were matched for age and sex. The peak ALT, AST and T.bil levels were significantly higher (3788 ± 2856 vs 2170 ± 1350 IU/L, $P < 0.001$; 3131 ± 3673 vs 1676 ± 1851 IU/L, $P < 0.05$; and 14.8 ± 8.6 vs 9.5 ± 9.8 mg/dL, $P < 0.01$, respectively), while HBeAg was less frequent (30% vs 56%, $P < 0.01$) in the FH-T patients than AHB. The level of HBcrAg was significantly lower (5.30 ± 1.32 vs 5.95 ± 1.13 log U/mL, $P < 0.01$), while HBV DNA loads were higher (5.97 ± 1.87 vs 4.98 ± 1.17 log copies/mL, $P < 0.005$), in the FH-T patients than AHB. The level of core protein in sera tended to be higher in the FH-T patients than AHB (3.21 ± 1.28 vs 3.01 ± 1.00 log U/mL). Death occurred more often in the FH-T patients than AHB (38% vs 0%, $P < 0.001$).

HBV Genotypes and enhancer II/core promoter/pre-core/core Mutations in Patients with FHB by transient HBV infection and AHB

Figure 1(a) compares the distribution of HBV genotypes/subgenotypes between the FH-T and the AHB patients. The subgenotype C2/Ce was most prevalent in both patients with FH-T and AHB (66% and 62%, respectively), whereas B1/Bj was more frequent in the FH-T patients than AHB (22% vs 6%, $P < 0.05$). Likewise, mutations in enhancer II/core promoter/pre-core/core regions are compared between the FH-T and AHB patients in Figure 1(b). A1762T/G1764A, G1896A, G1899A and A2339G mutation were more frequent in the FH-T patients than AHB (48% vs 16%, $P < 0.001$; 62% vs 6%, $P < 0.001$; 24% vs 4%, $P < 0.001$; and 8% vs 0%, $P < 0.05$, respectively).

Figure 2(a) compares various mutations between the 11 FH-T patients and the three AHB patients who were infected with B1/Bj. Only G1896A was significantly more frequent (73% vs 0%, $P < 0.05$), while the lack of any mutations was less common (0% vs 33%, $P < 0.05$) in the FH-T patients than AHB. In comparison with the 33 FH-T patients and the 31 AHB patients who were infected with C2/Ce (Fig. 2b), A1762T/G1764A (70% vs 19%, $P < 0.001$), G1896A (61% vs 6%, $P < 0.001$) and the combination of all three mutations (A1762T/G1764A and G1896A) (45% vs 6%, $P < 0.001$) were significantly more frequent, while the lack of any mutations was less common (9% vs 70%, $P < 0.001$) in the FH-T patients than AHB. Interestingly, all the AHB patients with both G1896A and A1762T/G1764A mutations suffered acute severe hepatitis B that was defined by prothrombin time less than 40% but without coma of grade II or higher.

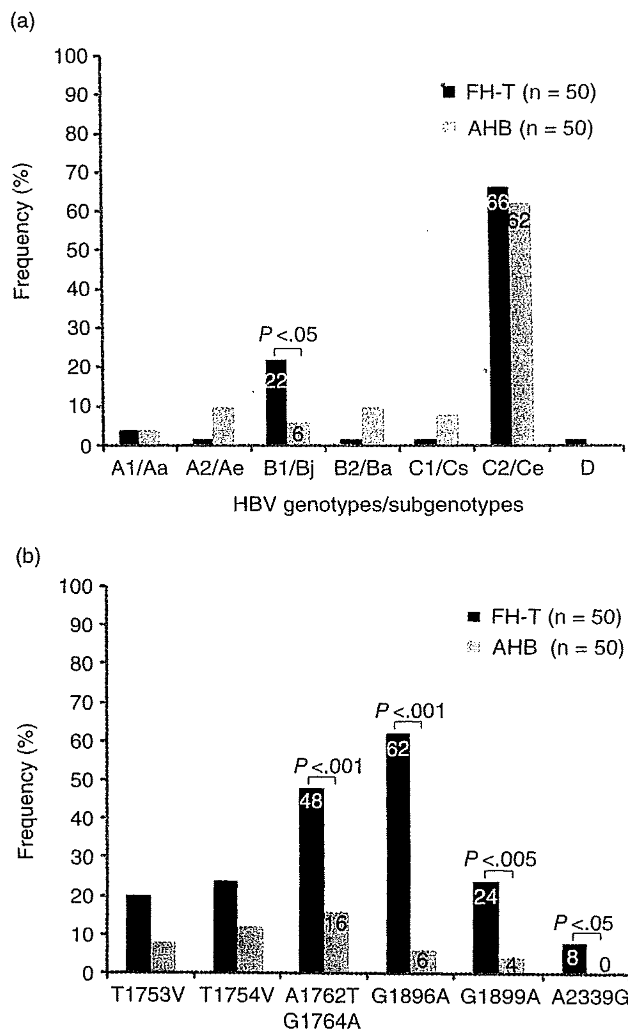

Figure 1 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 50 transient hepatitis B virus infection (FH-T) and the 50 acute self-limited hepatitis B (AHB) patients.

Factors independently associated with the development of FHB by transient HBV infection

The following independent factors, promoting the development of FHB, were evaluated by multivariate analysis: ALT, AST, T.bil, HBeAg, HBV DNA, core protein, HBcrAg, genotypes/subgenotypes (B1/Bj or not) and mutations (T1753V, T1754V, A1762T/G1764A, G1896A, G1899A and A2339G). T.bil more than 10.35 mg/dL (OR, 7.81 [95% CI, 1.77–34.51], $P = 0.0067$), G1896A mutation (OR, 13.53 [95% CI,

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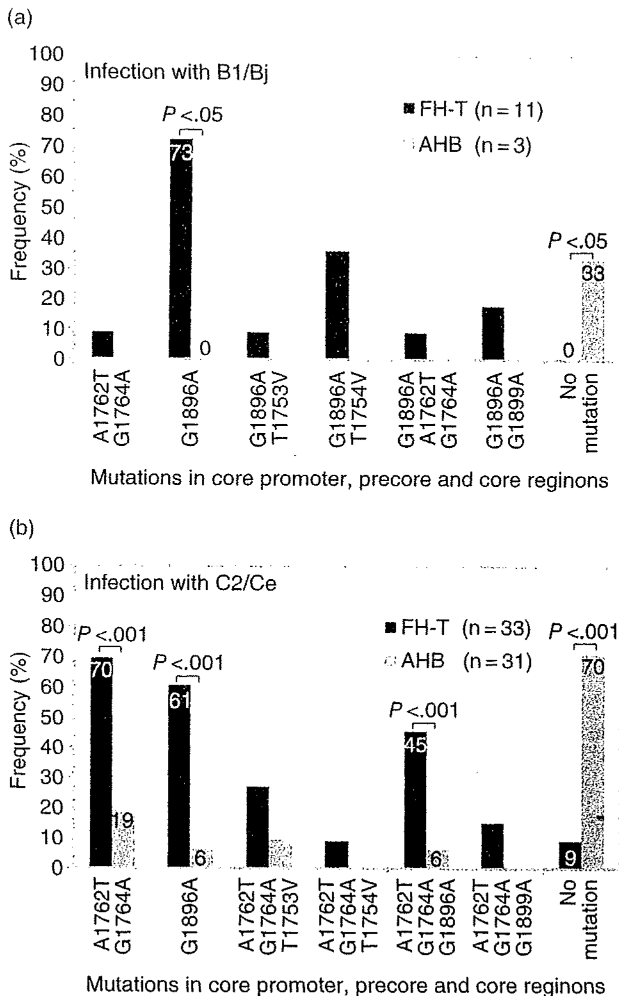

Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs. 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	*P*-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

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Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C (n = 12)	AE-C (n = 12)	Differences *P*-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.[31] Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.[13–16] Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.[19–21] It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.[22]

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,[32] and downregulated by the A1762T/G1764A double mutation at the transcription level.[33,34] Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.[35] Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,[35] the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tollerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.[36,37] In the absence or decrease of HBeAg, therefore, hosts would mount vigor cytotoxic T-cell responses to core epitopes excessively presented on hepatocytes, and develop severe liver injuries culminating in FHB.[38]

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case–control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.[8] These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;[13–16] these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.[18] Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient

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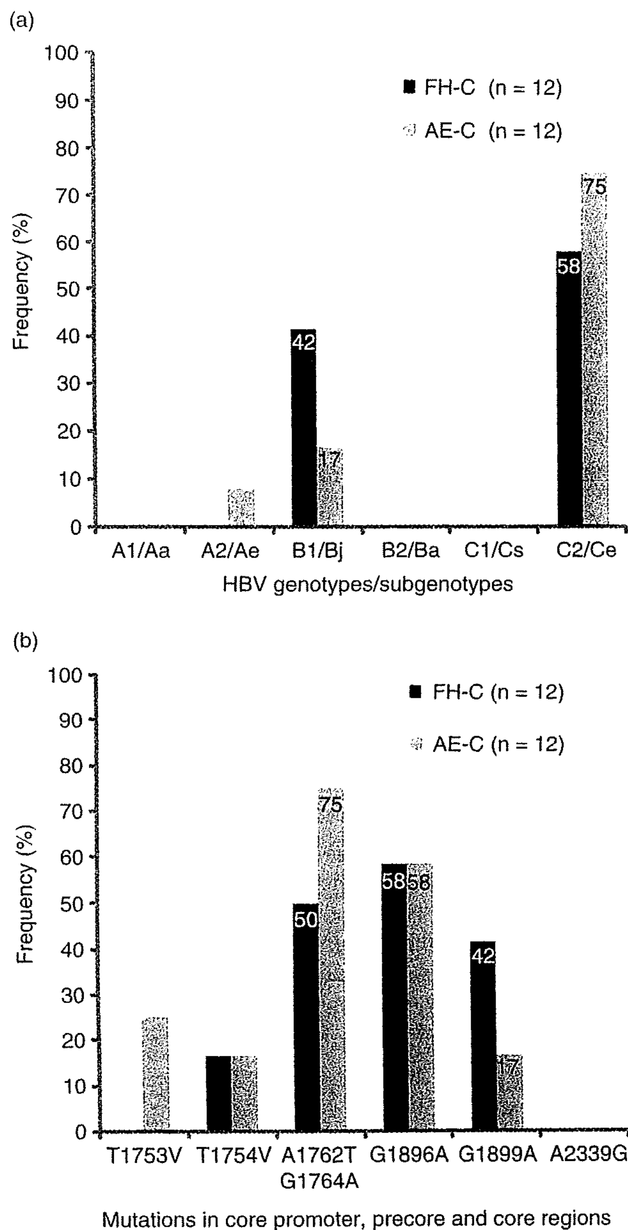

Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

HBV infection (Table 2). In particular, G1896A mutation was the most important factor associated with the development of FHB. Host responses, represented by T.bil, contributed to the development of FHB as well.

As for HBV genotypes, B1/Bj alone was significantly more frequent in the FH-T patients in univariate analysis. In the patients infected with B1/Bj, G1896A was more frequent in those with FH-T than AHB. In *in vitro* replication analysis, Ozasa *et al.*[15] observed extremely high expressions of intra- and extracellular HBV DNA in culture transfected with an HBV clone of B1/Bj genotype having the G1896A mutation; a high replication would be induced by this pre-core mutation for the induction of FHB. Our clinical results stand in support of this *in vitro* analysis. Taken altogether, chances for developing severe acute or FH would be high in the patients with acute hepatitis who are infected with HBV/B1 having the pre-core mutation. By contrast, in patients infected with C2/Ce, G1896A or A1762T/G1764A, or both was much more frequent in the FH-T patients than AHB. Of note, the co-occurrence of G1896A and A1762T/G1764A mutations was invariably accompanied by either FHB or acute severe hepatitis B in this study. Hence, these pre-core and core-promoter mutations might have addictive or synergetic effects for exacerbating hepatitis, when they emerge in the patients infected with C2/Ce. Such high-risk patients deserve special care and surveillance for signs and symptoms of fulminant or severe acute hepatitis B.

In the present study, serum levels of HBV DNA were significantly higher in the patients with FH-T than AHB. High serum levels of HBV DNA have been reported in patients with FHB;[39] they are followed by rapid decrease as the sequel of virus elimination operated by vigorous immune responses. Because of rapid and extensive elimination of HBV by the host immune system, HBV DNA in serum, in general, has decreased to low levels in patients with FHB at the presentation.[40] HBV DNA levels may be subject to the time that has elapsed from the onset of hepatitis to its measurement.[39] Also, serum levels of core protein (the product of the C gene) closely correlate with serum HBV DNA levels in patients with hepatitis B,[27] and they were compared between the FH-T patients and AHB. The core protein was determined by the newly developed CLEIA method; it is much easier and less expensive than the determination of HBV DNA. The level of core protein has turned out to be marginally higher in the FH-T patients than AHB (Table 1), and therefore might not contribute to an early diagnosis of FHB by transient infection.

Fulminant hepatitis B by AE of ASC is assumed as a different clinical condition from FHB by transient HBV infection. In this study, as there was no case–control study on virological factors associated with FHB for the patients with AE of ASC, we also attempted to identify virological factors associated with the development of FHB in the 12 FH-C and the 12 AE-C patients who were

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matched for age as well as sex. Disappointingly, no differences of virological factors such as HBV genotypes and pre-core mutations, which were strongly associated with the development of FHB by transient infection, were found between the FH-C and AE-C patients (Fig. 3a,b). Furthermore, there were also no significant differences about HBeAg-positive rate and the levels of serum HBV DNA or core protein (Table 3), suggesting that several host factors may play a more important role in the development of FHB in ASC instead of virological factors. In this case–control study, however, there seems to be some problems: a small number of patients, different duration of HBV infection, different clinical stage (ASC or CHB) at the onset of AE, and HBV quasispecies complexity. Further investigations are needed to identify factors associated with FHB precipitating in asymptomatic HBV carriers.

In conclusion, virological factors associated with enhancement of viral replication seemed to be important for the development of FHB in the patients by transient HBV infection. But no virological factors were identified for differentiation of the FH-C patients from the AE-C patients. Hence, the pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

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Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B☆

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See Editorial, pages 7–9

***Background/Aims*:** **To determine whether gender, age, hepatitis B virus genotype, core promoter and precore mutations, HBeAg/anti-HBe status, HBV DNA, ALT levels and cirrhosis on presentation were independent risk factors and derive a novel risk score for the development of HCC.**

***Methods*:** **CHB patients (820) were followed up (mean duration 76.8 months) for the occurrence of HCC.**

***Results*:** **The 5- and 10-year prevalence of HCC were 4.4% and 6.3%, respectively. Cox regression analysis showed that male gender ($p = 0.025$, RR 2.98), increasing age ($p < 0.001$, RR 1.07), higher HBV DNA levels ($p = 0.02$, RR 1.28), core promoter mutations ($p = 0.007$, RR 3.66), and presence of cirrhosis ($p < 0.001$, RR 7.31) were independent risks for the development of HCC. A risk score was derived and validated with sensitivity > 84% and specificity > 76% to predict the 5- and 10-year risks for the development of HCC. The AUC for the 5- and 10-year prediction were 0.88 and 0.89, respectively.**

***Conclusions*:** **The risk score, based on age, gender, HBV DNA levels, core promoter mutations and cirrhosis, can estimate the chance of development of HCC in 5 and 10 years after presentation. It can be used to identify high-risk CHB patients for treatment and screening of HCC.**

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***Keywords*:** **Chronic hepatitis B; Hepatocellular carcinoma; Risk factor; Prediction**

1. Introduction

Chronic hepatitis B (CHB) infection affects 350–400 million people worldwide [1]. These patients have a greater than 100-fold increased risk of development of hepatocellular carcinoma (HCC) compared to uninfected individuals [2]. HCC is one of the most aggressive malignancies associated with poor survival. It is very important to identify high-risk patients among carriers of CHB. This will have important implications for treatment allocation and strategic screening for HCC in CHB patients.

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Abbreviations: CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; AFP, alpha-fetoprotein; CT, computer tomography; MRI, magnetic resonance imaging; AST, aspartate aminotransferase.

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Several potential factors have been identified to be associated with a higher risk of development of HCC. These include patient factors including male gender, increasing age [3]; virological factors including hepatitis B e antigen positivity, high serum HBV DNA levels, genotype C compared to B, precore mutations and core promoter mutations [4–11]; and disease factors including alanine aminotransferase (ALT) levels and presence of cirrhosis [3,12]. These risk factors are identified mostly by cross-sectional or case–cohort studies with relatively limited numbers of study population. In addition, these various listed factors may have interacting relationships. Whether these risk factors are independent factors for the development of HCC are still uncertain. A large scale, longitudinal follow-up study examining all these potential risk factors for HCC is required for more accurate assessment of prognosis in CHB patients.

We therefore conducted the present large scale longitudinal study to determine (1) the risk factors for the development of HCC, (2) which risk factors were independent risk factors for the development of HCC, and (3) whether a projected risk estimation score for the development of HCC can be derived from these independent risk factors.

2. Patients and methods

2.1. Patients

During the period between January 1995 and December 2005, all CHB patients who were first seen in our Liver Clinic, Queen Mary Hospital, Hong Kong, were followed up for the development of HCC. Patients were recruited if they were positive for hepatitis B surface antigen (HBsAg) checked by radioimmunoassay (AUSRIA II, Abbott Laboratories, North Chicago, IL) for at least 6 months and had available baseline ultrasound findings and platelet counts on presentation. Hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) were determined by the same radioimmunoassay. Patients who had HCC on presentation or other concomitant diseases including hepatitis C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and fatty liver (diagnosed by ultrasonography) were excluded. Patients who had received or were receiving any form of established treatment for CHB were also excluded. A total of 820 patients were recruited in the present study. Eighty-eight patients were subsequently treated for CHB. The limited number of patients receiving the treatment was due to the fact that the cost of treatment was not reimbursed in our center. Their follow-up time was censored at the time of initiation of treatment. The study was approved by the Institute Review Board of the University of Hong Kong, Hong Kong.

2.2. Monitoring of patients

Patients were followed up every 3–6 months by monitoring HBsAg status, HBeAg/anti-HBe status, liver biochemistry and alpha-fetoprotein (AFP). Ultrasound of the liver was scheduled for patients with AFP levels greater than 20 ng/mL. Computer tomography (CT) and/or magnetic resonance imaging (MRI) and/or hepatic angiogram were performed if ultrasound showed suspicion of HCC. The diagnosis of HCC was made when the patients had one of the following criteria: (1) positive histology or (2) elevated AFP levels together with imaging features compatible with HCC by CT, MRI or hepatic angiogram.

2.3. Definition of cirrhosis

Liver cirrhosis is defined by the following combined parameters: (1) score greater than 2 according to the aspartate aminotransferase (AST) to platelet ratio (APRI) using the formula: [AST/upper limit of normal]/platelet count ($\times 10^9$/L) × 100 [13], (2) ultrasonographic evidence of small sized liver with and without splenomegaly/ascites, and (3) albumin level less than 35 g/L without other identifiable causes of hypoalbuminemia such as renal loss or gastrointestinal loss.

2.4. Determination of HBV genotypes, core promoter/precore mutations and HBV DNA levels

Sera from patients on presentation were taken for the following tests: (1) HBV genotyping performed by an enzyme-linked immunosorbent assay (ELISA) as described in a previous study [14], (2) core promoter (A1762T/G1764A) and precore (G1896A) mutations determined by direct sequencing described in another study [15], (3) HBV DNA levels determined by the Cobas Amplicor HBV Monitor test, Roche Diagnostics, Branchburg, NJ, with the lower limit of detection of 300 copies/mL. HBV DNA levels below the lower detection limit were regarded as 300 copies/mL for statistical calculations.

2.5. Statistical analysis

Statistical analyses to identify risk factors were performed using the SPSS 14.0 for Windows, SPSS Inc., Chicago, IL). Mann–Whitney U test was used to compare continuous variables with skew distribution. Kaplan–Meier analysis using log rank test was used to compare the cumulative risks of development of HCC in different groups of patients. Cox regression analysis was used to determine whether the identified variables associated with HCC were independent risk factors.

Statistical analyses to formulate a risk score for the development of HCC were performed by using the R 2.4.1 version (A language and environment for statistical computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org). This score was constructed as a weighted sum of sex, age, HBV DNA levels in copies/mL in log, cirrhosis, and core promoter mutations. The weights were taken as the corresponding estimated coefficients in a Cox regression analysis after divided by the smallest coefficient and rounded to the nearest integer.

The accuracy of using the derived HCC score for predicting the development of HCC at 5 and 10 years after presentation was examined by first estimating a time-dependent Receiver Operating Characteristic curve by the Nearest Neighbor Estimation method [16]. The area under curve (AUC) was then calculated for measuring the overall prediction accuracy. A 95% confidence interval for an AUC was obtained by sampling the 820 patients for 1000 bootstrap samples with the confidence limits calculated as the 2.5th and 97.5th percentiles. The score was assessed by the leave-one-out cross-validation in order to assess the performance of the HCC score in new data [17]. Specifically, the first of the 820 patients was dropped before we re-did the determination of the weights for calculating a risk score. The weights were used to calculate the score for the first patient. Similarly, the second patient was dropped before its score was calculated based on the other 819 patients. The process continued until all patients had their score calculated.

To ease clinical application of the risk score, cut-off values for the prediction of HCC development at 5 and 10 years were determined by maximizing the Youden index, i.e. sensitivity + specificity − 1, calculated from the time-dependent ROC analysis. Accuracy of using the optimal cut-off values was assessed by the sensitivity, specificity, predictive values and likelihood ratios. Their

Table 1
Baseline demographic and virological data of the study population

Number of patients	820
Sex (M:F)	573:247
Age (years)	40.6 (13.5–83.2)
HBeAg/anti-HBe	356:464 (43.4%:56.6%)
Albumin (g/L)	44 (22–56)
Bilirubin (μmol/L)	11 (1–77)
Alanine aminotransferase (U/L)	47 (5–1251)
Platelet count ($\times 10^9$)/L	175 (55–290)
HBV DNA (log copies/mL)	6.01 (2.5–13)
Genotype (B:C)	328:492 (40%:60%)
Core promoter mutations, WT: MT	312:508 (38%:62%)
Precore mutations, WT:MT	502:318 (61.2%:38.8%)
Cirrhosis	124 (15.1%)

Continuous variables are expressed in median (range). WT, wild-type; MT, mutant.

95% confidence intervals were again obtained by 1000 bootstrap samples. The cut-off values were also cross-validated by the leave-one-out method.

3. Results

3.1. Demographics

A total of 820 CHB patients were recruited. The baseline demographics, liver function tests, platelet counts and virological data are listed in Table 1. The mean and median follow-up duration were 76.8 months (standard deviation 36.2) and 67.4 months (range 6.4–221.4), respectively.

3.2. Characteristics of patients with HCC

Forty (4.9%) patients developed HCC. Thirty-five (87.5%) patients were male. The median age of development of HCC was 57.3 years (range 24.9–84.2). At the time of development of HCC, 12 (30%) patients were HBeAg positive and 24 (70%) were anti-HBe positive (5 were HBeAg positive on presentation and underwent HBeAg seroconversion on subsequent follow-up).

3.3. Patients' factors for the development of HCC

Male patients had a higher cumulative risk of development of HCC compared to female patients (Fig. 1, $p = 0.01$). The median age on presentation of patients with HCC was significantly older compared to that of patients without HCC [54.2 years (range 19.4–81.7) vs. 39.8 years (range 13.5–83.2), respectively, $p < 0.001$].

3.4. Virological factors for the development of HCC

There was no significant difference in the cumulative risk of development of HCC between patients with HBV genotype B and C (Fig. 2, $p = 0.26$) and between patients with precore mutations and wild-type (Fig. 2, $p = 0.64$). However, patients with core promoter mutations had a significantly higher cumulative risk of development of HCC compared to patients with core promoter wild-type (Fig. 2, $p < 0.001$). There was a stepwise increase in the cumulative risks of HCC with increasing levels of HBV DNA in log copies/mL starting from HBV DNA levels of $\geqslant 4$ logs (Fig. 3, $p = 0.028$).

For the HBeAg/anti-HBe status on presentation, there was no difference in the cumulative risk of HCC between patients who were positive for HBeAg ($n = 356$) and positive for anti-HBe ($n = 464$) on presentation ($p = 0.54$). Since 144 out of 356 HBeAg-positive

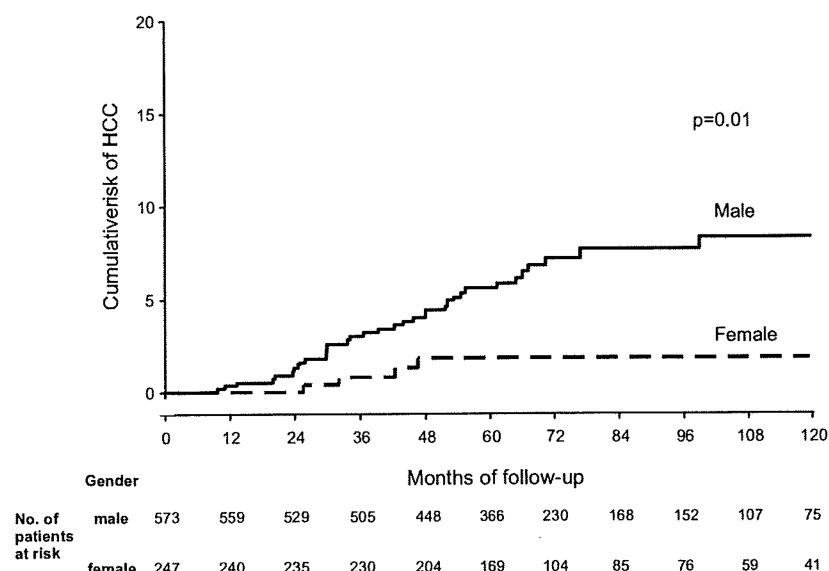


Fig. 1. Cumulative risks for the development of hepatocellular carcinoma in male and female patients.

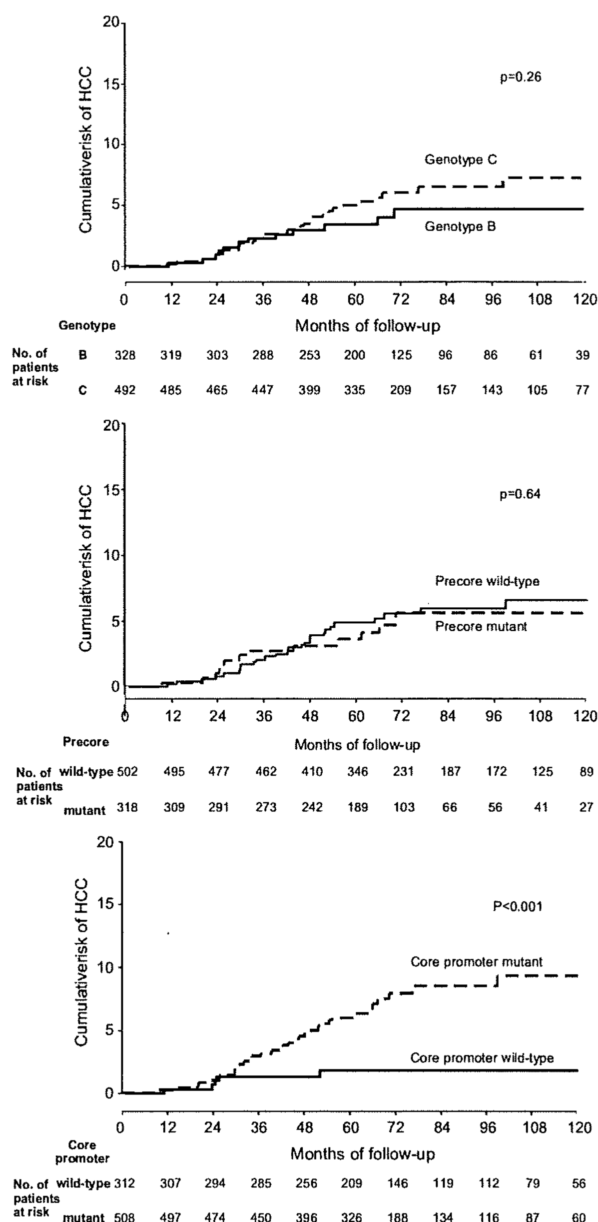


Fig. 2. Cumulative risks for the development of hepatocellular carcinoma in patients with genotype B and C (upper diagram), with precore mutant and wild-type (middle diagram), and with core promoter mutant and wild-type (lower diagram).

patients underwent subsequent HBeAg seroconversion to anti-HBe, patients are further classified into group 1 (persistent HBeAg positivity), group 2 (HBeAg positivity on presentation and with HBeAg seroconversion to anti-HBe during subsequent follow-up) and group 3 (persistent anti-HBe positivity). There were again no differences in the cumulative risk of development of HCC between these 3 groups of patients (Fig. 4, $p = 0.22$ for group 1 vs. group 2; $p = 0.94$ for group 1 vs. group 3; $p = 0.24$ for group 2 vs. group 3).

3.5. Disease factors for the development of HCC

Patients were stratified into 3 groups according to ALT levels on presentation [ALT < 0.5 upper limit of normal (ULN) vs. 0.5–1 × ULN vs. >1 × ULN]. Patients with ALT levels <0.5 × ULN had a significantly lower cumulative risk of development of HCC compared to patients with ALT levels between 0.5–1 × ULN ($p = 0.008$) and >1 × ULN ($p = 0.007$) (Fig. 5). There was no difference in the cumulative risk for the development of HCC between the latter two groups ($p = 0.95$).

Patients with pre-existing cirrhosis had a significantly higher cumulative risk of development of HCC compared to patients without cirrhosis (Fig. 5, $p < 0.001$).

3.6. Multivariate analysis for factors associated with development of HCC

Univariate analyses showed that male gender, increasing age, higher HBV DNA levels in log copies/mL, presence of core promoter mutations, ALT levels higher than 0.5 × ULN and presence of pre-existing cirrhosis were significant risk factors for the development of HCC. On further calculation using Cox Regression analysis, the followings were found to be independent risk factors for the development to HCC: male gender [$p = 0.025$, relative risk (RR) 2.98 [95% confidence interval (CI) 1.15–7.78]; increasing age ($p < 0.001$, RR 1.07, 95% CI 1.04–1.09); higher HBV DNA levels in log copies/ml ($p = 0.02$, RR 1.28, 95% CI 1.04–1.58); core promoter mutations ($p = 0.007$, RR 3.66, 95% CI 1.42–9.47); and presence of cirrhosis ($p < 0.001$, RR 7.31, 95% CI 3.76–14.21). ALT level was not an independent factor ($p = 0.35$).

3.7. Predictive score for the development of HCC

The 5-year and 10-year prevalence of HCC were 4.4% (95% CI 2.9–5.9%) and 6.3% (95% CI 4.2–8.3%). The derived HCC score was formulated as *16 ∗ sex (male = 1; female = 0) + age (in years) + 3 ∗ HBV DNA levels (copies/mL in log) + 19 ∗ core promoter mutations (mutant = 1; wild-type = 0) + 30 ∗ cirrhosis (presence = 1; absence = 0).* This score is abbreviated as "GAG-HCC" score from "Guide with Age, Gender, HBV DNA, Core promoter mutations and Cirrhosis". The hazard ratio of GAG-HCC score for the development of HCC was 1.07 (95% CI 1.05–1.08, $p < 0.001$) indicating that the risk of development of HCC increased by 7% with an increase of score value by one. The hazard ratio of the score by the leave-one-out cross-validation was 1.06 (95% CI 1.05–1.08, $p < 0.001$). By optimizing with the Youden's index, the optimal cut-off of the HCC score for the prediction of 5- and 10-year development of HCC was

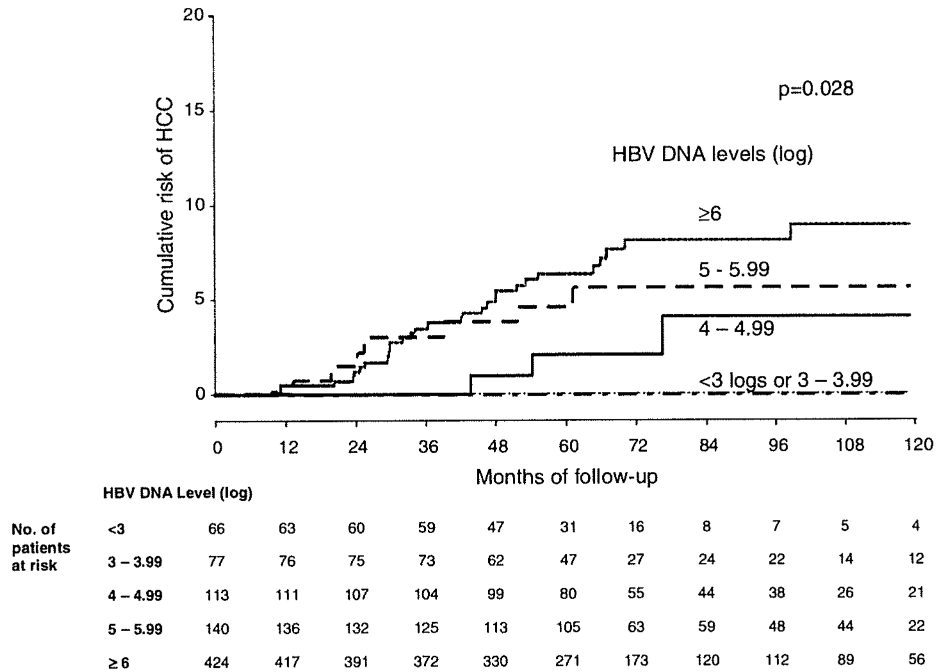


Fig. 3. Cumulative risks for the development of hepatocellular carcinoma in patients with different HBV DNA levels (copies/mL) in log.

101. This cut-off had good sensitivity and specificity and had been accurately validated by the leave-one-out-validation (Table 2). The AUCs were as high as 0.88 (95% CI 0.82–0.93) and 0.89 (95% CI 0.85–0.93) for 5- and 10-year prediction, respectively. The estimated chances of development of HCC at 5 and 10 years according to the score on presentation are depicted in Fig. 6.

Since the data on core promoter mutations may not be easily available in some centers, the score was formulated again without incorporating core promoter mutations to predict the 5- and 10-year risk of development of HCC. Using the same statistical methodology, the risk score was *14 ∗ sex (male = 1; female = 0) + age (in years) + 3 ∗ HBV DNA levels (copies/mL in log) + 33 ∗ cirrhosis (presence = 1; absence = 0).* The

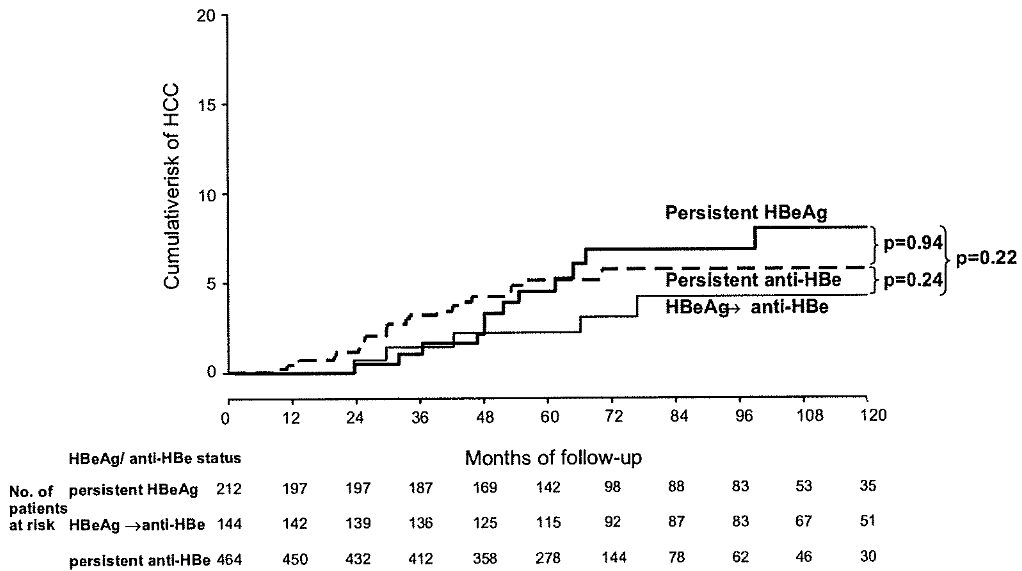


Fig. 4. Cumulative risks for the development of hepatocellular carcinoma in patients with persistently positive HBeAg, HBeAg seroconversion to anti-HBe and persistently positive anti-HBe.

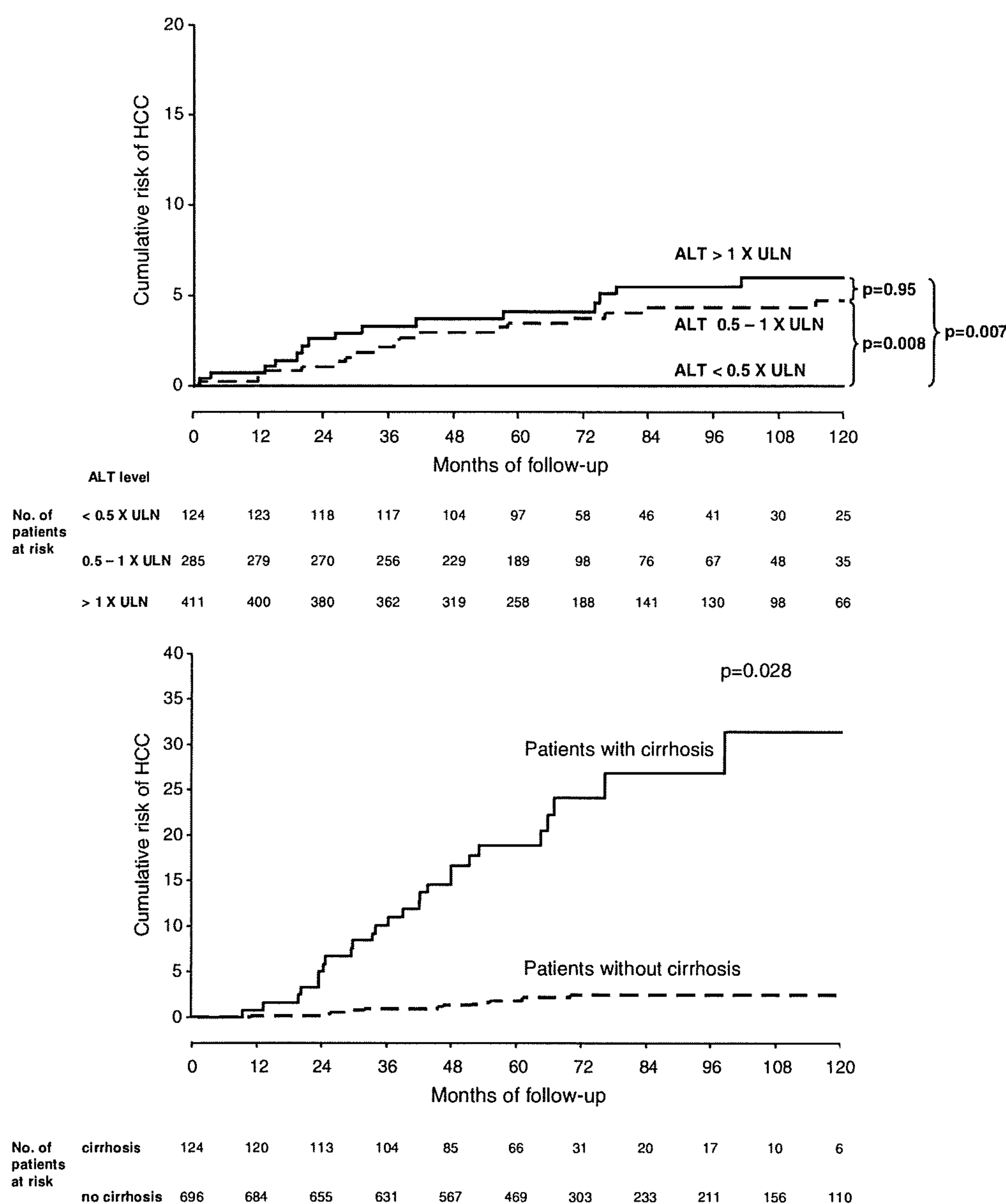


Fig. 5. Cumulative risks for the development of hepatocellular carcinoma in patients with different levels of alanine aminotransferase (upper diagram) and in patients with and without cirrhosis (lower diagram).

hazard ratio of this score without core promoter mutations was 1.06 (95% CI 1.05–1.08, $p < 0.001$). The hazard ratio of this score by the leave-one-out cross-validation was 1.06 (95% CI 1.05–1.08, $p < 0.001$). By optimizing with the Youden's index, the optimal cut-off of the HCC score without core promoter mutations for the prediction of 5- and 10-year development of HCC were 100 and 82, respectively. The sensitivity and specificity of these cut-off values are listed in Table 3. The AUCs were 0.87 (95% CI 0.82–0.93) and 0.88 (95% CI 0.82–0.92) for 5- and 10-year prediction, respectively. The estimated chances of development of HCC at 5 and 10 years according to the score without core promoter mutations on presentation are shown in Fig. 6.

4. Discussion

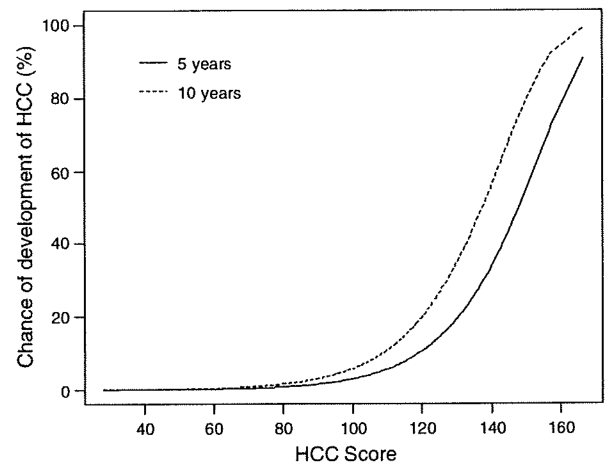
There are two main unsettling issues concerning study reliability and utility of the clinical risk factors for the development of HCC. First, risk factors may be inter-related or inter-dependent and thus confound the calculation of risk development. Second, when there is more than one risk factor for the development of HCC, the risk estimations are not simply additive. It

Table 2
Optimal cut-off values by maximizing Youden index and their accuracies for the HCC score derived from whole study population and validated with leave-one-out cross-validation

	5-year prediction		10 year prediction	
	Value	95% CI	Value	95% CI
Total study population				
Optimal cut-off	101		101	
Sensitivity	84.1%	(67.7%, 97.5%)	88.0%	(76.7%, 95.3%)
Specificity	76.2%	(60.8%, 90.7%)	78.7%	(73.8%, 87.7%)
Positive predictive value	14.0%	(10.0%, 26.3%)	21.7%	(18.0%, 32.9%)
Negative predictive value	98.3%	(99.5%, 99.9%)	99.0%	(98.0%, 99.6%)
Positive likelihood ratio	3.54	(2.38, 7.81)	4.13	(3.27, 7.10)
Negative likelihood ratio	0.21	(0.04, 0.38)	0.15	(0.06, 0.29)
Leave-one-out cross-validation				
Optimal cut-off	101		101	
Sensitivity	87.9%	(74.0%, 100%)	100%	(76.9%, 100%)
Specificity	76.2%	(73.3%, 79.1%)	79.1%	(75.3%, 83.5%)
Positive predictive value	14.6%	(12.2%, 17.2%)	25.7%	(18.1%, 35.6%)
Negative predictive value	99.3%	(98.4%, 100%)	100%	(98.0%, 100%)
Positive likelihood ratio	3.70	(3.02, 4.50)	5.18	(3.31, 8.25)
Negative likelihood ratio	0.16	(0, 0.34)	0	(0, 0.30)

has been shown that the risk for the development of HCC increases dramatically when two risk factors are present concomitantly [18]. This makes simple risk stratification for CHB patients very difficult. The present study tackled these difficulties. One of the limitations of the present study is the possibility of underestimation of the rate of development of HCC since abdominal ultrasound was performed only when patients had elevated AFP levels. Another limitation was that the definition of cirrhosis was not reached from liver biopsies. This may lead to an underestimation of subclinical cirrhosis of the study population. However using our criteria, which were easily available in clinical practice, patients with frank cirrhosis could be identified with reasonable accuracy. In addition, our criteria may be much easier to apply in clinical practice compared to adopting histological assessment for cirrhosis, thus reinforcing the usefulness of the proposed score.

In line with several studies [19,20], HBV genotypes B and C did not differ in the risk for development of HCC. The apparent association between genotype C and HCC reported in some studies [21–23] may be related to the close connection between genotype C and core promoter mutations [24]. In contrast, the presence of core promoter mutations was an independent risk factor for the development of HCC. This is in accordance with previous studies [9–11,18,25,26]. Two previous studies



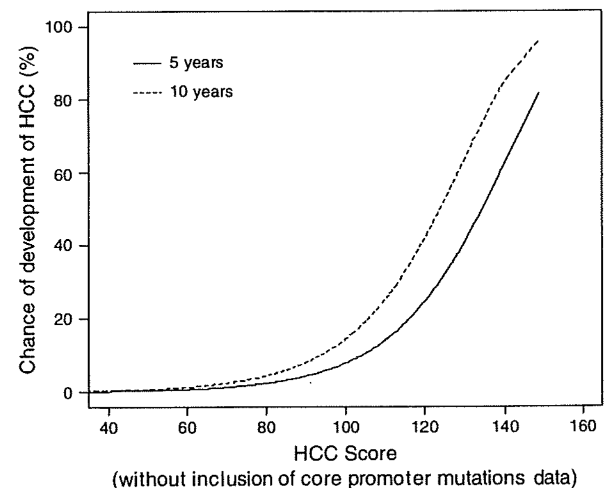


Fig. 6. Chance of the development of hepatocellular carcinoma at 5 and 10 years according to the different values of the HCC score on presentation using the GAG-HCC score (upper diagram) and the score without inclusion of the data of core promoter mutations (lower diagram).

showed that patients with core promoter mutations have higher HBV DNA levels compared to patients without core promoter mutations [27,28].

Apart from core promoter mutations, the present study also found that increasing HBV DNA levels from 4 to >6 logs copies/mL were independently associated with a stepwise increase in the risk for the development of HCC. Importantly, HBeAg and anti-HBe status were not risk factors for the development of HCC. This is true for both the HBeAg/anti-HBe status on presentation and during subsequent follow-up. Our findings that HBV DNA levels and not HBeAg/anti-HBe status were risk factors for the development of HCC are similar to the conclusion of a large scale study from Taiwan (The REVEAL study) [5]. This has obvious implication on whether HBeAg seroconversion *alone* is an adequate endpoint for treatment of HBeAg-positive patients.

Table 3
Optimal cut-off values by maximizing Youden index and their accuracies for the HCC score *without inclusion of the data of core promoter mutations* derived from whole study population and validated with leave-one-out cross-validation

	5-year prediction		10 year prediction	
	Value	95% CI	Value	95% CI
Total study population				
Optimal cut-off	100		82	
Sensitivity	67.8%	(62.4%, 99.3%)	86.6%	(69.7%, 96.3%)
Specificity	88.1%	(59.0%, 91.5%)	75.6%	(65.5%, 90.3%)
Positive predictive value	20.9%	(9.4%, 27.9%)	19.2%	(14.6%, 34.5%)
Negative predictive value	98.3%	(98.1%, 99.9%)	98.8%	(97.7%, 99.7%)
Positive likelihood ratio	5.72	(2.24, 8.37)	3.55	(2.56, 7.88)
Negative likelihood ratio	0.37	(0.01, 0.43)	0.18	(0.05, 0.35)
Leave-one-out cross-validation				
Optimal cut-off	100		82	
Sensitivity	69.6%	(51.1%, 86.7%)	100%	(74.9%, 100%)
Specificity	87.9%	(85.4%, 90.2%)	75.3%	(71.4%, 79.2%)
Positive predictive value	21.0%	(15.5%, 26.6%)	21.7%	(15.8%, 28.4%)
Negative predictive value	98.4%	(97.5%, 99.3%)	100%	(97.8%, 100%)
Positive likelihood ratio	5.75	(3.96, 7.86)	4.14	(2.80, 5.93)
Negative likelihood ratio	0.35	(0.15, 0.56)	0	(0, 0.34)

The present study confirmed that presence of cirrhosis at baseline was the most important independent risk factor because its relative risk for development of HCC for patients with cirrhosis was the highest among the five independent risk factors (male gender, increasing age, HBV DNA level, core promoter mutations and cirrhosis). Another virological factor, namely preS deletion has not been examined in the study population. It has been recently documented to be an important risk factor for the development of HCC [29]. The predictive power of scoring system for the development of HCC may be augmented if preS deletion can also be incorporated in the model in the future studies.

After identifying these five independent risk factors for the development of HCC, the present study derived a novel "GAG-HCC" score which was able to identify patients who were at risk of development of HCC if the score was greater or equal to the optimal cut-off value of 101. This score was validated by the stringent leave-one-out statistical analysis with high sensitivity and specificity of 88.0% and 78.7% for the prediction at 10 years, respectively. The chance of development of HCC increased exponentially once the score was $\geqslant$101 (Fig. 6). There is a slight decrease in the sensitivity (86.6%) and specificity (75.6%) for the 10 year prediction by using the score without inclusion of the data of core promoter mutations (Table 3). Using these risk scores, one can calculate the prognosis of patients on presentation which is important for a clinician in devising each individual patient's management. One can also identify very high-risk patients (e.g. $\geqslant$50% chance of developing HCC in 5 years and 10 years if the GAG-HCC score were $\geqslant$148 and 136, respectively) who should be strongly recommended for treatment by nucleoside/nucleotide analogues to lower one of the two remediable/preventable factors in the score, the HBV DNA levels. Treatment with nucleoside/nucleotide analogues can also potentially prevent or delay the development of cirrhosis (another remediable/preventable risk factor). Even for patients with established cirrhosis, nucleoside/nucleoside analogues have been shown to potentially revert it [30].

In clinical practice, we are not aware of any predictive score for the development of HCC in CHB patients with the integration of all possible independent factors. Our novel score may serve as a good reference for clinicians to decide who should have regular screening for HCC and who should be treated preferentially. It has been shown that early detection of HCC by screening is associated with a higher chance of receiving more curative treatment [31].

In conclusion, male gender, increasing age, HBV DNA levels, presence of core promoter mutations and cirrhosis on presentation are independently associated with development of HCC in CHB patients. A novel GAG-HCC score has been formulated. It is of great clinical use to identify CHB patients at high-risk for the development of HCC. These patients should be treated by drugs to lower the HBV DNA levels and carefully monitored for the development of HCC.

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Direct Cytopathic Effects of Particular Hepatitis B Virus Genotypes in Severe Combined Immunodeficiency Transgenic With Urokinase-Type Plasminogen Activator Mouse With Human Hepatocytes

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Background & Aims: Little is known about the direct cytopathic effect of hepatitis B virus (HBV) and its association with particular viral genotypes or genetic mutations. We investigate HBV genotype–related differences in viral replication, antigen expression, and histopathology in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mice harboring human hepatocytes. _Methods:_ Mice were inoculated with wild-type of different genotype strains (3 for each HBV/A2, B1, and C2) recovered from preinfected-mice sera or patient sera. _Results:_ Histologic analysis of mice infected with HBV/C2 for 22–25 weeks showed abundant ground-glass appearance of the hepatocytes and fibrosis in the humanized part of the murine liver owing to the activation of hepatic stellate cells mediated by oxidative stress through transforming growth factor-β1 signaling, whereas neither was observed with HBV/A2 and B1. The HBV-DNA level in sera was the highest in mice infected with HBV/C2 compared with those with HBV/A2 and HBV/B1 (10^9, 10^7, and 10^4 log copies/mL, respectively, $P < .05$) during 6–8 weeks postinoculation. HB core-related antigen excretion had a similar trend among the genotypes, whereas secretion of HB surface antigen was more pronounced for HBV/A2 followed by HBV/C2 and much less for HBV/B1. Introduction of precore stop-codon mutation in the HBV/B1 caused a significant increase in viral replication, antigen expression, and a histopathologic picture similar to HBV/C2. _Conclusions:_ By using a humanized in vivo model, we show that different HBV genotypes and even particular mutations resulted in different virologic and histopathologic outcomes of infection, indicating that particular genetic variants of HBV may be directly cytopathic in immunosuppressive conditions.

With an estimated 420 million chronic carriers, hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections of human beings. The chronic infection often leads to cirrhosis and/or hepatocellular carcinoma, which is responsible for at least 1 million deaths annually worldwide.[1] The precise mechanism by which chronic viral hepatitis results in hepatocellular carcinoma (HCC) is not known. However, evidence now is available concerning the direct effects of HBV in this process.[2,3] The important issue of a distinct impact of the various HBV genotypes on the virulence has not been addressed directly so far.[4,5]

Genotypes are subdivided further into subgenotypes on the basis of phylogenetic relationships.[6] Evidence for the influence of HBV genotypes/subgenotypes on liver diseases in acute, fulminant, and chronic infection have been reported increasingly.[7–13] Involvement of genetic mutations of HBV in its pathogenesis is another open question. Previous reports have indicated that mutations in basal core promoter, precore/core, envelope, and X coding regions may be associated with HCC.[14] The term *precore mutants* refers to HBV strains with nonsense frameshift or initiation codon mutation in the precore region that prevent translation of hepatitis B e antigen (HBeAg) precursor and are associated with an increase of viral replication via stabilization of the pregenomic encapsidation signal.[15] However, little is known about the histopathologic implication of the mutants. Complexity

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; PCm, precore stop-codon mutation; HBcrAg, antigens related to hepatitis B virus core; HSC, hepatic stellate cell; 8-OHdG, 8-hydroxydeoxyguanosine; PCR, polymerase chain reaction; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1.

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Table 1. Inoculum Profiles on HBV Isolates of Distinct Genotypes/Subgenotypes

Genotype (Subgenotype)	Isolates	Mice (n)	Accession No.	Precore (1896)	HBeAg
A (A2/Ae)	A2_US	4	AB246337	Wild	+
	A2_JPN1	3	AB246338	Wild	+
	A2_JPN2	3	AB362931	Wild	+
C (C2/Ce)	C2_JPN22	4	AB246344	Wild	+
	C2_JPNAT	4	AB246345	Wild	+
	C2_JPN31	3	AB362932	Wild	+
B (B1/Bj_wild)	B1_JPN35w	4	AB246341	Wild	+
	B1_JPN56w	3	AB246342	Wild	+
	B1_JPN58w	4	AB362933	Wild	+
B (B1/Bj_PCm)*	B1_JPN35m	3	[a]	Mutant	−
	B1_JPN56m	3	[a]	Mutant	−
	B1_JPN58m	3	[a]	Mutant	−

[a]Accession numbers are not shown because these 3 clones identical to the above described HBV/B clones were constructed with G1896A point mutation.

of the host and environmental factors complicates evaluation of the veritable virologic differences between genetic variants of HBV in a clinical study. Therefore, a model that eliminates these factors and allows a direct comparison of early dynamics of HBV genotypes is essential for such investigation.

Recently engineered severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator received human hepatocyte transplants (hereafter referred to as *chimeric mice*)[16-18] and are suitable for the experiments with hepatitis viruses in vivo,[19,20] and offer a rare opportunity in modeling the early kinetics of the HBV replication.[21]

In the present study, infecting human hepatocytes in chimeric mice, we show that different HBV genotypes and even particular mutations within the same genotype have distinct virologic characteristics that may have contributed to the distinct histologic outcomes.

Materials and Methods

Inoculation of Chimeric Mice With the Liver Repopulated for Human Hepatocytes

The chimeric mice were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Human hepatocytes were imported from BD Biosciences (San Jose, CA). The human serum albumin was measured by enzyme-linked immunosorbent assay using commercial kits (Eiken Chemical Co Ltd, Tokyo, Japan). The serum levels of the human albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HBV recovered from preinfected-mice sera or sera of patients as described in our previous report.[21] Briefly, a mixture of immature virions can be present in supernatants of cell culture transfected with plasmids expressing HBV[22,23]; therefore, to avoid direct use of the supernatants in experimental mice, the preinfected mice were infected instead, using the culture media, and then were used as a source of HBV inoculums for the experimental mice. Three clones for each HBV/A2, C2, B1_wild, or B1_PC mutant (precore stop-codon mutation [PCm]) were used in this study (Table 1), and each clone was inoculated to 3 or 4 mice.

Patients

Sera were obtained from 6 patients, 3 of whom had acute hepatitis B and the remaining 3 had fulminant hepatitis B. All sera were subjected to HBV extraction and direct sequencing, which determined genotype B (subgenotype Bj/B1) in all of them. HBV genome sequence analysis of the HBV clones isolated from 3 patients with fulminant hepatitis revealed both the presence of the PC mutation (G1896A) and the absence of any other featured mutations such as core promoter or tyrosine methionine aspartate aspartate mutations (Table 2). HBV strains isolated from the 3 acute hepatitis patients were wild type without core

Table 2. Characteristics of Patients From Whom HBV Isolates of Distinct Genotypes/Subgenotypes Were Recovered

Genotype/subgenotype	Isolates	Precore (1896)	Diseases	HBeAg	HBV (LGE[a]/mL)
B1/Bj_wild	B1_JPN1	Wild	AHB	+	6.8
	B1_JPN2	Wild	AHB	+	7.0
	B1_JPN3	Wild	AHB	+	6.7
B1/Bj_PCm	B1_JPN4	Mutant	FHB	−	8.7
	B1_JPN5	Mutant	FHB	−	8.0
	B1_JPN6	Mutant	FHB	−	8.6

AHB, acute hepatitis B; FHB, fulminant hepatitis B.
[a]Log genome equivalents.

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promoter, precore, and tyrosine methionine aspartate aspartate mutations. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committees of the participating institutions. Written informed consent was obtained from each patient.

Histopathologic Examination

Liver tissues were fixed in buffered formalin, embedded in paraffin, and stained with H&E, Masson's trichrome (MT), or orcein staining. To detect α-smooth muscle actin (α-SMA) and human nuclei, polyclonal antibodies against anti-α-SMA (Lab Vision Corp, Fremont, CA) and monoclonal antibody against anti-human nuclei (Chemicon International, Inc, Temecula, CA) were used as primary antibodies, respectively. The fibrosis stage was evaluated by an expert pathologist who was blinded to the nature of inocula (S.T.).

Dihydroethidium Labeling of Reactive Oxygen Species in Liver Tissue

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen, Carlsbad, CA) as previously reported with minor modification.[24] Briefly, in the presence of ROS, dihydroethidium is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA. The fluorescence was detected with laser scanning confocal microscopy. The relative stained area was quantified using National Institutes of Health image analysis for 5 randomly selected areas of digital images in each specimen.

Detection of 8-Hydroxydeoxyguanosine in Liver Tissue

Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHdG) was performed as previously reported with minor modification.[25] The detailed protocol is shown in the Supplementary Materials and Methods section (see Supplementary material online at www.gastrojournal.org).

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Results

Differences of Replication Efficiency Among HBV Genotypes

The inoculums, each containing approximately 10^5 copies of any 1 of the 4 clones: HBV/A2, C2, B1_wild, and B1_PC mutant (PCm), were inoculated to 3 or 4 mice. HBV DNA was quantified in murine sera weekly. One week after inoculation, HBV DNA was detected in both the HBV/A2 and C2 groups. The titer increased approximately by 2 logs within the next 2 weeks, and continued to increase until 7–12 weeks before reaching a plateau. HBV-DNA levels were 2 logs higher in the mice inoculated with HBV/C2 than HBV/A2 at 6–8 weeks postinoculation ($P < .05$) (Figure 1*A*).

To assess the role of the PC mutation, 2 variants of HBV/B1 were included in the comparison between the genotypes: the HBV/B1_wild and HBV/B1_PCm. Differently from HBV/A2 and HBV/C2, both of the HBV/B1 variants had shown a so-called *window* period; characterized by the HBV-DNA levels remaining undetectable until weeks 4–5 after the inoculation. However, after the window period, the HBV-DNA level of the B1_PCm detected at week 5 had rapidly increased in titer, reaching the levels of HBV/C2 and A2 by week 11 (Figure 1*A*). Interestingly, HBV-DNA levels of B1_wild did not show this rapid increment during the whole follow-up period (until week 25). HBV-DNA titer was 3 logs lower in mice inoculated with HBV/B1_wild compared with those with the other genotypes ($P < .01$). To evaluate the replication dynamics of the different genotypes, the time required for a 10-fold increment of the viral load (*log time*) was estimated. When the window periods of HBV/B1_PCm were excluded from the comparison, the log time was similar between the HBV/C2 and B1_PCm, ranging from 7.3 to 8.4 days, whereas HBV/A2 had a longer index (12.9 days), suggesting slower replication. However, the lowest replication efficiency was observed for HBV/B1_wild, with a log time of 27.7 days.

Distinct Characteristics on Antigen Production Among HBV Genotypes

The expression of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) (depicted in Figure 1*B*) did not correspond with that of HBV DNA (Figure 1*A*) for HBV/B1_PCm, which had rapidly increased antigen expression in the early phase, and then decreased sharply. HBeAg of HBV/B1_PCm was undetectable as expected to confirm the function of the stop codon mutation. In contrast, dynamics of HBcrAg and HBeAg expression by HBV/C2 and HBV/A2 resembled those of HBV DNA. The HBcrAg levels of HBV/B1_PCm without HBeAg expression revealed lower levels than those of HBV/A2 or C2. To detect core protein alone without detecting HBeAg, only hepatitis B core antigen (HBcAg) was assessed in each mice group at the peak point of HBcrAg by enzyme-linked immunosorbent assay. The value of HBV/B1_PCm shown was equal to that of HBV/C2, and higher than that of HBV/A2 (data not shown). HBV antigens of HBV/B1_wild group were detectable, although they had extremely low levels, suggesting a very low replication level for this group. Core protein levels in liver tissue, with adjustment for human albumin levels, showed a similar trend to that of sera (data not shown).

Confirmation of HBV/B1_Wild Infectivity by Using Human Sera

Virus titer of the HBV/B1_wild group was very low and the log time was long in the present study. To further confirm these findings, we used 6 sera: 3 from

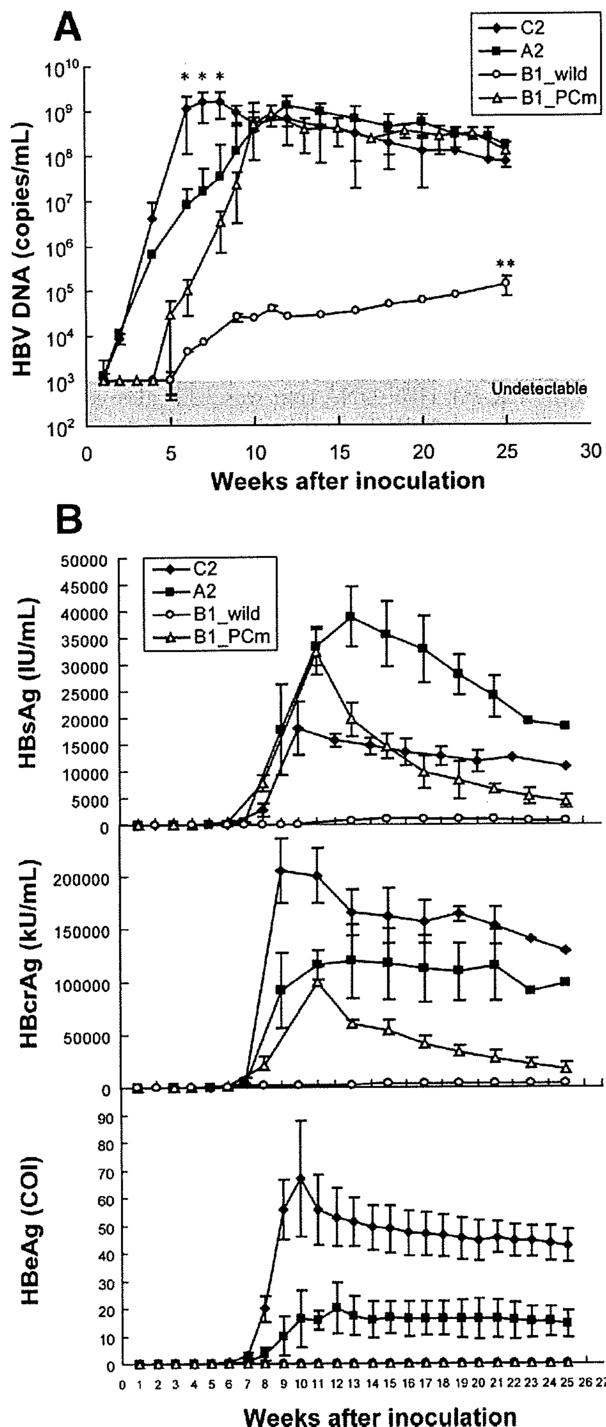

Figure 1. Comparative dynamic profile of HBV-DNA and antigen levels in sera of mice inoculated with preinfected-mice sera recovered from culture media transfecting HBV construct. (*A*) Levels of HBV DNA in sera of the chimeric mice inoculated with HBV/A2, C2, B1_wild, or B1_PCm. Shaded in *gray* is an area below the detection limit (<10^3 copies/mL) of the real-time detection PCR assay. *Statistical differences with a *P* value of less than .05. **Statistical differences with a *P* value of less than .01. (*B*) Dynamic profiles of HBV antigen expression, as revealed by quantification of HBsAg, HBeAg, and HBcrAg in sera of the chimeric mice (see Supplementary Materials and Methods section). For each group, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

acute hepatitis B patients harboring precore wild-type HBV/B1 and the other 3 from fulminant hepatitis B patients harboring precore nonsense-mutation B1 (B1_PCm) (Table 2). Three mice were inoculated with each one of the 6 serum specimens adjusted to contain approximately 10^6 copies of HBV DNA (Figure 2*A* and *B*). Serum HBV-DNA levels increased immediately after inoculation of HBV/B1_PCm and continued to increase until they reached a plateau at week 6 (Figure 2*A*), showing extremely high replication efficiency. The window period was shortened to 2 weeks in the acute hepatitis B serum group with HBV/B1_wild; however, the peak of mean HBV-DNA levels still was low (5×10^5 copies/mL), which was similar to the results by inoculation of preinfected-mice sera (Figure 1*A*). Neither serum levels of the human albumin nor the body weight differed among the mice groups. Based on direct sequencing, no mutations were detected in the HBV complete genomes from any mice 25 weeks after inoculation in comparison with those of inoculated strains.

HBV antigen expression levels of the groups inoculated with human serum samples were compared with those of the groups inoculated with the preinfected-mice sera (Figure 2*B*). HBV antigens of HBV/B1_PCm waxed and waned in profiles similar to that of the groups inoculated with the mice sera in the early phase.

Liver Pathology of Chimeric Mice Infected With Each Genotype

Figure 3 shows the histology of liver in representative chimeric mice infected with HBV/A2, C2, B1_wild, or B1_PCm during weeks 22–25. The immunofluorescence staining was performed using anti-HBcAg and anti-human albumin polyclonal antibody to confirm the location of HBV infection (Supplementary Figure 1; see Supplementary material online at www.gastrojournal.org). Colocalization of HBcAg and human hepatocytes was shown by double staining for HBcAg and human albumin. Almost all of the mice did not reveal apparent steatosis of hepatocytes with H&E stain. The majority of HBV/C2- or B1_PCm-infected human hepatocytes had a ground-glass appearance on H&E stain, fibrosis of stage 2 with MT stain, as well as neutrophil or monocyte invitation. In contrast, the mice infected with HBV/A2 or B1_wild had neither a ground-glass appearance nor fibrosis. To confirm the ground-glass appearance, these specimens were stained by orcein staining. The orcein staining clearly showed cytoplasmic positivity of human hepatocytes infected with HBV/B1_PCm or C2, but not the other group, including control mice.

Immunostaining Analysis on Expression of α-SMA

Active hepatic stellate cells (HSCs) express α-SMA in the early phase of fibrogenesis. To estimate the activation of stellate cells, we performed immunostaining

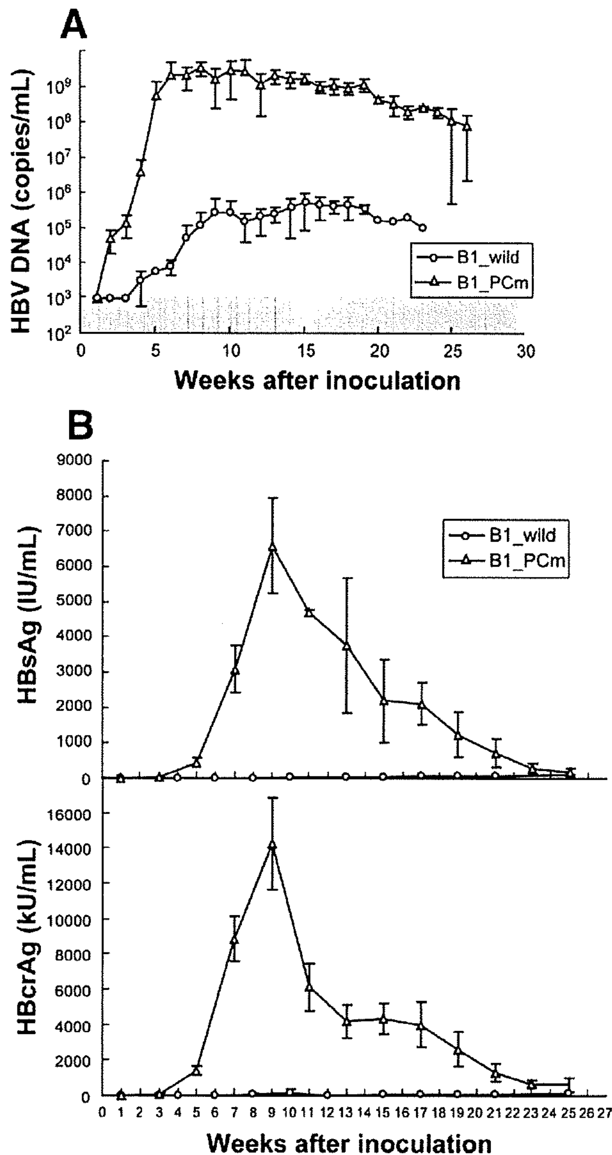

Figure 2. Comparative dynamic profile of HBV-DNA antigen levels in sera of mice inoculated with patient sera from HBV/B1_wild (PC wild-type) and HBV/B1_PCm (PC mutant). (*A*) Mice inoculated with sera from HBV/B1_wild-infected carriers developed acute hepatitis B or from HBV/B1_PCm-infected carriers developed fulminant hepatitis B and were assessed for levels of HBV DNA in mice sera with real-time detection PCR weekly. The area below the detection limit (<10^3 copies/mL) is shaded in *gray*. (*B*) Dynamic profiles of HBV antigens including HBsAg and HBcrAg in mice corresponding to panel *A*. For each genotype, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

using anti–α-SMA antibody. Immunostaining analysis showed strong staining of α-SMA around fibrosis, which was found by MT staining (Figure 4*A*). These results indicated that liver fibrosis of HBV/C2 and B1_PCm occurred via profibrotic cytokines from the activated HSCs but not artifacts. The specimen was double-stained for human nuclei and α-SMA to distinguish between human and mouse cells. As shown in Figure 4*B*, α-SMA and human nuclei did not stain in the same cells, suggesting that the active HSCs were of mouse origin.

Increased Oxidized State in Liver by HBV Infection

In the fibrosis process, current knowledge establishes that the production of ROS plays a critical role in HSC activation involving transforming growth factor-β1 (TGF-β1) signaling.[26] Because α-SMA expressed by HSCs was detected in chimeric mice liver, we next investigated ROS production in mice liver. The ROS production was confirmed by dihydroethidium staining (Figure 5*A*). The level of ROS production was increased statistically when mice were infected with HBV/B1_PC or C2 compared with HBV/A2 or B1_wild ($P < .01$) (Figure 5*B*). Figure 5*C* shows representative immunohistochemical staining for 8-OHdG, which is a marker of oxidative DNA damage, in liver; 8-OHdG–positive cells were recognized in both HBV/C2 and B1_PCm groups, whereas few 8-OHdG–positive cells were detected in the other groups. These data were consistent with those of ROS production.

Gene Expression of Fibrosis Markers in the Mice Liver

As for the change of factors associated with TGF-β1 signaling in the mice, serum alanine aminotransferase (ALT) and TGF-β1 levels were increased in the fibrosis group (B1_PC and C2) as compared with the nonfibrosis group (A2 and B1_wild) (Figure 6*A* and *B*). The TGF-β1 levels in the fibrosis group showed significant difference ($P < .01$). To determine whether the representative fibrosis-related genes were of human or mouse origin, we established species-specific primer sets. Polymerase chain reaction using the species-specific primers gave bands of specific size showing reliable specificity (Figure 6*C*) and dissociation curves (data not shown) (the detailed protocol is provided in the Supplementary Materials and Methods section). Gene expression levels of tissue inhibitor of metalloproteinase 1, matrix metalloproteinase 2, and collagen type 1α2 were quantified by real-time detection reverse-transcription PCR analyses. Specifically, gene expression of human tissue inhibitor of metalloproteinase 1 and mouse collagen type 1α2 represented significantly higher expression in the fibrosis group than that of the nonfibrosis or control groups ($P < .001$). Matrix metalloproteinase 2 and collagen type 1α2 messenger RNA (mRNA) of human origin were undetectable because these genes are produced predominantly in mesenchymal cells.[27]

Discussion

In the present study, the severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator mouse with human hepatocytes was applied to evaluate genotype-dependent differences in the