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Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qidong, China☆

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***Background/Aims*: To investigate the mutations in hepatitis B virus (HBV) that might be related to hepatocellular carcinoma (HCC) in the high-risk area Qidong, China.**

***Methods*: DNA sequences of HBV basal core promoter (BCP) and the overlapping X gene were determined in 58 HCC and 71 chronic hepatitis (CH) patients. In addition, a consecutive series of plasma samples from 15 HCC cases were employed to compare the CP/X sequences before and after the occurrence of HCC.**

***Results*: T1762/A1764 double mutation was frequently found in Qidong patients, regardless of clinical status (65.5% in HCC and 73.2% in CH, $P > 0.05$). Unexpectedly, the adjacent T1766/A1768 mutation significantly increased the risk of HCC ($P < 0.05$). Moreover, the prevalence of triple mutations in BCP was significantly higher in patients with HCC than those with CH ($P < 0.05$). The longitudinal study demonstrated that the mutations in BCP were gradually accumulated during the development of HCC. Colony formation assay showed while A1764 mutation alone did not alter the colony-inhibitory activity of HBx, double or triple mutations largely abrogated this effect.**

***Conclusions*: The complex mutation involving T1766/A1768 was closely related to HCC. The enhanced risk of HCC caused by BCP variants could be attributable partially to the aberrant activity of HBx.**

***Keywords*: Hepatitis B virus; Hepatocellular carcinoma; Mutation; Core promoter; X protein**

1. Introduction

Hepatitis B virus (HBV) core promoter (CP) resides in the overlapping X gene and plays a central role in HBV replication and morphogenesis [1]. Core promoter is composed of the core upstream regulatory region (URR) and the basal core promoter (BCP). A-to-T at HBV nucleotide (nt) 1762 (Galibert numbering system [2])and G-to-A at nt 1764 double mutation in BCP was reported to be associated with severe forms of liver disease [3–10]. Recently, C-to-T at nt 1653 in the box α of URR and T-to-V(C/A/G) at nt 1753 were also found to be frequent in HCC patients [9,11,12]. Although in cell transfection experiments, the T1762/A1764 double mutation has been found to up-regulate viral genome replication and down-regulate HBeAg expression [13,14], the mechanism underlying CP mutation and hepatocarcinogenesis is largely unknown.

The incidence of HCC in Qidong, China is amongst the highest in the world. While the national age-standardized incidence (ASR) for HCC was 38.9/100,000 for males and 14.5/100,000 for females [15], the ASR in Qidong was 79.6/100,000 for males and 23.1/100,000 for females [16]. HCC accounts for almost half

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of the malignancies and is the leading cause of cancer mortality in Qidong. The high incidence of HCC is the consequence of high prevalence of HBV infection [17] and exposure to aflatoxin B1 (AFB1) [18]. HBV poses a high level of risk for HCC in Qidong (odds ration [OR], 10.5–13.5). It has been reported that 100% of the HCC cases in Qidong showed the evidence of HBV infection [19]. By use of a mass spectrometry technology, SOMA, Chen et al. have reported that T1762/A1764 mutation could be measured in 53.3% of HCC plasma samples and 74.3% of HCC tissue specimens in Qidong. They also reported an acceleration of mortality in individuals infected with T1762/A1764 mutants [20]. However, to date, the epidemiological prevalence of T1762/A1764 mutation in Qidong chronic hepatitis (CH) patients has not been documented. It is also unclear whether there is any other type of CP mutation responsible for the worse outcomes of liver disease in Qidong. To this end, we examined the nucleotide sequence of entire CP/X region and analyzed the hotspot mutations through both cross-sectional and longitudinal studies.

2. Materials and methods

2.1. Patients and samples

Plasma samples were obtained from a prospective cohort started in 1992, where 852 HBsAg-positive individuals and 786 HBsAg-negative individuals residing in Qidong were recruited. Plasma samples of each individual were collected annually and stored at −40 °C. HCC diagnosis was made pathologically. For case-control study, 129 HBV DNA-positive plasma samples were selected from the cohort: 71 from CH patients and 58 from HCC patients. There were no differences in the median age and male to female ratio between these 2 groups (39.9 ± 9.1 years vs. 41.9 ± 10.4 years, $P = 0.24$ for age; 55:3 vs. 68:3, $P = 1.00$ for male to female ratio). For longitudinal study, 15 HCC patients with T1762/A1764 or T1766/A1768 mutation in the year of HCC diagnosis were selected. Of each patient, at least one PCR amplifiable DNA was available before HCC.

2.2. Amplification and sequence analysis of HBV DNA

DNA was extracted from 100 μl plasma by proteinase K digestion and phenol–chloroform extraction. HBV sequence (nt. 1266–1823) was amplified by PCR using the primer set HB1/R-dr, and semi-nested with primer HB2 (Table 1). PCR reaction was carried out in 50 μl containing 5 μl 10 × buffer, 4 μl 2.5 mmol/L dNTP, 2 μl 10 μmol/L sense and antisense primers, 1 U Pyrobest DNA polymerase (TaKaRa Bio Inc., Dalian, China). The PCR products were gel purified and then used as the template for sequencing by BigDye terminator cycle-sequencing reaction kit and Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were compared using the Clustal W program.

2.3. HBV genotyping

HBV genotypes were determined by comparing the sequence of HBx with a set of database-derived standard sequences. Phylogenetic tree was constructed by software MEGA3.1.

2.4. Site-directed mutagenesis and plasmid construction

The mutagenesis was carried out through PCR-mediated site-directed mutagenesis [21] with a set of designed mutagenetic primers (Table 1). Briefly, single base changes were introduced by PCR using internal primers containing the desired point mutation(s) (F2–4, F2–4.2 and F2–4.2.8) paired with the outside primer R2. Primer set F1/R1 was used to generate the products with the homologue ends overlapped with the above mutated fragments. The products of the first round PCR were purified and served as the templates for the second round PCR with the outside primer set F1/R2 to generate a full-length HBx. HBx was cloned into pcDNA3.1 (−) (Invitrogen, Carlsbad, CA) by XbaI and KpnI sites. All constructs were sequenced to confirm the desired sequences.

2.5. Colony formation assay

HuH7 cells and liver Chang cells were transfected with 2 μg of HBx constructs or empty vector pcDNA3.1(−) per 35 mm plate by Lipofectamine2000 (Invitrogen, Carlsbad, CA) reagent. Thirty-six hours after transfection, the cells were transferred to a 60 mm plate and fed with the selective medium containing G418 800 μg/ml. The cell culture medium was changed every 3 or 4 days until the colonies formed. Drug-resistant colonies were fixed with cold methanol, stained with crystal violet, and then scored.

2.6. Statistical analysis

Data values were analyzed with relative risk test (OR value), Chi-square test, Fisher's exact test or Student's *t*-test where appropriate. SPSS version 12.0 was used for statistical analysis. All of the tests of significance were two-tailed, and a P value of less than 0.05 was considered statistically significant.

3. Results

3.1. HBV CP/X mutation in patients with HCC and CH

In order to detect the genetic variations in CP/X region, DNA fragment from HBV nt 1266–1823 was

Table 1
Oligonucleotide primers used in the study

Primer	Sequence	Position
HB1	5′-GCCAAGTGTTTGCTGACGC-3′	1175–1193
R-dr	5′-AAAGTTGCATGGTGCTGGTG-3′	1823–1804
HB2	5′-CCATACTGCGGAACTCCTAG-3′	1266–1285
F1	5′-GAAtctaga**CCACC**ATGGCTGCTAGGGTGTGCTG-3′	1374–1393
R1	5′-CTAATCTCCTCCCCCAACTCCTCCCAGTC-3′	1728–1756
F2-4	5′-GTTGGGGGAGGAGATTAGGTTAAAGATCTTTGTAC-3′	1739–1773
F2-4.2	5′-GTTGGGGGAGGAGATTAGGTTAATGATCTTTGTAC-3′	1739–1773
F2-4.2.8	5′-GTTGGGGGAGGAGATTAGGTTAATGATCTATGTAC-3′	1739–1773
R2	5′-CATggtaccTTAGGCAGAGGTGAAAAAGTTGCATG-3′	1813–1838

The lowercase letters, restriction enzyme site; the bold letters, kozak sequence; the underlined letters, artificial mutations.