

Long-term tracking of hepatitis B viral load and the relationship with risk for hepatocellular carcinoma in men

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Little is known about the longitudinal course of hepatitis B virus (HBV) load and its relationship with the development of hepatocellular carcinoma (HCC). We conducted a case-cohort study nested within a cohort of 2874 HBV surface antigen-positive male Taiwanese government employees aged 30 years or older. HBV genotype and DNA levels (i.e. viral load) were tested using polymerase chain reaction-based assays on plasma samples from 112 cases and 1031 non-cases. Prediagnostic plasma levels of HBV DNA were measured in multiple samples collected from each man (total 7706 samples), taken over periods of up to 16 years before diagnosis. Baseline viral load influenced HBV genotype-specific HCC risks and predicted the persistence of high viral load (≥ 4.39 log copies/ml) that can cause HCC. Moderate to high tracking of viral load was observed within 9 years. Hepatitis B e antigen ($P < 0.0001$), genotype C HBV infection ($P = 0.0369$) and longitudinal alanine aminotransferase (ALT) elevation (defined as ALT abnormality in $\geq 50\%$ of the visits) ($P = 0.0005$) were positively related to longer duration of persistence for high viral load. After multivariate adjustment, HBV genotype C [odds ratio (OR) = 5.97, 95% confidence interval (CI) = 3.44–10.34], high viral load detected at $\geq 50\%$ of the visits (compared with sustained low viral load: OR = 5.04, 95% CI = 2.31–11.00) and longitudinal ALT elevation (compared with sustained normal ALT levels: OR = 2.84, 95% CI = 1.46–5.51) accounted for 43.5, 57.2 and 24.9% of HCCs, respectively. The results suggest that maintenance of viral load < 4.39 log copies/ml was associated with sustained normalization of ALT levels and decreased risk of HCC.

Introduction

Hepatitis B is a main cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (1). A high viral load of hepatitis B virus (HBV) seems crucial for the development of chronic liver disease. Prospective studies with the use of sensitive techniques for the detection of HBV DNA have demonstrated a dose–response increase in HCC risk with circulating HBV DNA levels (2,3). Furthermore, reducing HBV DNA levels by means of antiviral therapy was associated with improvement in histologic and biochemical findings and reduction in the incidence of advanced liver disease (4–6). It is possible that prolonged maintenance of high viral load may be more important than transient increases of viral load in predicting risk of HCC. However, little is known about the natural history of HBV viral load.

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio; PAR, population attributable risk; PCR, polymerase chain reaction.

In this study, we described the magnitude of tracking for HBV viral load by testing consecutive plasma samples collected from a cohort of hepatitis B surface antigen (HBsAg)-positive men who had been followed for up to 16 years. In particular, we assessed the influence of HBV genotype and hepatitis B e antigen (HBeAg) status, which are also important viral factors associated with clinical outcomes of HBV infection (2,6–9), on the change in viral load over time. We also investigated the relation of long-term patterns of HBV viral load with longitudinal alanine aminotransferase (ALT) levels and the risk of incident HCC.

Subjects and methods

Case-cohort study design

This study was conducted among 2874 HBsAg-positive male Taiwanese government employees aged ≥ 30 years, who are a subgroup in the cohort study involving 4841 HBsAg carriers that has been described previously (2). These male government employees were enrolled during routine free physical examination between 1989 and 1992. Study participants were invited to return for annual follow-up examinations such as α -fetoprotein, ultrasonography and liver biochemistry tests. Serum ALT levels were assayed at baseline and after August 1994. Information about vital status and cancer occurrence for those who did not participate in a follow-up examination were obtained through a data linkage to the national death and cancer registry systems. This study was approved by the research ethics committee at the College of Public Health, National Taiwan University. All subjects provided an informed consent.

We excluded subjects who met any of the following criteria: had a history of antiviral therapy ($n=86$); had only baseline sample ($n=149$); or missing or positivity for antibodies against hepatitis C virus ($n=229$). Therefore, a total of 2410 subjects were deemed eligible for this study. By 2005, we confirmed 112 incident cases of HCC on the basis of histologic findings or an elevated level of serum α -fetoprotein (≥ 400 ng/ml) combined with at least one positive image from angiography, sonography and/or computed tomography. Using the case-cohort approach, a random subcohort of 1084 men (45.0%) was drawn from the 2410 subjects. This random sample included 53 of the 112 cases.

Data collection

Venous samples were taken from fasting subjects at baseline and each follow-up examination. Samples were aliquoted and frozen at -80°C for subsequent analysis. Information on sociodemographic characteristics, lifestyle habits and medical history were also collected during an in-person interview given prior to each examination as described previously (2).

Laboratory assays

HBsAg was measured by radioimmunoassay (Abbott Laboratories, Chicago, IL). HBeAg (Roche Diagnostics, Indianapolis, IN) was tested by electrochemiluminescence immunoassay. Antibodies against hepatitis C virus were tested by enzyme immunoassay (Abbott Laboratories). After DNA extraction by the method described (10), plasma HBV DNA levels were analyzed by the real-time TaqMan polymerase chain reaction (PCR) with an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). We used the PCR primers and fluorescent probe described by Loeb *et al.* (11). To generate copy number standard curves, the plasmid pBR322-HBV with an insert of a full-length HBV genomic DNA derived from subtype adw HBV (ATCC #45020D) was purchased from American Type Culture Collection (Manassas, VA). The pBR322-HBV plasmid was transformed into ECOS 101 competent cells and purified using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The purified DNA concentration was determined with a spectrophotometer at 260 nm and the corresponding copy number of HBV was calculated. For each run, serial dilutions, ranging from 10^2 to 10^{10} copies/ml, of the plasmid stock were prepared. TaqMan PCR was performed in a 10 μl reaction mixture containing 3 μl of DNA template, 5 μl of $2\times$ BD QTM PCR Buffer, 0.2 μl of $50\times$ BD QTM DNA polymerase (Becton, Dickinson and Company, Palo Alto, CA), 500 nM each of the primers and 200 nM the probe. PCR conditions were 95°C for 5 min, and then 50 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was measured in duplicate and the results from two independent experiments were averaged. This assay has a detection limit of 215 copies/ml and a dynamic range up to 10^{10} copies/ml. The within-run

and between-run coefficients of variance were <5.6% for viral load of ≥1000 copies/ml.

HBV genotype was determined by a nested PCR. The first-round PCR was performed in a total volume of 30 µl containing 6 µl of DNA template, 166 nM each of the primers 5'-TTCACCTCTGCCTAATCATC-3' (HB8F-1824) and 5'-AACAGACCAATTTATGCCTA-3' (HB6R-1803), 83 nM deoxynucleoside triphosphate each, 3 µl of 10× TEMPase Buffer II and 2.5 U TEMPase Hot Start DNA polymerase (Ampliqon, Rødovre, Denmark). Reactions were initially denatured at 94°C for 4 min, and then 34 cycles of 94°C for 40 s, 55°C for 30 s and 72°C for 1.5 min followed by a final extension at 72°C for 10 min. The second-round PCR was a multiplex PCR based on the method as described with a different forward primer (5'-CCCAACAAGGATCACTGGCCA-GAGGCA-3') for genotype C amplification (12). The amplification reaction consisted of an initial denaturation step at 95°C for 4 min followed by 34 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, and then a final extension at 72°C for 10 min. All the forward primers were labeled with fluorescent dyes, and the derived PCR products were subjected to electrophoresis by an ABI 3130xl genetic analyzer (Applied Biosystems). To verify the genotyping results, direct DNA sequencing on both strands was also performed (2).

Statistical analyses

All statistical calculations were performed using SAS version 9.1 (SAS Institute, Cary, NC). Plasma HBV DNA levels were log₁₀ transformed. We assigned a value of 215 copies/ml, the detection limit of our quantitative assay, to samples with undetectable HBV DNA. The hazard ratios of incident HCC and 95% confidence intervals (CIs) were computed by means of a weighted proportional hazards regression, accounting for the case-cohort design by the Barlow method (13). We used the subcohort that was randomly selected from the entire cohort to estimate the cumulative incidences of HCC. The cumulative incidence of HCC estimated from the disease-specific survival curve was

assessed with the use of the Kaplan–Meier method. Comparison of cumulative incidence curves between groups was evaluated by the Wilcoxon test.

In longitudinal analysis, we examined the influence of factors on HBV viral load over the 16-year follow-up and explored the long-term stability of viral load. First, HBV viral load was treated as a continuous variable. We used linear mixed models adjusted for age at the time of assay for HBV DNA to analyze factors associated with change in HBV viral load over time. The covariance matrix of a first-order autoregressive process was used to account for dependence of measurements across time within subjects. Second, we assessed the tendency to maintain a high level of viral load over time. Viral load was dichotomized to a binary variable according to a predetermined cutoff. A generalized logit model was used to assess the influence of initial viral load on latter measurements of viral load over time. All models were adjusted for age at recruitment and the time of follow-up assay. We used an unstructured covariance matrix to account for dependence of observations across time within individuals. Based on parameter estimates from the model, we estimated the probability of having high viral load during a given follow-up period for subjects with initial viral load above or below the predetermined cutoff via inverse logit function. The estimates of the predicted probability were produced using the LSMEANS statement in PROC GLIMMIX. Third, we used the Kaplan–Meier method to compute the time to the first viral load of <4.39 log copies/ml, the threshold of viral load associated with increased HCC risk, for men with initial viral load ≥4.39 log copies/ml.

Finally, we used logistic regression to calculate the odds ratio (OR) of HCC associated with the frequency of having viral load ≥4.39 log copies/ml during the follow-up after adjusting for age at recruitment, total number of visits, HBeAg, HBV genotype and longitudinal ALT levels. The frequency of high viral load or ALT abnormality was categorized as 'zero', 'one', '≥2 visits but <50% of the visits' and '≥50% of the visits' to ensure that subjects with data on two time points could be counted for analysis. The population attributable

Table I. Characteristics of study subjects and their associations with HCC

Characteristic	Group	HCC (n = 112)	No HCC (n = 1031)	Hazard ratio of HCC (95% CI)
Age at onset (year)				
Median		59.2		
Range		39.8–78.7		
Total no. of samples		627	7079	
Median no. of samples per person		6	7	
Range		2–11	2–12	
Average interval between two consecutive visits (year)				
Median		1.5	1.7	
Interquartile range		1.1–1.8	1.5–2.0	
At baseline				
Age (year), n (%)	30–39	13 (11.6)	371 (36.0)	1.00 (reference)
	40–49	40 (35.7)	398 (38.6)	2.83 (1.50–5.34)
	50–59	29 (25.9)	161 (15.6)	5.14 (2.63–10.04)
	≥60	30 (26.8)	101 (9.8)	8.59 (4.38–16.85)
History of cigarette smoking	No	69 (61.6)	718 (69.6)	1.00 ^a (reference)
	Yes	43 (38.4)	313 (30.4)	1.18 (0.78–1.76)
History of alcohol consumption	No	86 (76.8)	818 (79.3)	1.00 ^a (reference)
	Yes	26 (23.2)	213 (20.7)	0.99 (0.62–1.57)
ALT (×upper limit of normal)	≤1	82 (74.6)	959 (93.3)	1.00 ^a (reference)
	>1 and ≤2	24 (21.8)	55 (5.4)	5.75 (3.43–9.65)
	>2	4 (3.6)	14 (1.4)	3.42 (1.04–11.29)
	Missing	2	3	
HBV genotype, n (%)	B	47 (42.3)	825 (81.3)	1.00 ^a (reference)
	B + C	6 (5.4)	45 (4.4)	1.73 (0.71–4.22)
	C	58 (52.3)	145 (14.3)	5.85 (3.82–8.96)
	Untypable	1	16	
Quintile levels of HBV DNA ^b (log copies/ml), n (%)	<2.83	6 (5.4)	214 (20.8)	1.00 ^a (reference)
	2.83–3.56	15 (13.4)	209 (20.3)	1.91 (0.73–4.99)
	3.57–4.38	11 (9.8)	211 (20.5)	1.38 (0.49–3.84)
	4.39–5.90	20 (17.9)	206 (20.0)	2.60 (1.02–6.61)
	≥5.91	60 (53.6)	191 (18.5)	9.18 (3.86–21.84)
HBeAg	Negative	93 (84.5)	914 (90.9)	1.00 ^a (reference)
	Positive	17 (15.5)	92 (9.1)	3.02 (1.67–5.47)
	Missing	2	25	

^aHazard ratios adjusted for age at recruitment (continuous variable).

^bPlasma HBV DNA quintiles were based on distribution of the subcohort (n = 1084).

risks (PARs) of HCC for varying categories of a HBV-related factor were calculated using the formula $PAR = (RR-1)P_c/RR$, in which RR is the relative risk associated with a given category and P_c is the prevalence of that category in HCC cases. The OR was assumed to approximate the relative risk. All reported P values are two sided.

Results

Cases and non-cases were followed for a median time of 7.3 (range = 0.6–15.0) and 13.4 (range = 2.1–16.0) years, respectively. Among the 112 cases, 66 (58.9%) were diagnosed with cirrhosis by ultrasonography during the follow-up, and a total of 54 deaths were observed. Fifty-two of the 54 deaths were attributable to liver-related disease, including 47 from HCC and 5 from cirrhosis. The total number of samples available for testing HBV viral load was 7706 (range = 2–12 samples per person): 627 for cases and 7079 for non-cases (Table I). Supplementary Table I (available at *Carcinogenesis* Online) shows no association between missing observations and baseline viral load.

Baseline characteristics and HCC risk

In case-cohort analysis, the risk of HCC was increased starting at the fourth quintile of HBV viral load (≥ 4.39 log copies/ml), as compared with the lowest quintile of viral load. HBeAg positivity, genotype C HBV infection and elevated ALT activity were also associated with increased risk for HCC (Table I).

In subcohort analysis, men infected with HBV genotype C had a higher cumulative incidence of HCC than men with B or B plus C mixed genotype infection ($P < 0.0001$). The cumulative incidence of HCC within 10 years was 14.49% (95% CI = 9.24–19.74) for genotype C subjects and 2.14% (95% CI = 1.19–3.09) for other genotype subjects. The difference in the cumulative HCC incidences between the two groups was greater if baseline viral load was ≥ 4.39 log copies/ml [e.g. 10-year cumulative incidence: 25.52% (95% CI = 15.86–35.18) for genotype C versus 3.76% (95% CI = 1.76–5.76) for other genotypes] and lesser if baseline viral load was < 4.39 log copies/ml [e.g. 10-year cumulative incidence: 5.32% (95% CI = 0.79–9.85) for genotype C versus 1.11% (95% CI = 0.23–1.99) for other genotypes] (Figure 1).

Longitudinal course of viral load

Cases had HBV viral load that were higher but decreased more rapidly with increasing age compared with non-cases (supplementary Figure 1, available at *Carcinogenesis* Online). As can be seen in supplementary Table II (available at *Carcinogenesis* Online), viral load of study subjects slowly declined with follow-up time. Using normal mixed models controlled for age at the time of assay on viral load, factors positively associated with viral load over the 16-year follow-up period included HCC status, HBeAg positivity, HBV genotype C, elevated ALT levels at baseline, longitudinal ALT elevation and initial viral load (Table II).

Table III shows that tracking for viral load of ≥ 4.39 log copies/ml was better than tracking for viral load in the highest quintile (≥ 5.91 log copies/ml). Within 9-year follow-up, the predicted probabilities of maintaining viral load of ≥ 4.39 log copies/ml among men with initial viral load ≥ 4.39 log copies/ml were between 0.58 and 0.90, whereas the probabilities of maintaining viral load in the highest quintile among men with initial viral load in the highest quintile were between 0.34 and 0.86. However, in the same follow-up period, whatever the cutoffs were set to classify subjects at risk, men with HBeAg positivity, HBV genotype C or longitudinal ALT elevation appeared to have higher probability of maintaining high levels of viral load when compared with men with HBeAg negativity, men with other genotype infection or men without longitudinal ALT elevation. As shown in supplementary Table III (available at *Carcinogenesis* Online), the probability of change from low to high viral load during the follow-up is relatively low.

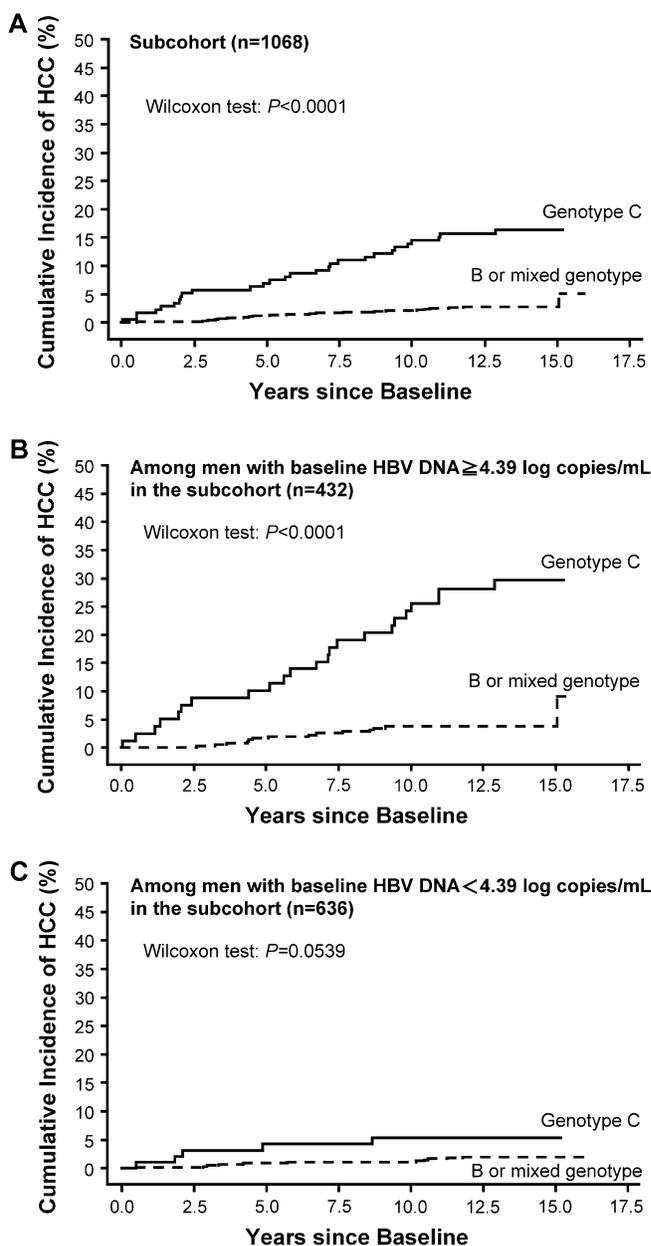


Fig. 1. Kaplan-Meier estimates of the cumulative incidences of HCC by HBV genotype. The analysis was based on the subcohort randomly selected from the entire cohort.

Among men with initial viral load ≥ 4.39 log copies/ml, the median time to first viral load of < 4.39 log copies/ml from the initial assay was 10.0, 4.0, 7.5, 4.5, 8.5 and 4.0 years, respectively, in subgroups with HBeAg positivity, HBeAg negativity, genotype C, B or mixed genotype, ALT elevation in $\geq 50\%$ of the visits and ALT elevation in $< 50\%$ of the visits. HBeAg positivity ($P < 0.0001$), HBV genotype C ($P = 0.0369$) and longitudinal ALT elevation ($P = 0.0005$) were associated with longer duration of persistence for high viral load (Figure 2).

Long-term patterns of viral load and HCC risk

In a logistic regression model controlled for age at recruitment, total number of visits and other potential risk factors, HBV genotype C (OR = 5.97, 95% CI = 3.44–10.34), high viral load (≥ 4.39 log copies/ml) detected at $\geq 50\%$ of the visits (compared with sustained low viral load: OR = 5.04, 95% CI = 2.31–11.00) and longitudinal ALT

Table II. Influence of various factors on change in HBV viral load over 16 years of follow-up

	Estimated change in HBV viral load (log copies/ml)	95% CI	P value
Model 1			
Age at the time of measurement (per 1-year increment)	-0.0169	(-0.0240 to -0.0099)	<0.0001
HBV genotype C versus B or B plus C mixed genotype	0.4726	(0.2730 to 0.6722)	<0.0001
Model 2			
Age at the time of measurement (per 1-year increment)	-0.0204	(-0.0273 to -0.0135)	<0.0001
Cases versus non-cases	1.3706	(1.1044 to 1.6367)	<0.0001
Model 3			
Age at the time of measurement (per 1-year increment)	-0.0154	(-0.0224 to -0.0084)	<0.0001
With versus without ALT elevation at baseline	0.6741	(0.3943 to 0.9538)	<0.0001
Model 4			
Age at the time of measurement (per 1-year increment)	-0.0148	(-0.0215 to -0.0081)	<0.0001
With versus without longitudinal ALT elevation ^a	1.5411	(1.3330 to 1.7492)	<0.0001
Model 5			
Age at the time of measurement (per 1-year increment)	-0.0119	(-0.0168 to -0.0070)	<0.0001
Initial HBV viral load (per 1-log ₁₀ copy/ml increment)	0.6006	(0.5753 to 0.6260)	<0.0001
Model 6			
Age at the time of measurement (per 1-year increment)	-0.00276	(-0.0090 to 0.0035)	0.3863
HBeAg positivity versus negativity	2.8514	(2.6268 to 3.0760)	<0.0001

^aLongitudinal ALT elevation was defined as ALT abnormality in ≥50% of the visits.

Table III. Predicted probability of maintaining high viral load during the follow-up for subjects with high viral load at baseline^a

Years since baseline	All subjects	HBeAg positivity	HBeAg negativity	Genotype C HBV	B or B + C mixed genotype of HBV	With longitudinal ALT elevation ^b	Without longitudinal ALT elevation ^b
No. of study subjects/no. of samples during given follow-up period							
≤3	1094/2445	102/233	966/2155	198/439	880/1969	139/312	839/1938
4-6	961/1358	92/132	848/1198	162/241	786/1098	135/198	822/1156
7-9	848/1307	77/117	753/1160	142/221	695/1071	113/178	735/1129
10-12	698/885	54/70	633/800	109/130	580/740	76/97	621/787
≥13	481/568	34/38	436/516	75/82	400/479	51/60	430/508
Predicted probability (95% CI) of maintaining HBV viral load ≥4.39 log copies/ml during the given follow-up period for men with viral load ≥4.39 log copies/ml at baseline							
≤3	0.78 (0.75-0.80)	0.90 (0.87-0.93)	0.75 (0.71-0.78)	0.84 (0.79-0.87)	0.76 (0.73-0.79)	0.85 (0.80-0.89)	0.75 (0.72-0.78)
4-6	0.71 (0.67-0.75)	NE ^c	0.69 (0.64-0.73)	0.80 (0.69-0.88)	0.69 (0.64-0.73)	0.86 (0.80-0.91)	0.66 (0.62-0.71)
7-9	0.63 (0.59-0.67)	NE ^c	0.63 (0.58-0.68)	0.66 (0.57-0.75)	0.63 (0.58-0.67)	0.79 (0.72-0.85)	0.58 (0.53-0.63)
10-12	0.58 (0.52-0.63)	NE ^c	0.60 (0.54-0.66)	0.41 (0.30-0.52)	0.61 (0.55-0.67)	0.73 (0.53-0.86)	0.54 (0.47-0.60)
≥13	0.44 (0.27-0.63)	NE ^c	0.44 (0.29-0.59)	0.49 (0.21-0.78)	0.46 (0.28-0.66)	NE ^c	0.41 (0.24-0.61)
Predicted probability (95% CI) of maintaining HBV viral load ≥5.91 log copies/ml during given follow-up period for men with viral load ≥5.91 log copies/ml at baseline							
≤3	0.64 (0.61-0.67)	0.86 (0.81-0.91)	0.48 (0.43-0.54)	0.78 (0.72-0.82)	0.59 (0.54-0.63)	0.71 (0.62-0.78)	0.61 (0.57-0.65)
4-6	0.52 (0.48-0.56)	0.65 (0.56-0.74)	0.46 (0.39-0.54)	0.68 (0.54-0.80)	0.48 (0.43-0.53)	0.70 (0.60-0.79)	0.47 (0.42-0.51)
7-9	0.41 (0.36-0.45)	NE ^c	0.40 (0.32-0.48)	0.56 (0.41-0.71)	0.36 (0.31-0.41)	0.61 (0.50-0.71)	0.34 (0.30-0.39)
10-12	0.32 (0.27-0.37)	NE ^c	0.32 (0.23-0.42)	0.16 (0.06-0.39)	0.32 (0.26-0.38)	0.44 (0.23-0.67)	0.29 (0.24-0.35)
≥13	0.17 (0.01-0.86)	NE ^c	0.00 ^d	0.56 (0.21-0.86)	0.14 (0.00-0.93)	NE ^c	0.15 (0.06-0.33)

^aPredicted probability was derived from generalized logit models, with age at recruitment (continuous variable), the time of follow-up examination (continuous variable) and baseline viral load (categorized according to a predetermined cutoff) as covariates and values (categorized according to a predetermined cutoff) of viral load measured during given follow-up period as dependent variable. The ORs of having high viral load during the given follow-up period for subjects with high viral load at baseline relative to those who had low viral load at baseline are shown in supplementary Table IV (available at *Carcinogenesis* Online).

^bLongitudinal ALT elevation was defined as ALT abnormality in ≥50% of the visits. Twenty-six cases and 91 non-cases were missing on longitudinal ALT levels because they had less than two measures on ALT.

^cNot estimated. Predicted probability was not estimated because baseline viral load was not statistically significantly associated with values of viral load measured during the given follow-up period.

^dPredicted probability is very small and its 95% CI is thus omitted.

elevation (compared with sustained normal ALT levels: OR = 2.84, 95% CI = 1.46-5.51) were each significantly associated with increased risk for HCC. HBeAg did not significantly associate with HCC after adjusting for other HBV-related factors. The PARs for genotype C HBV infection, high viral load detected in ≥50% of the visits and longitudinal ALT elevation were 43.5, 57.2 and 24.9%, respectively (Table IV).

Discussion

Higher circulating levels of HBV DNA have been associated with increased risk of HCC in prospective studies. However, previous such studies were based on single- or two-time-point measurements of viral load (2,3,14). HBV DNA in blood may change during long-term follow-up. This change in HBV DNA levels over time is likely to have resulted in measurement error in these studies.

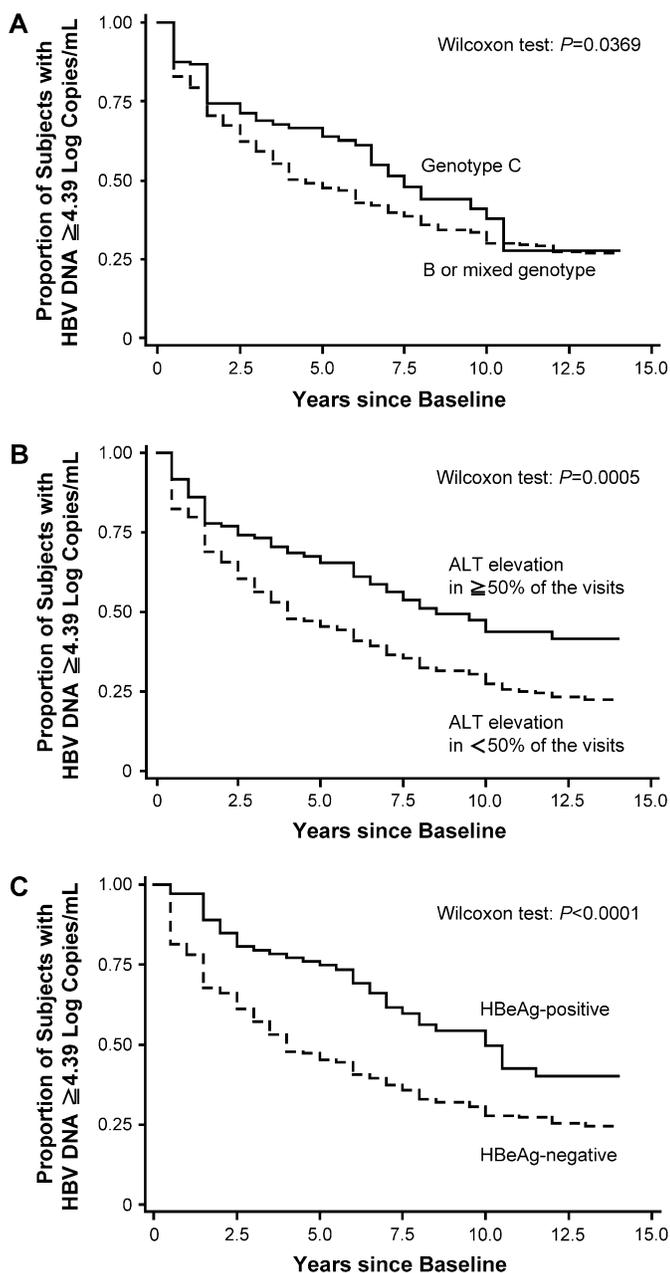


Fig. 2. Kaplan–Meier curves for the time to the first HBV viral load of < 4.39 log copies/ml in men with viral load ≥ 4.39 log copies/ml at baseline. The time to the first viral load of < 4.39 log copies/ml was defined as the time from enrollment to the midpoint between the last visits at which subjects had viral load ≥ 4.39 log copies/ml and the first visits at which subjects had viral load < 4.39 log copies/ml. The median (interquartile range) of the average time between every two consecutive visits for each subject was 1.63 (1.40–1.86), 1.71 (1.50–2.00), 1.67 (1.44–2.00), 1.75 (1.57–2.00), 1.63 (1.40–1.86) and 1.71 (1.50–2.00) years, respectively, in subgroups with HBV genotype C, B or mixed genotype, ALT elevation in $\geq 50\%$ of the visits, ALT elevation in $< 50\%$ of the visits, HBeAg positivity and HBeAg negativity.

This is the first report on the longitudinal course of HBV viral load and HCC in asymptomatic HBV carriers. Our assay for HBV DNA can measure levels to as low as 215 copies/ml. This assay demonstrated good reproducibility, with the coefficients of variation for both within- and between-run variability being comparable with those of previously reported quantitative methods (15,16). One limitation of our study, which is common to all longitudinal studies, was the intermittently missing observations due to non-participation in the

follow-up examinations. However, it does not appear related to the subjects' initial HBV viral load. We therefore believe that our results have a high internal validity.

HBeAg is an indicator of active replication of HBV (2). This study was restricted to HBsAg carriers aged ≥ 30 years, the majority of whom had lost HBeAg at recruitment. We saw moderate to high tracking of HBV viral load during 9-year follow-up, as evidenced by moderate to high probability of maintaining viral load ≥ 4.39 log copies/ml that was associated with increased risk of HCC, even in HBeAg-negative individuals. In contrast, the probability of change to ≥ 4.39 log copies/ml from lower viral load was relatively low. In subgroup analysis, we found that the tendency to maintain a high level of viral load was associated with HBeAg status, HBV genotype and the longitudinal course of ALT levels.

Eight genotypes of HBV have been identified previously (17). In Asia where genotypes B and C are most prevalent, genotype C has been associated with worse response to interferon and more serious liver disease than genotype B (2,6–9). In our samples, HCC affected $\sim 14\%$ of men infected with HBV genotype C within 10 years but only 2% of men with genotype B or mixed genotype infection. The difference in the 10-year cumulative HCC incidences between the two groups was reduced to $\sim 4\%$ when baseline viral load was < 4.39 log copies/ml and was enlarged to 22% when baseline viral load was ≥ 4.39 log copies/ml. This observation suggests that interventions aimed to decrease viral load can substantially reduce the risk of genotype C HBV-related HCC. Genotype C has also been associated with a higher circulating level of HBV DNA than genotype B (2,8). However, since previous investigations of HBV genotype and HBV DNA were cross-sectional studies, the impact of viral genotype on the longitudinal course of HBV DNA remains unclear.

From longitudinal analysis, we found that HBV genotype C was associated with higher viral load over the 16-year follow-up compared with genotype B or mixed genotype infection. In addition, the persistence for viral load of ≥ 4.39 log copies/ml was longer, and the probability of remaining in the highest quintile, which was associated with a 9-fold higher risk of HCC compared with the bottom quintile, was higher in men infected with genotype C HBV than in men with other genotype infection. Therefore, tracking for high viral load that can cause HCC was stronger in men with genotype C than in those with B or mixed genotype.

Our HBsAg carrier subjects were enrolled during regular health examination. Those HBsAg carriers with abnormal ALT at baseline had only mildly to moderately elevated serum ALT levels, and most of them had intermittent ALT elevation during the follow-up. Despite this, we found that men with longitudinal ALT elevation (defined as ALT abnormality in $\geq 50\%$ of the visits) had much higher risk of HCC than those without. Moreover, we have demonstrated an association between longitudinal ALT elevation and prolonged duration of persistence of high viral load that can cause HCC. These findings agree with other investigation demonstrating that upsurge of viral load often preceded ALT elevation (10). Also, they are compatible with clinical trial results of effective suppression of HBV replication by antiviral therapy in association with reduction in necroinflammatory activity and the subsequent development of HCC (4–6).

Approximately 37% (Table IV) of the cases in this study had normal ALT at all samples collected before diagnosis. Previous reports have indicated that a normal ALT level alone was not an accurate indicator of inactive liver disease (3,9,18). From multivariate logistic regression analysis with all the HBV-related factors including HBeAg, our data revealed that HBV genotype C, repeated detection of high viral load and longitudinal ALT elevation were significant predictors of the risk of HCC. The three factors are thus equally important for longitudinal evaluation to select HBV carriers for clinical trial and/or intervention.

The threshold of HBV viral load associated with increased risk of HCC in this study was 4.39 log copies/ml, similar to the levels suggested by the previous prospective studies (2,3). Although circulating HBV DNA levels may fluctuate over time, they remain sufficiently high in most of the time for a long term in some HBV carriers. On the

Table IV. ORs and PARs for the main risk factors of HCC

	HCC, n (%)	No HCC, n (%)	OR of HCC (95% CI)			PAR ^b (%)
			Age and total no. of visits adjusted	Fully adjusted ^a	P value	
Frequency of having HBV DNA levels ≥ 4.39 log copies/ml						
Zero	17 (15.2)	374 (36.3)	1.00 (reference)	1.00 (reference)		
At one visit	7 (6.3)	127 (12.3)	1.45 (0.58–3.66)	2.16 (0.72–6.49)	0.1723	3.4
At ≥ 2 visits but $< 50\%$ of the visits	8 (7.1)	122 (11.8)	2.31 (0.93–5.73)	3.19 (1.16–8.77)	0.0246	4.9
At $\geq 50\%$ of the visits	80 (71.4)	408 (39.6)	4.45 (2.55–7.78)	5.04 (2.31–11.00)	< 0.0001	57.2
HBV genotype						
B	47 (42.3)	825 (81.3)	1.00 (reference)	1.00 (reference)		
B + C	6 (5.4)	45 (4.4)	1.52 (0.59–3.92)	1.47 (0.40–5.34)	0.5587	1.7
C	58 (52.3)	145 (14.3)	7.14 (4.56–11.17)	5.97 (3.44–10.34)	< 0.0001	43.5
Untypable	1	16				
Frequency of ALT elevation						
Zero	32 (37.2)	551 (58.6)	1.00 (reference)	1.00 (reference)		
At one visit	11 (12.8)	176 (18.7)	1.63 (0.78–3.42)	1.12 (0.51–2.45)	0.7719	1.4
At ≥ 2 visits but $< 50\%$ of the visits	10 (11.6)	97 (10.3)	3.40 (1.51–7.65)	1.59 (0.64–3.91)	0.3160	4.3
At $\geq 50\%$ of the visits	33 (38.4)	116 (12.3)	6.41 (3.63–11.31)	2.84 (1.46–5.51)	0.0021	24.9
Not available ^c	26	91				
HBeAg						
Negative	93 (84.5)	914 (90.9)	1.00 (reference)	1.00 (reference)		
Positive	17 (15.5)	92 (9.1)	2.91 (1.57–5.40)	0.89 (0.40–1.94)	0.7616	
Not available	2	25				

^aORs adjusted for age at recruitment (continuous variable), total number of visits (continuous variable) and all other factors listed in the table.

^bPARs were calculated using the fully adjusted ORs.

^cSubjects who had less than two measures on ALT were excluded from analysis.

other hand, some of these fluctuations may be merely due to variability in the sampling or measurement procedure. Therefore, subjects with viral load ≥ 4.39 log copies/ml in $\geq 50\%$ of the visits had a much higher risk for HCC than subjects with such high viral load in $< 50\%$ of the visits, as compared with those who had sustained low viral load.

According to PAR calculation, we estimate that 57% of the HCCs occurring among male HBV carriers aged ≥ 30 years in high-incidence areas where perinatal transmission of HBV is common could be attributed to long-term high-titer viral replication. It can be expected that therapy effective in sustained viral suppression to the levels of $< 10\,000$ copies/ml would lead to a marked reduction in HCC incidence. Based on our data, however, $\sim 40\%$ (Table I) of the male HBV carriers aged ≥ 30 years who have random viral load of ≥ 4.39 log copies/ml would be considered receiving such therapy. The screening rules for determining HBV viral load after age 30 and the strategies to prevent HCC in high-risk population remained to be defined (1).

In conclusion, over a period of 16 years, this longitudinal study has provided the unique data on the long-term dynamics of HBV viral load among male HBV carriers aged ≥ 30 years in area where HBV infection is mostly acquired perinatally (1). Only men were included because incidence of HCC is two to four times higher in men than in women (19). Since subjects with antiviral therapy were excluded from the analysis, our results could infer the natural course of chronic HBV infection. The results suggest that HBV viral load influenced HBV genotype-specific HCC risks and was fairly stable for up to 9 years. HBeAg positivity, genotype C HBV infection and longitudinal ALT elevation were associated with longer duration of persistence of high HBV viral load that can cause HCC. Although most subjects had a fluctuating course of circulating HBV DNA levels, maintenance of a level < 4.39 log copies/ml was associated with sustained normalization of ALT levels and decreased risk of HCC.

Supplementary material

Supplementary Figure 1 and Tables I–IV can be found at <http://carcin.oxfordjournals.org/>

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