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ORIGINAL ARTICLE

A case–control study on sequence variations in the enhancer II/core promoter/precore and X genes of hepatitis B virus in patients with hepatocellular carcinoma

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Abstract
Purpose To evaluate the sequence variations in the enhancer II (EnhII)/basal core promotor (BCP)/precore (PC) and X genes of hepatitis B virus (HBV) in Thai patients with hepatocellular carcinoma (HCC) by conducting a cross-sectional case–control study.
Methods As much as 60 patients with HCC and 60 patients without HCC, who were matched for sex, age, hepatitis B e antigen (HBeAg) status, and HBV genotype, were included. Viral mutations in the EnhII/BCP/PC and X regions were characterized by direct sequencing in serum samples.
Results The prevalence of T1753C/A, A1762T/G1764A and G1899A mutations were significantly higher in the HCC group compared to the non-HCC group (43.3 vs. 23.3%, $P = 0.02$; 88.3 vs. 53.0%, $P < 0.001$; and 35.0 vs. 8.3%, $P = 0.001$, respectively). No significant difference between groups was found with respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C, and G1896A mutations. By multiple logistic regression analysis, the presence of cirrhosis, A1762T/G1764A and G1899A mutations were independently associated with the risk of HCC.
Conclusion These data suggested that A1762T/G1764A and G1899A mutations were associated with the development of HCC in Thai patients.

Keywords Hepatitis B virus · Enhancer II · Core promoter · Precore · X genes · Hepatocellular carcinoma

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Introduction

Hepatitis B virus (HBV) infection is a major public health problem, with more than 350 million HBV carriers estimated worldwide [1]. Chronic HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). In HBV endemic areas, such as Southeast Asia, more than 60% of HCC cases are attributable to chronic infection with the virus [2]. Although the association between chronic HBV infection and HCC is well established, the virological factors, particularly HBV mutations, contributing to tumor development remain uncertain.

Hepatitis B virus, a member of the family *Hepadnaviridae*, is a partially double-stranded DNA virus that contains four overlapping open reading frames (ORFs) encoding the surface, precore/core, polymerase, and X genes. The virus shows remarkable genetic variability and is currently classified into eight genotypes, designated A to H based on genomic sequence analysis [3]. HBV has a high mutation rate compared with other DNA viruses because it lacks proofreading capacity during the replication via reverse transcription of its pregenomic RNA [4]. The well-known naturally occurring HBV variants include the precore (PC) stop codon mutation (G1896A), which abolishes hepatitis B e antigen (HBeAg)

production. The other common HBV variants include double mutations in the basal core promotor (BCP) region (A1762T/G1764A), which overlap with the ORF of the X gene and result in substantial decreases in HBeAg production [5]. These dual mutants have been reported in up to 50–80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia [6], and have been implicated in HCC development [7–9]. Apart from these variants, other mutations, such as T1753C/A/G in the BCP region and C1653T in the enhancer II region (EnhII) have become increasingly recognized as being associated with the outcome of chronic HBV infection, including HCC development [10–12].

The X-ORF encodes a 154 amino acid protein called hepatitis B virus X protein. This protein plays an important role in the regulation of viral genome expression, and has also been implicated in hepatocarcinogenesis [13]. The X protein is a multifunctional regulator that modulates host transcription, cell cycle progress, protein degradation, apoptosis, and signal transduction pathways [14]. It has been shown that mutations in the X gene may contribute to the development of HCC in HBV-infected patients [15, 16]. In addition, mutations in the BCP region, which overlaps the coding sequence for the X gene, may result in amino acid changes in the X protein [5]. However, current knowledge regarding the mutational patterns in the entire X region among patients with HCC is rather limited. Therefore, the aim of the current study was to evaluate the association between the mutations within the EnhII/BCP/PC and X genes and the risk of HCC by conducting a case–control study among Thai patients.

Materials and methods

Patients

Serum samples obtained from 60 patients with HBV-related HCC and positive for HBV DNA were randomly selected from a pool of patients with chronic liver disease who were seen and followed-up at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between July 2002 and June 2006. The diagnosis of HCC was based on typical imaging studies and/or histopathology (fine needle aspiration, core liver biopsy or surgical resection) according to American Association for the Study of Liver Diseases (AASLD) guideline [17]. Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic CT or MRI. In cases without typical imaging features, liver biopsy was performed to confirm the diagnosis of HCC.

Among these, 55 patients had cirrhosis as underlying liver disease. As much as 52 were males and 8 were females, with the mean age (±SD) of 55.7 ± 9.8 years. A total of 18 patients were positive and 42 were negative for HBeAg.

To examine the role of molecular virological factors in the development of HCC, 60 hepatitis B s antigen (HBsAg)-positive patients, who matched for age (±5 years), gender, HBeAg status, and HBV genotype with the patients with HCC, were selected as control patients. These patients visited our clinic every 4–6 months during the same period of recruitment of the present study and none had HCC development during follow-up. Of these control patients, 32 cases had cirrhosis diagnosed based on clinical features and/or histological examination.

None of the patients enrolled in this study had a history of hepatitis C virus (HCV) infection or human immunodeficiency virus (HIV) co-infection. In addition, none of the patients had a history of heavy alcoholic drinking, or received any antiviral therapy when the serum sample was obtained. All patients were informed about the purposes of the study, and subsequently gave their written informed consent. Serum samples were collected from each patient at the time of their evaluation and frozen at –70°C until use. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Biochemical, serological, and virological assays

Serum alanine aminotransferase (ALT), total bilirubin (TB), and albumin levels were measured with a commercial assay using an automated analyzer (Hitachi 912). Sera tested for HBsAg and HBeAg were determined using commercially available ELISA tests (Abbott Laboratories, Chicago, IL, USA). Serum HBV DNA level was quantified using a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Tokyo, Japan). The detection range of this assay was 2.7–8.7 log copies/mL.

HBV DNA preparation, amplification, and direct sequencing

HBV DNA was extracted from 100 μL serum sample by incubation in lysis buffer (10 mM Tris–HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/mL proteinase K) and phenol–chloroform–isoamyl alcohol extraction. The DNA pellet was re-suspended in 30 μL sterile distilled water and subjected to amplification of the X/BCP/PC regions (nucleotides (nt) 1,287–2,038) by polymerase chain reaction (PCR) using the primers Xi1: 5′-AGCTTGTTTTGC TCGCAGC-3′ (forward primer, nt. 1,287–1,305), and Ci1: 5′-TTCCGGAGACTCTAAGGCC-3′ (reverse primer, nt. 2,020–2,038). The obtained sequences span the region which included the entire X protein ORF (nt. 1,374–1,836), the EnhII region (nt. 1,685–1,773), the basal core promoter

(BCP) (nt. 1,742–1,849), direct repeat 1 (DR1) (nt. 1,824–1,834), direct repeat 2 (DR2) (nt. 1,590–1,600), the precore (nt. 1,814–1,901), and a part of the core region (nt. 1,901–2,038).

Briefly, the reaction mixture comprised 2 μL re-suspended DNA, 0.5 μL of 25 mmol of each primer, 10 μL of 2.5× MasterMix® (Eppendorf, Germany) and sterile distilled water to a final volume of 25 μL reaction. The reaction was performed in a PCR thermocycler (Eppendorf AG, Hamburg, Germany) with the initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s (denaturing), at 55°C for 30 s (annealing), at 72°C for 1 min (extension) and concluded by a final 7 min extension at 72°C. The PCR products were segregated by 2% agarose gel electrophoresis. The PCR products were extracted from the agarose gel using the Perfectprep® Gel cleanup kit (Eppendorf, Hanburg, Germany). The sequencing reaction was performed using the AmpliTaq™ DNA Polymerase FS dye terminator from the ABIPRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's specification. Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and aligned with CLUSTAL_X (version 1.83) program as previously described [18].

HBV genotyping

HBV genotypes were determined from serum samples, using PCR-restriction fragment length polymorphism (PCR–RFLP) genotyping based on analysis of the surface gene, as previously described [19].

Statistical analysis

Data were presented as percentage, mean, and standard deviation. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann–Whitney test or Student's *t* test when appropriate for quantitative variables. Multiple logistic regression analysis was used to assess the influence of each clinical or viral factor on the risk of HCC development. *P* values below 0.05 were considered statistically significant. Data were analyzed by using the SPSS software for Windows 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of patients with and without HCC

The clinical features of patients with HCC and controls are showed in Table 1. Compared with the control group, patients with HCC had higher frequency of cirrhosis. In addition, patients with HCC had significantly poorer liver biochemical parameters (TB and albumin) compared to controls. However, there was no significant difference between groups in respect to ALT and HBV DNA levels (Table 1).

Comparisons of sequences in the EnhII/BCP/PC and X regions between the HCC and control groups

Based on direct sequencing of EnhII/BCP/PC regions, the mutations were found at nt. 1,613, 1,653, 1,753, 1,762, 1,764, 1,766, 1,768, 1,846, 1,858, 1,896, and 1,899. Compared with the controls, the HCC group had higher frequencies of T1753C/A, A1762T/G1764A, and G1899A mutations. However, no significant difference between groups was found with respect to G1613A, C1653T, C1766T/T1768A, A1846T/C, T1858C, and G1896A mutations (Table 2).

Single codon mutations were present in the X region, but with a generally scattered distribution, and without significant difference between the HCC and control groups. However, three mutational patterns including I127T/N, K130M, and V131I, corresponding to T1753C/A and double A1762T/G1764A mutations in the BCP region were found with significantly higher frequencies in the HCC group than in the controls. In contrast, no significant difference between groups was found with respect to A36T, P38S, A44L, and H94L mutations (Table 2).

In addition, four HBV variants in the HCC group showed the following deletions at or around nt. 1,762–1,764. One HBV variant had deletions at nt. 1,757–1,777, while another had deletions at nt. 1,756–1,764. One additional case had long deletions at nt. 1,594–1,827, while another had a deletion at nt. 1,762–1,776. Interestingly, one HBV variant in the HCC group had a 24-base insertion between nt. 1,674 and 1,675. All these cases belonged to the HBeAg-negative group.

Multivariate analysis of factors associated with HCC

To determine the independent contribution of clinical and virological features to the development of HCC, multiple logistic regression analysis was performed by using the significant factors identified in the univariate analysis. These factors included TB and albumin levels, the presence of cirrhosis, and nucleotide sequence variants list in Table 2 (C1653T, T1753C/A, A1762T/G1764A, and G1899A mutations). The significant factors associated with HCC development were A1762T/G1764A and G1899A mutations and the presence of cirrhosis (Table 3).

The cumulative effect of the mutations at A1762T/G1764A and/or G1899A, which were the significant factors

Table 1 Demographic and clinical characteristics of patients with or without HCC

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	*P*
Age (years)	52.9 ± 8.6	55.7 ± 9.8	0.096
Sex			1
Male	52 (86.7)	52 (86.7)	
Female	8 (13.3)	8 (13.3)	
Total bilirubin (mg/dL)	1.5 ± 1.2	2.1 ± 1.4	0.014
Albumin (g/L)	3.6 ± 0.6	3.3 ± 0.6	0.005
ALT (U/L)	139.7 ± 101.4	161.1 ± 116.9	0.285
Cirrhosis	32 (53.3)	55 (91.7)	<0.001
HBeAg positivity	18 (30.0)	18 (30.0)	1
HBV genotype			1
B	16 (26.7)	16 (26.7)	
C	44 (73.3)	44 (73.3)	
HBV DNA level (log copies/mL)	6.1 ± 1.3	5.9 ± 1.4	0.451

Data were expressed as mean ± SD, no (%)

Table 2 Virological characteristics of HBV in the HCC and control groups

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	*P*
Nucleotide sequences of EnhII/BCP/PC genes			
G1613A	18 (30.0)	24 (40.0)	0.339
C1653T	7 (11.7)	16 (26.7)	0.062
T1753C/A	14 (23.3)	26 (43.3)	0.02
A1762T/G1764A	33 (55.0)	53 (88.3)	<0.001
C1766T/T1768A	3 (5.0)	10 (16.7)	0.075
A1846T/C	14 (23.3)	16 (26.7)	0.833
T1858C	1 (1.7)	3 (5.0)	0.619
G1896A	17 (28.3)	26 (43.3)	0.127
G1899A	5 (8.3)	21 (35.0)	0.001
Amino acid sequences of X gene			
A36T	42 (70.0)	41 (68.3)	0.843
P38S	2 (3.3)	0 (0)	0.496
A44L	14 (23.3)	20 (33.3)	0.311
H94Y	7 (11.7)	16 (26.7)	0.062
I127T/N	18 (30.0)	39 (65.0)	<0.001
K130M	33 (55.0)	51 (85.0)	<0.001
V131I	33 (55.0)	52 (86.7)	<0.001

Data were expressed as mean ± SD, no (%)

Table 3 Multivariate analysis of factors associated with HCC

Factor	Odds ratio (95% CI)	*P*
A1762T/G1764A mutations	3.56 (1.16–10.89)	0.026
G1899A mutation	3.54 (1.09–11.47)	0.034
Presence of cirrhosis	8.44 (2.65–26.84)	<0.001

CI confidence interval, *OR* odds ratio

in multivariate analysis, was further examined. The odd ratio (OR) of HCC with A1762T/G1764A mutations was 6.19, while the OR with G1899A mutation was 5.92. With the presence of both A1762T/G1764A and G1899A mutations, the OR of HCC increased to 10.23. In setting of cirrhosis, the presence of A1762T/G1764A mutations substantially increased the OR of HCC to 15.00, while the presence of both A1762T/G1764A and G1899A mutations increased the OR to 13.44 (Table 4).

Comparison of clinical and virological features according to A1762T/G1764A mutations

The clinical and virological characteristics according to A1762T/G1764A mutations, which were the strongest mutations associated with HCC development, are shown in Table 5. Patients with HBV harboring A1762T/G1764A mutations had higher rates of cirrhosis and HBV genotype C

Table 4 Cumulative effect of factors on the risk of HCC

Characteristics	Control group ($n = 60$)	HCC group ($n = 60$)	Odds ratio (95% CI)	*P*
A1762T/G1764A	33 (55.0)	53 (88.3)	6.19 (2.43–15.83)	<0.001
G1899A	5 (8.3)	21 (35.0)	5.92 (2.06–17.06)	0.001
Cirrhosis	32 (53.3)	55 (91.7)	9.63 (3.38–27.41)	<0.001
A1762T/G1764A and G1899A	3 (5.0)	21 (35.0)	10.23 (2.86–36.67)	<0.001
Cirrhosis and G1899A	4 (6.7)	19 (31.7)	6.49 (2.05–20.51)	0.001
Cirrhosis and A1762T/G1764A	15 (25.0)	50 (83.3)	15.00 (6.12–36.74)	<0.001
Cirrhosis and A1762/G1764 and G1899A	2 (3.3)	19 (31.7)	13.44 (2.97–60.89)	<0.001

Data were expressed as no (%)

CI confidence interval, *OR* odds ratio

than those without such variants. In addition, the virus with A1762T/G1764A mutations had higher frequencies of T1753C/A, C1766T/T1768A, and G1899A mutations than the wild-type virus. However, no differences between groups were found with regard to other virological factors, including HBeAg positivity, HBV DNA level, C1653T, G1613A, A1846T/C, T1858C, and G1896A mutations.

Discussion

Identification of host and viral factors leading to the development of HCC may have important clinical implications in the management of patients with chronic HBV infection. There are now increasing data suggesting that HBV genotypes, HBeAg status, viral load, and emergence of genomic mutations may play an important role in causing different disease profiles in chronic HBV infection. This case–control study was aimed specifically to study the role of HBV mutations in EnhII/BCP/PC and X regions by excluding the confounding effects of viral factors, such as the status of HBeAg, HBV genotype, and viral load. This study also excluded the possibility of cohort effect that patients with chronic HBV infection are prone to have the evolution of viral mutations in advanced age. Thus, these results are more reliable than those of previous case series in which their confounding consequences from selection bias could not be avoided. Because host factors may vary among different populations, data from various ethnic groups and countries are needed to be compared before conclusions can be drawn. To our knowledge, the current case–control study is the first to reveal the association between HBV mutations and the development of HCC among Thai patients.

In this study, we found that double A1762T/G1764A mutations were an independent risk factor for the development of HCC, which was consistent with recent case–control studies conducted in China, Taiwan, and Korea [7, 12, 20, 21]. Also, the magnitude of the OR of HCC associated with the presence of the BCP double mutants in this study was approximately 3 to 4-fold, which was similar with reports of other studies. In fact, a prospective cohort of approximately 1,600 high-risk individuals in Qidong, China, showed that A1762T/G1764A mutations were detected in approximately 50% of HCC cases before cancer development, suggesting that these variants would indicate a high potential risk for hepatocarcinogenesis [22]. It has been reported that the development of A1762T/G1764A mutations is associated with HBV genotype and their prevalence is higher in genotype C than genotype B [8]. As expected, our data also demonstrated that A1762T/G1764A mutations were genotype C related. We also showed that the prevalence of T1753C/A mutation was significantly higher among the HCC group than the controls, although such mutant was not an independent risk factor of HCC in multivariate analysis. In this study, it should be noted that T1753C/A mutation always existed along with the presence of A1762T/G1764A mutations. Interestingly, previous data also demonstrated that T1753C/A mutation occurred later than A1762T/G1764A mutations in the course of chronic HBV infection [23]. These results suggested that A1762T/G1764A mutations might be the main HBV variants associated with the development of HCC, and T1753C/A mutation might also play an important, albeit lesser, role in hepatocarcinogenesis.

The association between the well-known G1896A mutation in the PC region and the risk of HCC development remains controversial. For instance, a Taiwanese study showed that the presence of the PC mutation significantly increased the risk for HCC [9], while another community-based cohort study with long-term follow-up conducted in the same country demonstrated that this mutant was associated with a decreased risk of HCC development [24]. In this study, our data showed that this common variant might not account for the increased risk of HCC among Thai populations. In contrast, point mutation at nt. 1,899 was an independent viral factor of HCC development. Our results were well matched with a recent study performed in Taiwan, which demonstrated that the prevalence of G1899A and not G1896A mutation was significantly higher among patients with HCC than those

Table 5 Comparison of clinical and virological characteristics according to A1762T/G1764A mutations

Characteristics	No A1762T/G1764A mutations ($n = 32$)	A1762T/G1764A mutations ($n = 88$)	P
Age (years)	52.9 ± 9.0	54.9 ± 9.4	0.268
Sex			0.385
Male	28 (87.5)	76 (86.4)	
Female	6 (12.5)	10 (13.6)	
Total bilirubin (mg/dL)	1.6 ± 1.2	1.9 ± 1.4	0.215
Albumin (g/L)	3.6 ± 0.6	3.4 ± 0.6	0.065
ALT (U/L)	145.2 ± 105.5	152.5 ± 111.5	0.74
Cirrhosis	20 (62.5)	67 (76.1)	0.043
HBeAg positivity	9 (28.1)	27 (30.7)	0.663
HBV genotype			<0.001
B	19 (59.4)	13 (14.8)	
C	15 (40.6)	73 (85.2)	
HBV DNA level (log copies/mL)	5.9 ± 1.5	6.1 ± 1.2	0.325
Mutations			
G1613A	8 (25.0)	34 (38.6)	0.137
C1653T	4 (12.5)	19 (21.6)	0.303
T1753C/A	0 (0)	40 (45.5)	<0.001
C1766T/T1768A	0 (0)	13 (14.8)	0.019
A1846T/C	8 (25.0)	22 (25.0)	0.815
T1858C	2 (6.3)	2 (2.3)	0.318
G1896A	14 (43.8)	3 (3.4)	0.527
G1899A	2 (6.3)	24 (27.3)	0.007

Data were expressed as mean ± SD, no (%)

without HCC [7]. In contrast, G1899A mutation was found at low prevalence with no clinical association in other previous reports [25, 26]. The reasons for these discrepancies among reports remain unclear and merit further studies to clarify the role of G1896A or G1899A mutant in HBV-related hepatocarcinogenesis.

Whether there are any additive or synergistic effects on the risk of HCC development with combinations of HBV mutations remain to be established. Recent studies demonstrated that certain complex HBV mutational patterns might be associated with the development of advanced liver diseases, including HCC [7, 27]. In this respect, our study showed that the risk of HCC was significantly increased in patients infected with HBV encoding both A1762T/G1764A and G1899A mutations. Of note, the risk of HCC was further increased among cirrhotic patients who were infected with HBV harboring A1762T/G1764A mutations or A1762T/G1764A and G1899A mutations in combination. These results suggest that these HBV mutations may serve as helpful virological markers for predicting the development of HCC, particularly in patients who already had cirrhosis. In agreement with our data, a recent prospective study demonstrated that A1762T/G1764A mutations were useful biomarkers for identifying a subset of male patients who were at increased risk of HCC [28].

Although the precise mechanism of A1762T/G1764A mutations in hepatocarcinogenesis remains uncertain, several hypotheses have been proposed. For instance, it has been shown that A1762T/G1764A mutants may enhance viral replication either by creating a hepatocyte nuclear factor 1 transcription factor-binding site or modulating the relative levels of precore and core RNAs [29]. Furthermore, the presence of BCP double mutants may be associated with decreasing T-cell immune responses [30]. In addition, mutations in the BCP region, which overlaps the coding sequence for the X gene, may result in amino acid changes in the X protein [5]. Thus, genomic variation in these regions could modify the oncogenic potential of the X protein and induce inactivation of p53-mediated apoptosis or impairment of DNA repair [31].

In this study, the rate of mutations affecting codons 130 (K130M) and 131(V131I) in the X protein, corresponding to double A1762T/G1764A mutations, significantly differed between patients with or without HCC. In addition, HBV with I127T/N mutation in the X protein, which corresponds to T1753C/A mutation was observed more frequently in patients with HCC than in the control group. These 'hot-spot' mutations are located in the carboxy functional region, and thus might be associated with the transactivating function of the X protein [32]. Previous

studies also reported that other amino acid substitutions, such as A36T, P38S, A44L, and H94Y were significantly associated with the risk of HCC [11, 15, 33, 34]. However, the prevalence of these mutations, except A36T, was found to be relatively low in our study and there was no significant difference in their prevalence between the HCC and non-HCC group. Thus, our data suggested that the emergence of these mutants might not lead to development of HCC in Thai patients. Instead, these mutants might occur during a long-standing inflammatory process of vertically transmitted chronic HBV infection among Thai populations.

In conclusion, our case–control study showed that A1762T/G1764A and G1899A mutations were independent virological factors associated with the risk of HCC. Identification of A1762T/G1764A and G1899A mutants may be valuable for predicting the development of HBV-associated HCC. Further large-scale prospective studies, which offer advantages over cross-sectional investigations, are needed to confirm these observations.

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