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RAPID COMMUNICATION

A case-control study of the relationship between hepatitis B virus DNA level and risk of hepatocellular carcinoma in Qidong, China

Tao-Tao Liu, Ying Fang, Hui Xiong, Tao-Yang Chen, Zheng-Pin Ni, Jian-Feng Luo, Nai-Qing Zhao, Xi-Zhong Shen

Tao-Tao Liu, Xi-Zhong Shen, Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai 200032, China
Ying Fang, Endoscopy Center, Huadong Hospital, Fudan University, Shanghai 200040, China
Hui Xiong, Chinese National Human Genome Center at Shanghai, Shanghai 201203, China
Tao-Yang Chen, Zheng-Pin Ni, Qidong Liver Cancer Institute, Qidong 226200, Jiangsu Province, China
Jian-Feng Luo, Nai-Qing Zhao, Department of Health Statistics and Community Medicine, Fudan University, Shanghai 200032, China
Author contributions: Liu TT, Xiong H and Shen XZ designed the research; Liu TT and Fang Y performed the research; Chen TY and Ni ZP contributed samples and materials; Zhao NQ and Ruo JF analyzed the data; and Liu TT and Shen XZ wrote the paper.
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Correspondents: Professor Xi-Zhong Shen, MD, Department of Gastroenterology, Zhongshan Hospital, Fudan University, 180# Fenglin Road, Shanghai 200032, China. shenxz99@yahoo.com
Telephone: +86-21-64041990 Fax: +86-21-64038038
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Abstract

AIM: To investigate the role of hepatitis B virus (HBV) replication in the development of hepatocellular carcinoma (HCC), a nested case-control study was performed to study the relationship between HBV DNA level and risk of HCC.

METHODS: One hundred and seventy cases of HCC and 276 control subjects free of HCC and cirrhosis were selected for this study. Serum HBV DNA level was measured using fluorescein quantitative polymerase chain reaction at study entry and the last visit.

RESULTS: In a binary unconditional logistic regression analysis adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases, the adjusted odds ratios (95% confidence intervals) of HCC in patients with increasing HBV DNA level were 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411) for HBV DNA levels $\geqslant 10^4$ to $< 10^5$; $\geqslant 10^5$ to $< 10^6$; $\geqslant 10^6$ to $< 10^7$; $\geqslant 10^7$ copies/mL, respectively. Forty-six HCC cases were selected to compare the serums viral loads of HBV DNA at study entry with those at the last visit. The HBV DNA levels measured at the two time points did not differ significantly.

CONCLUSION: The findings of this study provide strong longitudinal evidence of an increased risk of HCC associated with persistent elevation of serum HBV DNA level in the 10^4-10^7 range.

Key words: Hepatitis B surface antigen; Viral replication; Asymptomatic carriers; Viral load

Peer reviewer: Xin-Xin Zhang, Department of Infectious Disease, Ruijin Hospital, 197, Ruijin Er Road, Shanghai 200025, China

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is still a worldwide health problem[1], with approximate 400 million patients persistently infected[2,3]. Although most of the HBV carriers are asymptomatic, about one-third (25%-40%) die from cirrhotic complications or hepatocellular carcinoma (HCC)[4]. The relative risk of HBV carriers for the development of HCC is up to 200:1, which is one of the highest relative risks known for a human malignancy[5]. Due to the high incidence of recurrence and secondary primary tumor, the survival rate of HCC after any treatment is still low[6]. Therefore, looking for the predictive factors for HCC in patients with chronic Hepatitis B will have a profound impact on the prevention and treatment of chronic HBV infection.

The precise mechanisms by which chronic Hepatitis B leads to HCC are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in hepatocarcinogenesis[7-12]. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC[13-16]. Tang *et al*[17] have previously reported that adult HBV carriers who maintain high-titer serum HBV DNA are at higher risk for development of HCC. In Taiwan, a 12-year follow-up study of 4841 men who were Hepatitis B surface antigen

(HBsAg) positive has demonstrated that the risk of HCC is 2.7-10.7-fold higher in patients with baseline HBV DNA levels of 4.0 $\log_{10}$ copies/mL to $\geqslant$ 6.0 $\log_{10}$ copies/mL[18]. However, it is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is a geographic distribution of HBV genotypes. In particular, the data are largely lacking in mainland of China, where chronic HBV infection is highly endemic and accounts for half of the chronic hepatitis B in the world.

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China[19]. Between October 1996 and February 2006, we followed a total of 2387 HBsAg-positive adult residents in Qidong city. The aims of this study were to determine whether chronic HBV carriers who maintain high serum HBV DNA level are at higher risk for development of HCC in Chinese patients with chronic Hepatitis B.

MATERIALS AND METHODS

Study population

In October 1996, about 18 000 male residents between the ages of 20 and 65 yr living in 17 townships in Qidong county, China were invited to participate in a prospective study. All of those invited were tested for serum HBsAg, alanine aminotransferase (ALT) and α-fetoprotein (AFP). A total of 2387 participants who were seropositive for HBsAg and confirmed to be free of HCC by AFP level and abdominal ultrasonography were followed up with abdominal ultrasonography and serological tests including ALT, AFP, HBV serological markers (HBsAg) and anti-Hepatitis C virus (HCV) antibody until February 2006. Each study participant provided informed written consent and a structured questionnaire on sociodemographic characteristics, habits of alcohol and tobacco consumption and family histories. A serum specimen was collected from each participant at every interview. All of the serum samples were stored at -30℃ before analysis. This study was approved by the research ethics committee at Zhongshan Hospital, Fudan University, Shanghai, China.

Laboratory testing

Serum HBsAg and anti-HCV antibody were tested by commercially available enzyme immunoassay kits (Shanghai Kehua Bio-engineering Co. Ltd., China). Serum ALT level was determined by ultraviolet-lactate dehydrogenase (UV-LDH) method and serum AFP level was determined by ELISA (Shanghai Kehua Bio-engineering Co. Ltd).

Fluorescein quantitative polymerase chain reaction (FQ-PCR)

The serum HBV DNA levels were determined using the FQ-PCR detection system (Taqmen; Roche USA), according to the manufacturer's instructions. HBV DNA was extracted using the commercial Kit (Shanghai Shenyou Biotech Company) from 50 μL serum. The PCR reaction was carried out as follows: 37℃ for 120 s, 94℃ for 180 s, followed by 40 cycles of 94℃ for 10 s, 55℃ for 30 s and 72℃ for 40 s. The lower limit of detection of this assay was 500 copies/mL with a linear range of up to 10^8 copies/mL.

Statistical analysis

The χ^2 test was used to compare baseline characteristics between patients and controls subjects. Wilcoxon signed ranks test has been used to compare the constancy of the viral replication at two time points. For statistical comparisons, a value of 500 copies/mL was assigned, the detection limit of the assay, to samples that had undetectable levels of HBV DNA. Samples of the two groups were divided into six subgroups, according to the level of serum HBV DNA expressed as the logarithmic equivalent (LGE) per milliliter, subgroup 1 (< 500 copies/mL, undetectable), subgroup 2 (2.69 $\log_{10}$ to 3.99 $\log_{10}$ copies/mL), subgroup 3 (4.0 $\log_{10}$ to 4.99 $\log_{10}$ copies/mL), subgroup 4 (5.0 $\log_{10}$ to 5.99 $\log_{10}$ copies/mL), subgroup 5 (6.0 $\log_{10}$ to 6.99 $\log_{10}$ copies/mL), and subgroup 6 ($\geqslant$ 7.0 $\log_{10}$ copies/mL). Binary unconditional logistic regression analysis was used to evaluate relative risks. Potential confounders including age, cigarette smoking, alcohol consumption and family history of chronic liver diseases were adjusted. SPSS 13.0 for Windows was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic characteristic of HCC and control patients

No participants had any clinical evidence of HCC at study entry. By December 31, 2004, 243 participants died of HCC. The data were obtained from medical records and searches of computer files of death certification and cancer registry systems. To ensure complete ascertainment, we also contacted relatives by mail to identify cases. HCC was diagnosed on the basis of either surgical biopsy or an elevated serum AFP level ($\geqslant$ 400 ng/mL), combined with at least one positive image on sonography, computed tomography and/or magnetic resonance imaging. Seventy-three patients diagnosed with HCC within the first two years of our study probably had subclinical HCC at study entry, and were therefore, excluded from the analysis, which left 170 cases of HCC. The paired serum samples were available only in 46 cases, both at study entry and at the time of HCC, for determining the change in serum HBV DNA level over time. Two hundred and seventy-six subjects with chronic Hepatitis B infection and normal ALT level at each follow-up, and free of evidence of cirrhosis or HCC, were selected as controls.

At baseline, there were no significant differences in age, cigarette smoking and alcohol consumption between HCC and control patients, while the family histories of HBV-associated chronic liver diseases were significantly different between the two groups. 85/170 (50%) of cases had a family history, while only 92/276 (33.3%) of control subjects had (Table 1).

Baseline serum HBV DNA level in HCC patients and controls

186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA. Compared with those with undetectable levels of serum HBV DNA, the adjusted odds ratios of HCC for subjects with increasing

Table 1 Demographic data in HCC and control patients *n* (%)

	HCC patients (*n* = 170)	Control patients (*n* = 276)	χ^2	*P* value
Age at recruitment (yr)			8.347	$P > 0.05$
20-29	6 (3.5)	15 (5.4)		
30-39	52 (30.6)	88 (31.9)		
40-49	72 (42.4)	87 (31.5)		
50-59	35 (20.6)	68 (24.6)		
⩾ 60	5 (2.9)	18 (6.5)		
Smoking			0.131	$P > 0.05$
Yes	88 (51.8)	138 (50.0)		
No	82 (48.2)	138 (50.0)		
Alcohol use			0.989	$P > 0.05$
Yes	103 (60.6)	154 (55.8)		
No	67 (39.4)	122 (44.2)		
Family history			12.209	$P < 0.01$
Yes	85 (50.0)	92 (33.3)		
No	85 (50.0)	184 (66.7)		

Table 2 Association between HBV DNA level at study entry and subsequent risk of HCC *n* (%)

HBV DNA level (log_{10} copies/mL)	HCC patients (*n* = 170)	Control patients (*n* = 276)	Adjusted odds ratio (95% CI)
1 undetectable	44 (25.9)	186 (67.4)	1.000 (reference)
2 (2.69-3.99)	5 (2.9)	46 (16.7)	0.465 (0.172-1.259)
3 (4.00-4.99)	12 (7.1)	19 (6.9)	2.834 (1.237-6.492)
4 (5.00-5.99)	30 (17.6)	4 (1.4)	48.403 (14.392-162.789)
5 (6.00-6.99)	38 (22.4)	5 (1.8)	42.252 (14.784-120.750)
6 (⩾ 7.00)	41 (24.1)	16 (5.8)	14.819 (6.992–31.411)

Adjusted for age at enrollment (continuous variable), cigarette smoking, alcohol consumption and family history of chronic liver diseases.

HBV DNA level were 0.465 (95% CI 0.172-1.259), 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411). The analysis has been adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases. The risk of HCC was increased with increasing HBV viral load in 4.0 log_{10} to 7.0 log_{10} copies/mL (Table 2).

Change of serum HBV DNA level over time

All the control subjects in our study were followed up for 10 years with persistently normal ALT level, and had no history of interferon-α or nucleoside analogue therapy. HBV DNA levels were compared between entry and last visit in asymptomatic HBV carriers (controls). There was a statistically significant difference in serum HBV DNA level at the two time points (Table 3). For the 46 patients for whom the serum samples were collected both at study entry and at or after the time of HCC diagnosis, the time interval between collection of the two samples ranged from 24 to 94 mo. The log HBV DNA levels measured at the two time points did not have a statistically significant difference.

DISCUSSION

Family history of liver carcinoma is one of the main risk factors for HCC, especially in the Chinese population[20-22]. In our study, 85/170 (50%) of cases had a family history of HBV-associated chronic liver diseases. However, only 92/276 (33.3%) of control subjects did.

Table 3 Comparison of serum levels of HBV DNA at study entry and at last visit in asymptomatic HBV carriers (controls) *n* (%)

HBV DNA level (log_{10} copies/mL)	At study entry (*n* = 276)	At last visit (*n* = 276)	*Z*	*P* value
1 undetectable	186 (67.4)	221 (80.1)	- 4.904	$P < 0.01$
2 (2.69-3.99)	46 (16.7)	30 (10.9)		
3 (4.00-4.99)	19 (6.9)	9 (3.3)		
4 (5.00-5.99)	4 (1.4)	6 (2.2)		
5 (6.00-6.99)	5 (1.8)	3 (1.1)		
6 (⩾ 7.00)	16 (5.8)	7 (2.5)		

In China, HBV DNA levels > 5.0 log_{10} copies/mL have been considered clinically significant, and are suggested by clinical practice guidelines for making a decision on antiviral therapy in chronic carriers of Hepatitis B. The guidelines are supported by the findings of a meta-analysis of 26 trials of statistical significance and consistent correlations between viral load and histological grading, and biochemical and serological response[23]. However, the relationship between different levels, especially lower levels, of HBV DNA and risk of HCC remains uncertain.

During the past 10 years, longitudinal studies have been used to evaluate HBV DNA level as risk factors of HCC in HBV carriers. A significant biological gradient of HCC risk by serum HBV DNA level from 4.0 log_{10} to 7.0 log_{10} copies/mL was observed in our cohort. Similar to previous results[24], the HCC risk started to increase significantly at a serum HBV DNA level of 4.0 log_{10} copies/mL, which is much lower than the level of 5.0 log_{10} copies/mL suggested by clinical practice guidelines for making decisions on antiviral therapy in carriers of chronic Hepatitis B. Viral loads < 4.0 log_{10} copies/mL have been thought to be characteristic of an inactive carrier state and a much lower risk of HCC. Moreover, it is important to know that compared to viral loads between 5.0 log_{10} and 7.0 log_{10} copies/mL, patients with HBV DNA levels > 7.0 log_{10} copies/mL were at lower risk of developing HCC. Chronic HBV carriers with mid-high viral loads (4.0 log_{10} to 7.0 log_{10} copies/mL) tended to be in the phase of immune clearance, while the majority of those with viral load levels of 7.0 log_{10} copies/mL were immunotolerant and at lower risk of HCC. Our findings are partly consistent with studies in different areas. In Japan, Ohata *et al*[25] have investigated the risk factors for HCC in 73 patients with HBV-associated liver disease. A high viral load of HBV DNA, together with age and histological fibrosis, were found to be linked to the occurrence of HCC. Yang *et al*[26] have reported that HCC risk increased with the increasing HBV viral load above 7.5 log_{10} copies/mL. They have also found that HCC risk is associated with Hepatitis B e antigen (HBeAg) positivity among HBsAg-positive men in Taiwan. Based on these results, the serum level of HBV DNA may be used as a prominent risk predictor for HCC, independent of age, histological

fibrosis and HBeAg status.

To the best of our knowledge, there have been few studies on longitudinal stability of HBV DNA level in HBV carriers over time in mainland China. In the 276 control subjects in our study, all the HBV DNA levels in samples at the last visit were compared with those collected at study entry. 186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA at study entry, while 221/276 (80.1%) samples had undetectable levels of serum HBV DNA at the last visit. During a follow-up period of 10 years, the HBV DNA levels of those asymptomatic carriers tended to decrease. Forty-six case patients were selected whose serum samples were collected both at study entry and after the time of HCC diagnosis. Compared with serum HBV DNA levels at study entry, viral load after HCC onset remained at high levels. This implied that for chronic HBV carriers free of antiviral therapy, HCC was preceded by persistently high replication activity of HBV and viral levels did not decline with progression of HCC.

It is generally agreed that antiviral treatment is suitable in patients with active HBV replication ($\geqslant$ 5.0 $\log_{10}$ copies/mL) and elevated ALT level (at least twice the upper limit of the normal range)[27] or advanced fibrosis present upon liver biopsy. In clinical trials, among patients with chronic Hepatitis B and advanced stage fibrosis, longer term lamivudine therapy reduces the risk of HCC[28,29]. Although individuals with low viral load (< 4.0 $\log_{10}$ copies/mL) are at decreased risk for HCC, continued monitoring is essential because of the fluctuating nature of chronic HBV infection. Treatment choices for patients with serum HBV DNA levels < 5.0 $\log_{10}$ copies/mL are still controversial. In our study, HBV carriers with HBV DNA levels > 4.0 $\log_{10}$ copies/mL have 2.834 times excess risk of HCC compared with HBV carriers with lower HBV DNA levels. Therefore, among patients with HBV DNA levels > 4.0 $\log_{10}$ copies/mL, liver tests should be carefully monitored at 3-4-mo intervals, irrespective of age and ALT levels. Antiviral treatment should be advised when hepatitis flares and/or advanced fibrosis is present upon liver biopsy.

In conclusion, serum HBV DNA levels were found to be associated with increased risk of HCC. For chronic HBV carriers without antiviral therapy, HBV DNA levels changed little with the progression of HCC. Based on these findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring[30] and even antiviral treatment.

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COMMENTS

Background

Chronic Hepatitis B virus (HBV) infection is still a worldwide health problem. The precise mechanisms by which chronic Hepatitis B leads to hepatocellular carcinoma (HCC) are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in hepatocarcinogenesis. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC. However, the data are largely lacking in mainland China, where chronic HBV infection is highly endemic and accounts for half of the chronic Hepatitis B in the world. It is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is geographic distribution of HBV genotypes.

Research frontiers

Study on the prognostic factors in patients with chronic Hepatitis B, the relationship between Hepatitis B virus genotype and HBV DNA level, and HCC and treatment of chronic Hepatitis B patients who are resistant to antiviral therapy.

Innovations and breakthroughs

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China. However, this is believed to be the first study of the relationship between HBV replication and development of HCC in that region.

Applications

Base on our current findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring and even antiviral treatment. The results provide a data-supported approach to patients with Hepatitis B.

Peer review

This case-control study examined the relationship of HBV DNA quantitative levels and the risk of HCC in Qidong, China. They confirm other studies from Taiwan and elsewhere that demonstrate the risk of HCC occurs across a gradient of HBV DNA levels. This study is important.

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