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Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma

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***Background/Aims*: Hepatitis B virus genotype C (HBV/C) has been classified into two geographically distinct subgenotypes; HBV/C1/Cs (Southeast Asia) and HBV/C2/Ce (East Asia).**

***Methods*: Viral differences in enhancer II/core promoter and precore regions between the subgenotypes and their association with hepatocellular carcinoma (HCC) were assessed in a matched cross-sectional control study of 118 carriers (from Hong Kong) with HBV/C1/Cs (48.0 years, 81% male, 40% HBeAg+, 44% HCC) and 210 HBV/C2/Ce (172 from Japan, 38 from Hong Kong) (50.2 years, 78% male, 30% HBeAg+, 46% HCC).**

***Results*: Univariate analyses showed that mutation V1753 was predictive for HCC among HBeAg-positive-C1/Cs-carriers ($P = 0.0055$), and T1653 among HBeAg-positive-C2/Ce-carriers ($P = 0.018$), and T1653 or V1753 or T1762/A1764 among HBeAg-negative-C2/Ce-carriers ($P < 0.05$). In the multivariate analysis on all HBV/C subjects, independent predictive factors for HCC were subgenotype C2/Ce (odds ratio, 4.21; 95% confidence interval, 1.07–16.23), T1653 (3.64; 1.93–6.86), V1753 (3.07; 1.66–5.65) and T1762/A1764 (2.58; 1.21–5.49) mutations, age ($\geqslant$50 years), gender (male) and HBeAg (positive).**

***Conclusions*: Our data indicate that T1653 and/or V1753 mutations in addition to T1762/A1764 are differently associated with HCC in context of HBeAg status among HBV/C1/Cs and C2/Ce-carriers. HBV/C subgenotypes have specific mutation patterns, which is probably responsible for increased carcinogenesis of HBV/C2/Ce.**

***Keywords*: Hepatitis B virus; Hepatocellular carcinoma; Subgenotype C; Enhancer II; Core promoter; Precore genome**

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba (“a” standing for Asia) and Bj (“j”

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Abbreviations: HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; RFLP, restriction fragment length polymorphism; RTD-PCR, real-time detection polymerase chain reaction.

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for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Recently, a phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from Southeast Asia including Vietnam, Myanmar, Thailand and Hong Kong (named HBV/C1) and the other for strains from (Far) East Asia including Japan and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [8], and by recent independent studies in Hong Kong [9] and Japan [10]. The latter papers designated the 2 subgenotypes as HBV/Cs in Southeast Asia and HBV/Ce in the (Far) East Asia that have not only different epidemiological distributions but also different virological findings in precore regions [9,10].

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore region at nt 1896 (A1896) are associated with HBe antigen seroconversion (SC) and viral replication. It is noteworthy that the both BCP and precore stop-codon mutations are often found in patients with advanced liver disease such as hepatocellular carcinoma (HCC) [11–14]. Beyond these mutations, the C to T mutation in the upstream regulatory sequence (C1653T) is associated with fulminant hepatitis [15] and located in the alpha box, which is a strong activating element of both enhancer II and core promoter [16]. Takahashi et al. [17,18] reported that C-to-T1653 and T-to-V(not T)1753 mutants were more closely associated with the progression of liver disease from chronic hepatitis to cirrhosis and/or HCC in HBeAg-positive patients, compared with the BCP double mutation. Our recent case-control study supports that the addition of T1653 mutation in enhancer II to the BCP mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19].

To evaluate clinical and virological significances between HBV/C1/Cs and HBV/C2/Ce, in the present study, we performed a multi-center cross-sectional matched control study among HBV/C carriers [inactive carriers (IC), chronic hepatitis (CH), HCC] and determined the specific HBV mutations associated with disease progression.

2. Materials and methods

2.1. Serum samples

A total of 328 sera were obtained from chronic HBV/C carriers who visited Nagoya City University Hospital, Musashino Red Cross Hospital, Osaka National Hospital in Japan and Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each carrier.

2.2. Serological assays for HBV markers

HBeAg and anti-HBe were detected by Chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse f, FUJIREBIO INC., Tokyo, Japan). HBV Genotypes were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed to distinct epitopes on the preS2-region [20,21], with use of commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan).

2.3. PCR-RFLP for distinguishing between subgenotypes C1/Cs and C2/Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/C1/Cs or C2/Ce [10]. The first-round PCR was performed with a sense primer (HBV964F) and an antisense primer (HBV1272R) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2) and an antisense primer (HBV1272R). To determine HBV/C1/Cs, a portion (5 μL) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *BstE*II at 60 °C for 1 h each. For HBV/C2/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (wt/vol) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.4. Detection and quantification of serum HBV DNA

HBV DNA sequences spanning the S gene were amplified with real-time detection polymerase chain reaction (RTD-PCR) according to the method of Abe et al. [22] with a forward primer (HBSF2), a reverse primer (HBSR2), and Taq Man probe (HBSP2′) with an additional G at the 3′-end of the original HBSP2 [23]. The detection limit of this method was 100 copies/mL.

2.5. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [24], with slight modifications [23]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer. The sequences covered enhancer II/core promoter (Fig. 1A) and precore genes (Fig. 1B), which could be associated with HBeAg production, viral replication and disease progression.

2.6. A cross-sectional control study for clinical and virological differences between HBV/C1/Cs and C2/Ce

The clinical diagnosis was established after serum biochemistry tests ultasonography, computerized tomography (CT), the magnetic resonance imaging (MRI), and the liver biopsy. To compare the clinical differences between HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 210$), age-, sex-, HBeAg status-matched HBV carriers were enrolled (Table 1). The carriers were also matched according to the severity of liver disease in each group. The HBsAg-positive individuals with normal alanine aminotransferase (ALT) levels over 2 years (examined at least four times at 3-month intervals), and without the presence of portal hypertension were defined as IC. Individuals with persistent elevation of ALT levels (>1.5× upper limit of normal) [35 U/L] over