.4年追跡し(63,170人年)，50例の発癌を認めている．HBV単独感染によるHCCハザード比は17.1 (95% CI: 7.3-24.4)，HCV単独感染によるHCCハザード比は10.4 (95% CI: 4.9-22.1)であり，HBV/HCV共感染によるHCCハザード比は115.0 (95% CI: 32.5-407.3)であった(表2).

一方，住民コホートではないものの，無治療のHBVキャリアの自然史についてHBs抗原量の長期経過の観点から報告がある[9]．Matsumotoらによると，1999年～2009年まで101例のHBVキャリアのHBs抗原量を追跡したところ，20例でHBs抗原が陰性化し(2.1%)，その陰性化は高齢と低ウイルス複製状態と関連していた(表3).

さらに韓国のAhnらは，病院コホートではあるが無治療のHBVキャリア432名のHBs抗原自然消失に関し，組織学的比較も加えて報告している[10]．19.6カ月の観察の間，9.5%にあたる49名でHBs抗原が自然消失した.