Association between the basal core promoter mutation and the development of hepatocellular carcinoma in patients with chronic hepatitis B virus infection

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Abstract

It has been suggested that the A to T mutation at nucleotide 1762 and/or the G to A mutation at nucleotide 1764 (A1762T/G1764A) in the basal core promoter (BCP) in hepatitis B virus (HBV) might be associated with the development of hepatocellular carcinoma (HCC). This study investigated the association between the A1762T/G1764A mutations of HBV and the development of HCC by adjusting age, sex, and genotypes of HBV. A total of 106 HCC patients were studied. Age, sex, and genotype-matched patients were assigned in a 2:1:1 ratio (HCC: inactive HBsAg carrier: liver cirrhosis). The prevalence of A1762T/G1764A mutations was higher in LC (94.3%) (P=0.004) and HCC patients (96.2%) (P=0.001) than inactive HBsAg carriers (73.6%). There was no difference in the prevalence of A1762T/G1764A mutations between LC and HCC patients (P=0.69). In multivariate analysis, patients with cirrhosis (odds ratio [OR], 5.0: 95% confidence interval [CI], 1.6–22.3) and HCC (OR, 9.2; 95% CI, 2.8–29.5) had a greater likelihood of A1762T/G1764A mutations than in inactive HBsAg carriers. There was no increased likelihood of A1762T/G1764A mutations in HCC patients compared with LC patients (OR, 1.7; 95% CI, 0.4–8.4; P=0.5). These data suggest that A1762T/G1764A mutations themselves might not be associated with the development of HCC, especially in patients with genotype C. (J Med Life Sci 2009;6:113–118)

Key Words: hepatitis B virus, hepatocellular carcinoma, core promoter, HBV genotype

Introduction

Hepatitis B virus (HBV) infection is associated with various clinical outcomes such as acute self-limited hepatitis, asymptomatic inactive hepatitis B surface antigen (HBsAg) carrier, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). It has been suggested that the clinical outcomes of HBV infection depend on several factors. Age at infection, host factors, and viral factors such as mutation in the precore and the basal core promoter (BCP) and the genotype of HBV has been widely studied on the course of chronic hepatitis B infection.

BCP, mapping between nucleotide 1742 and 1849, controls the transcription of both precore mRNA and pregenomic RNA in HBV replication. Previous studies indicate that the A to T mutation at nucleotide 1762 and/or the G to A mutation at nucleotide 1764 (A1762T/G1764A), which usually occurs together and is the most frequent mutation observed in the BCP, might be associated with high levels of viral replication and linked to fulminant hepatitis. However, subsequent studies did not prove this. In addition, it has been postulated that A1762T/G1764A mutations have a role in the development of HCC. However, the possibility of cohort effect cannot be excluded in the previous studies because some problems, such as different age, sex, heterogeneous genotype of HBV, which affect the development of HCC, may exist. Moreover, some studies are small in sample size and included the patients with unknown or different age and unknown HBV genotype. Furthermore, A1762T/G1764A mutations depend on HBV genotypes and are highly prevalent in areas, where HBV genotype C is prevalent, such as Korea and Japan. In view of the above, A1762T/G1764A mutations may act as a confounding variable in the development of HCC. Therefore, association between A1762T/G1764A mutations and the development of HCC remains to be clarified by adjusting confounding factors.

The aim of this study was to elucidate the association between A1762T/G1764A mutations and the development of HCC under adjusting the age, the sex, and the genotypes of HBV with large sample size.
Patients

Between May 2001 and August 2003, consecutive patients, who were diagnosed as having HCC at Jeju National University Hospital, Jeju, Korea and Ulsan University Hospital, Ulsan, Korea, were screened. Age, sex, and genotype-matched controls were used in this study.

Patients were assigned in a 2:1:1 ratio (HCC: inactive HBsAg carrier: clinical liver cirrhosis). HCC was diagnosed either histologically or clinically on the basis of serum levels of α-fetoprotein and radiological findings as previously reported. Inactive HBsAg carrier was defined as detectable HBsAg, undetectable HBeAg and HBV DNA by hybridization, normal levels of serum alanine aminotransferase (ALT) over 6 months, and without any clinical evidence of liver cirrhosis and HCC for at least 6 months before enrollment. Clinical liver cirrhosis was diagnosed on the following criteria: having clinically relevant portal hypertension (esophageal varices and/or ascites, splenomegaly with platelet count 100,000/mm²) and/or imaging features suggestive of liver cirrhosis on ultrasonography without any evidence of HCC at least 6 months. Patients were excluded if they had any of the followings: acute hepatitis B, concomitant hepatitis C or D virus infection, human immunodeficiency virus, any history of antiviral therapy, history of immunosuppressive therapy, and history of heavy alcohol drinking. Ethics committee approved this study and patients gave written informed consent.

Serologic Markers

HBsAg, HBeAg and anti-HBe were tested by third generation microparticle enzyme immunoassays using commercial kits (Abbott, North Chicago, IL, USA). HBV DNA levels were measured by the Digene Hybrid Capture assay (Digene Corporation, Gaithersburg, MD, USA). The limit of detection of this assay was 0.5 pg/mL.

Genotyping of HBV and Sequencing

Nucleic acids were extracted from 200 μL serum that had been stored at 80°C using a High Pure Viral Nucleic Acid Kit (Roche, Penzberg, Germany). HBV genotype was determined by genotype-specific primers.

To detect the mutations in the BCP and the precore region of the HBV, we did bi-directional sequencing with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, USA) by automatic sequencing machine (ABI PRISM 3100 Genetic Analyzer, Tokyo, Japan). Polymerase chain reaction and direct sequencing were performed as described previously. We undertook all necessary precautions to prevent cross-contamination, and distilled water was used as a negative control at each PCR step.

Statistical Analyses

The results were expressed as mean SD (range) or percentage. The differences between categorical variables were analyzed by Fisher's exact test or chi-square test. For the continuous variables, Student's t-test was used. Logistic regression analysis was used to assess the independent predictive factors for A1762T/G1764A mutations. A P value of less than 0.05 (two-tailed) was considered to be statistically significant.

Results

During the study period, 113 consecutive patients were diagnosed as having HCC. Among them, 106 patients (88 men and 18 women: the mean age 53.8 yr) with HCC were studied. Seven patients with HCC were excluded because of the exclusion criteria. Demographic features of the study patients are presented in Table 1.

All patients were infected with HBV genotype C. The details of the mutation pattern in the BCP region are presented in Table 1. The prevalence of A1762T/G1764A mutations was 90.1%. Among these, 1 patient showed deletion from nucleotide 1749 to nucleotide 1771. The A to T mutation at nucleotide 1762 and the G to A mutation at nucleotide 1764 in the BCP accompanied in 189/192 (98.4%) patients. The prevalence of A1762T/G1764A mutations was higher in patients with LC (94.3%) (vs 96.2%) than in inactive HBsAg carriers (P=0.004) and HCC (96.2%) (vs inactive HBsAg carrier, P<0.001) than in inactive HBsAg carriers (73.6%). However, there was no difference in the prevalence of A1762T/G1764A mutations between the patients with LC and HCC (P=0.69). In patients with HCC, clinical liver cirrhosis was accompanied in 86 patients (81%). There was no difference in the prevalence of A1762T/G1764A mutations between the HCC patients with LC (83/86, 98.5%) and without LC (19/20, 95%) (P=0.8).

The G to A mutation at nucleotide 1896 (G1896A) was more frequent in patients with LC and HCC than inactive HBsAg carriers, however they did not differ between the patients with LC and HCC (Table 1).
Table 1. The Baseline Characteristics of the Study Patients

| Variables                  | Inactive HBsAg carriers (n=53) | Liver cirrhosis (n=53) | Hepatocellular carcinoma (n=106) | Total (n=212) |
|----------------------------|--------------------------------|------------------------|----------------------------------|---------------|
| Age (mean SD; years)       | 53.3 (10.9)                    | 53.3 (9.6)             | 53.6 (9.5)                       | 53.6 (9.6)    |
| Gender (M/F)               | 44/9                           | 44/9                   | 86/18                            | 176/36        |
| HBeAg-positive*            | 0 (0)                          | 21 (30.6)              | 26 (24.5)                        | 47 (22.2)     |
| HBV DNA†                   | Positive 0.5 pg/mL             | 0 (0)                  | 46 (86.8)                        | 114 (53.8)    |
|                            | Negative 0.5 pg/mL             | 53 (100)               | 7 (13.2)                         | 59 (28.0)     |
| Mutations in the BCP‡      | nt. 1762                       |                        |                                  |               |
|                            | Wild                           | 16 (30.2)              | 3 (5.7)                          | 24 (11.3)     |
|                            | Mixed                          | 7 (13.2)               | 3 (5.7)                          | 14 (6.8)      |
|                            | Mutation                       | 39 (50.6)              | 47 (88.7)                        | 174 (82.1)    |
|                            | nt. 1764                       |                        |                                  |               |
|                            | Wild                           | 14 (26.4)              | 3 (5.7)                          | 21 (9.9)      |
|                            | Mixed                          | 8 (15.1)               | 3 (5.7)                          | 12 (5.7)      |
|                            | Mutation                       | 31 (58.5)              | 47 (88.7)                        | 179 (84.4)    |
|                            | Overall mutations              | 39 (73.6)              | 50 (94.3)                        | 191 (89.8)    |
| Mutation in the precore nt. 1896 |                        |                       |                                  |               |
|                            | Wild                           | 37 (69.8)              | 28 (52.8)                        | 115 (54.2)    |
|                            | Mixed                          | 8 (15.1)               | 13 (20.8)                        | 43 (20.3)     |
|                            | Mutant                         | 8 (15.1)               | 14 (26.4)                        | 54 (25.5)     |
|                            | Overall mutation               | 16 (30.2)              | 25 (47.2)                        | 97 (45.8)     |
| Genotypes C                | 53 (100)                       | 53 (100)               | 106 (100)                        | 212 (100)     |

Data are number of patients (%).
* P < 0.001, inactive HBsAg carrier vs. liver cirrhosis; P < 0.001, inactive HBsAg carrier vs. HCC; P < 0.07, liver cirrhosis vs. HCC.
† P < 0.001, inactive HBsAg carrier vs. liver cirrhosis; P < 0.001, inactive HBsAg carrier vs. HCC; P < 0.03, liver cirrhosis vs. HCC.
‡ P < 0.04, inactive HBsAg carrier vs. liver cirrhosis; P < 0.04, inactive HBsAg carrier vs. HCC; P < 0.85, liver cirrhosis vs. HCC.

Factors that affect A1762T/G1764A and G1896A mutations

Among baseline characteristics, clinical status as above mentioned, HBeAg (P = 0.037) and HBV DNA (P = 0.001) were significant predicting factors for A1762T/G1764A mutations in univariate analysis (data not shown). When multivariate analysis was performed using logistic regression, patients with cirrhosis (odds ratio [OR], 6.0; 95% confidence interval [CI], 1.6–22.3; P = 0.008) and HCC (OR, 9.2; 95% CI, 2.8–29.5; P < 0.001) had a significantly greater likelihood of A1762T/G1764A mutations than in inactive HBsAg carriers (Table 2). There was no increased likelihood of T1762/A1764 mutations in patients with HCC compared with LC (OR, 1.7; 95% CI, 0.4–8.4; P = 0.5).

There was no increased likelihood of G1986A mutations in patients with LC (OR, 1.6; CI, 0.6–4.6; P = 0.35) and HCC (OR, 1.7; 95% CI, 0.7–3.0; P = 0.24) compared with inactive HBsAg carriers. G1896A mutation was only associated with HBeAg status (P = 0.02) (Table 2).

Table 2. Logistic Regression Analysis for the A1762T/G1764A Mutations and G1896A Mutation

| Variables                  | A1762T/G1764A mutations | G1896A mutation |
|----------------------------|--------------------------|-----------------|
|                            | OR (95% CI)              | P value         | OR (95% CI)              | P value         |
| HBeAg status               |                          |                 |                          |                 |
| Positive                   | 1                        |                 | 1                        |                 |
| Negative                   | 0.3 (0.04–3.1)           | 0.3             | 2.4 (1.2–4.9)            | 0.02            |
| Diagnosis                  |                          |                 |                          |                 |
| Inactive HBsAg carrier     | 1                        |                 | 1                        |                 |
| Liver cirrhosis            | 6.0 (1.6–22.3)           | 0.008           | 1.6 (0.6–4.6)            | 0.35            |
| HCC                        | 9.2 (2.8–29.5)           | 0.001           | 1.7 (0.7–3.0)            | 0.24            |

OR, odds ratio; CI, confidence interval.
In this study, A1762T/G1764A mutations were detected in most (90.1%) of the study patients. In addition, the prevalence of A1762T/G1764A mutations even in inactive HBsAg carriers was extraordinarily high (73.6%) compared with previous reports\cite{6-7}, in which the prevalence of A1762T/G1764A mutations was observed in 20%~40% in inactive HBsAg carriers. However, our data are in agreement with a study from Korea by Yoo et al.\cite{26}. In their study, 78.3% of the study patients were considered to be inactive HBsAg carriers and 21.7% were clinical liver cirrhosis. A1762T/G1764A mutations were found in 89.9% using direct sequencing in patients with HBsAg-negative. HBV DNA-negative by hybridization, and normal ALT. In addition, Shindo et al.\cite{27} also reported that the prevalence of A1762T/G1764A mutations was 97% in patients with HBsAg-negative. HBV DNA-negative by hybridization, and normal ALT, even though the proportion of the patients with liver cirrhosis and inactive HBsAg carriers were not assessed in their study. Taking those into account, it is unlikely that the discrepancy between the previous studies\cite{6-7,26} and our study is caused by methodological problem. A possible explanation for the discrepancy in the prevalence of A1762T/G1764A mutations in inactive HBsAg carriers is the different distribution of HBV genotype, because this is an important predictive factor for A1762T/A1764G mutations in the BCP\cite{18,29}

Previously, some case-control studies suggested that A1762T/G1764A mutations had a role in the development of HCC\cite{6-7}. Previous studies are potentially subjected to some sampling bias and the potential confounding effects of age, and HBV genotype. In the study by Fang et al.\cite{6}, as control group, HBsAg-positive asymptomatic carriers, who were young and considered to be in a state of immune tolerant phase in the natural course of chronic hepatitis B, were more selected than in HCC patients. In the study of Baptista et al.\cite{9}, there was no mention of the genotype of HBV. The age was different among the study groups in the study of Kao et al.\cite{7}.

In addition, Chan et al.\cite{9} reported that A1762T/G1764A mutations were not associated with the development of HCC in a prospective longitudinal cohort study. It has been also reported that A1762T/G1764A mutations were not associated with the development of HCC in patients with HBV genotype C\cite{60}. Therefore, these observations suggest that A1762T/G1764A mutations in the BCP have no pathogenic role in the development of HCC, especially infected with HBV genotype C.

Previously, a significant correlation has been found between liver damage and the presence of A1762T/G1764A mutations\cite{59}. Liver cirrhosis, surrogate of liver damage, is a major risk factor in the development of HCC\cite{11}. In this study, patients with clinical liver cirrhosis and HCC had a greater likelihood of A1762T/G1764A mutations than in inactive HBsAg carriers. However, there was no increased likelihood of A1762T/G1764A mutations in patients with HCC compared with LC, suggesting that these mutations might have no direct role in the development of HCC. Instead, A1762T/G1764A mutations were associated with the progression to liver cirrhosis, indicating that A1762T/G1764A mutations may be, in part, indirectly associated with the development of HCC. However, A1762T/G1764A mutations were also high even in patients with inactive HBsAg carriers. It is speculated that the clinical outcomes of HBV infection may be determined by multiple mutations along the HBV genomes\cite{28} or other viral factors in the development of HCC.

The correlation between the G1896A mutation and the development of HCC was also analyzed. However, there was no correlation between G1896A mutation and HCC as previously reported\cite{60}.

Recently, HBV genotypes may contribute to the development of HCC\cite{10,11}. In addition, mutation in the surface gene was also reported to be associated with hepatocarcinogenesis\cite{30}. Therefore, the possibility that other viral mutants contributed to the development of HCC could not be excluded. Therefore, to address these issues, prospective follow-up full-length viral genomic studies from the clinically silent stage to the development of HCC are needed in the future.

In conclusion, although this data showed that A1762T/G1764A mutations in the BCP were highly prevalent in patients with HCC, it was also commonly found in patients with LC and inactive HBsAg carriers. Therefore, the BCP mutations themselves might not be directly associated with the development of HCC, especially in patients infected with HBV genotype C.

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