行政院國家科學委員會補助專題研究計畫成果報告

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※ B型肝炎病毒基因型之病毒學特性研究 ※

※ Virological characteristics of hepatitis B genotypes ※

※ in patients with chronic hepatitis B ※

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一、 中文摘要

不同 HBV 基因型對慢性 B 型肝炎之預後和抗病毒治療成效之影響已有文獻報告，但不同基因型間之病毒特性仍未明瞭。本計劃在於研究台灣地區 HBV 基因型 B 和 C 感染之 B 肝表面抗原陽性自願捐血者的臨床和病毒學上之差異。共收集 300 例 B 肝帶原之捐血者，利用 PCR-RFLP 法決定 HBV 之基因型，並分析 50 例 e 抗原陽性和 50 例 e 抗原陰性者之 HBV 前核心序列。研究結果顯示 300 例 B 肝帶原捐血者中，10 %有有肝功能指數升高，27 %為 e 抗原陽性，264 例為 HBV DNA 陽性。HBV 基因型分佈如下：B 型 83.7 %，C 型 14.8 %，F 型 0.4%，混合型 1.1 %。基因型 C 之 B 肝帶原捐血者似乎比基因型 B 之帶原者有較高的 e 抗原陽性率和血清 HBV DNA 值。此外，e 抗原陰性帶原者比 e 抗原陽性者有較高之前核心突變率，並且和基因型無關。只有 5 %基因型 C 之帶原者帶有 C-1858 之病毒株。

關鍵詞：B 型肝炎病毒、基因型、B 肝帶原者、捐血者、病毒濃度、前核心突變

二、Abstract

Pathogenic and therapeutic differences among hepatitis B virus (HBV) genotypes have been documented. However, the association of virological characteristics with clinical differences among HBV genotypes remains unclear. We therefore studied the clinical and virological characteristics between Taiwanese volunteer blood donors infected with HBV genotypes B and C. HBV genotypes were determined in 300 candidate blood donors positive for hepatitis B surface antigen (HBsAg), and sequences of the precore gene of HBV genome were determined in 50 HBeAg-positive and 50 HBeAg-negative blood donors. Of 300 HBsAg-positive blood donors, 10% had elevated serum aminotransferase levels and 27% were positive for hepatitis B e antigen (HBeAg). HBV genotype distribution in 264 viremic carriers was as follows: B, 221 (83.7%); C, 39 (14.8%); F, 1 (0.4%) and mixed infection, 3 (1.1%). Blood donors with genotype C infection tended to have a higher frequency of HBeAg positivity and a higher serum HBV DNA level than those with genotype B infection. The frequency of precore stop codon mutation was significantly higher in HBeAg-negative blood donors than HBeAg-positive ones, irrespective of HBV genotypes. Meanwhile, only 5% of blood donors with genotype C infection had C-1858 strains. In conclusion, mixed infection of HBV genotypes indeed occurs, and genotype C has a higher serum HBV DNA level than genotype B. Precore stop codon mutation is common in HBeAg-negative HBV carriers, irrespective of HBV genotypes. In contrast, precore C-1858 strains are rarely identified in Taiwanese HBV genotype C.

Key words: Hepatitis B virus, genotype, hepatitis B carrier, blood donor, HBV DNA, precore mutant.

三、Introduction

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people of the world population are chronic carriers of the virus (7). The infection is associated with a wide clinical spectrum, ranging from acute or fulminant hepatitis to various forms of chronic infection including asymptomatic carrier, chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (3, 7). Although serological and genotypic classifications of HBV have been well documented (20, 24), the clinical significance of HBV genotypes in terms of clinical outcomes and therapeutic response to antiviral therapy in patients with chronic HBV infection remains largely unknown until recently. Our previous studies indicated that HBV genotypes B and C are the most prevalent viral strains in Taiwan, and genotype C is associated with the development of cirrhosis and HCC while genotype B may be associated the development of HCC in young patients (8). In addition, HBV genotype C is associated with a higher frequency of core promoter mutation and a lower response rate to interferon alfa therapy as compared to genotype B (10). Taken together, these data suggest the possible pathogenic and therapeutic differences among HBV genotypes. However, the association of virological characteristics including efficiency of viral replication as well as viral genome variability with these clinical differences among HBV genotypes remains unclear. We therefore studied the clinical features and virological characteristics, with special reference to the precore mutations, between Taiwanese volunteer blood donors infected with HBV genotypes B and C.

四、Materials and Methods

Subjects

Plasma samples from 300 volunteers (197 men and 103 women; mean age: 31±10 years) eliminated as blood donors because of positivity of hepatitis B surface antigen (HBsAg), were collected and stored at -70°C until use. These samples were negative for antibodies to hepatitis C and D viruses (anti-HCV and anti-HDV), and were selected from blood donations at Taipei (northern), Taichung (middle) and Kaohsiung (southern) Blood Donation Centers through the Blood Services Foundation of the Republic of China, which collects virtually all of the blood donations in Taiwan. The foundation is a non-government and non-profit organization, and has an annual voluntary donation of nearly 2 million units. Around 1.8% of them were HBsAg-positive in 2000.

Serological testings

Serum alanine aminotransferase (ALT) was tested with routine automated techniques (upper limit of normal: 40 U/L). Serum HBsAg and hepatitis B e antigen (HBeAg) were assayed by Ausria-II and IMx HBe 2.0 (Abbott Laboratories, North Chicago, IL, USA), respectively. Anti-HCV and anti-HDV were tested by commercially available assays (HCV EIA II, Anti-Delta, Abbott Laboratories).

Genotyping of HBV

HBV genotypes were determined by using the nested polymerase chain reaction (PCR)-restriction fragment length polymorphism of the surface gene of HBV as previously described (18). Six genotypes (A to F) of HBV could be identified by the restriction patterns of DNA fragments. To avoid false-positive results, instructions to prevent cross contaminations were strictly followed, and results were considered valid only when they were obtained in duplicate. The sensitivity of our first-round and second-round PCR assay was 10^5 and 10 copies of HBV DNA per specimen, respectively, by testing serial 10-fold dilutions of HBV DNA transcripts with known amounts (10^8 copies/ml).

Amplification and sequencing of the precore gene

The entire precore gene was amplified in 50 HBeAg-positive (34 with genotype B infection and 16 with genotype C infection) and 50 HBeAg-negative (27 with genotype B infection and 23 with genotype C infection) blood donors as previously described (10), and precore mutations at codons 1, 2, 28 and 29 as well as variability at nucleotide 1858 were subsequently analyzed. Nucleotide sequences of amplified products were directly determined by using fluorescence labeled primers with a 373A Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing conditions were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The inner primer pair was used as sequencing primers for both directions.

Statistical Analysis

Fisher's exact test, Chi-square test with Yates' correction, and Student's t test were used where appropriate. A P value of < 0.05 was considered statistically significant.

五、Results

Of 300 HBsAg-positive volunteer blood donors, 30 (10%) had elevated s ALT levels (> 40 U/L) and 82 (27%) were positive for HBeAg. Among the (88%) were positive for serum HBV DNA by the sensitive PCR assay a genotype distribution was as follows: B, 221 (83.7%); C, 39 (14.8%); F, 1 (mixed infection of B and C, 3 (1.1%). Accordingly, genotypes B and C predominant strains among these candidate volunteer blood donors i infected with HBV. The clinical and virological features of HBs volunteer blood donors infected with HBV genotype B or C are shown Those with genotype B or C infection were comparable in terms of ge age, seropositivity of HBeAg, mean serum ALT level and the f elevated serum ALT level. However, those with genotype C infe higher frequency of first-round PCR positivity than those with infection (77% vs. 55%, $P < 0.02$).

The prevalence of precore mutations (codons 1, 2, 28 and 29) and nucleotide 1858 (codon 15) were further analyzed in 50 HBeAg-p HBeAg-negative volunteer blood donors stratified by HBV genoty

None of these 100 volunteer blood donors had codons 1 and 2 mutations; however, the frequency of codon 28 mutation (precore stop codon mutation) was significantly higher in HBeAg-negative volunteer blood donors than HBeAg-positive ones, irrespective of HBV genotypes (74% vs. 12% for genotype B and 65% vs. 6% for genotype C, both $P < 0.001$). In contrast, codon 29 mutation was infrequent and all were found in genotype B strains and in combination with codon 28 mutation. Sequence analysis of the nucleotide variability of codon 15 showed that all genotype B and most genotype C strains had a T at nucleotide 1858 (T-1858). Only 2 (5%) of the 39 patients with genotype C infection had a C at nucleotide 1858 (C-1858), and the one with HBeAg negativity possessed G-1896.

六、Discussion

In addition to the serological classification of HBV isolates into nine subtypes according to the antigenic determinants of their HBsAg (4, 19), a genetic classification based on the comparison of complete genomes has recently defined seven genotypes of HBV (A to G) (20, 24). HBV genotypes have distinct geographical distributions (11, 21). In general, genotypes B and C are prevalent in Asia (8, 26), whereas genotypes A and D prevail in Western countries (17). Genotype E is restricted to Africa, and genotype F prevails in Central America. Genotype G has been identified in France and North America very recently (24). Our previous clinic-based study indicated that genotypes B and C are the most common HBV genotypes in Taiwan (8); however, the study population may not be well controlled and thus a selection bias may be present. To avoid the possibility of biased selection, studies based on general population such as first time blood donors should be more appropriate to address this important issue. By using a simple HBV genotyping method (18), the distribution of HBV genotypes was studied in the general population of Taiwan, and our data consistently showed that genotype B was the most predominant HBV followed by genotype C. Other genotypes only accounted for a minimal proportion. In addition, mixed infection of genotypes B and C was found in 1% of the HBsAg-positive candidate volunteer blood donors, suggesting coinfection of different HBV genotypes or superinfection of heterologous HBV strains on top of hepatitis B carriers may occur (6, 29), as is in chronic hepatitis C or D (5, 9, 30). In addition, the frequency of HBeAg positivity (27%) and abnormal serum ALT level (10%) in our HBsAg-positive volunteer blood donors was comparable to that reported in asymptomatic hepatitis B carriers (28).

The clinical, virological and therapeutic implications of HBV genotypes in patients with chronic HBV infection have been partially clarified. Our previous data suggested that HBV genotype C is associated with the development of cirrhosis and HCC as well as a lower response rate to interferon therapy as compared to genotype B (8, 10). Lindh et al. also indicated that genotype C, compared to genotype B, is associated with a higher frequency of HBeAg positivity and HBV DNA level, more pronounced liver inflammation, lower

frequency of precore mutants as well as a higher frequency of core promoter mutants (13, 15). Similarly, Orito et al. reported that HBeAg and core promoter mutants were less frequent in genotype B than C whereas the frequency precore mutants was comparable between genotypes B and C (22). Nevertheless, the association of molecular virological characteristics including efficiency of viral replication as well as viral genome variability with these differences among HBV genotypes remains to be established further.

In the present study, the results showed that the clinical and laboratory features were comparable between volunteer blood donors with genotype B or C infection (Table 1). Although HBeAg was less common in genotype B than C-infected volunteer blood donors (30% vs. 41%), the difference was not statistically significant. However, volunteer blood donors with genotype C infection had a higher frequency of first-round PCR positivity than those with genotype B infection (77% vs. 55%, $P < 0.02$), suggesting genotype C may yield a higher HBV DNA level than genotype B as previously reported (13, 15).

Mutations in the precore and basal core promoter regions of the HBV genome have been observed in patients with chronic HBV infection (1, 25). The major missense/nonsense mutations in the precore region are found in codons 1, 2, 28 and 29 (14, 16). Among them, a G-to-A change at nucleotide 1896 (codon 28), which creates a premature stop codon (precore stop codon mutant). This mutation prevents the translation of the precore protein and completely abolishes the production of HBeAg (27). In addition, nucleotide variability (T or C) at position 1858 (codon 15) is commonly observed (11). The relationship between HBV genotypes and types of precore mutation as well as nucleotide variability at position 1858 has been reported (6, 14). For example, genotypes other than A have a T at nucleotide 1858 (T-1858) which makes a wobble pairing with G-1896 in the stem of the 'ε'encapsidation signal. The mutation for A-1896 (precore stop codon mutant) tightens the stem structure by making a T-A pair. In contrast, genotype A possesses C-1858 making C-G pair with G-1896 in the wild type. Since mutation for A-1896 breaks this stable pair, it does not occur except in combination with another mutation from C-1858 to T-1858. Accordingly, precore stop codon mutant is restricted to HBV strains with T-1858 and rarely occurs in those with C-1858, and this may explain why genotype A rarely circulates as an HBe-minus mutant and why genotype D is the most frequent HBV genotype among the precore mutants in the Western countries (6, 12, 16, 23). Recently, C-1858 is also frequently observed in Chinese patients with genotype C infection (2); however, the sample size of the study was limited and further large studies are needed to confirm the findings. In the present study, we sequenced the precore region of the HBV genome in 50 HBeAg-positive and 50 HBeAg-negative volunteer blood donors with genotype B or C infection (Table 2). Our data showed that none of them had codon 1 or 2 mutation. In contrast, the frequency of precore stop codon mutation was significantly higher in HBeAg-negative volunteer blood donors than HBeAg-positive ones, irrespective of HBV genotypes, confirming that this mutation can

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Table 1. Clinical and virological features of volunteer blood donors infected with HBV genotype B or C [a]

Features	Genotype B	Genotype C	P Value
Number	221	39	-
Gender (M/F)	150/71	22/17	NS
Age (years, mean±SD)	31±11	30 ±9	NS
Positivity of HBeAg	66 (30%)	16 (41%)	NS
Mean ALT (U/L)	26±34	18±12	NS
ALT > 40 U/L	27 (12%)	2 (5%)	NS
Positivity of PCR			
First-round	122 (55%)	30 (77%)	< 0.02
Second-round	99 (45%)	9 (23%)	

[a] HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; PCR, polymerase chain reaction; NS, not significant.

Table 2. Prevalence of variability at nucleotide 1858 (codon 15) and precore mutations (codons 28 and 29) according to the positivity of HBeAg and HBV genotypes [a]

	HBeAg (+)		HBeAg (-)	
	B	C	B	C
Number	34	16	27	23
Codon 15 variability				
C-1858	0	1 (6%)	0	1 (4%)[b]
T-1858	34 (100%)	15 (95%)	27 (100%)	22 (96%)
Codon 28 mutation				
A-1896	4 (12%)[c]	1 (6%)[d]	20 (74%)[c]	15 (65%)[d]
Codon 29 mutation				
A-1899	1 (3%)[e]	0	3 (11%)[e]	0

[a] HBeAg, hepatitis B e antigen; (+), positive; (-), negative.
[b] This patient had a wild-type codon 28 (G1896).
[c] $P < 0.001$.
[d] $P < 0.001$.
[e] All had codon 28 mutations (A1896).