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Association of HBV Genotype and Viral Genomic Variation with the Development of Hepatocellular Carcinoma

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Abstract

Hepatitis B virus (HBV) infection is a major health problem in Taiwan. Currently, 4 subtypes and 8 genotypes of HBV are identified worldwide, and most of them have distinct geographic distributions. The impact of HBV genotypes on the clinical outcome of chronic HBV infection in Taiwan has been partially clarified. Our recent data showed that subtypes *adw* and *adr* or genotypes B and C are the predominant HBV strains in Taiwan. In addition, all *adr* strains are genotype C whereas 81% and 12% of the *adw* strains are genotype B and genotype C, respectively. Clinically, genotype C is associated with more severe liver disease including cirrhosis and hepatocellular carcinoma (HCC) whereas genotype B is associated the development of HCC in young non-cirrhotic patients. Serologically, genotype C tends to have a higher frequency of hepatitis B e antigen (HBeAg) positivity and a higher serum HBV DNA level than genotype B. Virologically, genotype C bears a higher frequency of core promoter mutation than genotype B. Although superinfection of HBV on top of hepatitis B carriers indeed occurs in Taiwan, it is rarely associated with acute exacerbations. As to the response to antiviral treatments, genotype C is associated with a lower response rate to interferon therapy as compared to genotypes B. Although pathogenic and therapeutic differences do exist among HBV genotypes in Taiwan, the molecular virological mechanisms contributing these clinical differences, especially hepatocarcinogenesis, deserve further exploration. In this study, we therefore investigated the prevalence of specific mutations of HBV genome in a cohort of genotype B or C-infected hepatitis B carries in different stages of chronic liver disease and its risk associated with the development of HCC. Our data showed that both HBV genotype C and basal core promoter mutant were

independent factors for HCC development. Therefore different HBV genotypes and viral mutants may have different contribution to the pathogenesis of HBV infection.

Key Words: chronic hepatitis B, hepatitis B virus, hepatocellular carcinoma, genotype, genomic variation.

Introduction

Hepatitis B virus (HBV) infection is a global health problem. About 2 billion people in the world have been infected by HBV, 350 million of whom are chronic carriers of the virus [1]. The infection can cause acute and chronic liver disease including cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. The natural history can be divided into three phases based on virus-host interactions [2, 3]. In the first immune tolerance phase, patients are hepatitis B e antigen (HBeAg) positive and have high serum HBV DNA levels, but have no symptoms, normal serum aminotransferase levels and minimal histological activities. During the second immune clearance phase, a proportion of previously symptomless HBV carriers start to have bouts of symptoms and signs suggestive of acute hepatitis, i.e., the so-called “acute exacerbations”, when previous immune tolerance no longer exists. In the third low-replicative or integration phase, serum hepatitis B surface antigen (HBsAg) persists, but HBeAg is no longer detectable and patients are usually asymptomatic and liver disease is inactive. It is known that early seroconversion from HBeAg to anti-HBe in the natural course of chronic HBV infection usually indicates a favorable outcome, because it is usually associated with the cessation of virus replication and non-progressive liver disease [2, 3]. In contrast, late seroconversion of HBeAg after multiple bouts of reactivation and remission may accelerate the progression of chronic hepatitis to liver cirrhosis and thus have a poor clinical outcome [4-6]. Thus the final outcome of chronic HBV infection depends on the frequency and severity of acute exacerbation in the second immune clearance phase, i.e. the more frequent and severe acute exacerbation in this phase, the more chance to develop liver cirrhosis and even HCC in later life.

Having only 3,200 base pairs in its genome, HBV is the smallest known DNA virus [1]. The partially double-stranded circular HBV DNA consists of four overlapping genes encoding the viral envelope (pre-S and S), nucleocapsid (precore and core), polymerase with error prone reverse transcriptase activity, and X protein. Because of the spontaneous error rate of viral reverse transcriptase, HBV genome evolves with an estimated rate of nucleotide substitution at $1.4\text{-}3.2 \times 10^{-5}$ / site / year [7]. After a long-time evolution, currently 4 major HBV serological subtypes (*adw*, *ayw*, *adr* and *ayr*) or nine minor subtypes are identified by the antigenic determinants of HBsAg and 8 HBV genotypes (A to H) are defined by divergence in the entire HBV genomic sequence > 8% [8-10]. The interrelation of subtypes to genotypes has been clarified [8]. In general, genomes encoding *adw* are found in genotypes A, B, C, F, and G, while the genomes encoding both *adr* and *ayr* occurs in genotype C alongside with *adw*. Most of the HBV genotypes have distinct geographic distributions. In brief, genotypes B and C are prevalent in Asia, whereas genotypes A and D prevail in Western countries and India. Genotype E is restricted to Africa, and genotype F in Central and South America. Genotype G has been recently identified in France and North America [8, 9].

Taiwan is an area endemic for HBV infection. Previous epidemiological survey indicated the carrier rate of HBsAg in the general population of Taiwan was as high as 15% to 20% [11], which is one of the highest in the world. Since chronic HBV infection will result in chronic hepatitis, cirrhosis, and HCC in the carriers, the liver cancer is therefore very common in Taiwan [12]. Actually, HCC has ranked first for cancer mortality in men and second in women since the early 1980s. In addition, chronic liver diseases rank sixth

among causes of death. In about half of the Taiwanese chronic HBsAg carriers, the infection is attributed to perinatal transmission of the virus from mothers to infants [13]. Fortunately, a mass immunization program against hepatitis B has been launched in Taiwan since 1 July 1984 [14], and the efficacy of universal immunization has been shown, with dramatic reductions of the prevalence of HBsAg carriage in children and adolescents [15, 16]. In addition, hepatitis B vaccination can protect children against HCC and fulminant hepatitis [17-20]. Nevertheless, there are still many carriers (~2.4 million) left in our population. And thus, the focus of hepatitis B research in the new century will be the search of factors, host or virus, that determine the clinical outcomes of patients with chronic HBV infection and the development of more effective therapies that can be applied to all hepatitis B carriers.

The clinical relevance of HBV genotypes in Taiwan remains unknown until very recently. Our previous data showed that subtypes *adw* and *adr* or genotypes B and C are the predominant HBV strains in Taiwan [21]. In addition, all *adr* strains are genotype C whereas 81% and 12% of the *adw* strains are genotype B and genotype C, respectively. Clinically, genotype C is associated with more severe liver disease including cirrhosis and HCC whereas genotype B is associated the development of HCC in young non-cirrhotic patients [22]. Serologically, genotype C tends to have a higher frequency of HBeAg positivity and a higher serum HBV DNA level than genotype B [23]. Virologically, genotype C bears a higher frequency of core promoter mutation than genotype B [24]. Although superinfection of HBV on top of hepatitis B carriers indeed occurs in Taiwan, it is rarely associated with acute exacerbations [25]. As to the response to antiviral treatments, genotype

C is associated with a lower response rate to interferon therapy as compared to genotypes B [24]. In addition, genotype B seems to have a better virological response to lamivudine as compared to genotype C but both genotypes have a similar risk in developing lamivudine resistance [26].

One of our major findings that HBV genotype C is associated with a poor response to IFN therapy and the development of HCC has been confirmed by subsequent studies from Japan and China [27-30]. Wai et al. studied 73 patients received IFN and 34 received no treatment (controls). Antiviral response was achieved in 39% and 17% of IFN-alpha-treated patients (P = 0.03) and in 10% and 8% of untreated controls (P = 0.88) with HBV genotype B and C, respectively [29]. Further multivariate analysis identified HBV genotype B, elevated pretreatment ALT levels, and low pretreatment HBV-DNA levels but not IFN treatment as independent factors associated with antiviral response. In the meantime, Orito et al. performed a large-scale survey of the geographic distribution of HBV genotypes in Japan and investigated the clinical characteristics among the patients with different genotypes [28]. They found that genotypes C and B are predominant in Japan, and there are significant differences in the geographic distribution. Compared with genotype C patients, genotype B patients were older, had a lower rate of positive HBeAg and a lower serum HBV DNA level. The number of patients with liver cirrhosis or HCC increased with age in the patients with genotype C, indicating genotype C is also closely associated with the development of HCC in Japan. However, none of their HCC patients younger than 35 years had genotype B. Similarly, Ding et al. have shown genotype C is associated with the development of HCC, while genotype B has a relatively good prognosis in China [27]. In summary, their data indicated genotype B is rarely

associated with the development of HCC in Japan and China. On the contrary, more than 50% of HBV-related HCC patients in Taiwan are infected with HBV genotype B [22]. Accordingly, The genotype B strains in Taiwan are somewhat different from those in Japan and China, and are proposed to divide into three subtypes based on the rate of liver disease progression [31]. The first is the slowly progressive subtype that is associated with a tendency for early disappearance of HBeAg during a carrier's lifetime or in the course of chronic hepatitis and subsequently leads to the low death rate from HCC as has been observed in Okinawa of Japan [32]. The second is the rapidly progressive subtype that is associated with the development of HCC in young hepatitis B carriers before their fourth decade even in the absence of cirrhosis, suggesting that an oncogenic potential of certain particular HBV strains may exist as is in the case of HCC in woodchucks. Chronic HBV infection in woodchucks never results in cirrhosis but contains viral DNA with preferential integration sites that activate *myc* family genes [33]. The third is the intermediately progressive subtype that runs a typical natural course of chronic HBV infection with the development of HCC usually in their sixth decade, as does genotype C. However, further large studies are awaited to prove or disprove these speculations. Of particular note is that Sumi et al. recently indicated that, although the patients with genotype B experience earlier HBe seroconversion, slower progression of liver fibrosis, and slower development of HCC, the life-long risk of progression to advanced fibrosis and development of HCC may not differ among genotypes B- and C-related chronic liver disease [30].

Similar situation has been observed in other HBV genotypes. Sanchez-Tapias et al. studied 258 Spanish patients with chronic hepatitis B

infected with different HBV genotypes, mostly genotype A, D and F [34]. They found that concomitant sustained biochemical remission and clearance of HBV DNA occurred at a higher rate in genotype A- than in genotype D- (log-rank, 14.2; P = 0.002) or genotype F-infected patients (log-rank, 4.2; P = 0.03). The rate of HBsAg clearance was higher in genotype A than in genotype D hepatitis (log-rank, 4.6; P = 0.03). Sustained remission and clearance of HBsAg were associated with infection with genotype A by Cox regression analysis. Seroconversion to anti-HBe was unrelated to HBV genotype, but the rate of sustained remission after seroconversion was higher in genotype A than in genotype D hepatitis both in patients who seroconverted to anti-HBe during follow-up (log-rank, 4.5; P = 0.03) and in patients with positive anti-HBe at baseline (log-rank, 6.66; P = 0.009). Death related to liver disease was more frequent in genotype F than in genotype A (P = 0.02) or genotype D (P = 0.002) hepatitis. Thus the long-term outcome of chronic hepatitis B was also is different in patients infected with HBV genotype A, D, or F.

The molecular virological mechanisms related to the different clinical outcomes among HBV genotypes remains largely unknown. Previous studies have indicated that several HBV variants display alteration of epitopes important in the host immune recognition, enhanced virulence with increased replication of HBV, resistance to antiviral therapies or facilitated cell attachment / penetration [35]. Among these variants, isolates with an adenine (A) to thymine (T) transversion at nucleotide 1762 together with a guanine (G) to adenine (A) transition at nucleotide 1764 (T1762 / A1764) mutations in the basal core promoter (nucleotides 1742-1849) are often present in hepatitis B carriers with chronic hepatitis, fulminant hepatitis and HCC, and less often in inactive carriers and immunosuppressed patients [36]. Recent studies have

indicated that HBV genotype C has a higher frequency of T1762/A1764 mutation as compared to genotype B [24, 37]. Furthermore, a higher frequency of basal core promoter mutant has been found in HCC patients as compared to asymptomatic carriers (66%-90% vs. 11-47%) [38, 39], and the frequency of this mutant has been reported to increase with progression of liver disease [40]. It is proposed that changes in the secondary structure of pregenome, given rise to by T1762/A1764 mutation, may increase viral replication through the enhancement of core protein synthesis and creation of a binding site for HNF1 (hepatocyte nuclear factor) transcription factor [41]. On the other hand, the X gene of HBV genome encodes two proteins that have potent transactivation activities on viral as well as cellular genes [42]. This property makes the X gene a candidate for a role in the development of HCC in patients with chronic HBV infection. Since its coding sequence overlaps regions of crucial importance for virus replication such as enhancer II and core promoter, mutations in this region may therefore induce not only an amino acid change in the X protein but also an alteration of HBV gene expression [35, 43]. For example, T1762/A1764 mutations in the basal core promoter are non-synonymous that may affect the structure of the X protein. These mutations convert amino acids 130 and 131 of the overlapping X coding region from lysine to methionine and valine to isoleucine, respectively. The amino acid changes caused by these mutations in the presence of serine/serine caused by changes at nucleotides 1809/1812 could therefore alter the structure of the X protein and thus contribute to hepatocarcinogenesis [39]. Taken together, the basal core promoter mutation may serve as a candidate molecular marker to account for the pathogenic differences among HBV genotypes. However, the hypothesis needs to be

confirmed and other common variants throughout the HBV genome that may also act as possible molecular markers await further examination.

Taken together, these lines of evidence indeed suggest pathogenic and therapeutic differences among HBV genotypes in Taiwan; however, the relationship between HBV genotypes and clinical phenotypes of patients with chronic hepatitis B as well as the molecular virological mechanisms contributing these clinical differences, especially hepatocarcinogenesis, remains to be further explored. In this study, we therefore investigated the prevalence of common subgenomic variants of HBV including precore stop codon mutation and basal core promoter mutation in a cohort of genotype B or C-infected hepatitis B carries with different clinical stages of liver disease. The association of HBV genotype and viral genomic variation with the development of HCC will be further analyzed.

Materials and Methods

Patients

Serum samples were obtained from a cohort of 250 genotype B or C-infected chronic HBV carriers (200 men, 50 women; 143 infected with genotype B and 107 with genotype C) with long-term follow-up at the gastroenterological clinics of the National Taiwan University Hospital since 1990. They included 60 hepatitis B e antigen (HBeAg)-negative hepatitis B carriers (44 men, 16 women; 35 infected with genotype B and 25 with genotype C; mean age: 45$\pm$12 years) with persistently normal serum alanine aminotransferase (ALT) levels for at least three years in periodic biochemical examinations (every 3 or 6 months), and were inferred as inactive HBsAg carriers, and 190 HBsAg-positive patients with histologically verified chronic

liver disease and HCC. Among them, 35 (25 men, 10 women; 20 infected with genotype B and 15 with genotype C; mean age: 38±9 years) had chronic hepatitis, 28 (22 men, 6 women; 13 infected with genotype B and 15 with genotype C; mean age: 46±11 years) had liver cirrhosis and 127 (109 men, 18 women; 75 infected with genotype B and 52 with genotype C; mean age: 55±12 years) had HCC. Of 127 patients with HCC, 48 (43 men, 5 women; 36 infected with genotype B and 12 with genotype C; mean age: 41±7 years) were younger than 50 years of age (young HCC) and the remaining 79 (66 men, 13 women; 39 infected with genotype B and 40 with genotype C; mean age: 61±7 years) were 50 or more (older HCC).[7] The mean age of genotype B and genotype C-infected young HCC patients was 38±7 years and 43±6 years, respectively. The histological diagnosis of chronic liver disease was based on standard criteria.[15] None of them had coinfection with hepatitis C virus (HCV) or hepatitis D virus (HDV). Serum samples from each subject were stored at -70 ^{O}C until use. For patients with histologically verified chronic liver disease, serum samples were collected at the time when liver biopsies were performed.

Genotyping of HBV

HBV genotypes will be determined by using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the surface gene of HBV as previously described [44].

Amplification and sequencing of HBV DNA

To amplify the full-length genome of the dominant HBV strains, we used the primers as previously reported [45]. If first-round PCR yielded only weak or no visible band on a 1.0% agarose gel after electrophoresis, we performed

second-round PCR with 3 different primer pairs yielding overlapping subgenomic fragments and covering the whole length of HBV genome. The first-round PCR condition of full-length HBV DNA were as in the following: 96 ^{o}C for 3 minutes; 94 ^{o}C for 1 minute, 60 ^{o}C for 30 seconds, and 72 ^{o}C for 150 seconds, for 10 cycles; 94 ^{o}C for 1 minute, 56 ^{o}C for 30 seconds, and 72 ^{o}C for 150 seconds, for 40 cycles; and finally 72 ^{o}C for 10 minutes. The second-round PCR condition was similar to that of the first-round PCR, except that the extension time of each cycle was 90 seconds. The polymerase we used for first- and second-round PCR was commercially available Combi-Pol (InViTek GmbH, Berlin, Germany). The PCR product was isolated by the electroelution method from 1.0 % agarose gel and purified by the phenol-chloroform extraction method. Then we performed PCR sequencing on these purified full-length and subgenomic PCR products with a set of 9 forward primers and 1 reverse primer. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 2.0; Foster City, CA) with an automated ABI DNA sequencer (Model 377A, Applied Biosystems).

Amplification and sequencing of subgenomic precore and basal core promoter genes

For the first stage PCR, a 25 贡l of reaction mixture containing 2 贡l of the cDNA sample, 1x PCR buffer (10 mM tris-HCl pH 9.0, 50mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100), 10 mM of each dNTP, 100 ng of each outer primer (outer sense: P1s 5'-CAGACGGTCTGGAGCAAACC-3', positions 1302-1321; outer antisense: P2a

5'-CAATGCTCAGGAGACTCTAAGGC-3', positions 2043–2021) and 1 unit of Taq DNA polymerase is amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. Each cycle entails denaturation at 95 ºC for 60 s, primer annealing at 55 ºC for 30 s and extension at 72 ºC for 60 s with a final extension step at 72°C for 7 min. After the first amplification, 1 贡l of the PCR products is reamplified for another 30 cycles with 100 ng of each inner primer (inner sense: P3s 5'-CTCATCTGCCGGACCGTGTG-3', positions 1562–1581; inner antisense: P4a 5'-GTCAGAAGGCAAAAAAGAGAG-3', positions 1966-1946). Nucleotide sequences of amplified products will be directly determined as described above.

Statistical Analysis

Data will be analyzed by Fisher's exact test, Chi-square test with Yates' correction where appropriate. Logistic regression analysis was used to assess the likelihood of specific subgenomic variations in different clinical stages of chronic HBV infection and the influence of various factors on the risk of HCC development, after adjusting for potential confounders.

Results and Discussion

Overall, the frequency of T1762/A1764 mutation increased with advancing clinical stages, from 3% in inactive carriers to 64% in HCC patients ($P < 0.001$). For patients with genotype B infection, the frequency of T1762/A1764 mutation was significantly higher in HCC patients compared to that in inactive carriers, chronic hepatitis and cirrhosis patients ($P < 0.001$). For those with genotype C infection, HCC patients also had a significantly higher prevalence of T1762/A1764 mutant than inactive carriers, chronic hepatitis and cirrhosis patients ($P < 0.001$). To determine whether the

likelihood of T1762/A1764 mutant differed by different clinical stages of liver disease, multiple logistic regression analysis was used. The logistic regression model was adjusted for potential confounding factors. For genotype B infection, only HCC patients had a significantly greater likelihood of T1762/A1764 mutant than inactive carriers (odds ratio, 17.79; 95% confidence interval [CI], 3.81-82.98; $P < 0.001$). For genotype C infection, both liver cirrhosis and HCC patients had a significantly greater likelihood of T1762/A1764 mutant than inactive carriers (odds ratio, 4.91; 95% CI, 1.13-21.33; $P = 0.034$ for liver cirrhosis and odds ratio, 18.70; 95% CI, 4.77-73.33; $P < 0.001$ for HCC). The logistic regression analysis also showed that genotype C was significantly associated with the presence of T1762/A1764 mutation than genotype B (52% vs. 29%; odds ratio, 5.18; 95% CI, 2.59-10.37; $P < 0.001$) after adjusting for sex, age and severity of liver disease. The difference was most remarkable in the stages of liver cirrhosis and HCC (53% vs. 8%, $P < 0.01$ and 83% vs. 51%, $P < 0.001$, respectively).

The attributable risk of multiple factors including sex, age, liver cirrhosis, T1762/A1764 mutation and HBV genotype for HCC in hepatitis B carriers was determined by multiple logistic regression analysis. Individuals with T1762/A1764 mutation were significantly associated with the development of HCC than those without this mutation (odds ratio, 10.60; 95% CI, 4.92-22.86; $P < 0.001$), and the risk was observed for both genotypes B and C. Statistically significant odds ratios were also obtained for male gender ($P = 0.033$), older age ($P < 0.001$), liver cirrhosis ($P < 0.001$) and HBV genotype C ($P = 0.002$).

Of 127 patients with HCC, 76 (60%) were cirrhotic and 54 (71%) of them had T1762/A1764 mutation, whereas only 27 (53%) of 51 non-cirrhotic patients had T1762/A1764 mutation. Therefore, cirrhotic patients tended to have a higher

frequency of T1762/A1764 mutant than non-cirrhotic patients (71% vs. 53%, P = 0.06). When stratified by age, the prevalence of T1762/A1764 mutant was comparable between young and older HCC patients, irrespective of HBV genotypes (54% vs. 46% for genotype B and 92% vs. 80% for genotype C). Nevertheless, the difference was significant between young HCC patients and age-matched inactive carriers (54% vs. 6% for genotype B, P < 0.001 and 92% vs. 20% for genotype C, P < 0.001).

With a mean follow-up period of 5.4+2.6 years (median: 6 years), 3 of 9 cirrhosis patients infected with of T1762/A1764 mutant virus developed HCC as compared to none of 19 with non- T1762/A1764 mutant virus (33% vs. 0%, P = 0.045). Of the 3 patients who developed HCC, one was infected with genotype B and 2 with genotype C.

Other mutations in the basal core promoter were further analyzed. Mutation from A to G or T at nucleotide 1752 was found in 51 (20%) patients (45, G1752; 6, T1752) and was linked to genotype but not to the progression of liver disease. Mutation from T to C at nucleotide 1753 was observed in 17 (7%) patients (all with genotype C) and always occurred together with T1762/A1764 mutation. A distinct genotype-specific polymorphism was noted from nucleotides 1726 to 1730: all 143 genotype B strains had CTGAG, whereas 90 (84%) of 107 genotype C strains had AAGAC and the remaining 17 (16%) had AGGAC. Another polymorphism at nucleotide 1799 (G or C) was also genotype-related: 127 (89%) genotype B strains had G1799, whereas 86 (80%) genotype C strains had C1799. Less-consistent genotype-associated mutation was seen at nucleotide 1773. Mutation from C to T or A at this position was found in 13 (9%) genotype B strains and in 17 (16%) genotype C strains. No correlation was found between these mutations and the severity of

liver disease or the development of HCC.

The correlation between the precore stop codon mutant or A1896 mutant and the development of HCC was studied in inactive carriers and HCC patients. The data indicated that HCC patients had a similar frequency of A1896 mutation to inactive carriers, irrespective of HBV genotypes.

In summary, our results indicate that the occurrence of T1762/A1764 basal core promoter mutation of HBV is more frequent in genotype C than B patients, and the likelihood of T1762/A1764 mutation parallels the progression of chronic liver disease. Meanwhile, patients with HBV genotype C and T1762/A1764 mutant are independently at increased risk for HCC development. These data suggest a possible role for genotype C and T1762/A1764 mutant in the pathogenesis and carcinogenesis of chronic HBV infection. Specific HBV genotype and viral mutant may serve as useful molecular markers in predicting the clinical outcomes of patients with chronic hepatitis B.

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