Hepatitis B Virus Genotype and DNA Level and Hepatocellular Carcinoma: A Prospective Study in Men

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Background: **Although chronic infection with hepatitis B virus (HBV) has been established as a cause of hepatocellular carcinoma (HCC), the roles of viral load and HBV genotype remain unclear.** ***Methods:*** **From 1988 through 1992, baseline blood samples were collected from 4841 Taiwanese men who were HBV carriers but had not been diagnosed with HCC. We used real-time polymerase chain reaction assays of plasma DNA samples to quantify HBV DNA levels (a measure of viral load) and determine HBV genotypes for 154 case patients who were diagnosed with HCC during 14 years of follow-up and 316 control subjects. Unconditional logistic regression was used to assess odds ratios (ORs) of HCC for HBV-related factors. All statistical tests were two-sided.** ***Results:*** **The risk of HCC increased with increasing HBV viral load (adjusted OR for the highest versus the lowest quintile of HBV DNA copies/mL = 7.26, 95% confidence interval [CI] = 3.54 to 14.89; P_{trend}<.001). Genotype C HBV was associated with an increased risk of HCC compared with other HBV genotypes (adjusted OR = 5.11, 95% CI = 3.20 to 8.18). Both viral load and genotype were positively associated with HCC within 10-year age categories among subjects aged 30 years old to older than 60 years. Genotype C HBV was associated with increased viral load, and associations of HBV genotype and viral load with HCC risk were additive. The adjusted OR of HCC for those carrying genotype C HBV and with viral load in the highest quintile was 26.49 (95% CI = 10.41 to 67.42) compared with HBV carriers with other HBV genotypes and viral load in the lowest two quintiles.** ***Conclusions:*** **Measurements of HBV viral load and genotype may help to define which male HBV carriers aged 30 years or older are at high risk for HCC.** **[J Natl Cancer Inst 2005;97:265–72]**

More than 350 million people worldwide have a chronic infection with hepatitis B virus (HBV) *(1)*. Among individuals infected with HBV, those who express HBV surface antigen (i.e., HBsAg carriers) are approximately 20 times more likely to develop HCC than those who do not *(2)*. Nonetheless, only some HBsAg carriers develop HCC, and the hepatocarcinogenic process seems to depend on many predisposing factors, including both viral and host factors *(3–9)*.

HBV induces HCC mainly by causing chronic necroinflammatory hepatic disease *(2)*. Liver injury associated with HBV infection is mediated by both viral factors and the host immune response. Positivity for HBV e antigen (HBeAg), a surrogate marker of active HBV replication, has been associated with higher hepatic inflammatory activity and an increased risk of HCC *(7,10,11)*. Although seroconversion from HBeAg to its antibody (anti-HBe) often coincides with subsidence of hepatic inflammatory activity, the prognosis after HBeAg seroconversion is generally, although not invariably, excellent *(10–16)*. Indeed, a proportion of HBsAg carriers who test positive for anti-HBe retain high levels of HBV DNA and display persistent necroinflammation in the liver *(13–16)*. Results of a small case–control study that was nested within another cohort study of Taiwanese men and used an HBV DNA assay with a detection limit of 7×10^5 copies/mL demonstrated an association between the presence of HBV DNA and the development of HCC in HBeAg-negative HBsAg carriers *(7)*. However, the clinical significance of different levels of HBV DNA remains uncertain.

Seven genotypes of HBV (designated A through G) have been identified on the basis of greater than 8% nucleotide divergence over the whole genome. Most of the HBV genotypes display remarkable geographic variation. For example, genotypes B and C are the predominant genotypes in Asia *(8,9,17–21)*. Although results of some studies suggest that genotype B may be associated with earlier seroconversion to anti-HBe, less serious clinical outcomes, and a better response to interferon therapy than genotype C *(22–25)*, insufficient data on the association between HBV genotype and the risk of HCC are available to draw clear conclusions *(8,9)*.

We report data on the independent and interactive associations between HBV DNA levels and genotype and the development of HCC among HBsAg carriers from a nested case–control study in which blood samples used for measuring the two viral factors and the status of HBeAg and anti-HBe were collected before the diagnosis of cancer.

Subjects and Methods

Study Population

The cohort consisted of 4841 male HBsAg carriers, aged 30 years or older, who had no history of HCC and who attended a

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clinic specific for asymptomatic HBsAg carriers at the Liver Unit of Chang-Gung Memorial Hospital (Taoyuan, Taiwan) or the Government Employee Central Clinics (Taipei, Taiwan) for regular health examinations from August 1, 1988, through June 30, 1992. All study participants provided informed written consent. This study was approved by the research ethics committee at the College of Public Health, National Taiwan University, Taipei, and the appropriate institutional review board. The general design of this cohort study has been described elsewhere *(5,6)*. At enrollment, we conducted in-person interviews with each participant by using a structured questionnaire to obtain information on demographic characteristics and lifetime habits of alcohol and tobacco use. A blood specimen also was collected from each participant at the end of the interview. Blood samples were collected in heparinized tubes and were fractioned into plasma, buffy coat, and erythrocytes, all of which were stored at −70°C before analysis.

Study participants were scheduled to undergo ultrasonography measurements and conventional liver function tests every 6–12 months. Information about vital status and cancer occurrence for those who did not participate in the follow-up examinations were obtained by telephone interview and by linking data with the computer files of national death certification and cancer registry systems. When a case of HCC was identified, we sought permission from the hospital in which the subject was diagnosed with cancer to obtain the medical charts. After 14 years of follow-up examinations, approximately 70% of the HBsAg carriers who were still alive continued to return for their examinations. Beginning in January 1, 2002, we expanded our follow-up interview questionnaire to include questions about any anti-HBV therapy the participant might have received to identify and exclude subjects who had such therapy from this study. The proportion of subjects in our study who returned for examinations after December 31, 2001, and had a history of antiviral therapy was only 0.4%.

Our analysis was restricted to HCCs diagnosed from August 1, 1988, to December 31, 2002. The average follow-up period per participant was 12 years. During the follow-up period, we confirmed 189 incident case patients with HCC. We excluded 35 of these case patients because plasma samples that had been collected from them at the time of enrollment were depleted. The 154 case patients included in this study and the 35 case patients not included were comparable with respect to the distributions of putative risk factors for HCC such as age and histories of cigarette smoking and alcohol consumption *(2–4)*. Thus, we have assumed that the data for these 35 case patients with no available plasma samples was missing completely at random and that the case patients included in this study represent a simple random sample for all eligible case patients identified in the cohort. A total of 114 case patients were included in our previous study on the role of hormonal markers in the development of HCC *(6)*. Five case patients who were included in the previous study were not included in the present study because of insufficient plasma samples.

Eighty-one case patients were diagnosed with HCC on the basis of a pathologic examination and 73 case patients were diagnosed with HCC on the basis of results of abdominal ultrasonographic, angiographic, or computed tomographic studies as well as an elevated serum α-fetoprotein level (≥400 ng/mL). The mean age at HCC diagnosis for the 154 case patients included in this study was 56.7 ± 9.0 years (± standard deviation). Their cancers developed during a median follow-up time of 6.7 years (range = 0.3–13.3 years). Seventy-six case patients also had blood samples collected at the time of diagnosis of cancer or within 2 years before diagnosis; those samples were used to test the longitudinal stability of the viral load and genotype of HBV.

For each case patient, we randomly selected up to three control subjects from the cohort of HBsAg carriers who were alive and had not been diagnosed with HCC throughout the follow-up period. The control subjects were then individually matched to the case patients by age at recruitment (within 5 years) and date of blood collection (within 6 months). A total of 316 control subjects were recruited, of whom 234 control subjects were included in the previous nested case–control study on hormonal markers and HCC *(6)*. Four control subjects who were included in the previous study were not included in this study because of depleted plasma samples. Subjects who were known to have a history of anti-HBV therapy were excluded from this study.

Laboratory Analyses

We used commercially available radioimmunoassay kits to test blood samples for HBsAg (Abbott Laboratories, Chicago, IL) and for HBeAg and anti-HBe (General Biologicals Corporation, Hsin-Chu, Taiwan).

HBV DNA was extracted from 200 μL of each plasma sample by using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics Applied Science, Mannheim, Germany) as previously described *(26)*. We used a real-time polymerase chain reaction (PCR)-based single-tube assay with fluorescent hybridization probes and LightCycler technology (Roche Diagnostics Applied Science, Mannheim, Germany) to determine the HBV viral load and genotype as described by Yeh et al. *(27)*. Briefly, the method consisted of two consecutive steps: the first step used real-time PCR to quantify the viral DNA (a measure of viral load) and the second step used melting curve analysis to genotype the virus. We used three sets of primers and fluorescence-labeled hybridization probes (one anchor probe and one sensor probe), which were synthesized by TIB MOLBIOL (Gerline, Germany) (probe and primer sequences in Supplementary Table 1, available at: http://jncicancerspectrum.oupjournals.org/jnci/content/vol97/issue4). For accurate quantification, the sequences of one set (set 1) of PCR primers and hybridization probes were selected from highly conserved regions of the viral genome to ensure equivalent amplification and hybridization of all HBV genotypes. At the annealing step of PCR, the anchor and sensor probes were hybridized to the target DNA, bringing the fluorescent dyes on each probe into close proximity to yield fluorescence signals generated by fluorescence resonance energy transfer. The final PCR products were further subjected to melting curve analysis. Each sensor probe contained a single-nucleotide polymorphism that allowed different HBV genotypes to be distinguished on the basis of their melting temperatures. The quantification results showed a broad linear distribution of HBV DNA that ranged from 10^2 to 10^{11} copies/mL, with a detection threshold of approximately 250 copies/mL. The within-run and between-run coefficients of variance for this quantification method were 8.9% and 14.3%, respectively.

The two other sets of primers and probes (i.e., sets 2 and 3) were designed for genotyping by melting curve analysis. They were used to confirm the genotype defined by set 1 primers and probes and differentiate infrequent genotypes in Taiwan. When direct sequencing and the phylogenetic analysis was used as the gold-standard genotyping method, our genotyping assay had an inconsistency rate of less than 1%, which was substantially lower than that of the traditional restriction fragment length

polymorphism method for genotyping (1.5%) *(8,18)*. Samples that gave equivocal genotyping results by this approach were subjected to DNA sequence analysis of the pre-S region of HBV genome by using ABI PRISM BigDye sequencing kits (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyzer (Applied Biosystems) as previously described to identify the correct genotypes *(28)*. All assays were conducted by laboratory personnel who were blinded to disease status.

Statistical Methods

Plasma HBV DNA levels were $\log_{10}$-transformed to normalize the data and categorized as quintiles based on their distribution among the control subjects. For statistical comparisons, we assigned a value of 250 copies/mL, the detection limit of our quantification assay, to samples that had undetectable levels of HBV DNA. We used one-way analysis of variance and paired or unpaired Student's *t* tests to compare $\log_{10}$-transformed HBV DNA levels between groups, as appropriate. The correlation between HBV DNA levels measured at two time points was examined by Spearman's rank-order correlation coefficient.

Odds ratios (ORs) and 95% confidence intervals (CIs) were used to evaluate relative risks. We used conditional and unconditional logistic regression models; the latter incorporated terms for the matching factors (e.g., age at recruitment and the date of blood collection). Other covariates that were considered to be potential confounders included histories of cigarette smoking and alcohol consumption and ethnic background (Mainland Chinese or other). Cigarette smoking was considered as a potential confounder because it may influence the immune system *(29)* and is a known risk factor for HCC whose effect on the development of HCC may be modified by alcohol consumption *(2–4)*. Thus, in the multivariable analyses of viral factors and HCC, cigarette smoking and alcohol consumption were also included in the logistic regression models as covariates. However, because the conditional and unconditional models gave similar results and 38 subjects (eight case patients and 30 control subjects) had missing data on viral genotype, we report only results obtained by using unconditional models, which permitted the inclusion of all study subjects with genotype data.

We performed tests for linear trends in the logistic regression models by assigning the medians of each category as the score. Formal tests of statistical interaction were conducted by using the likelihood ratio test to compare nested models with and without the interaction terms of interest. All analyses were conducted using SAS release 6.12 (SAS Institute, Cary, NC). All statistical tests were two-sided.

Results

Baseline Characteristics

At recruitment, there were no statistically significant differences in age, ethnic background, or the histories of cigarette smoking and alcohol consumption between case patients and control subjects (Table 1).

Compared with subjects in the lowest quintile of plasma HBV DNA level at baseline (i.e., those with $\leq$3.61 $\log_{10}$ copies/mL), subjects with plasma HBV DNA levels greater than the second quintile (i.e., $\geq$4.23 $\log_{10}$ copies/mL) had an increased risk of HCC. The adjusted odds ratios of HCC for subjects in increasing quintiles of HBV DNA were 1.00 (referent), 1.07 (95% CI = 0.44 to 2.60), 2.54 (95% CI = 1.16 to 5.59), 2.44 (95% CI = 1.12 to 5.28), and 7.26 (95% CI = 3.54 to 14.89), respectively (P_{trend}<.001). The adjusted odds ratio for subjects infected with genotype C HBV compared with subjects infected with other HBV genotypes (primarily genotype B) was 5.11 (95% CI = 3.20 to 8.18). Compared with control subjects, case patients were more likely to be positive for HBeAg (adjusted OR = 2.13, 95% CI = 1.09 to 4.18) but less likely to be positive for anti-HBe (adjusted OR = 0.28, 95% CI = 0.17 to 0.45) (Table 2).

Table 1. Baseline characteristics of case patients and control subjects*

Characteristic	Control subjects (n = 316), n. (%)	Case patients (n = 154), n. (%)
Age at recruitment, y		
30–39	47 (14.9)	22 (14.3)
40–49	114 (36.1)	58 (37.7)
50–59	91 (28.8)	42 (27.3)
≥60	64 (20.3)	32 (20.8)
Ethnic background		
Fukien Taiwanese	210 (66.5)	89 (57.8)
Hakka Taiwanese	33 (10.4)	15 (9.7)
Mainland Chinese	73 (23.1)	50 (32.5)
History of cigarette smoking		
No	195 (61.7)	90 (58.4)
Yes	121 (38.3)	64 (41.6)
History of alcohol consumption		
No	253 (80.1)	118 (76.6)
Yes	63 (19.9)	36 (23.4)

*Because of rounding, percentages do not always total 100.

To eliminate the possible influence of undiagnosed HCC on HBV DNA levels, we repeated the analysis after excluding the 30 case patients who were diagnosed with HCC within 3 years of the date their blood was drawn. The association between viral load (as defined by HBV DNA level) and HCC became even stronger after these patients were removed (adjusted ORs for increasing quintiles of viral load were 1.00 [referent], 1.09 [95% CI = 0.36 to 3.33], 3.04 [95% CI = 1.17 to 7.94], 3.76 [95% CI = 1.50 to 9.41], and 10.41 [95% CI = 4.38 to 24.73]) (data not shown).

We also examined the association between viral load and HCC within 10-year categories of age because we detected an inverse correlation between $\log_{10}$ HBV DNA levels and age (Spearman's rho = −.11; P = .0178). In all 10-year age groups, case patients had statistically significantly higher mean HBV DNA levels than control subjects (Fig. 1). In addition, the associations we observed for all subjects between an increased risk of HCC and baseline HBV DNA levels of at least 4.23 $\log_{10}$ copies/mL or infection with genotype C HBV were also observed for subjects in each of the 10-year age groups (Fig. 2).

Because HBeAg positivity is associated with an increased risk of HCC *(7)*, we also performed analyses in which subjects were stratified according to HBeAg and anti-HBe status. The adjusted odds ratios of HCC for subjects with genotype C HBV versus subjects with other genotypes of HBV were 2.01 (95% CI = 0.51 to 8.00) among subjects who were positive for HBeAg, 3.61 (95% CI = 0.90 to 14.43) among subjects who were negative for both HBeAg and anti-HBe, and 6.09 (95% CI = 3.34 to 11.10) among subjects who were positive for anti-HBe ($P_{interaction}$ = .2444). When we analyzed the association between viral load and HCC stratified by HBeAg and anti-HBe status, we treated HBV

Table 2. Associations between various serological markers of HBV measured at baseline and subsequent risk of HCC*

Variable	Control subjects (n = 316), n (%)	Case patients (n = 154), n (%)	Adjusted odds ratio (95% CI)†
Quintile of HBV DNA level, $\log_{10}$ copies/mL			
Undetectable	28 (8.9)	5 (3.2)	1.00 (referent)‡
1 (2.77–3.61)	35 (11.1)	7 (4.5)	
2 (3.62–4.22)	63 (19.9)	12 (7.8)	1.07 (0.44 to 2.60)
3 (4.23–4.90)	63 (19.9)	26 (16.9)	2.54 (1.16 to 5.59)
4 (4.91–5.90)	63 (19.9)	28 (18.2)	2.44 (1.12 to 5.28)
5 (5.91–10.81)	64 (20.3)	76 (49.4)	7.26 (3.54 to 14.89)
HBV genotype			
A	2 (0.7)	2 (1.4)	
A and B	1 (0.3)	0 (0.0)	1.00 (referent)
B	234 (81.8)	72 (49.3)	
B and C	5 (1.7)	1 (0.7)	
C	44 (15.4)	71 (48.6)	5.11 (3.20 to 8.18)
Nontypable	2	3	
DNA undetectable	28	5	
HBeAg status			
Negative	286 (93.2)	130 (86.7)	1.00 (referent)
Positive	21 (6.8)	20 (13.3)	2.13 (1.09 to 4.18)
Missing	9	4	
Anti-HBe status			
Negative	47 (15.3)	57 (38.0)	1.00 (referent)
Positive	260 (84.7)	93 (62.0)	0.28 (0.17 to 0.45)
Missing	9	4	

*Because of rounding, percentages do not always total 100. HCC = hepatocellular carcinoma; HBV = hepatitis B virus; CI = confidence interval; HBeAg = hepatitis B e antigen; anti-HBe = antibodies against HBeAg.

†Adjusted for age at recruitment (continuous variable), date of blood collection (continuous variable), ethnicity (Mainland Chinese or other), history of cigarette smoking, and history of alcohol consumption.

‡Two-sided $P_{trend}<.001$, derived from likelihood ratio test.

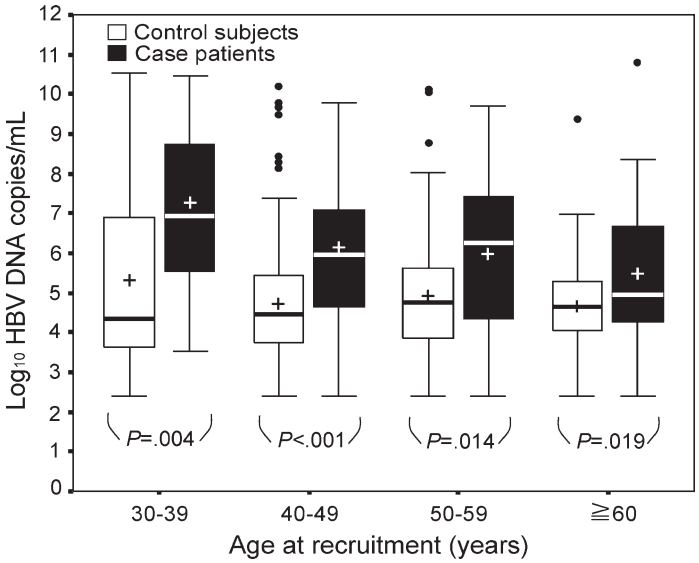

Fig. 1. Box plot of hepatitis B virus (HBV) DNA levels in case patients and control subjects according to age at recruitment. The **horizontal line** within each box represents the median, the **plus sign** shows the mean level, the **lower** and **upper borders** of each box represent the 25th and the 75th percentiles, respectively, and the **T bars** represent the differences between the lower and the upper borders of each box multiplied by 1.5. Outliers (values that exceed these boundaries) are depicted as **single points**. *P* values were derived from Student's t tests for the comparison of the mean levels between case patients and control subjects.

DNA levels as a continuous variable because no subjects who tested positive for HBeAg had HBV DNA levels less than 4.23 $\log_{10}$ copies/mL (i.e., the threshold of viral load that we found was associated with an increased risk of HCC in all subjects) and only 20 case patients and 21 control subjects were positive for HBeAg. The adjusted odds ratio of HCC for an increase of 1 $\log_{10}$ HBV DNA copy/mL was 0.68 (95% CI = 0.39 to 1.16) among subjects who were positive for HBeAg, 3.09 (95% CI = 1.74 to 5.49) among subjects who were negative for both HBeAg and anti-HBe, and 1.55 (95% CI = 1.30 to 1.85) among subjects who were positive for anti-HBe ($P_{interaction}<.001$) (data not shown).

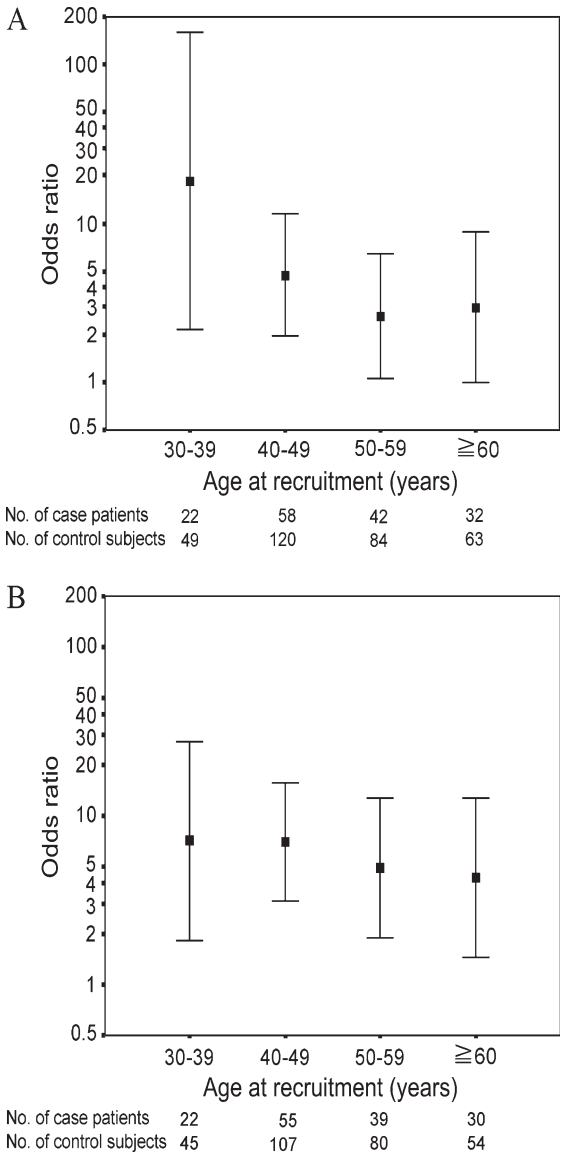

Fig. 2. Odds ratios of hepatocellular carcinoma (HCC) for hepatitis B virus (HBV) viral load and genotype according to age at recruitment. Odds ratios (**solid squares**) with 95% confidence intervals (**error bars**) were estimated by unconditional logistic regression analysis with adjustment for age at recruitment (continuous variable), date of blood collection (continuous variable), ethnicity (Mainland Chinese or other), and histories of cigarette smoking and alcohol consumption. **A**) odds ratios of HCC for HBV carriers with the baseline HBV DNA levels greater than the second quintile (i.e., ≥4.23 $\log_{10}$ copies/mL) compared with HBV carriers with lower HBV DNA levels. **B**) odds ratios of HCC for subjects with genotype C HBV versus subjects with other HBV genotypes (primarily genotype B).

Table 3. Combined risk of HCC associated with the DNA levels and genotype of HBV at baseline*

HBV genotype	Baseline HBV DNA level (log_{10} copies/mL)		
	≤4.22	4.23–5.90	≥5.91
A, B, or mixed genotype†			
No. of case patients/No. of control subjects	8/81	34/113	33/48
Adjusted odds ratio (95% CI)‡	1.00 (referent)	2.95 (1.29 to 6.75)	6.99 (2.97 to 16.49)
C genotype			
No. of case patients/No. of control subjects	11/15	18/13	42/16
Adjusted odds ratio (95% CI)‡	6.55 (2.23 to 19.27)	13.00 (4.65 to 36.31)	26.49 (10.41 to 67.42)

*Excludes 33 subjects (5 case patients and 28 control subjects) with undetectable HBV DNA levels and 5 subjects (3 case patients and 2 control subjects) for whom HBV genotype could not be determined. HCC = hepatocellular carcinoma; HBV = hepatitis B virus; CI = confidence interval.

†Mixed genotype includes genotypes A + B and B + C.

‡Adjusted for age at recruitment (continuous variable), date of blood collection (continuous variable), ethnicity (Mainland Chinese or other), history of cigarette smoking, and history of alcohol consumption.

Combined Association Between Viral Load and Genotype and Risk of HCC

Compared with subjects who had A, B, or mixed genotypes of HBV (A and B or B and C) and were in the bottom two quintiles of HBV DNA levels, the adjusted odds ratios of HCC were 6.99 (95% CI = 2.97 to 16.49) for subjects with A, B, or mixed genotypes of HBV and HBV DNA levels in the highest quintile and 6.55 (95% CI = 2.23 to 19.27) for subjects with genotype C HBV and HBV DNA levels in the lowest two quintiles. Subjects with genotype C HBV and HBV DNA levels in the highest quintile had the highest risk of HCC (adjusted OR = 26.49, 95% CI = 10.41 to 67.42) (Table 3). We used a likelihood ratio test to examine possible deviation from a multiplicative interaction model, which assumes independent effects for HBV genotype and DNA levels on the risk of HCC, and found no statistically significant interaction.

Longitudinal Stability of Viral Load and Genotype

For the 76 case patients for whom we had blood samples collected both at baseline (i.e., study recruitment) and at the time of HCC diagnosis or within 2 years before diagnosis, the time interval between the collection of the two samples ranged from 0.9 to 13.3 years (median = 7.5 years). The mean viral load (± standard deviation) for blood samples collected at baseline was 6.19 ± 1.93 log_{10} HBV DNA copies/mL compared with 5.43 ± 1.70 log_{10} HBV DNA copies/mL for blood samples collected at or within 2 years before HCC diagnosis (P = .0018). The log HBV DNA levels measured at the two time points were moderately correlated (Spearman's rho = 0.41; P<.001). The magnitude of this correlation was only slightly attenuated when we excluded the 17 case patients for whom the interval between the date of the baseline sample collection and the date of the subsequent sample collection was less than 5 years (Spearman's rho = 0.37; P = .0042). After adjustment for age at recruitment and the time interval between the collection of the two samples, case patients in the highest quintile of viral load at baseline had an approximately 20-fold higher risk of being in the highest quintile of viral load at the subsequent measurement than case patients in the three lowest quintile groups at baseline (Table 4).

For the 65 (91.5%) of the 71 case patients who had detectable levels of HBV DNA in both the baseline blood sample and the subsequent blood sample, the HBV genotype was the same in both samples (data not shown).

Association Between HBV Genotype and Viral Load at Baseline

Overall, the mean HBV DNA levels were statistically significantly different among HBeAg-positive subjects (8.87 log_{10} copies/mL, range = 4.39–10.53 log_{10} copies/mL), subjects who were negative for both HBeAg and anti-HBe (5.40 log_{10} copies/mL, range = ≤2.40–8.83 log_{10} copies/mL), and subjects who were positive for anti-HBe (4.84 log_{10} copies/mL, range = ≤2.40–10.81 log_{10} copies/mL) (P<.001). The differences in mean HBV

Table 4. Association between baseline HBV DNA levels and HBV DNA levels measured at the time of diagnosis of HCC or within 2 years before diagnosis*

Quintile of HBV DNA level at baseline, log_{10} copies/mL	HBV DNA levels at the time of diagnosis of HCC or within 2 years before diagnosis		Adjusted odds ratio (95% CI)†
	<5.91 log_{10} copies/mL, n (%)	≥5.91 log_{10} copies/mL, n (%)	
Undetectable	1 (100)	0 (0)	
1 (2.77–3.61)	4 (100)	0 (0)	1.00 (referent)
2 (3.62–4.22)	5 (100)	0 (0)	
3 (4.23–4.90)	12 (85.7)	2 (14.3)	
4 (4.91–5.90)	7 (50.0)	7 (50.0)	10.28 (1.70 to 62.08)
5 (5.91–10.81)	13 (34.2)	25 (65.8)	20.12 (4.05 to 100.12)

*A total of 76 case patients who had a baseline blood sample and a blood sample collected at the time of diagnosis of HCC or within 2 years before diagnosis were included in analysis. HCC = hepatocellular carcinoma; HBV = hepatitis B virus; CI = confidence interval.

†Adjusted for age at recruitment (continuous variable) and the interval between the dates the baseline sample and the follow-up sample were collected (continuous variable).

Table 5. Associations of HBV genotype with HBV DNA levels and the status of HBeAg/anti-HBe at baseline*

Variable	Control subjects (n = 286)			Case patients (n = 146)		
	C genotype (n = 44)	A, B or mixed genotypes (n = 242)†	*P*	C genotype (n = 71)	A, B or mixed genotypes (n = 75)†	*P*
Mean age, y (SD)	51.5 (8.7)	50.0 (9.0)	.308	50.4 (9.4)	50.4 (9.3)	.962
Mean viral load, $\log_{10}$ HBV DNA copies/mL (SD)	5.92 (2.39)	4.99 (1.39)	.016	6.34 (1.93)	5.99 (1.73)	.248
Positive for HBeAg, %‡	25.6	4.3	<.001§	20.3	8.2	.039
Positive for anti-HBe, %‡	65.1	89.4	<.001§	56.5	64.4	.338

*Excluding 33 subjects (5 case patients and 28 control subjects) with undetectable HBV DNA levels and 5 subjects (3 case patients and 2 control subjects) for whom HBV genotype could not be determined. All *P* values were derived from two-sided tests. HBV = hepatitis B virus; HBeAg = hepatitis B e antigen; anti-HBe = antibodies against HBeAg; SD = standard deviation.

†Mixed genotype includes genotypes A + B and B + C.

‡Data were not available for four case patients and eight control subjects.

§Derived from Fisher's exact test.

DNA levels between the three groups remained statistically significant when we considered case patients and control subjects separately ($P<.001$ for each). The percentages of case patients with a viral load greater than 10^5 copies/mL at baseline were 95.0% for those who were HBeAg-positive, 81.1% for those who were negative for both HBeAg and anti-HBe, and 52.7% for those who were positive for anti-HBe. The corresponding percentages for control subjects were 95.2%, 26.9%, and 34.6%, respectively. The mean age at baseline was not statistically significantly different for subjects who were infected with genotype C HBV and those who were infected with other HBV genotypes ($P<.4865$). However, subjects who were infected with genotype C HBV had a higher prevalence of HBeAg-positivity and a higher viral load than subjects who were infected with other HBV genotypes (Table 5).

Discussion

In this study, we found that the risk of HCC increased with increasing plasma HBV DNA levels. This association was stronger after we excluded data from case patients who were diagnosed with HCC within 3 years of the date of blood collection, which implies that HBV DNA levels may decline with the progression of premalignant liver disease. We also found that subjects who were infected with genotype C HBV were approximately five times more likely to develop HCC than subjects who were infected with other HBV genotypes, the majority of whom were infected with genotype B HBV. Subjects who were infected with genotype C HBV had a higher prevalence of HBeAg positivity and a higher HBV DNA level than subjects infected with other HBV genotypes, suggesting that they also had higher levels of HBV replication. However, HBV genotype was also associated with HCC risk independent of high viral load. These observations suggest additive associations of viral load and genotype C HBV with HCC risk. For example, subjects who were infected with genotype C HBV and had HBV DNA levels greater than 5.90 $\log_{10}$ copies/mL had a 26.5-fold higher risk of HCC than subjects who were infected with other HBV genotypes and had HBV DNA levels less than 4.23 $\log_{10}$ copies/mL. The magnitude of this relative risk suggests that HBV viral load and genotype may help to identify HBV carriers who are at a high risk of disease progression to HCC.

There have been few studies on HBV genotype and HCC. Two small studies of the association between HBV genotype and the risk of HCC have been conducted in two regions of Asia, where HBV genotypes B and C predominate, with the use of blood samples taken after the cancer was diagnosed *(8,9)*. Results of the Taiwanese study, which involved 80 case patients, suggested that genotype B HBV is associated with an increased risk of early-onset HCC (i.e., HCC diagnosed in patients younger than 50 years) *(8)*. However, results of the Japanese study, which involved only 58 case patients, suggested that genotype C was more common in HBsAg-positive patients with HCC than in HBsAg-positive control subjects *(9)*. Given that HBV carriers who live in areas where HBV infection is endemic can become superinfected with multiple genotypes of HBV, which can cause hepatitis flares *(30)*, and that some HBV carriers are infected with multiple HBV genotypes *(19)*, prospective studies such as ours are needed to establish the association between long-term stability of HBV genotypes and the development of HCC. We found a strong concordance between the HBV genotype observed in the baseline sample and that observed in the sample collected at the time the cancer was or was about to be diagnosed after a long-term follow-up, further supporting a role of persistent infection with genotype C HBV in hepatocarcinogenesis.

However, the risk of HCC might differ even among subjects infected only with genotype B HBV. HBV genotype B can be further classified into two distinct subtypes, Ba and Bj, based on sequence divergence in the precore region plus the core gene. Because Bj was detected exclusively in Japan in the only study reporting the geographical distribution of HBV/Ba and HBV/Bj *(31)*, it is likely that our results regarding the relative risks of HCC estimated for genotype C HBV were mainly derived from comparing genotype C with Ba.

In accord with previous results *(32)*, we observed that almost all the HBV carriers who were positive for HBeAg had HBV DNA levels greater than 10^5 copies/mL. Positivity for HBeAg was found to be associated with increased risk of HCC in another prospective study of Taiwanese men *(7)*. Our results confirmed that association and further established that anti-HBe positivity is associated with a decreased risk of HCC. Nevertheless, active hepatitis can still occur in some HBV carriers after they develop anti-HBe positivity *(11–16)*. Most HBV carriers eventually lose expression of HBeAg (i.e., become HBeAg-negative) and develop anti-HBe positivity *(33)*. In our study, most of the HBV carriers who subsequently developed HCC had already undergone seroconversion from HBeAg positivity to anti-HBe positivity at recruitment. However, we found that HBV DNA levels and infection with genotype C HBV were still associated with the

development of HCC among subjects who were positive for anti-HBe. In addition, as has been reported in other studies *(13–16)*, we observed that a proportion of HBV carriers who were positive for anti-HBe retained a high viral load. These results suggest that HBeAg status and anti-HBe status are less reliable markers than HBV DNA level for viral replication or disease risk.

Lok and McMahon *(13)* recommended that HBV DNA levels greater than 10^5 copies/mL be considered clinically significant. This recommendation is supported by the findings of a meta-analysis of 26 trials of anti-HBV therapy that evaluated correlations between viral load and hepatic inflammatory activity measured by hepatic histology and aminotransferase levels *(34)*. However, results of previous studies of HBV DNA levels and various chronic liver diseases, including HCC, were frequently based on relatively insensitive assays for which the threshold of detection of HBV DNA is limited to 10^5–10^6 copies/mL *(7,34)*. The pathogenic significance of different levels, especially lower levels, of HBV DNA remains uncertain.

The strengths of this study include the use of a more sensitive assay that can detect HBV DNA at levels as low as 250 copies/mL. We found that HBV carriers with HBV DNA levels greater than 4.22 $\log_{10}$ copies/mL had from two- to sevenfold higher risk of HCC than those with HBV DNA levels less than 3.62 $\log_{10}$ copies/mL. Furthermore, this association between HBV DNA level and HCC risk is unlikely to be influenced by the effect of antiviral therapy because the proportion of subjects in our cohort who received such therapy was very low and no subjects who were known to have a history of such therapy were included in the analysis. Some HBV carriers have HBV DNA levels that fluctuate over time *(32)*. However, during a median follow-up period of 7.5 years, we found that the odds ratio estimate of remaining in the highest quintile of viral load at the time around HCC onset was extremely high for case patients in the highest quintile of viral load at baseline compared with case patients in the three lowest quintile groups at baseline. This finding suggests that HBV DNA levels may remain high in some HBV carriers and that in those individuals, HCC is preceded by the persistently high replication activity of HBV. It has been reported that high activity of viral replication tends to persist longer in Chinese HBV carriers compared with HBV carriers of other ethnic groups *(35)*.

A potential limitation of this work is that our analysis of the stability of HBV DNA levels was based on measurements taken at only two time points. The stability might depend on the initial age of the study subjects, the number of repeated measurement, and the length of the total time period under consideration. Another limitation of this work is that the generalizability of the results is limited because all the study subjects were male. Because HCC is two to three times more frequent in men than in women *(2)*, a larger cohort and a longer follow-up time are needed for a similar study in females.

In conclusion, we found that higher plasma HBV DNA levels and infection with genotype C HBV were independently (and additively) associated with an increased risk of HCC among Taiwanese men. The longitudinal stability of these factors and their positive associations with HCC across 10-year age groups ranging from age 30 years to older than age 60 years suggest that they may be important markers for defining high-risk patients for antiviral treatment among HBV carriers aged 30 years or older. Our finding that HBV carriers with plasma HBV DNA levels greater than 4.22 $\log_{10}$ copies/mL have at least a twofold excess risk of HCC compared with HBV carriers with lower HBV DNA levels, irrespective of age, provides information to define a virologic response representing the long-term clinical improvement for future anti-HBV therapy.

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Notes

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