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Analysis of the Precore and Core Promoter DNA Sequence in Liver Tissues from Patients with Hepatocellular Carcinoma

To investigate the role of mutant hepatitis B virus (HBV) in the development of hepatocellular carcinoma (HCC), 20 patients with HCC were studied for precore and core promoter mutations in tumorous and nontumorous tissues. The precore and core promoter region was amplified and analyzed by direct sequencing. Among the 20 tumorous and nontumorous tissues, precore mutant HBV was found in 12 (60%) and 18 (90%), respectively. Of the 12 tumorous tissues with precore mutant, nine tissues had a single mutation (1896) and one tissue had another single mutation (1899). The remaining two tissues had a double mutation (1896 and 1899). A single mutation (1896) and a single mutation (1899) were found in 11 and two of the 18 nontumorous tissues with precore mutant, respectively. Among 20 tumorous and nontumorous tissues, HBV with a C to T mutation at nucleotide (nt) 1846 was detected in six and eight, respectively, and was associated with the virus carrying a mutation (1896 or 1899) except in two tumorous tissues. Mutations at nt 1762 and 1764 in core promoter were observed in 16 (80%) tumorous tissues and 18 (90%) nontumorous tissues. Mutations in the precore and core promoter region were found frequently in nontumorous tissue and in tumorous tissue (18/20 and 12/20 in precore region, 18/20 and 16/20 in core promoter respectively). The high prevalence of precore and core promoter mutations in liver tissue from patients with HCC suggests that these mutations may contribute to the development of HCC.

Key Words: *Hepatitis B virus; Mutation; Carcinoma, hepatocellular*

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INTRODUCTION

Mutations in the precore region of hepatitis B virus (HBV) have been detected mainly in hepatitis e antigen (HBeAg)-negative patients with active liver disease (1-3). An in-frame TAG stop codon in the distal precore region has been related to the absence of HBeAg secretion; in addition, other precore mutations have been observed rarely, including loss of start codon (4-6), or various other deletions and insertions (5, 7). As HBeAg is believed to be an important humoral and cellular immune target (8-11), this loss of HBeAg production may represent escape mutation, thus contributing to viral persistence (12).

In HBV, the cis-acting signal for encapsidation has been defined by a region of 85 nucleotides near the 5' end of pregenomic RNA, which is essential not only for the packaging of pregenomic RNA but also for the initiation of reverse transcription (13, 14). The preference for mutation at nucleotide (nt) 1896, as compared with other mutations that prevent HBeAg production, is explained by the fact that a U at nt 1858 may form a base pair with A in nt position of 1896, resulting in enhanced stability of a stem-loop structure (15). In strains with a C at nt 1858, however, a G to A mutation at nt 1896 does not evolve, because it would considerably impair encapsidation and replication (16). The G to A mutation at nt 1896 is the most prevalent mutation among patients with HBeAg-negative hepatitis. However, the high HBeAg-negative rate in South African black adult carriers was found not to be the result of a stop codon mutation at nt 1896, but of a missense mutation at nt 1862 in the bulge of the RNA encapsidation signal because of the high incidence of subtype adw with C at nt 1858 instead of T at nt 1858 (17).

Point mutations in the core promoter have been found in patients with fulminant or chronic hepatitis as an isolated event or in association with the TAG stop codon in the distal precore region (18). The most frequent mu-

tations involve an A to T and a G to A mutation at nt 1762 and 1764 in the core promoter. A recent study demonstrated that a HBV genome carrying the mutations at nt 1762 and 1764 displayed reduced levels of HBeAg synthesis and was associated with enhanced viral replication (19). Mutations in the precore and core promoter region have been found frequently in Korean patients with chronic HBV infection (47% in precore region and 91% in core promoter) (20, 21).

Persistent HBV infection has been associated with hepatocellular carcinoma (HCC). HBV persistence is involved in hepatocarcinogenesis by induction of liver cell necrosis and secondary proliferation of adjacent liver cells (22, 23), as well as by cis- and trans-activation effects of integrated HBV-DNA on cellular genes (24-27). There are few data on the role of genetic HBV variations in hepatocarcinogenesis. HBV-DNA with a distal precore stop codon has been reported in HCC (28). The variations of viral sequences have been mainly studied in the serum samples, and it has been reported that the analysis of the HBV strains in the serum does not necessarily reflect the situation in the liver where more mutations seem to be retained (29). In this study, we analyzed the precore and core promoter sequence of HBV in tumorous and adjacent nontumorous tissues from patients with HCC to investigate the pattern of HBV mutations and the relationship between HBV mutations and HCC in Korea, an endemic area for HBV.

MATERIALS AND METHODS

Patients

We studied 20 patients with HCC, comprised of 17 positive and three negative for serum HBsAg (Abbott Laboratories, North Chicago, IL). HBV-DNA was detected in liver samples obtained from three HBsAg negative patients. All the patients were anti-hepatitis C virus negative (Abbott Laboratories, North Chicago, IL). The patients' clinical data are summarized in Table 1. Liver tissues were obtained surgically and stored immediately at -70℃. Histological examination of nontumorous samples showed cirrhosis in all 20 patients studied. Tumorous liver samples were designated with a T, while the adjacent nontumorous liver samples were designated with an N.

Extraction and amplification of DNA

DNA was extracted from tumorous and adjacent nontumorous tissues. Frozen liver tissue was mechanically shattered and incubated for protein digestion in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 150 mmol/L NaCl, 2% SDS) containing proteinase K at 2 mg/mL for 18 hr at 37℃. After incubation, DNA was extracted twice with phenol chloroform/isoamyl alcohol. The DNA was precipitated by ethanol overnight at -20

Table 1. Clinical data from patients with hepatocellular carcinoma

No. of patient	Age (yr)	Sex	HBV serological markers				
			HBsAg	Anti-HBc	Anti-HBs	HBeAg	Anti-HBe
1	50	F	+	+	−	NA	NA
2	28	F	+	+	−	−	+
3	48	M	+	+	+	−	+
4	41	M	−	+	−	NA	NA
5	57	F	+	+	−	−	+
6	24	M	+	+	−	−	+
7	67	F	+	+	−	−	+
8	64	M	+	+	−	−	+
9	57	M	+	+	−	+	−
10	77	M	−	+	+	+	−
11	58	M	+	+	−	−	−
12	64	M	+	+	−	NA	NA
13	51	M	+	+	−	−	+
14	44	M	+	+	−	−	+
15	56	M	+	+	−	−	+
16	47	M	+	+	−	−	+
17	69	M	−	+	−	−	+
18	57	F	+	+	−	−	+
19	58	M	+	+	+	−	+
20	43	F	+	+	+	−	+

F, female; M, male; NA, information not available

℃, and resuspended in 10 mmol/L Tris-HCl (pH 8.0) containing 1 mmol/L EDTA. Amplification of the precore sequence of HBV was performed by nested PCR using 0.5 μg of DNA in a final volume of 20 μL containing 50 mmol/L Tris-HCl, pH 8.3, 40 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 250 μmol/L of each deoxynucleotide, 1 U of *Taq* polymerase (Korea Biotech, Inc, Korea) and 20 pmol of each external primer. The reaction was carried out in 30 cycles of 94℃ for one min, 55℃ for one min, and 72℃ for two min, with a ten-min extension step at 72℃ at the end. For the second round PCR, 2 μL of the first round PCR product was added to 18 μL of the reaction mixture with the same composition as the first round mixture except that 20 pmol of the internal primers was used. Two microliters of the second round PCR amplified products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide, and visualized under UV light. The external primers were 5'-CATAAGAGGACTCTT-GGACT-3' (sense, nt 1653 to 1672), and 5'-GGCGAGGGAGTTCTTCTTCTAGG-GG-3' (antisense, nt 2394 to 2369), and primers for the second PCR were 5'AATGTCAACGACC-GACCTTG-3' (sense, nt 1679 to 1698) and 5'-AGCTGAGGCGGTG-TCGAGGAGATC-3' (antisense, nt 1985 to 2009). To prevent cross-contamination, all precautions recommended by Kwok and Higuchi (30) were observed, and negative controls were included in each assay.

Direct sequencing of amplified DNA

From the extracted DNA, a second PCR product was obtained and analyzed by direct sequencing to define the precore sequence. Amplified DNA was purified by the QIA quick PCR purification kit (QIAGEN GmbH, Germany) and then used for direct sequencing using internal antisense primer. Dideoxynucleotide termination sequencing was performed with the sequenase PCR product sequencing kit (version 2.0, US Biochemicals, Cleveland, OH, U.S.A.), according to the manufacturer's instructions. Sequencing reactions were run on 6% polyacrylamide urea gels, and autoradiography was performed with intensifying screens at 4℃ for three days. To reduce the probability of an erroneous sequence determination due to *Taq* polymerase incorporation errors, whenever a HBV-DNA sequence differed from known wild-type HBV strains, the sequencing was repeated from a new PCR reaction.

RESULTS

HBV-DNA products were successfully amplified from all liver tissue samples tested by PCR. It is impossible to know whether the HBV DNA detected in liver tissues was amplified from integrated or episomal DNA. The failure of HBV amplification or appearance of multiple PCR bands may occur when the HBV DNA was amplified from integrated HBV DNA because the integrated HBV is often extensively rearranged as a result of deletion or insertion in the precore gene of HBV. A single clear band of the appropriate size was observed on the agarose gel in this study. Three major missense/nonsense mutations and two point mutations were found in the encapsidation signals and in core promoter, respectively (Table 2). The most frequently detected mutation was a G to A mutation at nt 1896 creating stop codon in the precore region. An A to T mutation at nt 1762 and a G to A mutation at nt 1764 were the most prevalent mutation in the core promoter. All patients studied had virus with a T at nt 1858. The nucleotide suquences of the precore genes and core promoter are given in Fig. 1 and Fig. 2, respectively. Comparison of the complete sequence of precore region, isolated from both tumorous and nontumorous tissues of the same patient, demonstrated a concordance with the presence of wild-type HBV and precore mutants in 11 out of 20 patients (55 %) (Fig. 2 and Table 2).

Among the 20 tumorous tissues, precore mutant HBV-DNA was found in 12 tissues (60%). Of these 12 tumorous tissues, nine had a single mutation at nt 1896 and one had a single mutation at nt 1899. Double mutations were detected in the remaining two tissues. In six of the 20 tumorous tissues, HBV with a C to T mutation at nt 1846 was found and associated with the virus carrying an A1896 or A1899, except in two tumorous tissues. Among the 20 nontumorous tissues, precore mutant HBV-DNA was detected in 18 tissues (90%). Of these 18 nontumorous tissues, 11 had a single mutation

Table 2. Mutations in the precore region and core promoter of hepatitis B virus DNA

Tissue	Total	Mutations - core promoter		Mutations - precore region		
		A to T at nt 1762	G to A at nt 1766	G to A at nt 1896	G to A at nt 1899	C to T at nt 1846
		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Tumorous tissue	20	16 (80)	18 (90)	11 (55)	3 (20)	6 (30)
Nontumorous tissue	20	18 (90)	19 (95)	16 (80)	8 (40)	8 (40)

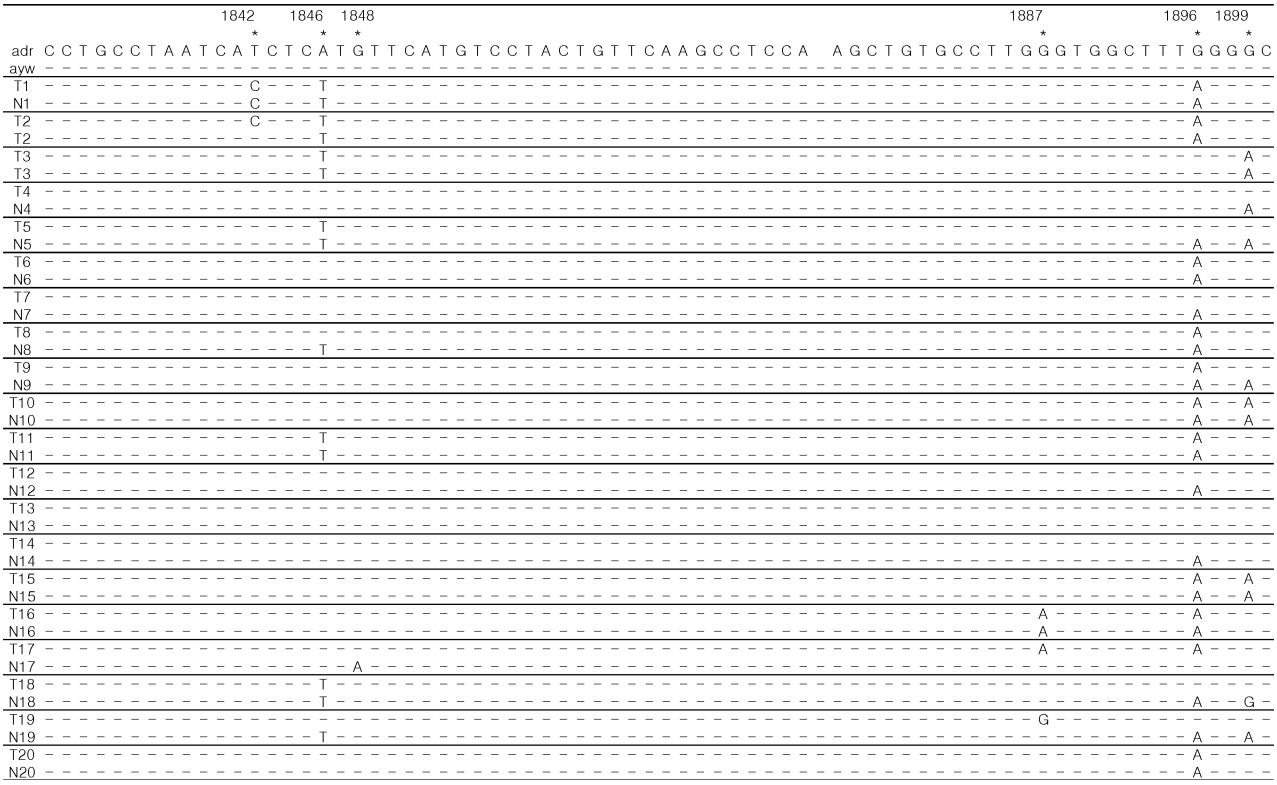

Fig. 1. Nucleotide sequence of the precore of HBV DNA. The wild type sequences of adr and ayw are shown. HBV sequences are obtained by direct sequencing from tumorous tissues (T) and adjacent nontumorous tissues (N).

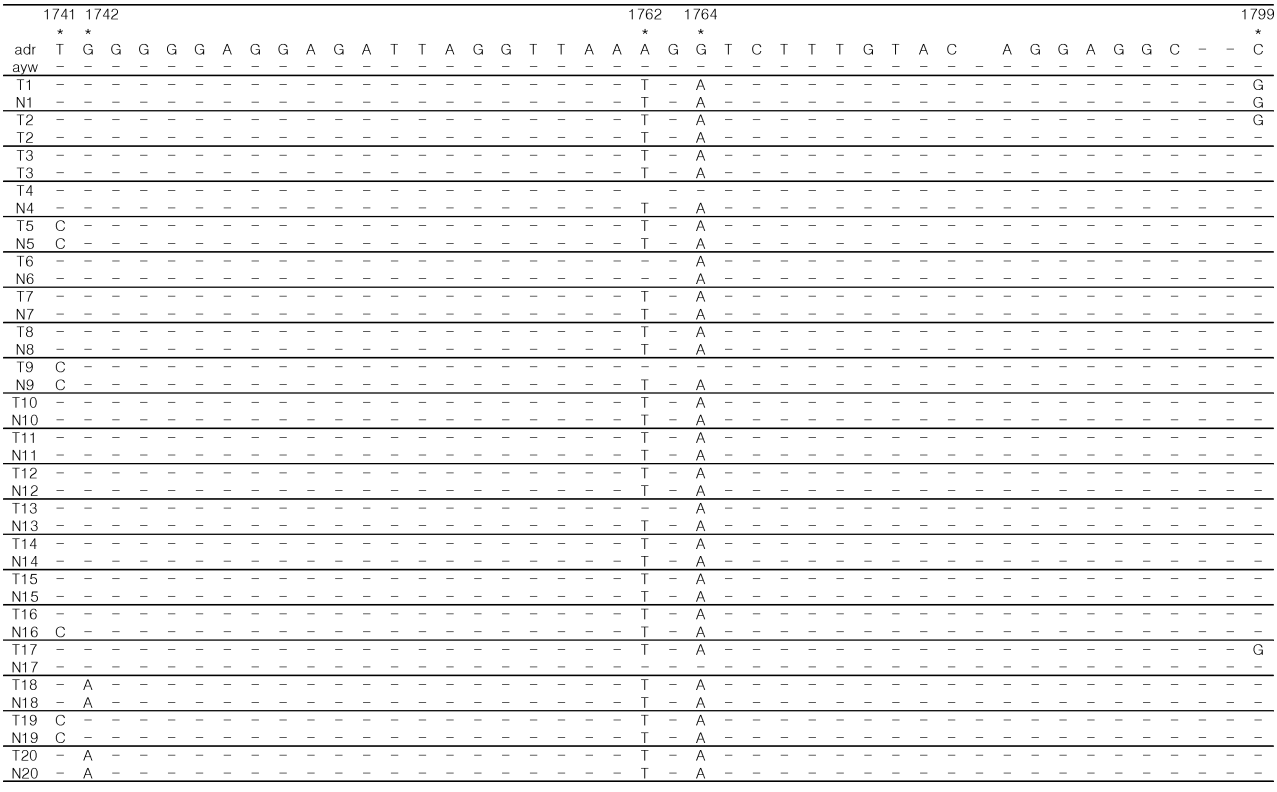

Fig. 2. Nucleotide sequence of the core premoter region of HBV DNA extracted from tumorous (T) and adjacent nontumorous tissues (N) compared with the most closely related wild-type strains. HBV DNA sequences were amplified and sequenced directly.

at nt 1896 and two had a single mutation at nt 1899. Double mutations at nt 1896 and nt 1899 were found in the remaining five tissues. When the mutation frequency of the HBV pre-core region in tumorous and nontumorous tissues from the same patient was analyzed, the mutation developed frequently in nontumorous tissue and in tumorous tissue (18/20 and 12/20; Table 2).

Mutations in the core promoter were detected in 18 and 19 of 20 tumorous and nontumorous tissues, respectively. Of the 18 tumorous tissues which had mutations in the core promoter, 16 tissues (80%) had the two point mutations, from A to T at nt 1762 and from G to A at nt 1764. The remaining two tissues had one point mutation at nt 1764. Of the 20 nontumorous tissues, 18 tissues (90%) had the two point mutations at nt 1762 and 1764. The remaining one tissue had one point mutation at nt 1764. The precore mutations at nt 1896 were detected in 10 (55.5%) and 16 (84.2) of 18 tumorous and 19 nontumorous tissues with the mutation in the core promoter, respectively (Table 2).

DISCUSSION

A TAG Stop codon mutation in the terminal part of the precore region develops during viral replication (31), and displacement of the wild-type by mutant can take several years (5, 32). HBV has been classified into four genotypes (A, B, C, and D) (33), and HBV genotypes may influence the rate of occurrence of precore mutants (16). Different genotypes may explain the uneven prevalence of HBe-minus mutants in the world.

We found three major missense/nonsense mutations in the precore region. A precore stop codon mutation was by far the most common and found in 16 (80%) of 20 cirrhotic tissues studied which is consistent with the study (5) in Japanese anti-HBe positive HBV carriers. In contrast, in a study (15) among Chinese hepatitis B patients in Hong Kong, of 62 HBeAg-negative patients, only 24 (39%) were infected with mutants in the precore region. This difference could be due to different circulating HBV strains. Sequence analysis of the precore regions indicates that the sequence of codon 15 was CCT in all samples from Korea and Japan, whereas codon 15 was CCC in 40% of the patients from Hong Kong. In strains with CCT sequence at codon 15, the precore stop codon mutation is tolerated, because a T-1858 may form a base pair with an A in nt 1896. Thus, the higher percentage of CCT at codon 15 could account for the higher incidence of precore mutation detected in Korea and Japan when compared with that in Hong Kong.

We found that 40% of cirrhotic tissues had an A to T mutation at nt 1846. This mutation was only found in the presence of 1896 or 1899 mutations. Although the mutation at nt 1846 destroys base pairing at this site, the combined mutation at 1896 or 1899 may stabilize stem-loop. In contrast to this study, Lok et al. (15) reported low frequency (11%) of combined mutation at nt 1896 in patients with mutation at nt 1846. The sequence analysis of wild ayw subtype revealed that ayw subtype had a T at nt 1846 (34). These findings suggest that this site is not critical for viral replication. Further in vitro transfection studies using site-directed mutants are necessary to confirm the significance of this mutation on viral replication.

Manzin et al. (35) reported that mutations leading to amino acid substitution at the level of distal cysteine residue were detected in 7 of 9 HBV DNA-positive samples from HCC tissues, and TAG stop codon mutation was not detected in tumorous tissues. This experiment suggests that TAG stop codon mutation has reduced oncogenic potential. Kramvis et al. (36) reported that the 1862 missense mutations which may disrupt HBV DAN replication were present more often in tumorous tissues, and TAG stop codon mutation was not detected in the tissue samples. Thus, they have claimed that disruption of viral replication caused by the missense mutation may promote integration of unencapsidated replicative intermediate and hence contribute to hepatocarcinogenesis. In contrast, in this study the TAG stop codon mutation was found mostly in tumorous and nontumorous tissues, and missense mutations were not detected. These discrepancies can be due to the different HBV genotypes. All of our patients belonged to genotype non-A. But, most of the patients in Minami's and Kramvis's study were genotype A. It is possible that the mechanism underlying development of HCC may be different according to HBV genotype. Maintenance of persistent infection and continuing liver cell necrosis through the accumulated TAG stop codon mutation may contribute to the development of HCC in patients infected with genotype non-A such as adr subtype.

It has been reported that fulminant hepatitis B is associated with mutations in the core promoter (37). However, mutations in the core promoter were frequently detected in patients with chronic hepatitis (18). We found the mutations at nt 1762 and 1764 in most cirrhotic tissues. The X protein seems to be immunogenic and immune response to X protein is mounted during infection (38). Thus, these changes might hypothetically represent immunologic escape mutants.

A lower incidence of the HBV mutation was reported in HCC tissues when compared with that of cirrhotic tissues (35). The reasons for this are not known. Since it is presently believed that the origin of these HBV mutants is a process requiring time and active viral

replication (31, 39), it is possible to hypothesize that an early HBV-DNA integration occurs in HCC cells and a selection pressure for the mutants is exerted weakly in tumorous tissues. Recently, Hosono et al. (28) found a lower frequency of mutations in the integrated HBV-DNA than that in replicative HBV-DNA in HCC.

In summary, we have identified mutations in the pre-core region and core promoter in the majority of HCC and surrounding cirrhotic livers. By favoring the escape from the immune response to HBeAg, they may also contribute to the persistence of HBV infected cells. The high prevalence of precore and core promoter mutations in liver tissue from patients with HCC suggests that these mutations may contribute to the development of HCC.

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