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BRIEF ARTICLE

HBV genotype C is independently associated with cirrhosis in community-based population

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Abstract

AIM: To determine the association of hepatitis B virus (HBV) genotypes with probable cirrhosis and fatty liver in community-based populations.

METHODS: A multi-stage cluster probability sampling method was applied to recruit 10 167 subjects aged between 6 and 72 years from our epidemiological bases in Eastern China. After excluding the subjects co-infected with hepatitis C or hepatitis D viruses, the hepatitis B surface antigen (HBsAg)-positive subjects were examined for HBV genotype, serum viral load, alanine aminotransferase (ALT), hepatitis B e antigen (HBeAg) status, and ultrasonographic changes. Logistic regression models were used to determine the factors associated with probable cirrhosis and fatty liver.

RESULTS: Of 634 HBsAg-positive subjects with HBV genotype determined, 82 had probable cirrhosis (ultrasonographic score $\geqslant$ 5), 42 had ultrasonographic fatty liver. Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently found in the subjects with genotype C than in those with genotype B (14.8% *vs* 8.0%, P = 0.018). In HBeAg-negative subjects, high viral load was frequently associated with abnormal ALT level, while ALT abnormality was more frequent in those with probable cirrhosis than those without (19.5% *vs* 7.8%, P = 0.001). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly related to ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75, P < 0.001). Multivariate analysis demonstrated that HBV genotype C, age ($\geqslant$ 45 years), male sex, and ALT abnormality were independently associated with probable cirrhosis (AOR = 2.30, 95% CI: 1.26-4.19; AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

CONCLUSION: A crude prevalence of probable cirrhosis is 12.9% in the community-based HBV-infected subjects. HBV genotype C is independently associated with probable cirrhosis in the HBeAg-negative subjects.

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Key words: Hepatitis B virus; Genotype; Viral load; Alanine aminotransferase; Probable cirrhosis; Ultrasonography

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem. Approximately 2 billion people have been exposed to HBV, and more than 300 million are chronically infected with HBV. Chronic HBV infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas[1]. Liver fibrosis, which is the natural wound healing process to necroinflammation frequently caused by chronic HBV infection, is the essential pathogenic process that leads to cirrhosis. Metabolic syndrome is also an independent risk factor of liver cirrhosis in the patients with chronic hepatitis B[2]. Subclinical liver cirrhosis diagnosed by ultrasonography is significantly associated with the risk for HCC[3].

HBV genotypes have distinct geographical distributions, and have been shown to differ with regard to clinical outcome and prognosis[4]. Genotypes B and C are endemic in most parts of Asia[5]. Genotype C is associated with HCC in the aged[6,7]. Genotype B is associated with HCC in the young, relapse of HCC, and acute hepatitis B in adults[8-10]. However, the relationship between HBV genotypes and liver cirrhosis remains controversial. Some studies suggested that genotype C had a higher risk of cirrhosis, whereas other studies indicated that the progression to cirrhosis did not differ among genotypes B- and C-related chronic liver diseases[11-13]. In addition, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based studies in the HBV endemic areas.

Our objective was to determine the prevalence of probable liver cirrhosis in community-based subjects who were seropositive for hepatitis B surface antigen (HBsAg), and to evaluate the viral and demographic factors contributing to subclinical cirrhosis.

MATERIALS AND METHODS

Study population and epidemiological survey

The study was carried out at our epidemiological bases in Eastern China, from February to July 2009. A multistage cluster probability sampling method was applied to select the study population. A total of 10167 residents aged between 6 and 72 years were involved in this study. The participants were interviewed by the trained research assistants using a standard questionnaire requesting information about sociodemographic characteristics. Fasting blood samples (4 mL) were collected with vacuum blood collection tube (BD Diagnostics, Plymouth, UK) without anticoagulant. The serum was separated by centrifugation at 4℃ at the Centers for Disease Control and Prevention, transported on dry ice and stored at -40℃ in the Department of Epidemiology, Second Military Medical University. Informed consent in writing was obtained from each participant or guardian. Each resident who agreed to participate in the study completed a questionnaire and provided blood samples. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of this university.

Examination of HBV serological markers, serum viral load, and serum alanine aminotransferase level

All participants received HBsAg examination. Those positive for HBsAg were examined for hepatitis B e antigen (HBeAg), serum viral load, and alanine aminotransferase (ALT). HBsAg was examined using enzyme linked immunosorbent assay (Kehua, Shanghai, China) according to the manufacturer's instructions. Serological testing for HBeAg, antibody to hepatitis C virus (HCV), and antibody to hepatitis D virus (HDV), liver function tests, and α-fetoprotein examination were performed as previously described[9]. Upper limit of normal ALT was 45 U/L. Viral load was measured in the LightCycler™ 480 (Roche, Basel, Switzerland) using quantitative HBV PCR fluorescence diagnostic kits (Fosun Diagnostics, Shanghai, China). The kit has a certified lower limit of detection of 500 copies/mL, which was standardized using the Abbott reagents (Abbott Laboratories, North Chicago, IL).

HBV genotyping

HBV DNA was extracted from 200 μL HBsAg-positive serum using High Pure Viral Nucleic Acid Kits (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. HBV genotype was determined using a multiplex PCR assay[9,14]. HBV genotypes of samples with low level of HBV DNA were identified by nested multiplex PCR. Outer primers were 5'-TTTGCGGGTCACCATATTCTTGG-3' and 5'-CGA ACCACTGAACAAATGGCACTAG-3'. An Autorisierter Thermocycler (Eppendorf AG, Hamburg, Germany) was programmed to initially denature the samples for 3 min at 95℃, followed by 35 cycles consisting of 94℃ for 60 s, 58℃ for 60 s, 72℃ for 60 s, followed by a final elongation step at 72℃ for 10 min. The products (2 μL) were used as templates for multiplex PCR[14].

Ultrasonographic examination of liver cirrhosis and fatty liver

With the use of a Philips iU22 scanner (Philips Medical Systems, Best, the Netherlands) equipped with a 2-4 MHz variable convex probe or Toshiba systems (SSA-340; Toshiba, Tokyo, Japan) with a 3.75 MHz convex probe, probable liver cirrhosis and fatty liver were determined. Each subject was examined by two independent operators who were blinded to the clinical details. Discrepancies were resolved by consensus. The ultrasonographic scoring system consisting of liver surface, parenchyma, vascular structure, and splenic size was used to describe the existence and the severity of cirrhosis. The scores ranged from 4 for a normal liver to 11 for advanced cirrhosis[15]. A score of 8 or more was used as the cutoff point for ultrasonographic cirrhosis. The subjects with the score from 5 to 7 were diagnosed as having cirrhosis-like ultrasonographic abnormality. A score of 5 or more was defined as probable cirrhosis. The subject with an ultrasonographic steatosis score of 2 or more was diagnosed as having fatty liver[16].

Statistical analysis

χ^2 test was used to determine the differences in categorical variables, such as HBeAg positivity and the percentage of HBV genotypes. Continuous variables, like serum viral load and ALT level with skewed distribution, were adjusted to normal distribution by transformation into logarithmic function, and then tested by Student's *t* test. Univariate and multivariate regression analyses were performed to obtain the odds ratio (OR) and adjusted odds ratio (AOR) of factors for the risk of probable liver cirrhosis or ultrasonographic fatty liver and their 95% confidence intervals (CI). All statistical tests were two-sided, and performed using the Statistical Program for Social Sciences (SPSS15.0 for Windows, SPSS, Chicago, IL). A *P* value of < 0.05 was considered as statistically significant.

RESULTS

Of the 10167 participants, 793 were HBsAg positive; 745 of the 793 subjects were free of antibodies to HCV or HDV; and 634 of 745 subjects had HBV genotyped. Ten of the 634 subjects (8 with genotype C, one with genotype D, and one with genotype B) were diagnosed as having ultrasonographic cirrhosis (score 8 or higher), while 72 had cirrhosis-like ultrasonographic abnormalities (scores 5-7). Of the 634 subjects, one was diagnosed as having HCC. A crude prevalence of probable cirrhosis (ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities) was 12.9%. Table 1 shows the demographic and viral characteristics and liver abnormalities of the 634 subjects. There were no significant differences in proportions of age, sex, ALT level, HBeAg positivity, and fatty liver between the subjects infected with genotype B and those with genotype C. Compared with genotype C, HBV genotype B was more frequently seen in those with a high viral load ($\log_{10}$ copies/mL ≥ 4). Of the 634 subjects, 39 were positive for HBeAg. The HBeAg-positive subjects were significantly younger than the HBeAg-negative subjects (25.4 ± 11.2 years *vs* 42.7 ± 12.4 years, $P < 0.001$). The subjects with probable cirrhosis were significantly older than those without probable cirrhosis (45.3 ± 10.5 years *vs* 41.1 ± 13.3 years, $P = 0.001$). Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently in the subjects with genotype C than in those with genotype B (14.8% *vs* 8.0%, $P = 0.018$). Serum viral load was significantly higher in the HBeAg-negative subjects with abnormal ALT levels than in the HBeAg-negative subjects with normal ALT levels (4.54 ± 2.16 $\log_{10}$ copies/mL *vs* 3.31 ± 1.36 $\log_{10}$ copies/mL; $P < 0.001$). However, this association was not found in the HBeAg-positive subjects. Serum ALT level was significantly higher in the subjects with high viral load ($\geq 1 \times 10^4$ copies/mL) than in those with low viral load ($< 1 \times 10^4$ copies/mL) (33.9 ± 2.4 U/L *vs* 22.4 ± 1.9 U/L, $P < 0.001$). Serum ALT level was significantly higher in the subjects with ultrasonographic cirrhosis (score ≥ 8) than in the HBeAg-negative subjects with ultrasonographic score less than 7 (41.1 ± 1.6 U/L *vs* 24.1 ± 2.0 U/L, $P = 0.026$). ALT abnormality was more frequent in HBeAg-negative subjects with probable cirrhosis than those without probable cirrhosis (19.5% *vs* 7.8%, $P = 0.001$).

Table 1 Demographic and viral characteristics and liver abnormalities of 634 subjects seropositive for hepatitis B surface antigen *n* (%)

Variables	Genotype B (*n* = 199)	Genotype C (*n* = 411)	Others (*n* = 24)[1]	*P* value[2]
Age (yr)[3]	42.1 ± 13.0	41.3 ± 12.8	43.8 ± 12.8	0.493
Gender				
Male	116 (58.3)	228 (55.5)	16 (66.7)	
Female	83 (41.7)	183 (44.5)	8 (33.3)	0.511
ALT ($\log_{10}$ U/L)[3]	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.2	0.238
≤ 45 U/L	174 (87.4)	373 (90.8)	23 (95.8)	
> 45 U/L	25 (12.6)	38 (9.2)	1 (4.2)	0.207
HBeAg				
Negative	186 (93.5)	385 (93.7)	24 (100.0)	
Positive	13 (6.5)	26 (6.3)	0	0.922
HBV DNA levels ($\log_{10}$ copies/mL)[3]	3.7 ± 1.7	3.5 ± 1.7	3.1 ± 1.1	0.194
< 4	151 (75.9)	347 (84.4)	23 (95.8)	
≥ 4	48 (24.1)	64 (15.6)	1 (4.2)	0.011
Fatty liver				
Yes	16 (8.0)	24 (5.8)	2 (8.3)	
No	183 (92.0)	387 (94.2)	22 (91.7)	0.303
Cirrhosis status				
Normal	183 (92.0)	350 (85.2)	19 (79.2)	
Probable cirrhosis[4]	16 (8.0)	61 (14.8)	5 (20.8)	0.018

[1]23 cases for genotype mixture, 1 case for genotype D; [2]Genotype C *vs* genotype B; [3]mean ± SD; [4]Ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score ≥ 5). ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

Table 2 shows the factors associated with probable liver cirrhosis in the HBeAg-negative subjects by univariate and multivariate regression analyses. Age (≥ 45 years *vs* < 45 years), sex (male *vs* female), HBV DNA (≥ 4 $\log_{10}$ copies/mL *vs* < 4 $\log_{10}$ copies/mL), ALT (> 45 U/L *vs* ≤ 45 U/L), and HBV genotypes (genotype C *vs* genotype B) were included in the models. It was found that age (≥ 45 years), male sex, genotype C, and ALT abnormality were independently associated with

Table 2 Univariate and multivariate regression analyses for the risk factors of probable liver cirrhosis in the 595 HBeAg negative subjects infected with HBV

Variables	Controls (*n* = 513)	Cases (*n* = 82)	OR (95% CI)	AOR (95% CI)
Age (yr)				
< 45	284 (55.4)	31 (37.8)		
≥ 45	229 (44.6)	51 (62.2)	2.04 (1.26-3.29)	1.81 (1.10-2.99)
Sex				
Female	235 (45.8)	25 (30.5)		
Male	278 (54.2)	57 (69.5)	1.93 (1.17-3.18)	1.74 (1.03-2.95)
ALT (U/L)				
≤ 45	473 (92.2)	66 (80.5)		
> 45	40 (7.8)	16 (12.9)	2.87 (1.52-5.41)	2.98 (1.48-5.99)
Viral load (Log_{10} copies/mL)				
< 4	432 (84.2)	67 (81.7)		
≥ 4	81 (15.8)	15 (18.3)	1.19 (0.65-2.19)	1.06 (0.54-2.08)
Genotype				
B	170 (34.4)	16 (20.8)		
C	324 (65.6)	61 (79.2)	2.00 (1.12-3.58)	2.30 (1.26-4.19)

AOR: Adjusted odds ratio; OR: Odds ratio.

probable cirrhosis (AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.30, 95% CI: 1.26-4.19; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

Forty-two (6.6%) of the 634 subjects had ultrasonographic fatty liver, including 11 with abnormal ALT levels. Ultrasonographic fatty liver was not found in the subjects with probable cirrhosis. In the subjects with high viral load (log_{10} copies/mL ≥ 4), ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C (12.5% *vs* 0.0%, $P = 0.005$). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly associated with ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75, $P < 0.001$).

DISCUSSION

This large epidemiological study for the first time described the prevalence of probable liver cirrhosis in community-based, HBV-infected subjects who were free of HCV or HDV infection. About 13% of HBV-infected subjects had probable cirrhosis. The subjects with probable cirrhosis were significantly older than the subjects without cirrhosis. Probable cirrhosis was only found in the HBeAg-negative subjects. The HBeAg-positive subjects were significantly younger than the HBeAg-negative subjects. These results indicate that age is an important determinant for the development of probable liver cirrhosis. High viral load and ALT abnormality are associated with liver fibrosis in the HBeAg-negative patients[17]. We further demonstrated that high viral load was associated with increased serum ALT levels in the HBeAg-negative subjects and high ALT levels were frequently found in the subjects with probable cirrhosis, indicating that continuing HBV replication and hepatocyte damage contribute to the development of liver cirrhosis.

Importantly, the occurrence of probable liver cirrhosis was significantly higher in the subjects with genotype C than in those with genotype B. Multivariate analysis indicated that genotype C was significantly associated with an increased risk of probable liver cirrhosis. This was probably related to the prolonged immune clearance and delayed HBeAg seroconversion[18,19]. Although genotype B is associated with acute hepatitis[10], it tends to be self-limiting and short-living. However, genotype C was associated with the longer duration of liver damage in the HBeAg-negative subjects[12,20], which may be the main reason for the development of liver cirrhosis. In addition, genotype C-specific viral mutations are associated with probable cirrhosis[11,21,22]. Our recent meta-analysis has shown that PreS deletion, C1653T, T1753V, and A1762T/G1764A are increasingly more prevalent as chronic HBV infection progressed from the asymptomatic HBsAg carrier to cirrhosis or HCC[23]. Further studies are needed to probe into the different mutation patterns between genotypes B and C and their roles in the development of liver cirrhosis.

Since metabolic syndrome increased the risk of liver cirrhosis in the patients infected with HBV[2], we evaluated the prevalence and possible risk factors of ultrasonographic fatty liver in the 634 HBV-infected subjects. Interestingly, ultrasonographic fatty liver was not found in those with probable cirrhosis, while ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C at high viral load levels. This suggests that ultrasonographic fatty liver is unlikely to be a late event during the development of probable cirrhosis.

In conclusion, this study found that HBV genotype C, age (≥ 45 years), ALT abnormality, and male sex are independently associated with an increased risk of probable cirrhosis. Ultrasonographic fatty liver is not found in the subjects with probable cirrhosis. Although cirrhosis-like ultrasonographic abnormalities are not clinical liver cirrhosis, it is an early event during the development of clinical cirrhosis. Genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas. Metabolic syndrome has been found to be an independent risk factor of liver cirrhosis in the

patients with chronic hepatitis B. The relationship between HBV genotypes and liver cirrhosis remains controversial. Furthermore, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based population.

Research frontiers

HBV genotypes have distinct geographical distributions and differ with regard to clinical outcome, prognosis, and response to interferon treatment. The role of genotype B and C, the two major HBV genotypes endemic in East Asia, in the development of liver cirrhosis has not been unequivocally addressed. In this study, the authors demonstrate that infection with HBV genotype C is closely associated with subclinical cirrhosis in the community-based subjects with increasing age.

Innovations and breakthroughs

Recent reports have highlighted the importance of HBV genotypes, alanine aminotransferase (ALT), age, and sex in hepatocarcinogenesis and the development of clinical liver cirrhosis. Metabolic syndrome has been found to be independently associated with liver cirrhosis in the patients with chronic hepatitis B. This is the first study to report that HBV genotype C, age ($\geqslant$ 45 years), male sex, and ALT abnormality are independently associated with probable cirrhosis in community-based HBV-infected subjects, especially with the subclinical liver cirrhosis. Furthermore, this study suggested that fatty liver may not be associated with probable liver cirrhosis.

Applications

This study suggests that genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

Terminology

Probable cirrhosis is referred to ultrasonographic cirrhosis (ultrasonographic score $\geqslant$ 8) and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score from 5 to 7). Probable cirrhosis is not histologically confirmed liver cirrhosis. Ultrasonography is an imaging examination which is widely accepted by the community-based HBV-infected subjects without apparent clinical manifestations.

Peer review

The results of this study provide sufficient experimental evidences or data from which scientific conclusions can be drawn. The discussion is well organized and an overall theoretical analysis is given.

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