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LETTER TO THE EDITOR

Hepatitis B virus with pre-S2 deletion is more prevalent in hepatocellular carcinoma than in chronic active hepatitis and asymptomatic carriers

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A significant health disparity exists among Pacific-Asian ethnic groups in the incidence and prevalence of hepatocellular carcinoma (HCC) (*1*). More than 600,000 people die of HCC each year and at least 300,000 of all deaths worldwide occur in China alone (*2*). Hepatitis B viruses (HBV) are responsible for the majority of the worldwide development of HCC and the lack of sensitive and specific biomarkers is a major reason for the high rate of HCC-related mortality. The cause of early oncogenesis in HBV-related childhood HCC remains unclear, because the reports about association between HBV mutations and hepatocarcinogenesis are conflicting (*3*). Recent studies dealing with the possibility that pre-S2 deletion mutation is a risk factor for HCC in children have revealed that pre-S2 deletion does not need a long time to occur (*4–5*). Accordingly, the knowledge about molecular epidemiology of pre-S2 deletion mutation in the HBV DNA of carriers and progressive liver disease patients is essential.

Nantong city, China has a particularly high rate of HBV infection and HCC incidence (*6–7*). There are many reports dealing with the association between HCC and some related factors such as HBV infection, aflatoxin $B_{1,}$ and trace elements. In this study, we investigated the prevalence of pre-S2 deletion mutation in asymptomatic HBV carriers (ASC), chronic active hepatitis (CAH), and HCC patients in Nantong. The study protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration and has been approved by the Nantong Centers for Disease Control and Prevention Institutional Review Board. Serum samples were stored at -80°C until tested. Later on, they were screened for the presence of alanine aminotransferase, hepatitis B virus surface antigen (HBsAg), and anti-hepatitis C virus (HCV) antibodies using commercially available kits. Patients with anti-HCV antibodies were excluded from the study in order to eliminate the confounding effect of HCV infection on the incidence of HCC. All cases of HCC diagnosed were confirmed at Nantong Cancer Hospital using criteria set by the Chinese Anti-Cancer Association. Clinical diagnoses of the study participants were as follows: asymptomatic HBV carriers (n = 25), chronic active hepatitis patients (n = 35), and HCC patients (n = 26). The results of genotype classification and plasma HBV DNA viral load were obtained using commercial HBV kits. Carriers of HBV without HCV infection, HBV vaccination and/or antiviral therapy were further examined for the presence of pre-S2 mutation.

HBV DNA was extracted from the serum using DNA isolation kit (Qiagen). For PCR amplification, the pre-S2 gene was amplified using PCR kit (Invitrogen) with pre-

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Abbreviations: ASC = asymptomatic HBV carriers; CAH = chronic active hepatitis; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; HBV = hepatitis B virus

Table. HBV pre-S2 deletion mutation in chronic active hepatitis and hepatocellular carcinoma patients

Case ID	Diagnosis	$Log^{HBV\ DNA}$	Deletion length (bp)	Pre-S2 protein amino acid deletion region 126–141
M38454.1 (wild strain)				TFHQALLDPRVRGLYF
46	CAH	5.97	30	TFHQAL––––––––––
98	HCC	5.34	45	––––––––––––––– I
104	HCC	6.12	36	TFHQ–––––––––––
92	HCC	4.78	30	TFHQAL––––––––––
80	HCC	4.23	33	TFHQA–––––––––L
85	HCC	4.68	30	TFHQAL––––––––––
81	HCC	5.26	39	TFH–––––––––––––
75	HCC	5.15	39	TFH–––––––––––––

S gene specific primers: forward: 5′-TCA CCA TAT TCT TGG GAA CA-3′ (nts 2817–2836), and reverse: 5′-GAG AGA AGT CCA CCA CGA GT-3′ (nts 252–271). The PCR products were purified and sequenced on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems). The sequences of all samples were analyzed using Clustal X 1.8 and MEGA 3.1 software and NCBI database. The sequences were aligned to the genotype-matched strains presented in the GenBank HBV Acc. No. M38454.1 (*10*). Statistical differences between groups were assessed by the Wilcox rank sum non-parametric hypothesis test using SAS version 8.2. All P-values were two-tailed and $P < 0.05$ was considered to be significant.

Group ASC consisted of 10 males and 15 females with an average age of 24.12 years (± 5.83 years). Group CAH consisted of 24 males and 11 females with an average age of 41.68 years (± 7.21 years). Group HCC consisted of 20 males and 6 females with an average age of 58.54 years (± 8.46 years). The genotype distribution of HBV found in Nantong was: B – 16.28%, C – 67.44%, and B+C mixed infection – 16.28%. Genotyping showed that among the ASC patients 6 samples were genotype B and 19 samples of genotype C. Among the CAH patients 4 samples were genotype B, 22 samples genotype C, and 9 samples belonged to mixed genotype B+C. Among the HCC patients 4 samples were genotype B, 17 samples genotype C, and 5 samples mixed genotype B+C. HBV loads of patients in the groups were between 10^3~10^8 copies/ml and there was no significant difference in the virus load between test groups ($P > 0.05$). Pre-S2 deletions were found in the HBV DNA of 7 patients from 26 HCC cases tested (26.92 %). In these 7 patients, all deletions were located in the hot deletion region nt 3223 ~ 3268 (aa 126 ~ aa 141). The length of pre-S2 deletions ranged from 30–45 bp. The average age of HCC patients with pre-S2 deletions was 60.25 years. Some of the HBV pre-S2 nucleotide sequences (patient ID 104, 92, and 81) were submitted to the Genbank and acquired Acc. Nos. HQ156938, HQ156939, and HQ156940, respectively.

The genotype C of HBV is most prevalent in China accounting for about 60% of the total cases. In this study, the prevalence of genotype C and mixed genotype B+C in Nantong was significantly higher than the prevalence of genotype B alone ($P < 0.05$). The detected prevalence of genotype C in Nantong was similar to the prevalence in other areas of China, in spite of the fact that not all reports were in agreement (*11–12*). We found a significant difference in patient age between the ASC, CAH, and HCC groups ($P < 0.005$) and in the percentage of male patients with HCC. The patients with HCC were elder than the patients with CAH and ASC and the disease was more prevalent in males than in females. These factors are known as the independent risk factors for HCC (*8*). However, no significant difference was found in serum levels of HBV DNA.

The outcomes of several studies (*9–10*) led to the hypothesis that pre-S2 deletion mutation may be a risk factor for the development of HCC. The epidemiology of pre-S2 deletion among the HCC patients in the high-risk regions such as Nantong City should be considered as important. In previous studies performed in the HCC high-risk regions, the prevalence of pre-S2 deletions ranged from 16.2-45.5 % with a weighted average of 22.5 % assessed in 312 patients (*11–12*). However, the pre-S2 deletion has a higher prevalence in HCC patients compared to CAH patients, but its role in the oncogenesis is unknown. Retrospective and longitudinal studies are necessary to elucidate completely the potential of pre-S2 mutation as a biomarker for the HCC development in the Asian population.

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