

Risk of Hepatocellular Carcinoma Across a Biological Gradient of Serum Hepatitis B Virus DNA Level

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MORE THAN 350 MILLION persons in the world are infected with chronic hepatitis B virus (HBV).¹

It is particularly endemic in Taiwan, where the infection is usually acquired perinatally or in early childhood.² Individuals with chronic hepatitis B infection are at an increased risk of developing liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma³; 15% to 40% of these individuals will develop these serious sequelae during their lifetime.^{3,4} Hepatitis B virus is not directly cytopathic, and the development of hepatocellular carcinoma in individuals with chronic hepatitis B is a multistage, multifactorial process including the interaction between host and environmental factors.⁵ Risk factors for chronic HBV-related hepatocellular carcinoma include sex, age, cigarette smoking, alcohol consumption, chemical carcinogens, hormonal factors, and genetic susceptibility.⁶

Seropositivity for the hepatitis B surface antigen (HBsAg) is one of the

Context Serum hepatitis B virus (HBV) DNA level is a marker of viral replication and efficacy of antiviral treatment in individuals with chronic hepatitis B.

Objective To evaluate the relationship between serum HBV DNA level and risk of hepatocellular carcinoma.

Design, Setting, and Participants Prospective cohort study of 3653 participants (aged 30-65 years), who were seropositive for the hepatitis B surface antigen and seronegative for antibodies against the hepatitis C virus, recruited to a community-based cancer screening program in Taiwan between 1991 and 1992.

Main Outcome Measure Incidence of hepatocellular carcinoma during follow-up examination and by data linkage with the national cancer registry and the death certification systems.

Results There were 164 incident cases of hepatocellular carcinoma and 346 deaths during a mean follow-up of 11.4 years and 41 779 person-years of follow-up. The incidence of hepatocellular carcinoma increased with serum HBV DNA level at study entry in a dose-response relationship ranging from 108 per 100 000 person-years for an HBV DNA level of less than 300 copies/mL to 1152 per 100 000 person-years for an HBV DNA level of 1 million copies/mL or greater. The corresponding cumulative incidence rates of hepatocellular carcinoma were 1.3% and 14.9%, respectively. The biological gradient of hepatocellular carcinoma by serum HBV DNA levels remained significant ($P < .001$) after adjustment for sex, age, cigarette smoking, alcohol consumption, serostatus for the hepatitis B e antigen (HBeAg), serum alanine aminotransferase level, and liver cirrhosis at study entry. The dose-response relationship was most prominent for participants who were seronegative for HBeAg with normal serum alanine aminotransferase levels and no liver cirrhosis at study entry. Participants with persistent elevation of serum HBV DNA level during follow-up had the highest hepatocellular carcinoma risk.

Conclusion Elevated serum HBV DNA level ($\geq 10\,000$ copies/mL) is a strong risk predictor of hepatocellular carcinoma independent of HBeAg, serum alanine aminotransferase level, and liver cirrhosis.

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most important risk factors for hepatocellular carcinoma.⁶ The risk of hepatocellular carcinoma associated with seropositivity for HBsAg ranges from

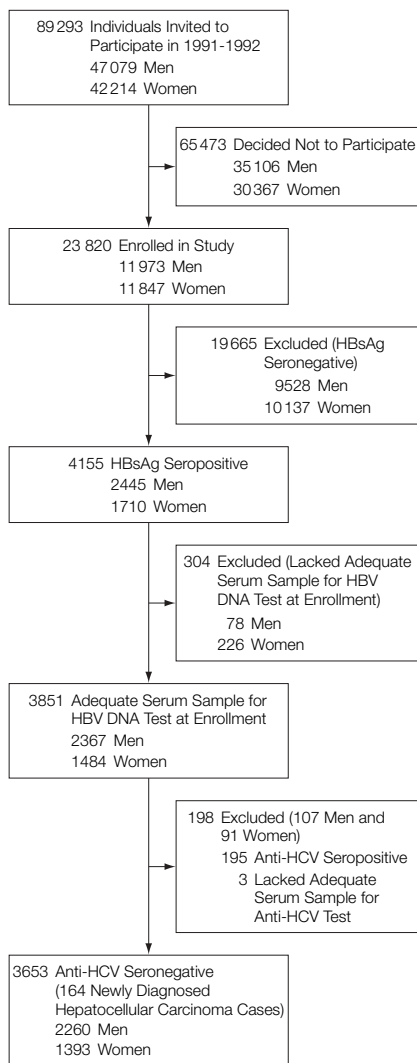
5-fold to 98-fold with a population-attributable risk of 8% to 94%.⁶ In our previous study, seropositivity for the hepatitis B e antigen (HBeAg) was

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Figure 1. Flow of Study Participants in the REVEAL-HBV Study

Anti-HCV indicates antibodies against hepatitis C virus (HCV); HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; REVEAL-HBV, Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus.

associated with an increased risk for hepatocellular carcinoma.⁷ The adjusted hazard ratio (HR) of developing hepatocellular carcinoma was 9.6 for individuals seropositive for HBsAg and 60.2 for those seropositive for both HBsAg and HBeAg compared with those seronegative for both HBsAg and HBeAg. The increased hepatocellular carcinoma risk in individuals seropositive for HBeAg remained significant regard-

less of serum level of alanine aminotransferase (ALT) and status of liver cirrhosis.^{8,9}

Highly sensitive testing methods based on polymerase chain reaction have become available for measuring serum level of HBV DNA. In current clinical practice guidelines,¹⁰⁻¹³ serum HBV DNA level is used as a criterion for antiviral treatment in patients with chronic hepatitis B. Effective suppression of serum HBV DNA is a marker of efficacy for antiviral therapy.¹⁴ Its implementation as the primary surrogate end point instead of liver histology in assessment of new antiviral therapies remains to be validated.¹⁵ There are no long-term follow-up data on the relationship between serum HBV DNA level and risk of liver complications. In a small nested case-control analysis,⁷ we reported a significant association between serum HBV DNA level at study entry and hepatocellular carcinoma risk among individuals seronegative for HBeAg. However, there were only a small number of hepatocellular carcinoma cases and controls included in this study. Neither hepatocellular carcinoma incidence nor change in serum HBV DNA level over time was studied.

In this population-based, long-term prospective study, we followed up a total of 3653 individuals who were seropositive for HBsAg and seronegative for antibodies against hepatitis C virus (anti-HCV) at study entry. The goals of this study were to assess the biological gradient of hepatocellular carcinoma risk by (1) serum HBV DNA levels at study entry and adjusted for other risk factors including age, sex, cigarette smoking, alcohol consumption, seropositivity for HBeAg, elevated serum ALT level, and presence of liver cirrhosis at study entry; and (2) persistent elevation of serum HBV DNA level at both study entry and follow-up examinations.

METHODS

Cohort Recruitment and Follow-up

FIGURE 1 shows the flow of participants through the Risk Evaluation of

Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) study. A total of 89 293 individuals aged 30 to 65 years, who were living in 7 townships in Taiwan, were invited to participate in the study. From 1991 to 1992, 23 820 individuals agreed to participate and provided written informed consent for interview, health examination, blood collection for serological and biochemical assays, and follow-up of health status through health examination, medical record review, and linkage with national health profiles on the national cancer registry and death certification systems. The health examination at study entry included abdominal ultrasonography and serological tests for HBsAg, HBeAg, anti-HCV, and serum levels of ALT and α -fetoprotein. A total of 4155 participants who were seropositive for HBsAg and free of hepatocellular carcinoma at study entry examination were followed up with abdominal ultrasonography and serological tests until June 30, 2004. Serum samples that had been collected and frozen at study entry were adequate for HBV DNA testing in 3851 participants. Among them, 3653 were seronegative for anti-HCV and were included in this study. This study was approved by the institutional review board of the College of Public Health, National Taiwan University in Taipei.

Interview and Blood Collection

All participants were interviewed in person using a structured questionnaire administered by well-trained public health nurses at recruitment. Information was collected on sociodemographic characteristics, dietary habits, cigarette smoking, alcohol consumption, medical and surgical history, and family history of hepatocellular carcinoma and liver cirrhosis. History of menarche, menopause, pregnancy, delivery, and use of oral contraceptives and hormone therapy was also collected from female participants. Using standard sterile techniques, a 10-mL blood sample was collected at study entry and at follow-up examinations.

Laboratory Examinations

Serological testing was performed as follows: HBsAg and HBeAg by radioimmunoassay with commercial kits (Abbott Laboratories, North Chicago, Ill); anti-HCV by enzyme immunoassay using second-generation commercial kits (Abbott Laboratories); ALT by serum chemistry autoanalyzer (Model 736, Hitachi, Tokyo, Japan) using commercial reagents (Biomérieux, Marcy L'Etoile, France); and serum HBV DNA was measured by polymerase chain reaction using the Cobas Amplicor HBV monitor test kit (Roche Diagnostics, Indianapolis, Ind), an in vitro nucleic acid amplification test for quantifying HBV DNA. It has a certified lower limit of detection of 300 copies/mL. At this level of HBV DNA, the test has a 98.1% positivity rate and a 100% clinical specificity rate. The manufacturer's procedures for sample preparation, storage, and testing were followed. Whole blood was collected with a vacuum blood collection tube without an anticoagulant, the serum was separated by centrifugation at room temperature and was stored in a sterile tube at -70°C . This procedure was accomplished within 6 hours of sample collection. Each sample run for HBV DNA included a replicate of the negative, low-positive, and high-positive controls, and each run was determined to be valid.

To examine the impact of changing serum HBV DNA level on hepatocellular carcinoma risk, the subset of participants with a study entry serum level of HBV DNA of 10 000 copies/mL or greater was evaluated. The follow-up serum samples were collected either at the last follow-up examination or at the follow-up examination preceding the diagnosis of hepatocellular carcinoma. Of the 1619 participants with serum HBV DNA levels of 10 000 copies/mL or greater at study entry, 1330 (82%) had adequate follow-up serum samples for testing.

Ascertainment of Newly Developed Hepatocellular Carcinoma

All study participants were without hepatocellular carcinoma at study entry.

Newly developed hepatocellular carcinoma cases were detected by follow-up health examination or by computerized data linkage with the profiles on the national cancer registry in Taiwan from January 1, 1991, through June 30, 2004. To ensure complete ascertainment, we also performed data linkage with the profiles on the national death certification system to identify cases not registered in the cancer registry system. The ascertainment of newly developed hepatocellular carcinoma was complete and accurate.

In total, 164 incident hepatocellular carcinoma cases occurred during the follow-up period. Medical record verification of all hepatocellular carcinoma diagnoses was based on the following criteria: a histopathological examination (66 cases); a positive lesion detected by at least 2 different imaging techniques (abdominal ultrasonography, angiogram, or computed tomography; 87 cases), or by 1 imaging technique and a serum α -fetoprotein level of 400 ng/mL or greater (35 cases). Several cases qualified based on more than 1 criterion.

Statistical Analysis

The person-years of follow-up for each participant were calculated from the date of recruitment to the date of the diagnosis of hepatocellular carcinoma, the date at death, or the last date of linked data available from the national cancer registry (June 30, 2004), whichever came first. Participants free of hepatocellular carcinoma at their death or at the end of follow-up were censored. Incidence rates of hepatocellular carcinoma were calculated by dividing the number of incident hepatocellular carcinoma cases by person-years of follow-up. Cumulative incidence of hepatocellular carcinoma by follow-up year was derived using the Nelson-Aalen method.¹⁶⁻¹⁸ STATA software version 7.0 (STATA Corp, College Station, Tex) was used for the statistical analyses.

The Cox proportional hazards model was used to analyze the associations between serum HBV DNA level and hepa-

tocellular carcinoma risk adjusted for sex, age, HBeAg status, serum ALT level, liver cirrhosis status, cigarette smoking, and alcohol consumption. The adjusted HRs with 95% confidence intervals (CIs) were derived to assess the magnitude of the association between various predictors and risk of hepatocellular carcinoma. Statistical significance levels were determined by 2-tailed tests ($P < .05$). The dose-response relationship between serum HBV DNA level and risk of hepatocellular carcinoma after adjustment for other risk factors was examined for statistical significance with a test for trend. The biological gradient of hepatocellular carcinoma risk by serum HBV DNA level was further examined in stepwise subgroup analyses by sequentially removing participants seropositive for HBeAg, with an elevated serum ALT level, and with liver cirrhosis at study entry from the analysis.

In the analysis of the risk of hepatocellular carcinoma by the change of serum level of HBV DNA during follow-up, the HBV DNA levels at baseline and follow-up examinations were combined to categorize study participants. Adjusted HRs were then derived for each category. $P < .05$ was considered significant.

RESULTS

The basic characteristics of the study cohort appear in TABLE 1. The majority were male (62%); older than 39 years (67%); never smoked (66%); never drank alcohol (87%); had a serum ALT level of less than 45 U/L (94%); were seronegative for HBeAg (85%); and did not have liver cirrhosis (98%). The frequency distribution of serum HBV DNA level at study entry of 3653 study participants appears in TABLE 2. There were 873 study participants (23.9%) with an undetectable HBV DNA level (< 300 copies/mL), while 976 participants (26.7%) had a level of 100 000 copies/mL or greater. Participants seropositive for HBeAg had a significantly higher serum level of HBV DNA than participants who were seronegative for HBeAg ($P < .001$). Of 565 participants seropositive for HBeAg, 523

(92.6%) had an HBV DNA level of 100 000 copies/mL or greater. Of 3088 participants seronegative for HBeAg, 453 (14.7%) had a HBV DNA level of 100 000 copies/mL or greater.

Incidence Rates

There were 41 779 person-years of follow-up with an average follow-up period of 11.4 years (TABLE 3). Through follow-up health examination and data linkage with the national cancer regis-

try and national death certification systems between January 1, 1991, and June 30, 2004, 164 new hepatocellular carcinoma cases and 346 deaths were identified. The incidence rates of hepatocellular carcinoma by serum HBV DNA level as well as other risk factors at study entry examination appear in Table 3. The incidence rates per 100 000 person-years increased from 108.3 for the undetectable HBV DNA level (<300 copies/mL) to 1152.0 for the HBV DNA level of 1 million copies/mL or greater in a dose-response relationship. The biological gradient of hepatocellular carcinoma risk by serum level of HBV DNA at study entry examination was significant ($P < .001$). Compared with participants having serum HBV DNA levels of less than 300 copies/mL, the crude HR was 1.0 (95% CI, 0.5-2.2) for participants with serum HBV DNA levels of 300 to 9999 copies/mL; 2.7 (95% CI, 1.3-5.6), 10 000-99 999 copies/mL; 8.9 (95% CI, 4.6-17.5), 100 000 to 999 999 copies/mL; and 10.7 (95% CI, 5.7-20.1), 1 million copies/mL or greater. Sex, age, cigarette smoking, alcohol consumption, seropositivity for HBeAg, elevated serum ALT level, and liver cirrhosis at study entry were also significantly associated with hepatocellular carcinoma development and were adjusted for in further multiple regression analyses.

Cumulative Incidence

The cumulative incidence of hepatocellular carcinoma during the fol-

low-up period by serum HBV DNA level at study entry examination appears in FIGURE 2. There was an increasing trend of cumulative hepatocellular carcinoma incidence with increasing serum HBV DNA level at study entry examination for all participants. The cumulative hepatocellular carcinoma incidence was further calculated in the stepwise analyses in which participants with seropositivity for HBeAg, elevated serum ALT level, and liver cirrhosis were removed sequentially. There was a dose-response relationship between cumulative hepatocellular carcinoma incidence and serum HBV DNA level for a subcohort of participants who were seronegative for HBeAg, had a normal ALT level, and did not have liver cirrhosis, which included most participants (80%) in this study.

TABLE 4 compares the cumulative hepatocellular carcinoma incidence at the end of the 13th year of follow-up derived from the stepwise analyses. Approximately 15% of all participants with serum HBV DNA levels of 1 million copies/mL or greater at study entry developed hepatocellular carcinoma by the 13th year of follow-up compared with 1.3% of participants with undetectable levels of HBV DNA. The biological gradient of cumulative hepatocellular carcinoma incidence by serum HBV DNA level remained prominent in all stepwise analyses. Among the 2925 participants seronegative for HBeAg with a normal ALT level and no liver cirrhosis, the cumulative hepatocellular carcinoma incidence was 13.5% for HBV DNA levels of 1 million copies/mL or greater and 0.7% for those with undetectable levels of HBV DNA.

Dose-Response Relationship

Seropositivity for HBeAg, elevated level of serum ALT, and presence of liver cirrhosis at study entry were significantly associated with hepatocellular carcinoma risk (Table 3). After adjustment for sex, age, cigarette smoking, and alcohol consumption, the HR for seropositivity for HBeAg was 6.9 (95% CI, 4.9-9.5; $P < .001$); elevated level of serum ALT, 3.8 (95% CI, 2.5-5.7;

Table 1. Baseline Characteristics of the Study Cohort

	No. (%) of Participants (N = 3653)*
Sex	
Female	1393 (38)
Male	2260 (62)
Age, y	
30-39	1216 (33)
40-49	1014 (28)
50-59	1058 (29)
≥60	365 (10)
Cigarette smoking†	
No	2416 (66)
Yes	1234 (34)
Alcohol consumption‡	
No	3195 (87)
Yes	451 (12)
Hepatitis B e antigen	
Seronegative	3088 (85)
Seropositive	565 (15)
Level of ALT, U/L	
<45	3435 (94)
≥45	218 (6)
Liver cirrhosis§	
No	3584 (98)
Yes	69 (2)

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus.

*Percentages may not add up to 100 due to rounding.

†Data were not available for 3 participants.

‡Data were not available for 7 participants.

§Diagnosed with ultrasonography within 6 months of cohort entry.

Table 2. Serum Level of HBV DNA and HBeAg Serostatus at Study Entry*

Level of HBV DNA, copies/mL	HBeAg Serostatus		Total (N = 3653)
	Negative (n = 3088)	Positive (n = 565)	
<300 (Undetectable)	865 (28.0)	8 (1.4)	873 (23.9)
300-999	372 (12.1)	0	372 (10.2)
1000-9999	773 (25.0)	16 (2.8)	789 (21.6)
10 000-99 999	625 (20.2)	18 (3.2)	643 (17.6)
100 000-999 999	298 (9.7)	51 (9.0)	349 (9.6)
1 to 9.9 million	96 (3.1)	58 (10.3)	154 (4.2)
10 to 99.9 million	42 (1.4)	58 (10.3)	100 (2.7)
≥100 million	17 (0.6)	356 (63.0)	373 (10.2)

Abbreviations: HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

*Values are expressed as number (percentage).

$P < .001$); and liver cirrhosis, 14.0 (95% CI, 9.4-21.0; $P < .001$).

Level of HBV DNA was significantly associated with seropositivity for HBeAg, elevated serum ALT level, and liver cirrhosis at study entry. The independent effects of serum HBV DNA level on hepatocellular carcinoma development after adjustment for other hepatocellular carcinoma risk factors also were analyzed. Compared with participants having serum HBV DNA levels of less than 300 copies/mL, the adjusted HR of developing hepatocellular carcinoma was 1.1 (95% CI, 0.5-2.3; $P = .86$) for participants with serum HBV DNA levels of 300 to 9999 copies/mL; 2.3 (95% CI, 1.1-4.9; $P = .02$), 10 000 to 99 999 copies/mL; 6.6 (95% CI, 3.3-13.1; $P < .001$), 100 000 to 999 999 cop-

ies/mL; and 6.1 (95% CI, 2.9-12.7; $P < .001$), 1 million copies/mL or greater (TABLE 5). The adjusted HR was 2.6 (95% CI, 1.6-4.2; $P < .001$) for seropositivity for HBeAg; 1.1 (95% CI, 0.7-1.7; $P = .64$) for elevated serum ALT level; and 9.1 (95% CI, 5.9-13.9; $P < .001$) for liver cirrhosis. In addition, male sex, increasing age, and habitual alcohol consumption were significantly associated with the development of hepatocellular carcinoma in this long-term follow-up study.

In the stepwise analyses removing participants with study entry status of seropositivity for HBeAg, elevated ALT level, and liver cirrhosis sequentially, the dose-response relationship ($P < .001$ for trend) between hepatocellular carcinoma risk and serum HBV DNA level at

study entry became increasingly stronger (Table 5). Among the 2925 participants seronegative for HBeAg who had a normal ALT level and no liver cirrhosis and compared with participants with serum HBV DNA levels of less than 300 copies/mL, the adjusted HR for participants with serum HBV DNA levels of 300 to 9999 copies/mL was 1.4 (95% CI, 0.5-3.8; $P = .56$); 10 000-99 999 copies/mL, 4.5 (95% CI, 1.8-11.4; $P = .001$); 100 000-999 999 copies/mL, 11.3 (95% CI, 4.5-28.4; $P < .001$); and 1 million copies/mL or greater, 17.7 (95% CI, 6.8-46.3; $P < .001$).

Multivariable-Adjusted HRs

We further examined the association between hepatocellular carcinoma risk and persistently elevated serum HBV

Table 3. Risk Factors for Hepatocellular Carcinoma at Study Entry

	No. (%) of Participants (N = 3653)	Person-Years of Follow-up	No. of Hepatocellular Carcinoma Cases	Incidence Rate Per 100 000 Person-Years	Crude HR (95%CI)*	P Value
Sex						
Female	1393 (38)	16 307	29	178	1.0	
Male	2260 (62)	25 472	135	530	3.0 (2.0-4.5)	<.001
Age, y						
30-39	1216 (33)	14 393	16	111	1.0	
40-49	1014 (28)	11 776	47	399	3.6 (2.0-6.4)	<.001
50-59	1058 (29)	11 837	67	566	5.1 (3.0-8.9)	<.001
≥60	365 (10)	3773	34	901	8.3 (4.6-15.0)	<.001
Cigarette smoking†						
No	2416 (66)	28 037	90	321	1.0	
Yes	1234 (34)	13 704	71	518	1.7 (1.2-2.3)	<.001
Alcohol consumption‡						
No	3195 (87)	36 779	121	329	1.0	
Yes	451 (12)	4928	42	852	2.6 (1.8-3.7)	<.001
Hepatitis B e antigen						
Seronegative	3088 (85)	35 584	94	264	1.0	
Seropositive	565 (15)	6195	70	1130	4.3 (3.2-5.9)	<.001
Level of ALT, U/L						
<45	3435 (94)	39 469	133	337	1.0	
≥45	218 (6)	2310	31	1342	4.1 (2.8-6.0)	<.001
Liver cirrhosis§						
No	3584 (98)	41 270	131	317	1.0	
Yes	69 (2)	509	33	6482	21.8 (14.9-32.0)	<.001
Level of HBV DNA, copies/mL						
<300 (Undetectable)	873 (24)	10 154	11	108	1.0	
300-9999	1161 (32)	13 518	15	111	1.0 (0.5-2.2)	.96
10 000-99 999	643 (18)	7404	22	297	2.7 (1.3-5.6)	.006
100 000-999 999	349 (9)	3845	37	962	8.9 (4.6-17.5)	<.001
≥1 million	627 (17)	6858	79	1152	10.7 (5.7-20.1)	<.001

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HBV, hepatitis B virus; HR, hazard ratio.

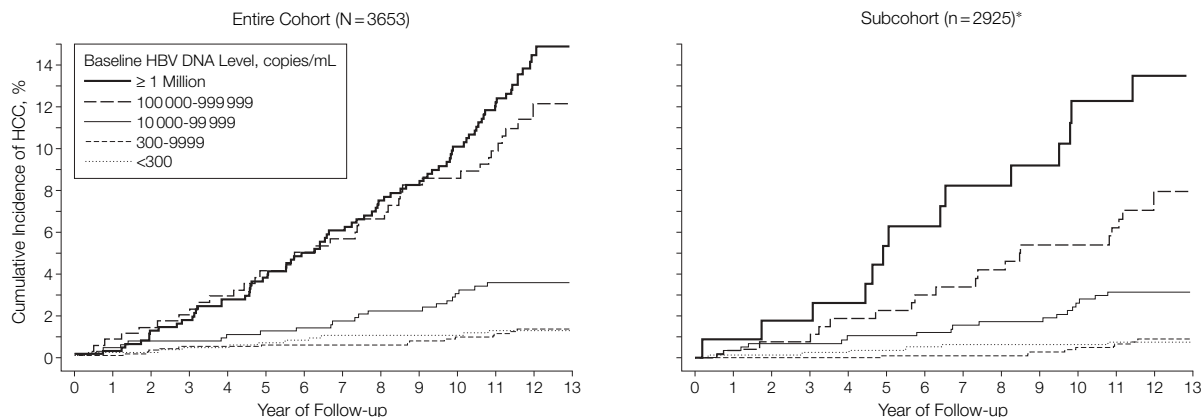
*Cox proportional hazard models were used.

†Data were not available for 3 participants.

‡Data were not available for 7 participants.

§Diagnosed with ultrasonography within 6 months of study entry.

Figure 2. Cumulative Incidence of Hepatocellular Carcinoma by Serum HBV DNA Level at Study Entry



No. at Risk																												
Baseline HBV DNA Level, copies/mL																												
≥1 Million	627	621	611	604	593	582	571	561	550	541	528	513	499	414	116	115	113	113	111	108	105	101	101	100	95	94	92	78
100 000-999 999	349	346	342	338	333	327	321	317	310	304	302	294	288	228	271	271	269	268	265	262	259	256	250	246	246	241	237	186
10 000-99 999	643	637	633	633	627	625	622	615	609	606	597	588	586	490	595	590	586	586	581	580	578	572	568	566	557	548	546	454
300-9999	1161	1155	1146	1139	1137	1131	1129	1123	1119	1113	1102	1091	1082	879	1104	1099	1095	1090	1088	1082	1080	1074	1070	1065	1055	1044	1036	847
<300	873	865	862	854	850	845	836	826	823	819	814	807	802	720	839	832	830	823	819	815	806	797	794	790	786	780	775	697

All participants were seropositive for the hepatitis B surface antigen (HBsAg) and seronegative for antibodies against the hepatitis C virus. Asterisk indicates these participants were seronegative for the hepatitis B e antigen and had a normal level of serum alanine aminotransferase and did not have liver cirrhosis at study entry. HBV indicates hepatitis B virus; HCC, hepatocellular carcinoma.

Table 4. Cumulative Incidence of Hepatocellular Carcinoma by HBV DNA Level at Study Entry

Level of HBV DNA, copies/mL	Cumulative Incidence of Hepatocellular Carcinoma (N = 3653)*	Seronegative for Hepatitis B e Antigen, %		
		Only (n = 3088)	Normal ALT Level (n = 2966)	Normal ALT Level and No Liver Cirrhosis (n = 2925)
<300 (Undetectable)	1.30	1.20	0.98	0.74
300-9999	1.37	1.21	1.25	0.89
10 000-99 999	3.57	3.68	3.42	3.15
100 000-999 999	12.17	9.54	8.55	7.96
≥1 million	14.89	17.88	19.51	13.50

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus.
*At the end of the 13th year of follow-up.

DNA levels at study entry and follow-up examinations. For participants who had a serum HBV DNA level of 10 000 copies/mL or greater at study entry, we retrieved their serum samples collected at the follow-up examination just preceding the hepatocellular carcinoma diagnoses or at the last follow-up examination for participants who had not developed hepatocellular carcinoma. Follow-up serum samples were inadequate for 289 (18%) participants with a serum HBV DNA level of 10 000 copies/mL or greater at

study entry examination. The median time between the study entry and follow-up serum samples was approximately 10 years (TABLE 6).

Among those who had serum HBV DNA levels at study entry of 10 000-99 999 copies/mL, a statistically significant increase in hepatocellular carcinoma risk (adjusted HR, 3.5; 95% CI, 1.4-9.2; $P = .01$) was observed for a follow-up level of 100 000 copies/mL or greater after adjustment for sex, age, cigarette smoking, and alcohol consumption. Among those with serum

HBV DNA levels at study entry of 100 000 copies/mL or greater, a significant biological gradient of hepatocellular carcinoma risk by follow-up HBV DNA level was observed ($P < .01$). The results remained unchanged after additional adjustment was performed for study entry HBeAg serostatus, serum ALT level, and presence of liver cirrhosis.

Changes in HBV DNA level may be due to HBeAg seroconversion, treatment effect, or fluctuations in viral HBV DNA between tests. Because HBeAg seroconversion is likely to have had the greatest impact on change in HBV DNA level in this cohort, this relationship was explored in additional analyses. Of the participants seropositive for HBeAg, approximately 42% were seronegative for HBeAg according to the last serum sample available for testing. Of those who were now seronegative for HBeAg, the HBV DNA level in a greater proportion (18.5% compared with 1.1% in the group remaining seropositive for HBeAg) went from 100 000 copies/mL or greater to less than 10 000 copies/mL. Of the group remaining seropositive for HBeAg, 95.2% (compared with 55.4% in the group now seronegative

for HBeAg) had an HBV DNA level remaining at 100 000 copies/mL or greater.

COMMENT

In Taiwan, HBV infection is mostly acquired perinatally. Many individuals infected with HBV are seropositive for HBeAg and have extremely high-level

viremia, normal serum ALT levels, and minimal liver disease.^{19,20} These individuals are considered to be in their immune-tolerant phase. Some of them develop chronic hepatitis B with an elevated serum ALT level later in life.²¹

Because treatment for HBV was not reimbursed by the universal national

health insurance in Taiwan until 2003, the participants in this study have not received any antiviral treatment with interferon alfa or nucleoside/nucleotide analogues. The cohort in this study therefore represents a natural history cohort. To ensure that participants received treatment that is the standard of care, individuals with abnormal α -fe-

Table 5. Regression Analysis of Risk Factors Associated With Hepatocellular Carcinoma

	Hepatitis B e Antigen Seronegative							
	All Participants (N = 3653)		Only (n = 3088)		Normal ALT Level (n = 2966)		Normal ALT Level and No Liver Cirrhosis (n = 2925)	
	Multivariable- Adjusted HR (95% CI)*	P Value	Multivariable- Adjusted HR (95% CI)*	P Value	Multivariable- Adjusted HR (95% CI)*	P Value	Multivariable- Adjusted HR (95% CI)*	P Value
Sex								
Male	2.1 (1.3-3.3)	.001	2.0 (1.1-3.6)	.03	1.6 (0.8-2.9)	.16	1.5 (0.8-2.9)	.24
Age in 1-y increment	1.09 (1.07-1.11)	<.001	1.08 (1.06-1.11)	<.001	1.09 (1.06-1.12)	<.001	1.11 (1.07-1.14)	<.001
Cigarette smoking								
Yes	1.0 (0.7-1.4)	.84	0.9 (0.6-1.4)	.69	1.1 (0.7-1.8)	.76	1.3 (0.7-2.3)	.35
Alcohol consumption								
Yes	1.6 (1.1-2.4)	.009	1.7 (1.0-2.8)	.05	1.8 (1.1-3.1)	.03	1.7 (0.9-3.1)	.08
Hepatitis B e antigen Seropositive	2.6 (1.6-4.2)	<.001						
Level of ALT, U/L ≥45	1.1 (0.7-1.7)	.64	0.8 (0.4-1.7)	.62				
Liver cirrhosis†								
Yes	9.1 (5.9-13.9)	<.001	7.9 (4.5-13.9)	<.001	11.7 (6.6-20.6)	<.001		
Level of HBV DNA, copies/mL‡								
<300 (Undetectable)	1.0	<.001§	1.0	<.001§	1.0	<.001§	1.0	<.001§
300-9999	1.1 (0.5-2.3)	.86	1.0 (0.5-2.4)	.94	1.3 (0.5-3.2)	.55	1.4 (0.5-3.8)	.56
10 000-99 999	2.3 (1.1-4.9)	.02	2.6 (1.2-5.6)	.01	2.7 (1.2-6.3)	.02	4.5 (1.8-11.4)	.001
100 000-999 999	6.6 (3.3-13.1)	<.001	6.1 (2.9-12.8)	<.001	7.2 (3.2-16.6)	<.001	11.3 (4.5-28.4)	<.001
≥1 million	6.1 (2.9-12.7)	<.001	10.6 (4.9-22.8)	<.001	14.3 (6.2-32.8)	<.001	17.7 (6.8-46.3)	<.001

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HBV, hepatitis B virus; HR, hazard ratio.

*Cox proportional hazard models were used.

†Diagnosed with ultrasonography within 6 months of study entry.

‡At study entry.

§For trend.

Table 6. Hepatocellular Carcinoma by Serum HBV DNA Levels at Study Entry and at Last Follow-up

Level of HBV DNA, copies/mL		No. of Participants (N = 3653)*	No. of Hepatocellular Carcinoma Cases	Median Time Between the Baseline and Last Follow-up Examination, y	Adjusted HR (95% CI)†	
At Study Entry	At Follow-up				Sex, Age, Cigarette Smoking, and Alcohol Consumption	Plus Seropositivity for HBeAg, Liver Cirrhosis, and ALT Level
<10 000	Not tested	2034	26		1.0	1.0
10 000-99 999	<10 000	256	6	10.7	1.6 (0.7-3.9)	1.3 (0.5-3.1)
10 000-99 999	10 000-99 999	161	1	9.2	0.5 (0.1-3.6)	0.4 (0.1-3.2)
10 000-99 999	≥100 000	110	5	9.9	3.5 (1.4-9.2)	2.9 (1.0-9.8)
≥100 000	<10 000	146	8	11.1	3.8 (1.7-8.4)	1.9 (0.8-4.4)
≥100 000	10 000-99 999	120	10	10.5	7.3 (3.5-15.3)	4.3 (2.0-9.3)
≥100 000	≥100 000	537	55	9.9	10.1 (6.3-16.2)	5.3 (2.9-9.7)

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio.

*There were 289 participants whose last follow-up serum samples were not available.

†Cox proportional hazard model was used.

toprotein, ALT, or ultrasound tests were informed in a letter of the results and instructed to see their physician for follow-up evaluation. Individuals were free to discontinue participation at any time for any reason. Additionally, all participants with suspected hepatocellular carcinoma were referred to a medical center of their choice for further evaluation. Based on the availability of reimbursement for HBV therapy in October 2003, individuals meeting the established treatment guidelines were referred to the National Taiwan University Hospital for evaluation.

There are few other examples of a population-based cohort with prospectively collected data on the development of hepatocellular carcinoma with detailed information on serostatus for HBeAg and serum levels of HBV DNA and ALT. To the best of our knowledge, this study is one of the largest and longest follow-up studies on the association between hepatocellular carcinoma risk and serum HBV DNA level at study entry and follow-up examinations.

Consistent with the findings we previously reported,^{7,9} there were significant associations between increased hepatocellular carcinoma risk and seropositivity for HBeAg, elevation of serum ALT level, and ultrasonographically detected liver cirrhosis within 6 months of entry in this study. Hepatitis B e antigen is a biomarker for the active replication of HBV in chronic hepatitis B patients. In this study, using a highly sensitive testing method based on polymerase chain reaction with a detection limit of 300 copies/mL for serum HBV DNA level, most participants with seropositivity for HBeAg (92.6%) had a highly elevated serum HBV DNA level of 100 000 copies/mL or greater. However, most participants seronegative for HBeAg (72.0%) still had a detectable serum HBV DNA level.

A significant biological gradient of hepatocellular carcinoma risk by serum HBV DNA level from 300 copies/mL (undetectable) to 1 million copies/mL or greater was observed in this

study. The hepatocellular carcinoma risk started to increase significantly at a serum HBV DNA level of 10 000 copies/mL, which is lower than the level (100 000 copies/mL) suggested by current clinical practice guidelines for making management decisions in the care of chronic hepatitis B patients.¹²

In this study, the majority of individuals with chronic hepatitis B (80%) were seronegative for HBeAg and had a normal serum ALT level at study entry and did not develop liver cirrhosis within 6 months of study entry. In this study, we found that serum level of HBV DNA was a prominent risk predictor of hepatocellular carcinoma independent of HBeAg status, serum ALT level, and the presence of liver cirrhosis. We also observed a significant dose-response relationship between serum HBV DNA level and hepatocellular carcinoma risk ($P < .001$). This biological gradient of hepatocellular carcinoma risk by serum HBV DNA level remained significant in all stepwise analyses using the Cox proportional hazards models. The consistency of a dose-response relationship between serum HBV DNA level at study entry and hepatocellular carcinoma risk during follow-up suggests the importance of closer clinical monitoring and even antiviral treatment for those who have an elevated serum HBV DNA level of 10 000 copies/mL or greater. The development of new antiviral agents capable of effectively lowering serum HBV DNA level regardless of HBeAg serostatus and serum ALT level may be important to lower the risk of hepatocellular carcinoma in chronic hepatitis B patients.

Serum level of HBV DNA is a dynamic parameter in patients with chronic hepatitis B. The importance of persistently elevated serum HBV DNA levels has seldom been elucidated. In this study, we analyzed the serum level of HBV DNA at last follow-up examination among those who had a serum HBV DNA level of 10 000 copies/mL or greater at study entry examination. For this analysis we used data from participants with a baseline HBV DNA level

of less than 10 000 copies/mL as the reference group and we did not retest their follow-up serum samples. Making these participants the reference group serves to bias the risk of hepatocellular carcinoma associated with persistent elevation of HBV DNA toward the null. This represents a conservative decision because some of these participants may have had elevated HBV DNA at follow-up examination. Among those who had a serum HBV DNA level of 100 000 copies/mL or greater, a significant reduction in hepatocellular carcinoma risk was observed with a decreasing serum HBV DNA level at follow-up examination in a reverse dose-response relationship ($P < .01$). Because most clinical trials of antiviral treatment of patients with chronic hepatitis B are limited by their short period of follow-up, virological, histological, and biochemical markers are used to assess the efficacy of the therapy.¹² It has been argued that serum HBV DNA level is a useful tool for comparing the antiviral potency of different antiviral drugs and strategies,¹⁴ but it is not the primary surrogate for clinical end points such as liver cirrhosis, liver decompensation, and hepatocellular carcinoma.¹⁵ The findings of this study provide strong longitudinal evidence of an increased hepatocellular carcinoma risk associated with the persistent elevation of serum HBV DNA level. This suggests that effective control of HBV replication indicated by the decrease in serum HBV DNA level following antiviral therapy can be expected to lower the risk of hepatocellular carcinoma in the long run.

Important consideration should be given to the impact of HBV genotype on the risk of hepatocellular carcinoma in this population. In Taiwan, the HBV genotypes B and C are the predominant genotypes, and genotype C has been shown to be associated with higher HBV DNA levels and a greater risk of hepatocellular carcinoma than genotype B.²² In unpublished data from our cohort, among participants with HBV DNA levels of 100 000 copies/mL or greater, the frequencies of genotypes B and C were 79.3% and

20.7% in participants who were seronegative for HBeAg and 41.7% and 58.3% in participants who were seropositive for HBeAg, respectively. Also, the rates of the precore stop codon (G1896A) mutation were 63.7% for those who were seronegative for HBeAg and 7.2% for those who were seropositive for HBeAg. The complete ascertainment of the different genotypes and mutation rates in this cohort as well as their impact on hepatocellular carcinoma risk is the subject of an ongoing study.

CONCLUSION

Serum level of HBV DNA may be used as a major risk predictor for hepatocellular carcinoma, independent of HBeAg serostatus, serum ALT level, and the presence of liver cirrhosis. Monitoring the change in serum HBV DNA level is recommended for the management

of chronic hepatitis B patients. Randomized controlled trials comparing different therapeutic strategies in patients with elevated serum HBV DNA level but a normal ALT level may further contribute to the development of appropriate treatment guidelines in these patients. These patients, especially those seronegative for HBeAg, account for an increasing majority of chronically infected individuals and are at an increased risk of future hepatocellular carcinoma.

Author Contributions: Dr Chen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Chen, Yang, Su, Huang, Iloeje.

Acquisition of data: Chen, Yang, Jen, You, Lu, Huang.

Analysis and interpretation of data: Chen, Yang, Su, Iloeje.

Drafting of the manuscript: Chen, Iloeje.

Critical revision of the manuscript for important intellectual content: Chen, Yang, Su, Jen, You, Lu, Huang, Iloeje.

Statistical analysis: Yang, Su, Iloeje.

Obtained funding: Chen, Su, Iloeje.

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